Universitat Autònoma de Barcelona

Activation of inflammator y resolution programs as a new threrapeutic a approach to promote neuroprotection after SCI

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Activation of inflammatory resolution programs as a new therapeutic approach to promote neuroprotection after SCI

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ACADEMIC DISSERTATION

To obtain the degree of PhD in Neuroscience of

the Universitat Autònoma de Barcelona 2016

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SUMMARY

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Inflammatory response plays an essential role to protect the body after injury or invasion by microorganisms, such as bacteria and parasites. However, inflammation must be a highly regulated process, otherwise, it may lead to tissue damage or even to inflammatory disease. In the nervous system, inflammatory response exerts a crucial role in promoting axonal regeneration after injury, as has been consistently shown in several models of peripheral nerve injury. Nevertheless, after spinal cord injury, an inefficient control of the inflammatory response occurs, which results in exacerbated tissue damage (death neurons and axonal disruption), in inefficient clearance of degenerating myelin, and does not remit over time.

Recent studies have highlighted the importance of anti-inflammatory cytokines and specialized proresolving lipid mediators in triggering inflammatory resolution. These molecules prevent excessive inflammation and promote removal of microbes and apoptotic cells, thereby expediting resolution and return to tissue homeostasis.

The present thesis reveals that the failure of the spinal cord to resolve inflammation after lesion is due, in part, to the inappropriate production of anti-inflammatory cytokines and specialized lipid mediators. Interestingly, it shows that administration of the anti-inflammatory cytokine IL-4, or the specialized proresolving lipid mediator, Maresin1, enhances inflammatory resolution after spinal cord injury in mice and promotes functional recovery and neuroprotection. Unexpectedly, treatment with two other specialized proresolving lipid mediators, resolvin D1 and lipoxin A4, does not show such therapeutic effect. Therefore, the work presented here provides new insights into the mechanisms that hampers the resolution of the inflammatory response after spinal cord injury, and suggest that IL-4 or Maresin1 might be novel useful candidates for the treatment of traumatic lesions in the central nervous system, and to other neurological conditions where inflammation contributes to the course of the pathology.

INTRODUCTION

INTRODUCTION

1- THE SPINAL CORD

I. Physiology and anatomy of the spinal cord

The spinal cord is a highly organized and complex part of the central nervous system (CNS) that represents a vital link between the brain and the body and *vice versa*. Spinal cord plays a role in the three most important functions of the individual: sensation, autonomic and motor control. It relays messages from the brain to different parts of the body in order to incur an action. Moreover, it receives sensory information from host tissues and send it to the brain to be processed. Finally, it also has neuronal networks involved in the generation of reflex responses.

Anatomically, the spinal cord is a white structure with tubular and spongy appearance. In an adult human, its average length is about 45 cm in males and from 42 to 43 cm in females. The spinal cord extends from the base of the brain (in medulla oblongata) through the foramen magnum of the skull to the firsts lumbar vertebras. In humans it occupies the upper two-thirds of the vertebral canal, below which the vertebral canal contains only the spinal nerve roots and meninges (Barson, 1970, Silva et al., 2014). The spinal cord is protected by the vertebral column, which is composed of individual vertebrae. The vertebrae can be organized into 31 segments that can be divided into five regions: cervical (C), thoracic (T), lumbar (L), sacral (S) and coccygeal (Co) **(Figure 1)**. Between the stacked vertebrae are discs of semi-rigid cartilage, and in the narrow spaces between them are passages through which the spinal nerves exit to the rest of the body.

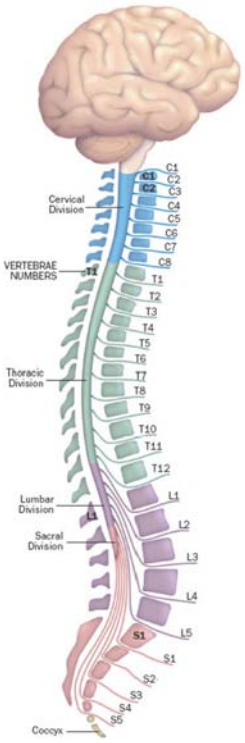

Fig 1. Spinal cord segments. Extracted from Scientific American Mind 16, 68 - 73 (2005)

Two consecutive rows of nerve roots emerge from both sides of each segment containing somatic efferent motor fibers (i.e. ventral roots) and the afferent sensory fibers (i.e. dorsal roots). The latter is distinguished by the presence of an oval swelling, the spinal ganglion, which contains numerous nerve cells. The location of the nerves in the spinal cord determines their function **(Figure 1)**: Cervical spinal nerves (C1 to C8) control signals to the back of the head, neck, shoulders, arms and the diaphragm. Thoracic spinal nerves (T1 to

T12) control signals to the chest muscles, some muscles of the back, and parts of the abdomen. Lumbar spinal nerves (L1 to L5) control signals to the lower parts of the abdomen and the back, the buttocks, some parts of the external genital organs, and parts of the leg. Sacral spinal nerves (S1 to S5) control signals to the thighs and lower parts of the legs, the feet, most of the external genital organs, and the area around the anus. The single coccygeal nerve carries sensory information from the skin of the lower back.

Like the brain, spinal cord is also protected by three membranes of connective tissue called meninges: the *pia mater*, firmly adhered to the spinal cord, *arachnoid mater*, the middle layer, and *dura mater*, closest to the vertebrae column. Between them and helping to protect the spinal cord, there are the subarachnoid space (between arachnoid and pia) filled with cerebrospinal fluid and the epidural space (between dura and periosteum) filled with loose fibrous and adipose connective tissues. Finally, in subarachnoid space, the spinal cord is supplied with blood by three arteries that run along its length starting in the brain (the anterior and right and left posterior spinal arteries), and many arteries that approach it through the sides of the spinal column. They form connections (anastamoses) which enter the spinal cord at various points along its length **(Figure 2).**

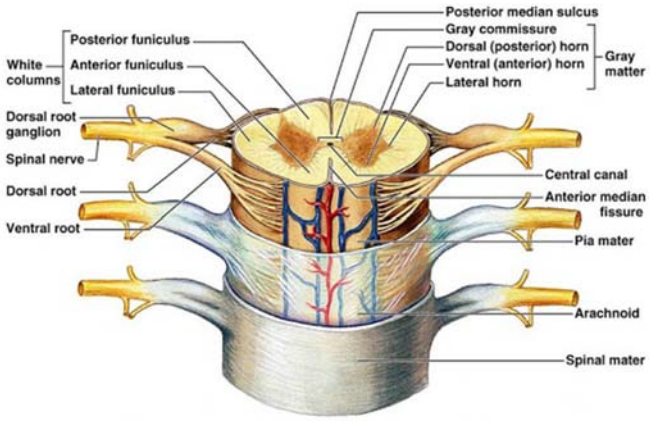

Fig 2 . Anatomy of the spinal cord. Extracted from Essential Essential Clinical Anatomy, Third Edition. ***Williams & Wilkins. p. 298.*** **(2006)**

Internal Structure of the Spinal Cord

A transverse section of the adult spinal cord shows white matter in the periphery, gray matter inside, and a tiny central canal, called ependymal canal, filled with cerebrospinal fluid (CSF) at its center.

Organization of the gray matter

The gray matter of the spinal cord is located centrally, shaped like the letter "H" or a "butterfly". This gray matter is composed by neuronal cell bodies, dendrites, axons, glial cells

and blood vessels. Depending on its H-shape, the grey matter can be divided macroscopically into dorsal and ventral horns, which correlates with the projecting arms of the H respectively, while the intermediate region of the H is called intermediate gray matter, which encloses the central canal. The grey matter at thoracic and upper lumbar segments has a small lateral projection known as intermediolateral horn, which contains the cells of origin of the autonomic nervous system. The shape and size of the gray matter varies according to spinal cord level. There are `limb´ enlargements (cervical and lumbar) where gray matter is enlarged due to the nerves of the limbs (brachial and lumbosacral plexuses). Furthermore, at the lower levels, the ratio between gray matter and white matter is greater than in higher levels, mainly because lower levels contain less ascending and descending nerve fibers.

Although the morphology of the spinal gray matter is very heterogeneous, it shows an intrinsic organization which permit to divide it in ten different layers called the Rexed laminae (Rexed, 1952). This division also coincides with a functional division as each layer has a different function. These different layers are organized from dorsal to ventral except for the tenth lamina that is composed by the cells surrounding the central (Anderson C, 2009) **(Figure 3)**. In this division, laminas I to IV are concerned with exteroceptive sensation, whereas lamina V and VI are concerned primarily with proprioceptive sensation. Lamina VII contains interneurons that communicate the dorsal and the ventral horns and mainly act as relay points in the transmission of visceral information. It is also involved in the regulation of posture and movement. Lamina VIII and IX form the final motor pathway to initiate and modulate motor activity. Finally, lamina X surrounds the central canal and this neurons project to the contralateral side of the spinal cord.

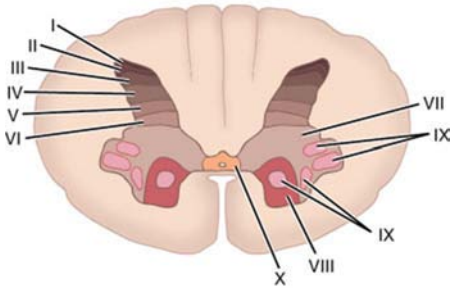

Fig. 3 Rexed laminae in a cross-section of the spinal cord at approximately the level of the seventh cervical vertebra .....

Organization of the white matter

Surrounding the grey matter area there is a region called "white matter." It contains glial cells and axons that are (most of them) covered with an insulating substance called myelin.

The white matter can be divided depending on its location, its origin or its function. By location criteria a large group of axons that are located in a given area is called funiculus and is due to the horns of the gray matter that white matter is divided into dorsal, lateral and ventral funiculus. Meanwhile those nerve fibers with the same origin, course and termination are called tracts. Finally, a group of tracts with a related function is known as a pathway.

Tract system connects the spinal cord to other parts of the CNS carrying sensory information

up (ascending tracts) sending signals from internal organs and external signals from the skin and extremities to the brain, or motor information down (descending tracts) sending signals from the brain to control voluntary and involuntary muscles through the spinal cord. The rest of the fibers are intrinsic pathways (propriospinal fibers) connecting different spinal cord segments between them.

The ascending tracts arise from primary neurons with soma are in the dorsal root ganglia or from interneurons in the dorsal horn, projects their axons to the brainstem, and transmit information concerning the body's interaction with the external and internal environment. Ascending tracts can be classified according to the functional components that they carry, as well as their anatomical localization. By functional classification are the general somatic afferent system (GSA), which transmit information from somatic structures such as pain, position sense, temperature and touch; and the general visceral afferent system (GVA), which transmits pressure, pain and visceral information from internal organs. Anatomically, the ascending spinal projections are located in the ventrolateral and dorsal funiculi on each side of the Spinal Cord **(Figure 4)**. In the ventrolateral funiculus ascend the anterolateral system (ALS) containing three major ascending tracts: spinothalamic, spinoreticular and Spinocerebellar. The Spinothalamic and spinoreticular tracts are involved in pain and temperature sensation, as well as non-discriminative touch, pressure and proprioceptive sensation. Finally, spinocerebellar tract transmits information for coordinated movement and posture but also some pain and pressure. Other ventrolateral ascending projections are the spinomesencephalic, spinotectal, spinoolivary and spinohypothalamic tract. In the dorsal funiculus ascend the dorsal column-medial lemniscal pathway (DCML) that includes the gracilis and the cuneatus tracts and transmit information related to tactile sense, vibratory sense and position sense.

Motor information travels from the brain down the spinal cord via **descending tracts (Figure 4).** There are two general descending systems, the lateral system and the medial system. The lateral descending system contains the corticospinal and rubrospinal tracts and influences lateral musculature and is focused on controlling fine movements of the distal parts of the limbs. On the other way, the medial descending system contains mainly the vestibulospinal and reticulospinal tracts and travels in the ventromedial part of the spinal white matter and influences motoneurons in the medial part of the spinal gray matter. The reticulospinal tract is involved in preparatory and movement-related activities, postural control and modulation of some sensory and autonomic functions. Vestibulospinal tract is involved in postural balance and locomotion, contracts and relaxes muscles to maintain postural stability when the head moves.

Finally, **<u>propriospinal tracts</u>** (fasciculi proprii) are intersegmental ascending, descending, crossed and uncrossed tracts that arise from neurons in the dorsal and ventral horns, travel in the white matter immediately external to the grey matter, and link nearby and distal segments of the spinal cord to integrate local reflex into coordinated body movement and visceral function.

The tract organization of the spinal cord is really conserved between species. Only a few differences are found between different species ranging from rodents, cats, primates and humans. One of the major differences is the location of the corticospinal which is originated from a number of cortical areas but while in humans the major bundle of nerves is placed in the lateral and ventral funiculus, rodents presents an important dorsal component of the corticospinal tract (Armand, 1982).

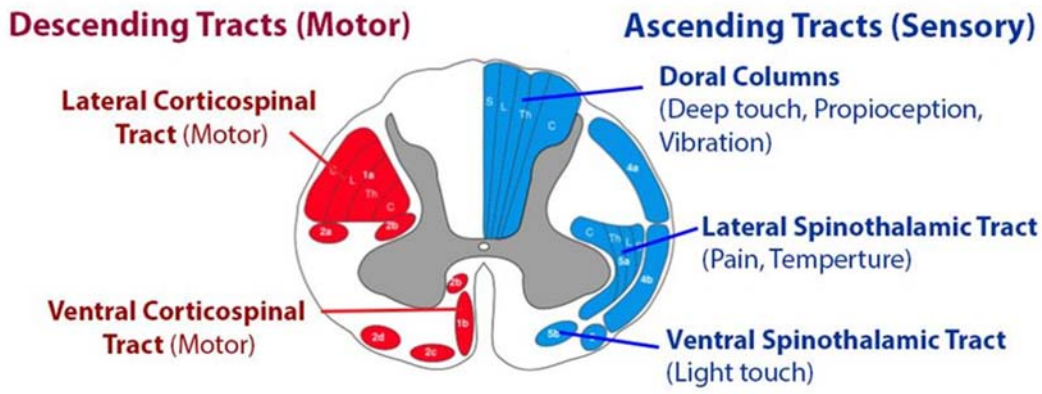

Fig 4. Diagram of the organization of the white matter of the spinal cord in humans.

2- <u>SPINAL CORD INJURY</u>

Spìnal cord injury (SCI) is defined by Kirshblum et al, (Kirshblum et al., 2011) as an impairment or loss of motor and/or sensory function due to damage to the neural elements within the spinal canal caused by an external force. Although the spinal cord is well protected by the bones of the spinal column, it can be damaged being trauma the most common cause and less frequently disease or infection. Any of these spinal cord injuries result in a disruption of the pathways that carry information between brain and body, leaving patients with deficits of motor, sensory, sexual and sphincteric function.

SCI is known in medicine for many centuries. The oldest known trauma textbook in history, the Edwin Smith papyrus (1700 BCE), described spinal cord injury (SCI) as a ''medical condition that cannot be healed" (van Middendorp et al., 2010). Now, more than 3700 years later, despite enormous progress in clinical medicine and preclinical research, damage from SCI is largely irreversible due to mainly that the CNS axons of adult mammals do not regenerate following lesion. This results in a dramatic functional loss below the site of injury that with the appearance of secondary complications as paralysis, sensory loss, pain, pressure sores, urinary and other infections, seriously diminish the quality of life for SCI patients (Rossignol et al., 2007).

I. Etiology and epidemiology of the spinal cord injury

SCI is one of the most prevalent and disabling conditions in the world. Although reliable information on the epidemiology for traumatic SCI is unavailable for many countries, it is clear that incidence, prevalence, and injury etiology vary considerably from region to region (Burns and O'Connell, 2012), depending, for example, on geographical and cultural differences, population characteristics, inclusion criteria and differences in data collection (Ackery et al., 2004). Worldwide, about 2.5 million people suffer from SCI, with more than 130,000 new cases reported each year (Rossignol et al., 2007). Incidence of traumatic SCI varies between 13.1 and 52.2 cases per million inhabitants in developed countries, and between 12.7 and 29.7 in developing countries (Chiu et al., 2010). In the US, the National Spinal Cord Injury Statistical Center (NSCISC) has reported the annual incidence of TSCI to be approximately 40 cases per million, whereas the number of people who currently suffer from SCI is estimated to be around 253,000, with 11,000-12,000 new cases occurring every year (Rosner et al., 2012, Silva et al., 2014). Importantly, this amount does not include injuries that result in death prior to hospitalization and therefore the incidence of SCI is underestimated (Rosner et al., 2012). According to B. B. Lee et al. (Lee et al., 2014), the median value for the incidence of SCI in Western Europe is 16 cases per million. The highest incidence of SCI in Europe is in Estonia with 39.7 per million (Sabre et al., 2012), followed by Romania, France and Spain. In Spain it is calculated that around 25.000 people are living with a SCI with 800-1000 new cases every year and an annual incidence of 23.5 per million (Pérez et al., 2012).

Among all types of SCI, traumatism is the most frequent (70-80%), and therefore the most studied. Motor vehicle accidents are the main cause (46%), followed by falls (18%), that is the most common cause in people over 60 years old, violence (17%) and sports (13%). The non-traumatic SCI have also an important impact in Spain (20-30%) and the main causes are tumors

(40%), infections (30%) and vascular accidents (20%), among others (Mazaira et al., 1998).

About demographic characteristics of SCI, the age of peak incidence is between 15-30 years of age or over 70 years and incidence is greater in males than in females (4:1 ratio of male: female) (Chen et al., 2013). This young onset of lifelong invalidation in addition of higher life expectancy in SCI patients results in particularly high personal and economic costs (Rosner et al., 2012).

II. Level and severity of the spinal cord injury

When the spinal cord is damaged, all its functions can be compromised below the lesion site, resulting in total or partial loss of movement (tetraplegia or paraplegia), sensation (anesthesia or hypoesthesia), autonomic deficits (sexual dysfunction, loss of control of the sphincters, etc.) and pain. The nature and extent of spinal cord injuries vary widely, depending on the level of the injury (cervical, thoracic, lumbar or sacral) as well as its severity (partial or complete) or the type of the injury (contusion, compression, transection, etc.) and will dictate its functional impact and prognosis.

In general, neurologists define the level of injury as the first spinal segmental level that shows abnormal neurological loss **(Figure 5). <u>Depending on the level of the injury</u>**, SCI can be differentiated in cervical, thoracic and lumbosacral injuries. C**ervical injuries** are the most frequent and severe injuries leading with tetraplegia but depending on the specific location, limited function may be retained. A patient with **high-cervical injury (C1-C4)** may not able to breathe on his own, cough or control bladder and bowel movements. Even speaking may be impaired or reduced. While if a patient presents an injury located at **low cervical level (C5-C8)**, breathing and speaking may not be affected, and allows a limited use of arms and limited wrist control but a complete hand function. **Thoracic injuries** result in paraplegia. Injuries in **high segments (T1-T8)**, although arm and hand is usually normal, lesions at this site result in the inability to control abdominal muscles and trunk stability is affected and lesions above T6 level can result in autonomic disreflexia. On the other hand, injuries at

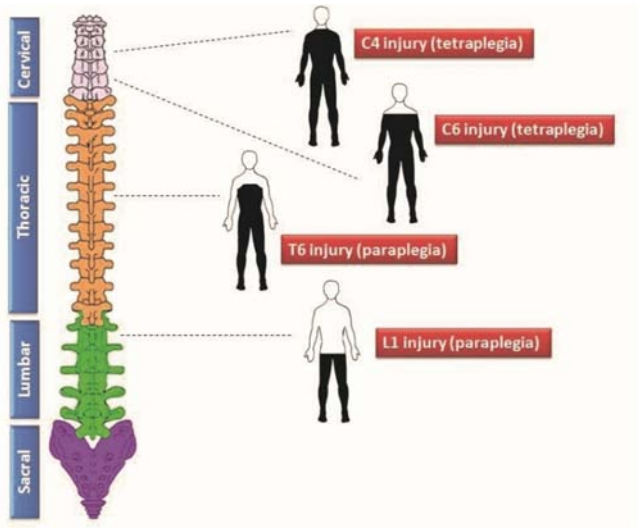

Figure 5. **Extent of injury due to damage of specific spinal segments (modified from Thuret et al., 2006).**

thoracic low levels (T9-T12) there is a fair to good ability to control and balance trunk while in the seated position and limited or absent control of bowel or bladder function. People with T1-T12 paraplegia have nerve sensation and function of all their upper extremities. They can become functionally independent, feeding and grooming themselves and cooking and doing light housework. These patients can transfer independently and manage bladder and bowel function. Finally, people with this type of injury can handle a wheelchair quite well and drive especially adaptive vehicles. The effects of **injuries at the lumbosacral** regions are decreased control of the legs and hips, urinary system, and anus. Remains little or no voluntary control of bowel or bladder function and sexual function is associated with the sacral spinal segments is often affected after injury. People with sacral or lumbar paraplegia can be functionally independent in all of their self-care and mobility needs. They can learn to skillfully handle a manual wheelchair and can drive specially equipped vehicles.

In addition to the level of the lesion, functional deficits observed after SCI depend on whether the **lesion is complete or incomplete**. Incomplete injuries are characterized by the remaining of some sensory and motor function due to the percentage of motor and sensory fibers preserved. Complete injuries leads with a total loss of function and the reflexes below the injury.

Much confusion surrounds the terminology associated with spinal cord injury levels, severity, and classification. In an effort to systematize the classification of spinal cord injuries, in 1992, the American Spinal Injury Association (ASIA) developed a uniform way to classify injuries according to the level and extend; The ASIA International Standards for Neurological Classification of the Spinal Cord Injury. This is based on the examination of neurological function to assess on a scale of 5 points (ASIA A to D) any improvement or deterioration throughout the course of the injury (Harvey and Graves, 2011, Kirshblum et al., 2014).

ASIA A indicates a “complete” spinal cord injury where no motor or sensory function is preserved in the sacral segments S4-S5. **ASIA B** indicates an “incomplete” spinal cord injury where sensory but not motor function is preserved below the neurological level and includes the sacral segments S4-S5.

ASIA levels A and B classification depend entirely on a single observation but for levels C and D the usefulness of lower limb function was added as quantitative criteria. This criteria but, ignored the arm and hand function in patients with cervical injury. To get around this problem was stipulated that a patient would be an ASIA C if more than half of the muscles evaluated had a grade of less than 3, which indicates active movements with full range of motion against gravity. If not, the person was assigned to level D.

ASIA C indicates an "incomplete" spinal cord injury where motor function is preserved below the neurological level, and more than half of key muscles below the neurological level have a muscle grade less than 3. **ASIA D** also indicates "incomplete" lesion where motor function is preserved below the neurological level, and at least half of key muscles below the neurological level has muscle grade of three or more. Finally, **ASIA E** indicates that motor and sensory functions are normal but even so it is possible to have a spinal cord injury with neurological deficits.

Finally is important to say that, the spinal cord normally goes into what is called spinal shock after it has been damaged, a transitory state in which all spinal functions and reflexes are abolished. As a consequence, the true extent of many incomplete injuries is not fully known until 6-8 weeks post injury. Someone who is completely paralyzed at the time of injury may get a partial recovery after spinal shock has subsided.

III. Pathophysiology of the spinal cord injury

Although tumor growth or other disorders such as infection can lead to SCI, the mechanical damage is the most common form of acute SCI in humans (Silva et al., 2014). These mechanical traumas promote a neurological damage known as "primary injury". Mechanical injury leads to a cascade of biological events described as "secondary injury", which occurs over the time course of minutes to weeks and leads to further neurological damage. Finally, there is the onset of a chronic phase, which can occur days to years after the injury, leading to neurological impairments in both orthograde and retrograde directions, including brain regions (Cramer et al., 2005, Yiu and He, 2006).

Primary injury

Primary damage is defined as the immediate effect of an injury to the spinal cord. Regardless of the cause (in human injuries usually is a contusion injury that implies the fracture of bones and disk displacement within the spinal canal compressing the spinal cord), the initial impact leads to disrupt of axons, myelin leading and a rapid cell necrosis in the immediate vicinity of the injury site. Furthermore, the death of endothelial cells and disruption of local blood vessels promoted by that mechanical trauma, results in an intraparenchymal hemorrhage, brain spinal barrier (BSB) dysfunction, and edema **(Figure 6).** As mentioned above, the initial trauma causes a transitory state called spinal shock in which all the spinal functions and reflexes are abolished due to the transient disruption of ionic homeostasis that provokes the interruption of the

generation of action potentials.

This primary injury is an uncontrollable and unpredictable injury since it depends on the trauma itself. Due to unexpectedness and its immediate effects on tissue damage, this cannot be prevented or treated (Kwon et al., 2004, Fehlings and Nguyen, 2010, Oyinbo, 2011).

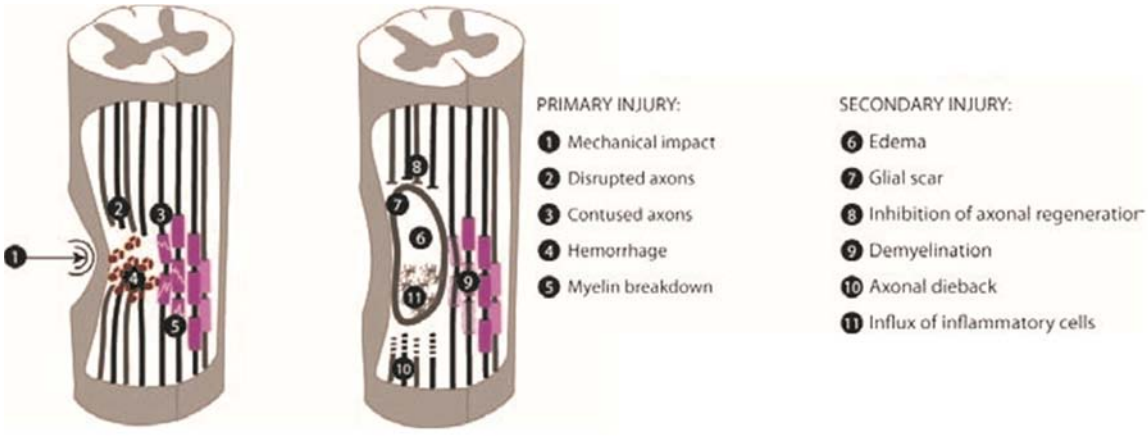

Fig 6: Pathophysiology of the spinal cord injury

Secondary injury

From hours to days after the initial trauma, a wide spectrum of secondary cellular and molecular events occur increase tissue damage, a process known as the secondary injury **(Figure 6)**. In this phase, the lesion is not restricted to the injury site itself but expands rostral and caudally to the lesion, increasing neuronal and glial cell death as well as axonal disruption, and consequently, aggravating neurological impairments (Oyinbo, 2011). Secondary injury is in most of the cases the major cause of tissue damage and functional deficits. Nowadays, more than 25 different mechanisms that contribute to secondary lesion have been described (Oyinbo, 2011). Importantly, these processes are interrelated and often positively influence one another to promote secondary damage. Finally, unlike the primary injury, the secondary injury events can be prevented, representing an important target in developing therapeutic strategies for treatment of SCI. Some of the most significant secondary mechanisms will be discussed below.

Blood flow changes and ischemia

Blood flow is intricately related to function in the spinal cord. Acute SCI results in rapid and permanent changes to the structure and function of the spinal cord blood supply. Local

changes at the injury site include hemorrhage and vasospasm of the superficial vessels that results in improper blood perfusion, and consequently, the blood flow in the spinal cord is impaired (Tator and Fehlings, 1991, Mautes et al., 2000, Figley et al., 2014).

Hemorrhage is initially localized in the highly vascularized central gray matter. Blood damages the nervous tissue due to the high levels of iron, which can generate radical oxygen species (ROS), and damage cell membranes and DNA (Sadrzadeh et al., 1987). In addition, recent studies also reveals that accumulation of iron in macrophages, due to phagocytosis of red blood cells, leads to cytotoxic activation of macrophages (Kroner et al., 2014). Damage of blood vessels also leads to accumulation of interstitial fluid and lack of drainage of promotes additional compression of nervous tissue (Oudega, 2012). Along with the hemorrhage, the intravascular thrombosis and systemic responses resulting from the loss of autoregulation, due to posttraumatic hypotension or decreased cardiac output (Tator and Fehlings, 1991, Mautes et al., 2000), result in a ischemic damage by hypoperfusion and hypoxia (Nelson et al., 1977, Armand, 1982). Ischemia, in turn, leads to a sharp decrease in oxygen (hypoxia) and glucose required for cell metabolism. Hypoxia increases the anabolic metabolism, leading to the production of acidic products such as lactic acid. The acidification of the environment would alter ATP production, stimulate pathologic ROS formation, and inhibit astrocytic glutamate uptake, contributing to excitatory neuronal damage (Chu and Xiong, 2013). Although this situation is transitory, the restoration of blood supply and return of oxygen to ischemic tissues causes an increase in free radicals and ROS, contributing to additional tissue damage known as reperfusion injury (Oudega, 2012).

Finally, another feature of vascular disturbances in the secondary injury phase is the breakdown of the BSCB. permeability that typically peaks at 24 hours following injury and lasts at least for 2-4 weeks and leads to the influx of leukocytes which in their own way contribute to enhance vascular permeability and secondary damage (McTigue et al., 2000, Profyris et al., 2004, Donnelly and Popovich, 2008)

Excitotoxicity and ionic imbalance

After injury the disruption of membranes results in an alteration of the ion equilibrium and an increase of glutamate into the extracellular space to neurotoxic levels (Mazaira et al., 1998). The increased extracellular glutamate levels lead to excessive stimulation of glutamate receptors, such as N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-isoxazolepropionate (AMPA)/kainate receptors that provokes persistent neuronal depolarization, which leads to neuronal death (Doble, 1999, Ondarza et al., 2003). Furthermore, excessive activation of glutamate receptors leads the influx of Na^{+} and Ca^{2+}

through the NMDA and AMPA/kainate channels producing osmotic cell lysis, as well as the consequent release of cell contents into the extracellular medium. Other factors that contributes to calcium overload after SCI are free radicals and oxidative stress (Xiong et al., 2007) resulting in inhibition of two enzymes extremely sensitive to free radical damage, Ca2+-ATPase and Na^+/K^+-ATPase. These enzymes are responsible for regulation of ionic homeostasis (Rohn et al., 1996). On the other hand, the rise in intracellular calcium causes the activation of proteases such as calpains, resulting in degradation of cytoskeletal proteins. In addition, high levels of intracellular calcium activate caspases and phospholipases, cause mitochondrial dysfunction and increased generation of ROS, ultimately leading to apoptotic death of the cells (Kwon et al., 2004).

Oxidative stress and lipid peroxidation

Compared to other organs, the CNS is particularly susceptible to oxidative stress and free radical damage due to its active oxygen metabolism and low anti-oxidant capacity (Lebel and Bondy, 1991, Andersen, 2004) (Li et al., 2013). Generation of ROS under physiological conditions is important for normal cellular redox reactions and sterilization, but after SCI there is excessive production of ROS, which causes oxidative stress and secondary cell death. There are several cellular events that results in ROS formation. As previously mentioned , re-exposure of endothelial cells to oxygen during reperfusion leads to an enzymatic reaction that gives rise to ROS formation (Basu et al., 2001). On the other hand, activation of glutamate receptors also results in increased intracellular calcium, which activates calcium-dependent phospholipases that leads to the formation of arachidonic acid and its subsequent metabolism in the cyclooxygenase pathway, producing prostaglandins and ROS (Hausmann, 2003). All these highly oxidizing compounds can induce damage to cells by oxidation of the lipids and proteins present in the plasma membranes and DNA, which contribute altogether to cell membrane disruption, cell death and generation of more ROS in a positive feedback loop (Profyris et al., 2004).

Cell Death, Apoptosis and Loss of Oligodendrocytes

Both necrotic and apoptotic events contribute to cell death after SCI. Primary injury results in initial necrosis affecting both, the grey and white matter, and induces swelling of neuronal bodies and glial cells, disruption of organelles and release of the intracellular contents through the ruptured membrane (Hausmann, 2003, Kwon et al., 2004, Profyris et al., 2004). On the other hand, programmed cell death known as apoptosis also occurs in the lesion core, preferably in the grey matter, over the first hours and days after injury and accompanies

necrosis in damaging multiple types of cells. By the end of the first week, the level of apoptosis in grey matter decreases, and a second wave of apoptotic cell death is predominant conducted in the white matter, affecting mainly to oligodendrocytes. This lasts for several weeks leading to persistent demyelination (Profyris et al., 2004, Mekhail et al., 2012). The loss of myelin sheaths results in impairment of axonal transmission and over time may lead to degeneration of demyelinated axons (Irvine and Blakemore, 2008).

Neuroinflammation

The Inflammatory response that occurs after SCI is probably the most important event that contributes to secondary injury. Inflammatory response is an essential aspect of the injury response to restore homeostasis of the tissue after injury or infection, as well as, to initiate wound healing. This inflammatory response must be tightly regulated and terminated when is no longer needed, otherwise, it may result in chronic inflammation and cellular destruction, as it occurs after SCI (David and Kroner, 2011). The damaging effects of inflammation have especially consequences in CNS due to the limited capacity of axons to regenerate and replace the damaged neurons and glial cells. Further details on the neuroinflammatory response in SCI will be discussed in following sections.

Failure of axon regeneration after SCI

Unlike peripheral nervous system (PNS), CNS of adult mammals has limited abilities for spontaneous self-repair. Many studies from the last decades have identified a number of important **environmental factors that inhibits axonal growth**. Early studies revealed that cultured sympathetic ganglion neurons extend neurites on PNS myelin but not on CNS myelin (Schwab and Thoenen, 1985). Characterization of CNS myelin led to identification of several components that exert potent inhibitory action on neurite outgrowth, such as Nogo, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMpg) (David and Lacroix, 2003, Fawcett et al., 2012). Extensive biochemical and cell culture studies have led to the current working model where the three inhibitors signal through multiple neuronal receptors including the shared ligand-binding receptors NgR1 and PirB as well as coreceptors such as p75NTR, TROY, and LINGO-1, with downstream effectors such as Rho and Rho-associated kinase (ROCK) (Thiede-Stan and Schwab, 2015). However, the role of Nogo, MAG, and OMgp in axon regeneration *in vivo* needs further studies, since gene deletion or pharmacology blockage of such inhibitors or their receptors has led to contradictory results (Kim et al., 2003, Zheng et al., 2003, Kim et al., 2004, Lee et al., 2009). In addition to these myelin components, repulsive guidance cues with roles in axon pathfinding during

development, such as ephrin B3 and Sema4D/CD100, are also present in CNS myelin and implicated in the failure of axonal repair in the adult (Thiede-Stan and Schwab, 2015).

In addition to degenerating myelin, another important source for the inhibition of axonal outgrowth is the glial scar. This is formed mainly by reactive astrocytes and meningeal cells that migrate to the lesion core. These cells, mostly hyperthophic astrocytes, release inhibitory extracellular matrix molecules known as chondroitin sulphate proteoglycans (CSPGs) (Galtrey and Fawcett, 2007, Fawcett et al., 2012). CSPGs (aggrecan, brevican, neurocan, versican, phosphacan and NG2) are a family of molecules characterized by a protein core to which large, highly sulphated glycosaminoglycan (GAG) chains are attached (Galtrey and Fawcett, 2007, Fawcett et al., 2012). CSPGs induce axonal retraction by signaling via receptor tyrosine phosphatase (RTPTσ), and activating the same downstream effectors than myelin inhibitors do, Rho and ROCK (Shen et al., 2009).

In addition to the CNS inhibitory environment, neurons show differential regenerative responses after PNS and CNS injury. In the search for differential neuronal cell programs after PNS and CNS lesions, studies from Dr He's laboratory have recently revealed that mTOR (Liu et al., 2010) and SOCS3 (Smith et al., 2009) are minimally activated in CNS neurons after axotomy. Interestingly, genetic approaches aimed at inducing the activation of mTOR or SOCS3 expression in CNS neurons have provided clear evidence on their key importance for successful axonal regeneration in models of CNS injury (Smith et al., 2009, Liu et al., 2010). Interestingly, activation of both molecular effectors in RGN have also showed additive effect on axonal regrowth after optic nerve injury (Sun et al., 2011, Jin et al., 2015). Future studies will likely target the environmental inhibitors and neuronal intrinsic factors in order to better induce axonal regrowth.

3- INFLAMMATORY RESPONSE AFTER SCI

I. Overview of the Immune System

The immune system is a system of many biological structures and processes within an organism that protects the body from diseases and infections. The immune system is tasked with three distinct and interrelated duties: i) Defense of the body from external invaders (pathogens and toxins); ii) Surveillance in identifying the body's cells, those have mutated and may become or have already become neoplasms (tumors); iii) Maintenance of tissue

homeostasis by removing cellular detritus to ensure uniformity of cells and function. In all three, the essential requirement for the immune system is the ability is to distinguish between what is self and what is foreign (non-self).

Immunity is divided into two subsystems based on the speed and specificity of this reaction: innate and adaptive immunity. However, the components that make up these divisions have overlapping roles and is impossible to describe one part of the immune system without using terms that belong in another part.

Innate immunity refers to nonspecific defense mechanisms (Janeway, 2001) that come into play immediately or within hours of an antigen's appearance in the body. Innate response does not require prior exposure to be effective (Parkin and Cohen, 2001). Before innate immunity becomes activated, pathogens have to cross mechanical and chemical barriers that keep harmful materials from entering your body. Some examples are the skin, saliva, stomach acids, mucus or cough reflex. Once microorganisms get through these barriers, innate immunity is activated mainly by pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) receptors, which recognize bacterial structures and molecules that indicate infection or cellular damage. The main functions of innate immunity are the identification and removal of foreign substances present in the body and the activation of the adaptive immune system through a process known as antigen presentation.

The most important innate immunity cells are the polymorphonuclear leukocytes (PMN), monocytes and dendritic cells. PMNs are crucial for the removal of bacteria and parasites from the body. Among the different PMN subsets, neutrophils are the most important and numerous, they engulf the foreign bodies and degrade them using lytic enzymes. Monocytes are rapidly recruited to tissues upon damage or infection, where they phagocytose the invaders and destroy them. Monocytes differentiate into macrophages or dendritic cells (DC) when infiltrate into tissues. These cells can activate the adaptive immune system acting like an antigen-presenting cell (APC) by processing large molecules into "readable" fragments (antigens) recognized by adaptive B or T cells. In the CNS, microglial cells play a similar role than host tissue macrophages and DC.

Innate immune cells are supported by soluble factors such as cytokines, opsonins and proteins of the complement cascade. Cytokines that includes several members of the colony-stimulating factor (CSF), interferon (IFN), interleukin (IL), chemokine, and tumor necrosis factor (TNF) family, are small proteins mainly secreted by leukocytes that affect the behavior of cells and modulates the immune system (Dinarello, 2007). Opsonins are molecules involved in the coating of pathogens or damage cells in order to be recognized and destroyed by the immune system (Parham, 2009). Finally, the complement system is a cascade of several lytic proteins

that aids pathogen destruction by direct lysis or by promoting phagocytosis (Walport 2001b, a).

Innate immunity is therefore a quick and effective reaction to control and to eradicate the infections. However many pathogens resist innate immunity and require a more powerful and specialized mechanisms to be removed, the adaptive immunity.

Adaptive immunity is a subsystem of the overall immune system that refers to antigen-specific immune response by lymphocytes (T and B cells). Whereas the innate response is rapid but sometimes damages normal tissues through lack of specificity, the adaptive response is more complex and precise response, but takes several days or weeks to develop as it relies on the coordination and expansion of specific adaptive immune cells. Adaptive immunity also leads to "memory" mechanisms, which makes future responses against a specific antigen to be more efficient.

There are two types of adaptive immune responses: humoral immunity, mediated by antibodies produced by B lymphocytes, and cell-mediated immunity, mediated by T lymphocytes.

B lymphocytes produce antibodies in response to foreign proteins of bacteria, viruses, and tumor cells. Each foreign protein is recognized by a unique B-cell receptor (BCR). Upon this recognition, the selective B cell becomes activated (now Plasma B cells) and secretes large amounts of antibodies that coats the surface of a pathogen and serve three major roles: neutralization (makes the pathogen unable to bind and infect host cells), opsonization (antibody-bound pathogen serves as a red flag to alert immune cells like neutrophils and macrophages, to engulf and digest the pathogen), and complement activation (for directly destroying, or lysing, foreign elements). In addition to antibody production, B cells can also act as antigen-presenting cells (APC) to T cells.

On the other hand, T lymphocytes action is linked to cell-mediated immunity, which serves as a defense mechanism against intracellular and phagocytosed microbes, proteins or viruses. T lymphocytes can be distinguished from other lymphocytes, by the presence of a T-cell receptor (TCR) on the cell surface. This receptor recognizes antigens when displayed as peptides bound to self-major histocompatibility complex (MHC), and together the presence of co-estimulator expressed on APCs, activate the T lymphocytes. Once active, T cells mediate different functions depending on the T lymphocyte subtype. Some of them send chemical instructions (cytokines) to the rest of the immune system (T helper or CD4 lymphocytes). Other types of T-cells recognize and kill virus-infected cells or tumors, directly (cytotoxic or CD8 lymphocytes).

Inflammatory response

The inflammatory response is the immune system response that involves immune cells, blood vessels, and molecular mediators to establish a physical barrier that isolate the damaged area, to remove the injury stimulus (pathogens or the initial cause of cell injury) and, in later stages, to promote healing of any damaged tissue. This process often causes temporary discomfort, resulting in what physicians refer to as the cardinal signs of inflammation: pain, heat, redness, swelling, and loss of function (Libby, 2007). Depending on its duration, two different phases of inflammation can be distinguished: acute and chronic inflammation.

Acute inflammation is a short-term response that can develop in minutes to hours and last for days and usually results in healing. Acute inflammatory response initiates with the recognition of infection or damage by the detection of PAMPs or DAMPs by the pattern recognition receptors (PRRs) present on innate immune cells (i.e. TLRs and NLRs). After recognition of ligands, PRRs respond by triggering activation of inflammatory transcription factors such as NF-kB, AP1, CREB, c/EBP, and IRF that induce expression of several pro-inflammatory cytokines, such as interleukin-1-beta (IL-1β), IL-6, tumor necrosis factor-alpha (TNF-α), among others. Together with chemokines and various costimulatory molecules, these soluble proteins facilitate the recruitment of effector cells such as monocytes and neutrophils to the site of disturbance. In addition, mast cells and tissue-resident macrophages present in the insulted tissue facilitate this migration by releasing histamine, leukotrienes, and prostaglandins, which have rapid effects upon the vasculature, including vasodilation and increased vascular permeability. Once in lesioned/infected region, macrophages and neutrophils create a cytotoxic environment by releasing of ROS and proteases that destroy the source of inflammation. These events must be tightly regulated, otherwise, they may lead to tissue damage. In addition, macrophages and dendritic cells also act as antigen presenting cells (APCs), and thus, participate in the activation and recruitment of adaptive immune cells (B and T cells) to better resolve the infection or tissue injury. The ultimate phase of acute inflammatory is known as resolution. During resolution, the recruitment of neutrophil is impeded, and instead, the infiltration of non-phlogistic monocytes is enhanced. This monocyte subset clean tissue and cell debris, creating a favorable environment for tissue repair and functional restoration (Serhan and Savill, 2005). Usually, inflammatory response is terminated in a properly fashion manner. Under certain circumstances, resolution of inflammation is hampered, and immune cells remain for several weeks or months in the disturbed tissue, resulting in **chronic inflammation**.

II. Inflammatory response after SCI

Inflammatory response is probably the event that mostly contributes to secondary damage after SCI. Inflammation is established immediately after the trauma with the initial necrosis and BSCB disruption. The BSCB is a monolayer of endothelial cells that form a physical barrier that tightly regulates the passages of molecules between the CNS and the blood, and limits the entrance of immune cells and immune mediators into the CNS (Muldoon et al., 2013). SCI triggers breakdown of BSCB, facilitating the invasion of immune cells to the spinal cord parenchyma. Highlight that damage to blood vessels also leads to ischemia and the extravasation of red blood cells (RBCs) and plasma components, such as fibrinogen, which can influence the activation of endogenous glial cells (microglia and astrocytes).

In parallel, mechanical trauma causes direct damage to neurons, glia, vasculature, and meningeal cells, leading to necrotic and apoptotic cell death. An important consequence of necrosis is the loss of plasma membrane integrity, thereby, allowing the release of intracellular material to the extracellular milieu. Some of these intracellular factors are considered as DAMPs, also called alarmins (Bianchi, 2007), and thus, can activate the inflammatory response when they are present in the environment (Chen and Nuñez, 2010). DAMPs include an extremely diverse class of molecules such as proteins (e.g HMGB1, IL-1α, IL-33 or heat shock proteins) (Scaffidi et al., 2002, Quintana and Cohen, 2005, Schmitz et al., 2005, Eigenbrod et al., 2008) nucleic acids and nucleotide derivatives (e.g. mitochondrial DNA or ATP) (Zhang et al., 2010, Lukens and Kanneganti, 2014), and extracellular located DAMPs (e.g. hyaluronan, heparan sulphate and biglycan). These alarmins activate different PRRs (Desmet and Ishii, 2012) that induce intracellular signaling cascades, such as nuclear factor-κB (NF-κB), mitogen-activated protein kinase (MAPK), and type I interferon pathways, which result in the upregulation of pro-inflammatory cytokines, chemokines and other factors, such as cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (Paxinos) and matrix metalloproteinases (MMPs) (Heiman et al., 2014). The upregulation of all of these pro-inflammatory mediators also promotes the activation of endogenous glial cells and recruitment of immune cells from circulation.

Microglial activation

Microglial cells are the macrophage resident cells in the CNS. In contrast to this leukocyte subset, they are not derived from the bone morrow but from the yolk sac during development

(Ginhoux et al., 2010). Under normal conditions, microglia display numerous cytoplasmic processes that extend and retract within seconds or minutes to monitor the tissue for detection of pathogens or other perturbations, and remove cellular debris without undergoing significant changes in morphology. In contrast, in response to injury or infection, microglia become activated and show dramatic morphological transformation and changes in gene expression.

Resident microglia is the first cell type to respond to CNS injury within the first few minutes (Popovich and Hickey, 2001, Sroga et al., 2003), in part, due to the presence of a wide number of PRRs on their membranes. After injury to the spinal cord, microglia reorients and extends their fine processes rapidly towards the injury site, sealing off the lesion to prevent the spread of damage. This process happens within minutes to hours after lesion, and is mediated, in part, by ATP released by astrocytes near the lesion site (Davalos et al., 2005). Other molecules, including DAMPs, cytokines and chemokines, and active lipid mediators, such as prostaglandins or lysophosphatidic acid, also elicit microglial activation and process extension (David et al., 2012a, David et al., 2012b). During this early response, microglial cells show elongated morphology with their processes aligned along degenerating axons. However, over the following hours and days post-injury, microglia retract their processes and adopt amoeboid morphology. Recent studies provide some insights into the mechanisms underlying this conversion. Among them, NO and ATP levels seem to play a key role in regulating these morphological changes. (Dibaj et al., 2010). Once microglia becomes amoeboid, they undergo proliferation (Greenhalgh and David, 2014), and participate in the engulfment of myelin and cellular debris (Dibaj et al., 2010).

Microglia also contribute to increase inflammation by producing several inflammatory mediators (Bartholdi and Schwab, 1997). DAMPs released from dying cells, induce activation of NFκB pathway, in microglia, which in turns, induces the expression of pro-inflammatory molecules, including CCL2, CCL3, GM-CSF, M-CSF, G-CSF IL-1β and TNFα (Pineau and Lacroix, 2007). M-CSF play a key role in triggering proliferation of microglial cells (Perry and Holmes, 2014), and thus facilitates the expansion of these cells within the injured CNS. Indeed, microglial cells peaks in numbers by day 7 post-injury (David et al., 2012b). Other mediators secreted by microglia, such as CCL2 and IL-1β, stimulate endothelial cells to increase synthesis of adhesion molecules (e.g. intercellular adhesion molecule (ICAM)-1, P- and E-selectin) that enhances leukocyte and endothelial production of chemotactic factors, and thus, facilitating leukocyte recruitment from circulation (Ma et al., 2002, Kułdo et al., 2005). In addition, DAMPs also trigger the expression of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (Paxinos), matrix metalloproteinases (MMPs), and other mediators (Heiman et al., 2014). COX-

2 gives rise to a variety of bioactive lipids such as prostaglandins and leukotrienes that promote inflammatory responses (David et al., 2012a). Nitric oxide generated via iNOS, a part from oxidative damage to cells, induces vasodilation and vascular leakage, which favors the entrance of leukocytes to the CNS. Furthermore, MMPs also increase vascular permeability, enhance leukocyte infiltration, and increase the extent of secondary damage (Noble et al., 2002) releasing more DAMPs that trigger the invasion of peripheral leukocytes in a positive feedback loop..

Cytokine Production, and Neutrophil Recruitment

As reported above, activation of microglia, but also astrocytes, results cytokine and chemokine expression, molecules that have a crucial role in the infiltration of circulating leukocytes into the injured site.

Cytokine expression

After SCI, there is upregulation of pro-inflammatory cytokines that orchestrate the inflammatory response. TNF-a, IL-1b, and IL-6 are considered the most important pro-inflammatory cytokines **(Figure 7)**. TNFα mRNA expression increases rapidly after spinal cord injury in mice, reaching a peak at 1 hour and decreasing by 24 hours. This first peak is expressed mainly by microglia, astrocytes, oligodendrocytes, and neurons (Kroner et al., 2014). There is a second peak of expression at 2–4 weeks post-injury, being microglia/macrophages the cell source (Pineau and Lacroix, 2007). IL-1β also increases in the injured spinal cord early after trauma. The expression profile of IL-1β is similar to TNFα, showing two peaks of expression at identical time points (Pineau and Lacroix, 2007). Microglia are the first cell type to produce IL-1β although astrocytes and neutrophils are also sources of this cytokine during this early peak expression. Contrary, endothelial cells are the main producers of IL-1β at the second expression peak following SCI. Moreover, astrocytes and microglia are the major cellular sources of IL-6 and LIF after SCI (Gruol and Nelson, 1997, Pineau and Lacroix, 2007).

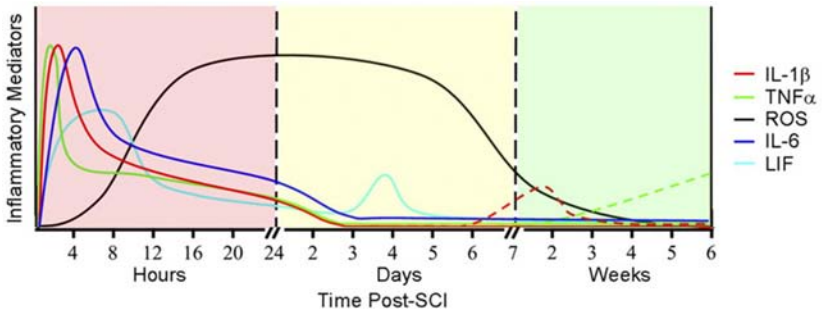

Fig 7. Expression of proinflammatory cytokines and reactive oxygen species (ROS) adapted from D.J. Donnelly, P.G. Popovich / Experimental Neurology 209 (2008)

Chemokines, together with cytokines, regulate the entrance of leukocytes to the CNS. Chemokines are small molecules of the group of cytokines that help to choreograph the transit of leukocytes out of the mainstream of blood and into tissues at sites of inflammation. They have been classified into four subfamilies CXC, CC, CX3C and XC, according to the presence and number of aminoacids between N-terminal cysteine residues. They signal through G-protein coupled to seven transmembrane receptors that are classified according to the chemokines they bind (CXCR, CCR, CX3CR and XCR). There is low but constitutive expression of chemokines in astrocytes, microglia, endothelial cells (Glabinski et al., 1996, Ma et al., 2002, Harkness et al., 2003) and neurons, which seem to play multiple pivotal roles in regulating homeostatic functions. However, after CNS injury, the presence of several inflammatory mediators, such as IL-1β, induce the production of chemokines and chemokine receptors, increasing the recruitment of leukocytes in the lesioned tissue.

Chemokines produce local vasodilation in the spinal cord, which leads to slow blood flow. They also induce the expression of adhesion molecules in endothelial cells, such as P- and E-selectin and various integrins, allowing the binding and diapedesis of circulating leukocytes **(Figure 8)** (Ley et al., 2007). Finally, chemokines cause leukocytes to move along a chemotactic gradient towards the source of inflammation. By this way, the recruitment of leukocytes is done sequentially, being neutrophils the first responders, followed by monocytes and other leukocytes such us eosinophils, basophils and lymphocytes.

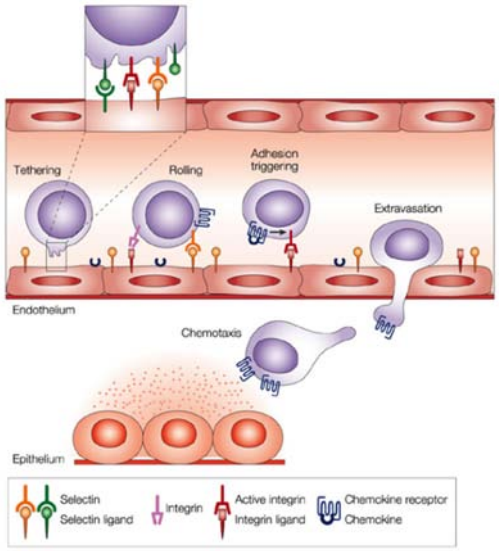

Fig 8. White cells rolling along the vascular endothelium and leaving the blood vessels. Adapted from Kunkel, Eric J. and Butcher, Eugene C. Nat Rev Immunol (2003)

Neutrophils

Neutrophils are the first peripheral responders after SCI, migrating rapidly into affected tissues reaching a peak at about 24 hours (Donnelly and Popovich, 2008). Once mobilized to a site of tissue damage, neutrophils participate in the communication networks, issuing instructions to practically all other immune cells and some non-immune cells.

Neutrophils can further increase the extent of the inflammatory response by producing pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 that promotes recruitment to other neutrophils via upregulation of endothelial adhesion factors and induce other cells to produce

neutrophil and other leukocytes (e.g. monocytes) chemoattractant. Neutrophils also release other signaling mediators, including chemokines, granule contents, reactive oxygen species (ROS), inducible nitric oxide synthase (Paxinos) and are a significant source of leukotrienes and prostaglandins, which enhances vasodilatation, vascular permeability, leukocytes attraction and cytokine production. Furthermore, neutrophils secrete MMP-9 that can degrade the collagen matrix of the BSCB and increase leukocyte infiltration.

All these reactions are essential to combat any invading pathogen (Amulic et al., 2012). Nevertheless, their impact on sterile injury (e.g. SCI) is less clear. Some studies report they exert harmful actions in SCI (Sewell et al., 2004, Gorio et al., 2007, Semple et al., 2010). However, depletion of neutrophils by administration of Gr1 antibodies resulted in worsened functional and histopathological outcomes after SCI, suggesting their beneficial actions in the pathology (Stirling et al., 2009).

Monocytes

Monocytes invade the injured spinal cord after neutrophil infiltration. They are divided into two subsets based on the expression of chemokine receptors and the presence of specific surface molecules (Shi and Pamer, 2011). In mice, high levels of Ly6C ($Ly6C^{high}$) identifies a monocyte subset that expresses high levels of CC chemokine receptor 2 (CCR2) but low amounts of CX3C-chemokine receptor 1 (CX3CR1). These monocytes are often referred to as "classical" or inflammatory monocytes. A second major subset of circulating monocytes in mice expresses high levels of CX3CR1 but low levels of CCR2 and Ly6C (Geissmann et al., 2003). This $Ly6C^{low}$ monocyte population is often referred as "non-classical" or resident monocytes. Similarly, human monocytes are divided into subsets based on CD14 and CD16 expression. $CD14^{+}$-$CD16^{-}$ monocytes, which are also referred as classical monocytes, are the most prevalent monocyte subset in human blood and, similarly to mouse $Ly6C^{high}$ monocytes, these cells express CCR2. Moreover, the $CD14^{+}$-$CD16^{high}$ subset, in terms of in vivo patrolling, is similar to mouse $Ly6C^{low}$ monocyte population (Ziegler-Heitbrock et al., 2010).

Recruitment of different monocyte subsets during non-CNS wound healing happens in a sequential manner mainly guided by different chemokines. Initially, $Ly6C^{high}$ monocytes are recruited to sites of tissue injury mainly by CCR2/CCL2 dependent manner, where they mediate acute inflammation (Nahrendorf et al., 2007, Crane et al., 2014). $Ly6C^{low}$ monocytes become dominant at resolution phases of inflammation, where they infiltrate into the tissue via CX3CR1-dependent manner. In contrast to $Ly6C^{high}$ monocytes, $Ly6C^{low}$ monocytes mediate tissue healing and repair (Nahrendorf et al., 2007, Crane et al., 2014). These two monocyte

populations are also present in the SCI, however their functional role in this pathology have not been fully characterized yet.

The infiltrated monocytes differentiate into activated macrophages in response to cytokines, chemokines and extracellular metabolites. These cells enter the spinal cord starting about day 2–3, reach maximum numbers by 7–10 days, and remain in the spinal cord for months in rodents (Kigerl et al., 2006) and even years in humans (Prüss et al., 2011). These monocyte-derived macrophages appear phenotypically similar to activated microglia. Both macrophages and microglia, possess many of the same myeloid markers including CD11b, Iba1, CSF1R and F4/80. In addition, activated microglia, adopt an amoeboid morphology very similar to macrophages, make them virtually indistinguishable in tissue section. Due to this phenotypic similarity after injury, many researchers have grouped these distinct cell types as macrophages/microglia. However, over the years, accumulating data demonstrated that these two cell types have some differences. One of these differences is that microglia and macrophages have distinct origins developmentally, arising from the primitive ectoderm of the yolk sac and from hematopoietic stem cells respectively (Ginhoux et al., 2010). In addition, despite of the similar surface markers, by FACS analysis is possible to distinguish these two cell types based on the expression of CD45. Microglia show $CD45^{low}$ expression while macrophages are $CD45^{high}$. A recent study using gene expression profiling, quantitative mass spectrometry and microRNA (miRNA) analysis has revealed differences in gene expression between these two cells types (Butovsky et al., 2014), which it suggest they may have different roles after injury (Ajami et al., 2011, Greenhalgh and David, 2014, Yamasaki et al., 2014). In fact, a mouse SCI study confirmed that microglia appear to be the first responders associated with phagocytosis of tissue debris acutely after injury while macrophages took over at later stages. In addition, macrophages and resident microglia differs in timing of proliferation and appears to process phagocytosed material differently, as tissue debris is cleared more efficiently in microglia than in macrophages *in vivo* (Greenhalgh and David, 2014). However, despite these significant advances in differentiation of both cell types, nowadays is very difficult to differentiate their own functions separately. By this reason, their functions and phenotype still are explained together.

Detrimental and beneficial effects of microglia/macrophages after SCI

Macrophages and microglia have been extensively studied in different models of nervous system disorders, but their different roles are still under debate showing beneficial and detrimental functions. A mentioned previously, these cells produce large amounts of pro-inflammatory cytokines, free radicals, extracellular matrix-degrading enzymes (e.g., MMPs) ,

several active lipid mediators, as well as glutamate, that exert damage to axons, neurons and glial cells. For these reason, microglia and macrophages are considered key contributors to secondary damage in SCI. Additionally, macrophages also impair axon regeneration via soluble factors and cell-axonal interaction (Horn et al., 2008, Evans et al., 2014) , contributing therefore, to impede the restoration of the injured spinal cord. This has led to develop various experimental approaches aimed to inhibit or deplete microglia/macrophages to minimize functional deficits in SCI. Counteracting macrophage responses during the first 1-2 weeks after SCI has consistently resulted in decreased tissue damage and improved functional recovery (Silver et al., 2015). These include use of anti-inflammatory drugs such as minocycline (Stirling et al., 2004) and FK506 (López-Vales et al., 2005) that inhibit macrophage activation, clodronate liposomes that systematically deplete monocytes and macrophages (Popovich et al., 1999), and mouse genetic manipulations such as myeloid-targeted deletion of the CD95-ligand which blocks macrophage infiltration (Letellier et al., 2010). Finally, there is also *in vivo* evidence that the area occupied by activated macrophages coincides with areas of axonal dieback, which is the retraction of the terminal cut ends of axons. Interestingly, clodronate-induced depletion of macrophages after SCI reduces axonal dying back (Horn et al., 2008).

Contrary, numerous studies reveals that macrophages also **contribute to tissue protection and axon regeneration** after CNS injury. Macrophages are very effective phagocytes that clean dead cells and myelin debris (Tofaris et al., 2002, Brosius Lutz and Barres, 2014). This is of crucial importance, since myelin contains several molecules that inhibits axonal growth (Silver et al., 2015). Indeed, axonal regeneration is significantly delayed after injury to the peripheral nerves when myelin phagocytosis is hampered (David et al., 2008, Martini et al., 2008). This may explain, in part, the failure of CNS axons to regenerate after injury, since myelin from the degenerating pathways is not efficiently cleared, and thus, inhibitory myelin-associate molecules remain in the CNS tissue for several month to years (Buss and Schwab, 2003, Buss et al., 2005), impeding axonal growth. Microglia and macrophage play also important roles in remyelination. Microglia, for instance, trigger oligodendrocyte maturation via mechanisms that include the release of IGF-I (Butovsky et al., 2006). In addition, studies reveal that counts of oligodendrocyte progenitor cells (OPCs) are significantly reduced when systemic monocytes are depleted after SCI, which results in impaired remyelination (Kotter et al., 2005). Highlight, that these immune cells also secrete growth-promoting molecules, such as CNTF, BDNF, NGF, NT-3, PDGF and oncomodulin after CNS injury (Dougherty et al., 2000, Donnelly and Popovich, 2008) that exert neuroprotective functions and can potentially support neural survival and sprouting of spared axons after SCI. These cells can release several anti-inflammatory cytokines such as TGFβ and IL-10. TGFβ1 acts as immunosuppressant and limits

oligodendrocyte toxicity (Merrill and Zimmerman, 1991), whereas IL-10 dampens and promotes resolution of inflammation (Shechter et al., 2009).

This double-edge sword of macrophages and microglia following SCI probably depends on the activation phenotype they acquire in the injured tissue, which depends on different factors present in the spinal cord milieu. This will be discussed in the following section.

Macrophage phenotype

Macrophages in non-neuronal tissues have remarkable plasticity that allows them to efficiently respond to environmental signals and change their phenotype and function following pathogen exposure or tissue damage (Gordon, 2003, Sica and Mantovani, 2012). This process is known as macrophage polarization, and enables the adaptive responses of innate immunity to take place. Two distinct macrophage polarization states have been described, mirroring the Th1/Th2 polarization concept of T cell activation, termed M1 also called pro-inflammatory or "classically" activated macrophages and M2 also known as anti-inflammatory or "alternative", which represent both ends of a spectrum of functional macrophage activation (Gordon, 2003, Sica and Mantovani, 2012). The concept of a pro-inflammatory/M1-like phenotype and an anti-inflammatory/M2-like phenotype were established using *in vitro* model systems, however macrophages do not display this phenotype dichotomy *in vivo* but multiple intermediate states. However, this classification is a useful tool to probe for responses to injury.

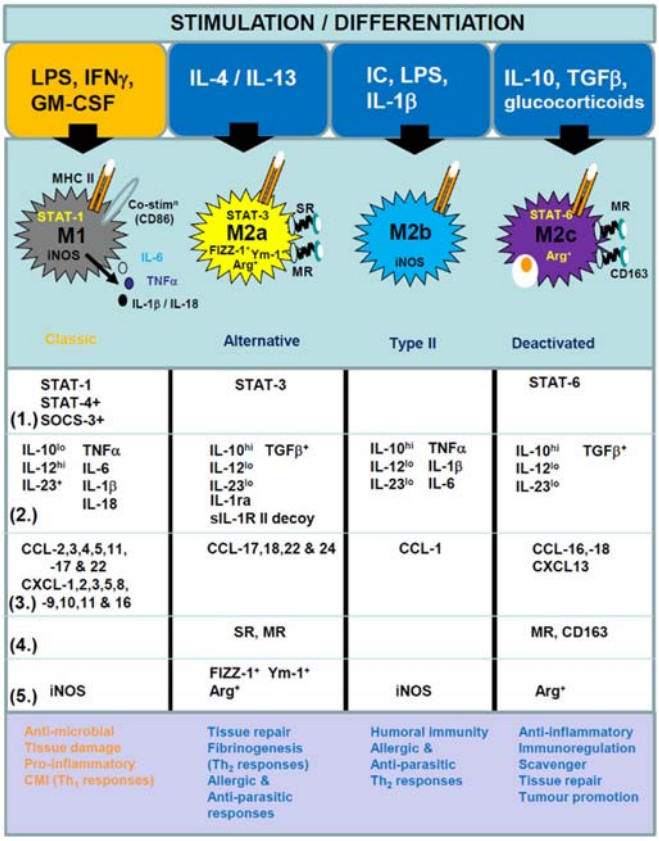

Fig 9. Macrophage phenotype. Extracted from Foey AD. Mucosal macrophages: phenotype and functionality in homeostasis and pathology. 2012.

M1 phenotype or "classical" macrophages are induced by stimulation with the prototypical Th1 cytokine IFN-γ or TLR signaling via LPS (Gordon and Martinez, 2010, David and Kroner,

2011) and their phenotype markers include CD16, CD32, CD86, MHC II, and iNOS. In vivo characterization of the functions of the M1 cells has been controversial, but it is largely believed that M1 macrophages are pro-inflammatory, possess higher phagocytic and antigen-presenting abilities and exert microbicidal functions (David and Kroner, 2011, Murray and Wynn, 2011b). These cells produce high levels of pro-inflammatory cytokines (IL-1β, IL-6, IL-12, IL-23, IL-1β, and TNF-α), chemokines (CCL2, CXCL9, CXCL10) and cytotoxic mediators (iNOS and NO) that are essential for host defense (Gordon, 2003, Sica and Mantovani, 2012). Pro-inflammatory M1 macrophages also release high amounts of active MMPs, such as MMP1 and MMP9. Therefore, the M1-like response might be beneficial at early stages of the pathology. However, dysregulated or excessive duration of M1-like macrophages might increase tissue damage in SCI **(Figure 9).**

M2 phenotype, also known as alternatively activated macrophage, express relatively low levels of pro-inflammatory cytokines, and play important regulatory roles in wound healing and repair (David and Kroner, 2011, Murray and Wynn, 2011b). The original alternatively activated macrophage was classified based on expression of the mannose receptor (CD206); since then, an assortment of different markers has been identified as 'M2' specific. One of the markers most characterized is the enzyme arginase 1 (Arg1), which converts arginine to polyamines, proline, and ornithine that can contribute to wound healing and matrix deposition. Interestingly, by using arginine, which is the same substrate used by iNOS, Arg1 can effectively outcompete iNOS to reduce the production of nitric oxide. Thus, iNOS and Arg1 represent a relatively straight forward set of markers to follow M1 versus M2 phenotypes. However, M2 macrophages are not a homogeneous population. Indeed, three different major subsets of M2 macrophages has been described *in vitro* (M2a, M2b and M2c) **(Figure 9)**. Furthermore, other different macrophage subsets have been identified in several pathologies, such as tumor-associated macrophages (Noy and Pollard, 2014) or resolution-phase macrophages (Bystrom et al., 2008, Stables et al., 2011), among others. This sub-classification is mainly based on the cell surface markers and polarizing signals they produce, mostly cytokines (Mantovani et al., 2004). Microglia and macrophages respond to Th2 cytokines such as IL-4 and IL-13 to induce an 'alternative' M2a state that signaling through IL-4Rα. M2a macrophages exert potent anti-inflammatory functions, remove parasites, recruit Th2 cells, and mediate tissue repair (Gordon, 2003, Sica and Mantovani, 2012). M2a macrophages, show low expression of NFκB and express CCL24, CCL17, CCL18, CCL22 and anti-inflammatory cytokines, such as IL-10. M2a macrophages are characterized by several phenotypic markers such as arginase 1, Ym1, Fizz1 in mice, CD206 (C-type mannose receptor 1), in both mouse and human (Porcheray et al., 2005) and increase production of scavenger receptors for phagocytosis (Gordon, 2003, Sica

and Mantovani, 2012).

A second sub-class of M2 activation has been observed following exposure to immune complexes and stimulation of TLR (Gordon, 2003, Sica and Mantovani, 2012). This class is termed 'M2b' and interestingly, they more closely resemble M1 macrophages, owing to the lack of any M2 specific markers, such as Arg1, YM1, or FIZZ1 and by the expression of TNFα, IL-1β and IL-6. However, they have an M2 cytokine profile since they produces high amounts of IL-10 and low levels IL-12. M2b macrophages modulate different aspects of the inflammatory response.

Finally, a third state of alternative activation is based on macrophages exposed to IL-10, glucocorticoids, or TGF-β. This phenotype is classified as 'M2c' (also called 'deactivated') and appears to be involved in tissue remodeling and matrix deposition once inflammation remits (Gordon, 2003, Sica and Mantovani, 2012). M2c-polarized cells upregulate phenotypic markers such as CD163, CD206, TGFβ, and Arginase-1 and express high levels of CXCL13, CCL16 and CCL18 (Murray and Wynn, 2011a).

It is important to mention that although the inflammatory properties of different monocyte subsets resemble the M1 and M2 macrophage polarization states, there is no direct evidence that M1/M2 macrophages are direct progenies of $Ly6C^{high}$/$Ly6C^{low}$ monocytes. In fact, studies have shown that either $Ly6C^{high}$ or $Ly6C^{low}$ monocytes could give rise to both M1 and M2 phenotypes (Misharin et al., 2014)

M1- and M2-like polarization after SCI

It is initially expected that M1- and M2-like microglia and macrophages would work in concert to fine-tune inflammatory responses after SCI to scavenge debris and promote tissue remodeling and repair. However, unlike other tissues, the inflammatory response after SCI is prolonged and fails to transit to the resolution phase (Prüss et al., 2011).

Experimental and clinical studies demonstrate predominant presence of M1-like phenotype in SCI, at least for the first 2 weeks (Kigerl et al., 2009, David and Kroner, 2011). The first detailed assessment of macrophage polarization under SCI context was reported by Popovich's group. They showed that at the site of injury, microglia and macrophages have mixed M1- and M2-like activation profiles. At early hours after injury (1–3 days), the M1 marker NOS2 (the gene that encodes iNOS) and the M2 marker Arg1 (the gene encoding Arg1) are upregulated at the injury site (Kigerl et al., 2009). However, three days post-injury, M1 markers continued to rise whereas M2 markers were downregulated, leading to a macrophage profile more skewed towards the M1-like state. Histological studies also showed that microglia/macrophages at the SCI site show predominant M1-like activation, since they are CD86 and CD16/32 positive,

whereas only a small fraction of macrophages/microglia express arginase 1- and CD206 (Kigerl et al., 2009). In addition, screening using a complementary DNA (cDNA) microarray and quantitative polymerase chain reaction (qPCR) analysis showed that M1 markers (CD16, CD32, CD86, CD64, and iNOS) are highly expressed in the first 2 weeks after SCI, whereas M2 markers (Arg1, CD163, CD206, CD14, and CD23) are expressed at a much lower level (Kigerl et al., 2009). Consequently, it is thought that low number of anti-inflammatory M2 macrophages and the abundant and prolonged presence of M1-like cells in the SCI triggers neuronal and glial cell death of oligodendrocytes, not only at the site of injury, but also distant from the lesion core (Davies et al., 2006).

There is currently limited information about the factors that impede macrophages to induce the expression of pro-repair M2 markers in SCI. This fact may be related to the post-injury milieu of the spinal cord where iNOS and pro-inflammatory cytokines such as TNFα, IL-1β and IL-6 are upregulated mainly by microglia and macrophages. A finding that provides strong evidence that the injured spinal cord environment favors M1 polarization is that bone-marrow-derived macrophages polarized with IL-4 to a M2 phenotype *in vitro* and transplanted into the contused spinal cord, lose the expression of the M2 marker Arg1 after 3 days (Kigerl et al., 2009).

One of that pro-inflammatory cytokines that hampers the switch to the M2 phenotype is the TNFα. *In vitro* studies have shown that phagocytosis of myelin by LPS-stimulated macrophages reduce the expression of pro-inflammatory cytokines (IL-12, TNF) (Boven et al., 2006). In addition, phagocytosis of myelin also reduces expression of the M1 markers CD16/32, CD86 and Ly6C and increases expression of M2 markers CD204, CD206 in macrophages and microglia (Kroner et al., 2014). However, despite of the abundant phagocytosis of myelin in the injured spinal cord, macrophages do not change toward M2 polarization, in part, blocked by TNF (Kroner et al., 2014). This was confirmed *in vivo* after SCI in TNF null mice where, as expected, there is greater number of M2 macrophages in the injured spinal cord. Besides to phagocytosis of damaged myelin, there is also abundant phagocytosis of red blood cells (RBCs) resulting from hemorrhage. Phagocytosis of RBCs by M1 macrophages induces reduction of some M1 markers (CD16 and CD86), but it retains expression of high levels of pro-inflammatory cytokines (TNF and IL-12). This fact may be due by the increase of iron loading of macrophages in the injured spinal cord by the phagocytosis of RBCs, favors the increase of TNF expression (Kroner et al., 2014). This was further confirmed *in vivo* in SCI when mice treated with intraperitoneal injections of iron dextran showed increased TNF expression in the injured spinal cord. On the other hand, as mentioned previously, although there is a preponderance of M1 macrophages after SCI, M2 macrophages are also present (Kigerl et al., 2009). M2

macrophages are also highly phagocytic cells (David and Kroner, 2011). Kroner et al. assessed the effects of RBC phagocytosis on M2 macrophages and show that RBC phagocytosis caused a rapid shift of IL-4-induced M2 macrophages to a M1 phenotype within 16 h (Kroner et al., 2014). An important aspect of the effects of iron on macrophage phenotype was revealed when M2 macrophages were either loaded with iron dextran or allowed to phagocytose RBCs and then transplanted into the injured spinal cord. Three days after transplantation, most cells expressed TNF and CD16, while the expression of the CD206 was markedly reduced (Kroner et al., 2014). These data clearly indicate that phagocytosis or RBC or uptake of iron from dying cells can transform M2 cells to a pro-inflammatory M1 phenotype. Taken together, these findings show that TNF can prevent the myelin phagocytosis-induced shift in polarization from M1 to M2. In addition, increased iron levels in M1 macrophages induce increased TNF expression that in M2 macrophages induce a shift to a pro-inflammatory M1 state.

All these evidence suggest that the pro-inflammatory environment after SCI promotes persistent M1-like phenotype that enhances the secondary damage.

Recruitment of Adaptive Immune Cells

SCI also elicits the activation of the adaptive immune system. After SCI, T and B lymphocytes are activated in the spleen and bone marrow within 24 hours, and they infiltrate the SCI site starting from a few days, peaks at day 7 (Donnelly and Popovich, 2008) and lasting until months after injury (Sroga et al., 2003, Beck et al., 2010). At the site of lesion, T-lymphocytes recognize the major histocompatibility complex from antigen presenting cells, and become activated. Activated T-lymphocytes increase in number and regulate the production of cytokines and antibodies via B-cells, which amplify the inflammatory response.

To date, the importance of T-lymphocytes after SCI remains elusive. Partly based on early dogma in the field of CNS injury, adaptive immune responses to the injury were assumed to be largely detrimental by default (Hickey et al., 1991, Popovich et al., 1996). In support of that idea, SCI leads to release of many self-antigens that activate autoreactive lymphocytes, causing autoimmune reactions, which may exacerbate the injury. It has been reported that autoantibodies against a range of CNS proteins such as GM1 gangliosides and myelin-associated glycoprotein were detected in serum of SCI patients (Davies et al., 2007). In mice, antibody-secreting B cells, immunoglobulins and complement component 1q were present in the cerebrospinal fluid and accumulated at sites of axon loss and demyelination in the injured spinal cord (Ankeny et al., 2009, Ankeny and Popovich, 2010). Strikingly, secondary degeneration is more extensive in rodents that lack both T and B cells (C. J. Serpe, 1999, Yoles

et al., 2001), suggesting an unknown neuroprotective role for adaptive immune cells in CNS injury. Future studies will be needed to elucidate the beneficial and pathologic effects of the adaptive immune response after SCI.

4- THE RESOLUTION OF ACUTE INFLAMMATION

Inflammatory response, as has been described before, is a highly regulated process that involves immune cells, blood vessels and molecular mediators necessary to eliminate the initial cause of cell injury. In order to prevent the progression from acute-resolving to persistent-chronic inflammation that could promote more tissue damage, the inflammatory reaction must be actively resolved (Gilroy et al., 2004) (Serhan et al., 2007). Resolution of inflammation is a coordinated and active process, encoded at the onset of inflammation and aimed at restoration of tissue integrity and function. It is defined as the interval from maximum leukocyte infiltration to the point when they are cleared from the tissue (Serhan et al., 2007) and involves different regulated steps:

1. Limitation or cessation of inflammatory cell recruitment by normalizing of chemokine gradients and the reduction of survival signals (Buckley et al., 2001).

2. The counter-regulation of pro-inflammatory factors such as TNF-α, IL-1β or iNOS by the suppression of the inflammatory pathways, such as NF-κB or activating protein-1 (AP1) (Gilroy et al., 2004). Furthermore, at the same time, anti-inflammatory cytokines such as IL-4 and IL-10 must be over-expressed (Iribarren et al., 2003).

3. Release of pro-resolving Factors. A shift in lipid-mediator biosynthesis from pro-inflammatory to pro-resolution lipid mediators accelerates resolution (Serhan et al., 2000, Levy et al., 2001).

4. The induction of apoptosis of inflammatory cells. Neutrophils undergoing programmed cell death lose the ability to degranulate after inflammatory stimuli (Serhan et al., 2008).

5. Promoting phagocytosis of apoptotic cells by macrophages (efferocytosis) (Mitchell et al., 2002, Serhan and Savill, 2005, Schwab et al., 2007).

6. Increasing Exit of Phagocytes. Following efferocytosis, macrophages leave the site of inflammation through the lymphatics system (Schwab and Serhan, 2006, Schwab et al., 2007). The emigration rate of macrophages through lymphatics is regulated by the state of macrophage activation and depends on adhesion molecule expression as shown in a peritonitis model (Bellingan et al., 1996, Bellingan et al., 2002).

7. Induction of tissue repair. These events culminate in a return to tissue homeostasis (Serhan and Savill, 2005) without fibrosis or scar formation marks the final step of resolution

Historically, it was believed that the resolution of inflammation was a passive process involving the dilution of chemokine gradients over time, thus circulating leukocytes would no longer sense gradients and be recruited to the site of injury. Instead, resolution is an active and coordinated anti-inflammatory, pro-resolving program aimed at restoring tissue homeostasis, integrity and function (Serhan, 2014) (Buckley et al., 2014). In last years, several local mediators called pro-resolving mediators (SPMs) has been identified as activators of the resolution mechanisms. This fact, demonstrate definitely that the resolution phase of acute inflammation is a biosynthetically active rather than a passive process (Serhan and Savill, 2005).

I. Pro-resolving mediators

In the last years, a new array of molecules that function in the resolution of inflammation has been identified and named specialized SPMs. Many of these SPMs are produced during the acute inflammatory response and can function at numerous steps of the resolution process. SPMs share fundamental properties to switch off leukocyte infiltration, terminate the inflammatory reaction and organize the 'cleaning phase' within the affected tissue, as required for the regain of homeostasis and return to normal physiological function.

Substances accumulated in resolution exudates, which fall under this umbrella, are diverse in nature and encompass SMP (e.g., lipoxins, resolvins, protectins and maresins) (Norling and Serhan, 2010), peptides/proteins (e.g., adrenocorticotropic hormone, annexin A1, chemerin peptides, galectin-1 or anti-inflammatory cytokines) (Bannenberg et al., 2005, Dalli et al., 2013a), gaseous mediators (e.g., H_2S (Caliendo et al., 2010) and CO (Chiang et al., 2013)), a purine (e.g., adenosine (Ehrentraut et al., 2013)), as well as a neuromodulator (neurotransmitter/neuropeptide) released under the control of the vagus nerve (Mirakaj et al., 2014).

Pro-resolving Lipids Mediators

In recent years, previously unrecognized chemical mediators derived from polyunsaturated fatty acids have been identified. These new lipids, called SPM, are biosynthesized within the resolution phase of acute inflammation (Serhan et al., 2000, Levy et al., 2001). This novel

genus of bioactive mediators has been identified by using systems of liquid chromatography tandem mass spectrometry (LC-MS/MS)-based analysis of self-limited inflammatory exudates (ones that resolve to homeostasis on their own) formed in vivo in animal models. SPMs are produced in a spatio-temporally regulated manner from essential polyunsaturated fatty acids (PUFAs) that are either released enzymatically by phospholipase A_2 (PLA_2) from cell membranes for secondary conversion by biosynthetic enzymes or delivered with edema fluid from plasma to exudates. The principal SPM families are lipoxins (LX) from arachidonic acid (C20:4n 6;), the E series resolvins (RvE) from eicosapentaenoic acid (EPA; C20:5n 3;) and D series resolvins (RvD), protectins (PD) and maresins (MaR) from docosahexaenoic acid (DHA; C22:6n 3). They each possess distinct chemical structures and regulate cellular pathways by their ability to activate pro-resolving G-protein coupled receptors (GPCRs) in a stereospecific manner (Serhan and Chiang, 2013).

Lipoxins

Lipoxins (LX) were the first anti-inflammatory LM recognized to possess pro-resolving actions (Serhan et al., 1984). Lipoxins are short lived endogenously produced non-classic eicosanoids whose appearance in inflammation signals the resolution of inflammation.

At present two lipoxins have been identified; lipoxin A4 (LXA4) and lipoxin B4 (LXB4). They are lipoxygenase interaction products derived from the enzymatic conversion of arachidonic acid (AA) during inflammation (Samuelsson et al., 1987) in three main pathways **(Figure 10)**:

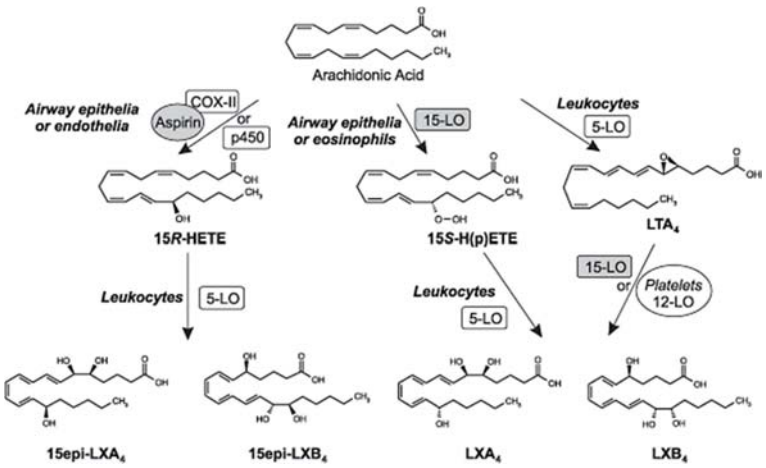

Fig 10. Synthesis of lipoxins. Extracted from Levy et al. Drugs Today 2003

- In humans, the first pathway is the sequential oxygenation of AA by epithelial cell, eosinophil or monocyte 15-Lipoxygenase (15-LO) and leukocyte 5-Lipoxygenase (5-LO), followed by enzymatic hydrolysis, leads to the biosynthesis of LXA4 and B4 in mucosal (Edenius et al., 1990, Levy et al., 1993).
- Blood vessels represent a second site for LX biosynthesis, with the conversion of 5-LO derived LTA4 into LXA4 and B4 by 12-LO in platelets (Serhan and Sheppard, 1990).

- The third LX synthetic pathway is initiated by aspirin. Although aspirin inhibits prostaglandin production, aspirin-mediated acetylation of cyclooxygenase 2 (COX2) leads to the conversion of arachidonic acid to 15(R)-hydroxyeicosatetraenoic acid (15(R)-HETE), which is a substrate of leukocyte 5-LO for the biosynthesis of 15R- epi-LXA4 and B4 (also known as aspirin-triggered (AT) lipoxins) (Clària and Serhan, 1995).

Lipoxins, as well as certain peptides, are high affinity ligands for the lipoxin A4 receptor (ALX), which was first identified based on sequence homology as the formyl peptide receptor like receptor (FPRL1). This receptor has recently been coined ALX/FPR2 by the international nomenclature committee in light to fits high affinity for LXA4 (Ye et al., 2009). The receptor for LXB4 has not been identified. Human FPR2/ALX is highly expressed in myeloid cells and at a lower extent in lymphocytes, dendritic cells, and resident cells (Chiang et al., 2006). Orthologues of the human FPR2/ALX have been identified in mice (Takano et al., 1997) and rats (Chiang et al., 2003). LXs have a number of immunomodulatory and anti-inflammatory actions. By signaling through the FPR2/ALX, LXA4 decrease vascular permeability, decrease neutrophil endothelium interactions and reduce neutrophil infiltration (Chiang et al., 2005). In addition, LXA4 can stimulate macrophage phagocytosis of microbes and apoptotic neutrophils (Maderna et al., 2002, Reville et al., 2006). Finally, LXA4 also can attenuate inflammation-induced pain implicated in spinal nociceptive processing (Svensson et al., 2007, Wang et al., 2014).

E-resolvins

E-series resolvins (e.g., RvE1, RvE2, and RvE3) are produced from EPA **(Figure 11)**. At the first time EPA is converted to 18-HEPE by aspirin-acetylated COX-2 or cytochrome P450 monooxygenase. In this point, 18-HEPE is further converted via sequential actions of lipoxygenases, which leads to the formation of E series resolvins.

Resolvin (Rv) E1 (5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16E-EPA) and RvE2 (5S,18R-dihydroxy-6E,8Z,11Z,14Z,16E-EPA) are biosynthesized by human PMN leukocytes via the 5-LO pathway from 18-HEPE (Arita et al., 2005,

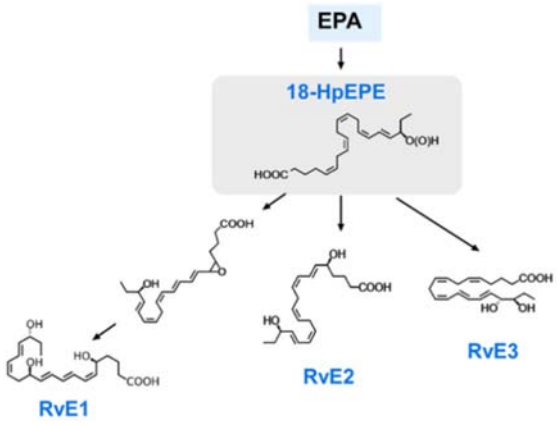

Fig. 11. Synthesis of E-resolvin series. Extracted from C.N. Serhan, et al., The resolution code of acute inflammation: Novel pro-resolving lipid mediators in resolution, Semin Immunol (2015),

Tjonahen et al., 2006).

RvE1 and RvE2, mediates their actions mainly binding to a GPCRs namely ChemR23 (Arita et al., 2005, Arita et al., 2007). RvE1 administered prior to a murine model of aspiration pneumonia is associated with a reduction in pro-inflammatory cytokines, decreased pulmonary PMN accumulation, enhanced bacterial clearance and improved survival (Seki et al., 2010). In addition, RvE1, enhances macrophage phagocytosis (Hong et al., 2008) and inhibit both ADP-stimulated activations of P2Y12 and the effects of TxB2, to prevent platelet aggregation. Furthermore, RvE1 can directly block the transient receptor potential (TRP) family of nociceptors on skin keratinocytes and peripheral sensory neurons to mute inflammatory pain. Finally, RvE1 and RvE2 are also endogenous receptor antagonists for the LTB4 receptor BLT-1, which most likely explains their ability to potently regulate PMN trafficking to sites of inflammation (Serhan and Chiang, 2013).

In addition to Rve1 and RvE2, another E series resolvin called RvE3 (17,18R/S-dihydroxy-5Z,8Z,11Z,13E,15E-EPE) is generated from 18-HEPE, via leukocyte-type 12/15-LOX pathway. (Isobe et al., 2012). Enzymatically generated RvE3 had the same physical properties as endogenously biosynthesized products and displayed a potent anti-inflammatory action by stopping PMN infiltration in zymosan-induced peritonitis.

D-resolvins

Resolvin D series, are generated from DHA. The biosynthetic conversion of DHA to D-series resolvins (RvD1 – RvD6) involves different lipoxygenation steps (Serhan et al., 2002). At the beginning, DHA is converted to 17S-HpDHA by 15-lipoxygenase (15-LO). At this point, there are 2 different pathways **(Figure 12)**:

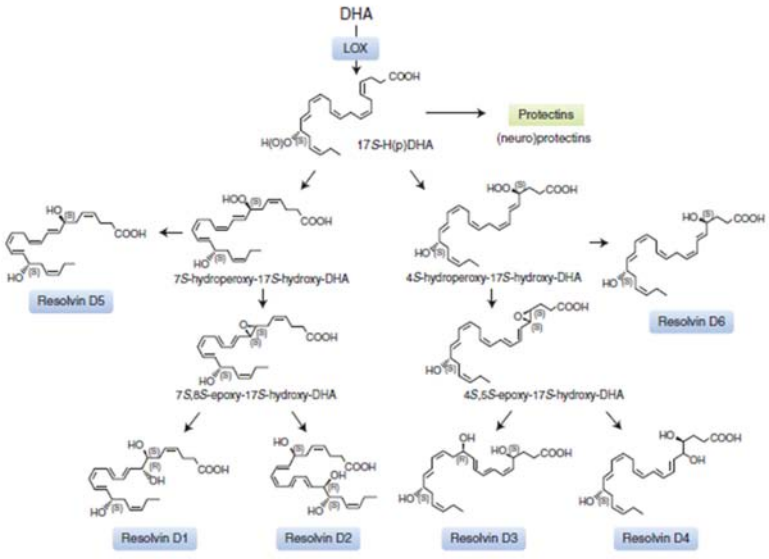

Fig 12. Synthesis of D-Resolvin series. Extracted from Cold Spring Harb Perspect Biol 2015;7:a016311

- Initial conversion of DHA to 17S-HpDHA catalyzed by 15-lipoxygenase (15-LO) followed a second lipoxygenation at the C-7 position of 17S-HDHA via 5-LO gives a peroxide intermediate that by peroxidase reduction forms RvD5 (i.e. 7(S),17(S)-

dihydroxy-4Z,8E,10Z,13Z,15E,19Z-DHA) or is transformed to the 7S,8S-epoxide which enzymatic hydrolysis generates trihydroxylated products RvD1 (i.e. 7(S),17(S)-dihydroxy-4Z,9E,11E,13Z,15E,19Z-DHA) and RvD2 (i.e. 7(S),17(S)-dihydroxy-4Z,8E,10Z,12E,14E,19Z--DHA)

- Alternatively, 5-LO lipoxygenation at the C-4 position of 17S-HDHA forms an intermediate product that is converted to RvD3 (i.e. 4,17(S)-dihydroxy-5Z,7E,9E,13Z,15E,19Z-DHA), RvD4 (i.e. 4,17(S)-dihydroxy-5Z,7E,9E,13Z,15E,19Z-DHA) by an hydrolase and RvD6 (i.e. 7(S),17(S)-dihydroxy-5E,7Z,10Z,13Z,15E,19Z-DHA) by peroxidation.

- In addition an aspirin-triggered 17R D-series resolvins were also identified (Hong et al., 2003). In that way, DHA is oxygenated to form 17R-HpDHA in the presence of aspirin via acetylated COX-2, or via a P450 pathway. Subsequent enzymatic transformations lead to AT-RvD1, AT-RvD2, ATRvD3 and AT-RvD4 the 17(R)-hydroxyl diastereomers of RvD1, RvD2, RvD3, and RvD4, respectively.

The mechanism by which each of the resolvins D activate cells has not been fully elucidated. However, many resolvins appear to operate at least in part by acting through the following G protein-coupled receptors. By one side, RvD1 and AT-RvD1 act through the Formyl peptide receptor 2 (ALX/FPR2) receptor, present on leukocytes and resident cells such us microglial, endothelial, and epithelial cells (Krishnamoorthy et al., 2010) and through human GPR32 receptor abundant on PMN, monocytes, macrophages and on vascular endothelial cells (Krishnamoorthy et al., 2010). Norling et al. (Norling et al., 2012) demonstrate that in humans, GPR32 mediates 'homeostatic' RvD1 functions and FPR2/ALX requires higher concentrations of RvD1 and can be modulated during PMN activation exposed to inflammatory stimuli. Furthermore, murine orthologue of DRV1/GPR32 is currently unknown and FPR2/ALX orthologue transduces all RvD1 bioactions. On the other hand RvD2, acts through the GPR18 receptor, expressed on human leukocytes (Krishnamoorthy et al., 2010, Chiang et al., 2015) and finally, RvD3, AT-RvD3, and RvD5 (Chiang et al., 2012) that appears later in the resolution phase also binds to that human GPR32 receptor (Dalli et al., 2013b).

RvD1 production by neutrophils has potent anti-inflammatory and pro-resolving actions (Sun et al., 2007, Serhan and Petasis, 2011) such us stop PMN infiltration (by reduction of human PMN-endothelial cell interactions) and enhances macrophage efferocytosis (Recchiuti et al., 2011). RvD1 also is involved in control pain (Xu et al., 2010, Ji et al., 2011). Furthermore RvD1 treatment prior to LPS-induced acute injury lung injury improves pathological indices and survival rates (Wang et al., 2011) and may be equally effective in central nervous system inflammation (Xu et al., 2013). The common mechanism appears to be suppression of NFkB activation in a partly PPARγ-dependent manner, with associated reduction in downstream signaling and alterations in transcriptomics (Arita et al., 2005, Liao et al., 2012).

On the other hand, RvD2 actions are multifaceted, targeting the modulation of leukocyte-endothelial interactions, altering the cytokine and eicosanoid profiles (reduce IL-17, prostaglandin [PG]E2 and LTB4). In addition, RvD2, as has been described to RvD1, is involved in reducing pain (Park et al., 2011). Furthermore, RvD2 possess the ability to enhance the innate immune response without rendering immune suppression of the host (Spite et al., 2009). RvD2 exerts diverse actions in host defense and pathogen interactions in a manner that is, paradoxically, conducive to optimal bacterial clearance (Chiang et al., 2012) and not immunosuppressive. In addition, recently the ability of RvD2 to restore directionality to neutrophil migration and prevent sepsis-induced immune dysfunction and thus increase survival from a secondary septic challenge post-burn injury has been demonstrated (Kurihara et al., 2013). Together, these results indicate potent roles for RvD2 in regulating resolution of bacterial infections and sterile inflammation.

Protectins

Protectin D1 also known as neuroprotectin D1 (when it acts in the nervous system) (PD1 or NPD1) is produced by the oxygenation of the ω-3 polyunsaturated fatty acid docosahexaenoic acid (DHA) as a response to inflammatory signals in many tissues such as the retina, the lungs and the nervous system (Mukherjee et al., 2004, Calandria et al., 2009).

The structure of the PD1, was first established as 10R,17S-dihydroxy-docosa-4Z,7Z,11E,13E,15Z,19Z-hexaenoic acid (Serhan et al., 2006). Overall, the biosynthesis of PD1 proceeds through three distinct steps throughout which the activity of 15-LO-1 is essential **(Figure 13**). Recently, a novel aspirin-triggered COX-2 driven pathway was reported that biosynthesizes the 17R-epimeric form of PD1 from DHA (Marcheselli et al., 2003) called AT-PD1 (10R,17R- dihydroxy-docosa-4Z,7Z,11E,13E,15Z,19Z-hexaenoic acid).

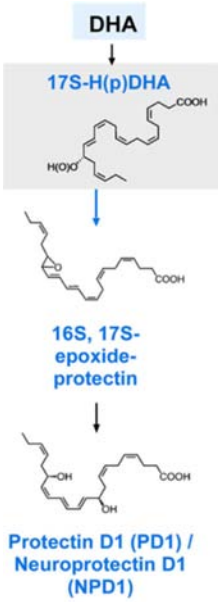

Fig 13 Synthesis of Protectin D1. Extracted from C.N. Serhan, et al., The resolution code of acute inflammation: Novel pro-resolving lipid mediators in resolution, Semin Immunol (2015),

Both PD1 and AT-PD1 reduced leukocyte infiltration in murine peritonitis, reduced PMN transmigration with endothelial cells, and enhanced efferocytosis of apoptotic PMN by human macrophages (Serhan et al., 2006). In addition to anti-inflammatory and resolutory role, PD1 has a significant role as anti-apoptotic and neuroprotective molecule. Studies in Alzheimer's disease animal models, in stroke patients and in human retina pigment epithelial cells (C. J. Serpe) have shown that PD1 can potentially reduce inflammation induced by oxidative stress and inhibit the pro-apoptotic signal, thereby preventing cellular

degeneration (Antony et al., 2010, Zhao et al., 2011). Finally, recent studies examining the pathogenicity of influenza viruses, including the avian flu (H5N1), have suggested that PD1 can potentially halt the proliferation of the virus, thus protecting respiratory cells from lethal viral infections (Morita et al., 2013).

Maresin

Maresin 1 (7(R)-MaR1 or 7R,14S-dihydroxy-4Z,8E,10E,12Z,16Z,19Z-DHA) is a macrophage-derived mediator of inflammation resolution. MaR1 is produced by human macrophages from endogenous docosahexaenoic acid (DHA) (Serhan et al., 2009) (**Figure 14**). 12-lipoxygenase converts DHA to its 14-hydroxperoxy intermediate, 14-HpDHA; next 14-HpDHA is converted enzymatically to 13(S),14(S)-epoxy-maresin which is then enzymatically hydrolyzed to 7(R)-Mar1 or its epimer 7(S)-MaR1 (Dalli et al., 2013c, Abdulnour et al., 2014). Furthermore, recent research show other Maresin family member called Maresin 2 (MaR2) (13(R),14(S)-dihydroxy-4Z,7Z,9E,11E,16Z,19Z-DHA), also synthesized by Macrophages (Deng et al., 2014).

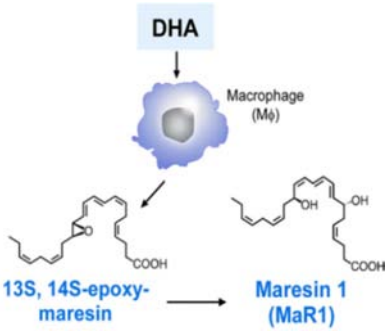

Fig 14. Synthesis of Maresin 1, Sintesis of E-resolvin series. Extracted from C.N. Serhan, et al., The resolution code of acute inflammation: Novel pro-resolving lipid mediators in resolution, Semin Immunol (2015)

MaR1 appears in the inflammatory response in the late stage with the entry of resolution phase macrophages that release it. MaR1 has potent actions in regulating inflammation resolution, tissue regeneration, and resolving pain (Serhan et al., 2012). MaR1 limits neutrophil (PMN) infiltration in murine peritonitis as well as enhances human macrophage uptake of apoptotic PMNs (Serhan et al., 2009). In addition, intermediate products posses regulatory functions. Intermediate 13(S),14(S)-epoxy-maresin stimulates M1 to M2 phenotype-switch and also inhibits the production of Leukotriene B4 (LTB4) (Dalli et al., 2013c) contributing to the resolution of inflammatory responses. Furthermore, MaR1 are potent stimulators of organ regeneration using a planaria regeneration system (Serhan et al., 2012). Finally, MaR1 is able to reduce neuropathic pain by inhibition a neuron ion channel, TRPV1, and thereby blocking capsaicin-induced inward currents and neuron excitation (Serhan et al., 2012).

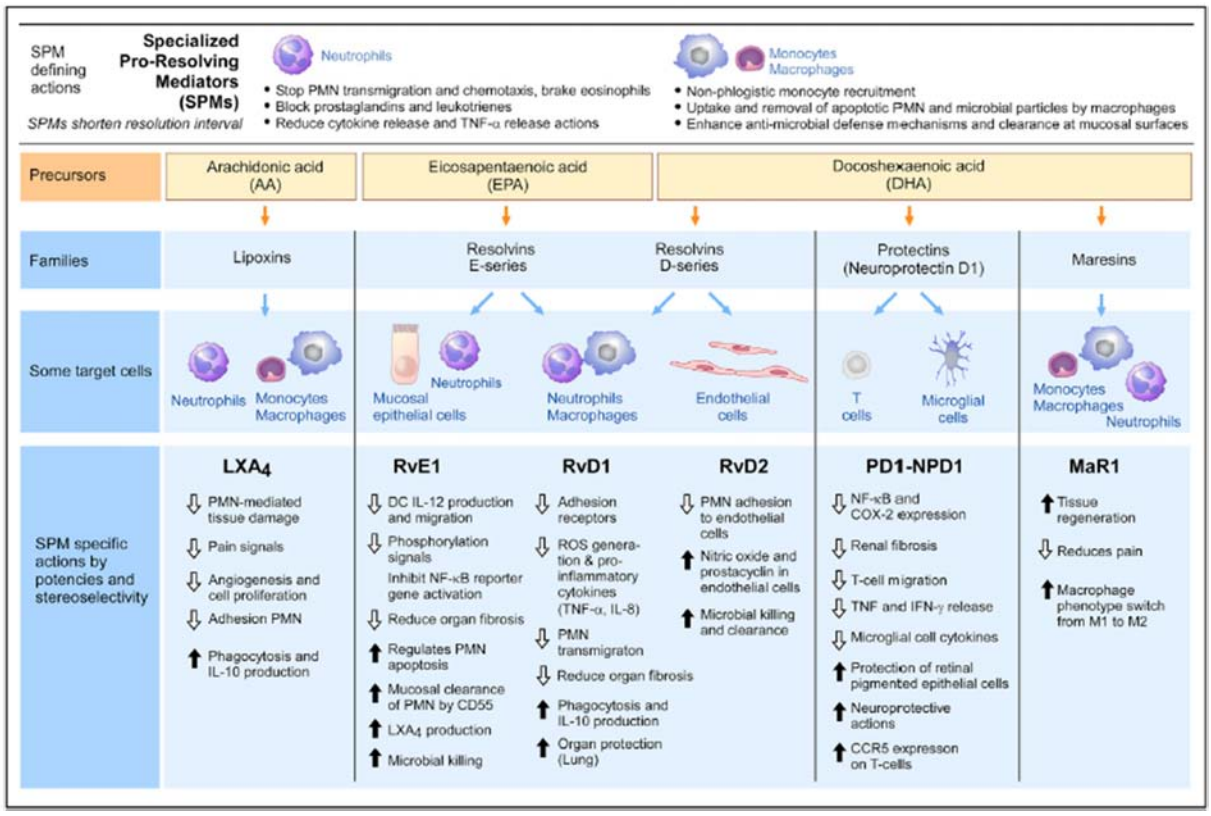

Fig 15 . SPM Actions and Target Cell Type. Extracted from Buckley et al. Immunity 40, March 20, 2014

II. Inflammatory resolution process

The inflammatory response is a terrain where lipid mediators (LM) such as eicosanoids (prostaglandins (PG) and leukotrienes (LT)) (Samuelsson, 2012) and novel pro-resolving mediators uncovered (Serhan et al., 2000, Serhan et al., 2002) play pivotal roles **(Figure 16).**

After tissue injury, the initiation of acute inflammation is controlled by a number of autacoids, including lipid mediators such as the eicosanoids (prostaglandins (PGs) and leukotrienes (LT)) (**Figure 17**). Eicosanoids are lipids formed from arachidonic acid (AA; omega 6; C20:4n 6) which play key roles in regulating blood flow, endothelial permeability, and PMN diapedesis (Samuelsson et al., 1987). At the starting stages, Prostaglandin E2 (PGE2) and Prostaglandin I2 (PGI2) increase vascular permeability (Samuelsson, 2012) and neutrophils transmigrate towards chemotactic gradients of LTB4 (Samuelsson, 1983, Lammermann et al., 2013). These lipid mediators, along with many cytokines, chemokines and complement components (C5a and C3b), stimulate the chemotaxis of neutrophils into tissues.

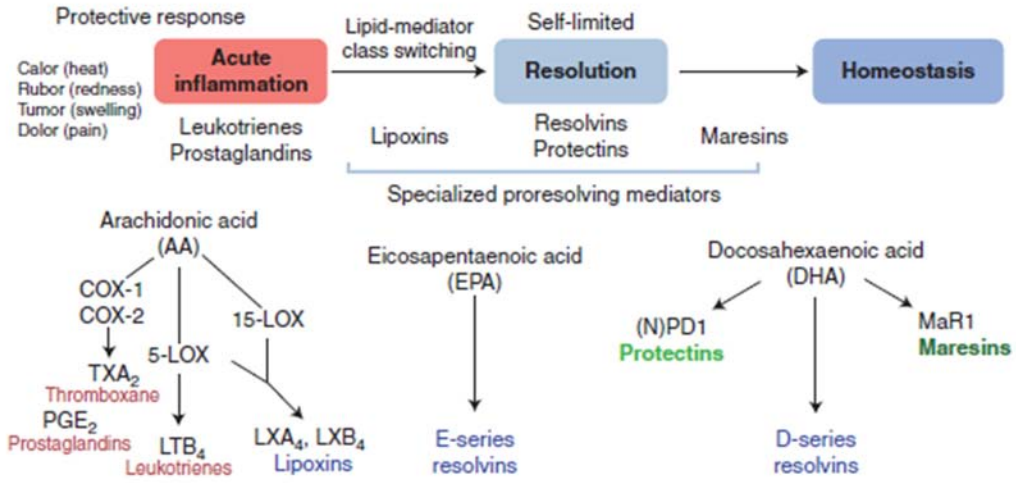

Fig 16 Time-course of lipid-mediator biosynthesis in exudate cell traffic in resolution of acute inflammation. Adapted from Cold Spring Harb Perspect Biol 2015;7:a016311

Next, after the initiation phase, in a self-limited inflammatory response, rapidly generated lipid mediators play a key role in orchestration of inflammatory resolution (Serhan et al., 2008). In particular, the arachidonic acid pathway synthesizes pro-inflammatory lipids such as prostaglandin (PG) E2 and D2, and, during the resolution phase, pro-resolving bioactive lipid mediators including lipoxins and the omega 3-unsaturated fatty acid-derivatives termed resolvins and protectins.

PGE2 and PGD2 each evoke pro-inflammatory and anti-inflammatory responses that depend on tissue location and timing. For example, as has been described above, PGE2 is involved in vasodilation, enhancing the LTB4-mediated neutrophil extravasation. However, in the context of resolution, local prostaglandin E2 initiate the lipid class switch by the stimulation of the processing of 15-lipoxygenase mRNA in leukocytes to produce functional enzyme for lipoxin A4 (Levy et al., 2001) and other SPMs (Serhan et al., 2000, Marcheselli et al., 2003) production. Furthermore, PGE2 enhance the production of IL-10 (Harizi et al., 2002). By the same way, PGD2 can elicit immunomodulatory and anti-inflammatory effects in the same manner as described for PGE2 via ligation to DP1, however, it can also act by other way when are converted into J2 series (e.g., PGJ2, D12,14-PGJ2 and 15-deoxy-D12,14-PGJ2 [15d-PGJ2]) (Diab et al., 2002). 15d-PGJ2 for example, via ligation to the nuclear receptor PPAR-g (Khan, 1995), decreases pro-inflammatory cytokine release and modifies gene expression (Ricote et al., 1998), as well as inhibits the activation of NF-kB (Straus et al., 2000, Cernuda-Morollón et al., 2001). In addition, independent of PPAR-g, 15d-PGJ2 promotes resolution actions, such as inhibition of neutrophil trafficking through differential regulation of cell adhesion molecule and chemokine expression and induction of leukocyte apoptosis through a caspase-dependent

mechanism (Gilroy et al., 2003). Therefore, prostaglandin production through COX-1 and COX-2 is crucial for initiation and timely resolution of inflammatory response.

At this time, the cells in the inflamed tissue, including neutrophils, start to produce pro-resolving lipid mediators. The successful progression of inflammation appears to hinge on a shift in the composition of secreted lipids, from high levels of prostaglandins and LTB4 to high levels of lipoxins and other pro-resolving lipid mediators, a process known as lipid-mediator class switching. It is now clear that neutrophils change their phenotype to produce different profiles of lipid mediators depending on cells and substrates present in their local environment. At early stages of inflammation, neutrophils synthesize pro-inflammatory lipid mediators, such as prostaglandins and leukotrienes. However, during the later stages of the inflammatory response, neutrophils interact with various cell types in their vicinity (epithelial cells, endothelial cells, fibroblasts, platelets, and leukocytes) and participate in the transcellular biosynthesis of lipid mediators with anti-inflammatory and pro-resolving activities, such as lipoxins (e.g LXA4). LXA4 not only inhibits neutrophil migration, also initiates non-phlogistic recruitment of monocytes and phagocytosis of apoptotic neutrophils by macrophages.

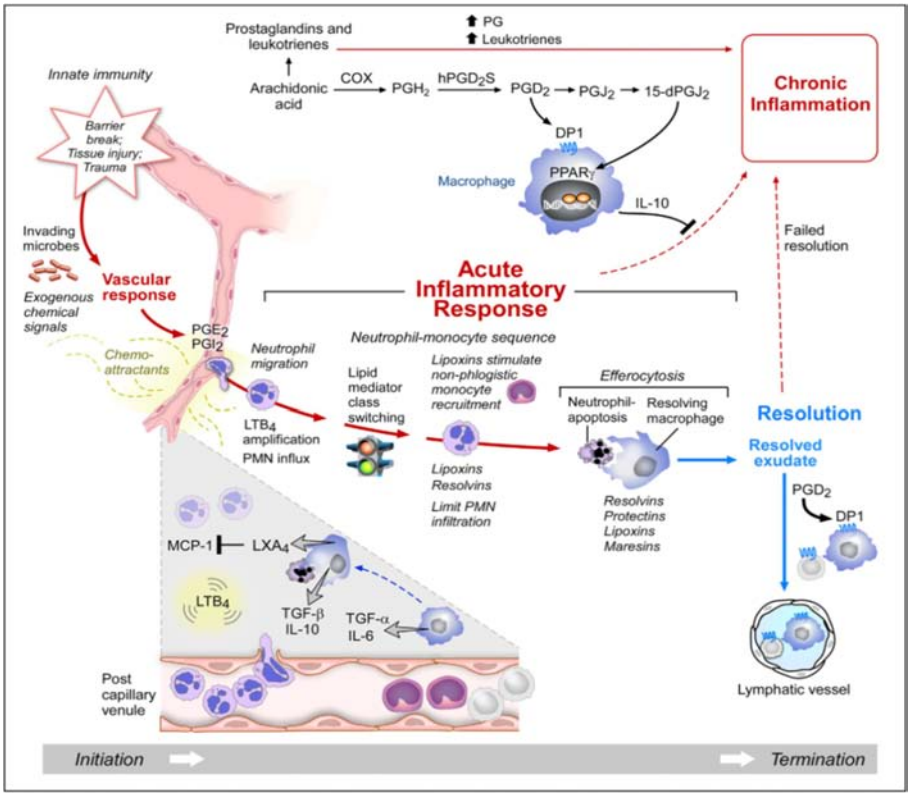

Fig 17. **Acute Inflammatory Response and the Role of Lipid Mediators in Resolution or its Failure. Extracted from Buckley et al. Immunity 40, March 20, 2014**

Closely related to LXA4, in later stages due to existence of new substrates such as DHA and EPA in the exudates from phospholipid stores (Hong et al., 2003), or via edema from peripheral blood (Kasuga et al., 2008), other specialized pro-resolving mediators are produced. These SPMs include resolvins and protectins produced by neutrophils and maresins synthesized by macrophages. These lipids reduce PMN infiltration, decrease pro-inflammatory mediator production (both lipid mediators and cytokines), regulate PMNs apoptosis and stimulate their uptake by macrophages to promote resolution (Serhan and Savill, 2005).

Equally important for the resolution of inflammation is that neutrophils undergo apoptosis after performing their action at the inflamed site (Fox et al., 2010) and macrophages ingest these apoptotic neutrophils.

Apoptosis of PMNs, is very important step because attenuate inflammation through distinct mechanisms. In one way, in addition to lipoxins, dying neutrophils secrete mediators that inhibit further neutrophil recruitment such as annexin A1 (AnxA1) which interacts with the formyl peptide receptor 2 (FPR2/ALX) to moderate leukocyte adhesion and migration (Perretti et al., 2002, Dalli et al., 2008). On the other hand, apoptotic neutrophils promote their own cleared by macrophages via efferocytosis. These macrophages are attracted by find me and eat me signals expressed by dying neutrophils. To date, four major find me signals have been described: lysophosphatidylcholine (LPC), sphingosine 1-phosphate (S1P), fractalkine (CX3CL1), and the nucleotides ATP and UTP (Lauber et al., 2003, Gude et al., 2008, Truman et al., 2008, Elliott et al., 2009). Find me signal gradients guide the macrophage towards the dead cell through the involvement of G2A, S1P1-5, CX3CR1, and P2Y2 receptors, respectively. Macrophage phagocytosis of apoptotic cells leads to the biosynthesis of pro-resolving lipid mediators, which act in an autocrine manner to facilitate. In addition, the engulfment of apoptotic neutrophils prompts a phenotypic switch in macrophages from a pro-inflammatory M1 to an anti-inflammatory M2 phenotype (Fadok et al., 1998, Michlewska et al., 2009), which is a prerequisite for macrophage egress via the lymphatic vessels favoring return to tissue homeostasis. Furthermore, M2 macrophages show up-regulation of arachidonate 15-lipoxygenase and COX1 (Stables et al., 2011) and alter their metabolism of lipids from pro-inflammatory prostaglandins and leukotrienes to pro-resolving autacoids such as lipoxins, resolvins, protectins and maresins (Ortega-Gomez et al., 2013). These bioactive lipid mediators suppress neutrophil and enhance monocyte recruitment, which aids in the further clearance of apoptotic cells (Ortega-Gomez et al., 2013). M2 macrophages also secrete anti-inflammatory cytokines such as IL-10 and TGF-b (Fadok et al., 1998), which inhibits pro-inflammatory cytokine production (such as TNF- a and IL-1 b) enhancing the resolution of inflammation.

Finally the phagocitosed cells, egress from the tissue via the lymphatic vessels (Schif-Zuck et

al., 2011), promoted almost in part by pro-resolution lipids such as Lipoxin A4 and Resolvin E1, returning the tissue to the pre-inflamed state.

Anti-inflammatory treatment versus resolution mediators

Importantly, resolution is now considered to be a distinct process from anti-inflammatory processes. This is because, in addition to serving as agonists to stop and lower neutrophil infiltration to inflamed tissues, pro-resolution molecules promote uptake and clearance of apoptotic cells by macrophages in inflamed sites **(Figure 18)**.

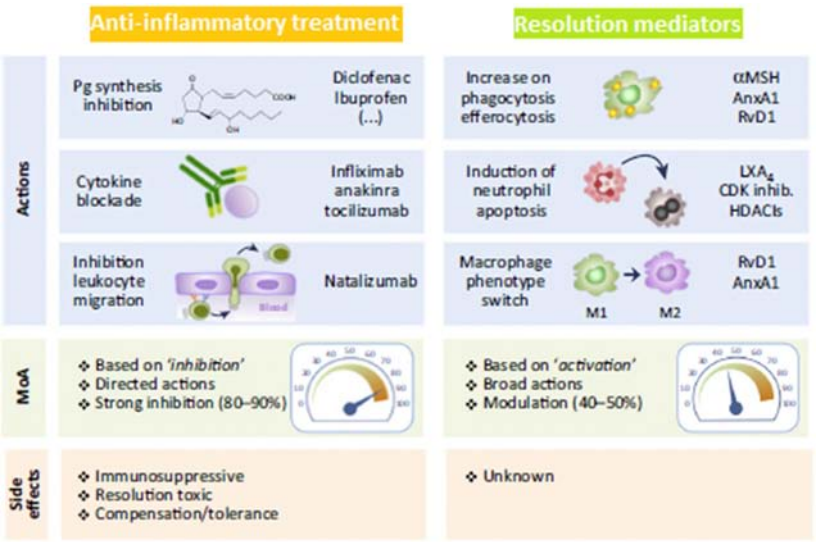

Fig 18 Differences between anti-inflammatory and pro-resolutory treatments. Adapted from Trends in Pharmacological Sciences, November 2015, Vol. 36, No. 11

Furthermore, in contrast to SPM, anti-inflammatory treatment such us inhibition of COX-2 (such us NSAID like ibuprofen) delays inflammatory resolution since prostaglandins have crucial roles in this process by initiating lipid-mediator class switching, as shown in animal disease models (Chan and Moore, 2010).

In addition, therapies that actively promote resolution may also have the advantage of enhancing innate immune responses to bacterial infections (Chiang et al., 2012), whereas established anti-inflammatory therapies such as anti-TNF strategies may be immunosuppressive (Bruns et al., 2009).

For these reasons, anti-inflammation and pro-resolution are therefore not equivalent. The agonists that actively promote resolution are fundamentally different from the antagonists that limit the duration and magnitude of the inflammatory response at both the molecular and cellular levels (Serhan, 2007, Ryan and Godson, 2010).

III. Resolution failure in CNS

The resolution phase at a histological level is defined as the interval from maximum leukocyte infiltration to the point when they are cleared from the tissue (Serhan et al., 2007). However, in CNS injury, resolution of inflammation is not complete, and blood-borne cells and microglia remain in the lesion site for prolonged periods, leading to chronic inflammation (David et al., 2012b). Sustained inflammatory response contributes to fibrotic scar formation, demyelination, chronic neurodegeneration, development of neuropathic pain, aberrant neuronal function and also the development of autoimmunity (David et al., 2012b). In addition, the propagation of injury and dysfunction of spared circuitry is especially important in CNS by its impaired endogenous regenerative capacity. Some of the reasons that impede the complete clearance of immune cells from CNS are:

- The immune privilege of the CNS may constitute a bidirectional shield in which inflammation proceeds behind the blood–brain barrier where immune cells are apparently trapped in the lesioned area (Lassmann, 2008, Lassmann et al., 2012).
- Clearance of leukocytes from the tissue is generally possible through returning to the systemic circulation via lymphatic drainage (Bellingan et al., 1996, Bellingan et al., 2002) or death by apoptosis and subsequent phagocytosis by macrophages that egress via the lymphatic vessels. However, the route of "lymphatic drainage" of the CNS is strikingly different compared to that of most organs of the human body and is represented by the perivascular spaces of capillaries and arteries, which are in continuity with the subarachnoid space (Weller et al., 2008, Ransohoff and Engelhardt, 2012). Here, in perivascular route, the cellular efflux is very limited and cellular drainage from the brain parenchyma to cervical lymph nodes is absent (Carare et al., 2008). However, a recent study has shown that CNS has also lymphatic vessels (Louveau et al., 2015), which may change this statement.
- After injury, microglia and macrophages do not always return to a resting state and become "primed." Primed microglia/macrophages are characterized by a dysfunctional exaggerated inflammatory response and constitute a well-documented pathoimmunological part in aging, neurodegeneration, and pain (Perry and Holmes, 2014).
- In addition and related with last reason, CNS appears unable to resolve inflammation effectively due to anatomical constraints but also due to its inability to generate an effective pro-resolution permissive milieu. Unlike other tissues where in late stages of inflammation pro-inflammatory cytokines are reduced an anti-inflammatory cytokines and SPM levels increase, in the injured spinal cord this might not occurs. However, this specific issue has not

been addressed yet after SCI. However, brains of individuals with Alzheimer's disease, NPD1 is reduced (Lukiw et al., 2005). In addition, LXA4 and RvD1 are reduced in cerebrospinal fluid and hippocampus, which correlated with lower scores on the mini-mental state examinations in these patients (Wang et al., 2014, Wang et al., 2015). Together, these human data point to an impaired resolution of inflammation in neurodegenerative disease, possibly due to poorly sustained resolution programs, which is associated with disease progression.

All these evidences suggest that SPMs and their receptors offer new reagents and targets, respectively, for the treatment of neurological conditions.

AIMS

AIMS

The general objective of the present thesis is to investigate whether activation of the resolution programs of inflammation mitigates functional deficits and secondary tissue damage after SCI. The thesis has been divided in five chapters according to the following specific aims:

Chapter 1. IL-4 drives microglia and macrophages towards a phenotype conducive for tissue repair and functional recovery after spinal cord injury

- To evaluate the dynamics of microglia and macrophage polarization after SCI.
- To assess whether the expression of pro-inflammatory cytokines is completely silenced over time in the injured spinal cord, and whether there is a switch to the production of anti-inflammatory cytokines during the evolution of inflammation after SCI.
- To evaluate whether increasing IL-4 levels in the injury milieu redirects microglial and macrophages towards a phenotype more conducive for tissue repair.
- To assess whether boosting IL-4 levels in the contused spinal cord leads to functional recovery and neuroprotection after SCI.

Chapter 2. Maresin-1 activates inflammation resolution programs after spinal cord injury and exerts therapeutic effects.

- To characterize the dynamics of microgliosis and leukocyte recruitment in the spinal cord after contusion lesion
- To assess whether there is inadequate synthesis of specialized pro-resolving lipid mediators after SCI

- To evaluate whether administration of Maresin-1 enhances inflammatory resolution in SCI, and if so, to elucidate its mechanisms of action.

- To evaluate whether exogenous administration of Maresin-1 improves locomotor skills and reduces tissue damage after spinal cord contusion injury.

Chapter 3. Effects of Resolvin D1 administration after spinal cord injury in mice.

- To evaluate whether administration of Resolvin-D1 induces the resolution of inflammation after SCI.

- To assess the therapeutic effects of Resolvin D1 on functional outcomes and tissue sparing after SCI

- To study whether Resolvin D1 activates differential gene expression programs after SCI than Maresin-1.

Chapter 4. Effects of Lipoxin A4 administration after spinal cord injury in mice.

- To evaluate the effects of Lipoxin A4 treatment in the resolution of inflammation after spinal cord contusion injury.

- To study whether Lipoxin A4 reduces secondary tissue damage and functional impairments after spinal cord injury.

Chapter 5. Effects of Maresin1 treatment in combination with delayed administration of Interleukin-4 after spinal cord contusion injury in mice.

- To evaluate whether combination of Maresin1 and interleukin-4 treatment results in therapeutic additive effects after spinal cord injury.

METHODOLOGY

METHODOLOGY

All the experimental procedures were approved by the Universitat Autònoma de Barcelona Animal Experimentation Ethical Committee (CEEAH 1188R3-DMAH 6131) and followed the European Communities Council Directive 2010/63/EU, and the methods for each procedure were carried out in accordance with the approved guidelines.

Spinal cord injury

Mice were housed in standard cages and feed ad libitum with a light-dark cycle of 12h. Adult (8-10 weeks old) female C57Bl/6 mice (Charles River) were deeply anesthetized with intraperitoneal injection of ketamine (90 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.), and the back of mice were shaved and disinfected with povidone iodine solution. A longitudinal incision was made and adipose tissue and muscle were removed in order to expose the vertebral column at the 11^{th} thoracic vertebrae level. Laminectomy was performed to expose the spinal cord.

Spinal cord contusion was performed using the Infinite Horizon Impactor Device (Precision Scientific Instrumentation, Fairfax Station, VA) (Figure 1). This instrument enables the application of standard-force injuries to the spinal cords of small rodents. Force levels are user-selectable between 30 and 300 kDynes, allowing to perform graded magnitudes of injury. Displacement produced to the spinal cord as a consequence of the impact is also registered. To induce injury the spinal column is rigidly stabilized with the aid of two Adson forceps attached to two articulated support arms and a contusive tip is placed in the middle of the spinal cord at selected spinal cord level. In this study contusion injury at the thoracic T11 level were performed by applying a force of 60 ± 5 kdynes and 500-700 µm in displacement producing a severe injury. These injuries leads to functional deficits below the level of the lesion but permits a partial recovery of hind limb locomotor function and leaves some intact tissue at the lesion epicenter allowing to detect changes produced by different treatments.

Figure1. Horizon impactor device.

Drug administration

Intravenous injection: One hour after injury, 100 µl of sterile saline or sterile saline containing 0,5, 1 or 2 µg of lipoxin A4, resolving D1 or maresin 1 (Cayman Chemical Company, Ann Arbor, MI) was injected intravenously and then repeated daily thereafter until day 7.

Intraspinal injection of IL-4: Intraspinal injections were performed using a glass micropipette (30 µm internal diameter, Eppendorf, Hamburg, Germany) coupled to a 10 µL Hamilton syringe (Hamilton #701, Hamilton Co, Reno, NV, USA). 1µl of saline or recombinant mouse IL-4 (100ng/µl) (eBioscience) was injected into the injured spinal cord at the lesion site. Injections were made at a perfusion speed of 2 µl/min controlled by an automatic injector (KDS 310 Plus, Kd Scientific, Holliston, MA, USA), and the tip of the needle was maintained inside the cord tissue 3 min after each injection to avoid liquid reflux.

Behavioral assessment

After injury, spontaneous locomotor recovery of mice was assessed by using the **Basso Mouse Scale (BMS)** (Table 1). BMS is a widely used 9-point scale in which two blinded observers score the animal's motor performance. Animals are allowed to move freely in a circular open field (90 cm diameter x 24 cm wall height) for 5 minutes, and then the consensus score is taken. When the animal is able to step frequently (5 points), a sub-score of 11-point scale is performed in parallel, in order to assess fine aspects of the locomotion (Table 2). BMS was performed prior to surgery (day 0) and at 1, 3, 5, 7, 10, 14, 21 and 28 days after injury.

0	No ankle movement
1	Slight ankle movement (less than 90°)
2	Extensive ankle movement (more than 90°)
3	Plantar placing of the paw with or without weight support of the body
4	Occasional plantar stepping
5	Frequent (> 50%) or consistent (less than 5 missed steps) plantar stepping, *no* coordination (between fore and hind limbs) **or** Frequent or consistent plantar stepping, *some* coordination and paws *rotated* at initial contact and lift off phases of the step
6	Frequent or consistent plantar stepping, *some* coordination and paws *parallel* (to the body) at initial contact of the step **or** Frequent or consistent plantar stepping, *mostly* coordinated and paws *rotated* at initial contact and lift off phases of the step
7	Frequent or consistent plantar stepping, *mostly* coordinated and paws *parallel* at initial contact and *rotated* at lift off phase of the step **or** Frequent or consistent plantar stepping, *mostly* coordinated, paws *parallel* at initial contact and lift off phases of the step, and *severe* trunk instability (lean or sway of the trunk)
8	Frequent or consistent plantar stepping, *mostly* coordinated, paws *parallel* at initial contact and lift off phases of the step, and *mild* trunk instability **or** Frequent or consistent plantar stepping, *mostly* coordinated, paws *parallel* at initial contact and lift off phases of the step, *normal* trunk stability, and tail *down* or *up & down*
9	Frequent or consistent plantar stepping, *mostly* coordinated, paws *parallel* at initial contact and lift off phases of the step, *normal* trunk stability, and tail *always* up (normal mouse locomotion).

Table 1. Scores for the Basso Mouse Scale (BMS), used in the evaluation of spontaneous locomotor recovery after spinal cord injury in mice.

Plantar stepping (score both paws)	Frequent	0
	Consistent	1
Coordination	None	0
	Some	1
	Most	2
Paw position (score both paws)	Rotated thru out	0
	Parallel and rotated	1
	Parallel thru out	2
Trunk instability	Severe	0
	Mild	1
	Normal	2
Tail	Down	0
	Up & Down	0
	Up	1

Table 2. Basso Mouse Scale (BMS) subscores, used to complement BMS scores. This scale evaluates more fine aspects of locomotion. Maximal score of 11 points.

Additional computerized assessment of locomotion was also performed at the end of the follow-up (day 28 post-injury) using the **DigiGait™ Imaging System** (Mouse Specifics Inc., Boston, MA). This system is constituted of a motorized transparent treadmill belt and a high-speed digital video camera that performs images to the underside of the walking animals. DigiGait™ software generates "digital pawprints" and dynamic gait signals, representing the temporal record of paw placement relative to the treadmill belt. This locomotor test allows for an easy and objective analysis of both static and dynamic locomotor parameters. DigiGait™ Imaging System device has also been used to evaluate the maximum speed at which animals were able to run in a forced locomotion situation. The device is provided with a motorized treadmill belt that allows the user to set a constant speed ranging from 0 to 99 cm/s. Animals were placed on the treadmill belt and allowed to explore the compartment for 5 min. Then, speed was gradually increased and stopped at the maximum speed at which animals were able to perform a minimum of 3 followed steps.

Electrophysiological tests

Electrophysiological tests were used to quantitatively assess central motor conduction after SCI. After lesion, few white matter is preserved at the epicenter of the lesion, as we can observe by histological analyses. However, motor evoked potentials (MEPs) are effective to directly quantify the functionality of those spinal tracts that are still preserved.

MEP monitoring requires transcranial electric stimulation of the motor cortex to produce a descending response that traverses the spinal cord tracts and peripheral nerves to eventually generate a measurable response in the form of muscle activity. The wave of depolarization

often only activates 4-5% of the corticospinal tract, which will descend from the motor cortex to the brainstem, where most fibers (90%) will cross the midline, descending in the contralateral spinal cord (Figure 2).

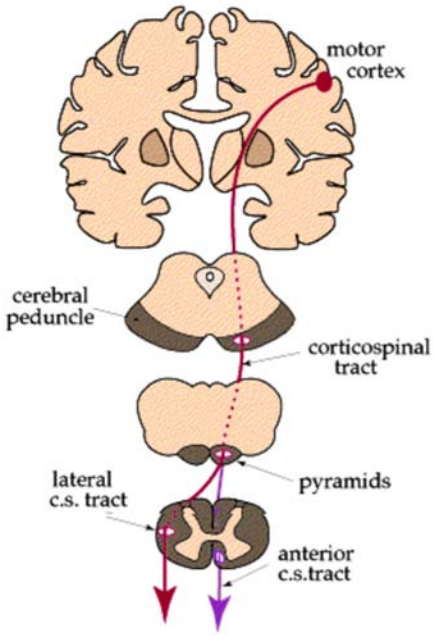

Figure 2. The pyramidal system, constituted by the corticospinal tract, is the basic motor pathway responsible for the voluntary movements of the body. It begins with the large pyramidal neurons of the motor cortex, and travels through the pyramids of the brainstem, where most fibers (90%) will cross over to the opposite side in the pyramidal decussation, and descend through the lateral funiculus of the contralateral spinal cord (lateral corticospinal tract). The remaining fibers (10%) will remain uncrossed and descend into the ipsilateral spinal cord (anterior corticospinal tract). An 8% of these fibers will cross the midline at the level they leave the spinal cord.

Conduction test were carried out at 28 days after injury, in order to detect differences between central conduction preservation on control and treated animals. Animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p., Sigma) and placed prone over a warmed flat coil controlled by a hot water circulating pump to maintain body temperature. Compound muscle action potentials (CMAPs) and MEPs from the tibialis anterior and gastrocnemius muscles were registered. In this case, the lesion does not affect peripheral nerve conduction. However, CMAPs were recorded for several reasons: for internal control of peripheral normal conduction, for normalization of MEPs values by individual's own CMAP for the same muscle, and for ensure right position of electrodes before MEPs register.

Central conduction. For evaluation of the motor central pathways, MEPs were elicited by transcranial electrical stimulation of the motor cortex by single rectangular pulses of 0.1 ms duration. Needle electrodes were inserted subcutaneously over the skull; the cathode overlaying the sensorimotor cortex and the anode at the nose. Recording electrodes were not moved after positioning for CMAP register. Acceptable MEP responses were polyphase with a consistent latency. Latency and maximum amplitude were taken (Figure 3).

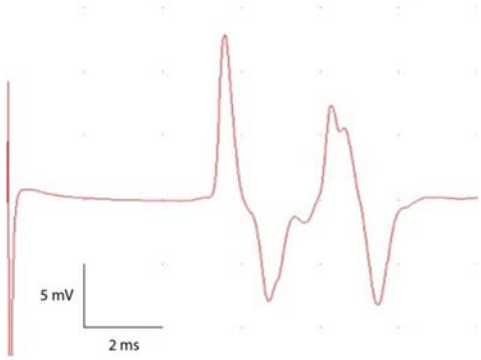

Figure 3. Representative recordings of a motor evoked potential (MEP) from tibialis anterior muscle of an intact mouse.

Histological assessment

Mice were deeply anaesthetized using Dolethal (pentobarbital sodium; Vétoquinol E. V. S. A.) and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Seven mm length of spinal cord containing the injection or the lesion site centered was harvested, post-fixed for 1 hour in 4% paraformaldehyde in 0.1 M PB and cryoprotected with 30% sucrose in 0.1 M PB at 4ºC, for a minimum of 48h. Spinal cords were fast-frozen at -60ºC in cryoembedding compound (Tissue-Tek® OCT, Sakura) and cut on a cryostat (Leica). Ten series of 10-µm-thick transversal sections were picked up on glass slides, so adjacent sections on the same slide were 100 µm apart.

Luxol Fast blue (LFB)

Tissue sections were placed in a hot plate during 15 min and then were rehydrated in PBS and gradually dehydrated in 50%, 70%, 80% and 95% ethanol. After that, sections were placed in a 1 mg/ml LFB solution in 95% ethanol and 0.05% acetic acid overnight at 37ºC. After incubation with LFB sections were sequentially washed with ethanol 95%, distilled water and placed during 1 minute into a solution of 0.5 mg/ml Li_2CO_3 and finally washed with distilled water, dehydrated and mounted in DPX mounting media (Sigma).

Fluoromyelin

Briefly, tissue sections were rehydrated in PBS and incubated with FluoroMyelin Green fluorescent myelin stain (1:300) (Invitrogen) in PBS for 20 min at room temperature. Then sections were washed and mounted in Mowiol mounting media containing DAPI (1 µg/ml; Sigma).

Fluorescent immunohistochemistry

Frozen spinal cord tissue sections were placed in a hotplate at 37ºC for 15 minutes and were

rehydrated in PBS and blocked with 5% FBS in PBST (blocking buffer, BB) for 1h at room temperature (RT). Sections were then incubated overnight at 4ºC with primary antibodies (Table 3) diluted in BB. After several washes in PBST, samples were incubated with secondary antibody bind to fluorocrom alexa-594 or alexa-488 (1:500 or 1:200 respectively, Invitrogen) for 1 hour at RT. Finally, sections were washed with PBST, PBS and PB and coverslipped in Mowiol mounting media containing DAPI (1 µg/ml; Sigma).

Antigen	Dilution	Manufacturer
Glial fibrilary acidic protein (GFAP)	1:500	Wako
Ionized calcium binding adaptator molecule (iba-1)	1:1000	Wako
Neuronal Nuclei (NeuN)	1:100	Millipore
Neurofilament (Nf)	1:1000	Millipore
Myelin Basic Protein (MBP)	1:100	Abcam

Table 3. List of primary antibodies with the correspond dilution and manufacturer.

For **histopathological analyses**, tissue sections were viewed with olympus BX51 microscope and images were captured using an olympus DP50 digital camera attached to it and using the Cell^A Image acquisition software. The NIH ImageJ (NIH, Bethesda, MD) software was used to quantify all the histological parameters.

- Determination of injury epicenter. Epicenter of intraspinal injection or injury impact was determined for each mouse spinal cord by localizing the tissue section with less tissue sparing along the spinal cord, regarding GFAP-positive staining or by localizing the tissue section with the greatest damage using LFB stained section.
- Quantification of tissue and myelin sparing after SCI. Tissue and myelin sparing were calculated by delineating total GPAP- and LFB-stained areas, respectively, on each spinal cord section. Quantifications were done at the epicenter and in sections every 200 µm until 1600 µm at both, rostral and caudal sides of the epicenter (17 sections per mouse). Myelin loss also was assessed using FluoroMyelin Green fluorescent myelin stain.
- Quantification of neuronal survival. Neuronal survival was assessed by manually counting $NeuN^+$ cells in the ventral horn of spinal cord slices corresponding to the epicenter, and every 200 µm until 1600 µm at both, rostral and caudal sides of the epicenter.

- Quantification of axonal sparing. Axonal sparing was calculated by counting the number of axons (Nf+) in the dorsal column at the injury epicenter, the most damage area of the spinal cord. The same sections were used to examine axonal demyelination in the dorsal column was by counting the fibers double stained for NF and MBP at the lesion epicenter.

Microarray

Uninjured control mice, and contused animals treated with different treatments were euthanized with Dolethal (pentobarbital sodium, Vetoquinol; 0.01ml/10g, intraperitoneal) at 1 and 7 days post-injury, and perfused with saline buffer. The segment of the spinal cord containing epicenter lesion was harvested and rapidly frozen and storage at -80ºC until mRNA extraction. For mRNA extraction, spinal cord was homogenized with Qiazol lysis reagent (Qiagen) and mRNA was extracted using RNeasy Lipid Tissue kit (Qiagen), according to the user's guide protocol. An additional step with DNase I digestion (Qiagen) was included to avoid genomic DNA contamination.

The microarray hybridation and the statistical processing of data were performed by the Scientific and Technical Support Unit and Statistics and Bioinformatics Unit at the Vall D'Hebron Research Institut (Hospital de la Vall d'Hebron, Barcelona). RNA samples were processed for Affimetrix MOUSE Exon/Gene 2.1 ST chip array according to the manufacturer protocol.

Real-time PCR

Uninjured and contused mice undergoing SCI were were euthanized with Dolethal (pentobarbital sodium, Vetoquinol; 0.01ml/10g, intraperitoneal), perfused with sterile saline and spinal cords were harvested at different time points (1 and 7 days post injury). Tissue was homogenized with QIAzol lysis reagent (Qiagen) and RNA extracted using RNeasy Lipid Tissue kit (Qiagen) as described above. 1 µg of obtained RNA was primed with random hexamers (Promega) and reverse transcribed using Omniscript RT kit (Qiagen). RNase inhibitor (Roche) was added (1 U/µl final concentration) to avoid RNA degradation. Quantitative RT-PCR analysis was performed using a MyiQ Single-Color Real-Time PCR Detection System (BIO RAD). RT-PCR reactions were performed using the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies) according to the manufacturer's instructions. The expression level of the target mRNA was normalized to the relative ratio of the expression of the GAPDH housekeeping gene.

Flow cytometry analysis

Immune cells from the injured spinal cord were analyzed by flow cytometry in different time points after SCI. Uninjured control mice, and contused animals were euthanized with Dolethal (pentobarbital sodium, Vetoquinol; 0.01ml/10g, intraperitoneal) and perfused with saline buffer. Briefly, spinal cords were cut in little pieces and mechanically dissociated through a cell strainer of 70 µm (BD falcon) and the cell suspension was centrifuged twice at 300g for 10 minutes at 4ºC. After cell counts, samples were divided, and cells alone and isotype-matched control samples were generated to control for auto-fluorescence and for nonspecific binding of antibodies. In the other samples, the following antibodies from eBioscience were used for extracellular staining (dilution 1:300) (table 4).

Antibodies	Antibodies	Antibodies	Antibodies
CD45-PerCP	CD3-FITC	Ly6C-FITC	IL-4Rα-APC (1:100 Miltenyi)
CD11b-PECy7 or PE	CD4-APC	Ly6G-PE	CD86-APC
F4/80-APC or PE	CD8-APC	CD16/32-PE	Rb-MHC-II unconjugated
Gr1-FITC	CD19-PE	CD206-FITC	Rb-CD11c unconjugated

Table 4. List of extracellular antibodies eBioscience 1:300.

After 30 min of incubation with combinations of antibodies at 4°C, the samples were washed and stained with Alexa488 or Alexa647 conjugated donkey secondary antibodies against rabbit (1:500 Molecular Probes) for 30 min when needed. Finally, the samples were washed and fixed in 1% paraformaldehyde.

For intracellular staining, cells were permeabilized with Permeabilization Wash Buffer (Biolegend) followed by staining with TGFβ-Alexa647 (1:200 Abcam), IL-10-Alexa488 (1:200 eBioscience) and unconjugated rabbit antibodies against 15-LOX (1:250 Bioss Biotechnology), COX-2 (1:250 Cayman Chemical), iNOS (1:200 Abcam) and goat antibodies against Arg1 (1:200 Santa Cruz). In the cases when intracellular cytokine staining was performed, cells were incubated with brefeldin-A (1:1000, Biolegend) at 37º for 4 hours before the incubation with the antibodies against cytokines. After 30 min of incubation with combinations of antibodies at 4°C, cells were washed and stained with Alexa488 or Alexa647 conjugated donkey secondary antibodies against rabbit or goat (1:750 Molecular Probes) for 30 min. Finally, the samples were washed and fixed in 1% paraformaldehyde.

Cells were analyzed on a FACSCanto flow cytometer (BD Biosciences) and results analyzed

using FlowJo® software version 10.0.7. To perform the analysis, cells were first gated for CD45 to ensure that only infiltrating leukocytes and resident microglia are selected, and then, the following combination of markers were used to identify CD4 T-Cells ($CD45^{+}$, $CD11b^{-}$, $CD3^{+}$, $CD4^{+}$), CD8 T Cells ($CD45^{+}$, CD11b, $CD3^{+}$, $CD8^{+}$), B cells ($CD45^{+}$, $CD11b^{-}$, $CD3^{-}$, $CD19^{+}$), microglial cells ($CD45^{low}$, $CD11b^{+}$, $F4/80^{+}$), macrophages ($CD45^{high}$, $CD11b^{+}$, $F4/80^{+}$), and neutrophils ($CD45^{high}$, $CD11b^{+}$, $F4/80^{-}$, $Ly6G^{+high}$). In addition microglia and macrophages were further differentiated based Ly6C, CD16/32, iNOS, CD206 and Arg1 expression. Since cell-associated fluorescence varies between the different immune cell subsets, the cut offs expression were defined based on isotype controls for each of the immune cell subsets and sample.

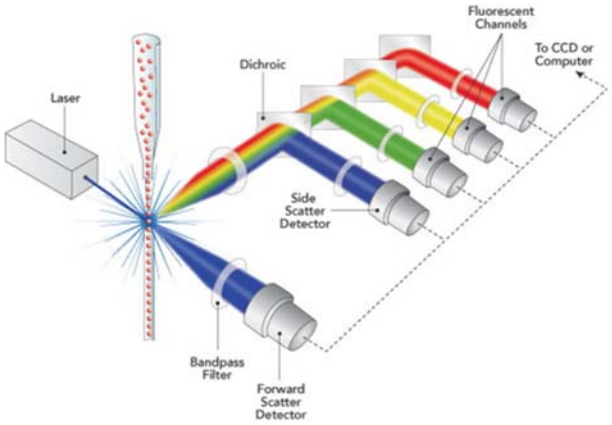

Figure 4. Schematic illustration of a Flow Cytometer

Assessment of cytokine protein levels

Control animals or contused animals treated with saline or SPMs were perfused with sterile saline and a 5 mm length of spinal cord centered on the lesion was collected at 12 and 24h after contusion injury and snap-frozen. Samples were prepared for protein extraction and homogenized in protein extraction buffer (25 mM HEPES; IGEPAL 1 %; 0,1 M MgCl2; 0,1 M EDTA pH=8; 0,1 M EGTA pH=8; 0,1 M PMSF adding 10 µl/ml of Protease Inhibitor cocktail (Sigma) and PhosphoSTOP phosphatase inhibitor cocktail (Roche)). Protein concentration was determined using the DC Protein Assay (Bio-Rad). Samples were concentrated to 4µg/µl using MicroCon centrifugation filters (Millipore) to ensure equal amounts of protein Low concentrations of cytokines in the sample result in binding to the filters whereas high concentrations of protein sustain fewer losses. The protein levels of cytokines and chemokines were then analyzed using the Milliplex MAP Mouse Cytokine/Chemokine magnetic bead panel (Millipore) on a Luminex (Millipore) as per manufacturers' protocol.

Western blotting

For protein extraction, contused animals were euthanized with Dolethal (pentobarbital sodium, Vetoquinol; 0.01ml/10g, intraperitoneal) and perfused with saline buffer. The segment of the spinal cord containing epicenter lesion was harvested. Samples were prepared for protein extraction and homogenized in protein extraction buffer (25 mM HEPES; IGEPAL 1 %; 0,1 M MgCl2; 0,1 M EDTA pH=8; 0,1 M EGTA pH=8; 0,1 M PMSF, adding 10 µl/ml of Protease Inhibitor cocktail (Sigma) and PhosphoSTOP phosphatase inhibitor cocktail (Roche)).

To perform western blot, 30 µg of protein of each sample were loaded in SDS-poliacrylamide gels and transferred onto PVDF membranes (Millipore). The transfer buffer was 25 mM trizma-base, 192 mM glycine, 20% (v/v) methanol, pH 8.4. The membranes were blocked with 5% BSA in PBS plus 0.1% Tween-20 for 2 hours, and then incubated with primary antibodies at 4°C overnight. The primary antibodies used were rabbit antibodies against phospho NF-kB p65 (1:1000; Cell Signaling), against the phosphorylated form of STAT1 (1:500; Cell Signaling), STAT3 (1:500; Cell Signaling), STAT5 (1:500; Cell Signaling) and STAT6 (1:500; Cell Signaling), phospho JNK (1:500; Santa Cruz), phospho ERK1/2 (1:1000; Cell Signaling), phosphor p38 (1:1000; Cell Signaling) and phospho-AKT (1:1000; Cell Signaling). Horseradish peroxidase–coupled secondary antibody (1:3000, BioRad) incubation was performed for 1 hour at room temperature. ß-actin (1:10.000; Sigma Aldrich;) was used to ensure equal loading of samples. The membranes were visualized using enhanced chemiluminiscence method and the images were collected and analyzed with Chemidoc apparatus (BioRad) and ImageLab software (BioRad), respectively.

Lipidomic analysis

Inflammatory and specialized por-resolving lipid mediation derived from ω-6 PUFA and ω-3 PUFA were identified and quantified by liquid chromatography (LC)-tandem mass spectrometry (MS/MS). In brief, 400 pg class-specific deuterated (-d) internal standards (AA-d8, DHA-d5, PGE2-d4, lipoxin A4-d5, leukotriene B4-d4, 15-hydroxyeicosatetraenoic acid-d8) were added to each spinal cord sample prior to processing to calculate the recovery of specific classes of PUFA, LOX, and COX metabolites. Spinal cord homogenates (30–50 µL) containing internal standards were combined with 2 mL methanol, dried under a gentle stream of nitrogen, immediately resuspended in high-performance LC mobile, and placed in a refrigerated autosampler for lipidomic analysis. Eicosanoids and docosanoids were identified

and quantified by LC-MS/MS–based lipidomics based on published methods.30,38 Processed tear samples were analyzed by a triple-quadrupole linear ion trap LC-MS/MS system (MDS SCIEX 3200 QTRAP; Applied Biosystems, Foster City, CA, USA) equipped with a Kinetex C18 mini-bore column (Phenomenex, Torrance, CA, USA). The mobile phase was a gradient of water/acetonitrile/acetic acid (72:28:0.01, vol:vol:vol) and isopropanol/acetonitrile (60:40, vol:vol) with a 450-µL/min flow rate. Tandem MS/MS analyses were performed in negative ion mode, and prominent fatty acid metabolites were quantified in multiple reaction monitoring mode using established and specific transitions as previously described.30,31,38–41 Calibration curves (1–1000 pg) and specific LC retention times for each compound were established with synthetic standards (Cayman Chemical, Ann Arbor, MI, USA). Structures were confirmed for selected autacoids by MS/MS analyses using enhanced product ion mode with appropriate selection of the parent ion in quadrupole 1.

RESULTS

CHAPTER 1

IL-4 drives microglia and macrophages towards a phenotype conducive for tissue repair and functional recovery after spinal cord injury

IL-4 drives microglia and macrophages towards a phenotype conducive for tissue repair and functional recovery after spinal cord injury

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ABSTRACT

Macrophages and microglia play a key role in the maintenance of nervous system homeostasis. However, upon different challenges, they can adopt several phenotypes, which may lead to divergent effects on tissue repair. After spinal cord injury (SCI), microglia and macrophages show predominantly pro-inflammatory activation and contribute to tissue damage. However, the factors that hamper their conversion to an anti-inflammatory state after SCI, or to other protective phenotypes, are poorly understood. Here, we show that inefficient induction of IL-4 after SCI favors microglia and macrophages to remain in a pro-inflammatory state. We also demonstrate that a single delayed administration of IL-4 48 hours after SCI redirects microglia and macrophages towards an anti-inflammatory/pro-repair phenotype. We also reveal that delayed injection of IL-4 leads to the appearance of resolution-phase macrophages and enhances resolution of inflammation. Interestingly, we provide clear evidence that delayed administration of IL-4 markedly improves functional outcomes and reduces tissue damage after contusion injury. These findings show that lack of sufficient amounts of IL-4 in the contused spinal cord prevents microglia and macrophages from acquiring a phenotype that is conducive for tissue repair. These data suggest that therapies aimed at increasing IL-4 levels could be valuable for the treatment of acute SCI, for which there is no effective treatments.

Keywords: IL-4, Macrophage, Neuroprotection, Polarization, Spinal Cord injury.

INTRODUCTION

SCI elicits an inflammatory response that comprises mainly of microglia and peripheral blood-derived macrophages (Alexander and Popovich 2009; David et al. 2012). These cells, which remain chronically in the spinal cord in humans after injury (David et al. 2012; Fleming et al. 2006), contribute directly or indirectly to tissue damage and functional loss in SCI, as well as, in other central nervous system disorders. However, they can promote repair in other experimental paradigms (Alexander and Popovich 2009; David et al. 2012; Mills et al. 2000; Popovich and Longbrake 2008; Yong and Rivest 2009). These paradoxically conflicting actions of microglia and macrophages may depend on their activation state and the signals in the lesion milieu. In response to IFNγ and/or LPS, macrophages undergo "classical" M1 activation (David and Kroner 2011; Dey et al. 2014; Gordon and Martinez 2010). However, upon IL-4 and IL-13 stimulation, they acquire "alternative" M2 differentiation (David and Kroner 2011; Dey et al. 2014; Gordon and Martinez 2010; Mills et al. 2000; Murray et al. 2014). M1 macrophages release high levels of pro-inflammatory cytokines and free radicals that are crucial for killing microbes and tumor cells, but also induce damage in healthy neighboring tissue, contributing to cell loss and pathology (David and Kroner 2011; Dey et al. 2014; Gordon and Martinez 2010). M2 macrophages, display anti-inflammatory features, and are involved in parasite containment, tumor progression, as well as, in promoting tissue repair (David and Kroner 2011; Dey et al. 2014; Gordon and Martinez 2010). Concerns have been raised recently about such a classification as macrophages in tissues are influenced by multiple factors that can influence their polarization state (Murray et al. 2014). The concept of a pro-inflammatory/M1-like phenotype and an anti-inflammatory/M2-like phenotype, however, is a useful tool to probe for responses to injury (Murray et al. 2014). Indeed, different macrophage subsets have been identified in several pathologies, including $Ly6C^{high}$ and $Ly6C^{low}$ monocytes (Arnold et al. 2007), tumor-associated macrophages (Noy and Pollard 2014) and resolution-phase macrophages (Bystrom et al. 2008), among others.

Microglia and macrophages display a predominantly pro-inflammatory/M1-like phenotype after SCI (Kigerl et al. 2009; Kroner et al. 2014). However, there is currently limited information about the factors that impede these cells to induce the expression of pro-repair M2-like markers in SCI. This is likely related to the post-injury milieu of the spinal cord, since M2 macrophages are rapidly lose such markers when transplanted into the contused spinal cord (Kigerl et al. 2009). A recent report reveals that phagocytosis of erythrocytes, which occurs between day 7 and 14 after SCI, prevents macrophages to adopt a pro-repair/M2-like

polarization via release of TNFα (Kroner et al. 2014). There is also evidence that TNFα can prevent the myelin phagocytosis-induced shift of M1 macrophages to M2 (Kroner et al. 2014). However, the existance of mechanisms occuring at early stages after SCI that may hamper the convertion of microglia and macrophages from pro-inflammatory towards a phenotype that is more conducive for tissue repair has yet to be elucidated. Moreover, it is also not known whether effective induction of such a phenotype in microglia and macrophages in the injured spinal cord attenuates functional deficits and tissue damage. Answers to these questions are highly relevant to advancing therapeutics to treat SCI in humans.

In the present work, we demonstrate that IL-4 levels expression are undetected after SCI. We reveal that a single delayed administration of IL-4 is sufficient to induce the expression of M2 markers in microglia and macrophages, as well as, to the appearance of a macrophage subset that has a phenotype compatible with resolution-phase macrophages. Finally, we show that such changes in microglia and macrophage phenotype triggered by delayed IL-4 confer protection against tissue and functional loss, indicating that IL-4 redirects microglia and macrophages from pro-inflammatory/cytotoxic to an anti-inflammatory/pro-repair state.

MATERIALS AND METHODS

All the experimental procedures were approved by the Universitat Autònoma de Barcelona Animal Experimentation Ethical Committee (CEEAH 1188R3-DMAH 6131) and followed the European Communities Council Directive 2010/63/EU, and the methods for each procedure were carried out in accordance with the approved guidelines.

Surgical procedure

Adult (8-10 weeks old) female C57Bl/6 mice (Charles River) were anesthetized with ketamine (90 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.). After performing a laminectomy at the 11th thoracic vertebrae, the exposed spinal cord was contused with a force of 50 kdynes using the Infinite Horizon Impactor device (Precision Scientific Instrumentation) (Klopstein et al. 2012). 15 minutes or 48 hours after SCI, 1µl of saline or saline containing 100 ng of recombinant mouse IL-4 (eBioscience) was injected into the injured spinal cord at the lesion site by means of a glass micropipette (30 µm internal diameter, Eppendorf, Hamburg, Germany) coupled to a 10 ml Hamilton syringe (Hamilton #701, Hamilton Co, Reno, NV, USA). 100ng of IL-4 was

chosen based on a recent report from our laboratory using another anti-inflammatory cytokine (Coll-Miro et al. 2016). Injections were made at a perfusion speed of 2 µl/min controlled by an automatic injector (KDS 310 Plus, Kd Scientific, Holliston, MA, USA), and the tip of the needle was maintained inside the cord tissue 3 min after each injection to avoid liquid reflux.

Cytokine Protein Expression

Adult female C57/Bl6 mice were perfused with sterile saline and a 5 mm length of intact or contused spinal cord was collected at 1, 6, 12 hours and at 1, 3, 7, 14 and 28 days after contusion (n=4 each group and time point) and snap-frozen. Spinal cords were homogenized and protein concentration was determined using the DC Protein Assay (Bio-Rad). Samples were concentrated to 4µg/µl using MicroCon centrifugation filters (Millipore) to ensure equal amounts of protein. Cytokine protein levels were then analyzed using the Milliplex MAP Mouse Cytokine/Chemokine magnetic bead panel (Millipore) on a Luminex (Millipore) as per manufacturers' protocol.

Flow Cytometry

Immune cells from the injured spinal cord were analyzed by flow cytometry. Briefly, spinal cords were cut in little pieces and mechanically dissociated through a cell strainer of 70 µm (BD falcon) and the cell suspension was centrifuged twice at 300g for 10 minutes at 4ºC. The following antibodies rat conjugated antibodies were used: CD45-PerCP (1:150 eBioscience), CD11b-PE-cy7 (1:150 eBioscience), Ly6G-PE (1:150 eBicoscience), F4/80-APC or PE, (1:150 eBioscience), CD16/32-PE (1:100 eBioscience) CD206-FITC (1:100 eBioscience), IL-4Rα-APC (1:100 Miltenyi Biotec) MHC-II (1:100 eBioscience), CD11c (1:100 eBioscience), TGFβ-Alexa647 (1:100 Abcam), IL-10-Alexa488 (1:100 eBioscience) and unconjugated rabbit antibodies against 15-LOX (1:100 Bioss Biotechnology), COX-2 (1:100 Cayman Chemical), iNOS (1:100 Abcam) and goat antibodies against Arg1 (1:100 Santa Cruz). After 30 min of incubation with combinations of antibodies at 4°C cell were then fixed in 1% paraformaldehyde. For intracellular staining, cells were permeabilized with Permeabilization Wash Buffer (Invitrogen) and followed by staining with Alexa488 or Alexa647 conjugated donkey secondary antibodies against rabbit or goat (1:500 Molecular Probes) for 30 min when needed. When intracellular cytokine staining was performed, cells were incubated with brefeldin (1:1000, Biolegend) at 37º for 4 hours before the incubation with the antibodies against cytokines. Cells were analyzed on a FACSCanto flow cytometer (BD Biosciences) and results analyzed using FlowJo® software

version 10.0.7.

To perform the analysis, cells were first gated for CD45 to ensure that only infiltrating leukocytes and resident microglia are selected, and then, the following combination of markers were used to identify microglia ($CD45^{low}$, $CD11b^{+}$, $F4/80^{+}$), macrophages ($CD45^{high}$, $CD11b^{+}$, $F4/80^{+}$), and neutrophils ($CD45^{high}$, $CD11b^{+}$, $F4/80^{-}$, $Ly6G^{+}$). Since cell-associated fluorescence varies between the different immune cell subsets, the cut offs expression were defined based on isotype controls for each of the immune cell subsets and sample.

Functional assessment

Locomotor recovery was evaluated at 1, 3, 5, 7, 10, 14, 21 and 28 days post-injury (dpi) in an open-field test using the nine-point Basso Mouse Scale (BMS), which was specifically developed for locomotor testing after contusion injuries in mice (Basso et al. 2006). The BMS analysis of hindlimb movements and coordination was performed by two independent assessors and the consensus score taken. In addition, at the end of the follow up (day 28 post-injury) the highest locomotion speed of the mice was evaluated on a belt of a motorized treadmill. Briefly, each mouse was allowed to explore the treadmill compartment, with the motor speed set to zero, for 5min. Then speed was gradually increased from 0 up to 35 cm/s and the maximum speed at which each mouse performed for at least 5 seconds was recorded (Santos-Nogueira et al. 2015).

At day 28, electrophysiological tests were used to evaluate spared motor central pathways after SCI. Motor evoked potentials (MEPs) were recorded from the *gastrocnemius* muscle (GM) with microneedle electrodes, in response to transcranial electrical stimulation of the motor cortex by single rectangular pulses of 0.1 ms duration. Pulses were delivered through needle electrodes inserted subcutaneously, the cathode over the skull overlying the sensoriomotor cortex and the anode at the nose (Santos-Nogueira et al. 2015). All potentials were amplified and displayed on a digital oscilloscope (Tektronix 450S)

Histology

At 28 days post-injury mice were perfused with 4% paraformaldehyde in 0.1M-phosphate buffer (PB) at 12h, 3 and 28 dpi. A 5mm length of spinal cord containing the lesion site was removed, cryoprotected with 30% sucrose in 0.1M PB at 4ºC, and 6 series of 10µm thick section were picked up on glass slides. Adjacent sections on the same slide were therefore

100µm apart. For demyelination analyses, sections were stained with Luxol fast blue (LFB) (Sigma). After graded dehydration, sections were placed in a 1 mg/ml LFB solution in 95% ethanol and 0.05% acetic acid overnight at 37ºC. Sections were then washed in 95% ethanol and distilled water before place them into a solution of 0.5 mg/ml Li2CO3 in distilled water for 1 min at RT. After washes in distilled water, sections were dehydrated and mounted in DPX mounting media (Sigma-Aldrich). For neuronal assessment, sections were incubated overnight at 4ºC with biotinilated antibodies against NeuN (1:500; Millipore). After several washes in PBS, sections were incubated with Alexa 594-conjugated streptoavidin, and then coverslipped in Mowiol mounting media (Sigma-Aldrich).

The epicenter of the injection or contusion injury impact was determined for each mouse spinal cord by localizing the tissue section with the greatest damage using LFB stained section. Myelin sparing after SCI was calculated by delineating the spared LFB stained tissue, whereas neuronal survival was assessed by counting the number of $NeuN^+$ cells in the ventral horns at the injury epicenter and at rostral and caudal areas. The NIH ImageJ software was used to quantify all the histological parameters.

Statistical analysis

All analyses were conducted with SPSS version19. Changes in the expression of M1 and M2 markers after spinal cord were analyzed by using one-way ANOVA with Tukey post-hoc comparisons. Functional follow-up for BMS score and subscore, histological analysis of myelin and neuronal sparing were analyzed using two-way repeated measure ANOVA with Bonferroni's correction for multiple comparison. Student t-test was used for single comparisons between two groups. Results are expressed as mean ± SEM. Differences were considered significant at $p < 0.05$.

RESULTS

Microglia and macrophages do not acquire anti-inflammatory phenotype after SCI

Previous reports describe that microglia and macrophages express predominantly M1 markers for the first 2 weeks after SCI, whereas the expression of M2 signatures is scarce (Kigerl et al. 2009; Kroner et al. 2014).

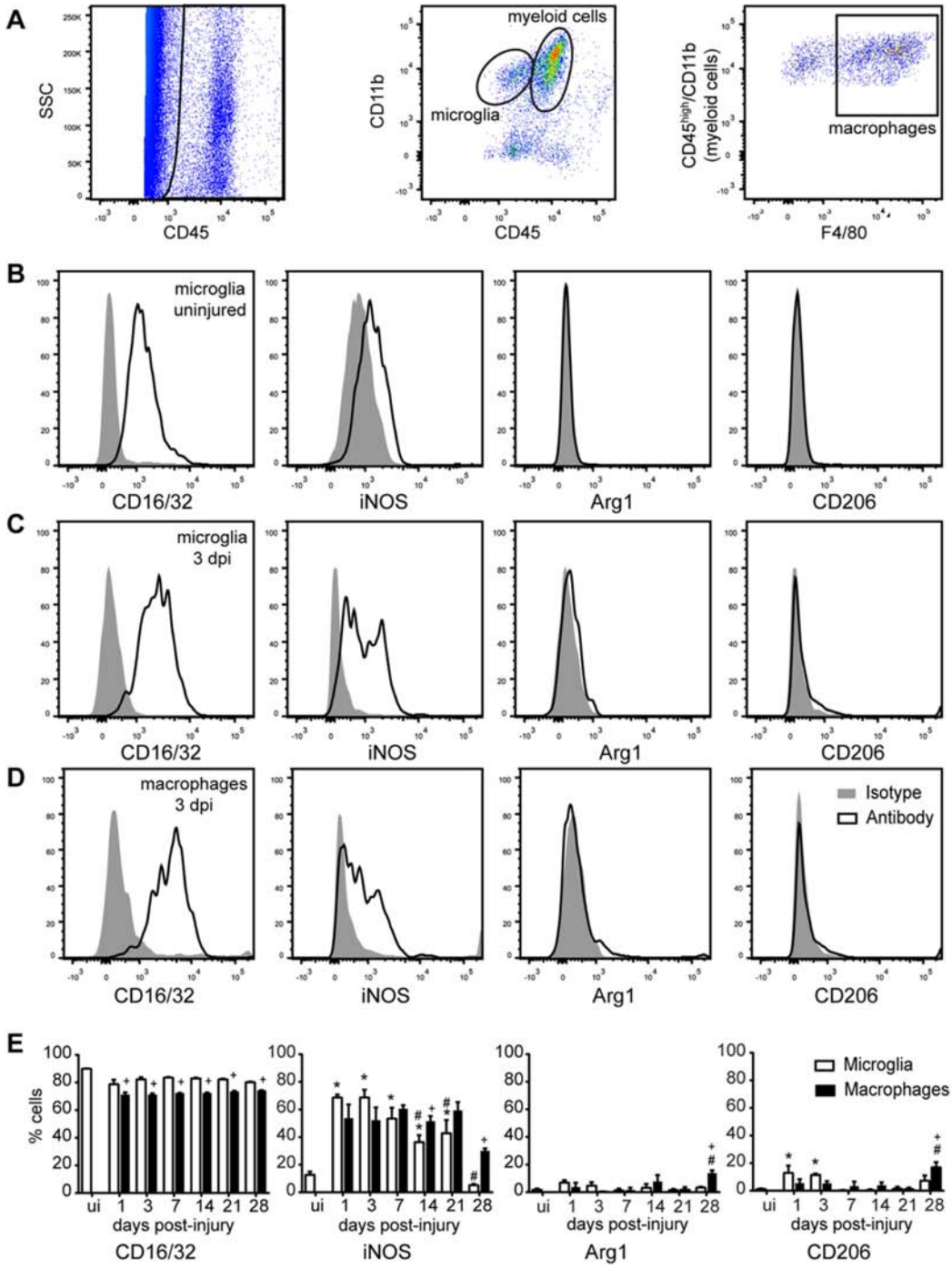

Figure 1. Microglia and macrophages show predominant expression of M1 markers after SCI. (**A**) Representative FACS dot plots of injured spinal cord showing how microglia and macrophages were gated. Note the CD45+ cells (panel I) were further gated in CD11b+ expression to distinguish between microglia ($CD45^{low}$ $CD11b^{+}$) and myeloid cells ($CD45^{high}$ $CD11b^{+}$) (panel II). Myeloid cells were further differentiated based on F4/80 expression to identify macrophages ($F4/80^{+}$) (panel III). (**B-D**) Representative FACS histograms plots of M1 and M2 markers in uninjured spinal cord for microglia (**B**) and of microglia (**C**) and macrophages (**D**) at 3 days after SCI. These plots show the labelling for different M1 and M2 markers, as well as, for their respective isotype controls. (E) Graphs showing the quantification of microglia and macrophages expressing M1 and M2 markers after SCI. Note the percentage of microglia and macrophages expressing M1 markers (CD16/32 and iNOS) is markedly higher as compared to the scarce expression of M2 markers (Arg1 and CD206). Mean ± SEM. (n=4 per group). *p<0.05 compared to microglia from uninjured spinal cord; # p<0.05 compared to day 1 post-injury; + p<0.05 compared to microglia at the same day post-injury. One-way ANOVA with Bonferroni's post hoc correction was performed for the analysis.

Here we first extended these observations using fluorescence-activated cell sorting analysis (FACS) to assess the temporal changes in expression of M1 (iNOS and CD16/32) and M2 (Arg1 and CD206) markers in microglia and macrophages for the first 4 weeks after spinal cord contusion injury in mice. We confirmed that these cells express mainly M1 markers after SCI, whereas the expression of M2 markers is restricted to a small population of microglia and macrophages (Figure 1A-E). However, the expression of the M1 markers did not showed a similar pattern. Most microglial cells and macrophages expressed CD16/32 for the entire 4 weeks period, however, this expression was more pronounced in microglia than macrophages (Figure 1E). The expression of iNOS, which was scarcely detected in microglial cells from uninjured spinal cords (~15%), was induced in ~70% of microglia and found in ~55% of macrophages for the first 3 days post-injury. However, we found that iNOS levels markedly dropped in microglia at day 28, when it reached basal levels, but also, although to lesser extent, in macrophages (Figure 1E).

These results suggest that microglia and macrophage express predominantly M1 markers during the first days after SCI, and fail to adopt an M2-like phenotype.

The lack of IL-4 expression in the injured spinal cord favors pro-inflammatory activation of microglia and macrophages

Since cytokines are one of main factors that regulate the functional phenotype of microglia and macrophage *in vitro* (Boche et al. 2013; David and Kroner 2011; Mantovani et al. 2013), we monitored the expression of the main cytokines in the spinal cord tissue following contusion lesion. We found that protein levels for most pro-inflammatory cytokines peaked between 6-12 hours post-injury (Figure 2). The reduction of cytokines was already evident at 24 hours post-injury, and most of them reached basal levels at day 3. A small number of cytokines, however, remained elevated up to day 28, although to much lower levels as compared to the first 12 hours post-injury (Figure 2). Interestingly, the protein levels of IL-4, one of the main M2 inducers, was undetected at any time point (Figure 2), which led us to hypothesize that the insufficient expression of IL-4 after SCI could account for the deficit of microglia and macrophages to adopt an M2-like phenotype.

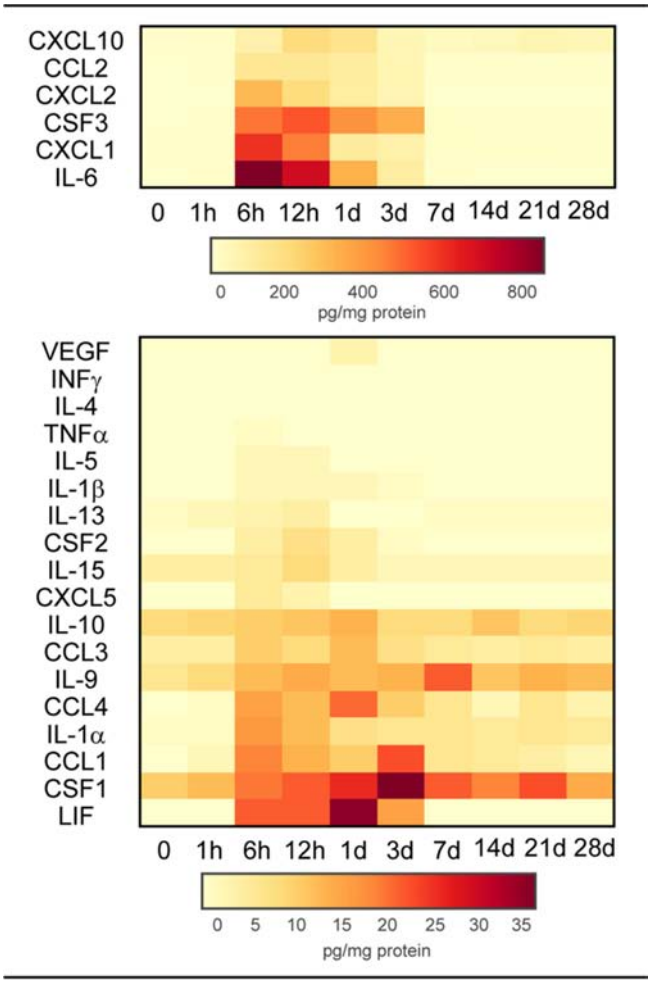

Figure 2. Dynamic changes in cytokine levels in the spinal cord after contusion injury. Heat map showing the changes in protein levels of relevant pro-inflammatory cytokines and anti-inflammatory in the injured spinal cord obtained by doing Luminex analysis. Mean ± SEM. (n=4 per time point).

To address this hypothesis, we first characterized the expression of IL-4Rα in the spinal cord by FACS analysis. We found that IL-4Rα was not expressed in the uninjured spinal cord spinal but was found after contusion injury (Figure 3A). Interestingly, we observed that ~90% of the IL-4Rα + cells are also CD45+, indicating the presence of this receptor is mainly restricted to microglia and infiltrating myeloid cells in the injured spinal cord. We then studied whether IL-4Rα was present in microglia and macrophages. Although microglia did not show constitutive expression of IL-4Rα, it was induced in ~55 % of microglia at 18 hours after injury, remaining at steady levels at day 2 (Figure 3B). In addition, peripheral macrophages, which migrate into the spinal cord after injury, showed similar proportion of IL-4Rα at the same time points (Figure 3B).

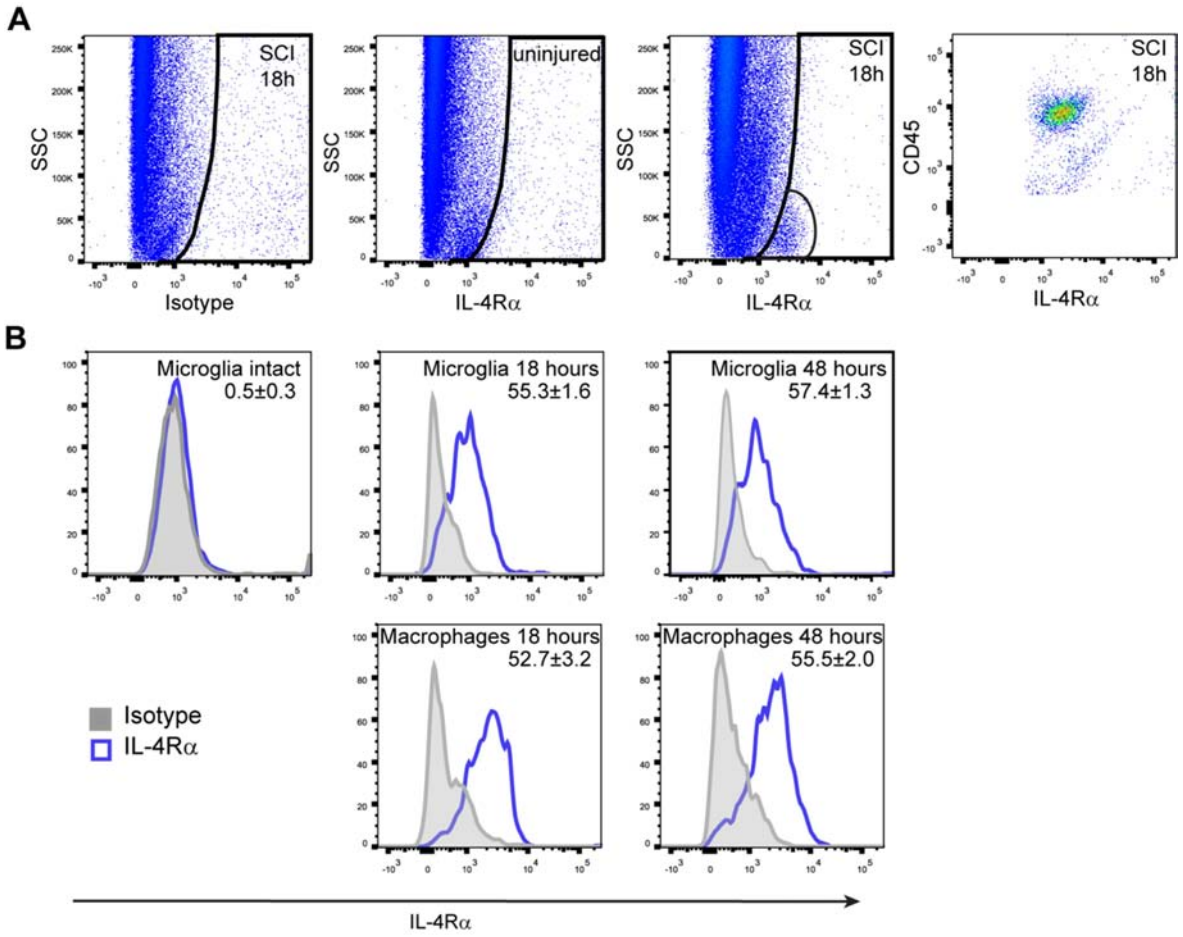

Figure 3. Expression of IL-4Rα after SCI. (**A**) Flow cytometry plots of showing the cut off of the isotype control (panel I), and the expression of IL-4Rα in the uninjured spinal cord (panel II), and at 18 post-injury (panel iii). Note that IL-4Rα is lacking in the uninjured spinal cord but it is rapidly detected after spinal cord injury, mostly in CD45+ cells (panel IV). (**B**) FACS plot histogram gated on the microglia and macrophage population (as shown in Fig. 1a) of intact and injured spinal cord at 18 hours and 48 hours post-injury. Note that IL-4Rα is not constitutively expressed in microglia, but it is found in >50% of microglia cells and infiltrated macrophages after SCI. Mean ± SEM (n=4 per time point).

Since IL-R4α was found in microglia and macrophages only after SCI, we then assessed the effects of increased levels of IL-4 in the contused spinal cord parenchyma, by injecting recombinant mouse IL-4 into the spinal cord, 15 minutes after contusion injury in adult mice. We observed that IL-4 induced the expression of Arg1 in ~30% of microglia and macrophages at day 3, but failed to modulate the levels of CD206 (Figure 4, Figure 5A). IL-4, however, did not reduce CD16/32 and iNOS expression in these cells (Figure 4, Figure 5A). This data shows that a single acute administration of IL-4 after SCI drives some aspects of M2 polarization in a small proportion of microglia and macrophages, indicating that the insufficient levels of this cytokine in the contused spinal cord contributes to hamper the conversion of microglia and macrophages towards a more pro-repair phenotype after SCI.

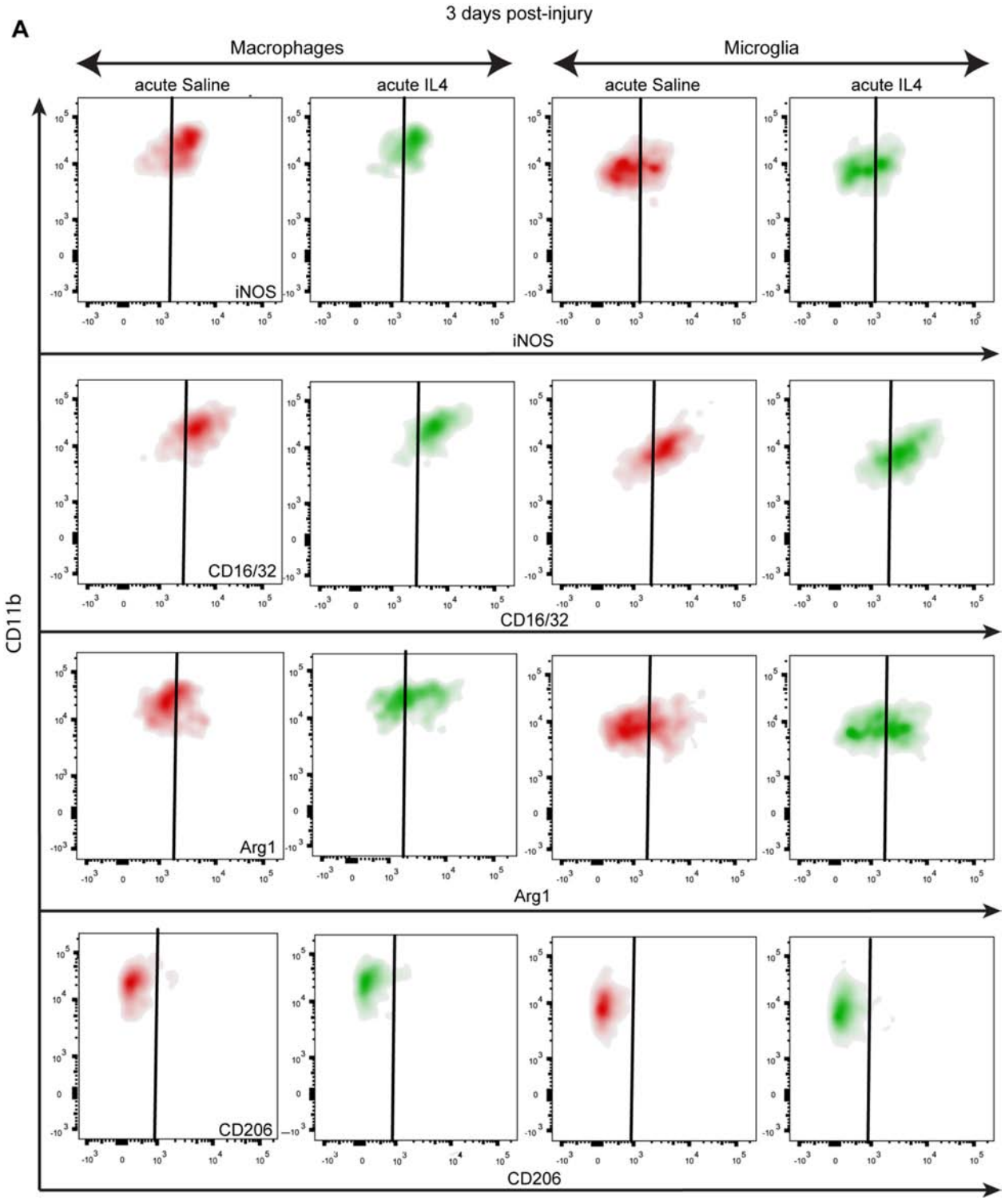

Figure 4. Acute administration of IL-4 induces Arg1 expression in microglia and macrophages at day 3 after SCI. (**A**) Flow cytometry density plots showing the effects of acute intraspinal injection of IL-4 in the expression of M1 markers (iNOS and CD16/32) and M2 markers (Arg1 and CD206) in microglia and macrophages at day 3 after SCI. Note that IL-4 increased the proportion of microglia and macrophages expressing Arg1, but did not modulate the expression of iNOS, CD16/32 or CD206.

Effects of delayed administration of IL-4 on microglia and macrophage phenotype

We hypothesized that the high levels of pro-inflammatory cytokines in the contused spinal cord within the first 6-12 hours, some of them being well known M1 inducers, as well as, the absence of IL-4R in the intact spinal cord, may have minimized the effects of *acute* administration of IL-4 (15 minutes after SCI) on the functional phenotype of microglia and macrophages. We therefore evaluated whether delaying the administration of IL-4 to 48 hours post-injury, when levels of most pro-inflammatory cytokines have markedly dropped and the expression of IL-4R is found in ~55% of microglia and macrophages (Figure 3B), would be more effective in inducing an anti-inflammatory/pro-repair phenotype. In contrast to Arg-1 being expressed in ~30% of microglia and macrophages after acute injection (15 minutes post-SCI), delaying the injection of IL-4 to 48 hours post-SCI, increased Arg-1 expression in ~55% macrophages and in about 35% of microglia (Figure 5B; Fig. S1). Moreover, in contrast to acute injection, delayed IL-4-administration, also induced the expression of CD206 in microglia and macrophages (~25%) (Figure 5B), indicating that delayed IL-4 treatment leads to rapid and more effective induction of M2 markers in a subset of microglia and macrophages.

We also found that the expression of CD16/32 is induced in microglia and macrophages after delayed injection of IL-4, whereas the expression of iNOS remained unaltered (Fig. 5B, Fig. S1).

Given previous reports showing that M2 macrophages lose Arg1 expression within 3 days after their transplantation into the injured spinal cord (Kigerl et al. 2009; Kroner et al. 2014), we assessed whether the responses of microglia and macrophages to delayed administration of IL-4 were acute or persisted for a few days. We found that 4 days post-injection (i.e., 6 days after SCI), the expression of Arg1 and CD206 in microglia and macrophages remained at similar levels as compared to day 1 post-injection (Figure 5C, Figure S2), indicating that the expression of M2 markers are maintained for up to 4 days. The expression of CD16/32 in microglia and macrophages, which was increased at day 1 post-delayed injection, was reduced back down to saline-injected mice, whereas the expression of iNOS was significantly reduced in both immune cell populations at 4 days after IL-4 injection (Figure 5C, Figure S2). These results therefore suggest that microglia and macrophages are more skewed toward anti-inflammatory/M2-like activation over time.

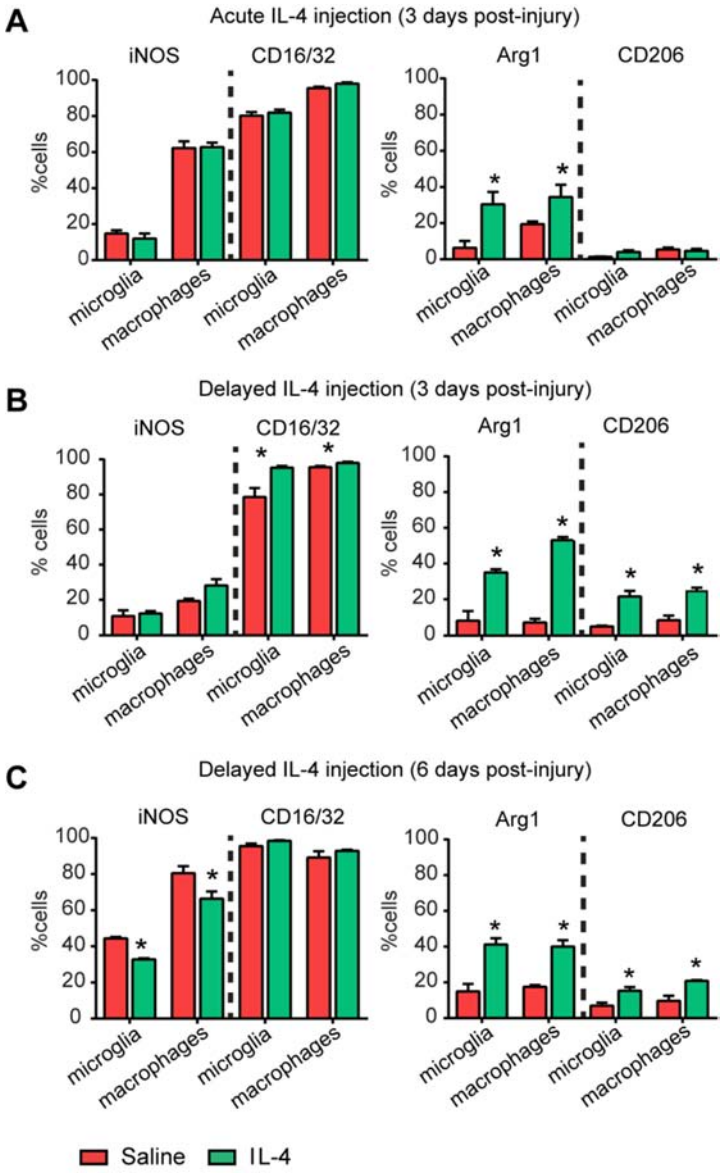

Figure 5. Delayed administration of IL-4 has greater effects in inducing M2 markers in microglia and macrophages than acute IL-4 injection. (**A-C**) Graphs showing the changes in the expression of M1 and M2 markers in microglia and macrophages at 3 days post-injury after acute IL-4 injection (**A**) or delayed IL-4 injection (**B**), and at 6 days post-injury after delayed IL-4 injection (**C**). Mean ±SEM. (n=4 per group) Note that microglia and macrophages adopt greater expression of M2 markers after delayed injection of IL-4, and the expression of M2 markers is maintained for at least 4 days. *p<0.05 vs saline. Student t test was used to analyze significant differences between IL-4 and saline injected mice.

Delayed administration of IL-4 induces the appearance of resolution-phase macrophages

One of the striking observations we found after doing FACS analysis on 6 days post-injury spinal cord receiving delayed administration of IL-4 spinal cords was the presence an immune cell population that showed high expression of CD45, but lower levels of CD11b than myeloid cells.

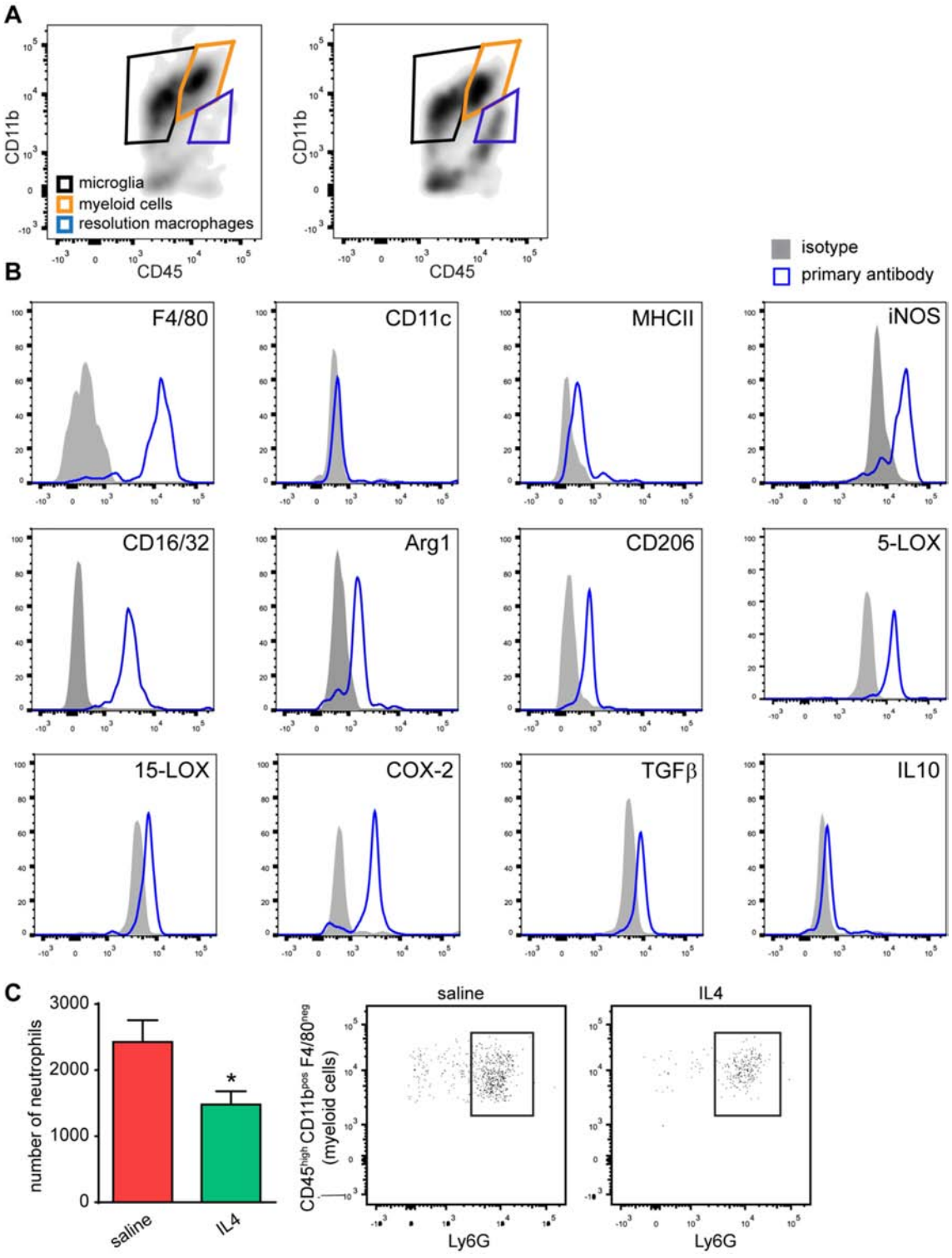

Figure 6. Delayed administration of IL-4 triggers the appearance of resolution-phase macrophages. (**A**) Flow cytometry density plot showing CD45+ and CD11b+ cells from 6 days post-injury spinal cords of mice receiving delayed injection of saline or IL-4. Note the presence of resolution-phase macrophages in the contused spinal cord only after delayed IL-4 administration. (**B**) Representative flow cytometry histograms characterizing the expression of resolution-phase macrophages. (**C**) Representative flow cytometry dot plots showing granulocytes in the contused spinal cord at 6 days post-injury. Note that neutrophil clearance is enhanced after delayed IL-4 injection. Mean ± SEM. (n=4 per group). *p<0.05 vs saline. Student t test was used to analyze significant differences between IL-4 and saline injected mice.

The presence of this immune cell subset ($CD45^{high}$, $CD11b^{low}$) was almost undetected in the spinal cords of mice injected with saline, or in those from our previous characterization of the changes in M1/M2 markers for the first 28 days post-injury (Figure 6A). We thus gated this immune cell subset in spinal cords receiving delayed injection of IL-4 for its characterization, and found that they expressed F4/80 and MHCII, but did not display CD11c, suggesting they were macrophages (Figure 6B). Moreover, they expressed iNOS, CD16/32, CD206 and Arg1, indicative of a mixed M1/M2 phenotype (Figure 6B). A variant activation form of macrophage was reported recently in the resolution phase of inflammation elicited by intraperitoneal administration of zymozan (Bystrom et al. 2008; Stables et al. 2011). These macrophages, known as resolution-phase macrophages express a mixed M1/M2 phenotype like the new macrophage subset we see after delayed IL-4 injection display MHCII but lack CD11c expression (Bystrom et al. 2008; Li et al. 2013; Stables et al. 2011). In addition, these cells were also shown to express anti-inflammatory cytokines as well as lipoxygenase (LOX) and cyclooxygenase (COX-2), key enzymes in the synthesis of the specialized pro-resolving lipid mediators, which turn on the resolution programs of inflammation (Bystrom et al. 2008; Li et al. 2013; Schwab et al. 2007; Serhan 2014). We therefore assessed whether the new macrophage subset found after delayed IL-4 injection also displayed these resolution-phase markers. Interestingly, these macrophages expressed 5-LOX, 15-LOX and COX-2 (Figure 6B), as well as the anti-inflammatory cytokines IL10, and TGFβ (Figure 6B; table 1). Note that IL-4 only increased the expression of these anti-inflammatory/resolvin markers in this new macrophage subset, but not in microglia or macrophages, with the exception for 15-LOX, which was increased in ~25% of macrophages after IL-4 administration (Table 1). Therefore, these data indicate that delayed administration of IL-4, which modulates microglia and macrophage phenotype after SCI, leads directly or indirectly to the appearance of a macrophage subset, which phenotypically resembles resolution-phase macrophages.

Since resolution-phase macrophages are important in resolving inflammation, we next investigated whether the delayed intraspinal injection of IL-4 enhanced the clearance of neutrophils in from contused spinal cord, a key step in the resolution of inflammation (Schwab et al. 2007; Serhan 2014). Neutrophil infiltration peaks in the contused spinal cord at day 1 after injury (David et al. 2012; Stirling and Yong 2008), which allowed us to assess whether their elimination was speeded by delayed administration of IL-4. Interestingly, we found that IL-4 injection reduced the number of neutrophils ($CD45^{high}$, $CD11b^{+}$, $F4/80^{-}$, $Ly6G^{+}$) by ~40% in the injured spinal cord at 6 days post-injury (i.e. 4 days post-injection) (Figure 6c), supporting the idea that the resolution of inflammation is enhanced by IL-4.

	Microglia		Macrophages		rMacrophages
	Saline	IL-4	Saline	IL-4	IL-4
IL-10	3.2±0.3	2.7±0.2	11.0±3.0	12.4±3.0	29.8±4.4 $^{\#*}$
TGFβ	34.2±1.7	34.3±3.6	52,1±2.7	54.7±1,7	61.1±4.1 *
5-LOX	67.1±0.7	63.9±5.0	71.3±2.8	72.8±3.7	82.4±1.9 $^{\#*}$
15-LOX	46.3±2.5	41.7±4.6	45.0±2.6	55.3±5.3 *	60.5±4.9 *
COX-2	0.2±0.2	0.6±0.2	2.1±0.5	5.4±1.5	84.0±5.3 $^{\#*}$

Table 1. **Quantification of resolution markers in microglia and macrophages at 4 days after delayed intraspinal injection into the contused spinal cord assessed by FACS analysis**. * p<0.05 vs macrophages saline; $^{\#}$ p<0.05 vs macrophages/microglia IL-4; one-way ANOVA with Tukey post hoc test was used for multiple comparison. “rMacrophages" mean "resolution phase macrophages".

Effects of delayed administration of IL-4 on functional and histopathological outcomes after SCI

We finally addressed whether the effects of delayed administration of IL-4 on microglia/macrophage activation led to functional recovery and reduced tissue damage after SCI. We found that delayed IL-4 treatment conferred significant protection against loss of locomotor function after SCI based the on the BMS scale (Figure 7A,B). At day 28 post-injury (the latest time point examined), 90% of saline-injected (control) mice showed plantar placement of the hindlimbs and only 40% displayed occasional stepping. However, all the IL-4-injected mice showed plantar placement and 83% of them showed occasional or frequent plantar stepping. In addition, mice treated with IL-4 were able to perform faster locomotion on the treadmill (Figure 7C). Additionally, electrophysiological evaluation of motor evoked potentials revealed that IL-4 treatment led to ~2.5-fold greater preservation of central descending axonal pathways, and conduction was slightly but significantly faster (Figure 7D-E), indicating that delayed IL-4 administration led to greater integrity of spinal motor pathways.

In line with the functional data, histopathological analysis revealed that IL-4 treatment led to significant enhancement in myelin sparing at the injury epicenter and in adjacent areas (Figure 7F-G), as well as, greater preservation of neurons but only at caudal regions to the injury epicenter (Figure 7H-I). Overall, this data provides clear evidence that delayed administration of IL-4 enhances functional and histological outcomes after SCI.

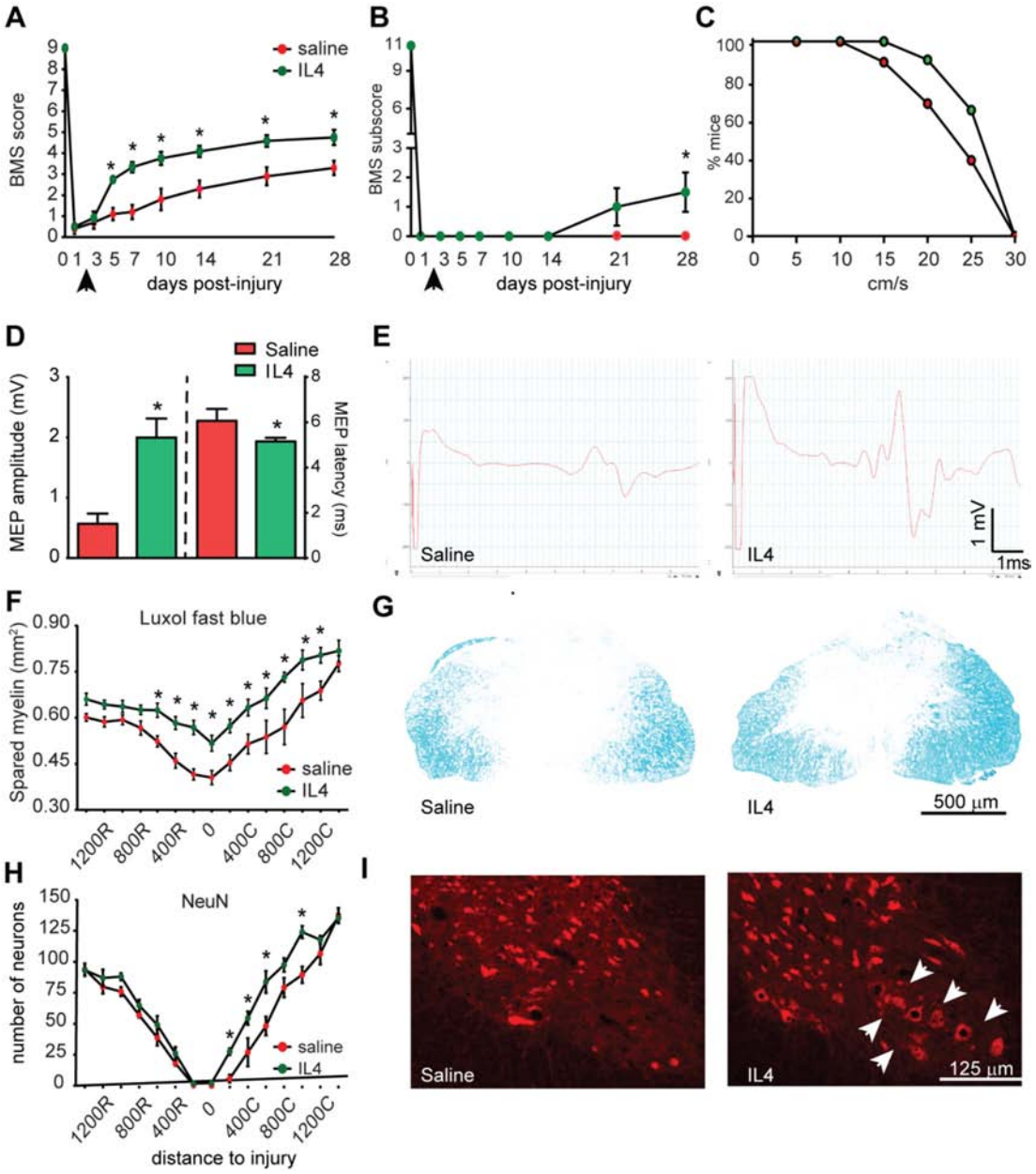

Figure 7. Delayed intraspinal injection of IL-4 reduces functional deficits and tissue damage after SCI. (**A-B**). Mice treated with of IL-4 at 48 hours post-injury showed significant improvement in locomotor skills using the (**A**) BMS score and (**B**) BMS subscore, as well as (**C**) faster locomotion on a treadmill. Arrows indicate the day IL-4 was injected in the spinal cord. (D-E) Administration of IL-4 led to greater preservation of MEPS. (**D**) Quantification MEPs recordings in the gastrocnemius muscle at day 28 post-injury and (**E**) representative MEPs recordings from saline and IL-4 treated mice. (**F-I**) Delayed IL-4 treatment reduced secondary tissue damage after SCI. (**F**) Quantification of myelin sparing at various distances rostral and caudal to the injury epicenter reveals significant reduction in myelin loss at the epicenter of the injury and in adjacent sections in mice treated with IL-4. (**G**) Representative micrographs showing myelin sparing at the injury epicenter in section stained against LFB from mice treated with saline or IL-4. (**H**) Quantification of ventral horn neuron survival at various distances rostral and caudal to the injury epicenter reveals significantly greater neuronal survival in caudal regions to the injury epicenter in mice treated with IL-4. (**I**) Representative micrographs showing sparing of ventral horn neurons in mice administered with saline or IL-4 in sections stained against NeuN at 1000 µm caudal to the injury epicenter. Mean ± SEM. (n=8 per group). *p<0.05 vs saline. Note the greater preservation of neurons after delayed IL-4, especially, in the most ventral region (white arrowheads). Two-way repeated-measures ANOVA with Bonferroni's post hoc correction was used to analyze significant differences between saline and delayed IL-4 injection in the BMS score and subscore, as well as, in histological parameters, and t-test for electrophysiological data.

DISCUSSION

At present, there is no effective treatment for acute SCI, however, a large body of experimental reports reveal that inflammation exacerbates tissue damage and functional recovery after SCI (David et al. 2012; Hawthorne and Popovich 2011; Ren and Young 2013). This is due, in part, to the release of numerous mediators from microglia and macrophages that exert cytotoxic effects on CNS cells that can lead to demyelination and neuronal loss (David et al. 2012; Hawthorne and Popovich 2011; Ren and Young 2013). Nevertheless, these cells also synthesize a variety of factors that promote cell survival and tissue healing, and under certain circumstances, may lead to protective effects in the injured CNS (David et al. 2012; Popovich and Longbrake 2008). These conflicting actions of microglia and macrophages are due to the plasticity of these cells that depends on factors in the immediate environment. Thus, understanding the mechanisms that drive microglia and macrophage activation toward a phenotype that is conducive for tissue repair can lead to therapies that are more effective to treat acute SCI than those aimed at limiting their bystander effects.

In agreement with previous studies, we show here that microglia and macrophages express predominantly M1 markers after SCI, whereas the expression of M2 markers is restricted to a small subpopulation, indicating that these cells adopt predominantly a pro-inflammatory phenotype after SCI (Kigerl et al. 2009; Kroner et al. 2014). Our data, in agreement with previous reports, highlights that microglia and macrophages after SCI cannot be defined within the simple M1-M2 classification described for cells in culture, but fall into a spectrum of activation states depending on multiple markers where the M1 and M2 phenotype are located at the opposite ends of this spectrum (David et al. 2015; Murray et al. 2014). This is reflected in our data, since the expression of the M1 markers (iNOS and CD16/32) was found in most microglial cells and macrophages for the first three days following injury, but the expression of iNOS, but not CD16/32, was progressively lost over time. Similarly, a recent study reports that the expression of CD86, another M1 marker, is also lost from microglia and macrophages over time (Kroner et al. 2014). We also observed, in agreement with a previous report (Kroner et al. 2014) that only a small subset of microglia and macrophages that expressed M2 markers. This is not surprising as microglia and macrophages *in vivo* in the injured spinal cord are influenced by a variety of both pro and anti-inflammatory stimuli (David et al. 2015). In contrast, a previous study showed that M2 macrophages are abundant in the spinal cord of irradiated mice at 2 weeks post-injury, and reach the injury site through the choroid plexus migrating along the central canal (Shechter and Schwartz 2013). However, these M2 macrophages were

classified based on the lack of Ly6C expression and not to the presence of classical M2 markers. These two factors may account for the difference seen between this study and our data or other recent papers. Thus, there is likely to be heterogeneity of microglia/macrophage phenotypes in the injured spinal cord, which undergo dynamic changes, going from more skewed to less skewed M1 polarization over the course of SCI physiopathology, but without being redirected toward the M2 edge of the spectrum.

Previous studies indicate that the environment of the injured spinal cord does not favor M2 polarization (Kigerl et al. 2009; Kroner et al. 2014) since M2 macrophages obtained in cell culture rapidly lose Arg1 expression when transplanted into the injured spinal cord (Kigerl et al. 2009). Polarization of microglia/macrophages into M1/M2 *in vitro* is induced upon stimulation with different cytokines (Mills et al. 2000; Murray et al. 2014; Sica and Mantovani 2012). Therefore, it is likely that the functional phenotype of microglia and macrophages after SCI might depend on the balance between the levels of pro-M1 and pro-M2 cytokines found in the injured spinal cord environment (David et al. 2015). Our analysis on cytokines expression shows that most pro-inflammatory cytokines are increased in the spinal cord for the first 24 hours post-injury, and most of them dropped to basal levels by day 3. Interestingly, the expression of cytokines that induce distinct alternative activation phenotypes was either undetected (i.e. IL-4) or expressed at low levels and only for the first 24 hours post-injury, but not at later stages (i.e. IL-10 and IL-13). Although IL-4 was not detected in the contused spinal cord, there is likely low but undetectable production of this cytokine, since a previous study showed that IL-4Rα null mice show greater functional deficits after SCI (Fenn et al. 2014). In the current work, however, we found that increasing the levels of IL-4 in the injured spinal cord induces the expression of M2 markers in microglia and macrophages, linking the low levels of IL-4 in the contused spinal cord with the failure of the CNS to redirect these cells towards the M2 end of the polarization spectrum following injury. Although previous studies reveal that activated microglia from adult and aged brain could be redirected toward an M2-like phenotype by IL-4 in *ex-vivo* experiments (Fenn et al. 2012), to our knowledge this is the first evidence that shows that this also occurs *in vivo*. Interestingly, we also reveal that ability of IL-4 to redirect microglia and macrophages is more effective when it is administered after a delay of 48 hours post-injury. This may be due to the high levels of pro-inflammatory cytokines that are present in the spinal cord for the first 24 hours post-injury, which might have hampered the phenotype changes in microglia and macrophages triggered by IL-4 when injected acutely after injury. Indeed, a recent work reveals that TNFα, which is increased in the injured spinal cord for the first 24 hours, prevents the induction of M2 markers in microglia

and macrophages after SCI (Kroner et al. 2014), as well as in tumor macrophages (Kratochvill et al. 2015). It is likely that other pro-inflammatory cytokines and/or mediators synthesized during the first hours post-injury could also exert a similar effect. Our data, along with another report(Fenn et al. 2014), indicates that microglia do not show constitutive expression of IL-4Rα. This may also limit the capacity of acute IL-4 administration to induce an anti-inflammatory phenotype, as the bioavailability of IL-4 could be already compromised by the time IL-R4 is expressed by microglia. In addition, since infiltration of blood borne monocytes into the spinal cord is scarce for the first 24 hours post-injury, this can also explain the low effectiveness of acute IL-4 to induce M2 markers in this immune cell population. However, at 48 hours post-injury, IL-4R is expressed on microglia, macrophage infiltration is abundant, and the levels of most of the pro-inflammatory cytokines are close to basal levels. At this point, the administration of IL-4 has greater effects in counteracting the pro-inflammatory polarizing environment of the injured spinal cord. This approach induced ~40% of microglia and ~55% of macrophages to acquire M2 markers, since only ~55% of these two immune cells expressed IL-4R at 48 hours post-injury. To our knowledge, this is currently the most effective strategy in inducing M2 markers in microglia and macrophages after SCI.

In many tissues, a switch in macrophage phenotype accompanies the resolution of inflammation. Transcriptomic profiling of isolated resolution-phase macrophages reveal that they show a mixed M1/M2 phenotype (Bystrom et al. 2008). Resolution-phase macrophages are enriched with MHCII antigens, but lack CD11c (Stables et al. 2011). They also express anti-inflammatory cytokines, such as TGFβ and IL-10 (Li et al. 2013; Stables et al. 2011). Importantly, they have elevated expression of COX-2 and LOX (Li et al. 2013; Stables et al. 2011), key enzymes in the synthesis of specialized pro-resolving mediators that actively turn off inflammation (Schwab et al. 2007; Serhan 2014). Our data reveal that only a small proportion of macrophages display M2 markers, suggesting that resolution-phase macrophages are scarce in SCI, which may explain, in part, why inflammation remains active for several months or even years in this pathology. However, we found that delayed IL-4 treatment induced the appearance of a new immune cell subset in spinal cord. Further characterization of this immune cell subset revealed that they were macrophages and that they had a phenotype compatible with resolution-phase macrophages (5-LOX, 15-LOX, COX-2, TGFβ, IL-10). Whether this is due to either direct or indirect effects of IL-4 on macrophages is not known. In line with these results, we also found that the clearance of neutrophils from the injured spinal cord, which is a key step in the resolution of inflammation (Li et al. 2013; Schwab et al. 2007; Serhan 2014), was markedly accelerated upon delayed IL-4 injection, linking the

lack of IL-4 expression with resolution deficit that occurs after SCI.

Delayed administration of IL-4 not only modulated microglia and macrophage phenotype after SCI, more importantly, this was accompanied with substantial reduction in tissue damage and functional deficits, which was demonstrated by a variety of functional, electrophysiological and histopathological analysis. Since IL-4Rα was mainly found in microglia and macrophages, our results indicate that the effects of delayed administration of IL-4 on microglia and macrophage exerted restorative effects after SCI.

Taken together, our data reveals that the insufficient induction of IL-4 after SCI is responsible, in part, to drive microglia and macrophages towards a phenotype that is not conducive to tissue repair and functional recovery. We show that a single, delayed injection of IL-4 induces the appearance of a resolution type macrophage subpopulation that is effective in reducing tissue damage and locomotor impairment after SCI. Since IL-4 is also undetected in human CSF samples after acute SCI (Kwon et al. 2010), these findings may also be of relevance to SCI in humans. Therefore, therapies that increase IL-4 levels in the injured spinal cord could be a good approach with a wide therapeutic window for the treatment of acute SCI.

SUPPLEMENTARY DATA

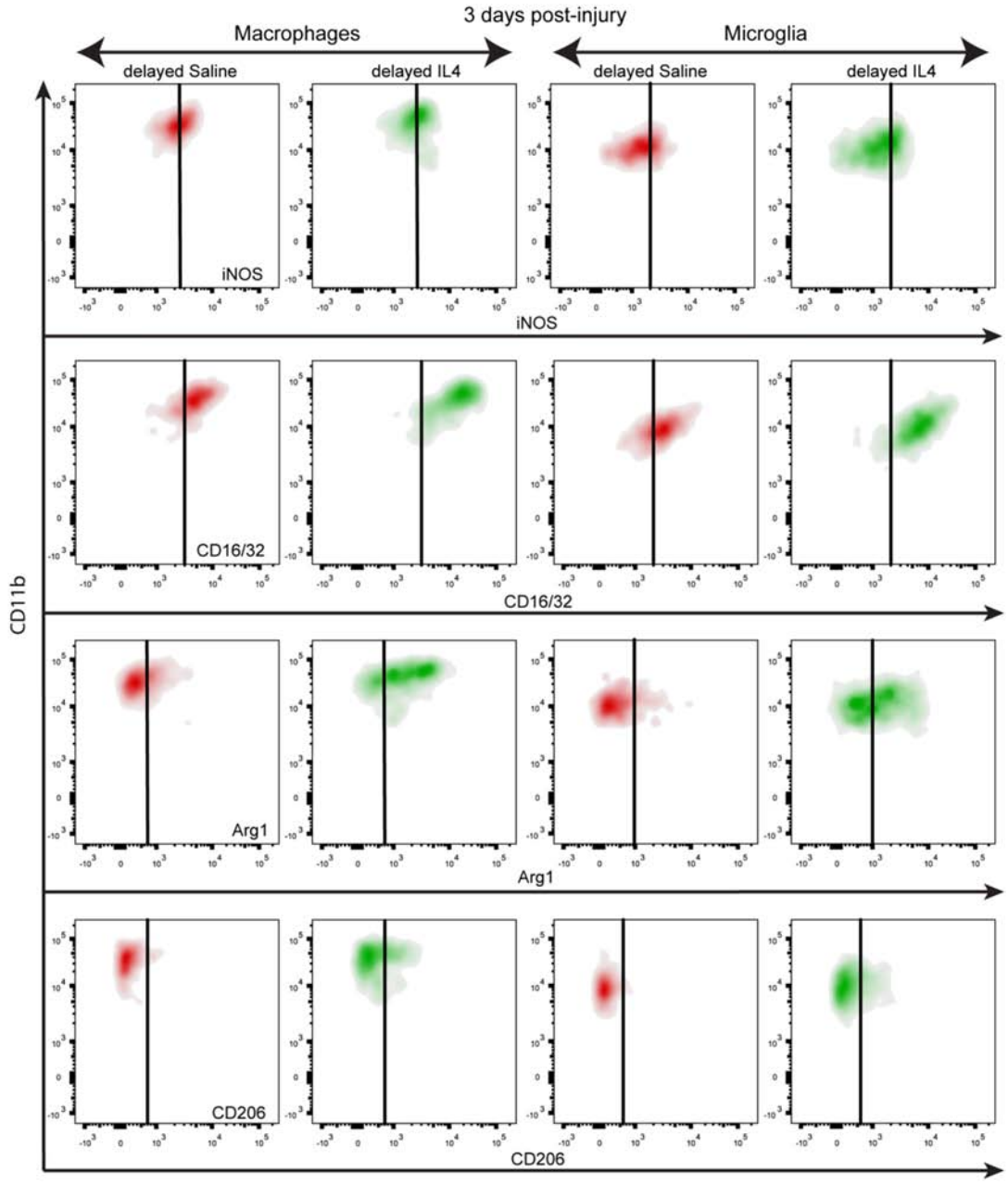

Fig. S1. Delayed administration of IL-4 increases the expression of M1 and M2 markers in microglia and macrophages at 3 days post-injury. (**A**) Representative density plots showing the changes in the expression of M1 and M2 markers in microglia and macrophages at day 3 post-injury. Note that delayed administration of IL-4 led to marked induction in the expression of Arg1 and CD206 in microglia and macrophages, but also increased the expression of CD16/32 in both immune cell populations.

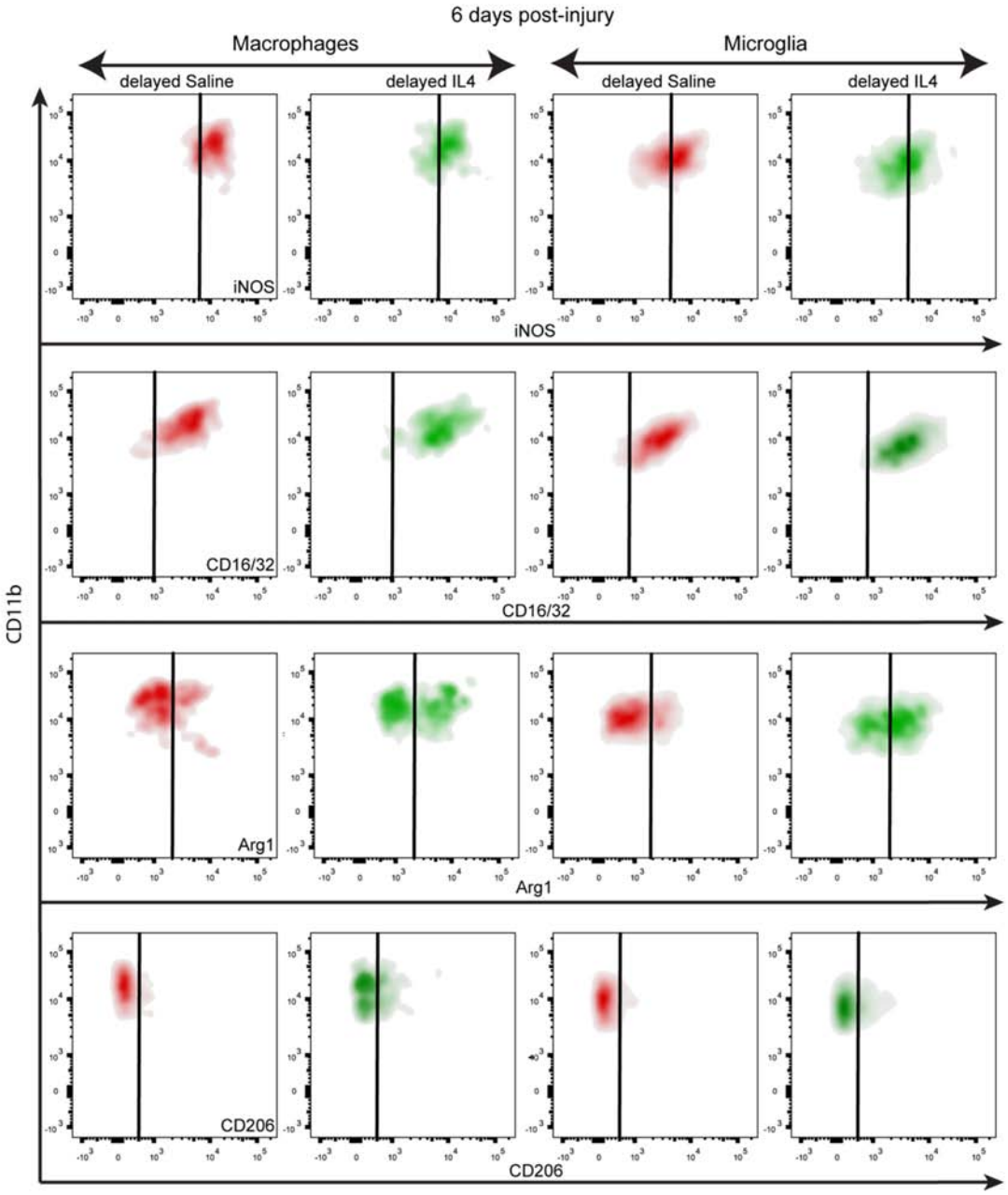

Fig. S2. Expression of M2 markers remained elevated in microglia and macrophages at 6 days-postinjury following delayed IL-4 administration. (**A**) Representative flow cytometry density plots showing the changes in the expression of M1 and M2 markers in microglia and macrophages at day 6 post-injury. Note that the expression of Arg1 and CD206 remains significant elevated in microglia and macrophages after delayed administration of IL-4, whereas the expression of iNOS is significant reduced

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CHAPTER 2

Maresin-1 activates inflammation resolution programs after spinal cord injury and exerts therapeutic effects

Maresin-1 activates inflammation resolution programs after spinal cord injury and exerts therapeutic effects

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ABSTRACT

It has been reported that inadequate synthesis of specialized pro-resolving lipid mediatiors (SPMs) leads to the development of chronic inflammatory disease. After spinal cord injury, inflammation is not properly resolved which leads to persistent presence of immune cells in the site of injury. However, it is currently unknown whether the failure of the spinal cord to properly resolve inflammation after trauma is due to the incapacity to produce SPMs. In the present study, we provide novel insights that link the defective synthesis of SPMs with the difficult of the injured spinal cord to resolve inflammation. In particular, we show that the metabolic precursors of different SPMs are not produced in the spinal cord until 2 weeks after contusion lesion. Interestingly, we found that administration of Maresin 1 (MaR1), a DHA-derived SPM, enhances different key aspects related with the inflammatory resolution, which include down-regulation of pro-inflammatory cytokine, inactivation of different inflammatory pathways, reduction in the accumulation of neutrophils and macrophages into the lesion site, polarization of macrophages, and efferocytosis of neutrophils. Interestingly, administration of MaR1 conferred protection against functional deficits and tissue loss, indicating that the inflammatory resolution deficits that occurs after SCI mediates detrimental actions on secondary tissue damage. Overall, these results suggested that therapies aimed at enhancing the resolution of inflammation could be a novel therapeutic approach for the treatment of acute SCI.

Keywords: Inflammation, Maresin 1, Spinal Cord Injury, Resolution, Neuroprotection

INTRODUCTION

A robust inflammatory response is observed in the central nervous system (CNS) in several pathological conditions (David et al., 2012b, Gomez-Nicola and Perry, 2015, Steinman, 2015). After spinal cord injury (SCI), inflammation is orchestrated by activated resident glial cells (microglia and astrocytes) and by circulating leukocytes (neutrophils, monocytes and lymphocytes) that enter the damaged spinal cord (Hawthorne and Popovich, 2011, Pruss et al., 2011, David et al., 2012b). These immune cells are required for effective clearance of cell and myelin debris, and for the release of key bioactive molecules that lead to tissue healing and repair (Popovich and Longbrake, 2008, David et al., 2012b). However, they also secrete several factors that may potentially mediate cytotoxicity to neurons, glia, axons and myelin (Popovich and Longbrake, 2008, David et al., 2012b). Hence, the inflammatory response exerts both, helpful and detrimental actions after SCI, and thus, its final outcome on this pathology will depend on the balance between mechanisms that regulate the different aspects of the inflammation.

Regardless of the tissue in which it occurs, the inflammatory response is normally self-limiting and undergoes resolution in a timely fashion. Insufficient or inadequate resolution leads to chronic inflammation that causes greater tissue damage and inappropriate tissue healing (Buckley et al., 2014, Serhan, 2014, Serhan et al., 2015). This is the case of many neurological disorders, including SCI, where inflammation fails to resolve properly leading to disproportionate harmful bystander side effects (Hawthorne and Popovich, 2011, Pruss et al., 2011, David et al., 2012b). The damaging consequences of inflammation are more pronounced in the spinal cord than other tissues due to the limited capacity of the CNS for axon regeneration and replacement of damaged neurons and myelinating glia, leading to irreversible functional disabilities (Fawcett et al., 2012, Lu et al., 2014, Stenudd et al., 2015).

Resolution of inflammation is not merely a passive event, but rather an active process regulated, in part, by a novel group of lipid mediators derived from poly-unsaturated fatty acid (PUFA) (Schwab et al., 2007, David et al., 2012a, Serhan, 2014). This family of specialized pro-resolving mediators (SPM) include: lipoxins, resolvins (RvD and RvE), protectins and maresins (Buckley et al., 2014, Serhan, 2014, Serhan et al., 2015). SPM actively turn off the inflammatory response by acting on distinct G protein coupled receptors expressed on immune cells that activates dual anti-inflammatory and pro-resolution programs (Buckley et al., 2014, Serhan, 2014, Serhan et al., 2015). SPM, for instance, suppress the secretion of pro-inflammatory

factors such as IL-10, IL-1 decoy receptors and IL-1 receptor antagonists, and induce the expression of scavenging molecules (Buckley et al., 2014, Serhan, 2014). In addition, SPM activate specific mechanisms that trigger the resolution of inflammation. Among these resolution mechanisms include: (i) clearance of the molecules that triggers the inflammatory storm, (ii) turn off the intracellular pathways that leads to inflammation, (Misharin et al.) phagocytosis of neutrophils by macrophages and (iv) normalization of immune cells counts to basal levels (Buckley et al., 2014, Serhan, 2014, Serhan et al., 2015). The importance of SPM in the resolution of inflammation is evident in many chronic pathological conditions where their synthesis is insufficient, delayed or event absent; and exogenous administration of SPM reduce inflammation and mediate tissue protection (Schwab et al., 2007, Buckley et al., 2014). However, it is currently not known whether chronic inflammatory in SCI is due to inadequate synthesis of SPM.

Here, we show that synthesis of SPM is impaired in the spinal cord after contusion injury. We demonstrate that administration of maresin 1 (MaR1) enhances resolution of inflammation after SCI and leads to improved functional and histopathological outcomes. These results demonstrate for the first time that failure to synthesize adequate levels of MaR1 hampers the resolution of inflammation after SCI, contributing to secondary tissue damage and impaired locomotor recovery.

MATERIALS AND METHODS

Spinal cord contusion injury

All surgical procedures were approved by the Universitat Autònoma de Barcelona Animal Care Committee and followed the guidelines of the European Commission on Animal Care, and the methods for each procedure were carried out in accordance with the approved guidelines. Adult (8 to 10 weeks old) female C57Bl/6 mice (Charles River) were anesthetized with ketamine:xylazine (90:10 mg/kg). After performing a laminectomy at the 11th thoracic vertebrae, the exposed spinal cord was contused using the Infinite Horizon Impactor device (Precision Scientific Instrumentation, Lexington, KY). Injuries were made using a force of 60 kdynes and tissue displacement ranging between 500-700 µm.

One hour after spinal cord injury, 1 µg of MaR1 (7S,14S-dihydroxy-4Z,8E,10E,12Z,16Z,19Z-docosahexaenoic acid; Cayman Chemical) was injected intravenously in 100 µl of sterile saline,

and then repeated daily thereafter until day 7. Control mice were injected with an equal volume of sterile saline following the same injection protocol.

Flow Cytometry

Immune cells from the laminectomized and injured spinal cord were analyzed by flow cytometry at 1, 3, 7, 14, 21 and 28 days post-injury as described previously to study the dynamics of immune cell in spinal cord injury as described previously (Santos-Nogueira et al., 2015). Similarly, spinal cord from mice treated with MaR1 or saline were also harvested at day 1, 3 and 7 post-lesion. Briefly, spinal cords were cut in little pieces and passed through a cell strainer of 70 µm (BD falcon) and the cell suspension was centrifuged twice at 300g for 10 minutes at 4ºC. After cell counts, samples were divided, and cells alone and isotype-matched control samples were generated to control for nonspecific binding of antibodies and for auto-fluorescence. The following antibodies from eBioscience were used at 1:100 concentration: CD45-PerCP, CD11b-PE-Cy7, Ly6C-FITC, Ly6G-PE, Gr1-FITC, F4/80-APC or PE, CD3-FITC, CD4-APC, CD8-APC, CD19-PE, CD206-FITC, CD16/32-PE. Samples were also stained with unconjugated rabbit antibodies against iNOS (1:100 Abcam), and goat antibodies against Arg1 (1:100; Santa Cruz). After 30 min of incubation with combinations of antibodies at 4°C, cells were then fixed in 1% paraformaldehyde. For intracellular staining, cells were permeabilized with Permeabilization Wash Buffer (Biolegend) followed by staining with Alexa488 or Alexa647 conjugated donkey secondary antibodies against rabbit or goat (1:500 Molecular Probes) for 30 min when needed. After 30 min of incubation with combinations of antibodies at 4°C, the samples were washed and fixed in 1% paraformaldehyde. To perform the analysis, cells were first gated for CD45 to ensure that only infiltrating leukocytes and resident microglia are selected (Fig. S1). Then, the following combination of markers were used to identify: microglia ($CD45^{low}$, $CD11b^{+}$, $F4/80^{+}$) ; macrophages ($CD45^{high}$, $CD11b^{+}$, $F4/80^{+}$); neutrophils ($CD45^{high}$, $CD11b^{+}$, $F4/80^{-}$, $Gr1^{high}$); CD4 T-Cells ($CD45^{+}$, $CD11b^{-}$, $CD3^{+}$, $CD4^{+}$); CD8 T Cells ($CD45^{+}$, CD11b, $CD3^{+}$, $CD8^{+}$); B cell ($CD45^{+}$, $CD11b^{-}$, $CD3^{-}$, $CD19^{+}$). Kinetics analysis of these immune cell types were calculated as described previously (Pruss et al., 2011). To study the phenotype of microglia and macrophages, these cells were further differentiated based on Ly6C, CD16/32, iNOS, CD206 and Arg1 expression. Cells were analyzed using FlowJo® software on a FACSCanto flow cytometer (BD Biosciences).

Lipid Mediator Metabololipidomics.

Inflammatory and SPM and specific ω-6 PUFA and ω-3 PUFA pathway markers were identified and quantified by liquid chromatography (LC)-tandem mass spectrometry (MS/MS). In brief, 400 pg class-specific deuterated (-d) internal standards (AA-d8, DHA-d5, PGE2-d4, lipoxin A4-d5, leukotriene B4-d4, 15-hydroxyeicosatetraenoic acid-d8) were added to each spinal cord sample prior to processing to calculate the recovery of specific classes of PUFA, LOX, and COX metabolites. Spinal cord homogenates (30–50 µL) containing internal standards were combined with 2 mL methanol, dried under a gentle stream of nitrogen, immediately resuspended in high-performance LC mobile, and placed in a refrigerated autosampler for lipidomic analysis. Eicosanoids and docosanoids were identified and quantified by LC-MS/MS–based lipidomics based on published methods.30,38 Processed tear samples were analyzed by a triple-quadrupole linear ion trap LC-MS/MS system (MDS SCIEX 3200 QTRAP; Applied Biosystems, Foster City, CA, USA) equipped with a Kinetex C18 mini-bore column (Phenomenex, Torrance, CA, USA). The mobile phase was a gradient of water/acetonitrile/acetic acid (72:28:0.01, vol:vol:vol) and isopropanol/acetonitrile (60:40, vol:vol) with a 450-µL/min flow rate. Tandem MS/MS analyses were performed in negative ion mode, and prominent fatty acid metabolites were quantified in multiple reaction monitoring mode using established and specific transitions as previously described.30,31,38–41 Calibration curves (1–1000 pg) and specific LC retention times for each compound were established with synthetic standards (Cayman Chemical, Ann Arbor, MI, USA). Structures were confirmed for selected autacoids by MS/MS analyses using enhanced product ion mode with appropriate selection of the parent ion in quadrupole 1. These analysis were done from spinal cord harvested from uninjured mice, as well as from animal receiving spinal cord contusion at 1, 3, 7 and 14 days post-injury (n=4 per time point).

Cytokine Protein Expression

Mice treated with saline or MaR1 were perfused with sterile saline and a 5 mm length of spinal cord centered on the lesion was collected at 12 and 24h after contusion injury and snap-frozen. Spinal cords were homogenized and protein concentration was determined using the DC Protein Assay (Bio-Rad). Samples were concentrated to 4µg/µl using MicroCon centrifugation filters (Millipore) to ensure equal amounts of protein. Low concentrations of cytokines in the sample result in binding to the filters whereas high concentrations of protein sustain less losses The protein levels of 32 cytokines and chemokines were then analyzed using the Milliplex MAP Mouse Cytokine/Chemokine magnetic bead panel (Millipore) on a Luminex

(Millipore) as per manufacturers' protocol.

Western blotting

Samples used for Luminex assay, were also used to for western blotting. Protein samples (30 µg) were separated by electrophoresis on a 10-15% polyacrylamide gel and transferred onto PVDF membranes (Millipore). The membranes were incubated with rabbit antibodies against phospho NF-kB p65 (1:1000; Cell Signaling), against the phosphorylated form of STAT1 (1:500; Cell Signaling), STAT3 (1:500; Cell Signaling), STAT5 (1:500; Cell Signaling) and STAT6 (1:500; Cell Signaling), phospho JNK (1:500; Santa Cruz), phospho ERK1/2 (1:1000; Cell Signaling), phosphor p38 (1:1000; Cell Signaling) and phospho-AKT (1:1000; Cell Signaling). Horseradish peroxidase–coupled secondary antibody (1:3000, BioRad) incubation was performed for 1 hour at room temperature. ß-actin (1:10.000; Sigma Aldrich;) was used to ensure equal loading of samples. The membranes were visualized using enhanced chemiluminiscence method and the images were collected and analyzed with Chemidoc apparatus (BioRad) and ImageLab software (BioRad), respectively.

Functional assessment

Locomotor recovery was evaluated at 1, 3, 5, 7, 10, 14, 21 and 28 dpi in an open-field test using the nine-point Basso Mouse Scale (BMS) (Basso et al., 2006), which was specifically developed for locomotor testing after contusion injuries in mice. The BMS analysis of hindlimb movements and coordination was performed by two independent assessors and the consensus score taken. In addition, at the end of the follow up (day 28 post-injury), a computerized assessment of locomotion was also performed using the DigiGait™ Imaging System (Mouse Specifics, Inc.). This system is constituted of a motorized transparent treadmill belt and a high-speed digital video camera that performs images on the underside of the walking animals. DigiGait™ software generates "digital pawprints" and dynamic gait signals, representing the temporal record of paw placement relative to the treadmill belt. This locomotor test allows for an easy and objective analysis of both static and dynamic locomotor parameters. Moreover, the highest locomotion speed that each mouse was to able locomote for at least 5 seconds was also recorded on the Digigait treadmill belt.

Histology

At 28 days post-injury, mice were perfused with 4% paraformaldehyde in 0.1M-phosphate buffer (Sun et al.). A 5mm length of spinal cord containing the lesion site was removed, cryoprotected with 30% sucrose in 0.1M PB at 4ºC, and 10 series of 10µm thick section were picked up on glass slides. Adjacent sections on the same slide were therefore 100µm apart. For demyelination analyses, sections were stained with Luxol Fast Blue (Sigma). For neuronal and axonal assessment, sections were incubated overnight at 4ºC with biotinylated antibodies against NeuN (1:200, Millipore) and NF (1:1000, Millipore), respectively. Double immunostaining for NF and MBP (1:100; Abcam) was done to assess the sparing of myelinated axons. Sections were incubated for 1 hour at room temperature with the streptavidin-Alexa 594 conjugated or donkey anti-rabbit Alexa 594-conjugated antibodies (Molecular Probes, 1:500), and then coverslipped in Mowiol containing DAPI to label nuclei.

The epicenter of the injection or contusion injury impact was determined for each mouse spinal cord by localizing the tissue section with the greatest damage using LFB stained section. Myelin sparing after SCI was calculated by delineating the spared LFB stained tissue. Neuronal survival was assessed by counting the number of $NeuN^+$ cells in the ventral horns at the injury epicenter and at rostral and caudal areas. Axonal sparing was calculated by counting the number of axons in the dorsal column at the injury epicenter, the most damage area of the spinal cord. The same sections were used to examine axonal demyelination in the dorsal column was by counting the fibers double stained for NF and MBP at the lesion epicenter. The NIH ImageJ software was used to quantify all the histological parameters.

Statistical analyses

Data are shown as mean ± standard error of the mean (SEM). Dynamics of immune cell recruitment and lipidomic profile after SCI were analyzed by using one-way ANOVA with post-hoc Bonferroni's test. Maximal speed on a treadmill was analyzed using the Mantel-Cox test. Functional follow-up for BMS score and subscore, as well as histological analysis of myelin and neuronal sparing were analyzed using two-way repeated measure ANOVA with post-hoc Bonferroni's post-hoc test for multiple comparisons. Two-tailed Student's t test was used for the single comparison between two groups. Differences were considered significant at $p<0.05$.

RESULTS

Inflammatory cell clearance is impaired after SCI

We first evaluated, by flow cytometry, the dynamics of the main inflammatory cell types in the contused spinal cord of C57/Bl6 mouse. We detected that neutrophils, macrophages and microglia cell reached maximal accumulation in the contused spinal cord at day 1, 3 and 7 post-injury, respectively (Figure 1 A-C), and then, their numbers dropped progressively up to day 7-14 post-injury, remaining at high and steady levels up to day 28 (Figure 1 A-C). We then quantify the resolution index (R_i: *time between maximum cell numbers and the point when they are reduced to 50%*) and the resolution plateau (R_p: *percent of persistent cellular component*) to provide quantitative measurements of the inflammatory resolution after SCI (Pruss et al., 2011). The R_i of neutrophils and macrophages was 2.5 and 9.5 days, respectively, reflecting the slower clearance of macrophages in SCI as compared to neutrophils. Microglia R_i could not be calculated owing to the rapid decline in their cell counts from 7 to 14 days post-injury, however, this is lower than 7. Interestingly, analysis on the R_p revealed the clearance of all three myeloid cell subsets after SCI was incomplete, with ~35% remaining neutrophils, macrophages and microglial cells at day 28 following lesion.

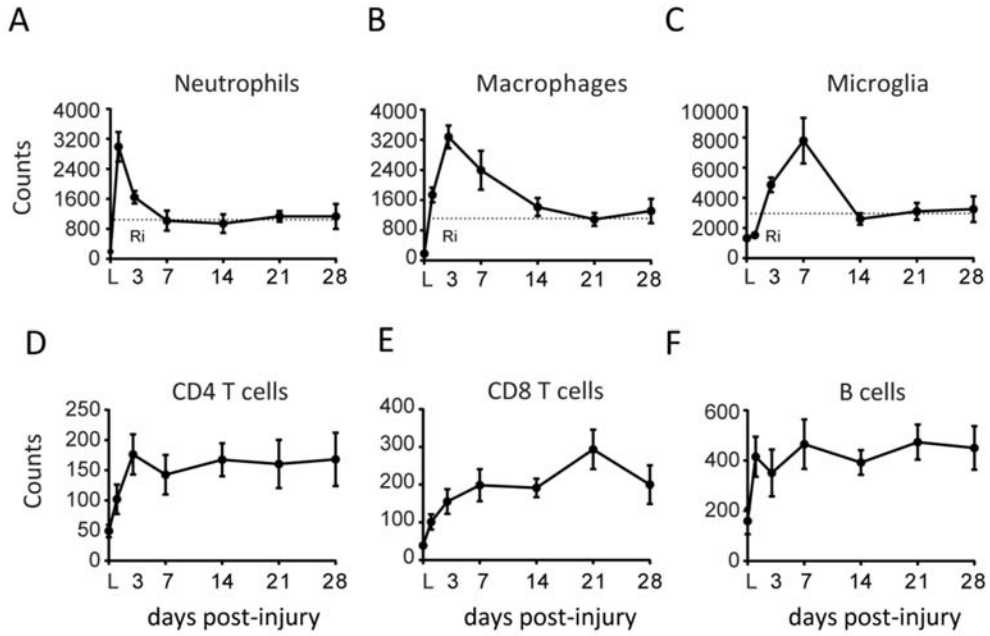


Figure 1. Dynamics of the immune cells after SCI. (A-F) Graph showing neutrophil (A) macrophage (B), microglial (C), lymphoid TCD4 cells (D), lymphoid TCD8 cells (E) and B cells (F) kinetics for the first 4 weeks after SCI. (n=6 per point)

We also studied the recruitment of lymphocytes in the contused spinal cord. We found infiltration of B cells and T cells, both CD4 and CD8, during the first few days after contusion injury, but at much lower numbers as compared to myeloid cells (Figure 1 D-E). R_p of the different lymphocyte subsets was >50% at day 28, indicating the persistent presence of lymphocytes in SCI.

These data provide clear evidence that immune cells are not efficiently eliminated from the contused spinal cord, highlighting that the resolution of inflammation is impaired after SCI.

Defective lipid mediator class switch as a classical hallmark of impaired resolution after SCI.

We investigated whether the impaired clearance of inflammatory cells is mirrored by failed induction of synthesis of specialized pro-resolution mediators (SPM), which have been identified as crucial for efficient resolution (Serhan, 2014). Lipidomic analysis of spinal cord revealed delayed synthesis of SPM after contusion injury. The levels of 12-HETE and 15-HETE, which are pathway markers of the synthesis of the arachidonic acid (AA) derived SPMs known as lipoxin A (LXA), did not increase until day 14 post-injury (Figure 2). Similarly, the synthesis of SPM derived from docosohexaenoic acid (DHA) was also delayed in SCI, since the levels of 17-HDHA, a pathway marker for the formation of resolvin D (RvD) and protectin D1 (PD1), and 14-HDHA, the precursor of maresin1 (MaR1), were not induced until day 14 (Figure 2). Moreover, SPM derived from eicosopentaenoic acid (EPA) were also impaired after SCI, since 18-HEPE, the pathway marker for the formation of resolvin E (RvE) series, was undetected in the injured spinal cord for the time period analysed (14 days).

Thus, the CNS lesion milieu is characterized by a defective and delayed induction of SPM, involving those derived from AA (omega-6), DHA and EPA (omega-3) pathways, which are required for orchestrating efficient resolution of inflammation. This inability to generate a resolution conducive milieu is contrasted by a full-blown early PGE2 response as a hallmark of pro-inflammatory activity (Figure 2). These data indicate that the class switch from pro-inflammatory to pro-resolution lipid mediators derived from AA, DHA and EPA does not occur in the injured spinal cord.

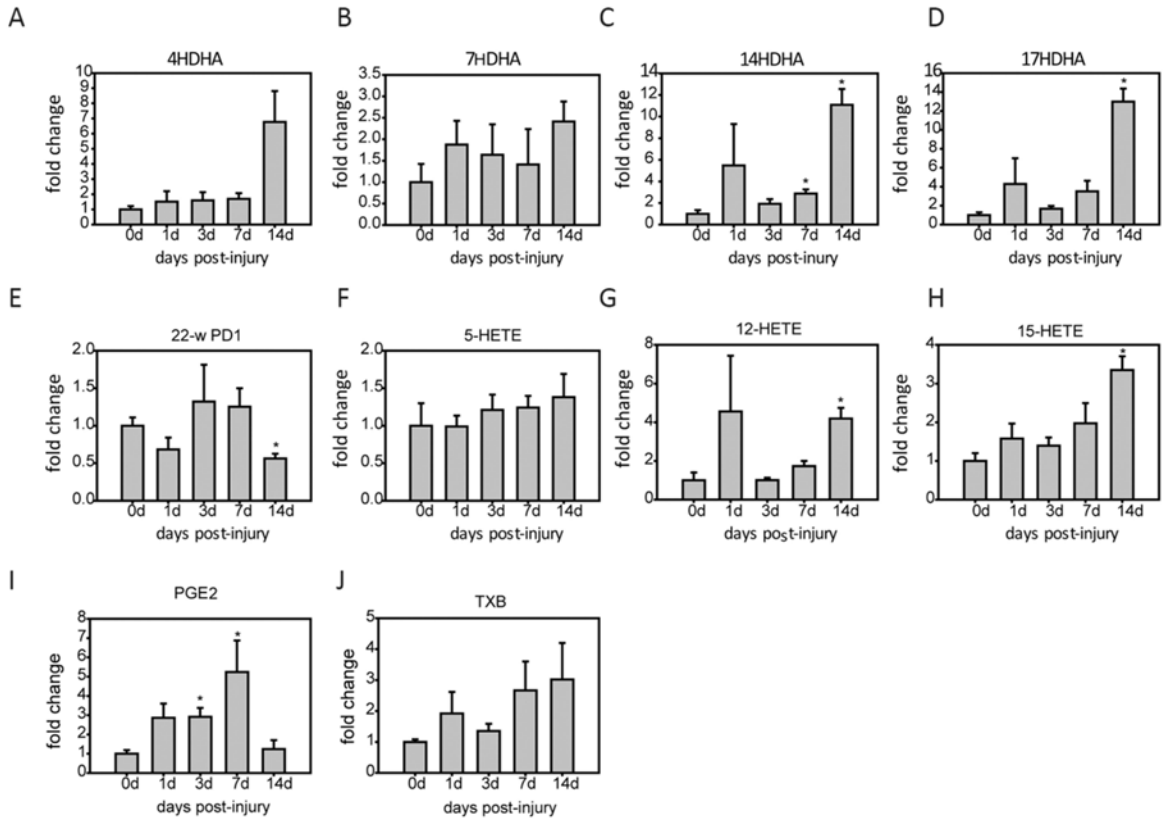
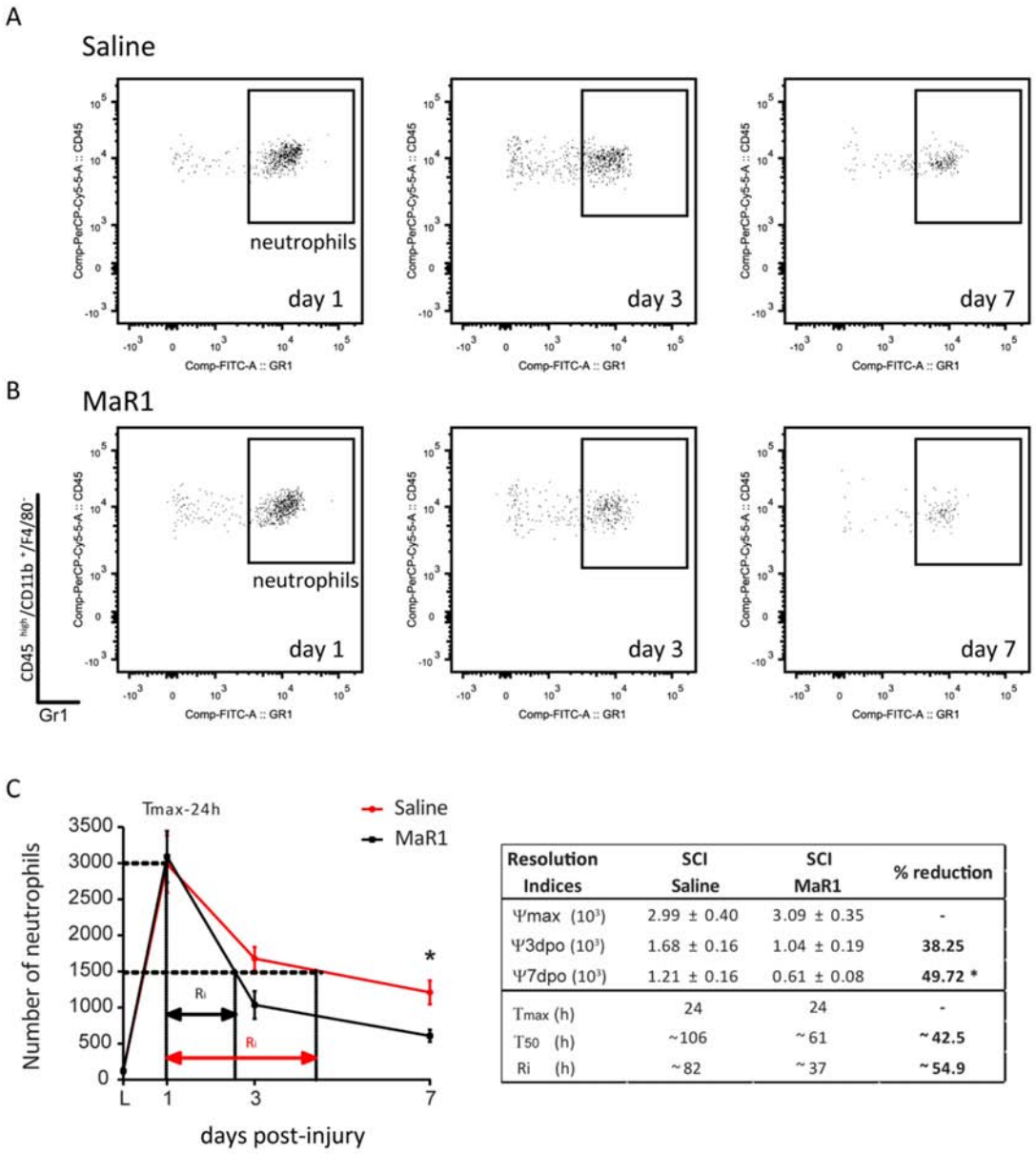


Figure 2. Lipidomic analysis. **(A-J)** Graph showing the induction of different eicosanoid and SPMs precursor markers in the injured spinal cord (n=4 per time point). *p<0.05 One-Way ANOVA Tukey's post-hoc test.

MaR1 regulates resolution of inflammation in the injured spinal cord

To assess whether the deficit in the resolution of inflammation after SCI is linked to impaired synthesis of SPM, we investigated whether systemic administration of MaR1 enhanced immune cell clearance from the contused spinal cord. Daily intravenous administration of MaR1 for 7 days starting 1 hour after SCI did not impede the infiltration of neutrophils in the contused spinal cord, as their counts at day 1 post-injury were unaltered by MaR1 treatment (Figure 3). However, MaR1 accelerated the clearance of neutrophils from the contused spinal cord based on several resolutions parameters, namely, the *Ri* for neutrophils as well as their counts in the injured spinal cord at day 7 post-lesion, was significantly reduced ~50% after MaR1 treatment (Figure 3C).



Resolution Indices	SCI Saline	SCI MaR1	% reduction
Ψmax (10^3)	2.99 ± 0.40	3.09 ± 0.35	-
Ψ3dpo (10^3)	1.68 ± 0.16	1.04 ± 0.19	**38.25**
Ψ7dpo (10^3)	1.21 ± 0.16	0.61 ± 0.08	**49.72 ***
Tmax (h)	24	24	-
T50 (h)	~106	~61	**~42.5**
Ri (h)	~82	~37	**~54.9**

Figure 3. Effects of MaR1 in the accumulation of neutrophils after SCI. (A-B) Representative density plots of FACS analysis showing neutrophils (CD45high, CD11b+, Gr1high) at 1, 3 and 7 days after the injury in the spinal cord of saline (A) and MaR1 (B) treated mice. (C) Graph showing neutrophil recruitment and resolution indices. (B-C) Graph showing microglial (B) and macrophage (C) in the injured spinal cord. Data are expressed as mean ±SEM. (*p<0.05 MaR1 vs saline; Student t test was used to analyze significant differences. n=10 at 1 dpi, n=6 at 3dpi and n=6 at 7 dpi).

We next studied whether the MaR1 interfered with the recruitment of macrophage after SCI. The entrance of blood-borne macrophages into the contused spinal cord was not different at day 1 after MaR1 treatment (Figure 4A-C). However, at day 3 post-injury, MaR1 treatment

showed reduction in the number of macrophages, reaching significance by day 7 (Figure 4A-C). However, MaR1 treatment did not attenuate microglial numbers in the contused spinal cord during the first week following contusion injury (Figure 4D). These results provide clear evidence that MaR1 accelerates the elimination of myeloid cells (neutrophils and macrophages) from the injured spinal cord, suggesting an important role for MaR1 in promoting resolution of inflammation after SCI.

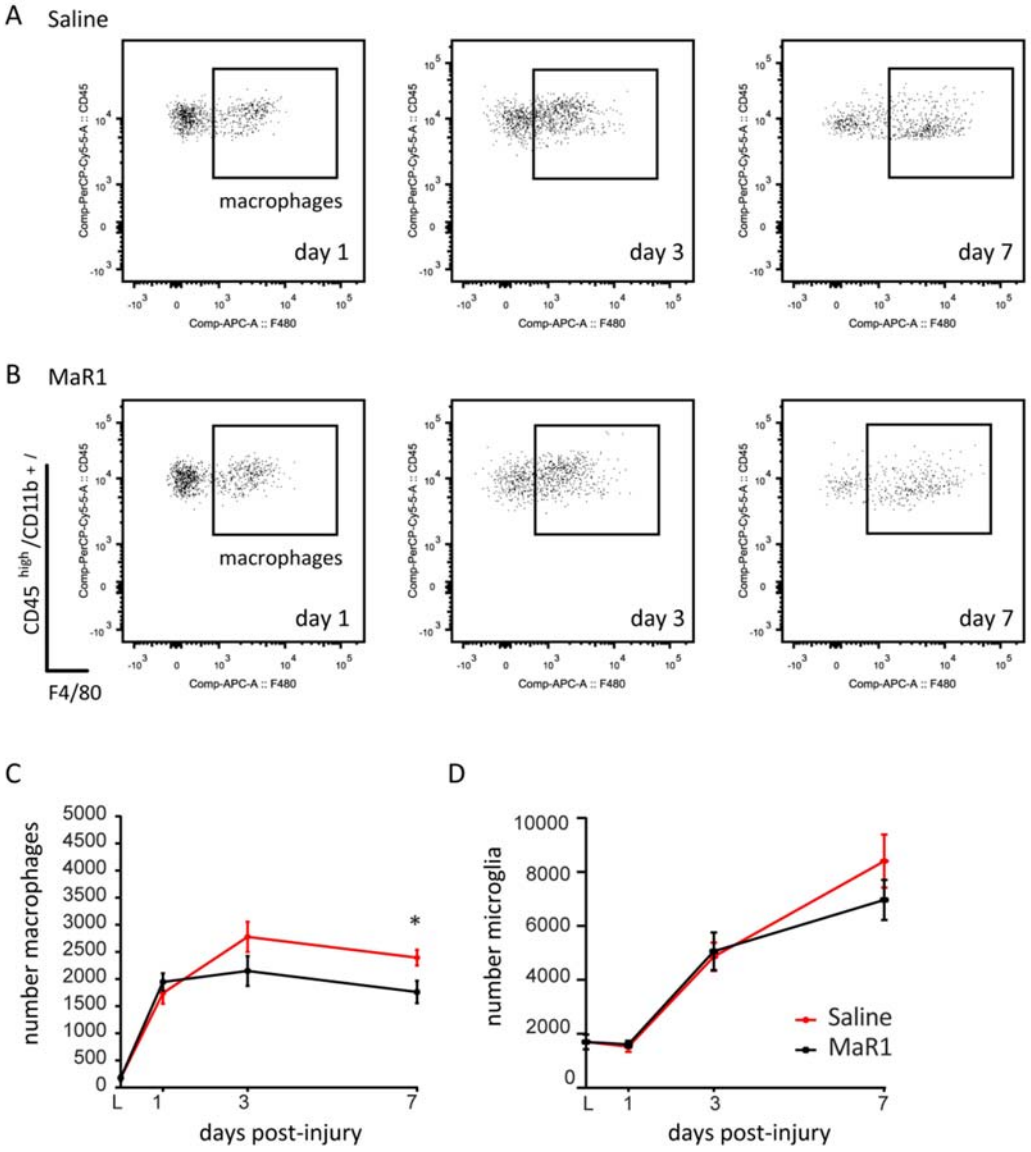


Figure 4. Effects of MaR1 in the accumulation of macrophages and microglial cells after SCI. (A-B) Representative density plots of FACS analysis showing macrophages (CD45high, CD11b+, F4/80+) at 1, 3 and 7 days after the injury in the spinal cord of saline (A) and MaR1 (B) treated mice. (C-D) Graph showing quantification of macrophage and microglial cells from FACS analysis. Data are expressed as mean ±SEM. (*p<0.05 MaR1 vs saline; Student t test was used to analyze significant differences. n=10 at 1 dpi, n=6 at 3dpi and n=6 at 7 dpi).

MaR1 silences cytokine expression in SCI

In an attempt to assess the mechanisms underlying the resolving actions of MaR1 in SCI, we assessed changes in expression of cytokines in the spinal cord 24 post-injury. The Luminex assay revealed that the protein levels of all the cytokines evaluated were increased in the spinal cord at 24 hours after injury (Figure 5A, Table 1), with the exception of IL-4, which was undetectable (data not shown). Interestingly, MaR1 treatment significant reduced the protein levels of CXCL1, CXCL2, CCL3, CCL4, IL-6, and CSF3 (Figure 5A; Table 1). In addition, the expression of IL-3, IL-13, IL-17 and CXCL5, which were found at low levels in contused spinal cord of mice treated with vehicle, were undetectable in those treated with MaR1 (Table 1). Note that MaR1 did not reduce the protein levels of the anti-inflammatory cytokine IL-10 after SCI.

	Naive	*Saline*	*MaR1*
CSF3	ND	1179± 182	741± 118*
IL-6	0.94± 0.03	671± 133	291± 56.7*
CXCL1	2.81± 0.23	219± 67.6	87.5± 21.3*
CXCL2	4.59± 0.30	119± 17.9	41.4± 7.50*
CCL4	ND	28.9± 3.47	17.7± 1.46*
CCL3	ND	21.5± 2.52	13.3± 1.18*
CXCL5	ND	4.80± 0.86	ND
IL-13	ND	4.19± 1.38	ND
IL-3	ND	0.56± 0.03	ND

Table 1. Multiplex analysis of cytokine protein levels from spinal cord. (A) Expression in pg/mg protein of significant cytokine reduced levels in MaR1 treated animals verus saline reated animals. Mean ±SEM. (n=5 per group) *p<0.05 vs saline. Student t test was used to analyze significant differences between MaR1 and control mice)

Since cytokines are regulated by multiple signal transduction pathways, we then investigated which of the main inflammatory signaling mechanisms were attenuated by MaR1 after SCI. Western blot analysis of spinal cord tissue taken 24 hours after SCI revealed that levels of pP65 and pAkt were up-regulated after contusion injury but these levels were not affected by MeR1 treatment (Figure 5B,C). In contrast, STAT and MAPK pathway, two of the main inflammatory signaling mechanisms, after SCI showed differences. STAT1, STAT3 and STAT5, as well as p38 and ERK1/2, were significantly increased at 24 hours post-injury in saline treated mice, and all of them were attenuated upon MaR1 treatment (Figure 5B,C). STAT6 and JKN, which were not significantly activated after SCI, remained unaltered after MaR1 administration.

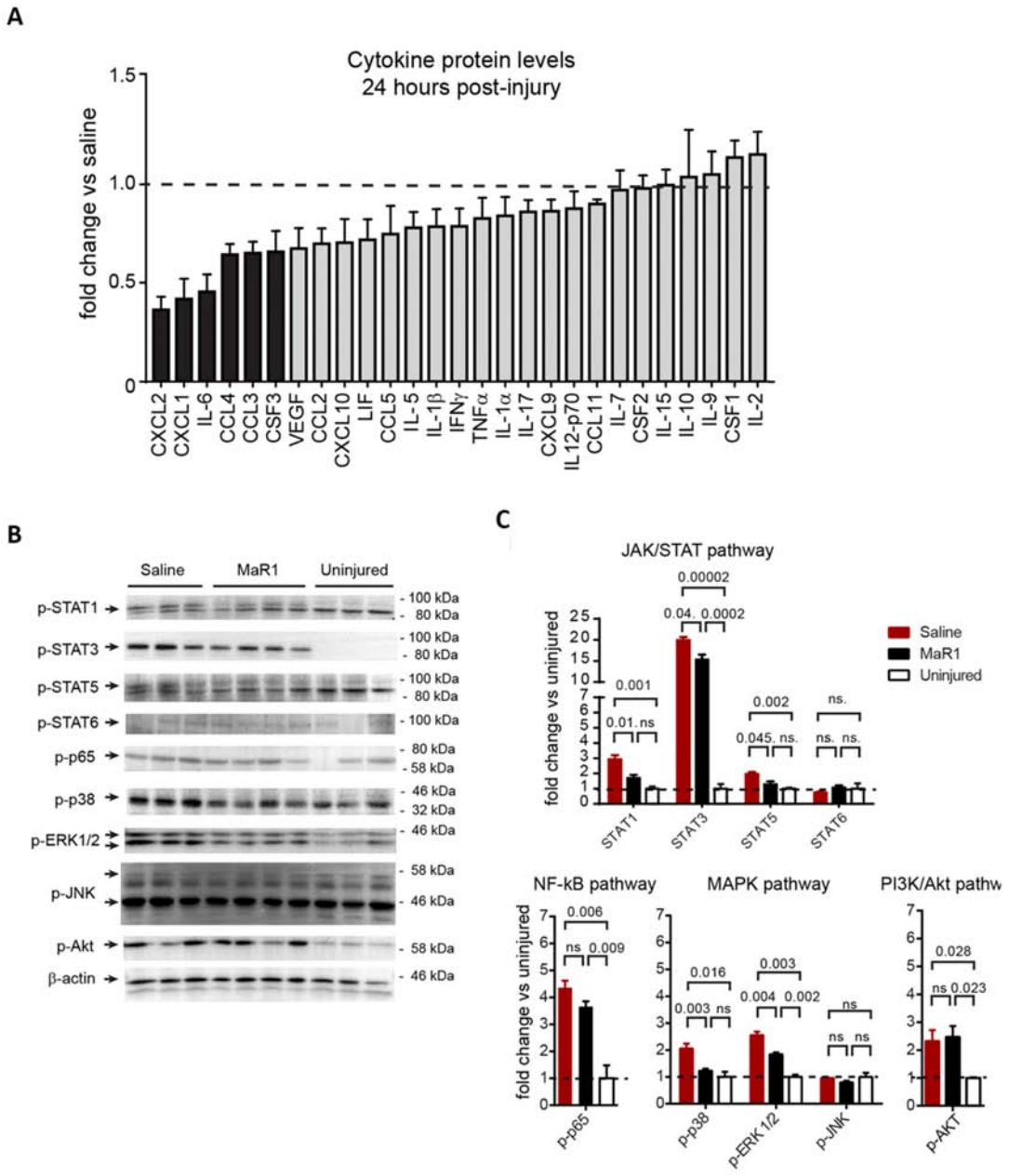


Figure 5. MaR1 turns off cytokine expression and attenuates the activation of several intracellular inflammatory pathways after SCI. (A) Multiplex analysis of cytokine protein levels from spinal cord of MaR1-treated and saline-treated mice at 24 hours post-injury. (B) Representative blots of phopho-STAT1, phopho-STAT3 , phopho-STAT5 , phopho-STAT6 , phopho-p65 , phopho-p38 , phopho-ERK 1/2 , phopho-JNK and phopho-AKT obtained from uninjured spinal cords or 24 hours injured spinal cords from saline- or MaR1 treated mice. (C) Graph showing the quantification of the western blots. Mean ±SEM. (n=3 in saline, n=4 in MaR1 and n =3 in control mice) *p<0.05 vs saline. One-way ANOVA, Bonferroni post hoc test to study significant differences between groups.

These data provide clear evidence that MaR1 silences cytokine expression and turns off the activation of some members of the STAT and MAPK pro-inflammatory signaling pathways, but does not limit NF-κB and PI3K/Akt signaling after SCI.

Effects of MaR1 on microglia and macrophage after SCI

Macrophages are a heterogeneous population of cells that exert divergent effects on damaged tissue depending on their phenotype. Ly6C^{high} macrophages exhibit phagocytic, proteolytic, and inflammatory functions, whereas Ly6C^{low} macrophages promote wound healing and repair (Arnold et al., 2007, Nahrendorf et al., 2007). Since cytokines plays a key function in regulating macrophage polarization(David and Kroner, 2011, Kroner et al., 2014), we studied whether MaR1 modulated the expression of Ly6C in macrophages at 7 days after SCI, the time point when MaR1 treatment reduced the number of these cells. The proportion of LyC6^{high} and Ly6C^{low} were somewhat similar at 7 days post-injury in vehicle treated animals, although the LyC6^{low} subset was slightly higher (giving a ratio LyC6^{low}/ LyC6^{high} of) (Figure 6A,B). MaR1 caused a significant impact on the expression of Ly6C in macrophages. MaR1 treatment reduced the number of Ly6C^{high} expressing and increased the number of Ly6C^{low} expressing macrophages. The ratio LyC6^{low}/ LyC6^{high} in saline treated SCI mice was 1.57±0.39. In contrast, this ratio was increased ~2.5 fold by MaR1 treatment relative to saline treated mice (3.83±0.17) (Figure 6A,B). These data indicate that this SPM changes the phenotype of macrophages in the injured spinal cord towards an anti-inflammatory state. MaR1 also significantly reduces expression of the pro-inflammatory, cytotoxic molecule iNOS (Figure 6C,E) and increased the expression of the pro-repair molecule Arg1, which was barely detectable in macrophages after SCI in saline treated mice was increased ~2 fold by MaR1, although it did not reach statistical significance (Figure 6C,E).

In contrast to macrophages, most microglial cells were Ly6C^{low} in SCI, and MaR1 did not reduce Ly6C^{high} in microglia (9.6%±0.8 and 10.6%±1.1, in saline- and MaR1-treated mice, respectively). MaR1 treatment tended to reduce the expression of iNOS, (Figure 6D, F). These results, therefore, suggest that MaR1 drives activation of macrophages but not microglia, from a pro-inflammatory towards a more pro-restorative state after SCI.

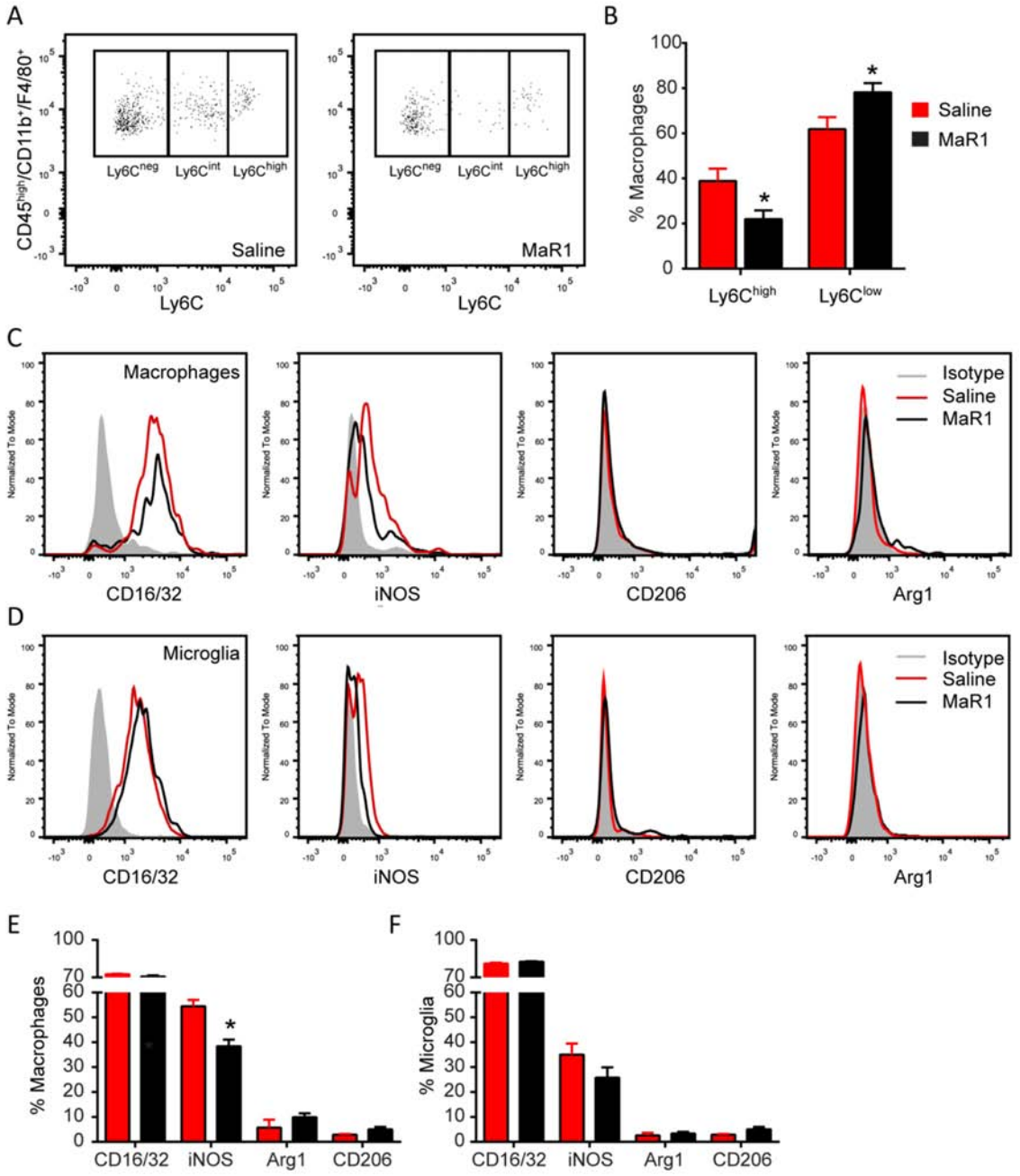


Figure 6. MaR1 treatment reduces M1 phenotype markers 7 days post injury. (A) Representative density plots of FACS analysis of Ly6C macrophages in saline and MaR1 treated mice at day 7 post injury. (B) Graph showing proportion of different macrophage subsets in the injured spinal cord 7 days after the injury. (C-D) Representative FACS histograms plots of M1 and M2 markers in injured spinal cord for macrophages (C) and microglial cells (D) at 7 days post injury. (E-F) Graph showing the quantification of macrophages (E) and microglial cells (F) expressing M1 and M2 markers after SCI. Mean ±SEM. (n=6 per group) *p<0.05 vs saline. Student t test was used to analyze significant differences between MaR1 and control mice.

As phagocytosis of neutrophils by macrophages is a crucial step for the resolution of inflammation (Schwab et al., 2007, Serhan, 2014, Serhan et al., 2015), we monitored whether MaR1 enhanced the ability of macrophages to phagocytose neutrophils (efferocytosis). We

found that the amount of the selective neutrophil marker Ly6G inside the macrophages was increased ~2 fold in the spinal cords of mice treated with MaR1 at 7 days post-lesion, indicating that this SPM enhanced neutrophil phagocytosis in SCI (Figure 7).

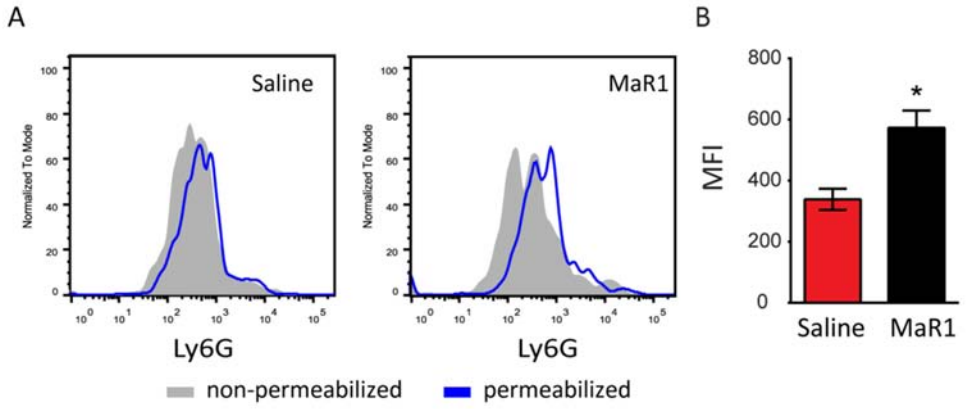


Figure 7. MaR1 promotes neutrophil phagocytosis by macrophages at 7 days post-lesion. (A) Representative FACS histograms plots of Ly6G marker in macrophages at 7 days post-lesion. (B) Graph showing the quantification of mean florescente intensity (MFI) of Ly6G marker in macrophages. Mean ±SEM. (n=4 per group) *p<0.05 vs saline. Student t test was used to analyze significant differences between MaR1 and control mice.

Administration of MaR1 reduces functional deficits and tissue damage after SCI

We finally examined whether MaR1 improves functional and histological outcomes after SCI. Mice treated with MaR1 showed significant improvement in locomotor recovery resulting in elevated BMS scores. Post hoc analysis revealed significant differences in BMS score starting at day 3 after injury and remaining significantly enhanced up the end of the follow up (Figure 8A).

At 28 dpi, 50% of mice treated with saline showed plantar placement with no stepping, whereas the 50% remaining performed occasional stepping (BMS score of 3.5±0.22). However, all the mice treated with Mar1 showed plantar placement with occasional or frequent stepping (score 4.58±0.22) (Figure 8A). Mice administered with Mar1 also showed significantly faster speeds of locomotion on the treadmill (Figure 8B). In addition, DigiGait analysis revealed that MaR1 improved specific parameters of locomotion such as gait symmetry and stance/width stepping variability after SCI (Figure 8C-D), further demonstrating improvement in locomotor control in mice treated with MaR1.

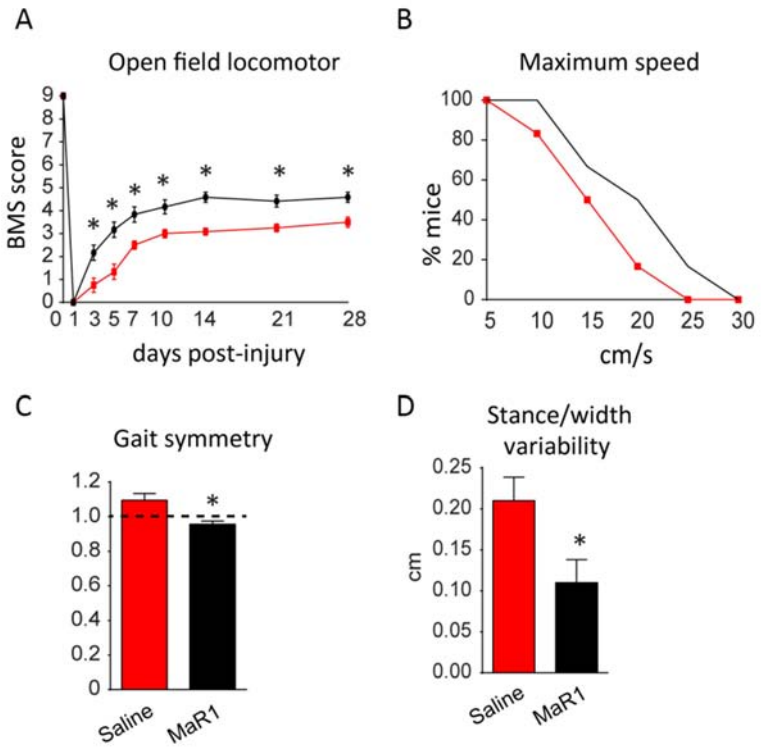


Figure 8. MaR1 enhances functional recovery after SCI. (A-D) Mice treated with MaR1 show significant improvement in locomotor skills assessed by (A) the 9-point Basso Mouse Scale (BMS) and (B-D) Digigait. Data are expressed as mean ±SEM (*p<0.05 Two-ways RM-ANOVA, Bonferroni's post hoc test in A, Mantel-Cox test in B and t test in C-D. n=10 per group).

We then assessed whether the improvement in locomotor skills of MaR1-treated mice was associated to reduction of secondary tissue damage after SCI. Histological sections stained with LFB revealed that MaR1 increased myelin sparing at the injury epicenter and in sections located at 200 µm rostral and caudal to the injury (Figure 9A-B). To determine whether this greater amount of myelin was due to reduced demyelination or reduced axonal damage or both, we quantified the number of axons (NF+) and those that had myelin sheath (NF/MBP+) in the dorsal columns at the injury epicenter, the most damaged area of the spinal cord. These analyses revealed that MaR1 enhanced both axonal sparing and reduced demyelination after SCI (Figure 9C-D). In addition, we also found that MaR1 improved neuronal survival in the ventral horn in caudal regions to the injury epicenter (Figure 9E-F).

Overall, these data demonstrates that treatment with MaR1 reduces secondary tissue damage and improves functional outcomes after SCI.

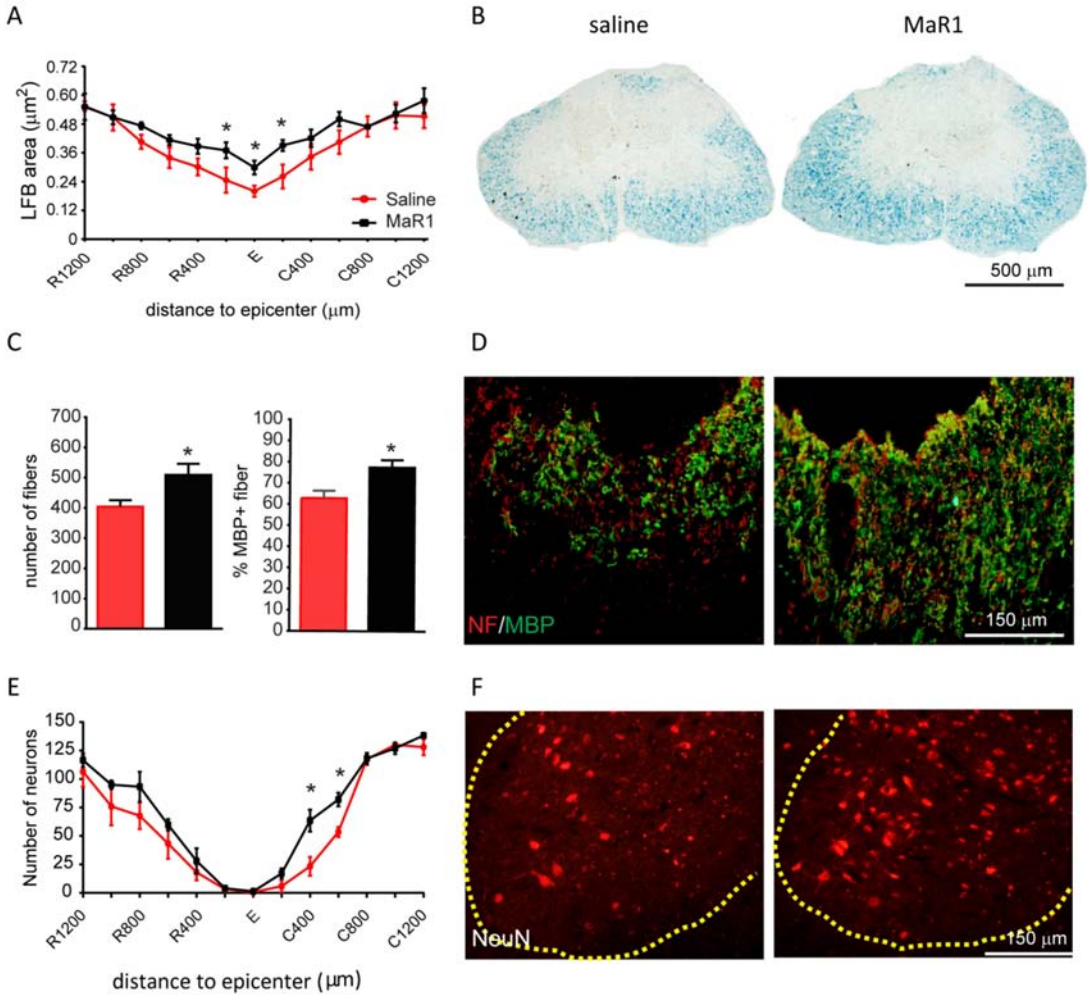


Figure 9. MaR1 treated mice attenuate tissue damage after SCI. (A) Quantification of myelin sparing at various distances rostral and caudal to the injury epicenter. (B) Representative micrographs showing myelin sparing at the injury epicenter in section stained with LFB from saline- and MaR1-treated mice. (C) Quantification of dorsal neurofilament and MBP immunoreactivity at the injury epicenter. (D) Representative micrographs showing dorsal neurofilament (red) and MBP (green) staining at the injury epicenter from saline- and MaR1-treated mice. (E) Quantification of ventral horn neuron survival at various distances rostral and caudal to the injury epicenter reveals the lack of effect of RvD1 in neuronal survival. (F) Representative micrographs showing sparing of ventral horn neurons in saline- and MaR1-treated mice tissue in sections stained against NeuN at 400 μm caudal to the injury epicenter. Data are expressed as mean ±SEM. (*p<0.05; two-ways RM-ANOVA, Bonferroni's post hoc test in A, and E; t-test in C; n=6).

DISCUSSION

Polyunsaturated fatty acids play a crucial role in regulating the inflammatory response. Among them, n-3 PUFA has brought to the attention of the scientific community due to its beneficial effects in several inflammatory diseases. Indeed, the n-3 PUFAs DHA and EPA, found in fish oils, are extensively used currently as dietary supplements and are thought to exert beneficial effects in a number of inflammation related diseases, including SCI (King et al., 2006, Huang et al., 2007, Lopez-Vales et al., 2010).

More recently, EPA and DHA lipid-derived mediators known as resolvins, protectins and maresins have been described as key mediators in the resolution of inflammation and regulators of normal homeostasis (Schwab et al., 2007, Buckley et al., 2014, Serhan, 2014, Serhan et al., 2015). It has been demonstrated that DHA lipid-derived mediators, such as resolvin D series (RvD), and protectins, as well as mediators such as RvE1 from EPA, are quite effective in preventing inflammation in several pathological conditions (Schwab et al., 2007, Serhan, 2014, Serhan et al., 2015), and that their lack or inappropriate synthesis, results in development of chronic inflammatory disease (Serhan, 2014).

Here, in agreement with previous studies (Fleming et al., 2006, Pruss et al., 2011), we show that inflammation is incompletely resolved after SCI, since there is residual presence of the different leukocyte subsets as well as microglial cells in the lesion spinal cord, even at 4 weeks post-injury. However, here we calculated different indices that assess resolution and that highlighted to what extend the clearance of the different immune cells is hampered in the injured spinal cord. Interestingly, we also performed lipidomic analysis of the injured spinal cord, which revealed that the synthesis of the different SPMs is substantially delayed after lesion. This data let us to conclude that the failure of the injured spinal cord to resolve inflammation could be due to the inappropriate production of SPMs. Indeed, exogenous administration of MaR1, confirmed the link between the impaired synthesis of SPMs and the failure of the spinal cord to resolve inflammation after injury.

MaR1 is a DHA-derived mediator that was recently described as an important modulator of inflammation resolution (Serhan et al., 2009). However, so far, little is known about its mechanism of action or its possible beneficial effects in the CNS. In this study, we report for the first time, to the best of our knowledge, the effects of MaR1 in a murine SCI model and demonstrate that systemic treatment with very low doses of MaR1 (1µg) consistently protected animals from tissue damage and functional impairments. Our data also give mechanistic insights into its protective mechanisms, some closely related to other lipid

mediators, but also new and relevant actions on inflammatory pathways and macrophage behavior.

A number of experimental evidence suggest that after SCI, the expression of chemokine and cytokine stimuli an excessive leukocyte recruitment plays an important role in the progression of tissue damage (Hawthorne and Popovich, 2011, David et al., 2012b). Previous data have described the role of MaR1 in reducing neutrophils counts in zymosan-induced peritonitis (Serhan et al., 2009, Serhan et al., 2012). Our study corroborates these data by demonstrating that MaR1 attenuates neutrophil accumulation in the injured spinal cord. Nonetheless, the mechanisms that underlie this process are not fully known and could involve reduction in chemokines/cytokines, activation of inflammatory pathways, and/or an effect on this leukocyte subset on macrophages.

It is now well documented that glial cells are responsible for producing a potent combination of broadly active pro-inflammatory cytokines, including IL-1β, TNF-α, IL-6, and INF-γ, which promote the recruitment of peripheral leukocytes, mainly neutrophils and monocytes, in the injured spinal cord (Pineau and Lacroix, 2007, David et al., 2012b). Our present data reveals that MaR1 reduced the protein levels of several pro-inflammatory cytokines and chemokines that has a key function in recruiting immune cells into the spinal cord. Although MaR1 did not attenuate the entrance of neutrophils and macrophages at the peak of infiltration, this could act as a brake for the infiltration of these leukocyte subsets at later time points, and thus, explain, at least in part, their reduced accumulation in the contused spinal cord upon MaR1 treatment.

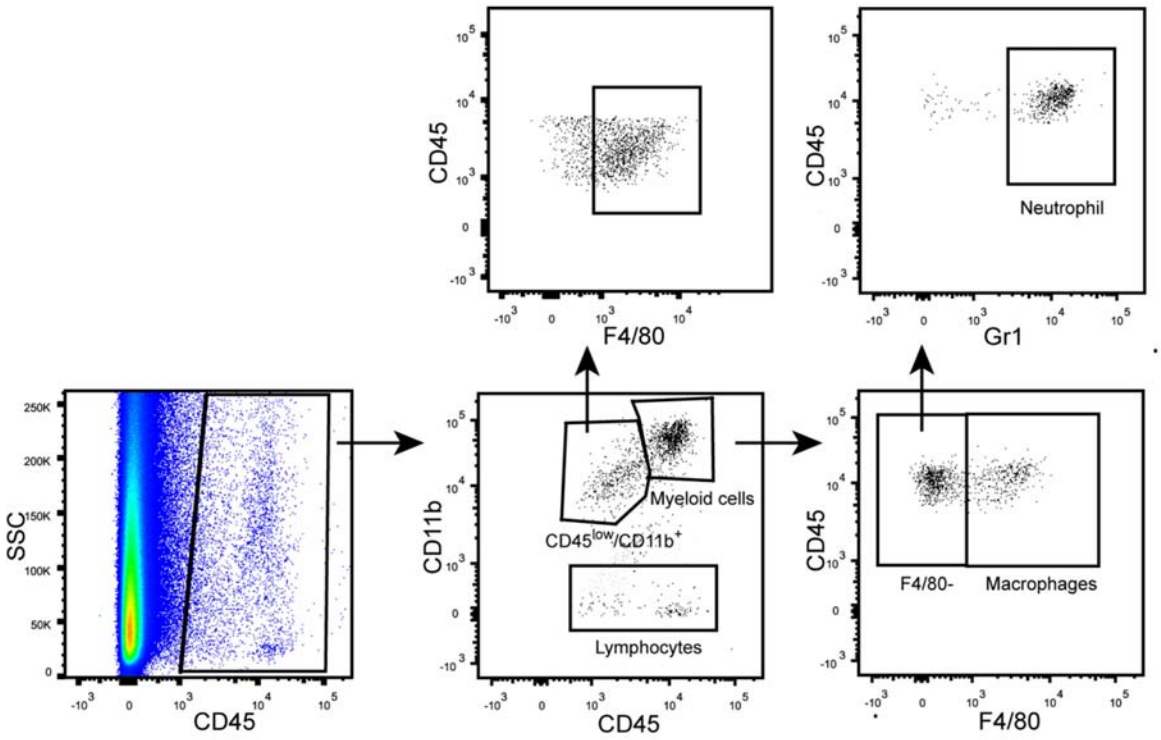
Since cytokine and chemokines trigger the activation of several inflammatory intracellular cascades to mediate inflammation, we decided to assess the effect of MaR1 on the activation on different key inflammatory pathways. In contrast to a previous study done in a mouse model of colitis (Marcon et al., 2013), MaR1 did not reduced NF-kB activation after SCI. Similarly, MaR1 did not interfere with the activation of the PI3K/Akt pathway. However, this SPM significantly turned off several MAPK and JAK/STAT pathways after SCI, which are known to exert important actions on inflammation in this pathology (Kerr et al., 2008, Ghasemlou et al., 2010, David et al., 2012c).

It is known that cytokines and some inflammatory pathways are strictly related to macrophage polarization. Macrophages can differentiate into two major types: M1 macrophages that display a pro-inflammatory profile, and M2 macrophages that have anti-inflammatory actions (Sica and Mantovani, 2012, Murray et al., 2014, David et al., 2015). As pointed out earlier, our results showed that MaR1 significantly silenced the expression and activation of these two polarizing factors after SCI. Although, we observed that MaR1 did not significantly induced the

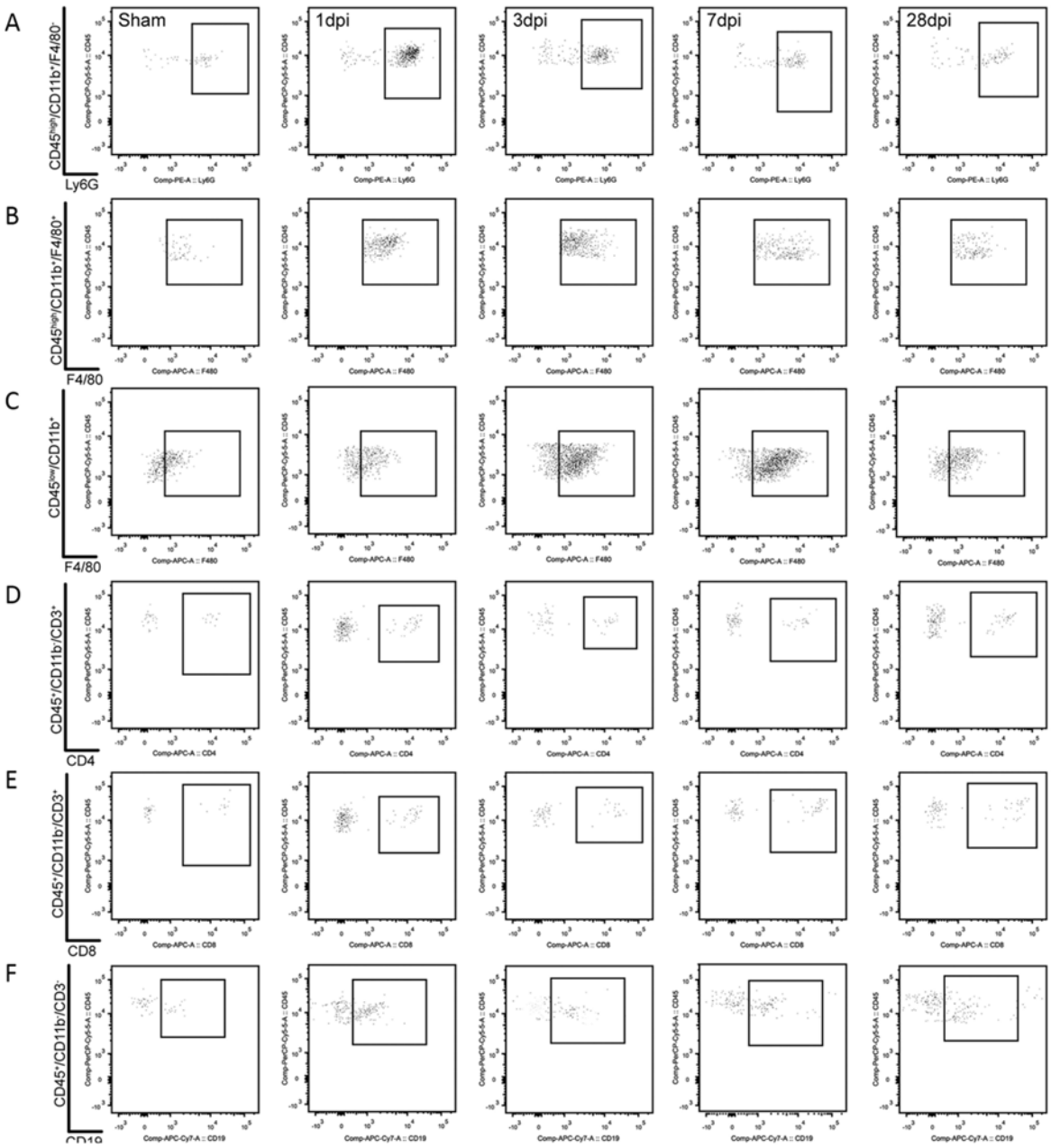
expression of M2 markers in macrophages, it led to significant reduction in the expression of M1 markers such as iNOS and Ly6C. These data suggest that MaR1 in SCI may redirect macrophage polarization towards a less skewed pro-inflammatory phenotype, and thus, reduce their bystander effects on tissue damage. The ability of MaR1 to redirect macrophage activation towards a phenotype more conducive for tissue repair has been also described in vitro and in a mouse model of colitis (Marcon et al., 2013). Interestingly, this is not the only effect that MaR1 exerted on this leukocyte subset. We also found that the administration of MaR1 after SCI triggered macrophages to increase neutrophil phagocytosis. Previous studies have shown that RvD and RvE also enhanced efferocytosis of neutrophils in a mouse model of peritonitis (Schwab et al., 2007). However, to our knowledge, this is the first study revealing that MaR1 also promotes this phagocytic activity in macrophages in vivo. This fact, together with the effects of MaR1 on cytokine expression and activation of inflammatory pathways, may explain, at least in part, the faster and greater elimination of neutrophils from the injured spinal cord observed upon treatment with this SPM.

In summary, the present results show for the first time, to the best of our knowledge, that MaR1, a pro-resolving DHA-derived mediator, effectively ameliorates functional deficits and tissue damage after SCI in mice by harnessing inflammatory resolution. MaR1 clearly proved effective in modulating multiple stages of the resolution of inflammation, which included, clearance of cytokines, silencing of inflammatory pathways, reduction of neutrophil and macrophages counts, redirection of macrophage phenotype, and induction of neutrophil phagocytosis by macrophages. Therefore, the present data support the notion the uncontrolled inflammatory response and its harmful effects after SCI are due, in part, to inappropriate synthesis of SPM, and suggest that MaR1 may constitute a novel therapeutic strategy for treatment of acute SCI.

SUPPLEMENTARY FIGURES



Supplementary Figure 1. Gates of different immune cell population in FACS analysis.



Supplementary Figure 2. Recruitment of immune cells after SCI. (A-F) Representative density plots of FACS analysis showing the dynamics of neutrophil ($CD45^{high}$, $CD11b^{+}$, $F4/80^{-}$, Gr1+) (A), macrophage ($CD45^{high}$, $CD11b^{+}$, $F4/80^{+}$) (B), microglial cell ($CD45^{low}$, $CD11b^{+}$, $F4/80^{+}$) (C), lymphocyte TCD4 ($CD45^{+}$, $CD11b^{-}$, $CD3^{+}$, $CD4^{+}$) (D), lymphocyte TCD8 ($CD45^{+}$, CD11b, $CD3^{+}$, $CD8^{+}$) (E) and B cell ($CD45^{+}$, $CD11b^{-}$, $CD3^{-}$, $CD19^{+}$) (F) recruitment in the spinal cord at 1, 3 , 7 and 28 days after the injury.

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CHAPTER 3

Effects of Resolvin D1 administration after spinal cord injury in mice

Effects of Resolvin D1 administration after spinal cord injury in mice

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ABSTRACT

Inflammatory response is an essential step of the injury response to restore tissue homeostasis and to initiate wound repair after injury or infection. However, to prevent unnecessary tissue damage, inflammation must be actively resolved when is no longer needed. Recently, several specialized pro-resolving lipid mediators (SPM) derived from polyunsaturated fatty acids (PUFA) has been identified to actively turn on the resolution programs of inflammation. Resolvin D1 (RvD1), one of the most well characterized SPM, has been shown to exert therapeutic effects in different inflammatory challenges. However, the whether RvD1 mediates beneficial effects in neurological conditions has not been addressed yet. In current work, we unexpectedly show that administration of RvD1 for the 1 week after spinal cord injury (SCI), and contrary to MaR1, fails to enhance inflammatory resolution, as well as, functional and histological outcomes. Transcriptomic analysis of spinal cord harvested from injured mice treated with RvD1 and Maresin1 (MaR1) reveals that there is differentially enriched expression of genes related to fibrosis and inflammation upon RvD1 as compared to MaR1 administration. Overall, this work reveals for the first time that the delivery of RvD1 for one week does not mediate therapeutic effects in SCI.

Keywords: Resolvin D1, Maresin 1, Inflammation, Inflammatory resolution, Neuroprotection, Spinal Cord injury, MicroArray.

INTRODUCTION

Traumatic SCI is a devastating neurological condition that causes irreversible axonal damage and neuronal death, resulting in permanent disability. In addition to the initial mechanical injury, a wide range of secondary cellular and molecular events occurs, constituting the secondary injury. In this phase, the damage expands to rostral and caudal regions to the lesion core, aggravating cell death, axonal disruption, and consequently, functional impairments (Oyinbo, 2011).

Inflammation is one of the most important events that trigger secondary tissue damage after SCI. Although this response is essential to restore homeostasis of the tissue and initiate wound repair, this physiological mechanisms is not properly conducted after SCI, leading to excessive tissue damage (Buckley et al., 2014, Serhan, 2014, Serhan et al., 2015).

In chapter 2, we revealed that after SCI there is inappropriate inflammatory resolution and production of SPMs. Interestingly, we demonstrate that administration of MaR1, a SPM derived from the docosahexaenoic acid (DHA), results in therapeutic effects, linking the failure of the spinal cord to produce SPMs to tissue damage. However, whether the administration of other SPMs derived from DHA, such as RvD1, results in similar beneficial effects in the injured spinal cord remains to be elucidate yet.

RvD1 (7S,8R,17S-trihydroxy- 4Z,9E,11E,13Z,15E,19Z-DHA, RvD1) is synthesized by 15- and 5-lipoxygenases (LOX) from docosahexaenoic acid (DHA) (Hong et al., 2003). RvD1 acts through the binding to its receptors, orphan receptor G protein coupling receptor 32 (GPR32) and the lipoxin A4 receptor/formyl peptide receptor 2 (ALX/Fpr2) (Krishnamoorthy et al., 2010), and actively turns off the inflammatory response (Recchiuti et al., 2011) by limiting leukocyte infiltration and the production of pro-inflammatory cytokines (Rogerio et al., 2012), by inducing macrophage phagocytosis (Krishnamoorthy et al., 2010) and redirection of macrophage polarization toward an M2-like phenotype (Titos et al., 2011).

In the present study, we therefore investigated the effects of the exogenous delivery of RvD1 after spinal cord contusion injury in mice. We found that, in contradistinction to MaR1, administration of RvD1 fails to improve inflammatory resolution and to confer protection against tissue damage and functional impairments after SCI.

MATERIALS AND METHODS

All the experimental procedures were approved by the Universitat Autònoma de Barcelona Animal Experimentation Ethical Committee (CEEAH 1188R3-DMAH 6131) and followed the European Communities Council Directive 2010/63/EU, and the methods for each procedure were carried out in accordance with the approved guidelines.

Surgical procedure

Adult (8-10 weeks old) female C57Bl/6 mice (Charles River) were deeply anesthetized with intraperitoneal injection of ketamine (90 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.). After performing a laminectomy at the 11th thoracic vertebrae, the exposed spinal cord was contused using the Infinite Horizon Impactor device (Precision Scientific Instrumentation) (Klopstein et al., 2012). Injuries were made using a force of 60 kdynes and tissue displacement ranging between 400 and 600 μm.

One hour after SCI, 100 μl of sterile saline or sterile saline containing 0,5, 1 or 2 μg of RvD1 (7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid; Cayman Chemical Company, Ann Arbor, MI) or 1 μg of MaR1 (Cayman Chemical Company, Ann Arbor, MI) was injected intravenously and then repeated daily thereafter until day 7.

Cytokine Protein Expression

Mice treated with saline or RvD1 were perfused with sterile saline and a 5 mm length of spinal cord centered on the lesion was collected at 12h after contusion injury and snap-frozen. Spinal cords were homogenized and protein concentration was determined using the DC Protein Assay (Bio-Rad). Samples were concentrated to 4μg/μl using MicroCon centrifugation filters (Millipore) to ensure equal amounts of protein. The protein levels of 20 cytokines and chemokines were then analyzed using the Milliplex MAP Mouse Cytokine/Chemokine magnetic bead panel (Millipore) on a Luminex (Millipore) as per manufacturers' protocol.

Flow cytometry

Immune cells from the injured spinal cord were analyzed by flow cytometry. Spinal cord from mice treated with RvD1 or saline were harvested at day 1 and 7 post-lesion. Briefly, spinal

cords were cut in little pieces and passed through a cell strainer of 70 µm (BD falcon) and the cell suspension was centrifuged twice at 300g for 10 minutes at 4ºC. After cell counts, samples were divided, and cells alone and isotype-matched control samples were generated to control for nonspecific binding of antibodies and for auto-fluorescence. The following antibodies from eBioscience were used at 1:250 concentrations: CD45-PerCP, CD11b-PE-Cy7, Gr1-FITC, Ly6C-FITC, F4/80-APC, CD3-FITC, CD4-APC, CD8-APC and CD19-PE. After 30 min of incubation with combinations of antibodies at 4°C, the samples were washed and fixed in 1% paraformaldehyde. Moreover, samples were also stained with unconjugated rabbit antibodies against iNOS (1:200 Abcam), and goat antibodies against Arg1 (1:200; Santa Cruz). After 30 min of incubation with combinations of antibodies at 4°C, cells were then fixed in 1% paraformaldehyde. For intracellular staining, cells were permeabilized with Permeabilization Wash Buffer (Biolegend) followed by staining with Alexa488 or Alexa647 conjugated donkey secondary antibodies against rabbit or goat (1:500 Molecular Probes) for 30 min when needed. After 30 min of incubation with combinations of antibodies at 4°C, the samples were washed and fixed in 1% paraformaldehyde. Cells were analyzed on a FACSCanto flow cytometer (BD Biosciences) and results analyzed using FlowJo® software version 10.0.7.

To perform the analysis, cells were first gated for CD45 to ensure that only infiltrating leukocytes and resident microglia are selected, and then, the following combination of markers were used to identify CD4 T-Cells ($CD45^{+}$, $CD11b^{-}$, $CD3^{+}$, $CD4^{+}$), CD8 T Cells ($CD45^{+}$, CD11b, $CD3^{+}$, $CD8^{+}$), B cells ($CD45^{+}$, $CD11b^{-}$, $CD3^{-}$, $CD19^{+}$), microglial cells ($CD45^{low}$, $CD11b^{+}$, $F4/80^{+}$), macrophages ($CD45^{high}$, $CD11b^{+}$, $F4/80^{+}$), and neutrophils ($CD45^{high}$, $CD11b^{+}$, $F4/80^{-}$, $Ly6G^{+high}$). Kinetics analysis of these immune cell types were calculated as described previously (Prüss et al., 2011). To study the phenotype of macrophages, these cells were further differentiated based on Ly6C, CD16/32, iNOS, CD206 and Arg1 expression. Cells were analyzed using FlowJo® software on a FACSCanto flow cytometer (BD Biosciences).

Functional assessment

Locomotor recovery was evaluated at 1, 3, 5, 7, 10, 14, 21 and 28 days post-injury (dpi) in an open-field test using the nine-point Basso Mouse Scale (BMS), which was specifically developed for locomotor testing after contusion injuries in mice (Basso et al., 2006). The BMS analysis of hindlimb movements and coordination was performed by two independent assessors and the consensus score taken. The final score is presented as mean ± SEM.

In addition, at the end of the follow up (day 28 post-injury), a computerized assessment of locomotion was also performed using the DigiGait™ Imaging System (Mouse Specifics Inc., Boston, MA). This system is constituted of a motorized transparent treadmill belt and a high-speed digital video camera that performs images to the underside of the walking animals. DigiGait™ software generates "digital pawprints" and dynamic gait signals, representing the temporal record of paw placement relative to the treadmill belt. This locomotor test allows for an easy and objective analysis of both static and dynamic locomotor parameters. Finally, the highest locomotion speed which each mouse was to able locomote was also recorded on the Digigait treadmill belt. Briefly, each mouse was allowed to explore the treadmill compartment, with the motor speed set to zero, for 5min. Then speed was gradually increased from 0 up to 35 cm/s and the maximum speed at which each mouse performed for at least 5 seconds was recorded (Santos-Nogueira et al., 2015).

Electrophysiological analysis

At day 28, electrophysiological tests were used to evaluate spared motor central pathways after SCI. Motor evoked potentials (MEPs) were recorded from the tibialis anterior (TA) and gastrocnemius (Buckley et al.) muscles with microneedle electrodes, in response to transcranial electrical stimulation of the motor cortex by single rectangular pulses of 0.1ms duration. Pulses were delivered through needle electrodes inserted subcutaneously, the cathode over the skull, overlaying the sensoriomotor cortex, and the anode at the nose.

Compound Muscle Action Potential (CMAP) M waves from tibialis anterior and gastrocnemius muscles were recorded for internal control of peripheral normal conduction. In this case the sciatic nerve was stimulated percutaneously by means of single pulses of 0.02ms duration (Grass S88), delivered through a pair of needle electrodes placed at the sciatic notch (Verdú et al., 2003).

All potentials were amplified and displayed on a digital oscilloscope Tektronix 450S (Tektronix, OR) at settings appropriate to measure the amplitude from baseline to the maximal negative peak. To ensure reproducibility, the recording needles were placed under microscope to secure the same placement on all animals guided by anatomical landmarks. During the tests, the mice body temperature was kept constant by means of a thermostated heating pad.

Histological analysis

At 28 days post-injury mice were deeply anaesthetized using Dolethal (pentobarbital sodium; Vétoquinol E. V. S. A.) and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (Sun et al.). A 10 mm length of spinal cord containing the injection or the lesion site centered was harvested, post-fixed for 1 hour in 4% paraformaldehyde in 0.1M PB and cryoprotected with 30% sucrose in 0.1 M PB at 4ºC, for a minimum of 48 hours. Spinal cords were fast-frozen at -60ºC in cryoembedding compound (Tissue-Tek® oCT, Sakura) and cut on a cryostat (Leica). Ten series of 10µm thick section were picked up on glass slides. Adjacent sections on the same slide were therefore 100µm apart.

For demyelination analyses, sections were stained with Luxol Fast Blue (Sigma). For neuronal and axonal assessment, sections were incubated overnight at 4ºC with biotinylated antibodies against NeuN (1:200, Millipore) and NF (1:1000, Millipore), respectively. Moreover, double immunostaining against NF and MBP (1:100; Abcam) was done to assess the sparing of myelinated axons. Sections were incubated for 1 hour at room temperature with the streptavidin-Alexa 594 conjugated or donkey anti-rabbit Alexa 594-conjugated antibodies (Molecular Probes, 1:500), and then coverslipped in Mowiol containing DAPI to label nuclei.

Tissue sections were viewed with olympus BX51 microscope and images were captured using an olympus DP50 digital camera attached to it and using the Cell^A Image acquisition software. The epicenter of the injection or contusion injury impact was determined for each mouse spinal cord by localizing the tissue section with the greatest damage using LFB stained section. Myelin sparing after SCI was calculated by delineating the spared LFB stained tissue. Neuronal survival was assessed by counting the number of $NeuN^+$ cells in the ventral horns at the injury epicenter and at rostral and caudal areas. Axonal sparing was calculated by counting the number of axons in the dorsal column at the injury epicenter, the most damage area of the spinal cord. The same sections were used to examine axonal demyelination in the dorsal column was by counting the fibers double stained for NF and MBP at the lesion epicenter. The NIH ImageJ software was used to quantify all the histological parameters.

RNA isolation, reverse transcription and real-time PCR

Uninjured control mice, and animals treated with saline, RvD1 or MaR1 were euthanized with Dolethal (pentobarbital sodium, Vetoquinol; 0.01ml/10g, intraperitoneal) at 7 days post-injury,

and perfused with saline buffer. A 5 mm segment of spinal cord containing epicenter lesion was harvested and rapidly frozen and storage at -80ºC until mRNA extraction. For mRNA extraction, tissue was homogenized with Quiazol lysis reagent (Qiagen) and mRNA extracted using RNeasy Lipid Tissue kit (Qiagen), according to the users guide protocol. An additional step with DNase I digestion (Qiagen) was included to avoid genomic DNA contamination.

1μg mRNA of each sample was primed with Random Hexamers (Promega) and reverse transcribed using Omniscript RT kit (Qiagen). RNase inhibitor (Roche) was added (1U/ μl final concentration) to avoid RNA degradation. RT-PCR reactions were performed using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies) according to the manufacturer's instructions. Data analysis was performed using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad laboratories). Primer sequences included the following: iNOS forward 5'- GGCCAGCCTGTGAGACCTTT-3', iNOS reverse 5'-TTGGAAGTGAAGCGTTTCG-3'. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene: GAPDH forward 5'-TCAACAGCAACTCCCACTCTTCCA-3', GAPDH reverse 5'-ACCCTGTTGCTGTAGCCGTATTCA-3'. The amount of cDNA was calculated based on the threshold cycle (Basso et al.) value, and was standardized by the amount of housekeeping gene using the 2-ΔΔC method (Livak and Schmittgen, 2001).

Microarray

Microarray hybridation and the statistical processing of data were performed by the Scientific and Technical Support Unit and the Statistics and Bioinformatics Unit at the Vall D'Hebron Research Institut (Hospital de la Vall d'Hebron, Barcelona). mRNA samples of spinal cords harvested from uninjured mice, as well as from 7 days post-injured animal treated with saline, RvD1 and MaR1 were processed for Affimetrix MOUSE Exon/Gene 2.1 ST chip array according to the manufacturer protocol. The optical images of the hybridized chip were processed with the Expression Console software (Affimetrix). CEL files containing the intensity values associated to probes and grouped into probesets were obtained. Then, RMA method (Irizarry et al., 2003) was used in order to transform intensity values to expression values. RMA is a three step-method that integrates background adjustment, scaling and aggregation of the probe sets to remove non-biological elements of the signal and genes with low signal (those genes whose mean signal in each group did not exceed a minimum threshold) and low variability (genes whose standard deviation between all samples didn't exceed a minimum threshold). The selection of differentially expressed genes between conditions was based on a

linear model analysis with empirical Bayes moderation of the variance by Smyth (Smyth Gordon, 2004) and implemented in the limma Bioconductor package. To asses changes in gene expression as an effect of injury, gene expression profile of was compared between (C57Bl/6) injured and non-injured mice and a cut-off P-value <0.01 and 2 fold change (Log2 FC>1) were applied to select the differentially expressed genes. To determine the gene expression changes after injury between saline, RvD1 and MaR1 treated mice was applied a cut-off P-value <0.05 and 1.5 fold change (Log2 FC>0.64).

All the statistical analysis were done using the free statistical language R and the libraries developed for microarray data analysis by de Bioconductor Project (www.bioconductor.org)

Analysis of the biological meaning

To investigate the biological meaning, term enrichment analysis in the Gene Ontology (Ashburner et al., 2000)(GO; www.geneontology.org), functional annotation GO term clustering analysis (Huang et al., 2009) and KEGG pathway mapping were performed (http://www.genome.jp/kegg/pathway.html) (Kanehisa et al., 2006) to identify biological functions that were significantly enriched . The Database for Annotation, Visualization and Integrated Discovery (DAVID, v6.7) (National Institute of Allergy and Infectious Diseases (NIAID); http://david.abcc.ncifcrf.gov/home.jsp) was used for DAVID's GO biological process FAT (GOTERM_BP_FAT) or KEGG analysis (KEEG_PATHWAY). The GO terms were classified by functional annotation clustering analysis, where the list of selected genes (the sample) was compared to a reference set (the whole probes in the Affimetrix chip used). The functional annotation cluster was ranked from largest to smallest enrichment score (ES). After analysis every cluster was labeled with a representative name of the GO terms included in the cluster. Finally genes were mapped to the KEGG database for pathway analysis using GOseq.

Statistical analysis

All analyses were conducted through GraphPad Prism 6.0. Data are shown as mean ± standard error of the mean (Semple et al.). Maximal speed on a treadmill was analyzed using the Mantel-Cox test. Functional follow-up for BMS score, as well as histological analysis of myelin and neuronal sparing were analyzed using two-way repeated measure ANOVA with post-hoc Bonferroni's post-hoc test for multiple comparisons. Two-tailed Student's t test was used for the single comparison between two groups. Differences were considered significant at $p<0.05$.

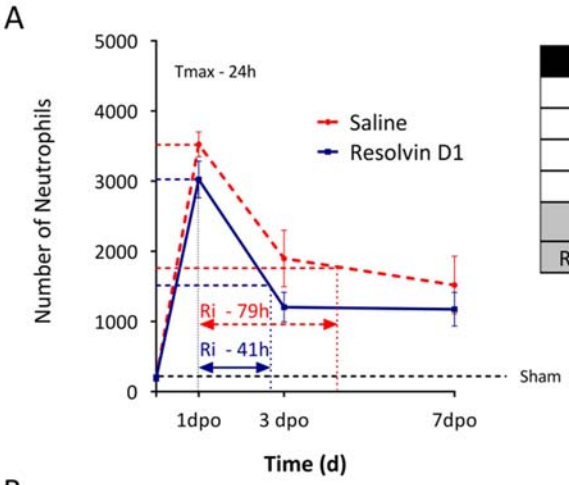
RESULTS

Effects of RvD1 treatment in the resolution of inflammation after spinal cord contusion.

Since our data from chapter 2 revealed that deficit in the resolution of inflammation after SCI may be linked to impaired synthesis of SPMs, we investigated whether systemic administration of RvD1 enhanced immune cell clearance from the contused spinal cord. For this purpose, we firstly determined the effects on different doses of RvD1 on functional recovery to choose the ideal amount of RvD1 to conduct our experiments (Supplementary Figure 1). Since all 3 doses resulted in similar effects, we chose to administrate 1 μg per mouse and day, as we did in chapter 2 with MaR1.

We then evaluated, by flow cytometry, whether there was a difference in the accumulation of immune cells between saline- and RvD1-treated animals following SCI. We found that RvD1 treatment slightly reduced the infiltration of neutrophils at day 1 post-injury (Figure 1 A) although it did not reach statistical significance. Interestingly, RvD1 accelerated the clearance of these cells from the contused spinal cord at day 3 post-injury (Figure 1 A). This is clear from the data obtained upon analyzing several resolutions parameters (Pruss et al., 2011). Indeed, we observed that the Ri for neutrophils, which indicates the time needed to drop the counts of this leukocyte subset to 50% from the peak of maximal accumulation, was reduced ~48% after RvD1 treatment (Figure 1A). Therefore, this data indicates that RvD1 treatment enhanced resolution of inflammation. Nevertheless, and in contrast to our expectations, we found that the clearance of neutrophils from the injured spinal cord was completely interrupted at later stages upon RvD1 treatment. Indeed, the counts of neutrophils in contused spinal cord were very similar in RvD1-treated animals at day 3 and 7 post-injury, and no significant differences were observed relative to vehicle-treated mice at 1 week post-injury (Figure 1).

We next studied whether the RvD1 interfered with microgliosis and the different leukocyte subsets after SCI. We observed that RvD1 did not attenuate microglial or macrophage counts after lesion (Figure 1B,C), although it tended to reduce their numbers. RvD1 did not impede the entrances of lymphocytes in the spinal cord (Supplementary Figure 2)



Resolution Indices	SCI + saline	SCI + RvD1	% reduction
Ψ_{max} (x 10^3)	3.53 ± 0.18	3.02 ± 0.26	-
$\Psi_{7\ dpo}$ (x 10^3)	1.52 ± 0.41	1.17 ± 0.24	-
T_{max}; (h)	24	24	-
T_{50}; (h)	~ 103	~ 65	-
R_i ; (h)	**~ 79**	**~ 41**	**~ 48.1**
Reduction 7 dpo ; (%)	**56.9**	**61.3**	**~ 4.4**

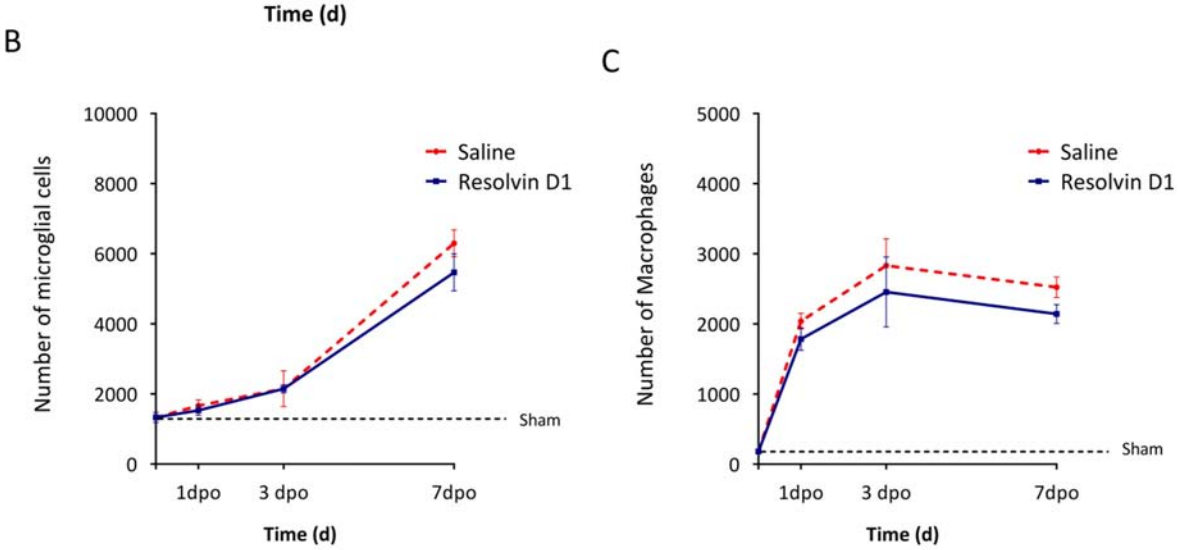


Figure 1. Effects of RvD1 in the infiltration of immune cells after SCI. (A) Graph showing neutrophil recruitment and resolution indices. (B-C) Graph showing microglial (B) and macrophage (C) in the injured spinal cord. Data are expressed as mean ±SEM. (Student t test was used to analyze significant differences. n=9 at 1 dpi, n=4 at 3dpi and n=4 at 7 dpi).

RvD1 does not modulate macrophage phenotype after SCI

Macrophages are a wide heterogeneous population of cells that exert divergent effects on damaged tissue depending on their phenotype. Ly6C^{high} macrophages exhibit phagocytic and inflammatory functions, whereas Ly6C^{low} macrophages promote wound healing and repair (Arnold et al., 2007, Nahrendorf et al., 2007). Thus, we wanted to evaluate whether RvD1 modulated macrophage polarization after SCI. We did not find differences in the progression of monocyte conversion from inflammatory (LyC6^{high}) to anti-inflammatory (LyC6^{low}) phenotypes in RvD1-treated animals (Figure 2A). Indeed, at day 7 post-injury, the proportion of macrophages that showed LyC6^{high} expression tended to be higher in mice treated with RvD1, whereas the proportion of Ly6C^{low} macrophages tended to be lower upon the treatment

with this SPM (35.2±5.1% vs 39.1±5.4% LyC6^{high}; 53.7±5.5% vs 49.8±4.4% Ly6C^{low} in saline- and RvD1-treated mice, respectively) (Figure 3 B-C).

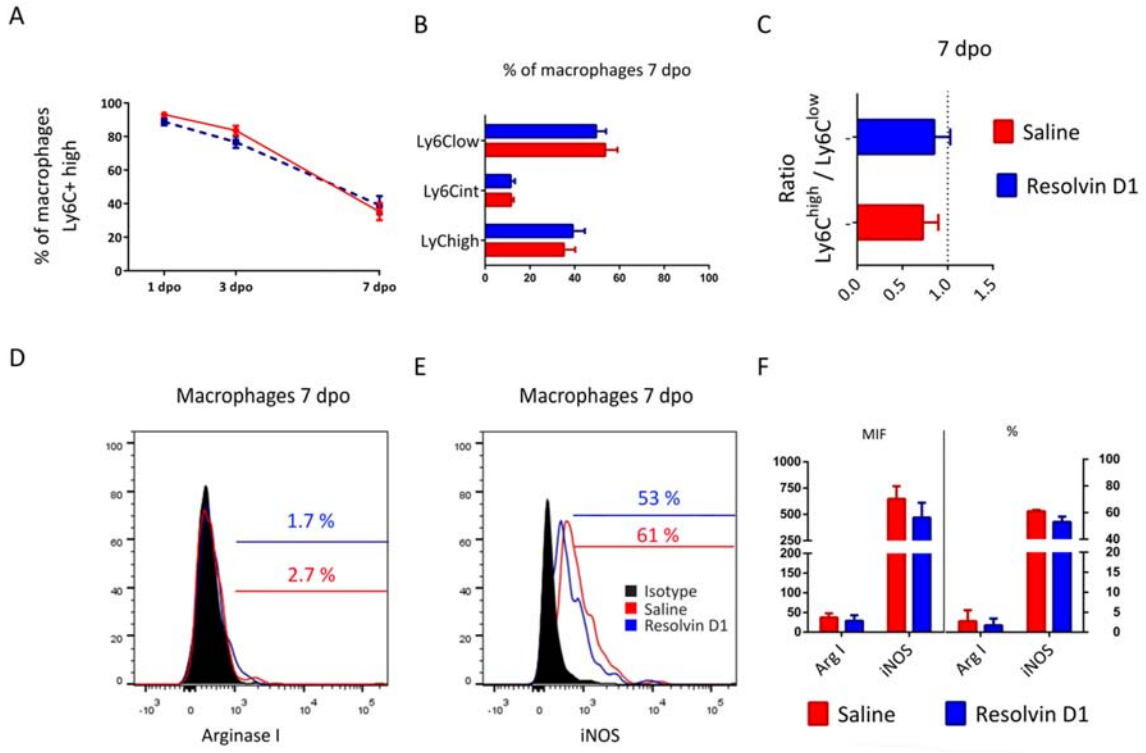


Figure 2. RvD1 does not modulate macrophage phenotype after SCI. (A) Graph showing the evolution of LyC6^{high} macrophages in the injured spinal cord after RvD1 treatment. (B) Graph showing proportion of different macrophage subsets in the injured spinal cord at 7 days after the injury. (C) Graph showing the effects of RvD1 in the ratio of LyC6^{high}/LyC6^{low} macrophages in the injured spinal cord at day 7 after lesion (D-E) Representative FACS histograms plots of Arg I and iNOS expresion in macrophages at 7 days post injury..(F) Graph showing the quantification of macrophages expressing Arg1 and iNOS after SCI. Mean ±SEM. (n=4 per group) *p<0.05 vs saline. Student t test was used to analyze significant differences between RvD1 and control mice.

We then assessed whether RvD1 modulated the expression of M1/M2 markers after SCI. The expression of the M2 marker Arg1 was barely detectable in macrophages after 7 days post injury (~3%) in both control and RvD1 treated mice (Figure 2 D-F). The expression of iNOS (M1 marker) was slightly reduced after RvD1, in both percentage (61±1 vs 53±4 % in saline- and RvD1-treated mice, respectively) and median fluorescence intensity (MFI; 648±120 vs 470±141in saline- and RvD1-treated mice, respectively), although it not reach statistical significance (Figure 2 E-F). In agreement with this data, RT-PCR analysis confirmed that iNOS mRNA levels was not reduced in the injured spinal cord after RvD1 treatment (Figure 3). The expression of other M1 (CD16/32) and M2 (CD206) markers remained unchanged after RvD1 treatment (4.2±4 vs 4.4±3.8% CD206; 72.5±1.7 vs 70.8±0.7% CD16/32 in saline- and RvD1-treated mice, respectively) (Supplementary Figure 3).

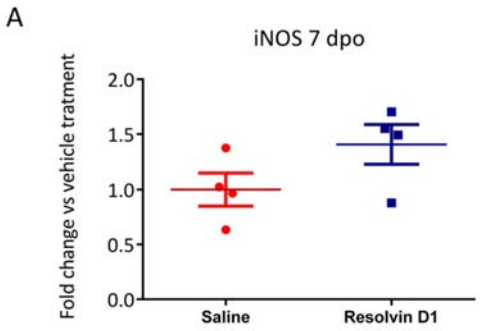


Figure 3. Effects of RvD1 treatment in the mRNA levels of iNOS in the spinal cord at 7 days post-injury assessed by real time PCR. Mean ±SEM. (n=5 per group)

These results, therefore, suggest that RvD1 failed to drive macrophages activation from a pro-inflammatory towards a more anti-inflammatory/pro-repair state after SCI.

Effects of RvD1 on cytokine expression in SCI

We study whether RvD1 regulated the protein levels of different cytokines in the spinal cord at 24 hours post-injury. Luminex assay revealed that injured spinal cord harvested from mice treated with RvD1 tended to have, although not significantly, lower levels of most pro-inflammatory cytokines (Figure 4). However, the expression of two pro-inflammatory chemokines involved in recruitment of immune cells, CCL11 and CXCL10, were significantly increased upon RvD1 treatment (27.3±4.5 vs 38±2 pg/mg protein for CCL11; and 1517.7±236.4 vs 2302.1±136.6 pg/mg prot for CXCL10 in saline and RvD1 groups, respectively). On the other hand, the anti-inflammatory cytokine IL-13 was significantly reduced in animals treated with RvD1 (4.6±0.8 vs 2.9±0.2 pg/mg prot). Therefore, in contrast to MaR1 treatment shown in chapter 2, RvD1 does not silence pro-inflammatory cytokine expression after SCI.

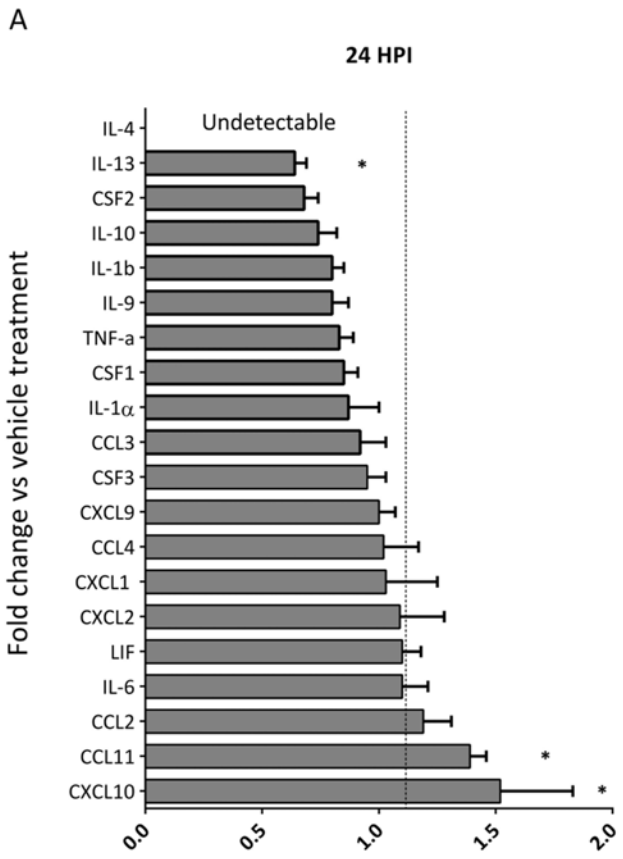


Figure 4. RvD1 does not turn off the expression of pro-inflammatory cytokines in SCI. (A) Multiplex analysis of cytokine protein levels from spinal cord of RvD1-treated and saline-treated mice at 24 hours post-injury. Mean ±SEM. (n=5 per group) *p<0.05 vs saline. Student t test was used to analyze significant differences between RvD1 and control mice.

Treatment with RvD1 does not reduce secondary tissue damage after SCI

We then assessed whether treatment of RvD1 resulted in beneficial action on tissue damage after SCI. Histological sections stained with luxol fast blue (LFB) revealed that RvD1 treated mice did not attenuate myelin loss ($p>0.05$; two-way ANOVA) (Figure 5 A-B). Similarly, quantification of NeuN+ cells in the ventral horns of the spinal cord did not reveal greater neuronal after RvD1 treatment ($p>0.05$; two-way ANOVA) (Figure 5 C-D). Finally, to determine whether RvD1 treatment led to greater axonal and myelin preservation, we quantified the number of axons (NF+) and those that had spared myelin sheath (NF/MBP+) in the dorsal columns at the injury epicenter, the most damaged area of the spinal cord. This analysis revealed that RvD1 treatment did not lead attenuated axonal or myelin sparing after SCI (Figure 5 E-G; $p>0.05$; two-way ANOVA). Overall, this data provides clear evidence that administration of RvD1 does not enhance histological outcomes after SCI.

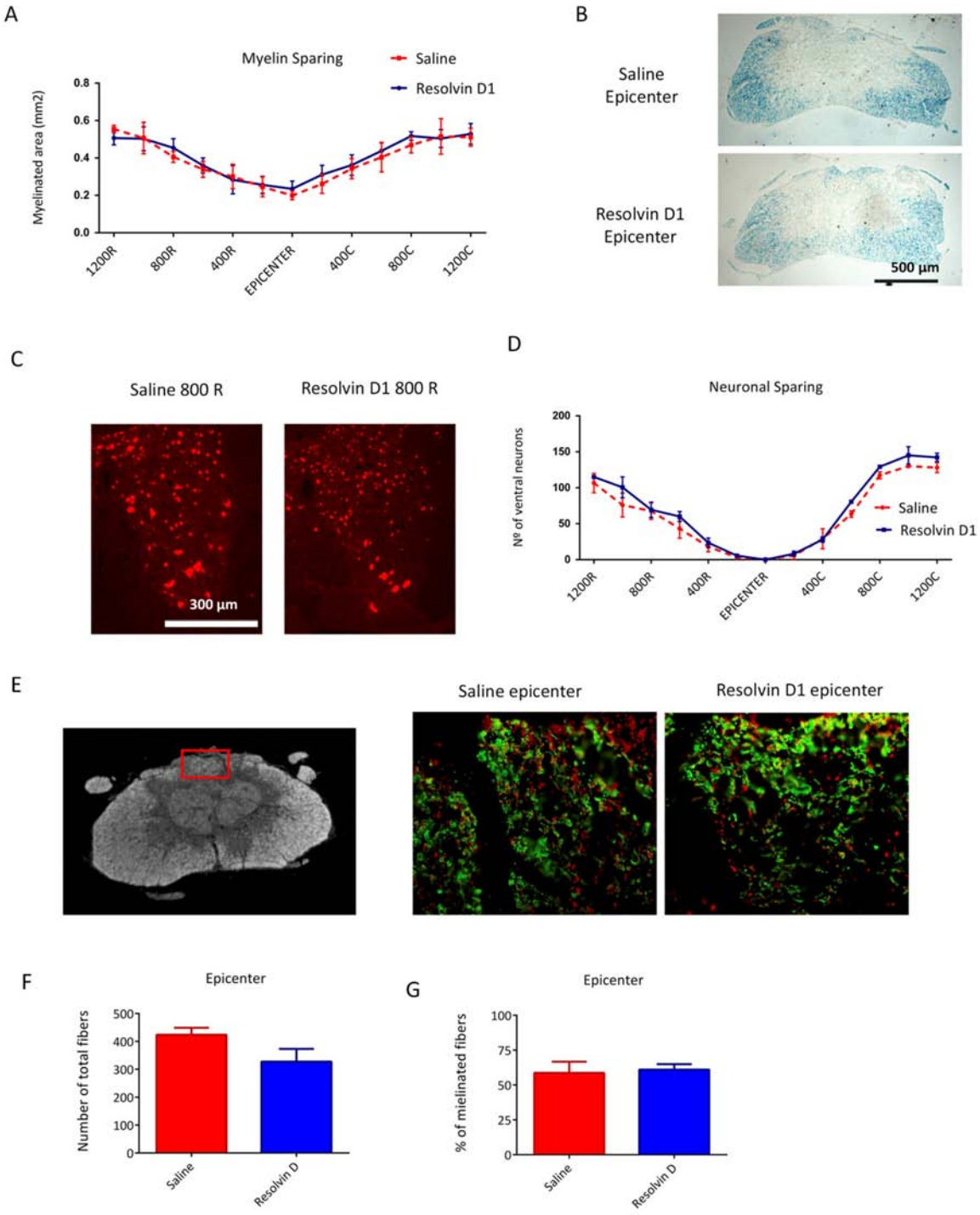


Figure 5. RvD1 treated mice does not attenuate tissue damage after SCI. (A) Quantification of myelin sparing at various distances rostral and caudal to the injury epicenter. (B) Representative micrographs showing myelin sparing at the injury epicenter in section stained with LFB from saline- and RvD1-treated mice. (C) Representative micrographs showing sparing of ventral horn neurons in saline- and RvD1-treated mice tissue in sections stained against NeuN at 800 µm rostral to the injury epicenter. (D) Quantification of ventral horn neuron survival at various distances rostral and caudal to the injury epicenter reveals the lack of effect of RvD1 in neuronal survival. (E) Representative micrographs showing dorsal neurofilament (red) and MBP (David et al.) staining at the injury epicenter from saline- and RvD1-treated mice. (F-G) Quantification of dorsal neurofilament and MBP immunoreactivity at the injury epicenter. Data are expressed as mean ±SEM. (*p<0.05; two-ways RM-ANOVA, Bonferroni's post hoc test in A, and D; t-test in F-G; n=5).

Treatment with RvD1 does not reduce functional deficits after spinal cord injury.

We finally examined whether RvD1 treatment enhanced functional recovery after SCI. In line with histopathological data, functional analysis revealed that RvD1 treatment did not confer significant protection against loss of locomotor function after SCI based the on the BMS scale (score 3.8±0.19 vs 3.55±0.26 % in control and RvD1, respectively) (Figure 6 A-B).

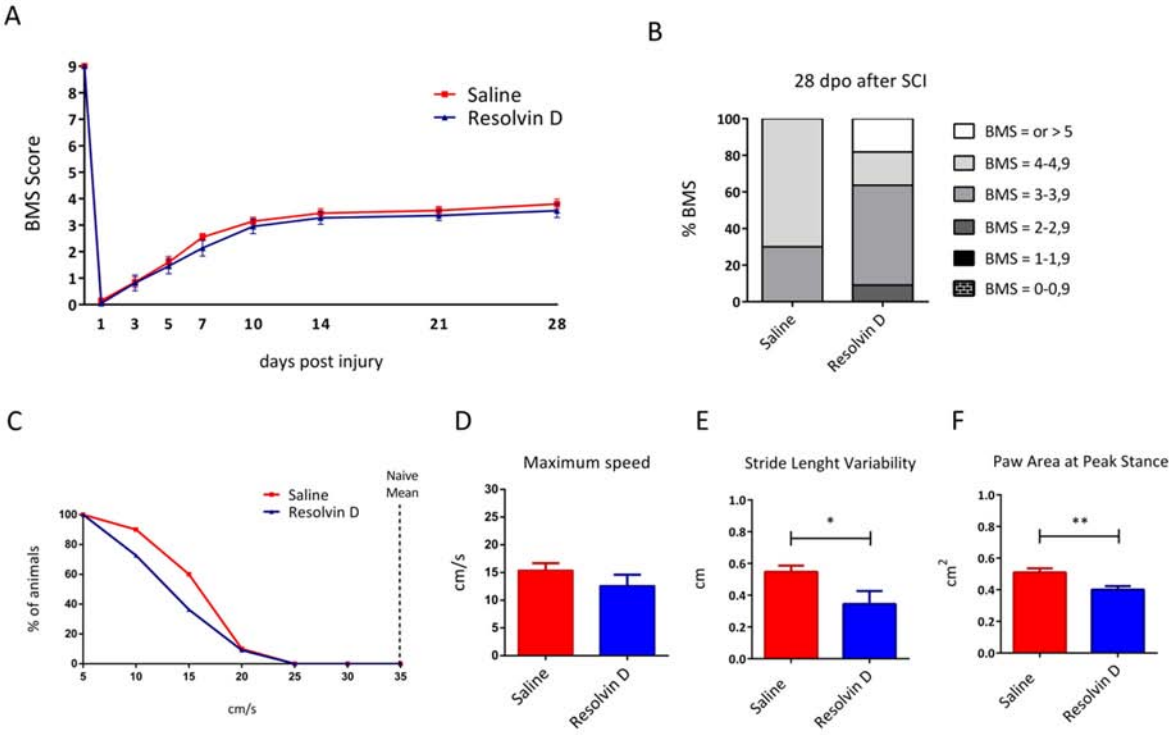


Figure 6. Animals treated with RvD1 does not show enhanced functional recovery after SCI. (A-F) Mice treated with RvD1 did not show significant improvement in locomotor skills assessed by (A-B) the 9-point Basso Mouse Scale (BMS) and (C-F) the Digigait. Data are expressed as mean ±SEM (*p<0.05 Two-ways RM-ANOVA, Bonferroni's post hoc test in A, Mantel-Cox test in C and t test in D-F. n=10 in saline group, n=11 in RvD1 group).

Further behavioral test using the Digigait analysis revealed that mice treated with RvD1 tended to show slower locomotion on a treadmill and to modulate some specific parameters of locomotion such as stride length variability or paw area at peak stance after SCI (Figure 6 C-F). Finally, electrophysiological evaluation of motor evoked potentials revealed no significant differences in the preservation of spinal cord descending pathways in mice treated with RvD1 (Figure 7 A-B).

Taken together, this data provides clear evidence that administration of RvD1 does not enhance significantly functional outcomes after SCI.

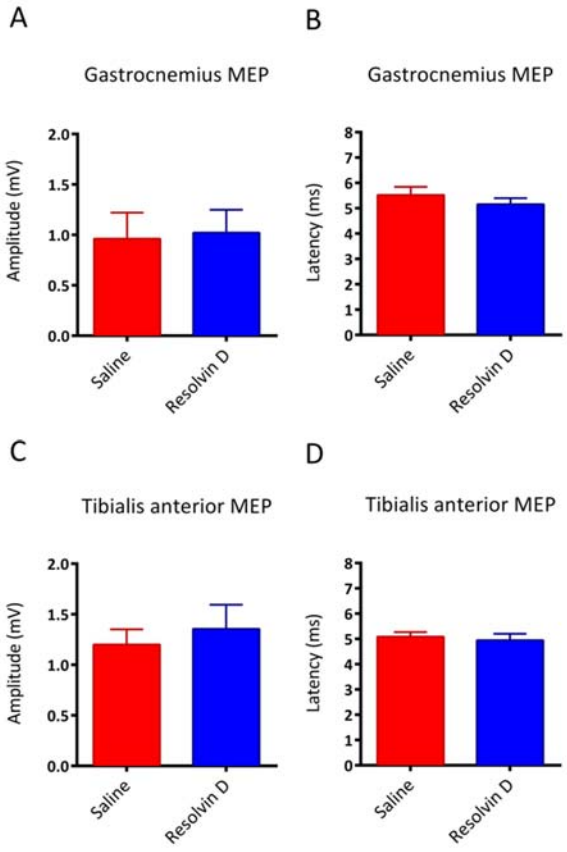


Figure 7. RvD1 treatment does not improve MEPs after SCI. (A-D) Quantification of MEPs amplitude and latency recorded from the gastrocnemius (A-B) and tibialis anterior muscles (C-D) after SCI. Data are shown as mean ±SEM. (*p<0.05; t-test; n=8 in saline- and n=10 in RvD1-treated mice).

Differences in gene profile of contused spinal cord in RvD1 and MaR1 treated mice at 7 days post-injury

As shown in Chapter 2, MaR1 treatment enhanced inflammatory resolution and exerted neuroprotection and functional improvement after SCI. Although RvD1 is generated from the same metabolic precursor that MaR1, here we show this SPM does not result in similar helpful effects. In order to gain new insights into the different mechanisms regulated by these two DHA-derived SPMs in SCI, we performed microarray-based expression profiling experiments from injured spinal cord at day 7, when the differences on the resolution of inflammation were markedly evident.

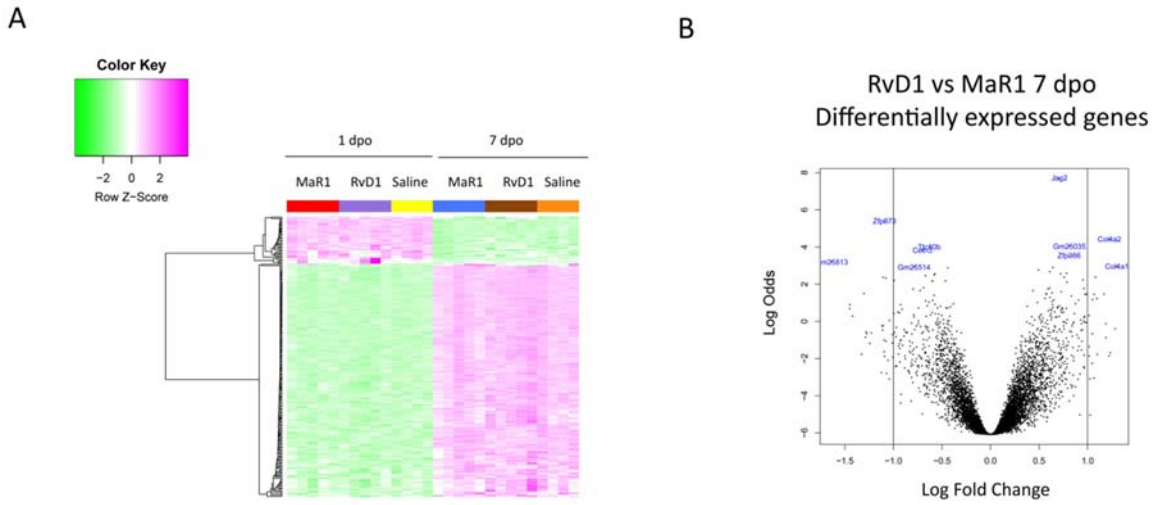


Figure 8. Gene expression changes by RvD1 treatment. (A) Heat-map of gene expressions of injured spinal cord at 1 and 7 days post-injury after saline, RvD and MaR1 treatment. (B) Volcano plot showing the differences in gene expression in the spinal cord from mice treated with MaR1 and RvD1 at day 7 after the injury

Microarray analysis revealed that 156 genes were down-regulated, for at least 1.5 fold and show p values <0.01, in RvD1- as compared to MaR1-treated mice. On the other hand, 194 genes, with similar a cut off, were up-regulated RvD1-treated mice relative to those injected with MaR1. The most top 20 down- and up regulated genes in RvD1 vs MaR1 are displayed in Table 1 and Table 2. The top-20 genes down-regulated in RvD1 treatment have unknown functions (Table 1). However, within the top 20-genes up-regulated in the RvD1 treated animals, we found genes that encode for fibrilin and for 6 different collagens, are all them involved in fibrosis. In addition, there were other genes involved in the motility of monocyte, such as, Actin-alfa 2 (Acta2), and genes related to matrix remodeling that facilitates the infiltration leukocytes such as, elastase and matrix metallopeptidase 14 (Mmp14) (Klose et al., 2013). Highlight that the second most up-regulated gene in RvD1 versus MaR1 treatment at day 7 was the lymphocyte antigen 6 complex 1 (Ly6C1), bolstering flow cytometry results where RvD1 treatment failed to reduce the macrophages LyC6^{high} subset after SCI, whereas administration of MaR1 resulted in significant reduction of this pro-inflammatory macrophage population (Table 2).

DOWN-regulated genes in RvD1 mice vs MaR1 at 7 dpi								
	Gene	logFC	P.Value			Gene	logFC	P.Value
1	Gm25813	1,63646	1,30E-05		11	Gm26175	1,22765	8,73E-04
2	Gm25360	1,45023	2,05E-04		12	Gm22270	1,12407	9,60E-03
3	Gm22155	1,4492	1,63E-04		13	Snora44//Snhg12	1,1167	5,71E-04
4	Gm25939	1,42145	3,21E-04		14	Gm25394	1,11116	7,38E-04
5	Gm22767	1,3283	3,16E-03		15	n-R5s151	1,10691	3,23E-05
6	Gm22866	1,30448	8,34E-05		16	Snora62	1,10469	5,71E-04
7	Gm25989	1,28841	1,14E-03		17	Gm25156	1,0967	1,34E-03
8	Gm25820	1,27982	8,31E-04		18	Gm25107	1,09539	2,71E-03
9	8842//Gm8841//Gm10420//G	1,2748	9,21E-04		19	Snord58b//Gm22354	1,09219	3,56E-03
10	Gm22486	1,24407	1,63E-03		20	Zfp873	1,09184	1,18E-06

Table 1. List of the top-20 down-regulated genes in the spinal cord of RvD1 vs MaR1 treated mice at day 7 after the injury

UP-regulated genes in RvD1 mice vs MaR1 at 7 dpi								
	Gene	logFC	P.Value			Gene	logFC	P.Value
1	collagen type IV alpha1	1,30181	1,67E-05		11	matrix metallopeptidase 14	1,08109	3,23E-05
2	lymphocyte antigen 6 c1	1,28454	6,86E-04		12	Ighg3	1,06796	8,86E-05
3	elastin	1,2328	2,88E-03		13	collagen, type X, alpha 1	1,06553	1,35E-02
4	collagen type IV alpha2	1,22457	3,33E-06		14	Gm24009	1,05084	1,21E-04
5	collagen type I alpha2	1,22077	3,49E-03		15	fibrillin 1	1,04842	9,21E-04
6	Gm23422	1,18932	1,16E-03		16	Gm23716	1,04707	5,48E-03
7	microRNA 598	1,18909	5,06E-04		17	collagen type VIII alpha2	1,02313	1,41E-02
8	microRNA 370	1,16883	8,73E-05		18	Gm22882	1,01648	3,50E-03
9	Gm8247	1,09964	3,16E-03		19	collagen type I alpha2	1,01521	2,00E-03
10	alanyl-tRNA synthetase	1,09685	1,14E-04		20	Actin-alpha 2	1,01463	3,11E-04

Table 2. List of the top-20 up-regulated genes in the spinal cord of RvD1 vs MaR1 treated mice at day 7 after the injury

The differentially expressed genes identified in the genearray were significantly enriched in several KEGG pathways (Table 3). Within the top-20 biological meaning pathways enriched upon RvD1 treatment, we found several relevant pathways involved in immune cell infiltration, such as, “focal adhesion” or “cell adhesion molecules (CAMs)”. In addition, some pathways related to non-resolved inflammation disease like “Rheumatoid arthritis” and CNS diseases such as “Alzheimer´s disease” and “Amyotrophic lateral sclerosis” were also increased in RvD1 treated mice

KEGG Pathways UP-Regulated in RvD1 mice vs MaR1 at 7 dpi										
	Term	Exp.Count	P.Value	KEGG ID			Term	Exp.Count	P.Value	KEGG ID
1	Pathways in cancer	23,2309	3,82E-02	5200		11	ECM-receptor interaction	6,2238	3,45E-12	4512
2	Neuroactive ligand-receptor	20,0466	4,34E-02	4080		12	Small cell lung cancer	6,1515	1,00E-03	5222
3	Focal adhesion	14,2570	4,70E-08	4510		13	Hypertrophic cardiomyopathy	5,9343	2,10E-04	5410
4	Calcium signaling pathway	12,8819	1,60E-04	4020		14	Ribosome	5,8620	4,52E-12	3010
5	Alzheimer's disease	12,0859	4,24E-03	5010		15	Rheumatoid arthritis	5,7896	1,18E-02	5323
6	Cell adhesion molecules (CAMs)	10,3490	2,85E-02	4514		16	Glycerophospholipid metabolism	5,7173	4,04E-03	564
7	Axon guidance	9,4082	2,47E-02	4360		17	Protein digestion and absorption	5,6449	7,71E-06	4974
8	Amoebiasis	8,3226	3,51E-03	5146		18	Cardiac muscle contraction	5,5001	7,89E-03	4260
9	Pancreatic secretion	7,3818	3,16E-02	4972		19	Arrhythmogenic cardiomyopathy	5,2830	4,87E-05	5412
10	Dilated cardiomyopathy	6,3686	1,00E-04	5414		20	Amyotrophic lateral sclerosis	3,9804	5,44E-03	4720

Table 3. List of the top-20 KEGG pathways enriched in the spinal cord of RvD1 vs MaR1 treated mice at day 7 after the injury

Overall, our data provided indicate that exogenous administration of RvD1, in contradistinction to MaR1, fails to resolve inflammation after SCI in mice.

DISCUSSION

Timely resolution is the ideal outcome of acute inflammation, as it is required for ensuring return to homeostasis and host health. It is now appreciated that resolution of inflammation is not as passive process as it was initially believed. This is indeed controlled, in part, by the biosynthesis of SPMs derived from poly-unsaturated fatty acid (PUFA) (Serhan and Petasis, 2011). SPMs actively turn off the inflammatory response by acting to distinct G protein couple receptors expressed on immune cells that mediate dual anti-inflammatory and pro-resolution programs (Buckley et al., 2014, Serhan, 2014, Serhan et al., 2015). Nevertheless, when the synthesis of SPMs is defective or delayed, inflammation may become chronic, cause bystander effects, and contribute to the pathogenesis of many highly burdening diseases (Lawrence and Gilroy, 2007, Serhan et al., 2007). This is the case of SCI where, as we demonstrated in chapter 2, lesion milieu is characterized by an impaired induction of SPMs, and the administration of

the DHA-derived SPM, MaR1, enhanced resolution of inflammation after SCI and improved functional and histopathological outcomes.

Besides MaR1, there are other DHA-derived SPMs, which include the different members of the RvD series (RvD1-RvD6) (Serhan et al., 2008, Bannenberg and Serhan, 2010). Among them, RvD1 has been the most studied member of this lipid family, and has consistently demonstrated to mediate potent effects on boosting inflammatory resolution in a wide spectrum of pathologies (Norling et al., 2012, Norling et al., 2016), but not in neurological conditions yet. Therefore, in the present report, we wondered whether systemic RvD1 administration reproduces the results shown by MaR1.

By doing a different experimental methodologies, we show that systemic administration of RvD1 (1ug) for one week diary does not result in overall enhancement in the resolution of inflammation after SCI. However, closer analysis on the data reveals that this SMP, similar to other studies, markedly accelerates the clearance of neutrophils from the injured spinal cord milieu (Norling et al., 2012). However, this effect is only limited to early stages after SCI (day 3), but surprisingly, interrupted from this time point. In addition, this SPM also failed to attenuate microglia and macrophage counts after SCI, and to redirect these cells towards a more anti-inflammatory phenotype. Indeed, it seems that RvD1 led to opposite effects, since macrophages tended to be closer to the pro-inflammatory state, although not significantly, as revealed the ratio of $Ly6C^{high}/Ly6C^{low}$ macrophages. Similarly, RvD1 increased the amounts of the pro-inflammatory cytokines, CCL11 and CXCL10, and reduced the levels of the anti-inflammatory cytokine IL-13. This surprising data suggest that continuous administration of RvD might not be effective for resolving inflammation after SCI.

We also found that although this SPM had not significant impact on tissue damage and functional impairment, mice treated with RvD1 tended to show worst locomotor skills during the period they were treated with RvD1, and underwent a slight in the BMS score when the administration of this SPM was discontinued. This observation might even suggest that daily administration of RvD1 may even result in harmful effects after SCI. We do not know yet the mechanisms underlying the inability of RvD1 to promote inflammatory resolution after SCI. However, we cannot discard that this could be due to the delivery of SPM for several days. In most of the studies where RvD1 mediate beneficial actions, this SPM is given in a single bolus. Indeed, clearance of neutrophils is importantly accelerated after RvD1 treatment during the first 3 days post-injury, but fully discontinued at later stages of the pathology. We can not therefore discard that RvD may exert dual effects, pro-resolving or pro-inflammatory actions, depending on the time point where it is administrated. Further studies are needed to elucidate this issue, which will be performed in the laboratory. In particular, we will compare the effects

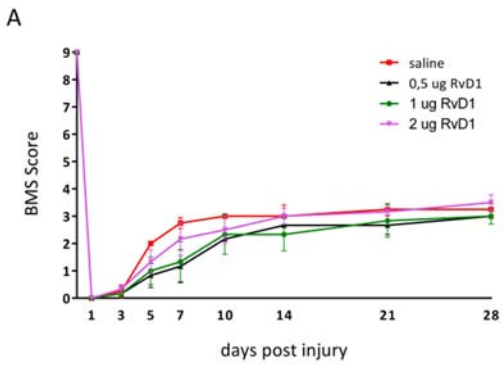
of RvD1 when given for the first 3 days post-injury or when the treatment is initiated at day 3 post-injury. These experiments may reveal that the resolution of inflammation is enhanced in the first group of mice, while significantly impaired in the later. However, due to time restrains I have not started these experiments yet.

RvD1 and AT-RvD1 act through the Formyl peptide receptor 2 (ALX/FPR2) receptor, present on leukocytes, endothelial and epithelial cells and through human GPR32 receptor abundant on PMN, monocytes, macrophages and on vascular endothelial cells (Krishnamoorthy et al., 2010). Previous reports showed that in a model of stroke FPR2 increased after subarachnoid hemorrhage (SAH) and peaked at 24 hours reducing later their expression considerably (Guo et al., 2016). Our preliminary data (data not shown) reveals that this receptor is also induced in the injured spinal cord parenchyma at day 1 post-injury, and tended to normalize its mRNA levels by day 7. Our data also shows that RvD1 treatment reduced by approximately 2 fold the expression of FRP2 in the injured spinal cord at 24 post-injury. This decrease in the mRNA levels of this RvD1 receptors may be due, in part, to the slight reduction of neutrophils induced by this SPM at this time point. However, it is possible that RvD1 may act on different mechanisms that silence the expression of FRP2. Indeed, the production of RvD1 is seems to be restricted to the early stages of inflammation in several pathologies (Buckley et al., 2014, Serhan, 2014, Serhan et al., 2015), in contrast to other RvD members and to MaR1, which are produced at more late stages. This might be, in part, an endogenous mechanism to minimize potential deleterious actions of RvD1 in later stages of inflammation.

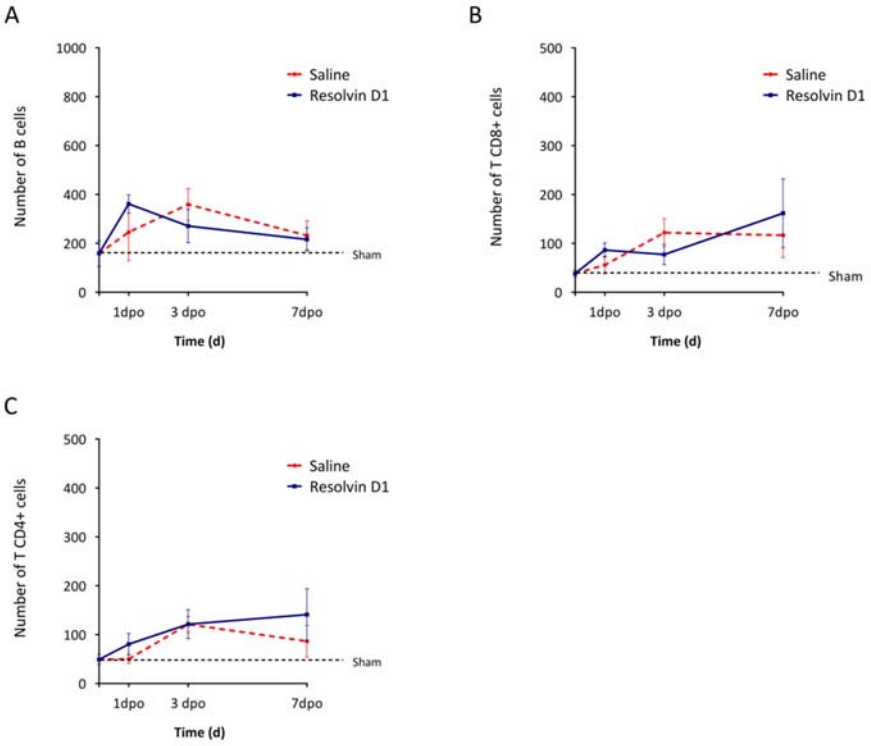
Despite MaR1 and RvD1 are both derived from DHA, in the present thesis we demonstrate that they exert different effects on inflammatory resolution after SCI, and consequently, on tissue damage and functional impairments. In order to know more mechanistic insight into the divergent effect exerted by these two SPMs in SCI, we performed a microarray-based expression profiling studies. These experiments revealed that spinal cords from mice treated with RvD1 were significantly enriched in genes related to fibrosis and inflammation. In addition, analysis on enriched biological processes also revealed that RvD1 favored the increase of biological pathways related to inflammation. Interestingly, injured spinal cord of mice treated with RvD1 were also enriched in biological pathways involved in chronic inflammation, further indicating the resolution of inflammatory after SCI is unsuccessfully resolved after RvD1 treatment as compared to MaR1 administration. Although further analysis and validation experiments are needed to confirm the genearray data, these results are in agreement with our FACS analysis experiment, where we demonstrated that RvD1, in contrast to MaR1, did not result in better resolution of inflammation after SCI, and consequently, did not confer protection against tissue damage and functional disabilities.

To our knowledge this is the first report suggesting that daily administration of RvD1 fails to accelerate the resolution of inflammation, and highlights the potential divergent actions of the different SPMs in SCI.

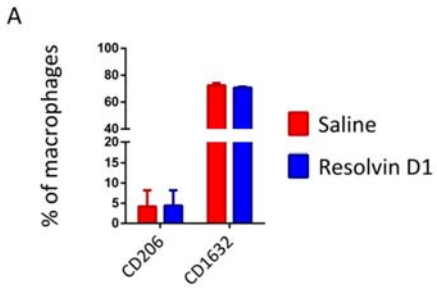
SUPPLEMENTARY DATA



Supplementary Figure 1. Effect of different doses of RvD1 on functional recovery after SCI. (A) Assessment of locomotor recovery by 9-point Basso Mouse Scale (BMS) in mice treated with different doses (0.5, 1 and 2 ug) of RvD1 and saline controls after SCI. Data are expressed as mean ±SEM; n=3 per group.



Supplementary Figure 2. Effects of RvD1 in the accumulation of lymphocytes after SCI. (A) Graph showing the effects of RvD1 on the recruitment of B cell, (B) T CD8+ cells(B) and T CD4+ cells (C) in the injured spinal cord. Data are expressed as mean ±SEM. (Student t test was used to analyze significant differences. n=9 at 1 dpi, n=4 at 3dpi and n=4 at 7 dpi).



Supplementary Figure 3. RvD1 does not modulate macrophage phenotype after SCI. (A) Graph showing the quantification of macrophages expressing CD206 and CD1632 in the spinal cord of mice treated with RvD1 or saline at day 7 post-lesion. Mean ±SEM. (n=4 per group) Student t test was used to analyze significant differences between RvD1 and control mice.

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CHAPTER 4

Effects of Lipoxin A4 administration after spinal cord injury in mice

Effects of Lipoxin A4 administration after spinal cord injury in mice

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ABSTRACT

Specialized pro-resolving mediators (SPMs) are endogenous bioactive lipid molecules that play a fundamental role in the regulation of inflammation and its resolution. The first SPMs recognized to possess pro-resolving actions was lipoxin A4 (LXA4). This SPM has accumulated several evidences that stimulate resolution of inflammation in different animal model of human disease, including in some neurological conditions. However, it remains unknown whether LXA4 results in therapeutic effects in spinal cord injury (SCI). In this study, we assessed the effects of exogenous administration of LXA4 in inflammatory resolution and tissue and functional outcomes after SCI. These experiments revealed that LXA4 fails to reduce the recruitment of the different leukocyte subsets in the injured spinal cord, as well as microgliosis. Moreover, LXA4 did not enhance the elimination of neutrophils from the injured spinal cord. Finally, we demonstrate that administration of LXA4 did not results in amelioration of tissue damage and functional deficits after SCI. Overall, this study indicate for the first time the lack of therapeutics effects of LXA4 in SCI

Keywords: Lipoxin A4, Inflammation, Inflammatory resolution, Neuroprotection, Spinal Cord injury.

INTRODUCTION

Inflammatory response is an essential step of the injury response that involves immune cells, blood vessels, and molecular mediators to restore homeostasis of the tissue and initiate wound repair. However, in order to prevent unnecessary tissue damage, inflammatory response must be actively resolved when is no longer needed. As an active rather than a passive process (Levy et al., 2001, Serhan et al., 2007), resolution phase is characterized by orderly neutralization of noxious materials, the inhibition of leukocyte recruitment and the infiltration of non-phlogistic monocytes that clean the rest of debris and cell death creating a favorable environment that facilitates tissue repair and restoration of function (Serhan and Savill, 2005). An insufficient or inadequate resolution leads to chronic inflammation, excess of tissue damage and inappropriate tissue healing (Buckley et al., 2014, Serhan, 2014). This is the case of many clinical relevant disorders, including SCI where blood-borne cells persist in the lesion site for prolonged periods leading to disproportionate harmful bystander side effects (Hawthorne and Popovich, 2011, Prüss et al., 2011, David et al., 2012). Additionally, the damaging consequences of inflammation are especially important in CNS by its impaired endogenous regenerative capacity (Fawcett et al., 2012, Lu et al., 2014, Stenudd et al., 2015). Thus, enhancing the inflammatory resolution after SCI is therefore expected to exert therapeutic effects.

In recent years, previously unrecognized chemical mediators, called specialized pro-resolving lipids mediators (SPMs), have been identified (Serhan et al., 2008, Norling and Serhan, 2010). This novel genus of bioactive mediators are biosynthesized from polyunsaturated fatty acids during the acute inflammatory response (Serhan et al., 2000, Levy et al., 2001, Schwab et al., 2007, Serhan, 2014). Acting by distinct G protein couple receptors expressed on immune cells, SPMs contribute to control the inflammatory response and allow inflamed tissues to return to homeostasis once the need for inflammation is over (Serhan et al., 2008, Buckley et al., 2014). To the present, it has been described several families of these SPMs including: lipoxins, resolvins (RvD and RvE), protectins and maresins (Buckley et al., 2014, Serhan, 2014, Serhan et al., 2015).

Lipoxins (LXs) were the first anti-inflammatory lipid mediator recognized to possess pro-resolving actions (Serhan et al., 1984). LXs, in contrast to the other SPMs, is generated from arachidonic acid (Samuelsson et al., 1987) via sequential actions of lipoxygenases. LXA4, the

most well-characterized LX, appears to act at both temporal and spatially distinct sites from other pro-inflammatory eicosanoids produced during the course of an inflammatory response, stimulating natural resolution of the process (Serhan, 2005). LXA4 has a number of immunomodulatory and anti-inflammatory actions in many inflammatory disorders such as cystic fibrosis, nephritis, periodontitis, arthritis, inflammatory bowel disease (Machado et al., 2006, Higgins et al., 2015, Van Dyke et al., 2015). By signaling through the FPR2/ALX receptor, LXA4 decreases vascular permeability, decrease neutrophil endothelium interactions and reduce neutrophil infiltration (Chiang et al., 2005). In addition, LXA4 can stimulate macrophage phagocytosis of microbes and apoptotic neutrophils (Maderna et al., 2002, Reville et al., 2006). Thus and given their importance in inflammation resolution, LXA4 treatment represents a novel therapeutic agent for inflammatory diseases. However, to our knowledge, there is no information yet about its effect after SCI. Therefore, the present study was designed to investigate whether LXA4 exerts immunomodulatory and neuroprotective effects in this neurological condition. Unexpectedly, we show that LXA4 fails to boost inflammatory resolution after SCI and, consequently, does not enhance functional and histopathological outcomes.

MATERIALS AND METHODS

All the experimental procedures were approved by the Universitat Autònoma de Barcelona Animal Experimentation Ethical Committee (CEEAH 1188R3-DMAH 6131) and followed the European Communities Council Directive 2010/63/EU, and the methods for each procedure were carried out in accordance with the approved guidelines.

Surgical procedure

Adult (8-10 weeks old) female C57Bl/6 mice (Charles River) were deeply anesthetized with intraperitoneal injection of ketamine (90 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.). After performing a laminectomy at the 11th thoracic vertebrae, the exposed spinal cord was contused using the Infinite Horizon Impactor device (Precision Scientific Instrumentation)(Klopstein et al., 2012). Injuries were made using a force of 60 kdynes and tissue displacement ranging between 400 and 600 µm.

One hour after SCI, 100 µl of sterile saline or sterile saline containing 0,5, 1 or 2 µg of LXA4 (5S,6R15S-trihydroxy-7E,9E11Z13E-eicosatetraenoic acid; Cayman Chemical Company, Ann Arbor, MI) was injected intravenously and then repeated daily thereafter until day 7.

Flow cytometry

Immune cells from the injured spinal cord were analyzed by flow cytometry. Spinal cord from mice treated with LXA4 or saline were harvested at day 1 and 7 post-lesion. Briefly, spinal cords were cut in little pieces and passed through a cell strainer of 70 µm (BD falcon) and the cell suspension was centrifuged twice at 300g for 10 minutes at 4ºC. After cell counts, samples were divided, and cells alone and isotype-matched control samples were generated to control for nonspecific binding of antibodies and for auto-fluorescence. The following antibodies from eBioscience were used at 1:250 concentrations: CD45-PerCP, CD11b-PE-Cy7, Gr1-FITC, Ly6C-FITC, F4/80-APC, CD3-FITC, CD4-APC, CD8-APC and CD19-PE. After 30 min of incubation with combinations of antibodies at 4°C, the samples were washed and fixed in 1% paraformaldehyde. Cells were analyzed on a FACSCanto flow cytometer (BD Biosciences) and results analyzed using FlowJo® software version 10.0.7.

To perform the analysis, cells were first gated for CD45 to ensure that only infiltrating leukocytes and resident microglia are selected, and then, the following combination of markers were used to identify CD4 T-Cells ($CD45^{+}$, $CD11b^{-}$, $CD3^{+}$, $CD4^{+}$), CD8 T Cells ($CD45^{+}$, CD11b, $CD3^{+}$, $CD8^{+}$), B cells ($CD45^{+}$, $CD11b^{-}$, $CD3^{-}$, $CD19^{+}$), microglial cells ($CD45^{low}$, $CD11b^{+}$, $F4/80^{+}$), macrophages ($CD45^{high}$, $CD11b^{+}$, $F4/80^{+}$), and neutrophils ($CD45^{high}$, $CD11b^{+}$, $F4/80^{-}$, $Ly6G^{+high}$). Kinetics analysis of these immune cell types were calculated as described previously (Prüss et al., 2011). To study the phenotype of microglia and macrophages, these cells were further differentiated based on Ly6C expression. Cells were analyzed using FlowJo® software on a FACSCanto flow cytometer (BD Biosciences).

Functional assessment

Locomotor recovery was evaluated at 1, 3, 5, 7, 10, 14, 21 and 28 days post-injury (dpi) in an open-field test using the nine-point Basso Mouse Scale (BMS), which was specifically developed for locomotor testing after contusion injuries in mice (Basso et al., 2006). The BMS analysis of hindlimb movements and coordination was performed by two independent assessors and the consensus score taken. The final score is presented as mean ± SEM.

In addition, at the end of the follow up (day 28 post-injury), a computerized assessment of locomotion was also performed using the DigiGait™ Imaging System (Mouse Specifics Inc., Boston, MA). This system is constituted of a motorized transparent treadmill belt and a high-speed digital video camera that performs images to the underside of the walking animals. DigiGait™ software generates "digital pawprints" and dynamic gait signals, representing the temporal record of paw placement relative to the treadmill belt. This locomotor test allows for an easy and objective analysis of both static and dynamic locomotor parameters. Finally, the highest locomotion speed which each mouse was able to run was also recorded on the Digigait treadmill belt. Briefly, each mouse was allowed to explore the treadmill compartment, with the motor speed set to zero, for 5min. Then speed was gradually increased from 0 up to 35 cm/s and the maximum speed at which each mouse performed for at least 5 seconds was recorded (Santos-Nogueira et al., 2015).

Electrophysiological analysis

At day 28, electrophysiological tests were used to evaluate spared motor central pathways after SCI. Motor evoked potentials (MEPs) were recorded from the tibialis anterior (TA) and gastrocnemius (Buckley et al.) muscles with microneedle electrodes, in response to transcranial electrical stimulation of the motor cortex by single rectangular pulses of 0.1 ms duration. Pulses were delivered through needle electrodes inserted subcutaneously, the cathode over the skull, overlaying the sensoriomotor cortex, and the anode at the nose.

Compound Muscle Action Potential (CMAP) M waves from tibialis anterior and gastrocnemius muscles were recorded for internal control of peripheral normal conduction. In this case the sciatic nerve was stimulated percutaneously by means of single pulses of 0.02 ms duration (Grass S88), delivered through a pair of needle electrodes placed at the sciatic notch (Verdú et al., 2003).

All potentials were amplified and displayed on a digital oscilloscope Tektronix 450S (Tektronix, OR) at settings appropriate to measure the amplitude from baseline to the maximal negative peak. To ensure reproducibility, the recording needles were placed under microscope to secure the same placement on all animals guided by anatomical landmarks. During the tests, the mice body temperature was kept constant by means of a thermostated heating pad.

Histological analysis

At 28 days post-injury mice were deeply anaesthetized using Dolethal (pentobarbital sodium; Vétoquinol E. V. S. A.) and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (Sun et al.). A 10 mm length of spinal cord containing the injection or the lesion site centered was harvested, post-fixed for 1 hour in 4% paraformaldehyde in 0.1 M PB and cryoprotected with 30% sucrose in 0.1 M PB at 4ºC, for a minimum of 48 hours. Spinal cords were fast-frozen at -60ºC in cryoembedding compound (Tissue-Tek® oCT, Sakura) and cut on a cryostat (Leica). Ten series of 10µm thick section were picked up on glass slides. Adjacent sections on the same slide were therefore 100µm apart.

For histological analysis after SCI, sections were stained using FluoroMyelin Green fluorescent myelin stain (Invitrogen) for assessing myelin loss. Briefly, tissue sections were rehydrated in PBS and incubated with FluoroMyelin (1:300) in PBS for 20 min at room temperature. Then sections were washed and mounted in Mowiol mounting media containing DAPI (1 µg/ml; Sigma).

For axonal assessment, sections were incubated overnight at 4ºC with biotinylated antibody against NF (1:1000, Millipore). Sections were incubated for 1 hour at room temperature with the streptavidin-Alexa 594 conjugated antibody (Molecular Probes, 1:500), and then coverslipped in Mowiol containing DAPI to label nuclei.

For neuronal assessment, sections were incubated overnight at 4ºC with biotinilated antibodies against NeuN (1:500; Millipore). After several washes in PBS, sections were incubated with Alexa 594-conjugated streptoavidin, and then coverslipped in Mowiol mounting media (Sigma-Aldrich).

Tissue sections were viewed with olympus BX51 microscope and images were captured using an olympus DP50 digital camera attached to it and using the Cell^A Image acquisition software. The epicenter of the injection or contusion injury impact was determined for each mouse spinal cord by localizing the tissue section with the greatest damage using Fluoromyelin stained section. Myelin sparing after SCI was calculated by delineating the spared Fluoromyelin stained tissue. Neuronal survival was assessed by counting the number of $NeuN^+$ cells in the ventral horns at the injury epicenter and at rostral and caudal areas. Axonal sparing was calculated by counting the area of neurofilament immunoreactivity in the dorsal column at the injury epicenter, the most damage area of the spinal cord. The NIH ImageJ software was used to quantify all the histological parameters.

Statistical analysis

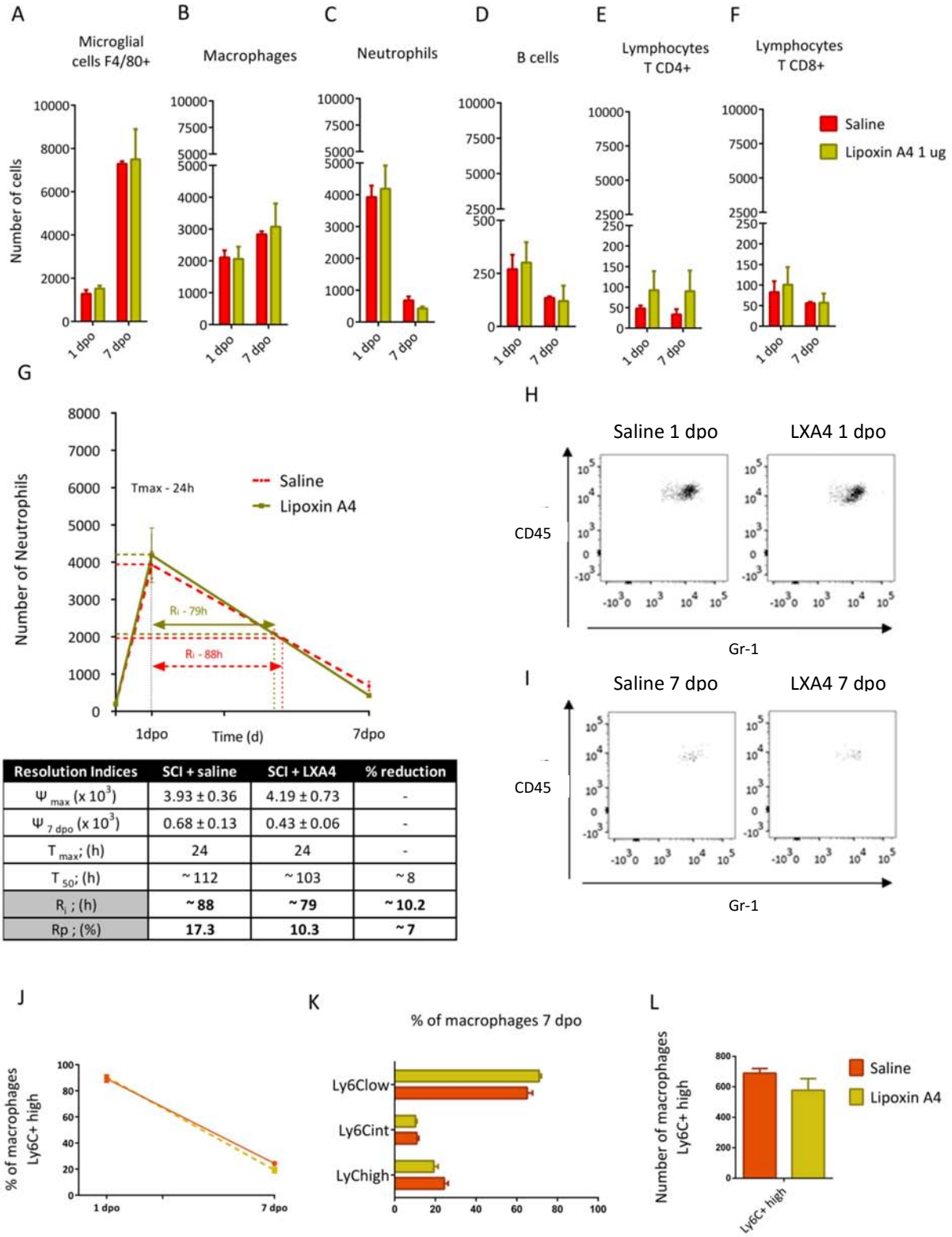
All analyses were conducted through GraphPad Prism 6.0. Data are shown as mean ± standard error of the mean (Semple et al.). Functional follow-up for BMS score and subscore, histological analysis of myelin and neuronal sparing were analyzed using two-way repeated measure ANOVA with Bonferroni's correction for multiple comparisons. Maximal speed on a treadmill was analyzed using the Mantel-Cox test. Finally, two-tailed Student's t test was used for the single comparison between two groups. Differences were considered significant at $p<0.05$.

RESULTS

LXA4 fails to activate a complete inflammation resolution program in SCI.

Since LXA4 exert several pro-resolution and anti-inflammatory actions, we wanted to evaluate the pro-resolutory effects of LXA4 in SCI. For this purpose, we firstly determined the optimal dose of LXA4 in this pathology (Supplementary Figure 1) and then we evaluated, by flow cytometry, it enhances the resolution of inflammation in SCI.

FACS analysis revealed that LXA4 did not impede the infiltration of leukocytes in the contused spinal cord since the cell counts for activated microglia (CD45low, CD11b+, F4/80+), blood borne macrophages (CD45high, CD11b+, F4/80+), neutrophils (CD45high, CD11b+, F4/80-, Gr1high), CD4 T cells (CD45+, CD11b-, CD3+, CD4+) CD8 T cells (CD45+, CD11b-, CD3+, CD8+) and B cells (CD45+, CD11b-, CD3-, CD19+) were not attenuated by this SPM (Figure 1 A-F).



Resolution Indices	SCI + saline	SCI + LXA4	% reduction
Ψ_{max} (x 10^3)	3.93 ± 0.36	4.19 ± 0.73	-
$\Psi_{7\ dpo}$ (x 10^3)	0.68 ± 0.13	0.43 ± 0.06	-
T_{max}; (h)	24	24	-
T_{50}; (h)	~ 112	~ 103	~ 8
R_i ; (h)	**~ 88**	**~ 79**	**~ 10.2**
Rp ; (%)	**17.3**	**10.3**	**~ 7**

Figure 1. LXA4 does not enhance inflammatory resolution under SCI. (A-F) Graph showing quantification of the different immune cell populations in the injured spinal cord 1 and 7 days after the injury. (G) Neutrophil resolution index. (H-I) Representative density plots of FACS analysis showing neutrophils (CD45high, CD11b+, Gr1high) at 1 (H) and 7 (I) days after the injury in the spinal cord of control and LXA4 treated mice.(J) Graph showing proportion of LyC6high phenotype in monocytes in LXA4-treated animals versus control animals along the treatment. (K) Graph showing proportion of different macrophage subsets in the injured spinal cord 7 days after the injury. (L) Graph showing quantification of Ly6Chigh macrophages in the injured spinal cord 7 days after the injury. Data are expressed as mean ±SEM. (Student t test was used to analyze significant differences n=5 at 1 dpi and n=3 at 7 dpi).

Since neutrophil clearance is a crucial step for the resolution of inflammation (Schwab et al., 2007, Serhan, 2014), we assessed whether LXA4 accelerated the elimination of this leukocyte subset from the injured cord. We found that at the end of the treatment (7 dpo), spinal cords from LXA4 treated-mice exhibited lower numbers of neutrophils, although this reduction did not reach statistical significance (Figure 1 C and I). We then quantified the resolution index (Ri) and the resolution plateau (Rp) of neutrophils to provide quantitative measurements of the inflammatory resolution after SCI (Prüss et al., 2011) (Figure 1 G). These parameters indicated the time between maximum cell numbers and the point when they were reduced to 50%, and the percent of persistent cellular component, respectively. The Ri of neutrophils was ~10.2% lower in LXA4-treated animals. In addition, analysis on the Rp revealed a higher reduction of neutrophils in LXA4 treated animals (~7% lower) but without statistical significance in total cell number (678.9±125.5 saline vs 424.8±60.7 LXA4) (Figure 1 C, G and I). These data indicates that LXA4 slightly accelerates the clearance of neutrophils from the contused spinal cord, although not to the statistical level.

Since macrophages exert divergent effects on damaged tissue depending on their phenotype, we also assessed whether LXA4 modulated macrophage polarization after SCI. We demonstrated that at day 1 after contusion injury, $LyC6^{high}$ was the main macrophage population in spinal cord (Figure 1 J). At this time point, the $LyC6^{high}$ subset counts were slightly lower in LXA4-treated animals, but did not reach statistical significance (Figure 1 J). Moreover, we did not find differences in the progression of monocyte conversion from inflammatory ($LyC6^{high}$) to anti-inflammatory ($LyC6^{low}$) phenotype after LXA4 treatment (Figure 1 J). However, LXA4 tended to trigger slightly reduction in the proportion and counts of pro-inflammatory monocytes along time (24.4±1.9 saline vs 19.3±2.1 LXA4) (Figure 1 K-L). These results, therefore, suggest that LXA4 treatment does not drives macrophages activation from a pro-inflammatory towards a more pro-restorative state after SCI.

These results provide clear evidence that LXA4 treatment fails to effectively enhance inflammatory resolution after SCI.

Treatment with LXA4 does not reduce secondary tissue damage after SCI

Since inflammation contributes to pathophysiology of SCI, we evaluated whether LXA4 treatment let to reduced tissue damage after SCI. Histological sections of the spinal cord stained with FluroMyelin revealed that LXA4 treated mice did not have reduced myelin loss at the injury epicenter neither in adjacent rostral and caudal regions ($p < 0.05$; two-way ANOVA) (Figure 2 A-B). In addition, to determine whether LXA4 treatment led to greater axonal preservation, we quantified the number of axons (NF+) in the dorsal columns at the injury epicenter, the most damaged area of the spinal cord. This analysis revealed that the LXA4 treatment did not lead to greater axonal sparing after SCI (Figure 2 C-D). Finally, quantification of NeuN+ cells in the ventral horns of the spinal cord did not reveal greater neuronal survival in mice treated with LXA4 ($p < 0.05$; two-way ANOVA) (Figure 2 E-F). This data provides clear evidence that administration of LXA4 does not enhance histological outcomes after SCI.

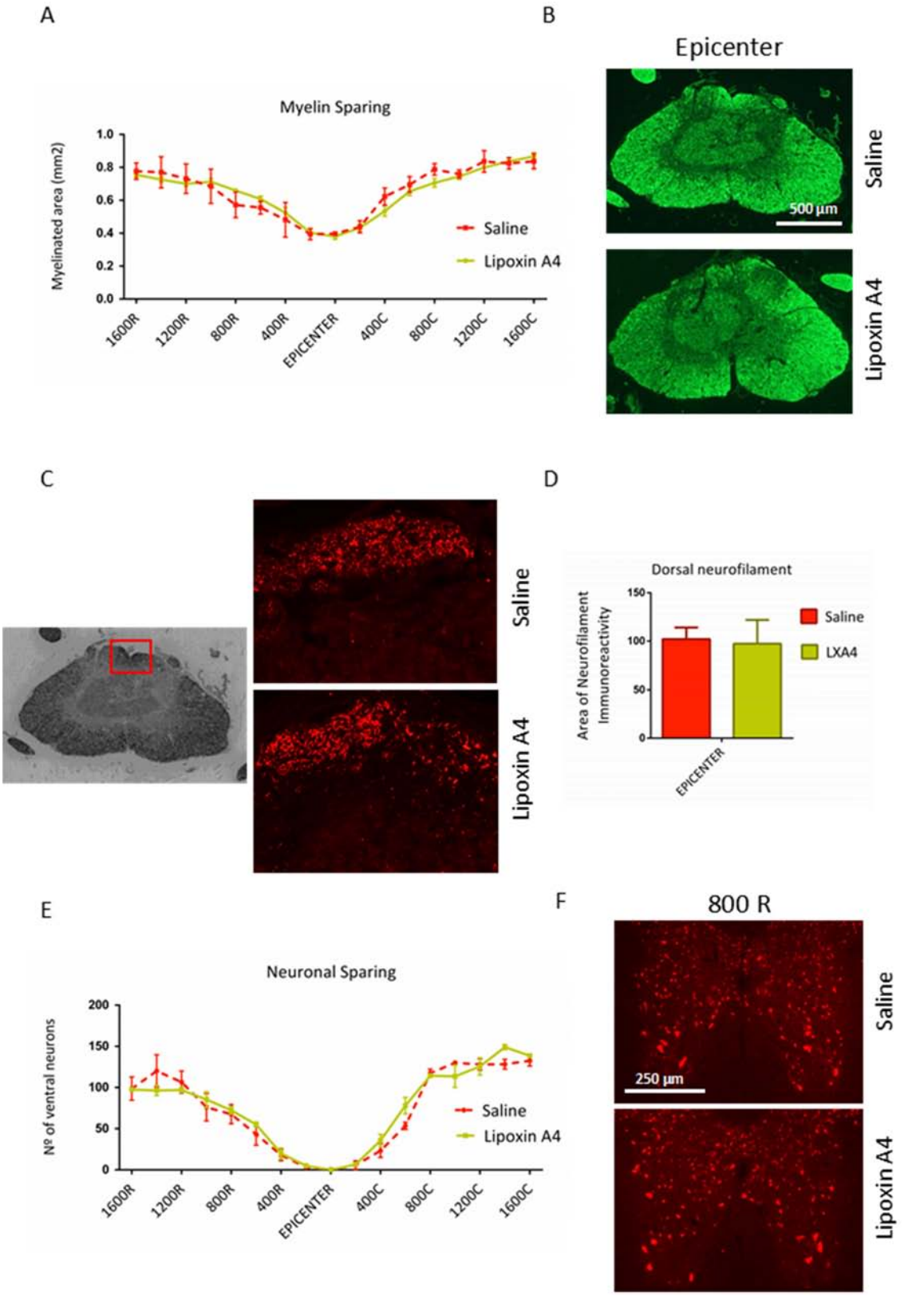


Figure 2. LXA4 treated mice does not show reduced tissue damage after SCI. (A) Quantification of myelin sparing at various distances rostral and caudal to the injury epicenter does not reveal significant reduction in tissue loss in LXA4 treated mice at the epicenter of the injury and in caudal regions. (B) Representative micrographs showing myelin sparing at the injury epicenter in section stained with FluoroMyelin from untreated and LXA4 treated mice. (C) Representative micrographs showing dorsal neurofilament staining at the injury epicenter from untreated and LXA4 treated mice. (D) Quantification of dorsal neurofilament immunoreactivity at the injury epicenter reveals no differences in axonal sparing after SCI between saline or LXA4 treated mice. (E) Quantification of ventral horn neuron survival at various distances rostral and caudal to the injury epicenter reveals the lack of significantly greater neuronal survival in LXA4 treated mice. (F) Representative micrographs showing sparing of ventral horn neurons in saline- and LXA treated mice in sections stained against NeuN at 800 µm rostral to the injury epicenter. Data are expressed as mean ±SEM. (*p<0.05; two-ways RM-ANOVA, Bonferroni's post hoc test in A, and E; t-test in D; n=4 in Saline group and n=6 in LXA4 group).

Treatment with LXA4 does not reduce functional deficits after spinal cord injury.

We finally assessed whether LXA4 treatment ameliorated motor impairments after SCI. In line with histopathological data, functional analysis revealed that LXA4 treatment did not confer significant protection against the loss of locomotor function after SCI, based the on the BMS scale (score 3.43±0.23 in saline animals vs 3.86±0.21 in LXA4-treated animals) (Figure 3 A). At day 28 post-injury, all the saline-injected (control) mice showed plantar placement of the hindlimbs (BMS 3) but only 28,6% displayed occasional stepping (BMS 4). However, 57,14% of LXA4-treated animals showed occasional plantar stepping (Figure 3 B), indicating an slight but not significant improvement in functional recovery.

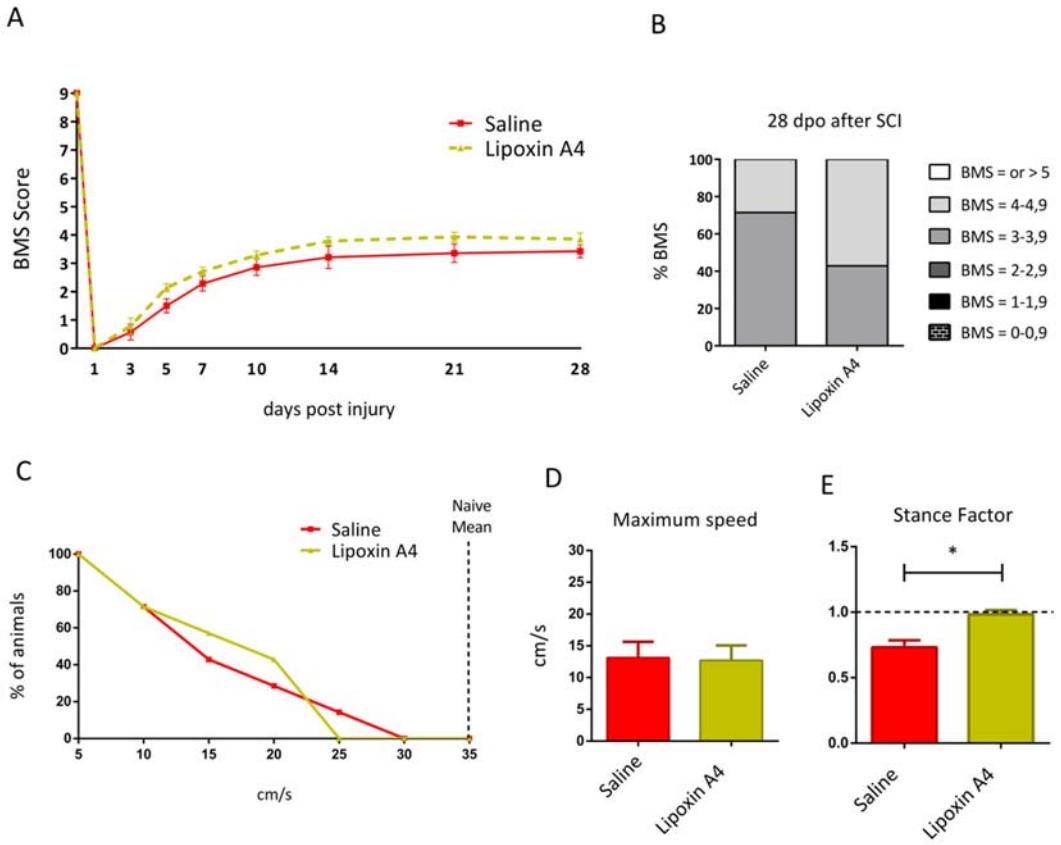


Figure 3. Animals treated with LXA4 does not enhances functional recovery after SCI. (Abdulnour et al.) Mice treated with LXA4 did not show significant improvement in locomotor skills assessed by (A-B) the 9-point Basso Mouse Scale (BMS) and (C-E) treadmill. Note that mice with LXA4 treatment only show significantly improvement in stance factor coordination. Data are expressed as mean ±SEM (*p<0.05 Two-ways RM-ANOVA, Bonferroni's post hoc test in A, Mantel-Cox test in C and t test in D and E. n=5 in saline group, n=6 in LXA4 group).

Digigait analysis revealed that LXA4 improved specific parameters of locomotion such as stance factor after SCI (Figure 3 E). Nevertheless, mice administered with LXA4 did not show significant faster speeds on a treadmill (Figure 3 C,D), indicating only slight functional improvements. We finally performed electrophysiological evaluation of motor evoked potentials, which revealed that LXA4 did not exert any significant improvements in the preservation of MEPs after SCI (Figure 4 A-D). Overall, this data provides clear evidence that administration of LXA4 does not enhance significantly functional outcomes after SCI.

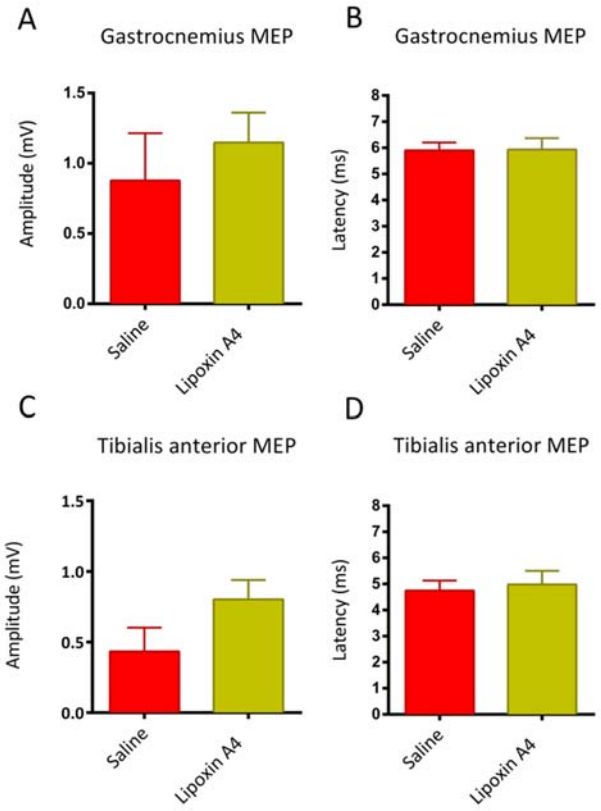


Figure 4. Evaluation of MEPs in mice treated with LXA4 after SCI. (A-D) Graph showing the amplitude and onset latency of MEPs registered from (A-B) from gastrocnemius (C-D) and tibialis anterior muscles. Data are shown as mean ±SEM. (*p<0.05; t-test; n=7per group).

DISCUSSION

Inflammation is a critical response to microbial invasion and tissue injury, and is completely necessary to restore homeostasis of the tissue and initiate wound repair (Medzhitov, 2008). To make this successfully, the acute inflammatory response must be terminated during the resolution phase. A failure of any step in this process may lead to chronic inflammation and lead to potential bystander effects on tissue damage (Hawthorne and Popovich, 2011, Prüss et al., 2011). This is the case of SCI where blood-borne cells persist in the lesion site for prolonged periods leading to exacerbated inflammatory response that promotes tissue and functional loss (Hawthorne and Popovich, 2011, Prüss et al., 2011, David et al., 2012). For this reason, many studies have focused their attention on uncovering the underlying regulatory mechanisms and strategies to modulate inflammation and treat chronic inflammatory diseases.

Recent advances in knowledge of the mechanisms of inflammatory resolution have identified LXs as attractive therapeutic tools to treat diseases in which inflammation is involved. LXs are endogenous anti-inflammatory, pro-resolving molecules that play a vital role in reducing excessive tissue injury and chronic inflammation. LXA4 is generated from arachidonic acid (AA) via the lipoxygenase pathway during cell-cell interactions in inflammatory conditions. LXA4 potentiate inflammatory resolution by means of potent agonistic actions at the G-protein coupled receptor, termed LXA4 receptor (ALX/FPR2) (Ye et al., 2009). Activation of ALX by LXA4 reduces many endogenous processes, such as chemokine and cytokine production, vascular permeability and neutrophil infiltration (Chiang et al., 2005). Likewise, in the nervous system, LXA4 or other LXs, such as aspiring-triggered lipoxin (AT-LXs) exerts neuroprotective effects in experimental stroke models and Ab42 toxicity by modulating inflammation (Wu et al., 2011) and in Alzheimer's disease (Dunn et al., 2015). However, the ability of LXA4 to modulate the neuroinflammatory response after SCI was not addressed until the present.

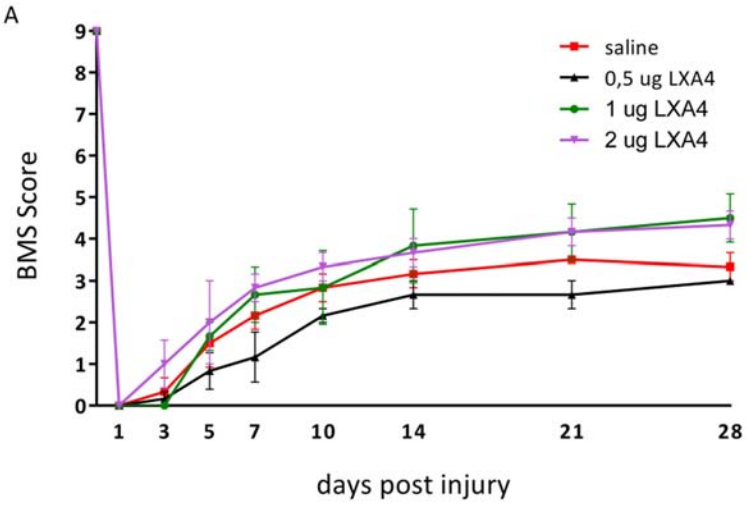
Previous reports have shown that LXs act as anti-inflammatory mediators by inhibiting neutrophil infiltration (Chiang et al., 2005) and by enhancing their clearance for the challenged tissue (Takano et al., 1997, Filep et al., 1999) and transmigration (Colgan et al., 1993, Kucharzik et al., 2003). In addition, LXA4 can stimulate macrophage phagocytosis of apoptotic neutrophils (Maderna et al., 2002, Reville et al., 2006). Here, we revealed that LXA4 triggered slight reduction in the infiltration of neutrophils in the injured spinal cord as compared with

saline-treated mice. In addition, we observed mild reduction in the neutrophil resolution index, indicating slight acceleration of neutrophilic clearance by LXA4. Nonetheless, these changes did not reach statistical significance. Similarly, LXA4 treatment slightly attenuates the transmigration of leukocytes and microgliosis after SCI. However, and similar to neutrophils, this is effect was not potent enough to reach statistical significance, indicating that our LXA4 treatment protocol was ineffective to resolve inflammation, and therefore, to attenuate histological and functional outcomes.

We do not know yet why LXA4 does not result in enhance inflammatory resolution after SCI. FPR2/ALX, the LXA4 receptor, is a chemotactic receptor for monocytes but has a limited role in resident tissue macrophages (Waechter et al., 2012), and is not found in microglial cells (Luo et al., 2013). However, the expression of FPR2 can be modulated depending on the environment. Guo and collaborators showed that in a model of stroke FPR2 increased after subarachnoid hemorrhage (SAH) and peaked at 24 hours reducing later their expression considerably (Guo et al., 2016). Our preliminary data (data not shown) also indicate that the expression of FPR2 is increased at 24 hours following SCI, and normalized by day 7. Interestingly, the group of Catherine Godson, using an *in vitro* model, showed that stimulation with LXA4 induced a time-dependent internalization of the receptor from the plasma membrane to the intracellular space (Maderna et al., 2010). Therefore, we cannot discard that treatment with LXA4 after SCI could lead to similar actions. Indeed, as discussed in the previous chapter, treatment with RvD1 decreased in two fold the mRNA levels of FPR2. If this occurs after LXA4 treatment, it may explain the poor effectivity of this SPM when administered after SCI. Another possibility that we cannot discard is the need to administrate greater amounts of LXA to promote resolution. LXA is easily degraded in host tissues, which may directly interfere with its pro-resolutory actions. Indeed, to overcome this limitation it has been synthesized different receptor peptide agonist, such as BML-111, which has resulted in neuroprotection after stroke (Hawkins et al., 2014, Smith et al., 2015). This may also explain the lack of effectivity of LXA4 in SCI. However, similar amounts of AT-LX resulted in beneficial actions in mouse models of Alzheimer's disease (Dunn et al., 2015, Wang et al., 2015).

In summary, here were evaluated the potential therapeutic effects of LXA4 in SCI, and showed that our dosage protocol was unable to enhance the resolution of inflammation in this neurological condition, and therefore, did not confer protection against functional and tissue loss. Further studies using different doses or FPR2 peptide agonist compounds are needed to collect further evidences on whether LXA4 has therapeutic potential in SCI.

SUPPLEMENTARY DATA



Supplementary Figure 1. Dose-effect functional recovery after SCI. (A) Assessment of locomotor recovery by 9-point Basso Mouse Scale (BMS) in mice treated with different doses (0.5, 1 and 2 ug) of LXA4 and saline controls. Data are expressed as mean ±SEM; n=3 per group.

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CHAPTER 5

Effects of Maresin1 treatment in combination with delayed administration of Interleukin-4 after spinal cord contusion injury in mice

Effects of Maresin1 treatment in combination with delayed administration of Interleukin-4 after spinal cord contusion injury in mice

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Inflammatory response is an essential process of the injury response to restore tissue homeostasis and initiate wound repair. This response, however, must be actively terminated when is no longer needed otherwise it may cause unnecessary tissue damage. The present thesis provides clear evidence that the synthesis of anti-inflammatory cytokines and specialized pro-resolving lipid mediators (SPMs) is inadequate after spinal cord injury (SCI), which leads to excessive inflammation and tissue damage. Interestingly, experiments from chapter 1 and 2 reveals that administration of interleukin-4 (IL-4) or maresin-1 (MaR1) enhances resolution of inflammation after SCI and minimize functional impairments and tissue damage. Nevertheless, there is currently no information on the impact of the combination of anti-inflammatory cytokines and SPMs in inflammatory challenges. In the present chapter, we studied whether treatment with IL-4 and MaR1 leads to additive beneficial effects in functional outcomes after SCI. Our results show that this combined treatment accelerates locomotor recovery after SCI in mice, but does not result in greater therapeutic effects as compared to IL-4 or MaR1 treatment alone. Overall, this study reveals the lack of additive effects of the combination of IL-4 and MaR1 in SCI, suggesting that these molecules may share similar mechanisms of action in this pathology.

Inflammatory response is a highly regulated process resulting from the orchestrated action of immune cells, blood vessels and molecular mediators in response to cell injury. This response is necessary to eliminate the initial cause of cell injury. However, in order to avoid excessive tissue damage, inflammation must be actively resolved through specialized active programs (Gilroy et al 2004) (Serhan et al 2007). Resolution phase is characterized by the inhibition of leukocyte recruitment, the clearance of neutrophils from the inflamed tissue, and the infiltration of non-phlogistic monocytes that clean the debris and death cells, creating a favorable environment that facilitates tissue repair and restoration of function (Serhan & Savill 2005).

Resolution of inflammation was initially thought to be a passive process that involved the dilution of cytokine gradients over time, and consequently, attenuated the accumulation of immune cells to the site of lesion or infected. However, it is currently known that resolution of inflammation is an active program coordinated by several molecules that are mostly produced by the immune cells (Serhan 2014). In a well-resolved inflammatory response, when immune cells accumulate in the lesion site, there is a switch in the production of cytokines. Specifically, the expression of pro-inflammatory cytokines is turned off while those with anti-inflammatory activity, such as IL-4 and IL-10, are induced. Besides, resident and infiltrated macrophages undergo several phenotype changes that allow their redirection from “classical” M1-like (pro-inflammatory) state towards “alternative” M2-like (anti-inflammatory) activation, which protect tissue to further damage. In this sense, in chapter 1 we show that the synthesis of anti-inflammatory cytokines is impaired in the injured spinal cord, which correlated with the inability of microglia and macrophages to adopt M2-like phenotype.

Resolution phase of inflammation also coincides with the switch in the production of several active lipid mediators. In particular, the synthesis of several pro-inflammatory lipids such as prostaglandins, leukotrienes and thromboxanes, is switched to the production of pro-resolving lipid mediators (SPMs) (Serhan et al 2008). In chapter 2, we reveal that the synthesis of these SPMs is hampered after SCI, as it occurs, for instance, in individuals with Alzheimer disease or multiple sclerosis (Lukiw et al 2005). However, one of the most striking results shown in the present thesis is that the administration of selective anti-inflammatory cytokines (IL-4) or SPMs (MaR1) improves functional and histopathological outcomes in SCI, as we revealed in chapter 1 and 2.

In the present chapter, we address a novel therapeutic approach to reduce inflammation by combining the administration of anti-inflammatory cytokine, IL-4, and the SPM, MaR1. We hypothesize that this combined treatment will generate a more permissible environment for resolve inflammation after SCI, and will therefore results in enhanced motor skills.

We first examined whether this combined treatment enhanced functional outcomes after SCI. Similarly to chapter 1 and 2, mice treated with MaR1 or IL-4 showed significant improvement in motor skills resulting in elevated BMS scores (Figure 1 A-C), further confirming our previous data. Combination of both treatments did not improve locomotor abilities at day 28 post-injury as compared to IL-4 or MaR1 administration alone, based on the BMS score. At this time point, BMS scores were score 2.0±0.39 for saline, 3.67±0.28 for MaR1, 3.71±0.15 for IL-4, and 3.93±0.25 for the combined-treated animals (Figure 1A). Nevertheless, recovery of hindlimb functions tended to be faster in mice receiving the combined treatment than in those administered with IL-4 MaR1 (Figure 1A). Moreover, combination of IL-4 and MaR1 was the only approach that resulted in mice with frequent stepping (BMS≥5) (Figure 1B). We also found that mice administered with MaR1, IL-4 or both showed significant faster locomotor speed on a treadmill than saline-treated mice, especially those receiving the combined treatment (Figure 1 C,D).

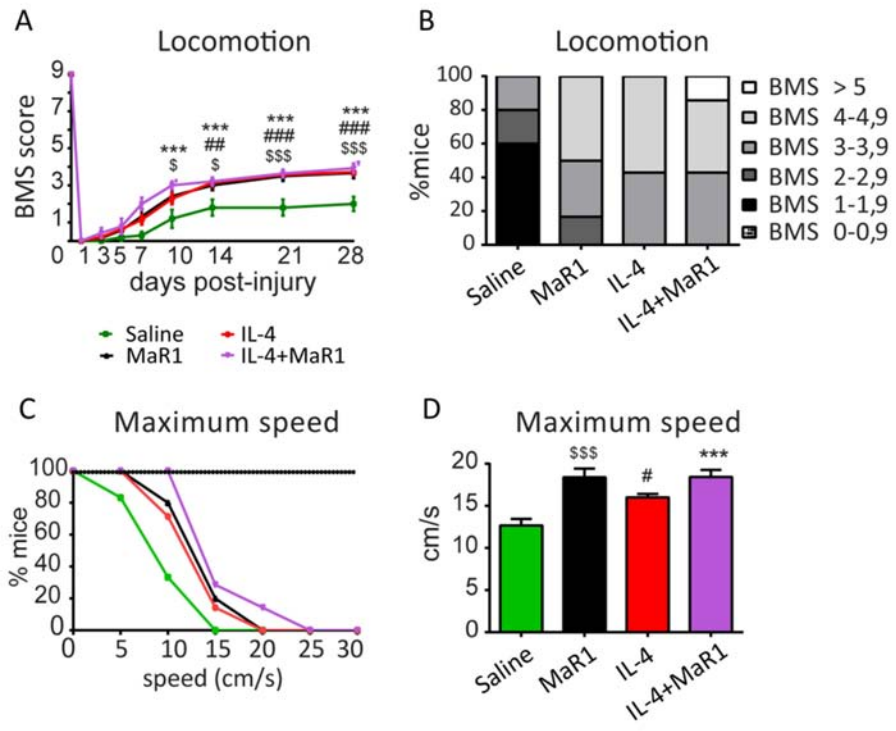

Figure 1. Animal treated with IL-4, MaR1 or combination of both enhances functional recovery after spinal cord injury. (A-D) Mice treated with IL-4, MaR1 or combination of both showed significant improvement in locomotor skills assessed by (A-B) the 9-point Basso Mouse Scale (BMS) and (C-D) treadmill. Note that mice with the combined treatment recovered the locomotor functions earlier (A) and were able to run at slight faster speeds than IL-4 or MaR1 treatment alone. Data are expressed as mean ±SEM (***p<0.001 IL-4 + MaR1 vs saline; ##p<0.01 ###p<0.001 IL-4 vs saline; $p<0.05 $$$p<0.001 MaR1 vs saline) Two-ways RM-ANOVA, Bonferroni's post hoc test in A and B and One-way ANOVA, Tukey´s post hoc test in D. n=6 in saline group, n=6 in Maresin group and n=7 in IL-4 and IL-4+Maresin groups.

We next performed motor evoked potential (MEP) test to assess the preservation of descending motor pathways of the spinal cord (Figure 2 A-B). At 28 dpi, mice administered with MaR1, IL-4 or with the combined treatment, showed greater amplitude in MEPs recorded in the gastrocnemius (Buckley et al) and tibialis anterior (TA) muscles compared to non-treated mice. This was especially evident in mice receiving the combined treatment, which showed ~7 fold greater amplitude in MEPs recorded in both muscles. Although, single treatments resulted in enhancement of MEPs, it did not reach statistical significance. These data therefore suggest that the integrity of spinal cord descending pathways were better preserved after combination of IL-4 and MaR1. No differences were observed in MEPs latency between the experimental groups (data not shown).

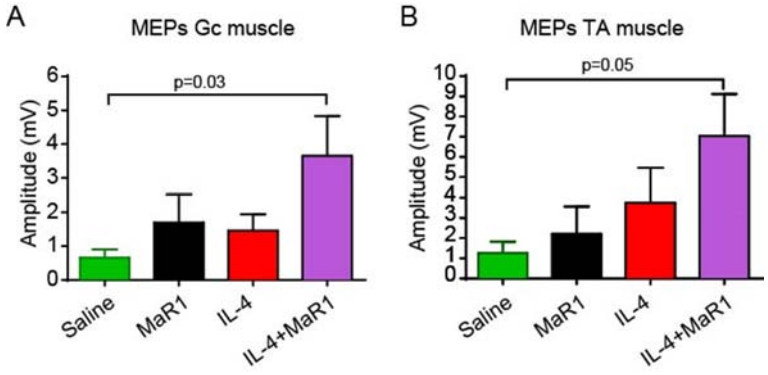

Figure 2. Slight tendency to MEPS preservation in treated animals (A-B). Preservation of descending axonal tracts after spinal cord injury was evaluated by recording motor evoked potentials (MEPs) from gastrocnemius (A) and tibialis anterior (B) muscles. Data are shown as mean ±SEM. (*p<0.05; One-way ANOVA, Dunnet´s post hoc test).

In the present chapter, we therefore replicated the therapeutic potential of MaR1 and IL-4 in SCI. Indeed, we observed that both treatments results in similar helpful effects after SCI. However, since IL-4 treatment was initiated 48 hours after spinal cord contusion lesion, this anti-inflammatory cytokine may have higher potential to be translated to the clinic, due to its therapeutic window. Nevertheless, intraspinal injection of this cytokines is unlikely to be a reliable delivery method for clinical application, and thus, studies assessing the effects of IL-4 when injected systemically or intrathecally need to be done.

The current chapter also reveals that the combination of IL-4 and MaR1 after SCI results in slight additive therapeutic effects, although did not reach statistical significance as compared to IL-4 and MaR1 treatment alone. In chapter 1 and chapter 2 we showed that IL-4 and MaR1 regulate two key events involved in the resolution phase of inflammation. In particular, IL-4 and MaR1 accelerated the clearance of neutrophils from the injured spinal cord, and

modulated the phenotype of macrophage driving them towards a more skewed anti-inflammatory state. These share mechanisms might be due, in part, to the appearance of resolution-phase macrophages after IL-4 treatment. This macrophage subset secretes several SPMs, and thus, it is likely that the delivery of this anti-inflammatory in the injured spinal cord resulted in the production of SPMs. Hence, combination of IL-4 and MaR1 may have fail in generating a remarkable enhanced favorable environment for the resolution of inflammation than IL-4 and MaR1 alone. This may explain mild beneficial additive effects of this combined treatment in in reducing functional deficits after SCI. Further studies are needed to elucidate whether the administration of other anti-inflammatory cytokines or treatments that does not modulate any of the key events occurring during the resolution phase of inflammation results in additive effects when combined with MaR1. Due to time constrains I have not been able to carry out these experiments, but they will be done in the laboratory in the future.

METHODS

Surgical procedure

All surgical procedures were approved by the Universitat Autònoma de Barcelona Animal Care Committee and followed the European Communities Council Directive 2010/63/EU, and the methods for each procedure were carried out in accordance with the approved guidelines.

Adult (8-10 weeks old) female C57Bl/6 mice (Charles River) were anesthetized with intraperitoneal injection of ketamine (90 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.). After performing a laminectomy at the 11th thoracic vertebrae, the exposed spinal cord was contused using the Infinite Horizon Impactor device (Precision Scientific Instrumentation). Injuries were made using a force of 60 kdynes and tissue displacement ranging between 400 and 600 µm. 1 hour after SCI, 100 µl of sterile saline or sterile saline containing 1 µg of 7-(S)-MaR1 (7S,14S-dihydroxy-4Z,8E,10E,12Z,16Z,19Z-docosahexaenoic acid; Cayman Chemical Company, Ann Arbor, MI) was injected intravenously. Injections were repeated daily for 1 week.

48 hours after SCI, 1µl of sterile saline or sterile saline containing 100 ng of recombinant mouse IL-4 (eBioscience) was injected into the injured spinal cord at the lesion site by means of a glass micropipette (30µm internal diameter, Eppendorf, Hamburg, Germany) coupled to a 10µl Hamilton syringe (Hamilton #701, Hamilton Co, Reno, NV, USA). 100ng of IL-4 was chosen based on results from chapter 1. Injections were made at a perfusion speed of 2µl/min controlled by an automatic injector (KDS 310 Plus, Kd Scientific, Holliston, MA, USA), and the tip of the needle was maintained inside the cord tissue 3 min after each injection to avoid liquid reflux.

Functional assessment

Locomotor recovery was evaluated at 1, 3, 5, 7, 10, 14, 21 and 28 days post-injury (dpi) in an open-field test using the nine-point Basso Mouse Scale (BMS) (Basso et al 2006), which was specifically developed for locomotor testing after contusion injuries in mice. The BMS analysis of hindlimb movements and coordination was performed by two independent assessors and the consensus score taken. The final score is presented as mean ± SEM.

In addition, at the end of the follow up (day 28 post-injury) the highest locomotion speed of the mice was evaluated on a belt of a motorized treadmill using the DigiGait Imaging system (Mouse Specifics Inc., Boston, MA). Briefly, each mouse was allowed to explore the treadmill compartment, with the motor speed set to zero, for 5min. Then speed was gradually increased from 0 up to 35 cm/s and the maximum speed at which each mouse performed for at least 5 seconds was recorded (Santos-Nogueira et al 2015).

Electrophysiological analysis

At day 28, electrophysiological tests were used to evaluate spared motor central pathways after SCI. Motor evoked potentials (MEPs) were recorded from the tibialis anterior (TA) and gastrocnemius (Buckley et al) muscles with microneedle electrodes, in response to transcranial electrical stimulation of the motor cortex by single rectangular pulses of 0.1 ms duration. Pulses were delivered through needle electrodes inserted subcutaneously, the cathode over the skull, overlaying the sensoriomotor cortex, and the anode at the nose.

Compound Muscle Action Potential (CMAP) M waves from tibialis anterior and gastrocnemius muscles were recorded for internal control of peripheral normal conduction. In this case the sciatic nerve was stimulated percutaneously by means of single pulses of 0.02 ms duration (Grass S88), delivered through a pair of needle electrodes placed at the sciatic notch (Verdú et al 2003).

All potentials were amplified and displayed on a digital oscilloscope Tektronix 450S (Tektronix , OR) at settings appropriate to measure the amplitude from baseline to the maximal negative peak. To ensure reproducibility, the recording needles were placed under microscope to secure the same placement on all animals guided by anatomical landmarks. During the tests, the mice body temperature was kept constant by means of a thermostated heating pad.

Statistical analysis

All analyses were conducted through GraphPad Prism 6.0. Functional follow-up for BMS score and subscore were analyzed using two-way repeated measure ANOVA with Bonferroni's correction for multiple comparisons. One-way ANOVA, Tukey´s or Dunnet's post hoc test was used for single comparison between various groups. Results are expressed as mean ± SEM. Differences were considered significant at $p < 0.05$.

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GENERAL DISCUSSION

DISCUSSION

Immune response plays an essential role after injury, since it avoids infection to tissues, helps to clear cellular debris, and initiates responses that are important for wound healing and for the restoration of homeostasis (Shi and Pamer, 2011, Perretti et al., 2015, Basil and Levy, 2016). This response must be tightly regulated; otherwise, it could lead the bystander effects on tissue damage.

Inflammatory response that occurs after SCI is one of the main contributors to secondary cell death and functional impairments (Popovich and Longbrake, 2008, David et al., 2012b). This response is orchestrated by activated endogenous glial cells (microglia and astrocytes) and by circulating leukocytes (neutrophils, monocytes, lymphocytes) that are recruited into the damaged spinal cord. Although inflammation is required for the clearance of cell and myelin debris and for wound healing, immune cells secrete several factors such as free radicals, proteases, eicosanoids that cause damage to neurons, glia, axons and myelin (Popovich and Longbrake, 2008, David et al., 2012b). In addition, these toxic mediators can further activate and recruit glial cells and leukocytes, mainly macrophages, into the spinal cord and thus spread the damage to adjacent areas to the injury core that were not previously affected by the initial insult. Highlight, that the damaging effects of inflammation are more pronounced in the CNS than other tissues because of the limited capacity of the CNS for axon regeneration and replenishment of damaged neurons and glial cells (Liu et al., 2011, Fawcett et al., 2012, Filli and Schwab, 2012), leading to irreversible functional disabilities.

Apart from tissue damage, reactive glia and macrophages contribute to the failure of axon regeneration in the CNS. Reactive astrocytes, for instance, synthesize proteoglycans which have potent effects in inhibiting axonal outgrowth in the CNS (Bradbury et al., 2002). Moreover, activated microglia/macrophages trigger retraction of growing axons by cell-cell interaction (Horn et al., 2008). It is noteworthy that the inflammatory response does not remit in the injured spinal cord contributing to the development of chronic neuropathic pain a (Eaton, 2006). This is of special interest, since pain severely compromises the quality of life in nearly 70% of the SCI patients (Eaton, 2006).

Although therapies aimed to stimulate the outgrowth of axons or the replacement of lost cells after SCI are important goals, preventing the secondary damage to axons, neuronal cell bodies, myelin and glial cells is likely to be more easily amenable to treatment. Therefore, targeting inflammation represent can be expected to substantially reduce the functional disabilities in SCI.

One important aspect of inflammation is the actions leading to its resolution. It was traditionally considered that a decrease in the levels of pro-inflammatory mediators was sufficient to turn off the inflammatory response. Works from the last decade, however, indicate that resolution of inflammation is not merely a passive event, but rather an active biochemical and metabolic process (Schwab et al., 2007, Serhan et al., 2009, Xu et al., 2010, Buckley et al., 2013, Buckley et al., 2014, Serhan, 2014, Serhan et al., 2015). Usually, inflammatory response undergoes resolution by switching the synthesis of cytokines, the production of active lipid mediators, and the phenotype of macrophages (Schwab et al., 2007, Serhan et al., 2009, Xu et al., 2010, Buckley et al., 2013, Buckley et al., 2014, Serhan, 2014, Serhan et al., 2015). These biological and biochemical processes are strictly regulated, otherwise, immune cells may cause tissue damage and remain in the insulted tissue for long periods, leading to the generation of a chronic inflammatory response, as it occurs after SCI (Pruss et al., 2011, David et al., 2012b).

In the present thesis, we have focused our efforts in investigating whether the detrimental actions of the inflammation response are mediated, in part, to inappropriate activation of the resolution programs. We provide clear evidence that the different immune cells remain in the injured spinal cord for the first 28 days post-injury. Although previous studies had already shown that immune cells remained in the spinal cord tissue for several months or years in rodents and humans (Arnold et al., 2007, Alexander and Popovich, 2009, David et al., 2012b), here we performed a cinematic analysis on the recruitment and clearance of the distinct immune cells in the contused spinal cord in mice, and calculated different resolution indices. A previous study has already described these parameters after SCI in rats (Pruss et al., 2011). However, these counts were calculated based on histological procedures. This is an important limiting factor since most of the markers used to distinguish the different immune cells subsets, are commonly expressed in most leukocytes. For instance, the markers Gr1, 7/4 and MPO, which are used to label neutrophils, are also widely expressed on macrophages. Similarly, the macrophage markers CD11b, CD68, ED1 are also found in neutrophils and microglia. In addition, the CD4 and CD8 markers that are used to identify helper and cytotoxic T cells, respectively, are also found in some macrophage subsets. Therefore, the characterization of the dynamics of immune cells and their resolution indices calculated based on histological data are likely to be imprecise. Since FACS analysis allows the use of multiple markers simultaneously, this is currently the only technique that allows to precisely identify the different immune cell subsets. By using FACS analysis, we found a similar order of leukocyte infiltration into the spinal cord as found by immunohistochemical methods (Basso et al., 2006, Hawthorne and Popovich, 2011, David et al., 2012a), being neutrophils the first cell

to peak in numbers in lesioned the spinal cord, followed by macrophages, microglia and lymphocytes. Despite such similarities, there are substantial differences compared to those using immunohistochemical analysis. One of them involved the infiltration of macrophages and microglia. Histological studies on immune cells infiltration in the lesioned spinal cord suggest that both, microglia and macrophages, reach maximal counts at day 7 post-injury. As previously mentioned, both immune cell subsets share most of the cell markers, and thus, these cell counts are based on cell morphology. However, microglial cells adopt macrophage-like morphology once activated and phagocytic, and therefore, it is virtually impossible to distinguish macrophages from phagocytic microglia in spinal cord tissue sections (David and Kroner, 2011, Boche et al., 2013, Gomez-Nicola and Perry, 2015). Here, by combining simultaneously several antibodies for FACS analysis, we reveal that macrophages peak in the contused spinal cord at day 3 post-injury, whereas microglial cells reach maximal accumulation at day 7. However, the most striking difference between histological studies and our FACS data relies on the clearance of immune cells from the lesioned spinal cord. Indeed, previous works suggested that neutrophils were efficiently eliminated from the injured spinal cord at day 7 (Pruss et al., 2011). However, our FACS analysis reveals that there are still numerous counts of this leukocyte subset in the spinal cord at 1 week post-injury. Interestingly, we also found that neutrophils remain at similar high numbers from 1 to 4 weeks post-injury, clearly indicating a failure of CNS to eliminate neutrophils from the injured spinal cord. Indeed, our resolution parameters indicate that only 60-65% of neutrophils are cleared from the injured spinal cord, while 35-40% of this leukocyte subset remain chronically in the SCI. The incapacity of the CNS to efficiently eliminate neutrophils after contusion injury is not specific for this leukocyte subsets, since resolution indices for microglia and macrophages were identical. Moreover, the counts of T and B cells, which are less numerous after SCI, remain even at higher proportion in the spinal cord at 4 weeks post-lesion. Since lifespan of immune cells is restricted to hours or few days in the insulted tissues, it is therefore likely the persistent presence of leukocytes and microglia in the contused spinal cord is due to continuous replacement of these cells from the injured spinal cord, rather than to the extremely long survival of these cells. To our knowledge, this fundamental issue has not been addressed in CNS pathologies yet. Indeed, in the present thesis, we provide clear evidence that several pro-inflammatory cytokines remain expressed in the injured spinal cord for the first 28 days post-injury, although to lower amounts than those detected at early hours after contusion. This failure to turn off the production of pro-inflammatory cytokines may contribute to low but continuous recruitment of leukocytes and microglia at the injury site, and to the replacement of the immune cells that progressively died in the spinal cord over time, leading, consequently, to chronic inflammation. Independently, in

which is the cause, these results provide key evidence suggesting that the resolution of inflammation is severely impaired after SCI.

As previously mentioned, a key component for the efficient resolution of inflammation is the switch in the production of pro-inflammatory cytokines to anti-inflammatory cytokines (Kigerl et al., 2009, Sica and Mantovani, 2012, Fenn et al., 2014). In chapter 1, we reveal for the first time that this mechanism is not driven efficiently after SCI. In particular, the results from chapter 1 indicate that the injured spinal cord in unable to synthesize anti-inflammatory cytokines (i.e. IL-4) or they do it for at very low levels and only for the first 24 hours post-injury (i.e. IL-10 and IL-13). This fact may play a key role in hampering the resolution of inflammation, since the expression of some of these inflammatory mediators at later stages after injury, such as IL-10, has shown to exert a critical action in inducing the resolution of inflammation after lesion in the PNS (Siqueira Mietto et al., 2015).

Anti-inflammatory cytokines contribute to mitigate the inflammatory response by suppressing several intracellular pathways that trigger inflammation, and by activating different events that a crucial to turn off this physiological process (Dinarello, 2003, Palin et al., 2008, David et al., 2012b). Among them, highlight the ability of some anti-inflammatory cytokines to change the phenotype of macrophages, a phenomenon known as macrophage polarization. As I have extensively described in the introduction section, macrophage phenotype is in tight equilibrium within the lesion environment. Whether they differentiate into cells that exacerbate tissue damage, –"classically activated" M1 macrophages–, or promote tissue repair, –"alternatively activated" M2 macrophages–, depends on signals in the lesion environment (Mills et al., 2000, Mantovani et al., 2013, Murray et al., 2014). In *in vitro* studies, macrophages adopt M1 activation when they are stimulated with pro-inflammatory cytokines or other pro-inflammatory mediators, such as LPS (Mills et al., 2000, Mantovani et al., 2013, Murray et al., 2014). Contrary, macrophages undergo M2 phenotype when activated with several anti-inflammatory cytokines such as IL-4, IL-10, and IL-13 (Mills et al., 2000, Mantovani et al., 2013, Murray et al., 2014). Highlight that this classification is based on cell culture studies, in which macrophages are stimulated with a single factor. However, *in vivo*, macrophages and microglia are influenced by multiple additional factors. This leads to a wide spectrum of intermediate phenotypes, where the M1 and M2 archetypal states are located at the ends of this range (Murray et al., 2014).

Recent studies suggest that microglia and macrophages adopt M1-like activation for the first 2 days after SCI and fail to polarize towards the M2 state (Kigerl et al., 2009, Kroner et al., 2014). Here, I extended these studies up to day 28 post-injury and corroborate that macrophages express preferably M1 markers after SCI, such as iNOS and CD16/32, whereas the presence of

M2 markers (Arg1 and CD206) is restricted to a minor subset of these cells, suggesting that these cells are more skewed towards the M1 state after lesion. The incapacity of the injured spinal cord to drive macrophage polarization to the M2 edge of this spectrum is due, in part, to the spinal cord environment, since transplantation of M2 macrophages in the contused spinal cord rapidly lose the expression of M2 markers (Kigerl et al., 2009, Kroner et al., 2014). It is known that the release of TNFα by macrophages upon phagocytosis of red blood cells that extravasate into the spinal cord after lesion prevents the redirection of microglia and macrophages towards the M2 state (Kroner et al., 2014). However, the existence of other mechanisms underlying the failure of microglia and macrophages to adopt M2-like phenotype was unknown until the present thesis. In chapter 1, we provide clear evidence that the lack or insufficient expression of anti-inflammatory cytokines after SCI prevents microglia and macrophages to express M2 markers. In particular, we demonstrate that the administration of IL-4 into the spinal cord lesion site immediately or at 48 hours after injury induces the expression of the M2 markers, Arg1 and CD206, in microglia and macrophages. We also observed that redirection of these immune cells is preferable achieved upon delayed injection of IL-4. This is probably due to the lack of IL-1R4 in the uninjured spinal cord, which may limit the capacity of acute IL-4 administration to induce an anti-inflammatory phenotype, as the bioavailability of IL-4 could be already compromised by the time when IL-R4 is expressed by microglia and macrophages. Additionally, the high levels of pro-inflammatory cytokines expressed for the first 24 host-injury in the injured spinal cord could also dilute the polarizing effects of IL-4 when administered acutely after lesion. Therefore, at 48 hours post-injury, when microglia and macrophages express IL-4R and the levels of most pro-inflammatory levels reach basal levels, may explain the greater M2 polarizing effects of delayed IL-4 administration. We also show that delayed administration of IL-4 trigger a subset of macrophages to adopt a phenotype which is compatible with resolution-phase macrophages, since they express several higher levels of anti-inflammatory cytokines, such as IL-10 and TGFβ1, and the enzymes COX-2, 5-LOX and 15-LOX, which are needed for the synthesis of SPMs (Bystrom et al., 2008, Stables et al., 2011). However, without the injection of IL-4, the expression of this macrophage subset is almost undetectable. Resolution-phase macrophages were initially described in resolution exudates where they play a key role in terminating the inflammatory response. In agreement with these findings, we demonstrated that the clearance of neutrophils from the injured spinal cord, a fundamental event for inflammatory resolution, was markedly accelerated upon delayed IL-4 injection, linking the lack of IL-4 expression with resolution deficit that occurs after SCI. Importantly, we showed that delayed IL-4 injection led to greater functional and histopathological outcomes, indicating that the actions of this anti-inflammatory cytokine are

beneficial for spinal cord repair.

Besides anti-inflammatory cytokines and macrophage polarization, another crucial factor needed for proper resolution of the inflammatory response is the synthesis of SPMs, which are a novel genus of bioactive lipid mediators derived from poly-unsaturated fatty acid (PUFA) by the sequential actions of lipoxygenases (LOX) and other enzymes such as cyclooxygenases (COX) (David et al., 2012a, David et al., 2012b, Buckley et al., 2013, Serhan et al., 2015). Among SPMs include lipoxins (LXA) resolvins (RvD and RvE), maresins (MaR1) and neuroprotectins (NP). LXAs are synthesized from arachidonic acid, whereas RvD, RvE, MaR1 and NP are derived from omega-3 PUFA (DHA and EPA) (Buckley et al., 2013, Buckley et al., 2014, Serhan, 2014). SPMs actively turn off the inflammatory response by acting to distinct G protein coupled receptors expressed on immune cells that activates dual anti-inflammatory and pro-resolution programs. SPMs, for instance, suppress the secretion of pro-inflammatory factors and induce the expression of scavenging molecules such as IL-10, IL-1 decoy receptors and IL-1 receptor antagonists (Buckley et al., 2013, Buckley et al., 2014, Serhan, 2014). In addition, PLM activates specific mechanisms that trigger the resolution of inflammation. Among these resolution mechanisms include the induction of: (i) apoptosis in neutrophils; (ii) a phenotypic switch in macrophages, from a classical pro-inflammatory M1 activation profile towards an alternatively anti-inflammatory M2-activation state; (Misharin et al.) clearance of immune cells from inflamed tissue. It is important to highlight that enzymes involved in the synthesis of SPMs (COX and LOX) are also crucial for the production of pro-inflammatory eicosanoids (prostaglandins, leukotrienes and thromboxanes) (Buckley et al., 2013, Buckley et al., 2014, Serhan, 2014). Although COX and LOX inhibitors reduce the amplitude of inflammation by inhibiting eicosanoid biosynthesis, they have now been shown to impair the tissue programs of resolution, thereby delaying the return to homeostasis (Schwab et al., 2007, Serhan et al., 2008). This is important since many anti-inflammatory therapies interferes with the expression of COX and LOX enzymes, which may explain, in part, their low efficacy in protecting against chronic inflammatory diseases.

The importance of SPMs in the resolution of inflammation is evident in many inflammatory diseases such as atherosclerosis, asthma, arthritis, Crohn's disease, ulcerative colitis, among others (Schwab et al., 2007, Serhan et al., 2008, Serhan et al., 2009, Xu et al., 2010, Buckley et al., 2013, Li et al., 2013, Buckley et al., 2014, Serhan, 2014, Serhan et al., 2015). In such pathological conditions there is absence, insufficient or delayed production of SPMs (Serhan et al., 2008, Serhan et al., 2009, Serhan et al., 2015). Importantly, the exogenous administration of SMPs reduces inflammation and prevents the detrimental effects exerted by immune cells, linking the pathogenesis of many several chronic inflammatory diseases with impairment in

the production of SPMs (Schwab and Serhan, 2006, Schwab et al., 2007, Buckley et al., 2013). Highlight, that recent evidences indicates that the synthesis of SPMs are also inadequate in multiple sclerosis (MS) and Alzheimer's disease, two neurological condition where inflammation contributes detrimentally to the pathology (Pruss et al., 2013, Wang et al., 2015). However, whether SPMs production is impaired in SCI, and if so, whether this is responsible, in part, to hamper the resolution of inflammation in this pathology has not been addressed until the present thesis.

In chapter 2 we show for the first time that the switch in the production of bioactive lipid mediators does not occurs in the injured spinal cord. In this chapter, together with other works, we demonstrated that production pro-inflammatory eicosanoids is induced in the spinal cord parenchyma during the first days after lesion (Redensek et al., 2011, Hanada et al., 2012). Lipidomic analysis also showed increase production of several intermediate metabolites in the synthesis of SPMs, such as 14-HDHA, 17HDHA, 15-HETE, which are the precursor of MaR1, RvD and LXA4, respectively. However, these metabolites were only increased at 2 weeks. This data suggest that the persistent inflammatory response that occurs in SCI might be due, in part, to the inappropriate or delayed production of SPMs in the injured spinal cord.

In chapter 2, 3 and 4, we studied the effects of the exogenous administration of several SPMs on inflammation and functional outcomes after SCI. By doing several immunological studies, we revealed that administration of MaR1 has a strong impact in enhancing resolution of inflammation after SCI. In particular, we demonstrated that the administration of this SPM boosted several key events that are needed to efficiently terminate the inflammatory response. They included the clearance of pro-inflammatory cytokines from the injured microenvironment; inhibition of some pro-inflammatory intracellular pathways; redirection of macrophages towards a phenotype more skewed towards an anti-inflammatory phenotype; acceleration of inflammatory cells from the injured spinal cord; and enhancement of neutrophil phagocytosis at the lesion site. These results provided clear evidence that increasing the levels of MaR1 in the injured spinal cord induces some aspects of inflammatory resolution, linking the lack or low levels of MaR1 with the failure of the spinal cord in terminating the inflammatory response after lesion. Importantly, administration of MaR1 also resulted in significant protection against functional deficits and tissue damage, proving further evidence that the uncontrolled inflammatory response that occurs after SCI leads to detrimental outcomes.

Despite the beneficial actions of MaR1 in SCI, in the present thesis we provided striking data indicating that not all the SPMs mediate therapeutic effects in this pathology. Surprisingly, we found that the administration of RvD1 and LXA does not lead to greater inflammatory

resolution, and consequently, they did not promote improvement in functional outcomes after SCI. This is especially relevant for RvD1, since this bioactive lipid is generated from the same precursor (DHA) than MaR1.

Accumulated evidence over the last 10 years highlight the importance of RvD1 in activating the resolution programs of activation (Xu et al., 2010, Buckley et al., 2014, Serhan, 2014, Serhan et al., 2015). Remarkable number of studies demonstrate that RvD1 mitigates inflammatory response after several inflammatory challenges in non-CNS tissues, as well as to mediate repair (Schwab et al., 2007, Xu et al., 2010, Buckley et al., 2014, Serhan, 2014, Serhan et al., 2015). However, in the present thesis, and contrary to our initial hypothesis, we found that administration RvD1 does not exert therapeutic actions after SCI. Indeed, animals treated with RvD1 showed lower, although not significant, locomotor skills during the period they were treated (first week post-injury), and reached locomotor performance similar to vehicle-treated mice once the administration of this SPM was terminated. Moreover, RvD1 fails to confer protection against tissue damage after SCI. Interestingly, we did not observed any significant change in cytokine expression triggered by RvD1 in the injured spinal cord, in exception to CCL11 and CXCL10, which were up-regulated by RvD1, and IL-13 levels that were down-regulated. However, similarly to MaR1 treatment, administration of RvD1 accelerated in 2 fold the clearance of neutrophils in the injured spinal cord at day 3 post-injury. The ability of RvD1 to boost the elimination of neutrophils from inflamed tissue has been extensively shown in several animal model of inflammatory challenges (Schwab et al., 2007, Xu et al., 2010, Buckley et al., 2014, Serhan, 2014, Serhan et al., 2015). Unexpectedly, we also observed that after day 3, administration of RvD1 completely impeded the clearance of this leukocyte subset in the injured spinal cord. This was also accompanied by an inability of this SPM to reduce microglia and macrophage counts from the injured site, and to switch the redirection of macrophages towards a phenotype more skewed towards an anti-inflammatory state. This data may suggest that although RvD1 may harness resolution of inflammation when given during the first 3 days post-injury, administration of this SPM from this critical period may activate different molecular events that exert detrimental effects for tissue homeostasis. Indeed, RvD1 is synthesized during the early stages of inflammation in challenged tissues, whether MaR1 and other RvD family members are produced at later stages of inflammation, coinciding the remitting phase of this physiological response. Indeed, most of the studies in which RvD1 shows potent pro-resolving properties, this SPM is delivered in a single bolus acutely after the inflammatory challenge (Schwab et al., 2007, Xu et al., 2010, Li et al., 2013). Future experiments need to be to elucidate whether administration of RvD1 acutely after injury and only for the first 2-3 days enhances resolution and mediates beneficial effects on functional

and histological outcomes. However, due to time constrains I have been unable to carry out this experiments yet.

Since MaR1 and RvD1 are produced from the same lipid precursor (DHA), but exert differential effects on inflammation resolution and functional deficits when delivered continuously after SCI, we performed a transcriptomic analysis from 7 days post-injury spinal cords of mice in order to gain some insights into the differential mechanisms regulated by these two SPMs. Preliminary analysis of these experiments revealed that among the list of top20 differentially up-regulated genes by RvD1 there were several genes related with extracellular matrix, including different members of the collagen family (Col4a1, Col4a2, Col10a1, Col8a1, Col1a2), fribilin 1, elastase and matrix metalloprotease 14, suggesting that RvD1 favors fibrosis after SCI. In addition, the second most up-regulated gene in RvD1 relative to MaR1 treated mice was Ly6C, a marker of pro-inflammatory monocytes. Interestingly, our FACS analysis revealed that RvD1 tended to increase the proportion of pro-inflammatory ($Ly6C^{high}$) macrophages after SCI, while MaR1 treatment led to significant reduction. This data, although preliminary, suggest that daily delivery of RvD1 might favor a more suitable environment for fibrosis and inflammation at 7 days post-injury, reinforcing the idea that RvD1 may have a dual role, beneficial or detrimental, in SCI depending on the time. Interestingly, similar dual effects have been observed after LXA administration after sepsis (Sordi et al., 2013).

Besides SPMs derived from 3-PUFA, we also studied the effects of the administration of LXA after SCI. Several previous works reveal that administration of LXA enhances inflammation resolution in different inflammatory pathologies, including asthma, colitis, peritonitis, among other (Schwab and Serhan, 2006, Schwab et al., 2007, Serhan et al., 2008, Li et al., 2013). This SPM is generated from AA acid by action of different LOX enzymes. Our lipidomic analysis revealed that the metabolic precursor (15-HETE) is produced at 2 weeks after spinal cord after lesion, suggesting that the inappropriate/delayed synthesis of this SPM may contribute to the uncontrolled immune response that occurs in this pathology. Indeed, human samples from Alzheimer's disease individual show reduced levels of this SPM (Medeiros et al., 2013, Dunn et al., 2015), and the administration of aspirin-triggered lipoxin (AXL) results in beneficial effects in inflammation and cognitive functions (Medeiros et al., 2013, Dunn et al., 2015).The therapeutics effects of LXA4 has been also shown after brain ischemia or trauma in mice (Luo et al., 2013). Despite these promising findings, we showed that the administration of LXA4 after SCI tended to improve, although not statistically, the locomotor performance in mice. Similarly, LXA4 failed to accelerate neutrophil clearance and to reduce the microglia and macrophage counts in the injured spinal cord. Although we cannot discard that the lack of therapeutic effects of LXA4 is due to the low amount administered (1µg per day), the

beneficial effects of this SPM is brain ischemia were achieved at doses ranging from 0.3 and 0.7 µg when delivered into the CSF (Sobrado et al., 2009, Luo et al., 2013). However, it is known that LXA is enzymatically inactivated very rapidly and for this reason, more stable solutes has been generated, such as the BTL111 (Zhang et al., 2007). Administration of BML111 has demonstrated therapeutic effects in brain ischemia in mice and rats (Sobrado et al., 2009, Hawkins et al., 2014). To our knowledge, no study has addressed yet whether BML111 has greater efficacy in resolving inflammation and conferring protection as compared to LXA or AXL, and will be tested in the laboratory in the future. Another possibility that we cannot discard is that the expression of FPR2, a common receptor to RvD1 and LXA4, could reduce their expresion after 24 hours as occur in a model of stroke (Guo et al., 2016), explaining, in part the poor effectivity of these SPM when administered after SCI.

We finally assessed whether functional deficits could be further minimize by combining the anti-inflammatory cytokine, IL-4, and the SPM, MaR1. These experiments reconfirmed that both, IL-4 and MaR1, mediate marked locomotor recovery after SCI. Interestingly, administration of IL-4 resulted in similar beneficial effects than treatment with MaR1, despite the anti-inflammatory cytokines was given at 48 hours post-injury. Therefore, these data support the therapeutic potential of both, MaR1 and IL-4, to treat acute SCI individuals, especially the use of IL-4 due its wide therapeutic window. Further studies are needed to elucidate whether delayed treatment with MaR1 also results in similar restorative effects. Nevertheless, and contrary to our initial hypothesis, combination of both drug approaches did not lead to additive effects as compared to mice receiving IL-4 or MaR1 alone, despite they tended to show slight helpful effects. Indeed, in chapter 1 and 2, we showed that IL-4 and MaR1 shared some biological actions in the injured spinal cord, such as the acceleration of neutrophil recruitments, and the redirection of macrophages towards a more anti-inflammatory phenotype. Since injection of IL-4 in the contused spinal cord induced the appearance of a macrophage subset phenotypically compatible with resolution-phase macrophages, this myeloid population may have resulted in production of some SPMs, such as MaR1.

Overall, this thesis provides clear evidence that the inadequate expression of anti-inflammatory cytokines and SPMs of the injured spinal cord parenchyma is responsible, in part, of the uncontrolled detrimental inflammatory response that occurs when this region of the CNS is injured. We demonstrate that harmful effects that immune cells mediate in the injured spinal cord can be partially avoided by the administration of IL-4 or MaR1, although combination of both approaches do not result in additive actions. Since inflammation contribute detrimentally to the course of many neurological conditions, administration of IL-4

or SPMs may lead to the development of a new pharmacological approach to treat a wide range of CNS pathologies.

CONCLUSIONS

CONCLUSIONS

Chapter 1. IL-4 drives microglia and macrophages towards a phenotype conducive for tissue repair and functional recovery after spinal cord injury

- Macrophages and microglia adopt M1-like phenotype after SCI, and only a small subset show the expression of M2 markers.

- Protein levels of pro-inflammatory cytokines are up-regulated in the injured spinal cord, whereas the expression of anti-inflammatory cytokines is absent (IL-4) or very low (IL-10 and IL-13).

- IL-4R is not expressed constitutively in microglia but it is induced in this glial population and infiltrating macrophages after SCI.

- Administration of IL-4 into the lesion site redirects microglia and macrophages towards an M2-like phenotype after SCI, especially, when the injection of this anti-inflammatory cytokines is delayed for 48 hours after lesion.

- Delayed administration of IL-4 leads to the appearance of a macrophage subset compatible with resolution-phase macrophages, and enhances the resolution of the inflammatory response.

- Delayed treatment with IL-4 confers protection against functional deficits and secondary tissue damage after SCI.

Chapter 2. Maresin-1 activates inflammation- resolution programs after spinal cord injury and exerts therapeutic effects.

- Immune cells are not effectively eliminated from the spinal cord after lesion.

- The synthesis of pro-resolving lipid mediators is impaired in the contused spinal cord.

- Administration of Maresin-1 enhances the clearance of neutrophils from the lesioned spinal cord, and reduces the infiltration of peripheral macrophages.

- Maresin-1 turns off cytokine expression and attenuates the activation of several intracellular inflammatory pathways after SCI.

- Maresin-1 switches macrophages activation towards an anti-inflammatory phenotype and induces the phagocytosis of neutrophils.

- Administration of Maresin-1 results in enhanced locomotor skills and reduced tissue damage after SCI.

Chapter 3. Effects of Resolvin D1 administration after spinal cord injury in mice.

- Administration of Resolvin D1 accelerates the clearance of neutrophils from the injured spinal cord but only for the first 3 days post-injury, but not at 1 week after lesion.

- Resolvin D1 fails to attenuate macrophages and microglia cell counts after spinal cord contusion injury.

- Treatment with Resolvin D1 does not modulate the phenotype of macrophages after spinal cord contusion.

- Resolvin D1 tend to reduce the expression of pro-inflammatory cytokines after SCI, but only attenuates significantly the levels of IL-13. Contrary, Resolvin D1 induces significant production of CCL11 and CXCL10.

- Administration of Resolvin D1 fails to confer protection against tissue damage and functional deficits.

- Resolvin D1 treatment enriches the expression of genes predominantly involved in fibrosis and inflammation after spinal cord injury as compared to Maresin1 administration.

Chapter 4. Effects of Lipoxin A4 administration after spinal cord injury in mice.

- Administration of Lipoxin A4 does not result in enhanced resolution of inflammation after spinal cord contusion injury

- Treatment with Lipoxin A4 fails to reduce secondary tissue damage and functional impairments after spinal cord injury.

Chapter 5. Effects of Maresin1 treatment in combination with delayed administration of Interleukin-4 after spinal cord contusion injury in mice.

- Acute administration of Maresin1 and delayed injection of interleukin-4 results in similar therapeutic effects after spinal cord injury

- Combination of Maresin1 and interleukin-4 treatment does not result in significant additive effects after contusion injury to the spinal cord.

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ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AMPK	AMP activated protein kinase
AKT	Protein kinase B or Akt
ANOVA	Analysis of variance
AP-1	Activating protein-1
APC	Allophycocyanin
ASIA	American spinal injury association
ATP	Adenosine triphosphate
BB	Blocking buffer
BCR	B-cell receptor
BDNF	brain-derived neurotrophic factor
BMP	Bone morphogenetic protein
BMS	Basso mouse scale
BSCB	Blood spinal cord barrier
C/EBF	CCAAT-enhancer-binding proteins
CD	Cluster of differentiation
CCL	Chemokine CC motif ligand
CCR	Chemokine CC motif receptor
CNS	Central nervous system
CREB	cAMP response element-binding
CSF	Cerebrospinal fluid
CSFs	Colony-stimulating factors
CSPG	Chondroitin sulfate proteoglycan
CXCL	Chemokine CXC motif ligand
CXCR	Chemokine CXC motif receptor
DAVID	Database for annotation, visualization and integrated discovery
DAMPS	Damage-associated molecular pattern molecules
DC	Dendritic cell
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DPI	Days post injury

DRG	Dorsal root ganglia
ECM	Extracellular matrix
ES	Enrichment score
EGF	Epithelial grow factor
ERK 1/2	Extracellular signal-regulated protein kinases 1 and 2
EPA	Eicosapentaenoic acid
FACS	Fluorescence activated cell sorting
FC	Fold change
FITC	Fluorescein isothiocyanate
FPR	Formyl peptide receptors
GABA	γ aminobutiric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GDNF	Glial cell derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GO	Gene ontology
GPCRs	G protein–coupled receptors
Gr1	Myeloid differentiation antigen
GSA	General somatic afferent fibers
GVA	General visceral afferent fibers
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
Iba-1	Ionized calcium binding adaptor molecule 1
IFNγ	Interferon γ
Ig	Immunoglobulin
IGF	Insulin-like growth factor-binding protein
IL	Interleukin
ILR	Interleukin 1 receptor family
IP-10	interferon γ induced protein 10

IRF	Interferon regulatory factors
JNK	c-Jun N-terminal kinases
KC	Keratinocyte chemoattractant
KEGG	Kyoto encyclopedia of genes and genomes
LFB	Luxol fast blue
LIF	Leukemia inhibitory factor
LIX	Lipopolysaccharide induced CXC chemokine
LPS	Lipopolysaccharide
LXA4	Lipoxin A4
MAPK	Mitogen activated protein kinase
MaR1	Maresin 1
MCP	Monocyte chemoattractant protein
MHC	Major histocompatibility complex
MIG	Monokine induced by interferonγ
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MyD-88	Myeloid differentiation primary response 88
Myoc	Myocilin
NeuN	Neuronal nuclei
NF-κB	Nuclear factor κ-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NgR	Nogo receptor family members
NMDA	N-methyl-D-aspartate receptor
NP	Neuropathic pain
NT-3	Neurotrophin-3
NTR	Neurotrophin receptor
OL	Oligodendrocytes
OPC	Oligodendrocyte precursor cell

PAMPs	Pathogen-associated molecular patterns
PB	Phosphate buffer
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PBST	Triton phosphate buffer saline
PCR	Polymerase chain reaction
PD1	Protectin 1
PE	Phycoeritrin
PerCP	Peridinin chlorophyll
PFA	Paraformaldehyde
PMNs	polymorphonuclear leukocytes
PNI	Peripheral nerve injury
PNS	Peripheral nerve system
PRRs	Pattern recognition receptors
RANTES	Regulated upon activation normal T cells expressed or secreted
RBCs	Red blood cells
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
RT-qPCR	Real time quantitative PCR
RvD	Resolvin D
RvE	Resolvin E
SC	Schwann cells
SCI	Spinal cord injury
Sel-P	Selectin P
STAT	Signal transducer and activator of transcription
TCR	T-cell receptor
TGFβ	Transforming growth factor β
TLR	Toll like receptor
TNFα	Tumor necrosis factor α

VEGF	Vascular endothelial growth factor