Therapeutic role of IL-37 after injury to the nervous system

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This is my fight song
Take back my life song
Prove I'm alright son
Starting right now I'll be strong
Because I've still got a lot of fight left in me

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Summary

Spinal cord injury (SCI) is a traumatic event that impacts in a patient's physical and psychological welfare. SCI is characterized by the loss of sensorial, motor and autonomic functions below the site of injury. The pathophysiology of SCI involves two stages of degeneration: the primary and secondary injury. Primary injury results from the mechanical trauma, which directly causes cell death, damage to axons, loss of myelin. Secondary injury occurs over a period of several weeks after lesion. It involves a series of cellular and molecular events, which are triggered in the spinal cord parenchyma by the initial trauma. Of these events, the inflammatory response is the major contributor to this secondary injury.

The inflammatory response plays and important role in the injury response and a means to curb infections and also initiate wound healing. Inflammation must be a highly regulated process, otherwise, it may increase the initial damage or even develop an inflammatory disease. After SCI an inefficient control of the inflammatory response occurs, which exacerbates tissue damage and functional impairments. These detrimental effects become particularly harmful due to the limited capacity of the central nervous system (CNS) to promote axonal regeneration or replace damaged neurons. Minimize the inflammatory response could be a valuable approach to promote neuroprotection and functional recovery after SCI.

Interleukin 37 (IL-37) is a member of interleukin 1 (IL-1) family that has been described as a potent suppressor of the inflammatory response in vitro and in vivo

conditions. IL-37 is the only IL-1 family member for which mouse homolog has yet not to be found. For that, to study IL-37 in vivo conditions a transgenic mouse overexpressing the human form of IL-37 (hIL37tg) has been create. After toxic stimuli such as LPS injection, hIL37tg mouse shows a reduction of pro-inflammatory cytokines and chemokines without affect the anti-inflammatory cytokines. Actually, hIL37tg mouse showed a marked improvement in lung and kidney function, and a reduction in liver damage after LPS-induced shock. These results suggest that IL-37 might therefore be a suitable candidate to reduce inflammation and thus mediate protection and functional recovery after SCI.

Our results reveal that IL-37 is expressed after SCI and correlates with a decrease of the inflammatory response, leading to an improvement of the tissue preservation, functional recovery and with a reduction of neuropathic pain. Moreover the injection of a recombinant form of IL-37 (IL-37r) in wild type mice showed a improvement of the functional recovery suggesting that could be use as a therapeutic drug to treat deficiencies after SCI.

INTRODUCTION

THE SPINAL CORD: structure and function.

The central nervous system (CNS) consists of the brain and the spinal cord. The brain is placed inside the skull whereas the spinal cord is housed into the vertebral column and has a tubular shape that extends from the *foramen magnum* to the level of the first or second lumbar vertebrae. It represents a vital link between the brain and the body and *vice versa*. It controls the voluntary muscles of the limbs and trunk, and receives sensory information from these regions and controls most of the viscera and blood vessels of the thorax, abdomen and pelvis.

Structurally, the spinal cord is composed of 31 segments that can be divided into five regions: cervical (C), thoracic (T), lumbar (L), sacral (S) and coccygeal (Co). Two consecutive rows of nerve roots emerge from both sides of each segment containing afferent sensory fibers (*i.e.* dorsal roots) and the somatic efferent motor fibers (*i.e.* ventral roots). Moreover, it presents two enlarged different regions: the lower cervical and lumbar enlargements, which control the upper and lower extremities, respectively. The spinal cord parenchyma is organized in gray and white matter. In a transverse section the gray matter is centrally placed, arranged in the form of an H and surrounded by the white matter. The shape and size of the gray matter varies according to spinal cord level. At the lower levels, the ratio between gray matter and white matter is greater than in higher levels

Together with the vertebral column, the spinal cord is also protected by three layers of connective tissue, known as meninges, that are also surrounding the

brain: the *pia mater*, firmly adhered to the spinal cord, *arachnoid mater*, the middle layer, and *dura mater*, closest to the vertebrae column. Through these three layers and the bone there are three different spaces: (i) The subarachnoid space that exist between the arachnoid and the pia mater and is filled with cerebrospinal fluid; (ii) the subdural space, between the arachnoid and dura mater; (iii) and although in the brain the dura mater is attached to the skull, in the spinal cord the dura mater is separated from the bone by the subdural space filled with lymphatic tissue, small arteries and venous plexus.

Organization of the gray matter

Gray matter is consisting in neuronal cell bodies, dendrites, axons and glial cells. 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 and in the thoracic and upper lumbar segments, it has a small lateral projection, known as intermediolateral horn, which contains the pre-ganglionic neuros of the autonomic nervous system.

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. These different layers are organized from dorsal to ventral except for the tenth lamina that is composed by the cells surrounding the central (Anderson et al., 2009) (Figure 1). Below there is a brief description of the function of each lamina:

Lamina I firstly was known as marginal nucleus of spinal cord it consist of a thin layer of cells located on the top of the dorsal horn. Its receive input from Aδ and C-fibers innervating the skin, viscera and muscle and joint. These fibers carry information about noxious and thermal stimuli. Lamina 1 neurons also respond to innocuous mechanical stimuli conveyed by Aβ fibers. Lamina 1 axons join the contralateral spinothalamic tract.

Lamina II is commonly known as substantia gelatinosa and is composed by tightly packed interneurons. Along with lamina 1, lamina 2 is the principal region of termination of from Aδ and C-fibers modulating as well the nociceptive transmission in the dorsal horn though the integration the primary afferent input and modulating the output of ascending projections in the surrounding lamina.

Lamina III is thicker than the first two layers, cells are less densely packed and while lamina II is myelin-free, lamina III is characterized by the presence of myelinated fibers. Neurons in lamina III respond to tactile stimulation via Aβ fibers.

Lamina IV is also known as the head of the dorsal horn is the thickest lamina of the first four layers. The cells of this layer are still receiving Aβ fibers and responds different ranges of mechanical stimuli. From this lamina arise ascending projections to the thalamus via spinothalamic tract.

Lamina V is located at the neck of the dorsal horn. The neurons in this layer receives information from Aβ, Aδ and C-fibers which carry nociceptive information from visceral organs. Lamina V cells projects to the brain stem and the thalamus via spinothalamic tract. Moreover, descending corticospinal and rubrospinal fivers synapse upon its cells.

Lamina VI is best developed in the cervical and lumbar enlargements and is located at the base of the dorsal horn. In this layer proprioceptive connections from muscle spindles arrive and where the spinocerebelar tract is originated. It contains numerous propiospinal interneurons involved in reflex pathways, while descending brainstem pathways project to the lateral zone of this layer.

Lamina VII is also known as zona intermedia. Lamina VII interneurons communicates the dorsal and the ventral horn. It also receives information from lamina II to VI as well as visceral afferent fibers, and they serve as intermediary relay in transmission of visceral motoneurons impulses. Neurons in this lamina are involved in the regulation of posture and movement.

Lamina VIII is located in the ventral horn. The size and shape of lamina 8 varies at different levels, being smaller at the cervical and lumbar enlargements, since the motoneuron pools from lamina IX are larger there. In the cord enlargements, the lamina occupies only the medial part of the ventral horn, where descending vestibulospinal and reticulospinal fibers terminates. The neurons of lamina VIII modulate motor activity via γ-motoneurons, which innervate the intrafusal muscle fibers.

Lamina IX is located at the base of the ventral horn, is mainly occupied by columns of large motoneurons (α-motoneurons) and also by smaller motoneurons (β and γ). The α- motoneurons are somatotopicically organized and it give rise to ventral roots fibers to supply extrafusal skeletal muscle fibers, while the small γ-motoneurons give rise to the intrafusal muscle fibers. Lamina IX is specially enlarged in the cervical and lumbar spinal cord, since motoneurons responsible for limb muscle control are allocated there.

Lamina X corresponds to the area around the central canal and the neurons of this layer project to the contralateral side of the spinal cord. It is also known as central gray matter.

In summary, lamina I-IV are concerned with exteroceptive sensation, lamina V and VI are concerned primarily with proprioceptive sensation, whereas lamina VIII and IX form the final motor pathway to initiate and modulate motor activity.

Organization of the white matter

Surrounding the gray matter, it is found the white matter containing glial cells and both myelinated and unmyelinated axons. It can be divided depending on its function or its location. By location criteria, a large group of axons that are found within a given area is called funiculus, and similarly to gray matter, white matter is also divided into dorsal, lateral and ventral funiculus. Meanwhile, those nerve fibers with the same origin, course and termination are called tracts. Tracts can conduct sensory information to the brain (ascending tracts), or motor information from the brain down (descending tracts) through the spinal cord. Besides, along with the different sensory and motor tracts, there are the propiospinal fibers that connect one spinal cord segment with another.

Few differences are found between the tract organization of the spinal cord in 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. While in humans the major bundle of corticospinal axons are placed in the lateral and ventral funiculus, rodents presents an important dorsal component of this tract (Figure 1).

The **ascending tracts** arise from primary neurons with soma are in the dorsal root ganglia, or from interneurons in the dorsal horn that 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 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, in the dorsal column 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. The **anterolateral system (ALS),** which includes spinothalamic, spinoreticular and spinotectal tracts, is involved in pain and temperature sensation, as well as non-discriminative touch, also known as protopathic touch. Finally, the somatosensory pathways including spinocerebellar, relay primarily proprioceptive information but also some pain and pressure.

Motor information travels from the brain down the spinal cord via **descending tracts**. The best known of these descending tracts are the corticospinal and the rubrospinal tracts both involved in the voluntary movements. The reticulospinal and vestibulospinal tracts mediate control of balance and posture. Here is a brief description of each descending tract:

The **corticospinal tract** is responsible for coordinate limb movements. Costicospinal tract arises from the primary motor cortex, premotor and somatosensory cortical areas, reach the caudal end of the brain stem where most

of them cross the opposite side in the pyramidal decussation remaining in the ventral part of the dorsal column. These nerves fibers travel along the spinal cord until sacral segments in most of the mammals. The main area of termination is the medial parts of the base of the dosal horn and the intermediate gray matter (Lamina III to VI).

Along with the Corticospinal tract, **rubrospinal tract** also has influence in general locomotion, but playing a cooperative role together with corticospinal tract in controlling more skilled motor tasks. In the spinal cord is located at the dorsal part of the lateral funiculus and continues on to reach the dorsal part of the lateral column, and mostly terminates in cervical and lumbosacral enlargements in lamina V and VI but some fibers can travel until reaching the motoneurons in lamina IX. In humans, cortico and rubrospinal tracts merge in the **descending lateral system**, focused on controlling fine movements of the distal parts of the limbs.

Other descending tracts (reticulospinal, vestibulospinal, raphespinal and tectospinal tracts) constitute the **descending medial system** where neurons are placed in the brainstem and axons travel in the medial part of the ventral funiculus. The **reticulospinal tract** is involved in preparatory and movement-related activities, postural control and modulation of some sensory and autonomic functions. **Vestibulospinal tract** is the main initiators of coordinated postural extensor activity in the limbs and trunk.

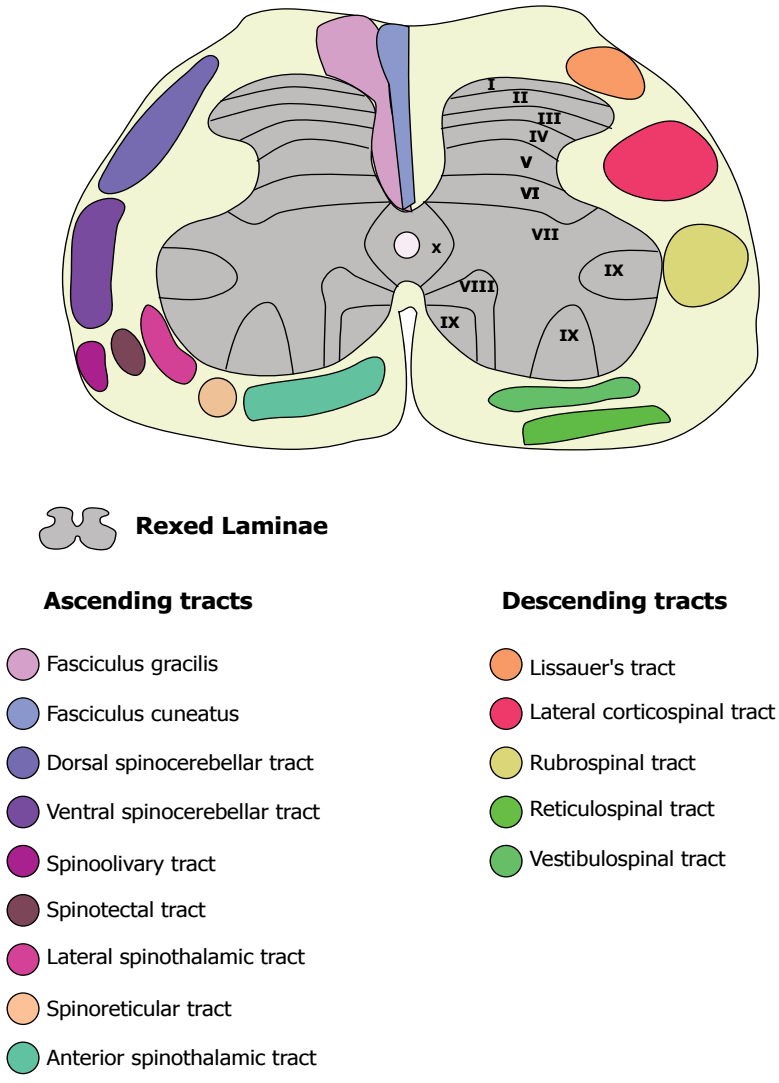

Figure 1. Diagram of the organization of the white and gray matter of the spinal cord

SPINAL CORD INJURY: causes and description

Spinal cord injury (SCI) is a devastating event that occurs unexpectedly being trauma the most common cause and less frequently disease or infection. Although limited return of function may occur in the weeks and months following spinal cord injury, in the CNS there is a lack of functional and extensive appropriate spontaneous regeneration of injured axons resulting in permanent loss of motor, sensory and autonomic function below the injury site.

Epidemiology of the spinal cord injury

SCI is one of the most prevalent and disabling conditions in the world. The most common cause of SCI is traffic accidents (47%) followed by: falls (31%) that is typically the second most common etiology in elderly population, sport accidents (10%) and violence injury (4.6%) (Mazaira et al., 1998). The highest incidence of SCI in Europe is in Estonia, followed by Romania, France and Spain with an annual incidence of 23.5 per million (Furlan et al., 2013; PCITATION {"citatio . The incidence is greater in males than in females (4:1 ratio of male: female) and the age of peak incidence is between 15-30 years of age or over 70 years. Most of the studies related with the incidence of SCI are from developed countries concluding that SCI incidence lies between 10.4 million per year to 83 million per year, depending on the study (Chiu et al., 2010).

Level and severity of the spinal cord injury

When the spinal cord is damaged all its functions can be compromised below the lesion site, ranging from total or partial loss of movement (tetraplegia or paraplegia), sensation (anesthesia or hypoesthesia), and autonomic deficits (sexual disfunction, control loss of the sphincters, etc.). Along with loss of sensation, pain is a frequent problem in the majority of the SCI population and can occur not only above the level of injury but also at or below the level of injury, being this one of the most devastating symptoms, which severely affects the quality of life of the patient (Ondarza et al., 2003; Rintala et al., 1998) . The nature and extent of spinal cord injuries vary widely, depending on the level of the injury, 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.

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 despite there is still the presence of some spared fibers cord tissue. Indeed, complete transection injuries are not common in humans.

Cervical injuries are more frequent than thoracic, lumbar or sacral injuries and are the most severe, 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 breath 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 **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.

The effects of **injuries at the lumbosacral** regions are restricted to the control of the legs and hips, urinary and system, and anus and sexual function. Remains little or no voluntary control of bowel or bladder function and sexual function when sacral spinal segments are affected after injury.

In a effort to systematize the classification of spinal cord injuries, 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, based on the examination of neurological function to asses 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 a 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.

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.

Animal models for the study of spinal cord injury

Although there are many differences between the nervous system of human and animals, several different animals models (specially models developed in rodents), have been developed to study of SCI, allowing exploration of mechanisms and properties of specific pathways and the study of potential therapies. To obtain the most useful data, the experimental animal design must accomplish two important precepts: must be as closely as possible to human situation and as reproducible as possible (Profyris et al., 2004). In 2000 the International Spinal Research Trust published guidelines that describes four characteristics that are required for an optimal model of SCI:

1. The nature and the extent of the lesion should be precisely defined.
2. A histological method should be available to detect the growth of axons through the lesion
3. A method should be available to analyze the functional synaptic transmission beyond the lesion by measuring the electrical activity that neurons use to communicate with one another
4. A behavioral measure should be available that is capable to detect restoration of known circuits.

Currently there are range of models that are use that include contusion, compression and transection-based model. Below there is a brief description of different SCI models.

Contusion model is the oldest and the most widely used SCI. It produce lesions similar to those seen in human patients of SCI. Thoracic injury contusion models are more common than cervical models, which is rarely reported due to life-threatening adverse effects can occur. Thoracic contusion models facilitate the assessment of locomotor recovery and postural activities and are induced with an impactor device or a weight-drop from specific height (Anderson et al., 2009). The first controlled contusion model was design by Allen in 1911 (Sharif-Alhoseini and Rahimi-Movaghar, 2014). Allen thought that when a known weight is dropped from a constant height onto a dog exposed spinal cord it shall produce same impact force on all occasions, delivering a defined amount of energy causing injury to the spinal cord as a result of compression and displacement (Sharif-Alhoseini and Rahimi-Movaghar, 2014).

Since Allen's first designs, the model has suffered several variations to be adapted for rodents. Besides. In the last decade, some computer-controlled contusion devices have been developed to create contusion in a controlled way to limit the variation between animals and allow compare between results obtained in different laboratories. In example, Ohio State University (OSU) has produce a weight-drop device controlled by a computer to closely control a particular level of spinal cord compression, in a similar way operates the only commercial available device, the Infinite Horizon (IH) impactor in which a stepping motor applies a defined force with a high precision to the cord. Once the force is reached, the impactor tip immediately withdrawn from the exposed spinal cord. Software displays the actual force applied, the amount of displacement, velocity of the impounder peak force measurement, and plots a graph of changes in displacement and force as a function of time from sequence initiation.

In return, **compression models** involve "placement" of a static weight on the cord without impact and contribute to simulate and study the effects of persistent compression or the optimal time of decompression. Different models of compression injury have been described including clip or balloon compression (Poon et al., 2007). In the clip compression, he injury is induced using clips calibrated to exert a specific force and is dorsoventrally closed over the entire cord for variable time periods. Adjustment of the closing force of the clip and the duration of compression produces different severities of SCI. The balloon-induced method it's a simple method that doesn't cause any damage to the surrounding structures, even the most recent methods doesn't require laminectomy. This type of injury involves pressure and volume controlled inflation of a micro-balloon in the epidural space. Spinal cord damage is graded by increasing the volume of saline used to inflate the balloon and treatment last typically from one minute resulting in a 'reversible' injury to 24 hours, which tend to be 'irreversible'.

Transection model is referred to as a complete spinal transection or an incomplete section of the dorsal or lateral aspects of the cord (hemisection). Spinal transection is made with fine surgical scissors producing a laceration of the cord. Despite the complete transection model reflects a complete SCI in patients, this type of lesion is barely observed in the clinic. Even so, the model provides an idealized setting for studying hypotheses that concern regeneration, degeneration or plasticity on axonal levels and usually are combined with neuroanatomical tract tracing.

Hemisection is used to selectively interrupt certain pathways and hold a tissue bridge between the proximal and distal ends of the cord, and maintain tissue continuity. Dorsal hemisection for selective transection of the corticospinal tract can be performed with some feedback from the change in color and texture between the white and gray matter giving a sign of the entirety of the hemisection. Dorsolateral quadrant lesions are used to interrupt the rubrospinal tract, and lateral hemisections disrupt all tracts on one side but spare some or all tracts on the opposite side.

Pathophysiology of the spinal cord injury

The pathophysiology of acute SCI involves two different neurodegenerative phases with distinct events in each phase. The first is known as the **primary injury,** characterized by the mechanical trauma in where bone or disk displacement within the spinal column compresses and contusions the spinal cord. This primary injury is restricted to the site of the impact itself and disrupts axons, blood vessels, membranes and myelin leading hemorrhage, axonal and neuronal necrosis and to an important edema at the impact site (Tator and Fehlings, 1991). This is an uncontrollable and unpredictable injury so, little can be done to reduce or avoid the events of the primary phase. From hours to days after the initial trauma a range of secondary cellular and molecular events occur, constituting the **secondary injury.** The secondary phase features a continuation of some events from the acute phase such as ionic imbalance or edema, exitotoxicity, free radical production, inflammation, apoptosis, lipid peroxidation and the release of extracellular matrix components to eventually form a glial scar that represents a physical and a chemical barrier for axonal regeneration and remyelination

(Rowland et al., 2008; Silver and Miller, 2004; Yiu and He, 2006) During this phase, the area of trauma distinctly enlarges rostral and caudally to the lesion, increasing the affected area and the cell death of neurons and glial cells (Oyinbo, 2011; Rowland et al., 2008; Silver and Miller, 2004). This makes the secondary injury the major cause of tissue damage and functional deficits (Figure 2). Unlike the primary injury, the secondary injury processes occur within hours to weeks after lesion, representing an important target for the development of protective strategies.

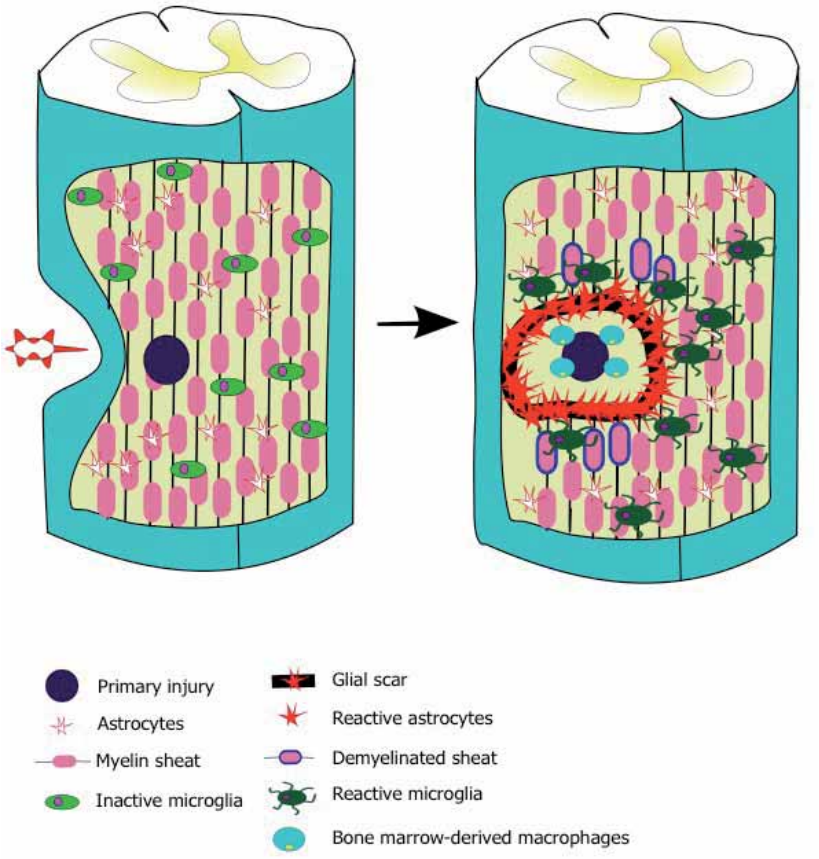

Figure 2. Schematic evolution of the spinal cord injury. From primary to secondary injury.

Blood flow changes and ischemia

Blood flow is intricately related to function in the spinal cord, but acute SCI results in rapid changes in the blood circulation within the spinal cord. Gray matter results highly affected due to the high vascularization, which makes gray matter more vulnerable than white matter when blood flow is altered(Tator and Fehlings, 1991). Mechanical trauma causes disruption of microvasculature with significant implications in secondary damage. Local changes at the injury site include hemorrhage and vasospasm of the superficial vessels that impedes the correct perfusion and consequently, the blood flow into the spinal cord is reduced (Figley et al., 2014; Mautes et al., 2000). Along with the impaired blood flow, the intravascular thrombosis and the breakdown of the blood-spinal cord leads to the formation of a vasogenic edema and the release of several vasoactive factors, resulting in a ischemic damage by hypoperfusion and hypoxia (Mautes et al., 2000; Tator and Fehlings, 1991). Severed vascular network and ischemia results in an increase of cell death in the tissue, initiating a signaling cascades that will also expand the area of tissue damage (Dumont et al., 2001; Mautes et al., 2000). Although this situation is transitory, reperfusion of the ischemic tissue implies the production of reactive oxygen species (ROS), leading to further the initial ischemia damage (Basu et al., 2001; Park et al., 2004). Besides, the reduction of the blood flow leads with hypoxia and a consequent increase of the anabolic metabolism that results in the production of acidic products such as lactic acid, which reduce the pH in the injured tissue. Acidification on the environment may affect the ATP

levels, which would eventually affects ion pumps and ion homeostasis (Chu and Xiong, 2013; Park et al., 2004)

Excitotoxicity

After injury the disruption of membranes results in alteration of the ion equilibrium and in increase of glutamate release into the extracellular space (Mazaira et al., 1998; Park et al., 2004). High concentration of glutamate results in excessive and prolonged activation of presynaptic and postsynaptic glutamate NMDA and AMPA receptors (Doble, 1999; Ondarza et al., 2003). Persistent activation of NMDA-receptor leads to excessive calcium influx that stimulates several intracellular mechanisms, which can release reactive oxygen species and other proteins that are important triggers of apoptosis. Activation of AMPA receptor, however, leads to the opening of sodium voltage-dependent channels, which results in a sodium influx followed by a passive influx of chloride and water to maintain ionic equilibrium (Doble, 1999; Kirshblum et al., 2011, 2014). These ionic alteration may produce osmotic cell lysis, enhancing apoptosis and necrosis of neurons (Doble, 1999; Park et al., 2004).

Oxidative stress

In SCI, production of reactive oxygen species (ROS) is enhanced by different processes such as reperfusion of ischemic tissue, the increase of intracellular calcium by glutamate increase or the inflammatory response (Basu et al., 2001; Guha and Tator, 1988). Microglia and macrophages express phospholipase A2

enzymes that leads the formation of arachidonic acid, the precursor of prostaglandins, thromboxans and leukotriens (David et al., 2012a). In normal conditions, proteolytic and oxidative enzymes produced by neutrophils sterilize the damaged area and prepare it for repair. However, the excess of neutrophils can cause tissue damage by oxidative stress as side effect (Carlson et al., 1998; Dumont et al., 2001), since free radicals induce peroxidation of lipids, proteins and DNA.

Inflammation

Inflammatory response is an essential aspect of the injury response to control infections, clear tissue debris, restore homeostasis of the tissue and initiate wound repair. Immune cells also secrete several factors that causes cell and tissue damage as a result of the normal side effect of the inflammatory response (Popovich and Jones, 2003). Therefore, inflammation must be actively terminated when is no longer needed in order to prevent unnecessary tissue damage. Different chemokines, cytokines and lipid mediators have an essential role regulating the inflammatory response, since they regulated the recruitment and activation of immune response and well as their clearance from the injured tissue. Failure of this resolution mechanisms results in chronic inflammation and tissue damage.

The inflammatory response that occurs after SCI is an important contributor to secondary tissue damage and functional impairments. The detrimental effects of inflammation in the CNS are more pronounced than in other tissues due to the limited capacity of the central nervous system (CNS) for axon regeneration and replaces damaged neurons, and consequently, leads to irreversible functional deficits (David and Kroner, 2011).

Early after SCI, the initial necrosis and the disruption of the blood spinal cord barrier leads to extravasation of peripheral inflammatory cells as well as activation of microglia and astrocytes, which up-regulates the production of pro-inflammatory cytokines and chemokines, such a IL-1β and TNFα and release other vasoactive substances that enhances vascular permeability (Donnelly and Popovich, 2008). In addition, damaged endothelial cells up-regulate and express leukocyte adhesion receptors and guide leukocytes to inflamed tissues. Neutrophils are one of the first blood-derived inflammatory cells to infiltrate into the tissue and reach a peak at 24 hours and disappear within 5 next days (Donnelly and Popovich, 2008). Increased neutrophil influx correlates with greater tissue destruction and more severe clinical deficits. Several studies has reported better functional recovery and tissue protection after neutralize neutrophil infiltration (Gris et al., 2004; Lee et al., 2011; Neirinckx et al., 2014; Taoka et al., 1997). Another study, however, showed opposite effect after neutrophil depletion (Stirling et al., 2009). This may suggest that a small influx of neutrophils may be necessary for an effective immune response and might contribute to the non-destructive effect by the clearance of microbial intruders and tissue debris.

Neutrophils trigger the leakage of the blood spinal cord barrier via the release of inflammatory mediators and cause the recruitment of more inflammatory cells to the site of lesion (Aubé et al., 2014; Schnell et al., 1999). Neutrophils also release cytokines, proteases and free radicals that activate other inflammatory and glial cells (Schnell et al., 1999; Taoka et al., 1997). During the first hours and during the first week microglia enter a transition phase in which their processes becomes shorter, thicker and they cell body larger due to activation, adopting a macrophage morphology that makes indistinguishable from blood derived macrophages (David

et al., 2012b). Blood derived monocytes infiltrate to injury site and turns into macrophages reaching a peak at 7 days after injury (Donnelly and Popovich, 2008). Activated microglia and macrophages secretes different cytokines, free radicals and growth factors, which promotes injury (Piani et al., 1991). On the other hand, macrophages are very effective phagocytes that clean myelin and cell debris. Moreover, they release some cytokines such as TGF-β, which may limit oligodendrocyte cytotoxicity and promote axonal growth (McTigue et al., 2000; Merrill et al., 1993). This dual role of macrophages may be attributed to molecules found in the tissue microenvironment that modulate macrophages activation. Indeed, two different phenotypes of macrophages has been described: classical cytotoxic (M1) and alternative activated (M2) macrophages (Mantovani et al., 2013). Macrophages adopt M1 polarization when stimulation with interferon γ (IFNγ), lipopolysaccharide (LPS), and some pro-inflammatory cytokines (Mantovani et al., 2013). M1 macrophages phagocytize cell debris but produces high levels of oxidative metabolites that may exacerbate the initial cell death as a collateral effect. On the other hand, after stimulation with IL-4 and IL-13 (IL-4, IL-13), macrophages adopt protective M2 phenotype. M2 macrophages promote angiogenesis, matrix re-modelation and axon regeneration while suppressing destructive immunity(Mantovani et al., 2013). After SCI, macrophages adopt predominantly the M1 cytotoxic phenotype, and the presence of M2 macrophages is scarce(Kigerl et al., 2009). Interestingly, when M2 macrophages are transplanted into the injured spinal cord rapidly switch to the M1 phenotype, indicating that the damage spinal cord milieu is conductive of M1 polarization (Kigerl et al., 2009).

Together with immune blood cells and microglia, astrocytes have an important role after SCI as well. Astrocytes are the largest cell population in the CNS, and play

an essential role in the control of the energy supply to neurons, the turnover of neurotransmitters and in the formation of new synapses, among others (Matute et al., 2006) . After injury, pro-inflammatory moleculues, such as IL-1β or TNFα, stimulate astrocytes to become hypertrophic exhibiting a high expression of glial fibrillary acidic protein (GFAP), nestin and vimentin. Astrocytes can remain hypertrophied for several months to years. Activated astrocytes important producers several cytokines and chemokines such as MCP-1, MIP-1α, IL-1β or TNFα that can activate the influx of immune cells to the CNS (Cregg et al., 2014). Importantly, astrocytes form a glial scar that inhibits axon regeneration and cellular repair. The scar tissue is characterized by excess deposition of extracellular matrix (ECM), a dense network of hypertrophic astrocytes, as well as infiltration of macrophages and fibroblasts (Okada et al., 2006; Wanner et al., 2013). Unlike peripheral tissue, this scar fails to resolve over time and remains chronically after SCI. Although glial scar play some beneficial roles in SCI, such as limiting the infiltration of leukocytes and the spread of tissue damage to adjacent unaffected regions (Bush et al., 1999), it also inhibits axon regeneration and remyelination (Fitch and Silver, 2008; Harlow and Macklin, 2014) since contain inhibitory molecules such as chondroitin sulfate proteoglycans (CSPG) (McKeon et al., 1995).

Cytokines and the spinal cord injury

Cytokines are mainly secreted by astrocytes and immune cells in SCI, but can be also expressed by other cell type such as oligodencrocytes, endothelial cells or fibroblast (Khan, 2008). Cytokines are a category of small proteins that function as key modulators of the immune system with a pro-inflammatory or anti-

inflammatory profile or both depending on the cells present and their sensitivity to the cytokines (Dinarello, 2007). Cytokines include chemokines, interferons (IFNs), interleukins (ILs), growth factors (GF), the tumor necrosis factor alpha (TNFα) and colony stimulating factors (CSF) (Khan, 2008). Below there is a brief description of each type of cytokines .

- **TNFα** is known as a major pro-inflammatory cytokine and mRNA expression is rapidly and transiently increased in mouse after SCI with two peaks at 1 hour and at 14-28 days after injury (Pineau and Lacroix, 2007). Is mainly secreted by microglia, astrocytes, oligodendrocytes and neurons during the first peak while at the second peak is secreted by microglia and macrophages. The inhibition of TNFα reduces neuronal cells death, tissue damage and enhances lomocotor recovery after SCI. At later times after injury TNFα triggers death and oligodencdrocytes that could contribute to de demyelination of intact axons, enhacing motor and sensory loss (Ferguson et al., 2008; Genovese et al., 2008).

- **Colony stimulating factors (CSF)** are a group of hematopoietic growth factors including macrophage colony stimulating factor (M-CSF) Granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF), and stimulates the proliferation and maturation of myeloid progenitors, precursors of neutrophils and monocytes in the CNS. GM-CSF contributes to myelin phagocytosis, promotes locomotor recovery and tissue sparing (Dittgen et al., 2012; Huang et al., 2009).

- **Chemokines** are small molecules with a chemotactic cytokines that are important for leukocyte migration and recruitment to the site of injury. Action of chemokines is mediated via specific G-protein-coupled-receptor. These receptors are either expressed constitutively on some cells or are induced after stimulation on others. These receptors can indude signals that are not limited to cell migration. Indeed chemokine have other important biological functions as immune cells polarization, angiogenesis and lymphopoiesis (Mackay, 2001)

  The mRNA levels of some chemokines such as macrophage inflammatory protein 1 (MIP-1α and MIP-1β), MIP-2, monocyte chemoattactive protein 1 (MCP-1) and interferon inducible protein (IP-10) are up-regulated after SCI reaching a peak at 6 hours after injury (Ghirnikar et al., 2000). Little is known about their contribution to cell death and functional loss in SCI, but the administration of viral macrophage inflammatory protein II (an antagonist for chemokine receptors) leads to reduction in neuronal cell death and astrogliosis (Ghirnikar et al., 2000). Morevoer, the lack of the receptor CCR2, the main receptor of MIP-1 and MIP- 2 correlates with a decreased macrophage accumulation in the spinal cord parenchyma at 7 days post-injury but not in later times.

- **Interleukins** are a group of cytokines that were first seen to be expressed by leukocytes. Structural homogeneity has been able to partially distinguish between interleukines and classificate in different families. One of the most important family of interleukins is the Interleukin 1 (IL-1) family (Dinarello, 2013). The IL-1 family has 11 different members, which are associated with

acute and chronic inflammation, and their biological properties are typically pro-inflammatory. Two of their members are IL-1α and IL-1β that are increased in the injured spinal cord early after trauma and reach a peak expression at 6-24 hours (Garlanda et al., 2013). Along with TNFα, they seem be involved in the recruitment and activation of peripheral and the activation of microglia and astrocytes. Treatment with the interleukin 1 receptor antagonist (IL-1ra), which inhibits IL-1α and IL-1β signaling, markedly reduce injury-induced apoptosis and reduce neuronal death after brain ischemia, while exogenous administration of IL-1β exacerbates ischemic and exitotoxic brain injury (Nesick et al., 2001; Loddick and rothwell et al., 1996; Telton et al., 1996). The other members of IL-1 family also have a pro-inflammatory profile. An exception is the newest member of this family recently discovered, interleukin 37 (IL-37) (Garlanda et al., 2013)

INTERLEUKIN 37

IL-37, initially known as IL-1 family member 7 (IL-1F7), was identified by different independent groups for the first time in 2000 by computational cloning (Busfield et al., 2000; Kumar et al., 2000; Pan et al., 2001). IL-37 was defined as one of the members of the IL-1 family with anti-inflammatory properties.

Structure of IL-37 gene

Like other members of IL-1 family (except IL-18 and IL-33) is encoded by a gene in chromosome 2 at 2q13. It presents a 21% of amino acid similarity with IL-1β gene, and a 29% of similarity with IL-1Ra (Taylor et al., 2002) gene, and it's secondary structure has the same domain of 12 β-strand connected by loop regions present in IL-1α, IL-1β and IL-1Ra.

The IL-37 gene is 3,617kb and it's composed by 6 exons although alternative splicing give rise to five different isoforms: from IL-37a to IL-37e (Boraschi et al., 2011; Dinarello and Bufler, 2013; Taylor et al., 2002).

- **IL-37a** contains exons from 3 to 6. That makes that this isoform has unique N-terminus encoded by exon 3 while in the other isoforms this exon is missing. The exons 4 to 6 encodes forms the putative 12β-strands required for the typical IL-1 secondary structure, suggesting that IL-37a could be a functional cytokine (see figure 3).
- **IL-37b** encodes the longest transcript variant. The N-terminals formed by the two first exons. Each exon contains a different cleavage site for cytokine

maturation. IL-37b also contains exons 4 to 6 encoding for the 12β-strands structure. For this reason, IL-37b is expected to be biologically functional.

- **IL-37c** is identical to isoform b but lacks exon 4, which encompasses a portion of the β-strand structure. For that, it is supposed that isoform c won't function as a cytokine (see figure 3)..
- **IL-37d** is also identical to isoform b except for the lacking of exon 2 but its sequence include exons from 4 to 6 that makes that the isoform d could represent another functional form of the protein (see figure 3).
- **IL-37e** consist in exons 1, 5 and 6 and like isoform c lacks exon 4 that makes along with isoform c questionable if those cytokine is functional (see figure 3).

Those isoforms that can be considered as functional cytokines (a, b and d) are characterized by the presence of the complete sequence encoding the 12β-strands structure and the presence of at least one cleavage site required for the maturation of the cytokine (Bulau et al., 2014; Murzin et al., 1992) IL-37 possesses three cleavage sites (see figure 3): one cleavage site is located in the exon 1 between amino acids D20 and E21 (present in isoforms b and d)(Kumar et al., 2002). The second site is located in the exon 2 between amino acids F45 and V46 (present in isoform b and c)(Pan et al., 2001). The first cleavage site is recognized mainly by caspase-1, which is also responsible for conversion into active mature peptides of different members of the IL-1familyl including IL-1β and IL-18, and in a less significant manner by caspase-4 (Kumar et al., 2002). The presence of a second cleavage site suggests that it is possible that other proteases may process IL-37. This hypothesis is supported by the fact that the use of caspase inhibitors only

inhibits partially IL-37 maturation and in cell lines overexpressing the complete form of IL-37b yield a soluble cytokine starting at V46 can be detected. By the other side, the presence of the third cleavage site located in exon 3 between amino acids L21 and R22 which is recognized by an elastase (Bulau et al., 2014; Kumar et al., 2002; Sharma et al., 2008) (Figure 3).

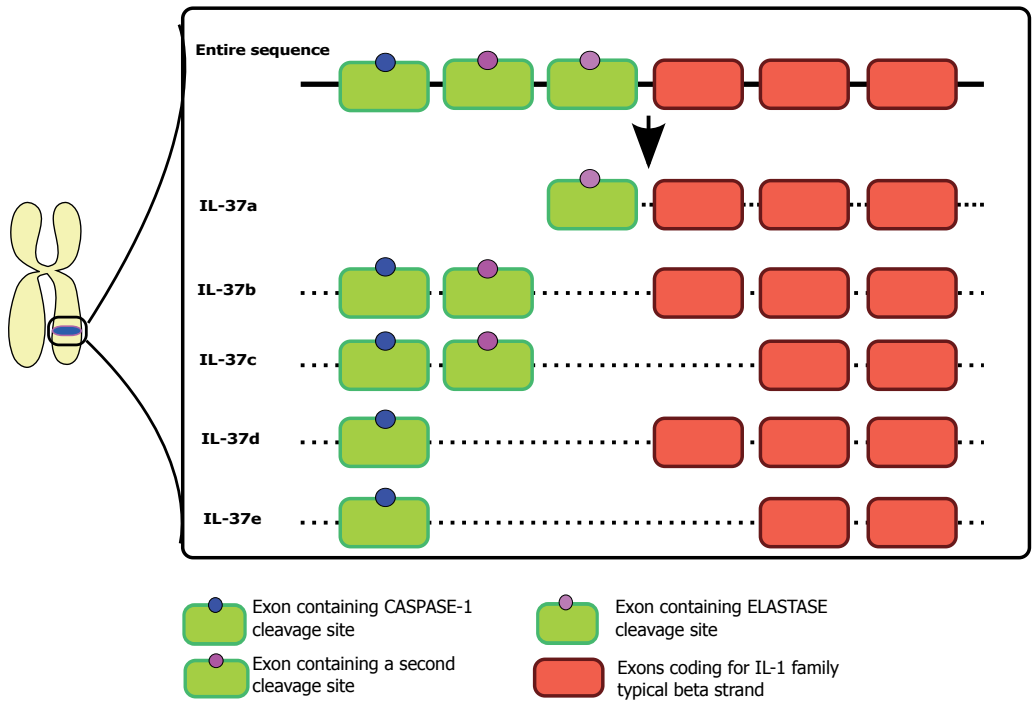

Figure 3. Structure of IL-37 with the different cleavage sites

In normal conditions, IL-37 mRNA is barely detected due to a conserved A-rich homology box in exon 5, which flanks a coding region of instability elements that are also found in IL-18, IL-1β or in a variety of other mRNA, such as plasminogen activator receptor or vascular endothelial growth factor (Tierney and Medcalf,

2001). These instability elements overlaps with binding sites for proteins associated with mRNA stability and limits the half life of IL-37 mRNA. However, after inflammatory stimuli mRNA levels increase due to the activation of mechanisms or sequences within the 3'-UTR (untranslated region) for mRNA stability (Bufler et al., 2004).

Function of IL-37

One of the problems when investigating in vivo the function of IL-37 is that no mouse genomic sequence corresponding to human IL-37 has been found yet. It was thought that mouse IL-36 could be the orthologous for human Il-37 because the gene is located at the same place where human IL-37 is, and because it is known that the order orientation of the IL-1 genes is conserved in both species. Nevertheless, mice IL-36 is more similar to human IL-36 (62% of similarity) than to human IL-37 (28% of similarity). It is currently thought that IL-37 locus in mice is found elsewhere or has been lost (Taylor et al., 2002).

To solve this limitation for in vivo studies of IL-37, a transgenic mice overexpressing human IL-37 isoform b (hIL37tg) was generated via transfection of pIRES IL-37b expression plasmid under constitutively active CMV promoter. Fertilized eggs from C57BL/6 were injected with the plasmid and implanted into C57BL/6 females. hIL37tg mice do not present differences compared with wild-type mice in growth, behavior, reproduction and gender ratio over generations (Nold et al., 2010). Although the expression of IL-37 is under CMV promotor, there is absent/low expression of IL-37 in the hIL-37tg mice due to the presence of instability region (Bufler et al., 2004). However, after systemic LPS-stimuli, the expression of IL-37 increases in the hIL37tg mice resulting in decreased circulating

cytokines, less acidosis, hyperkalemia, hepatitis, dehydration and hypothermia compared with the WT mice (Bufler et al., 2004; Nold et al., 2010).

IL-37 mRNA is detected constitutively expressed in a variety of normal human tissues such as testis, colon, placenta, lung, lymph nodes and can be induced in peripheral mononuclear blood cells (PBMC) and dendritic cells (DC). However, some isoforms seems to be expressed in a tissue-specific fashion suggesting that each isoforms may possess specific regulatory elements. The isoform a is the only one expressed in the brain, whereas the isoform b is the only expressed in kidney and the isoform c is found in the heart. IL-37 protein has been detected in monocytes, tonsil plasma and breast carcinoma cells, and although it is assumed that IL-1 family cytokines act primarily as a secreted mediators, some members of IL-1 family including IL-1α, IL-33 and IL-37 can be translocated to the nucleus (Busfield et al., 2000; Luheshi et al., 2009; Pan et al., 2001; Taylor et al., 2002).

Nuclear role of IL-37

Il-37 is found intracellularly in monocytes in very low amounts and is rapidly up-regulated after inflammatory stimuli. Although the intracellular effects of the IL-37 are poorly understood, to elucidate the intracellular role of IL-37 different experiments in PBMC cultures or mouse macrophages cell lines expressing IL-37 (RAW-IL-37) demonstrate that for its nuclear translation after LPS stimulation, the cleavage by caspase-1 is essential (Bulau et al., 2014; Sharma et al., 2008). Around a 20% of the total IL-37 protein is translocated to the nucleus where interacts with phospo-SMAD3 and acts suppressing the transcription of pro-inflammatory cytokines without affecting the expression of anti-inflammatory cytokines (Dinarello and Bufler, 2013; Nold et al., 2010) by the down-regulation TLR-4/NF-

κB signaling pathway (Wu et al., 2014). The inhibition of one or both elements using pan-caspase or SIS3 (an specific inhibitor of SMAD3) suppresses nuclear translocation and activity of IL-37, respectively.

Extracellular role of IL-37

IL-37 can be externalized in both forms: pro (full length)- or mature (cleaved at V46), being the release of the mature form ATP and caspase-1 dependent. Both forms of IL-37 bounds to IL-18Rα in a dose dependent manner, but the binding efficiency of mature IL-37 is higher than the full length IL-37 (Bulau et al., 2014; Kumar et al., 2002). The binding site of IL-37 to IL-18Rα contains two amino acids, Glu-42 and Lys-89, which are found in IL-18, and are critical for the interaction of IL-18 to IL-18Rα and IL-18 binding protein (IL-18BP), the natural antagonist of IL-18 (Novick et al., 1999) (Figure 4).

In general, cytokine receptors are composed by at least two receptor chains that oligomerize during ligand-induced signaling. IL-18 binds to the subunit α of IL-18R in presence of different co-stimulants (IL-2, IL-12 or IL-15). Then, forms a heterodimeric complex with the subunit β, promoting the expression of IFN-γ in T cells or Natural Killers (NK) by the activation of the NF-κB pathway (Bufler et al., 2002). When IL-37 binds to the subunit α of the IL-18R the recruitment of the subunit β fails (Bufler et al., 2002). This could suggest that IL-37 could work as an antagonist of IL-18. However this idea is dismissed because of the affinity to bind the subunit α of both forms of IL-37 (full length and cleaved) is much lower compared with the affinity of the own IL-18 to bind it, and seems to have little physiological relevance in the release of IFN-γ (Bufler et al., 2002). Even increasing

the concentration of IL-37 there is neither IL-18 like activity nor inhibition of IFN-γ, suggesting that IL-37 does not work as an antagonist (Bufler et al., 2002; Dinarello and Bufler, 2013; Kumar et al., 2002). On the other hand, silencing of IL-18Rα results in increase of the inflammation, which may indicate that IL-37 binding to IL-18Rα is not completely useless, suggesting that this receptor when binds to IL-37 can recruit another co-receptor that may delivers an anti-inflammatory signal (Boraschi et al., 2011; Li et al., 2015; Nold-Petry et al., 2015). To find a corresponding co-receptor for the complex IL-37-IL-18Rα, different studies focused in the binding to another protein in common with IL-18, the IL-18BP (Figure 4).

The two conserved amino acids in IL-37 and IL-18 required for the binding to the IL-18Rα are also required to bind IL-18BP. IL-18BP is not a part of signaling complex of IL-18, but binds and neutralizes IL-18 with high affinity. Mature and pro-IL-37 are also able to bind IL-18BP at the same Immunoglobulin-like (Ig-like) domain site where IL-18 does, and together forms a complex with the IL-18Rβ that prevents the formation of the active IL-18 receptor, depriving IL-18 signal transduction with more efficiency that IL-18BP alone. However, different studies suggest that the union between IL-37 and IL-18BP is very weak and the increasing efficiency of IL-18BP by the effect of IL-37 is only significant when the union is with the mature IL-37 (Azam et al., 2003; Boraschi et al., 2011; Bufler et al., 2002).

IL-18BP contains an Ig-like domain that is similar to the only Ig-like domain present in IL-1R8, an orphan member of the IL-1 receptor family (IL-1R). A common property along with the extracellular Ig-like domain IL-1R family is the presence in the intracellular domain of a Toll-IL-1R (TIR) domain, also found in Toll-like Receptors (TLR), which is involved in the initiation of NF- κB and MAPK

signaling pathway, leading to the production of pro-inflammatory mediators. Unlike other members of IL-1R, in the TIR domain of IL-1R8 lacks two conserved amino acids (Ser447 and Tyr536, replaced by Cys222 and Leu305) that are essential for IL-1R1 inflammatory signaling, which give to IL-1R8 a negative regulatory function on IL-1R and TLR mediated pathways. Interesting, studies with IL-1R8 deficient mice showed and exacerbated inflammatory response (Garlanda et al., 2009, 2013; Li et al., 2015; Nold-Petry et al., 2015). Therefore, IL-1R8 has been proposed as the co-receptor that forms a complex with IL-37 and IL-18Rα and leads the anti-inflammatory effect of IL-37 (Figure 4). To confirm this hypothesis, in vitro studies with bone marrow derived macrophages (BMDM) from WT mice, IL-1R8 deficient mice and IL-18Rα deficient mice showed that treated with recombinant IL-37 did not lead to IL-37 binding when these receptors were absent (Nold-Petry et al., 2015). Moreover, immunoprecipitation followed by staining of IL-18Rα of A549 cells transfected with IL-37, a band with different weight could be observed through time. Initially, the band corresponded with IL-18Rα alone, but along time, the band became of higher molecular weight, and corresponded to the complex IL-18Rα-IL-37-IL-1R8 (Nold-Petry et al., 2015).. Similar results were observed in human PBMC, and the silencing of IL-1R8 and IL-18Rα impaired IL-37 (Li et al., 2015; Nold-Petry et al., 2015). In fact, vivo studies in hIL37tg x IL-1R8 deficient mice showed that the lack of this receptor abolishes the anti-inflammatory effects of IL-37 after LPS treatment. The formation of complex IL-37-IL-1R8 results in a variety of different effects, but an effect that may be pivotal for IL-37's role in the inhibition of the innate immune response is the inhibition of *Fyn* and *TAK1* kinases. While *fyn* promotes inflammation and a phenotype away from M2, *TAK1* is an adaptor kinase with the ability to relay the

pro-inflammatory signals of TLR, different IL-1R and TNF-R, and their downstream effector pathways, including NF-κB, MKK3-p38 and *Akt* (Li et al., 2015; Nold-Petry et al., 2015). Another effect described is the inhibition of the insulin receptor-mTOR-kinase S6K, and the increasing activity of the AMP-activated kinase (AMPK), STAT6 and transcription factors of the *foxo* family, which results in a phenotype M2 in macrophages.

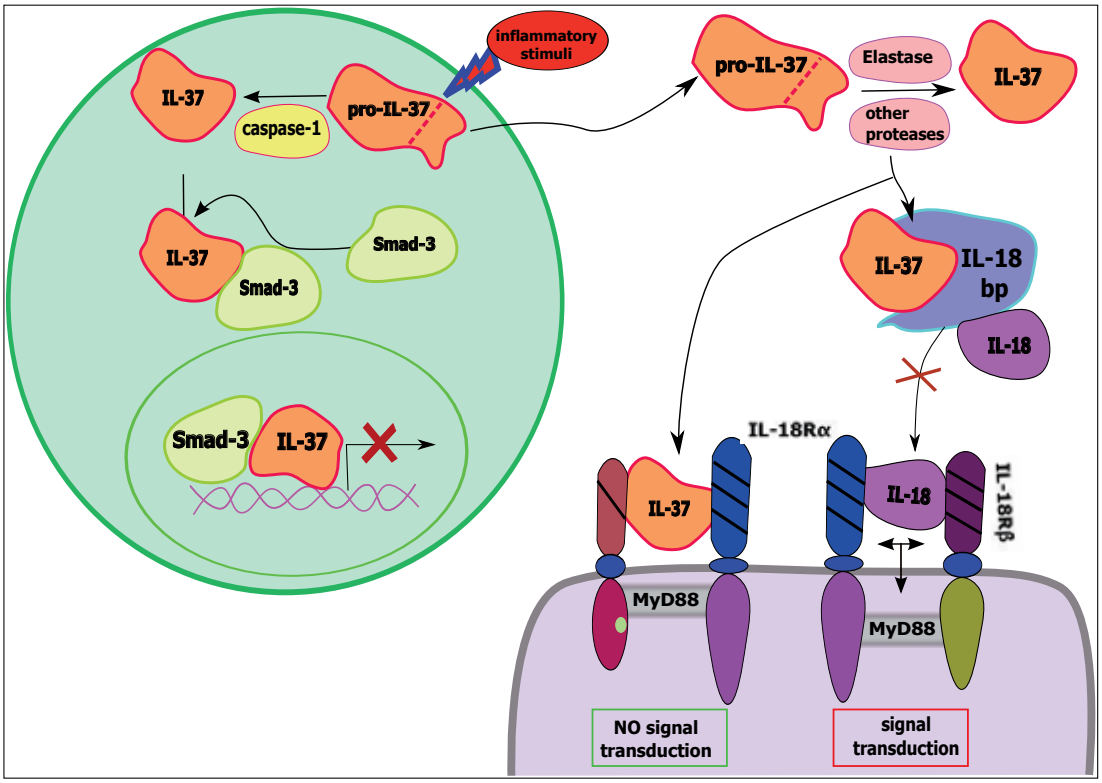

Figure 4. Resume of intracellular and extracellular role of IL-37.

Effect of IL-37 in inflammatory diseases

IL-37 has been described in a variety in human diseases and the effect of IL-37 has been studied in different animal models of human diseases.

Colitis is an inflammatory bowel disease characterized by an imbalance of the regulatory mechanisms of the intestinal immunity, with a preponderance of pro-inflammatory cytokines (McNamee et al., 2011). hIL37tg mice in where colitis was induced through the administration of dextran sulfate sodium (DSS) showed an increase of IL-37 protein levels and the inflammatory response of DSS-colitis induced was lower in hIL37tg mice compared with their counterparts administered with water as vehicle (McNamee et al., 2011; Weidlich et al., 2014). IL-37 decreases the expression of TNFα, IL-1β and increases the levels of IL-10 being this increase unique to this in vivo model. The lower inflammatory response was also accompanied by a decrease of the leukocyte recruitment in the lamina propia and a better preservation of the epithelial cell integrity and less edema (McNamee et al., 2011). In other physiological aspects, hIL37tg mice showed lower scores in all the compromising clinical signs of colitis: weight loss, stool consistency, bleeding and colon length (used as a macroscopic marker of colon injury) was higher in wild type (WT) mice (McNamee et al., 2011).

In a different models of ischemic damage, hIL37tg mice showed a reduced infarct size and improved left ventricular function and a decreased activation of monocyte chemoattractive protein-1 (MCP-1). This was linked to less accumulation of mononuclear cell in the myocardial tissue, and to reduced NF-κB intranuclear translocation (Yousif et al., 2011). In a well-studied model of brain ischemia, hIL37tg mice showed a reduced cytokine expression (including TNF-α, IL-6, IL-1β

and MCP-1), smaller infarcted area and better neurological and locomotor outcomes, although IL-37 seems to not affect cell death (Patel et al., 2014). Unlike brain ischemia, in hepatic ischemia, expression of IL-37 reduces cell death and promotes a reduction of neutrophil infiltration, a decrease of different cytokines such as Macrophage inflammatory protein-2 (MCP-2), keratin chemokine (KC) or TNF-α, and a reduction of the ROS levels (Sakai et al., 2012).

In other models of hepatic disease induced by concanavalin A (concA), mice transiently expressing IL-37 after hydrodynamic tail vein injection of plasmid-DNA showed reduced levels of IL-1α, IL-5, IL-6 and IL-9 2 hours after ConcA administration but this reduction was not maintained 24 hours after injection. Expression of other cytokines like IL-1β, TNFα and IL-10 did not show differences between control mice and mice expressing IL-37 (Bulau et al., 2011). In this model, alanine aminotransferase (ALT) levels were equal in IL-37 mice and control mice and the same with the histologic evaluation and both showed the same extent of liver injury(Bulau et al., 2011). One of the differences of this study is that the model does not use a transgenic mice and the expression of IL-37 is located in hepatocytes and not in the immune cells showing that the expression of IL-37 in non-immune cells can modulate immune response as well (Bulau et al., 2011).

The effect of IL-37 has been studied in other diseases, such obesity as effect of type-2 diabetes. hIL37tg mice with a hiperlipidic diet showed reduced adipose tissue macrophages, improved insulin sensitivity, less lipid accumulation and increased glucose tolerance (Ballak et al., 2014). Moreover, treatment with recombinant IL-37 in hepatocyte cell line results in a AMPK expression and preserved insulin signaling (Ballak et al., 2014)

The information of IL-37 and it role in other systems is barely known. Indeed, there is currently no knowledge whether IL-37 has in CNS conditions, where the deregulation of the immune response results in a worsening of the initial deleterious conditions.

THE DEVELOPMENT OF NEUROPATHIC PAIN AFTER NERVOUS SYSTEM INJURY

Pain is described as an unpleasant sensory and emotional experience that is associated with actual or potential tissue damage. Nevertheless, it is considered as a protective mechanism. This type of pain, known as *nociceptive pain,* is caused by normal activity of the peripheral terminals of primary nociceptors. It prevents injury by the generation of an unpleasant sensation that provokes a withdrawal reflex to avoid contact with injurious stimuli. The ultimate aim of pain is to promote healing of the injured body part by creating a situation that discourages physical contact and movement (Latremoliere and Woolf, 2009). Nociceptive pain is supported by *inflammatory pain* that is related to inflammation affecting peripheral tissues that are supplied with nociceptive fibers. Nociceptive pain usually ends when injury is healed (Costigan et al., 2009). In contrast, the development of chronic pain is considered a disease that affects seriously quality of life. *Neuropathic pain* is a kind of chronic pain that is a result of peripheral or central nervous system injury. It can persist in time even if the injury has been healed, and leads with secondary changes that affect the entire nervous system. Like nociceptive pain, neuropathic pain is also associated with excessive inflammation that can be involved in both, the initiation an the maintenance of neuropathic pain (Echeverry et al., 2013; Liu et al., 2000; Marchand et al., 2005; Scholz and Woolf, 2007) (Figure 5).

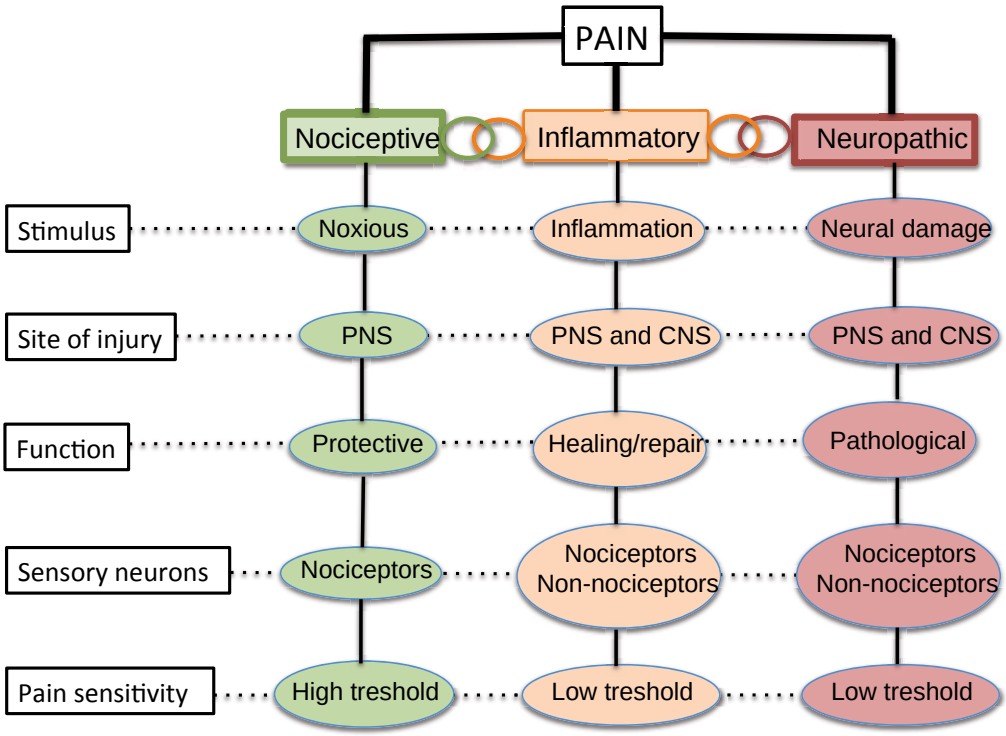

Figure 5. Classification of different types of pain.

Neuropathic pain can arise **spontaneously** or it can be **evoked**. When it is evoked, pain can be divided in **allodynia** (pain elicited by a stimuli that normally does not cause pain) or **hyperalgesia** (increase pain response produced by a stimuli that normally causes pain) (Figure 6).

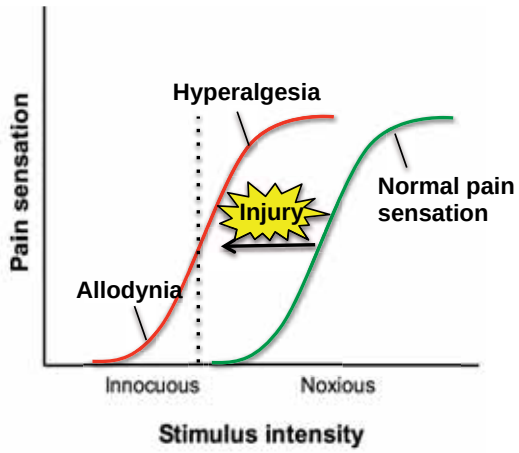

Figure 6. Shift of pain sensation after injury.

Neuropathic pain after peripheral nerve injury

Peripheral nerve injury is the most common model used to study Neuropathic pain. The most frequently models involving different lesions of the sciatic nerve are described below:

- ***Axotomy*****:** consists on the transection of the sciatic nerve.
- ***Spinal nerve ligation (SNL)*****:** involves a distal ligation of spinal nerves corresponding to L5 and L6 levels of the spinal cord.
- ***Partial sciatic ligation (PSL)*****:** involves the ligation of the sciatic nerve at high-thigh level, so that ⅓-½ thickness of the sciatic nerve is trapped in the ligature.
- ***Chronic constriction injury (CCI)*****:** this model loosely ties the sciatic nerve with four chronic silk ligatures at the mid-thigh level, causing swelling and strangulation of the nerve.
- ***Spared nerve injury (SNI)*****:** the ligation of the tibial and peroneal nerve branches while sparing the sural branch of the sciatic nerve.

All these models result in evoked and spontaneous pain (Zimmermann, 2001). Evoked pain can be assessed by standardized sensory testing methods. Spontaneous pain is more difficult to detect but the presence of autotomy (a self-mutilation of the denervated part) is generally considered a sing of spontaneous pain, along with the form of paw guarding and excessive grooming behavior on the injury site (Kauppila, 1998; Rodin and Kruger, 1984) (Figure 7).

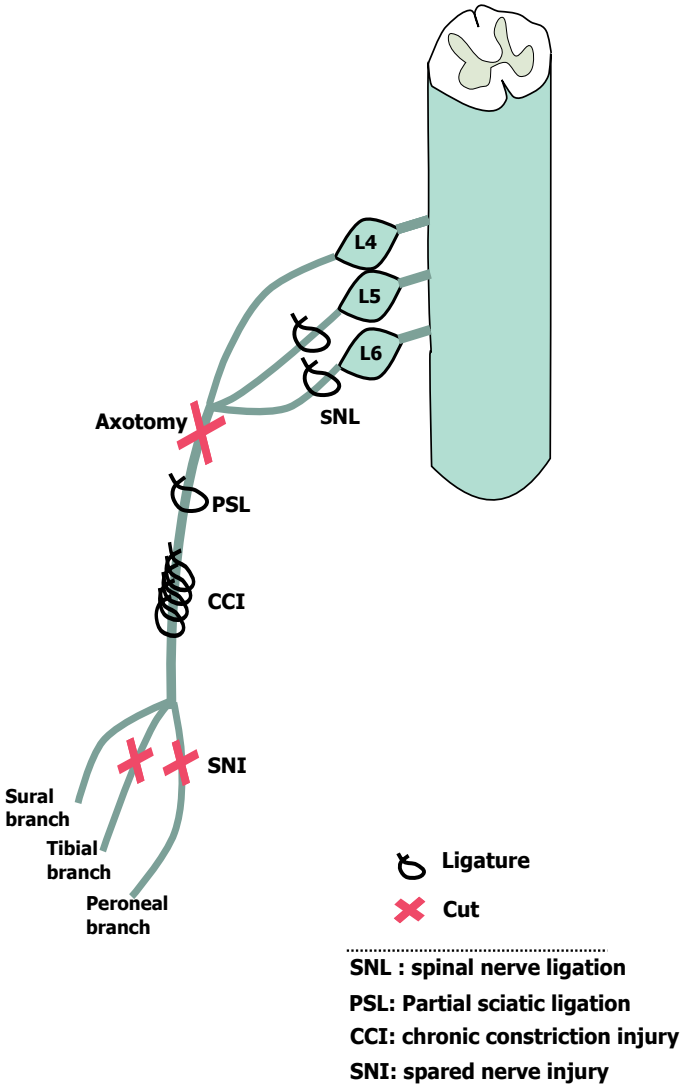

Figure 7. Different models used to study peripheral neuropathic pain.

Nerve injury results in a direct cell death and compromise the transduction of sensory information. Injury leads to peripheral sensitization that involves a reduction in threshold and an increase in the excitability of the peripheral terminals of nociceptors in response to inflammatory mediators. These mediators are mainly released by infiltrating macrophages from blood torrent, glia cells and Schwann cells (Scholz and Woolf, 2007).

The first cells to react are Schwann cells and microglia. Schwann cells start to degrade myelin sheaths and secrete metalloproteases, inflammatory mediators and different growth factors. Metalloproteases, such as MMP2 and MMP9 (Kawasaki et al., 2008a), attack the basal lamina of blood vessels leading to the disruption of the blood nerve barrier, and boosting the migration of circulating macrophages from blood to the injury site (Kawasaki et al., 2008a). Macrophage invasion is also supported by the release of chemokines like CCL2 and CCL3 (Biber and Boddeke, 2014), released by Schwann cells, activated glia and neurons. Moreover, injured axons present an increased excitability due to the effect of different growth factors released by Schwann cells, such as NGF and GDNF. These growth factors can migrate to cell bodies of injured axons, located at the dorsal root ganglia (DRG), and promote the expression of different vasoactive mediators, including calcitonine gene related peptide (CGRP) and substance P. Those mediators are released by DRG neurons and can increase the invasion of circulating macrophages and sensitize primary afferent neurons (von Hehn et al., 2012; Scholz and Woolf, 2007).

After peripheral nerve injury, inflammation is found at different levels: at the site of the injury, in the DRGs and at the spinal cord. The injury generates nociceptive inputs in both, injured and surrounding uninjured axons, which are transmitted to the sensory neurons in the DRG and activate microglia. DRG neurons among other molecules release CCL2, CCL3 and CCL21 (Biber and Boddeke, 2014; Thacker et al., 2009; Zhang et al., 2007), which promote microglia activation at DRGs. Chemokines are also transported to the dorsal horn, where activate glial cells and sensitize dorsal horn neurons. This process is known as *central sensitization*. Glial

activation and sensitized neurons release several inflammatory mediators that enhances the initial immune response at dorsal level (Latremoliere and Woolf, 2009).

All together leads to the release of pro-inflammatory cytokines at different levels of injury. The cytokines most involved in the development of pain are IL-1β, TNFα and IL-6 (Kawasaki et al., 2008b). Different experiments demonstrate that the inhibition of these cytokines leads to a reduction of pain (Clark et al., 2013; Echeverry et al., 2013; Kawasaki et al., 2008b; Marchand et al., 2005; Woolf and Salter, 2000). The pro-inflammatory environment persists at the original nerve injury and progresses to distant regions, leading to pain hypersensitization and long-persisting pain.

Neuropathic pain after central nerve injury

Neuropathic pain can develop as a consequence of injury or disease of the central nervous system. Initially, central neuropathic pain was classified according to its location*: above-*, *at-* and *below-level* of injury. While at- and below-level pain were strictly referred to a SCI context, above-level pain definition was more global, including pain derived from pathological conditions different than SCI, such as compressive neuropathies, surgery or multiple sclerosis (Bryce et al., 2012; Finnerup and Jensen, 2004). The problem with this terminology was that above–level injuries could develop below and at level pain as well. For that reason, a new classification was considered in which above-level pain is changed by the term *other neuropathic pain.* Then, other neuropathic pain is a pain located above-, at- or below-injury level, that occurs as an indirect cause or is unrelated to SCI (Bryce et al., 2012; Finnerup and Jensen, 2004).

Injury to the spinal cord produces a cascade of reactions including changes in receptors, ion channels, disruption of blood spinal cord barrier (BSCB), glial activation along with strong immune response. All these changes results in a hyperexcitablity of the dorsal horn neurons and the development of central neuropathic pain (Finnerup and Jensen, 2004; Hains and Waxman, 2006; Hains et al., 2003; Walters, 2014). After SCI, at-level pain is the first to develop. But the disruption of the blood spinal cord barrier (BSCB) allows infiltration of peripheral immune cells not only at the injury site but also far from injury leading to glial activation and the development of below-injury pain (Detloff et al., 2008; Hains and Waxman, 2006; Siddall et al., 2003; Walters, 2014).

Like in peripheral nerve injury, activation of glial cells and infiltrating macrophages plays a pivotal role in the development of central neuropathic pain. The release of pro-inflammatory cytokines and chemokines acts as potent neuromodulators that sensitize nociceptors promoting pain (Detloff et al., 2008; Kawasaki et al., 2008b; Reeve et al., 2000).

Although both, astrocytes and microglia, are involved in neuropathic pain, each population seems to play different roles. While is strongly extended that microglia is important for the initial stages of neuropathic pain (Coull et al., 2005; Hains and Waxman, 2006). Astrocytes are more involved in the maintenance pain. It is assumed that microglia presents a fast activation after injuries, and that astroglia progressively replaces microglia. The release of inflammatory mediators acts as a amplifying feedback loop between activated glia and persistent inflammation with the final outcome of indefinitely SCI pain (Byrnes et al., 2011; Detloff et al., 2008; Nesic et al., 2005).

AIMS

The general objective of the present thesis is to invesitgate whether interleukin 37 (IL-37attenuates the inflammatory response after spinal cord injury and promotes functional recovery,

This thesis is divide in three chapters according to the following particular objectives:

Chapter 1: Beneficial effects of IL-37 after spinal cord injury.

- To asses the levels if IL-37 in transgenic mice in the intact and injured spinal cord
- To asses whether IL-37 supresses inflammation after spinal cord injury.
- To evaluate the involment of IL-37 in minimizing tissue damage and functional deficits after spinal cord injury.
- To assess whether IL-37 leads to axon regeneration following lesion to the spinal cord.
- To asses the effects of the injection recombinant injection of IL-37 in functional recovery after spinal cord injury.

Chapter 2: Modulatory effects of IL-37 in gene expression after spinal cord injury in mice.

- Analysis of the transcriptome after spinal cord injury
- To study wheter IL-37 the mechanisms underlying to the effects of IL-37 through the comparative analysis of the transcriptome.

Chapter 3: Effect of IL-37 in the modulation of neuropathic pain after peripheral nerve injury.

- To study the expression of IL-37 after peripheral nerve injury in different anatomical regions of the peripheral nervous system.
- To asses whether IL-37 suppresses de inflammatory response after peripheral nerve injury.
- To study the effect of IL-37 in the development of neuropathic pain after peripheral nerve injury and spinal cord injury.

METHODOLOGY

Animals

Transgenic mice expressing human IL-37 (hIL37tg) or wild type (C57BL6) mice were used in the different experiments. Genotyping PCR was used to detect the presence of IL-37 allele. Using ArchivePure DNA purification System (5PRIME), genomic DNA was extracted from a piece of the tail. The protocols consist on digest tail tissue with 0.1 mg/ml proteinase K solution diluted in lysis buffer at 56ºC overnight. Then, proteins were removed using a protein precipitation buffer and centrifugation 3 min at 15000g. Supernatants were collected and mixed with 2-propanol (Panreac) to allow DNA precipitation. Samples were centrifuged and pellets washed with ethanol 70% and allowed to dry completely and finally DNA was solubilized by adding 50 μl DNA hydration solution at 65ºC for 1 hour. DNA was stored at 4ºC until genotyping.

For genotyping, allele amplification was performed by PCR reaction using the Taq DNA Polymerase kit (invitrogen), and PCR products were analysed by electrophoresis in 2% agarose gels. Primer sequences, PCR conditions and product size are resumed in the following table:

Forward primer	5 '- CTTAGAAGACCCGGCTGGAAG - 3'	
Reverse primer	5' - TGTGATCCTGGTCATGAATGCT - 3'	
PCR conditions		94ºC 3 min
	35 cycles	94ºC 3 min
		60.7ºC 45 seg
		72ºC 2 min
		72ºC 7 min
Product size	474bp	

Table 1. Sequence primers, PCR conditions and product size for IL-37 genotyping.

Surgical procedures

All animal procedures were approved by the ethical committee of the Universitat Autònoma de Barcelona in accordance with the European Comission on Animal Care.

Mice were housed in standard cages and feed ad libitum with a light-dark cycle of 12h. For the different surgical procedures, animals were anesthetized with a mixture of ketamine:xilacine (90:10mg/kg, intramuscular), and the back (for spinal cord injury) or the right hindpaw (for peripheral nerve injury) of mice were shaved and disinfected with povidone iodine solution.

Spinal cord injury

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. Two different types of lesions were made:

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 200 kDynes and the 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 50 ± 5 kdynes and 400-600μm in displacement, or a force of 60 ± 5 kdynes and 500-

700μm in displacement, producing a moderate or severe injury, respectively. 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.

Complete spinal transection: In this injury model, after exposing the spinal cord at T11 level, a complete section of the spinal cord was made with a fine scissors, and sharp needle was then used to asses the complete disruption of the spinal cord tracts.

Figure1. Horizon impactor device.

Peripheral nerve injury

A longitudinal incision in the skin and muscle was made at the right paw in order to expose the sciatic nerve and their 3 main branches: tibial, peroneal and sural branches. Tibial and peroneal branches were ligated and cut, leaving only the sural branch intact. Muscle and skin were sutured and disinfected with povidone.

After surgery, mice were kept in in a warm environment. Buprenorphine (0.01mg/Kg) was administered when mice were awake, and then, once a day for the following 48 hours. Mice undergoing SCI, bladders were manually emptied until reflex voiding was re-established.

Intraspinal injection of IL-37

Spinal cord contusing the spinal cord with a force of 60 Kdynes, two different batches of human recombinant IL-37 (full length or cleaved form IL-$37_{v46-218}$) was intraspinally injected. Intraspinal injections were performed using a glass needle (tip 30 μm diameter) coupled to a 10 μL Hamilton syringe (Hamilton #701, Hamilton Co, Reno, NV, USA). 1μl of saline or human recombinant IL-37 (100ng/μl) protein was injected in the dorsal funiculi. Injections were made at a perfusion speed of 2μl/min and the tip of the needle was maintained in the injured spinal cord for three minutes to avoid liquid reflux.

Behavioural assessment

Locomotor recovery

The Basso Mouse Scale (BMS) was used to analyse recovery of locomotor function after spinal cord injury. This scoring method uses a 9-point score with an 11-point sub-score to evaluate finer aspects of locomotor control (table 1 and table 2). Animals are placed in an open field for 5 min under the supervision of 2 blinded trained individuals and then consensus score is taken. BMS was done prior to surgery (day 0) and post-injury days 1, 3, 5, 7, 10, 14, 21 and 28.

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).

2.a.

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. Scores (a) and subscores (b) used in the Basso mouse scale to assess locomotor recovery.

DigiGaitTM Imaging System (Mouse Specifics, Inc., Boston, MA) 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/. 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 5 seconds. This test was performed before the injury and at day 28 post-injury (end of the follow-up).

Assessment of neuropathic pain.

One week before injury, animals were habituated to experimental devices used for assessing nociceptive tests, and baseline measurements for mechanical and thermal stimuli were taken in both hindpaws. After SCI and SNL, these test were done at:

- For spinal cord injury at 7,14,21,28 dpi.
- For sciatic nerve ligation at 4, 7, 10, 14, 21 dpi.

Mechanical sensitivity was measured with an electronic Von Frey algesimeter (Bioseb, Chaville, France). Mice were placed on a wire net platform in plastic chamber 45 min before the experiment for habituation. Mechanical nociceptive threshold was taken as mean of three measurements of each paw, with 5 min interval between measurements, and expressed as the force (in grams) at which mice withdrew their paws in response to the stimulus

Thermic stimuli were determined using a plantar algesimeter (Ugo Basile, Comerio, Italy). Mice were placed into a plastic box with an elevated glass floor 45 min before experiment for habituation. The beam of a projection lamp was focused onto the hindpaw plantar surface. Thermal nociceptive treshold was taken as a mean of three trials per paw with 5 min resting between each trial, and expressed as a latency (in seconds) of paw response.

Histological analysis

Mice were anesthetized with Dolethal (sodium pentobarbital; Vétoquinol E. V. S. A. 0.01ml/10g, intraperitoneal) and were perfused with 4% paraformaldehyde (Sigma) in 0.1 M phosphate-buffered (PB).

Contused spinal cords segment containing the lesion epicenter were removed and post-fixed in 4% paraformaldehyde for 1 hour, and then cryoprotected in 30% sucrose solution for at least 48 hours. Spinal cord were frozen at -60ºC in cryoembedding compound (Tissue-Tek, OCT, Sakura) and cryostat (Leica) cross-sections of 15 μm thickness were serially cut and picked up on gelatin-coated glass slides until reach a distance of 1200 μm from the epicenter to rostral and caudal parts. To study axonal regeneration, spinal cords with complete transection were cut on the sagittal plane. Lumbar segments (L4-L5) of mice undergoing SNL were processed similarly and cross-cut at 15 μm. In addition, in these mice, sciatic nerves and the L4-L5 dorsal root ganglia (DRG) were also collected cut in the sagittal plane, as described above. Tissue sections were kept at -20ºC until their use.

Luxol Fast blue (LFB)

Tissue sections were placed in a hot plate during 15 min and then were gradually dehydrated in 50%, 70%, 80% and 95% ethanol and placed in a 1mg/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).

Immunohistochemistry

Frozen spinal cord, nerve and DRG tissue sections were placed in a hotplate at 37ºC for 15 minutes and washed with Phosphate buffer Saline (PBS), then permeabilized with 0.3% triton-PBS and blocked with blocking buffer (BB: 5% Fetal Bovine Serum un 0.3%triton-PBS) for 1 hour and incubated with correspondent primary antibodies (Table 3) overnight at 4ºC. 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 room temperature and after washes with PBST, PBS and PB samples were mounted with Mowiol mounting media with DAPI (1μg/ml)

Antigen	Dilution	Manufacturer
Glial fibrilary acidic protein	1:500	Wako
Ionized calcium binding adaptator molecule (iba-1)	1:1000	Wako
Neuronal Nuclei (NeuN)	1:100	Millipore

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

BDA retrollabelling

To assess axon regeneration, Dextran Biotin Amine (BDA 10000 MW, Invitrogen) diluted in sterile PBS was injected into the corticospinal motor cortex 66 days after spinal cord complete transection. 14 days after the injection, animals were euthanized and the spinal cord collected and sagittal sections of 15μm thickness were cut as described above and then incubated with a streptavidine- alexa594 (1:500) for 1 hour at room temperature to visualize the corticospinal spinal tract.

Microarray

Uninjured WT (C57Bl/6) and 7 days contused (WT and hIL-37tg) mice were euthanized with Dolethal 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 were homogenized with Qiazol lysis reagent (Qiagen) and mRNA was 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.

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 1.1 ST chip array was used according to the manufacturer protocol.

For microarray validation, Real Time-PCR (RT-PCR) was performed.

Real time PCR

Mice undergoing SCI or SNL were perfused with sterile saline spinal cord, sciatic nerve and DRG were harvested at different time points. 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

Immune cells from the injured spinal cord were analyzed by flow cytometry at 1 and 7 dpi. 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 900g for 10 minutes at 4ºC. 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 were also purchased from eBioscience: CD45-PerCP, CD11b-PE-Cy7, Gr1-FITC, F4/80-APC or PE, CD3-FITC, CD4-APC, CD8-APC, CD19-PE. After 30 min of incubation with combinations of antibodies at 4°C, the samples were washed and fixed in 1% paraformaldehyde. The following combination of marker was used to identify activated microglia ($CD45^{low}$, $CD11b^{+}$, $F4/80^{+}$), granulocytes as ($CD45^{high}$, $CD11b^{+}$, $F4/80^{-}$, $Gr\text{-}1^{high}$), macrophages ($CD45^{high}$, $CD11b^{+}$, $F4/80^{+}$), CD4 T-Cells ($CD45^{+}$, $CD11b^{-}$, $CD3^{+}$, $CD4^{+}$) and CD8 T-Cells ($CD45^{+}$, CD11b, $CD3^{+}$, $CD8^{+}$). In addition microglia and macrophages were further differentiated based on CD16/32 and CD206 and to assess M1 and M2 polarization, respectively. Cells were analyzed using FlowJo® software on a FACSCanto flow cytometer (BD Biosciences).

Assesment of cytokine protein levels

Mice were perfused with sterile saline and a spinal cord epicenter segment was collected at different at two time points after injury (12 and 24h from hIL-37tg and WT mice) and frozen at -80ºC. Spinal cords were homogenized and protein concentration was determined using the DC Protein Assay (Bio-Rad). Samples were concentrated to 4 µg/µl using MicroCon centrifugation filters (Millipore) to ensure equal amounts of protein. 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.

RESULTS

CHAPTER 1.

Beneficial effects of IL-37 After Spinal Cord Injury in Mice

Beneficial effects of IL-37 After Spinal Cord Injury in Mice

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ABSTRACT

Interleukin 37 (IL-37), a member of the IL-1 family, broadly reduces innate inflammation as well as acquired immunity. However, it remains unknown whether the anti-inflammatory properties of IL-37 include the central nervous system. In the present study, we subjected mice to spinal cord compression injury. We compared mice that are transgenic for human IL-37 (hIL-37tg) to wild-type (WT) mice; we also treated WT mice with recombinant IL-37. In hIL-37tg mice, the expression of IL-37 was barely detectable in the uninjured cords, but strongly induced 24 and 72 hours after the injury. Compared to WT mice, hIL-37tg mice exhibited increased myelin and neuronal sparing and protection against locomotor deficits including a 2.5 fold greater speed under forced treadmill challenge. Reduced levels of cytokines, such as an 80% reduction in IL-6, were observed in the injured cords of hIL-37tg mice as well as lower numbers of blood-borne

neutrophils and macrophages but also reduced activated microglia. Therapeutically, we treated WT mice with single intraspinal injection of either full-length or processed recombinant IL-37 after the injury. We observed significantly enhanced locomotor recovery compared to vehicle-treated mice, as well as when IL-37-treated mice were subjected to forced treadmill locomotion. Both forms of recombinant IL-37 led to similar beneficial effects on locomotor recovery after the injury without additional IL-37. Overall, this study novel data that IL-37 functions to suppress inflammation in a clinically relevant model of spinal cord injury and conclude that recombinant IL-37 may have a therapeutic role in the treatment of spinal cord injuries.

INTRODUCTION

The inflammatory response plays an essential role in protecting the body after injury or invasion by microorganisms (David et al., 2012a; Serhan, 2014). Regardless of the tissue in which it occurs, inflammation must be highly regulated, otherwise there is the development of a chronic disease with loss of function (David et al., 2012a; Serhan, 2014). This is particularly the case with spinal cord injury. After spinal cord contusion or compression injury there is a rapid initiation of inflammation in rodents and in humans (David et al., 2012a). This response is orchestrated by endogenous microglial cells and by circulating leukocytes, especially monocytes and neutrophils, which invade the lesion site (David et al., 2012a, 2012b; Popovich, 2014). Although these cells are required for the clearance of cellular and myelin debris, they also release cytokines and cytotoxic factors, which are harmful to neurons, glia, axons and myelin, and which result in

secondary tissue damage to adjacent regions of the spinal cord that had been previously unaffected by the insult (Alexander and Popovich, 2009; David et al., 2012a; López-Vales et al., 2011). Indeed, it is currently well accepted that inflammation is a major contributor to secondary cell death after spinal cord injury (SCI). Moreover, the damaging effects of inflammation are more pronounced in the central nervous system (CNS) than in other tissues because of the limited capacity for axon regeneration and replenishment of damaged neurons and glial cells, which leads to irreversible functional disabilities (Rowland et al., 2008; Silver et al., 2014). Therefore, targeting inflammation is a valuable approach to promote neuroprotection and functional recovery in SCI.

Cytokines are key players in regulating the initiation, progression and suppression of inflammation. Although several members of the interleukin-1 (IL-1) family are known for their pro-inflammatory properties (Dinarello et al., 2012; Garlanda et al., 2013), IL-37 has broad suppressive effects on innate inflammation and acquired immunity (Bufler et al., 2004; Dinarello and Bufler, 2013; Luo et al., 2014; Nold et al., 2010). Since a complete open reading frame for the mouse homolog of IL-37 has not been found, it was necessary to generate a strain of transgenic mice overexpressing human IL-37 (hIL-37tg mice). These mice exhibit resistance against several diseases with a marked inflammatory component, such as endotoxin shock, colitis, hepatitis, myocardial infarction (Ballak et al., 2014; Bulau et al., 2011; Garlanda et al., 2013; McNamee et al., 2011; Nold et al., 2010; Teng et al., 2014). However, there is no information on the role of IL-37 after CNS trauma. In the current study, we subjected hIL-37tg to SCI and studied subsequent functional impairments in comparison to wild type (WT) mice. We also administered recombinant IL-37 to WT mice in order to provide a rationale for

clinical use of IL-37 as a therapeutic. We provide direct evidences for the first time that IL-37 exerts marked anti-inflammatory properties for the contused spinal cord, and confers protection from tissue damage and functional loss.

MATERIALS AND METHODS

Surgical procedure

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. Adult (8-10 weeks old) female C57BL/6 mice (Charles River) and hIL-37tg mice (13) 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 60 kdynes using the Infinite Horizon Impactor device (Precision Scientific Instrumentation) (Klopstein et al., 2012) or was completely transected using a microscalpel. At 56 days after complete spinal cord transection, corticospinal tract was labelled by injecting a 10% solution of the axonal tracer biotinylated dextran amine (BDA) in the sensory-motor cortex (Fry et al., 2010).

Administration of recombinant human IL-37 protein (b isoform) was performed intraspinally by means of a glass needle (30 µm internal diameter, Eppendorf, Hamburg, Germany) coupled to a 10 ml Hamilton syringe (Hamilton #701, Hamilton Co, Reno, NV, USA). One µl of saline, saline containing 100 ng of full length (rIL-$37_{1\text{-}218}$) or recombinant human IL-$37_{V46\text{-}218}$ was injected into injured spinal cord at the lesion site 5 minutes after lesion. 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.

RNA isolation, reverse transcription and real-time PCR

Mice were perfused with sterile saline and 5mm length of uninjured spinal cord was removed. Tissue was homogenized with QIAzol lysis reagent (Qiagen) and RNA extracted using RNeasy Lipid Tissue kit (Qiagen), according to the manufacturer's protocol. RNA was treated with DNaseI (Qiagen) to eliminate genomic DNA contamination. 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. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene

Cytokine Protein Expression

Mice were perfused with sterile saline and a 5 mm length of spinal cord centered on the lesion was collected at 12 and 24h after surgery from hIL-37tg and WT mice and snap-frozen. Spinal cords were homogenized and protein concentration was determined using the DC Protein Assay (Bio-Rad). Samples were concentrated to 4 µg/µl using MicroCon centrifugation filters (Millipore) to ensure equal amounts of protein. 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.

Flow Cytometry

Immune cells from the injured spinal cord were analyzed by flow cytometry at 1 and 7 dpi as described previously (Stirling et al., 2009). Briefly, spinal cords were cut in small pieces, passed through a cell strainer of 70 µm (BD Falcon) using saline and the cell suspension was centrifuged at 300g for 10 minutes at 4ºC. The pellet was resuspended and centrifuged for a second time. 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 were also purchased from eBioscience: CD45-PerCP, CD11b-PE-Cy7, Gr1-FITC, F4/80-APC or PE, CD3-FITC, CD4-APC, CD8-APC, CD19-PE. After 30 min of incubation with combinations of antibodies at 4°C, the samples were washed and fixed in 1% paraformaldehyde. The following combination of marker was used to identify activated microglia ($CD45^{low}$, $CD11b^{+}$, $F4/80^{+}$), granulocytes as ($CD45^{high}$, $CD11b^{+}$, $F4/80^{-}$, $Gr\text{-}1^{high}$), macrophages ($CD45^{high}$, $CD11b^{+}$, $F4/80^{+}$), CD4 T-Cells ($CD45^{+}$, $CD11b^{-}$, $CD3^{+}$, $CD4^{+}$) and CD8 T-Cells ($CD45^{+}$, CD11b, $CD3^{+}$, $CD8^{+}$) (Klopstein et al., 2012). In addition, microglia and macrophages were further differentiated based on CD16/32 and CD206 and to assess M1 and M2 polarization, respectively. 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 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 and blinded assessors and a 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 was to perform for at least 5 seconds was recorded.

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). For neuronal

assessment, sections were incubated overnight at 4ºC with biotinylated antibodies against NeuN. After several washes in PBS, sections were stained using the ABC kit (Vector labs) then a coverslip was applied in DPX mounting media (Sigma).

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.

10 days after BDA tracer injections (66 days post-injury) mice were perfused, and the transected spinal cord harvested and cut on 20 μm thick sagittal sections as described above. Visualization of corticospinal fibers of done by incubating the spinal cord sections with Alexa 594-conjugated streptavidin. The NIH ImageJ software was used to quantify all the histological parameters.

Statistical analysis

All analyses were conducted through IBM SPSS Statistics v19. Two-tailed Student's *t* test was used for the single comparison between two groups. 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 as well as BDA+ fibers were analyzed using two-way repeated measure ANOVA. *Post-hoc* comparisons were carried out only when a main effect showed statistical significance. *P* values for multiple comparisons were adjusted using Bonferroni's correction. Results are expressed as mean and standard error. Differences were considered significant at $p < 0.05$.

RESULTS

hIL-37tg mice exhibit reduced functional deficits and tissue damage after SCI

Since inflammation contributes to pathophysiology of SCI, we evaluated whether functional deficits and tissue loss are ameliorated in hIL-37tg mice after contusion injury. As shown in Figure 1A, hIL-37tg mice displayed significant improvement in locomotor recovery after SCI. There were significant differences in BMS scores starting at day 7 after injury and remaining significantly different until the end of the follow-up period (Fig. 1A). At the 28th day post-injury (dpi), all WT mice showed extensive ankle movement, but only 50% of these mice showed plantar paw placement without weight support, whereas none showed stepping (score 2.5). In contrast, all hIL-37tg mice showed extensive ankle movement, plantar paw placement with weight support, and the majority displayed occasional stepping (score 3.8) (Fig. 1A). Moreover, hIL-37tg mice ran at significantly faster velocities on the treadmill (Fig. 1B), further demonstrating the protective effect of IL-37 against functional loss in SCI.

We then assessed whether the improvement in motor skills of hIL-37tg mice was associated with reduced secondary tissue damage after SCI. Histological sections from the injury epicenter stained with luxol fast blue revealed enhanced myelin sparing at the injury site but also in caudal regions of hIL-37tg compared to WT mice (Fig 1C, D). Assessment of neuronal sparing in the ventral horns also demonstrated attenuated neuronal loss at rostral and caudal regions of the injury epicenter in hIL-37tg mice (Fig. 1 E, F).

Since infiltrating macrophages inhibit axonal outgrowth by releasing soluble factors and by cell-cell interaction (Evans et al., 2014; Horn et al., 2008), we sought to evaluate whether there was enhanced axonal regeneration in hIL-37tg mice

after complete spinal cord transection. hIL-37tg and WT mice presented complete hindlimb paralysis after the injury and lacked functional improvement at 10 weeks post-lesion (BMS score 0).

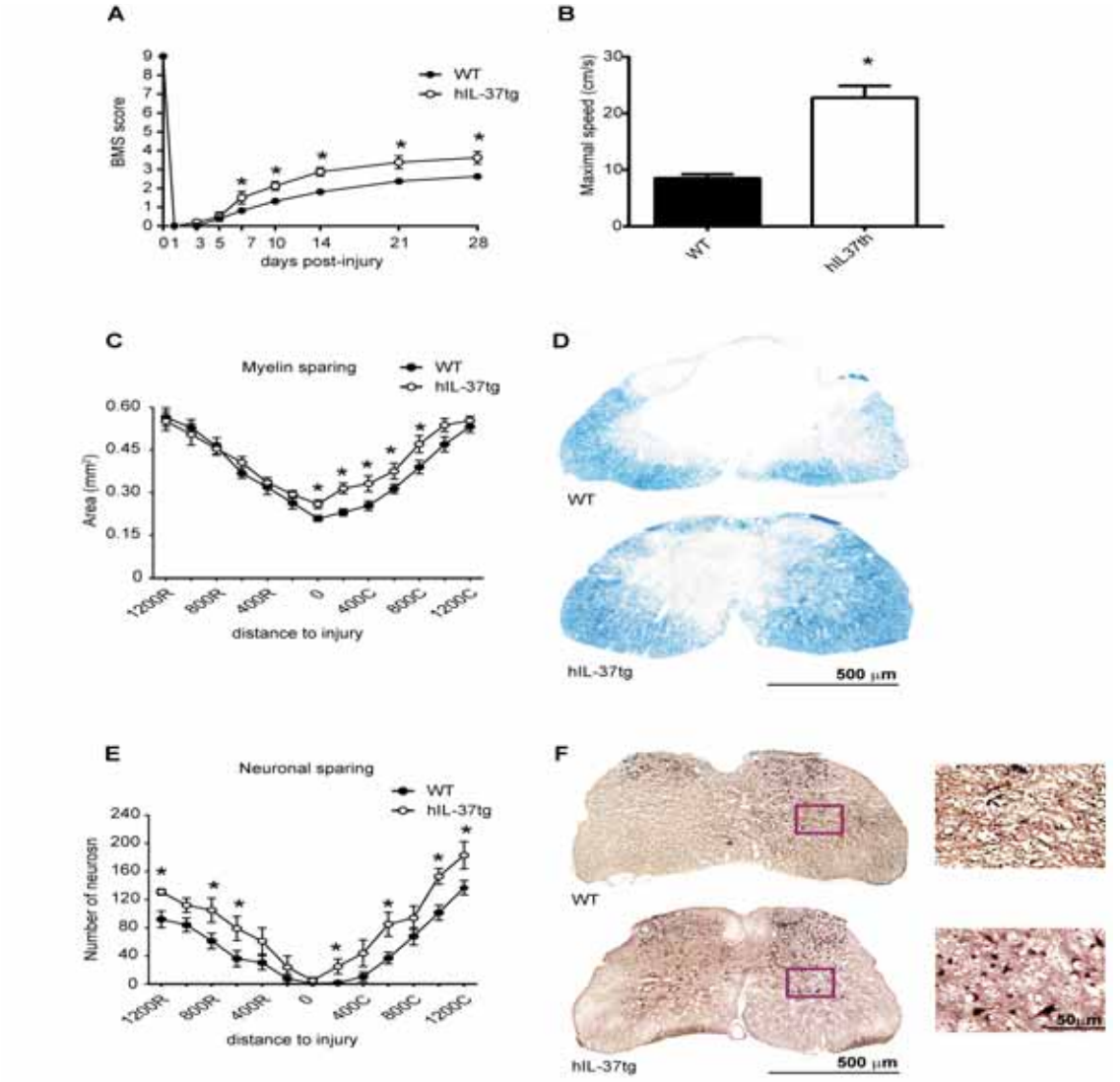

Figure 1. hIL-37-tg mice show enhanced functional outcomes and reduced tissue damage after SCI. (A) Assessment of locomotor skills assessed in the 9-point Basso Mouse Scale (BMS) as well as (B) on a treadmill. (C) Quantification of myelin sparing at various distances rostral and caudal to the injury epicenter reveals significant reduction in tissue loss in hIL-37tg mice at the epicenter of the injury and in caudal regions. (D) Representative micrographs showing myelin sparing at the injury epicenter in section stained against luxol fast blue from WT and hIL-37tg mice. (E) Quantification of ventral horn neuron survival at various distances rostral and caudal to the injury epicenter reveals significantly greater neuronal survival in hIL-37tg mice. (F) Representative micrographs showing sparing of ventral horn neurons in WT and hIL-37tg mice in sections stained against NeuN at 200 µm rostral to the injury epicenter. The area outlined in the box is shown in higher magnification in panels (insert). Data are expressed as mean ±SEM. (*$p<0.05$; two-ways RM-ANOVA, Bonferroni's post hoc test in A, C and D; t-test in B; n=8 per group).

Histological assessment of sagittal spinal cord tissue sections revealed that in hIL-37tg mice, expression did not promote regeneration nor sprouting of corticospinal axons (Fig. 2). Overall, these data provide clear evidence that IL-37 confers protection from functional disabilities and secondary tissue damage after spinal cord contusion injury, but does not promote axonal outgrowth.

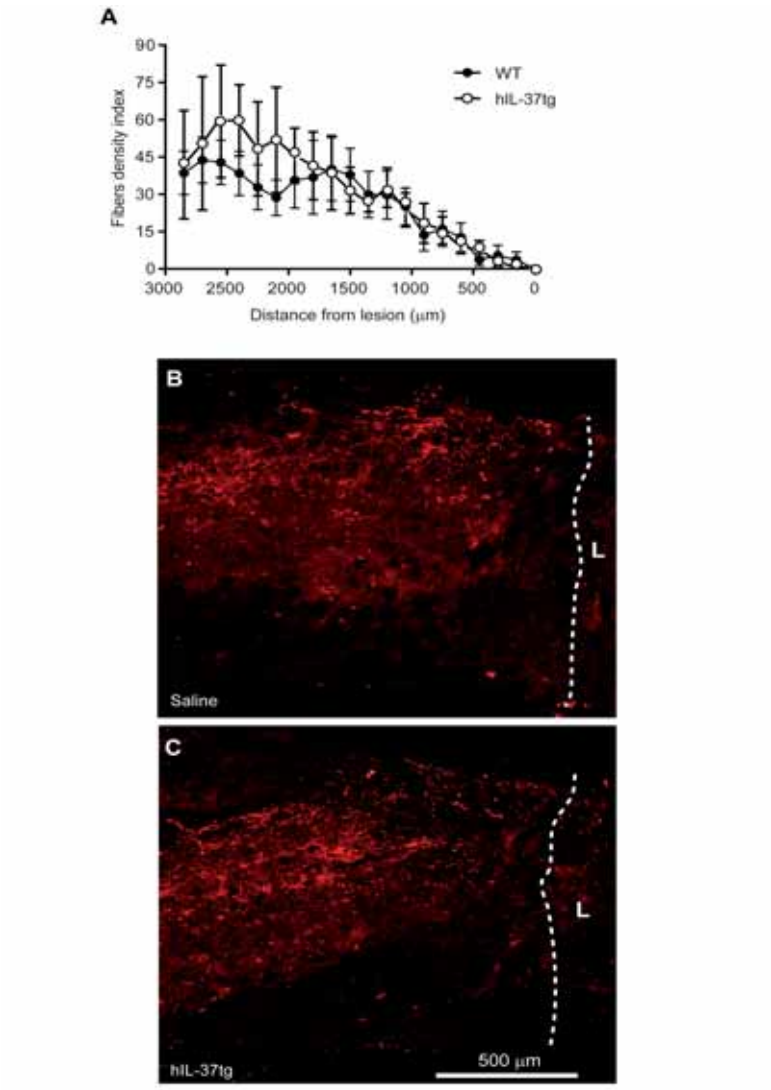

Figure 2. hIL-37tg expression does not promote axonal regeneration. (A) Quantification of BDA-labelled corticospinal axons at different distances to the transection site. (B-C) Low magnification images of complete transected spinal cord showing BDA-labelled corticospinal fibers in WT (B) and hIL-37tg mice (C). Lines define the transection site. L=lesion. Data are expressed as mean ±SEM. (*$p<0.05$; one-way ANOVA, Bonferroni's post hoc test; n=8 per group)

IL-37 is induced in hIL-37tg mice after SCI

Since hIL-37tg mice were markedly protected against functional disabilities and tissue loss after SCI, we studied the inflammatory response in hIL-37tg mice. We first assessed the expression profile of IL-37 in the spinal cord of hIL-37tg mice determined by real time PCR. IL-37 expression was not detected in the intact or damaged spinal cord obtained from WT C57Bl/6J mice. As expected, hIL-37tg mice exhibited a markedly low level of constitutive expression of IL-37 in the spinal cord. Low levels of constitutive IL-37 expression have also been observed in other studies showing minimal transcripts of IL-37 in the colon, skin, circulating leukocytes or in cell lines transfected with IL-37 (Luo et al., 2014; McNamee et al., 2011; Nold et al., 2010). The low level of constitutive expression is due to the instability sequence in human IL-37 (Bufler et al., 2004). However, after the contusion injury, induction of IL-37 in the spinal cord parenchyma was observed (Fig. 3A). IL--37 expression profile occurred at 2 peaks of expression; after 12 hours and at day 3 post-injury, the levels of IL-37 increased ~17 and ~35 fold, respectively (Fig. 3A). The early peak of IL-37 coincides with maximal induction of cytokines in the injured spinal cord (6-24 hours post-injury), whereas the latter correlates with the infiltration of blood monocytes (day 3) (David et al., 2012a).

Inhibition of cytokine and chemokine expression after SCI

We next sought to examine whether the early increase in IL-37 modulated gene expression of cytokines in the contused spinal cord. Injured spinal cords harvested at 12 and 24 hours post-contusion, which are time periods when the protein levels of most cytokines and chemokines reach maximal concentrations

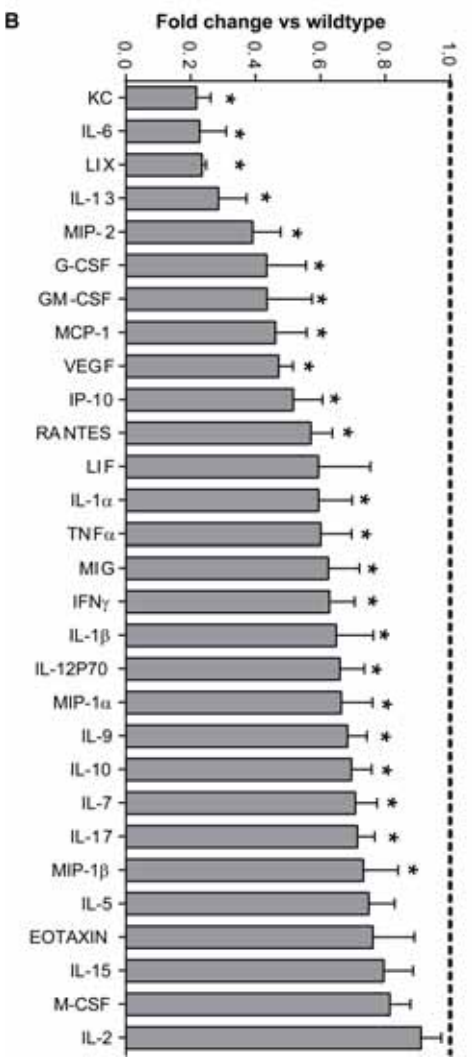

Figure 3. Expression of IL-37 after SCI. (A) Time course of IL-37 transcripts in the spinal cord of hIL-37tg mice after contusion injury (*p<0.05; one-way ANOVA, Bonferroni's post hoc test; n=4 per time point). (B) Multiplex analysis of cytokine protein profiles from spinal cord of WT and hIL-37tg mice at 24 post-injury (*p<0.05; t-test; n=4 per group). Data are expressed as fold change vs WT mice (mean ±SEM).

after SCI (David et al., 2012a). We evaluated the protein levels of 32 cytokines in the injured spinal cord. At 12 hours post-injury, cytokine levels were unchanged in hIL-37tg relative to WT mice (Fig. 4). However, at 24 hours post-injury, we observed a significant reduction in the expression of 23 out of the 32 cytokines in hIL-37tg mice (Fig. 3B). Moreover, the expression of IL-3, which was detected at low levels in the spinal cord homogenates of WT mice (0.56±0.03 pg/mg protein), was undetectable in hIL-37tg mice (<0.45 pg/mg protein). The levels of 6 cytokines (LIF, M-CSF, IL-2, IL-5, IL-15, and eotaxin) did not change in IL-37tg

mice, whereas the expression of IL-4, IL-12p40 was not detected in the contused spinal cord of both experimental groups (Fig. 3B)

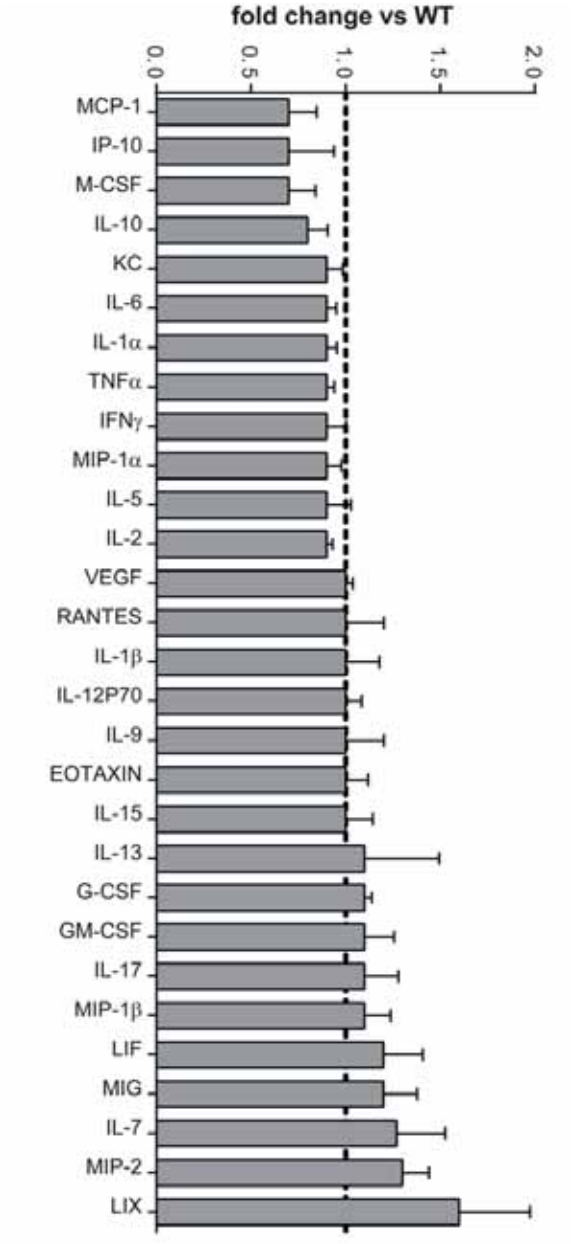

Figure 4. Multiplex analysis of cytokine protein profile from spinal cord of WT and hIL37tg mice at 12 hours post-injury. Data are expressed as fold change vs WT mice (mean ± SEM)

Since cytokine and chemokines play a role in the activation and recruitment of inflammatory cells, we next evaluated whether there was a difference in the accumulation of inflammatory cells between WT mice and IL-37tg mice following SCI. At day 1 post-injury, when granulocyte infiltration reaches peak levels, the

spinal cord of hIL-37tg mice showed ~40% reduction in the number of granulocytes ($CD45^{high}$, $CD11b^{+}$, $F4/80^{-}$, $Gr1^{high}$) (Fig. 5). There were no differences in the cell counts for activated microglia ($CD45^{low}$, $CD11b^{+}$, $F4/80^{+}$), blood borne macrophages ($CD45^{high}$, $CD11b^{+}$, $F4/80^{+}$), CD4 ($CD45^{+}$, $CD11b^{-}$, $CD3^{+}$, $CD4^{+}$) and CD8 T cell ($CD45^{+}$, $CD11b^{-}$, $CD3^{+}$, $CD8^{+}$) at this time point (Fig. 4).

Seven days post-injury, when the accumulation of activated microglia and macrophages reaches peak levels in the injured spinal cord and accounts for ~80% of total immune cells, spinal cords from hIL-37tg mice exhibited significantly lower numbers of these two cell subsets (Fig. 6). The number of granulocytes, which were reduced by 90% compared to day 1, were slightly increased in hIL-37tg, but did not reach statistical significance, whereas counts for CD4 and CD8 lymphocytes were unchanged (Fig. 3). We also assessed whether IL-37 modulated macrophage and microglia polarization after SCI. However, FACS analysis from contused spinal cords revealed no differences in IL-37tg mice for the expression of CD16/32 and CD206 on microglia (CD16/32: 76.2±4.2 vs 74.6±3.8; CD206: 14,1±3.1 vs 15.2±3.1 in WT and IL37tg, respectively) or macrophages (CD16/32 82.2±6.7 vs 79.4±5.3; CD206 14.5±1.9 vs 16.6±3.2 in WT and IL-37tg, respectively), two known markers of M1 and M2 activation, respectively.

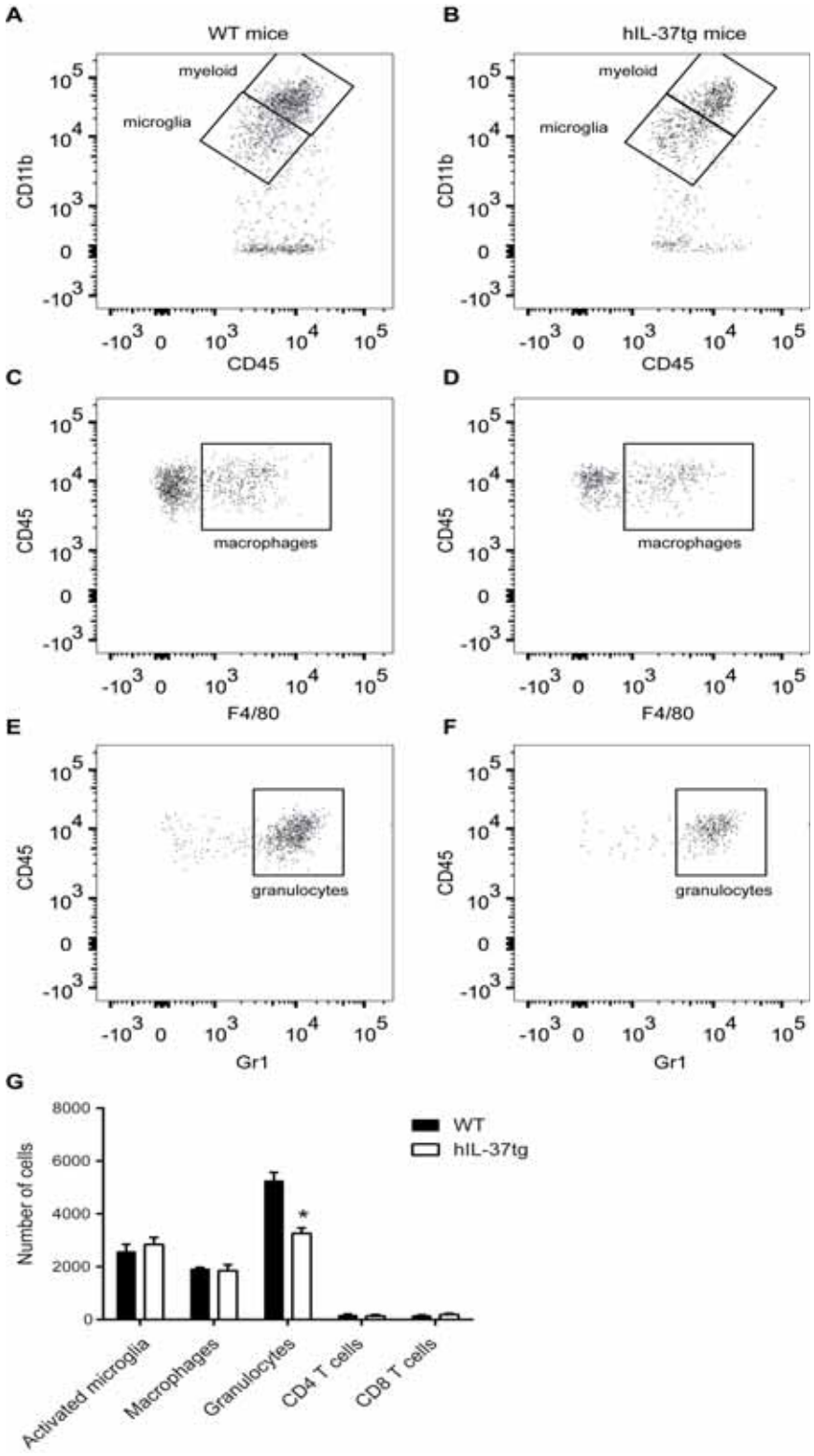

Figure 5. _Effects of hIL-37tg expression on spinal cord immune cell counts at day 1 post-injury. (A-F) Representative density plots of FACS analysis showing myeloid cells and microglia (A, B), macrophages (CD45high, CD11b+, F4/80+) (C, D), and granulocytes (CD45high, CD11b+, Gr1high) (E, F) in the spinal cord of WT and hIL-37tg mice. (G) Graph showing quantification of the different immune cell populations in the injured spinal cord. Data are expressed as mean ±SEM. (*p<0.05; t-test; n=4 per group).

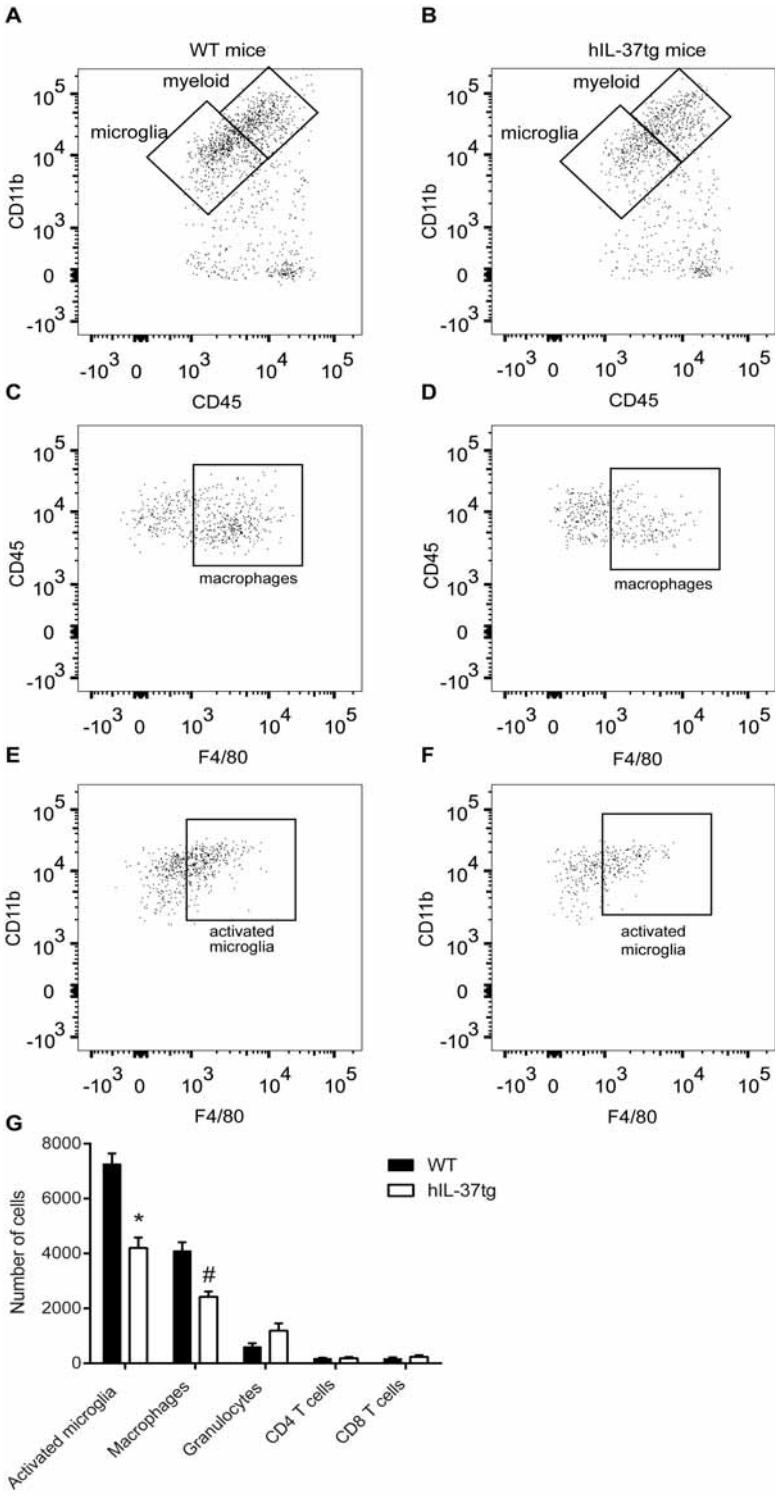

Figure 6. Effects of IL-37 on the recruitment of immune cells within the spinal cord at day 7 post-injury. (A-F) Representative density plots of FACS analysis showing myeloid cells and microglia (A, B), macrophages (CD45high, CD11b+, F4/80+) (C, D), and activated microglia (CD45low, CD11b+, F4/80+) (E, F) in the spinal cord of WT and hIL-37tg mice. (G) Graph showing quantification of the different immune cell populations in the injured spinal cord. Data are expressed as mean ±SEM. (*p<0.05 *p<0.001; # p=0.001; t-test; n=4 per group).

Intralesional administration of recombinant IL-37 improves functional outcomes after SCI

To assess a possible therapeutic use of IL-37 in SCI, we administered two forms of recombinant human IL-37 (rlL-37) to determine the *in vivo* effects in SCI. We first tested the IL-37 precursor (full-length of IL-37 isoform b), as previous studies have shown efficacy *in vivo* (Moretti et al., 2014) and *in vitro* (Li et al., 2015). We also administered a processed form of IL-37 with the N-terminus at valine 46 (Li et al., 2015; Pan et al., 2001). Since the blood-brain barrier prevents the entry of most molecules into the CNS, we infused rIL-37 into the lesion site 5 minutes after contusion injury, using a glass microneedle (30µm diameter). We observed that intraspinal injection of either full length or processed rIL-37 enhanced locomotor recovery in the open field test using the BMS score, as well as under forced locomotion on a treadmill (Fig. 6). Both forms of rIL-37 led to similar beneficial effects on motor skills (Fig. 7).

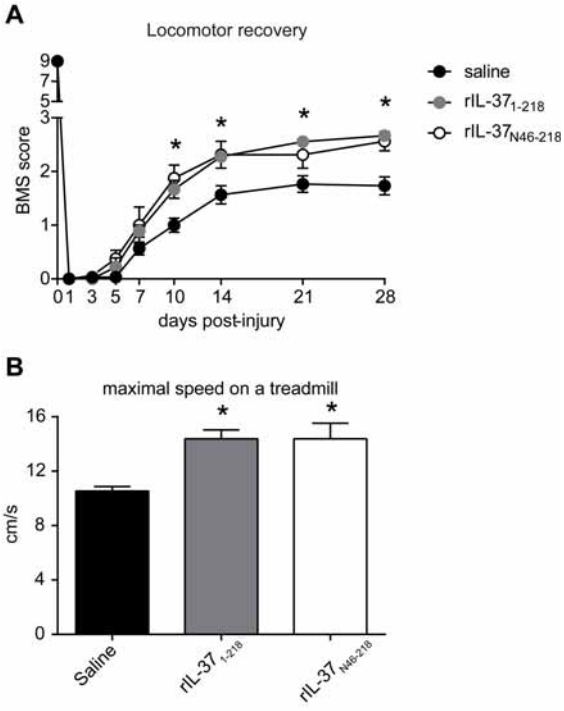

Figure 7. Infusion of recombinant human IL-37 promotes functional recovery after spinal cord injury. Graph showing locomotor performance of C57BL/6 mice treated with intraspinal injection of saline, full length ($IL\text{-}37_{1\text{-}218}$) or processed form of IL-37b ($IL\text{-}37_{V46\text{-}218}$) after spinal cord injury using the BMS score scale (A) and (B) treadmill. Data are shown as mean ±SEM. (*p<0.05; two-way RM-ANOVA, Bonferroni's post hoc in A and t-test in

DISCUSSION

Cytokines are key molecules that impact upon the onset and remission of inflammatory responses. Although IL-37 is lacking in rodents, transgenic mice expressing human IL-37 are protected against several inflammatory challenges, indicating that human IL-37 is fully functional in the mouse. Using the IL-37tg mouse, previous studies have consistently demonstrated the suppressive properties of IL-37 on inflammation. (Luo et al., 2014; McNamee et al., 2011; Nold et al., 2010) However, whether IL-37 exerts a similar anti-inflammatory effect in the CNS trauma remained unknown. Here we demonstrate for the first time that IL-37 suppresses inflammation and limits locomotor deficits and tissue damage in spinal cord contusion injury in the mouse. This conclusion is based on observations in IL-37tg mice as well as in WT mice treated with either recombinant IL-37 precursor or processed IL-37.

In IL-37tg mice, expression is regulated by a constitutive CMV promoter (Nold et al., 2010); however, constitutive expression of IL-37 in the spinal cord is low owing to the instability sequence that limits the half-life of the IL-37 transcript (Bufler et al., 2004). This has been also shown in other models using these mice (Luo et al., 2014; McNamee et al., 2011; Nold et al., 2010). However, we observed that mRNA levels were induced 12 hours after SCI and then a secondary increase occurs 3 days after the injury. It remains unknown, however, whether endogenous IL-37 is expressed in human SCI. Cytokines such as IL-1β, TLR agonist and TGFβ are known to induce IL-37 in vitro (Nold et al., 2010). Cytokine expression reaches maximal levels in the spinal cord within 6-24 hours post-injury (David et al., 2012a), which may account for the first peak of IL-37. Although IL-37 is mainly expressed in macrophages in hIL-37tg mice after several inflammatory challenges (McNamee et al., 2011; Nold et

al., 2010; Quirk and Agrawal, 2014), endogenous glial cells (astrocytes and microglia) are probably the early source of IL-37 due to the low amount of infiltrated leukocytes at this time point. However, the later expression peak of IL-37 (3dpi) coincides with the entrance of blood borne monocytes into the spinal cord, suggesting that macrophages are the main source of the second peak of IL-37 (David et al., 2012a).

IL-37 reduces the expression of several pro-inflammatory cytokines in cell cultures and in different inflammatory disorders (Ballak et al., 2014; McNamee et al., 2011; Nold et al., 2010; Teng et al., 2014; Ye et al., 2014). In line with this observation, we also found that IL-37 attenuated the protein levels of most pro-inflammatory cytokines evaluated in contused spinal cord, IL-6 being one of the cytokines most markedly effected. However, IL-10 protein levels were also reduced in the injured spinal cord by IL-37, although to lesser extend as compared to most pro-inflammatory cytokines. The capacity of IL-37 to reduce cytokine production after SCI directly impacted on the infiltration and activation of immune cells. Indeed, IL-37 reduced the recruitment of neutrophils and macrophages into the injured spinal cord, but also the activation state of the microglia. However, IL-37 did not affect the infiltration of T cells.

Regardless of the species or type of SCI, most studies in which inflammation is pharmacological targeted, or myeloid cells are depleted, consistently report reduced tissue damage consistently and greater functional outcomes (David et al., 2012a; Popovich, 2014). In the current study, we show that hIL-37tg mice exhibited enhanced locomotor function that was associated with attenuated tissue damage after SCI, linking decreased inflammation by IL-37 to improved function. In addition to tissue damage, which limits function, blood borne monocytes infiltrating into the

injured spinal cord mediate axonal retraction (Evans et al., 2014; Horn et al., 2008). This deleterious effect is due to factors released by macrophages as well as by integral macrophage membrane proteins that inhibit the growth and guidance of axons (Evans et al., 2014; Horn et al., 2008). Other inhibitory molecules include chondroitin sulphate proteoglycans, Nogo, Ephrins and semaphorins, which are expressed at the lesion site by astrocytes, oligodendrocytes and some precursor cells (Silver et al., 2014). There is also a limited ability of adult CNS neurons to switch on the intrinsic regeneration machinery after axotomy (Liu et al., 2010). However, despite reduced macrophage numbers in spinal cords of hIL-37tg mice, axonal outgrowth was not observed as these other factors, which hamper the ability of axons to regenerate in the injured CNS, may not be altered in the IL-37tg mouse. Therefore, our data indicate that attenuation of inflammation in the IL-37tg mouse is insufficient to overcome the extrinsic and intrinsic factors that curtail the relatively limited regenerative potential of injured CNS axons.

The anti-inflammatory actions of IL-37 are mediated at both the nuclear and extracellular levels. Once synthesized following activation, up to 30% of the IL-37 precursor will be cleaved by activated caspase-1 and translocate to the nucleus. IL-37 also engages Smad3 (Bulau et al., 2011; Nold et al., 2010). Although IL-37 binds to Smad3, it remains unclear whether Smad3 is a chaperone for IL-37 nuclear translocation (Bulau et al., 2014; Nold et al., 2010). Indeed, the anti-inflammatory effects of IL-37 are abolished in LPS-stimulated macrophages when incubated with caspase-1 inhibitors, or when the caspase-1 site (D20) of IL-37 is mutated (Bulau et al., 2014). Similarly, *in vivo* studies also reveal that the anti-inflammatory actions of IL-37 are lost when Smad3 is silenced after LPS challenge (Nold et al., 2010).

On the other hand, the precursor form of IL-37 is released from human monocytes, or cell lines transfected with IL-37 following LPS stimulation (Nold et al., 2010). The release of IL-37 is, however, independent on caspase-1 cleavage at D20 (Bulau et al., 2014). The administration of neutralizing antibodies against IL-37 enhances cytokine production in *in vitro* and *in vivo*, highlighting the extracellular anti-inflammatory function of IL-37 (Bulau et al., 2014; Li et al., 2015). These observations are further supported by our present studies showing the ability of recombinant IL-37 protein to reduce cytokine production (Li et al., 2015). Recently, it has been shown that extracellular IL-37 carries out its anti-inflammatory actions via hijacking IL-18Rα signaling using the decoy receptor IL-1R8 (Lunding et al., 2015; Nold-Petry et al., 2015; Yang et al., 2015). We observed that the exogenous administration of recombinant IL-37 improves functional recovery after SCI, indicating that extracellular IL-37 is sufficient for its beneficial effects in the CNS. Nevertheless, we cannot rule-out the possibility that the translocation of IL-37 to the nucleus is also playing a role in suppressing inflammation in the transgenic mouse but also in human diseases.

Processing of the IL-37 precursor appears to take place extracellularly. Edman degradation of supernatants from cells lines transfected with IL-37b revealed a processed form starting at valine 46 (IL-37$_{V46-218}$) (Pan et al., 2001). Both forms of recombinant IL-37, full length and processed, exhibit anti-inflammatory effects, although processed IL-37 exerts greater suppression of cytokine production (Li et al., 2015). We observed that both IL-37 forms exert similar beneficial effects after SCI, suggesting that the IL-37 precursor may be processed when administered in the injured spinal cord.

In summary, we report that IL-37 exhibits anti-inflammatory actions in SCI and prevents functional deficits and secondary tissue damage. Our data reveal that treatment with recombinant IL-37 exerts beneficial actions in a clinically relevant model of human SCI, and that IL-37 could be an effective therapy during the acute phase after SCI, for which there is currently no effective treatment.

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CHAPTER 2.

IL-37 modulates gene expression after spinal cord injury

IL-37 modulates gene expression after spinal cord injury

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ABSTRACT

Spinal cord injury (SCI) initiates a cascade of multifactorial events that results in secondary tissue damage and functional impairments. Understanding the mechanisms involved in secondary injury will lead to better knowledge of SCI pathophysiology and to design new therapeutic strategies. In chapter 1, we showed that IL-37 exerted potent suppressive actions on inflammation in the contused spinal cord, resulting in increased tissue preservation and improved locomotor recovery. However, it is still unknown whether the beneficial actions of IL-37 in SCI are limited to its anti-inflammatory effects, or by contrast, it also modulates other degenerative responses or even activates some restorative processes. Here, we performed a microarray analysis of the injured spinal cord harvested from hIL-37tg and WT mice at day 7 post-injury. We obtained a list of genes that were differentially modulated by IL-37 expression. This study provides new insights into the physiological actions of IL-37 in SCI.

INTRODUCTION

Traumatic SCI causes permanent functional deficits at anatomical regions located below the injury site. The pathophysiology of SCI can be divided in two phases of tissue degeneration known as primary and secondary injury. Primary injury is caused immediately after lesion due direct mechanical trauma and leads to hemorrhage, neuronal and glial cell death, as well as to axon disruption. Contrary, secondary injury mechanism are developed for the first hours and days following the initial damage, and include several biochemical and cellular events, such as vascular dysfunction, excitotoxicity, free radical formation, apoptosis, and inflammation (David et al., 2012; Rowland et al., 2008).

Knowledge on the changes in gene expression profile after SCI is important to understand the mechanisms underlying the pathophysiology of SCI, since it can lead to identification of key molecules triggering secondary damage, and thus, to develop new therapeutic strategies. The use of traditional techniques in molecular biology show several limitations to this aim, especially, since they only permit to study simultaneously a few genes. However, genomic approaches, such as microarray analysis, allows to measure the changes and regulation of the entire genome. Under certain biological conditions, gene array produce a large list of altered genes, that together with the biological knowledge accumulated in public databases, can be further analyzed and associated into biological functions.

Interleukin 37 (IL-37), also known as IL-1 family member 7 (IL-1F7), is a member of the IL-1 cytokine family which was discovered in 2000 by computational cloning by different independent groups (Busfield et al., 2000; Dunn et al., 2001; Kumar et al., 2000). Unlike other members of the IL-1 family, IL-37 acts as a suppressor of the inflammatory response under certain inflammatory challenges (Nold et al., 2010).

In chapter 1, we described for the first time that IL-37 mediates strong anti-inflammatory actions after SCI, conferring protections against functional deficits and tissue loss. Here, we performed a gene array analysis of contused spinal cords of hIL37tg and WT mice in order to get deeper knowledge on the physiological actions of this cytokine in SCI. The results obtained suggest that the actions of IL-37 are not limited to modulation of the inflammatory response after SCI, but also to a widespread spectrum of processes.

MATERIAL AND METHODS

Spinal cord injury

All animal procedures were approved by the ethical committee of the Universitat Autònoma de Barcelona in accordance with the European Commission on Animal Care.

8 weeks old female hIL37tg mice and wild type (WT; C57Bl/6) mice were use in this experiment. Animals were anesthetized with a combination of Ketamine and Xylacine (90 mg/kg and 10 mg/kg respectively, intramuscular). Laminectomy at thoracic level 11 was performed and the spinal cord was exposed and contused using the Infinite Horizon Impactor (Precision Scientific Instrumentation, Fairfax Station, VA). The impact force used was 50 kdynes, obtaining tissue displacement ranged between 400-600μm. After injury, animals were placed in a warm environment until they were fully awake. Subcutaneous buprenorphine was administered once a day during the following 48 hours. Bladders were manually emptied until reflex voiding was re-established.

mRNA extraction

Injured animals, both tgIL37 and WT (n=4 per group), and a group of uninjured mice (n=4) were euthanized with Dolethal (pentobarbital sodium, Vetoquinol; 0.01ml/10g, intraperitoneal) 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.

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 were processed for Affimetrix MOUSE Exon/Gene 1.1 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 probesets 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, 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 WT (C57Bl/6) injured and non-injured mice and a cut-off P-value <0.01 and a $\log_2$ fold change (FC) =1 (2 fold change) were applied to select the differentially expressed genes. To determine the gene expression changes after injury in hIL37tg mice relative to WT mice was applied a cut-off P-value <0.01 and a $\log_2$FC=0.64 (1.5 fold change).

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)

Microarray validation.

Real time-PCR (RT-PCR) targeting the SCI differential expressed genes (Table 1) was performed to validate the results obtained by microarray analysis. 1μg RNA of each sample was primed with Random Hexamers (Promega) and reverse transcribed using Omniscript RT kit (Qiagen). RNase inhibitor 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). Data analysis was performed using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad laboratories). Expression of target genes was normalized using the GAPDH values as a housekeeping gene.

GABA-ARα2	Forward: … AAGCCACTGGAGGAAAACATCT… (22 bp) Reverse: … CACACCAGAAGAACAAGCAGC… (21 bp)
Hba	Forward: … AATATGGAGCTGAAGCCCTGG… (21 bp) Reverse: …AACATCAAAGTGAGGGAAGTAGGTCT… (26 bp)
Hbb	Forward: … GTGAGCTCCACTGTGACAAGC… (21 bp) Reverse: … GGTGGCCCAGCACAATCACGATC… (23 bp)
Alas2	Forward: …TTAGTATTGGACGCTGCCCC… (20 bp) Reverse: … GGACAATGGCTCTTAGCCCA… (20 bp)
GAPDH	Forward: …TCAACAGCAACTCCCACTCTTCCA… (24 bp) Reverse: … ACCCTGTTGCTGTAGCCGTATTCA… (24 bp)

Table 1. List of genes and respective primers used for the RT-qPCR validation

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.

RESULTS

Gene profile after SCI

Microarray analysis showed that spinal cord contusion resulted in significant alteration in the expression of 852 genes at day 7 post-injury. This included 765 and 87 genes that were significantly up-regulated or down-regulated, respectively, in injured relative to uninjured spinal cord from WT mice (Fig.1).

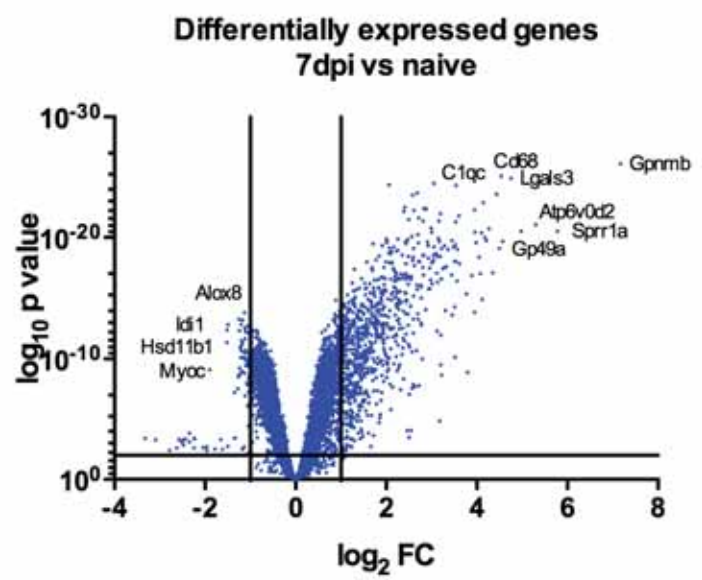


Figure1. Volcano plot showing the differences in gene expression in the spinal cord of C57/bl6 mice at day 7 after contusion injury.

Gene ontology (GO) was then used to classify genes into a biological functions. This revealed 42 biological processes were significantly up-regulated (Table 2) after SCI, while only 2 biological processes were down-regulated (Table 3). Most down-regulated genes were mainly related to lipid metabolism, which included 3-

hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, low density lipoprotein (LDL) receptor, or mevalonate descarboxilase, all them involved in biosynthesis of different steroids. Most of the up-regulated genes were related to immune response. Genes such as Toll like receptor (TLR), Myd88 and Interleukin 1 family receptor (ILR) were highly up-regulated after SCI, along with caspase 1, 4 or 12 that are involved in cytokine production and maturation. Processes involved in cell adhesion or cell-cell communication were also up-regulated. Besides, we found up-regulation in several genes that play a key role in triggering apoptosis, such caspase 8, Fas-associated protein with death domain (FADD) and TNF receptors. Other up-regulated genes were related to oxidative stress, such as heme oxigenase, and glutathione peroxidase. There was also up-regulation of genes related to response to hypoxia, regulation of body fluid levels, angiogenesis and platelet activation, or bone mineralization, although to lesser extent as compared to the involved in inflammation.

UP- GENES FUNCTIONAL CLUSTERING IN INJURED MOUSE

CLUSTER	nº GO	ES	CLUSTER	nº GO	ES
1. **Cell adhesion**	2	17.95	**22. Response to fungus**	3	2.56
2. **Behavior/taxis**	4	11.67	**23. Response to organic substances**	6	2.48
3. **Vasculature development**	4	9.89	**24. Regulation of cell motion**	8	2.46
4. **Immune effector process**	16	7.04	**25. Response to virus**	13	2.45
5. Response to bacterium	3	6.89	**26. Cell-substrate adhesion**	2	2.31
6. Defense response to bacterium	2	6.22	**27. Regulation of mRNA stability**	2	2.20
7. Extracellular structure organization	3	5.85	**28. Cell foam differentiation**	2	2.18
8. Skeletal system development	3	5.73	**29. Regulation of coagulation**	4	2.15
9. Immune cell motion	8	5.32	**30. Regulation of cellular biosynthesis**	11	2.01
10. Defense response	126	5.25	**31. Myeloid mediated immunity**	3	2.00
11. Immune cell activation	23	4.98	**32. Actin cytoskeleton organization**	4	1.89

12. Regulation of cell adhesion	4	4.50	**33. Biomineral formation**	2	1.86
13. Regulation of cell proliferation	3	4.25	**34. Type I interferon production**	2	1.84
14. Regulation of angiogenesis	2	3.54	**35. Integrated-mediated signaling pathway**	2	1.76
15. Regulation of cytokine production	26	3.48	**36. Response to hypoxia**	7	1.75
16. cell-cell adhesion	3	3.33	**37. Lipid localization**	3	1.70
17. Cell activation during immune response	7	3.23	**38. Response to stimulus (nutrients)**	3	1.57
18. Positive regulation of apoptosis	9	3.22	**39. Glycolipid catabolic process**	1	1.56
19. Fatty acid metabolic process	11	3.21	**40. Regulation of protein localization**	3	1.53
20. Regulation of body fluids	6	3.19	**41. Regulation of cell morphogenesis**	2	1.42
21. Programmed cell death	4	2.96	**42. Positive regulation of signal transduction**	2	1.37

Table2. List of biological up-regulated processes in injured spinal cords of C57Bl/6 mice.

DOWN-GENES FUNCTIONAL CLUSTERING IN INJURED MOUSE		
CLUSTER	**nº GO**	**ES**
1. Muscle contraction	**2**	**4.46**
2. Lipid metabolism	**9**	**3.25**
3. Muscle development	**6**	**1.98**

Table 3. List of biological down-regulated processes in the spinal cord of C57Bl/6 mice after contusion injury.

Along with GO, genes included within the different up or down regulated processes also shared different KEGG pathways. The most up-regulated pathways were involve in inflammation, such as "complement and coagulation cascades", "prostaglandins and nitric oxide biosynthesis", "lymphocyte and monocyte activation" or "antigen processing and presentation" (Table 4). The down-regulated pathways were related to "dilated cardiomyopathy", "hypertrophic cardiomyopathy" and "regulation of actin cytoskeleton" (Table 5).

KEGG PATHWAYS UP-REGULATED AFTER SCI AT 7dpo
1. ECM-receptor interaction
2. Focal adhesion
3. Complement and coagulation cascades
4. Systemic lupus erythematosus
5. Antigen processing and presentation
6. Cell receptor signaling pathway
7. Fc gamma R-mediated phagocytosis
8. Fc epsion RI signaling pathway
9. Cell adhesion molecules (CAMs)
10. Small cell lung cancer
11. Pathways in cancer
12. Cytosolic DNA sensing pathway
13. RIG-I-like receptor signaling pathway
14. Glycosphingolipid biosynthesis

Table 4. List of KEGG pathways up-regulated in the contused spinal cord of C57Bl/6 mice

KEGG PATHWAYS DOWN-REGULATED AFTER SCI AT 7dpo
1. Dilated cardiomyopathy
2. Hypertrophyc cardiomyopathy
3. Regulation of actine cytoskeleton

Table 5. List of KEGG pathways down-regulated in the contused spinal cord of C57Bl/6 mice

Gene profile of contused spinal cord in WT and hIL37tg mice

We next assessed whether transgenic expression of human IL-37 modulates gene expression profile in the contused spinal cord at day 7 post-injury. We found that there were 302 genes differentially expressed in the injured spinal cords of hIL37tg relative to C57Bl6 mice. Specifically, we found 173 and 129 genes that were up-regulated and down-regulated, respectively, in the spinal cords of hIL37tg (Fig. 2). Most of the genes found in top10 down-regulated list have unknown functions. However, serum amyloid 3 (Saa3) is involved in inflammation (Table 6a).

a.

TOP-10 DOWN REGULATED GENES IN hIL37tg MOUSE		
	log2FC	Pvalue
1700112E06Rik	-2.294	1.54E-13
Dock2	-2.074	2.02E-08
Scgb3a1	-1.988	0.000131
Rnu1b1//Rnu1b2//Rnu1b6	-1.863	0.0000005
Sfrp4	-1.748	0.0177737
Selp	-1.709	0.0031432
H19	-1.664	0.0049956
Saa3	-1.628	0.0450900
H19//Mir675	-1.625	0.0019623
8430408G22Rik	-1.480	0.0041943

b.

TOP-10 UP REGULATED GENES IN hIL37tg MOUSE		
	log2FC	Pvalue
Gabra2	2.197	1.08E-16
Myoc	2.127	1.54E-08
Kcnj13	2.060	0.00026
Cldn19	1.927	0.00110
Mpz	1.837	0.01036
Pmp2	1.802	0.01844
Hbb-b1//Hbb-b2	1.762	0.00057
Hbb-b2//Hbb-b1	1.752	0.00062
Hba-a1//Hba-a2	1.721	0.00322
Hba-a2//Hba-a1	1.717	0.00353

Table 6. List of top-10 of most down-regulated (a) and up-regulated genes (b) in the spinal cord of hIL-37tg mice .

Functional clusters classification of altered genes revealed that there were 4 functional clusters downregulated. Indeed, many of the genes present within these 4 functional clusters are involved in different aspects of inflammation, further supporting the anti-inflammatory actions of IL-37 described in chapter 1 (Table 7).

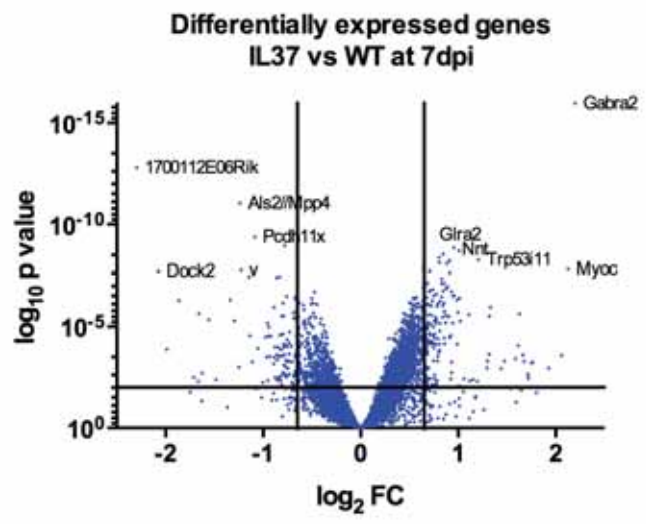


Figure 2. . Volcano plots showing gene expression differences in the injured spinal cord of hIL-37tg vs WT mice.

Within the top10 up-regulated genes in the hIL37tg spinal cords we found the 4 genes that encode for the 4 hemoglobin chains, and 3 that encodes for the peripheral nervous system (PNS) myelin, such as Myelin protein zero (MPZ), peripheral myelin protein 2 (pmp2) and Claudin 19 (Cld19). The most up-regulated gene triggered by the transgenic expression of human IL-37 was the α2 subunit of the $GABA_A$ receptor (Table 6b).

DOWN-REGULATED GENES IN IL37tg MOUSE			
		GO TERMS	ES
1.	Cell adhesión	3	4,74
2.	Response to stimulus	5	3,43
3.	Development (blood vessels)	9	3,09
4.	Regulation cell response (immunity)	3	1,76

Table7. List of biological down-regulated processes in injured spinal cords of hIL37tg mice.

UP-REGULATED GENES IN IL37tg MOUSE			
		GO TERMS	ES
1.	Lipid metabolic process	13	6,29
2.	Vitamin, hormone metabolic process	12	2,77
3.	Transport	4	2,54
4.	Cell communication	3	2,25
5.	Development	34	2,04
6.	Other go terms	9	

Table8. List of biological up-regulated processes in injured spinal cords of hIL-37tg mice.

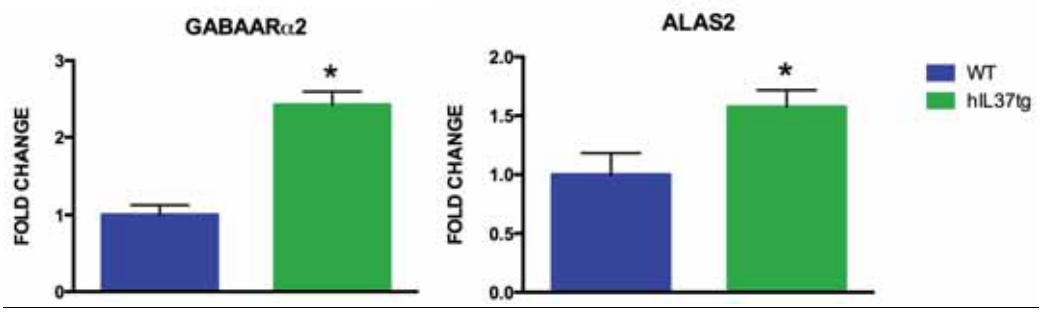
Classification of the up-regulated genes in functional clusters revealed the existence of six biological processes that were enriched in hIL-37tg mice. (Table 8). In addition, there were also 3 enriched KEGG pathways (Table 9).

KEGG PATHWAYS UP-REGULATED hIL37TG MOUSE
1. Terpenoid backbone biosynthesis
2. Hedgehog signaling pathway
3. Nicotine and nicotinamide metabolis

Table 9. KEGG pathways up-regulated in hIL37tg mouse

Microarray validation

Microarray data was validated by Real Time-PCR (RT-PCR). To validate gene expression changes in the mice spinal cord 6 genes identified in the array were analyzed (table 7). The expression of targeted genes was compared groups. RT-PCR results (fig. 3) showed significant correlation between micro array and RT-PCR, validating our microarray analysis.



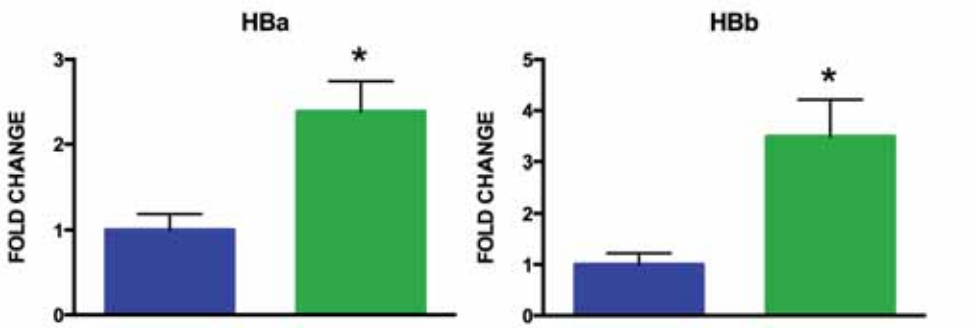


Figure 3. Validation of some gene by RT-PCR indicates good correlation between data obtained by microarray analysis. Bars indicat SEM. (*p<0.05 vs WT) (n=4 per group)

DISCUSION

In the present work we studied the changes in gene expression that occurs in the contused spinal cord upon transgenic expression of IL-37. Our results reveal that in the contused spinal cord of C57bl/6 mice there was enrichment of genes related with inflammation at day 7 post-injury. Microglial cells are considered the endogenous immune cells of the CNS. However, after SCI, there is also infiltration of immune cells from circulation, especially macrophages and granulocytes (David et al., 2012). These cells are recruited into the injury site from the circulation by the chemotaxis mediated by different chemokines and cytokines, being some of them up-regulated, such as CCL2, CCL3 or CCL5 (Abraham, 2003). In order to invade the spinal cord, immune cells need to interact with several cell adhesion molecules present on endothelial cells (Farooque et al., 1999; Kim et al., 2006; Schreiber et al., 2013; Smyth et al., 2009). Several genes that codifies for adhesion molecules were up-regulated in the spinal cord after lesion, including different types integrin member, selectin P (SelP) and tenascin C (TNC) among others. We found was also up-regulation of various members of the Toll-like receptor (TLR) family and their downstream effector Myd88, indicating that TLR signaling was

activated in the spinal cord at 7 days post-lesion. Moreover, several genes related with the complement system, presentation of antigens, and enzymes that mediate potent oxidative burst were also up-regulated.

In chapter 1, we demonstrated that IL-37 exert potent anti-inflammatory actions in SCI. These results are further confirmed in the transcriptomic profile of the contused spinal cord of hIL37-tg mice. Indeed, one of four the biological processes down-regulated by IL-37 was inflammation. Moreover, two more biological processes attenuated in the hIL-37tg mice was "response to stimuli" and "cell adhesion", both of them endorsing genes with high inflammatory component. Interestingly, the last biological function down-regulated by IL37 was the development of blood vessels, suggesting that angiogenesis was reduced in the hIL-37tg mice. This is, in principle, a negative factor, since destruction of blood vessels in the spinal cord parenchyma after injury lead to hypoxia and cell death (Mautes et al., 2000; Tator and Fehlings, 1991; Zipfel et al., 2000). However, reduction of angiogenesis is not necessarily an undesirable side effect, since it could be consequence of reduced blood vessel destruction and of enhanced spinal cord tissue preservation. In this line, as we showed in chapter 1, IL-37 increased myelin and neuronal sparing after SCI, indicating that tissue damage was attenuated in the hIL-37tg mice. This may account, in part, the reduced neoangiogenic response observed in hIL-37tg mice.

A part from gene suppression, we also found that IL-37 stimulated the expression of more that one hundred genes, which can be clustered in six biological functions. Two of them were related with lipid metabolism and synthesis, which englobed several genes involved in steroid synthesis, a hormone with suppressive actions on inflammation. Interestingly, IL-37 also induced the expression of genes involved in

the formation of myelin (MPZ, PMP2, Cld19). These three genes, which are not present in the CNS but in PNS myelin, were found within list of the top5 up-regulated genes in the hIL-37tg mouse. It is well established that Schwann cells from the dorsal and ventral roots migrate into the injured spinal cord parenchyma where re-myelinate demyelinated axons to restore the functional integrity of axonal pathways (Brook et al., 1998; Bruce et al., 2000; Wiliams and Bunge, 2012). This may suggest that of IL-37 stimulates, directly or indirectly, the migration of Schwann cells into the injured spinal cord, enhancing therefore re-myelination. Interestingly, as we showed in chapter 1, the amount of myelin was enhanced in the transgenic mice, which may be due, in part, by increased re-myelination of axons by Schwann cells. Highlight that the second gene more up-regulated in the contused spinal cord of hIL-37 mice was the glaucoma-associated gene, also known as myocilin (Myoc). Myoc is a secreted glycoprotein that belongs to the family of olfactomedin domain-containing proteins (Anholt, 2014). A recent work reports the Myoc has important role in the re-myelination of the PNS after injury, and thus, may support that re-myelination by Schwann cells might be enhanced in the spinal cord of hIL-37tg mice. Myoc expression is induced by glucocorticoids (Clark et al., 2001; Kirstein et al., 2000; Kwon et al., 2013; Ohlmann et al., 2003; Shepard et al., 2001), linking steroid-Myoc-and formation of PNS myelin. A part from Schwann cells, unmyelinated axons can be also re-myelinated by oligodendrocytes generated by endogenous oligodendrocyte pre-cursor cells (OPCs), which proliferate and migrate to the region of the injury site (Keirstead et al., 1999; Zawadzka et al., 2010). Accumulation of OPC is dependent, in part, of WNT and Hedgehog signaling pathway. Interestingly, the contused spinal cord of hIL37tg animals showed up-regulation several members of the WNT and hedgehog

family, including WNT4 and FRIZZLED, which both are involved in re-myelination (McTigue et al., 2006; Rodriguez et al., 2014). Therefore, all these data suggest that the enhanced amount of myelin observed after SCI in the hIL-37tg mouse might be due to reduction of secondary tissue damage, but also, to increased activation of re-myelination processes.

The biological role of WNT and Hedgehog family members is not limited to OPC accumulation, since it also controls the elongation of axons (Hollis and Zou, 2012; Onishi et al., 2014). However, as we showed and discussed in chapter 1, the corticospinal axons fail to regenerate in the spinal cord of hIL-37tg, suggesting that the induction of the genes by IL-37 is insufficient to overcome the inhibitory milieu of the damage CNS.

The most up-regulated gene in the contused spinal cord of hIL-37tg mice codifies for the subunit $\alpha 2$ of the GABA receptor. The GABA receptor is a heteropentameric ligand-gated ion channel. Different GABA receptors subtypes can be found in the spinal cord, which relies on the composition of these 5 subunits. Receptors is formed from two copies of a single α (from $\alpha 1$ to $\alpha 6$), two copies of a singles β (from $\beta 1$ to $\beta 3$) and one copy of another subunit, such as γ, δ or ε (Olsen and Sieghart, 2008). Different GABA receptors subtypes can be found in the spinal cord. In the spinal cord, $\alpha 2$-GABA receptor is expressed in the superficial layers of the dorsal horn (Bohlhalter et al., 1996). The activation of the GABA receptors selective conducts Cl^- through its pore leading to neuronal hyperpolarization. After SCI, GABAergic inhibition is attenuated, contributing to neuropathic pain (Drew et al., 2004; Gwak et al., 2006). Some experiments has showed that analgesia can be achieved by targeting GABA receptors formed by subunit $\alpha 2$ (Knabl et al., 2008). Besides, it has been also demonstrated that knockout mice for

the α2-GABA subunit show hyperalgesia (Witschi et al., 2011). Therefore, these results suggest that the over expression of α2- GABA receptor subunit in hIL-37tg animals, together with the attenuated inflammatory response, which is also a strong inducer of neuropathic pain after CNS and PNS injury (Kawasaki et al., 2008; Marchand et al., 2005; Scholz and Woolf, 2007), may result in reduced hyperalgesia observed after SCI.

Interestingly, we found that the genes encoding for the 4 polypeptides chains of the hemoglobin were found within the top10 up-regulated genes in the hIL-37tg. Since hemoglobin is synthesized by immature forms of red blood cell in the bone morrow, but not in mature erythrocytes, we discard that the source of these genes are the erythrocytes that extravassate into the spinal cord parenchyma due to hemorrhagic events that occurs after SCI. A part from immature red blood cells, recent data demonstrate that other cell types, including macrophages and neurons, are able to synthesize hemoglobin (Biagioli et al., 2009; Liu et al., 1999; Schelshorn et al., 2009). Although the role of hemoglobin in these cells is currently unknown, it is believe that it could act as scavenger of ROS species, and thus, play a protective role against oxidative stress. Since the expression of hemoglobin is induced by erythropoietin (EPO), this could explain the neuroprotective effects of EPO when administered after CNS injury, including after spinal cord contusion (Celik et al., 2002; Cetin et al., 2006; Gorio et al., 2002; Utada et al., 2015).

In conclusion, IL-37 regulates a great variety of physiological processes that are not only limited to inflammation, but also to other mechanisms involved in the restoration of the tissue after damage. This data further supports the therapeutic action of IL-37 in SCI.

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CHAPTER 3.

Anti-inflammatory role of IL-37 attenuates neuropathic pain after peripheral nerve injury

Anti-inflammatory role of IL-37 attenuates neuropathic pain after peripheral nerve injury.

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ABSTRACT

Development of neuropathic pain is common feature that occurs after injury into the central and peripheral nervous system. Inflammation is believed to play an important role in the origin and the maintenance of the neuropathic pain after injury, and thus, targeting the inflammatory response is expected to attenuate hyperalgesia and improve life quality in patients that undergo such conditions. Here, we investigate whether IL-37, a cytokine with potent anti-inflammatory actions attenuates neuropathic pain after spinal cord injury and sciatic nerve injury. Similar to that observed in chapter 1, we found that IL-37 exerts anti-inflammatory effects in the sciatic nerve and dorsal root ganglia after spared nerve injury, but did not prevent inflammation in the lumbar spinal cord. However, transgenic expression of IL-37 resulted in upregulation of the α2 subunit of $GABA_A$ receptor in the lumbar spinal cord upon spared nerve injury. Interestingly, hIL-37tg mice were protected against thermal, but not mechanical, hyperalgesia after spinal cord injury and spared nerve ligation injury. Overall, our data provide new insight about the beneficial actions of IL-37 after nervous system conditions.

INTRODUCTION

Injury to the peripheral nervous system results in a direct cell death, axon disruption and activation of glial cells and Schwann cells which release a number of inflammatory mediators. Some of these mediators acts as promoters of the macrophage infiltration from the blood torrent increasing the initial immune response (Scholz and Woolf, 2007). Inflammatory mediators released by immune and glial cells, such as several cytokines, chemokines or growth factors, are central to orchestrate inflammation. However, these factors can also induce hyperexcitability in DRG neuronal bodies, primary afferent, and changes in excitability of CNS neurons related in the transition of pain responses. This leads to peripheral and central sensitization, resulting in development of neuropathic pain (Marchand et al., 2005; Schäfers and Sorkin, 2008; Sommer and Kress, 2004; Thacker et al., 2009). Thus, the ability to modulate the immune response after nerve injury may be considered a target to treat neuropathic pain (Abbadie et al., 2003; Biber and Boddeke, 2014; Nadeau et al., 2011; Ramer et al., 1998). In this line, several studies showed that several anti-inflammatory approaches reduce pain sensation after central nervous system (CNS) and peripheral nervous system (PNS) injury (Echeverry et al., 2013; Liu et al., 2000; Myers et al., 1996; Rutkowski et al., 2000; Sommer and Schäfers, 1998).

IL-37 is an anti-inflammatory cytokine of the IL-1 family that acts as a suppressor of the innate immune response (Nold et al., 2010). Although the mouse homolog of IL-37 has not been found yet, the transgenic expression of IL-37 in mice overexpression (hIL37tg) leads to reduced inflammatory response after different inflammatory stimuli (Nold et al., 2010). As shown in chapter 1, we also demonstrated that IL-37 is able to attenuate the inflammatory response after

spinal cord injury (SCI), resulting in enhanced functional recovery and tissue preservation. Here we examined whether IL-37 modulates inflammation after PNS injury, and if so, whether it results in protection against neuropathic pain. Moreover, we also studied the effects of IL-37 in neuropathic pain responses after SCI.

MATERIALS & METHODS

All animal procedures were approved by the ethical committee of the Universitat Autònoma de Barcelona in accordance with the European Commission on Animal Care.

Spared nerve injury (SNI)

8 weeks old hIL-37tg mice and WT littermate mice were anesthetized with a combination of Ketamine and Xylacine (90mg/kg and 10mg/kg respectively, intramuscular). A longitudinal incision in the skin and muscle was made at the right paw in order to expose the sciatic nerve and the tibial, peroneal and sural branches. To perform the spared nerve injury (SNI), the tibial and peroneal branches were ligated and sectioned afterwards, leaving only the sural branch intact. Muscle and skin were there sutured and disinfected with povidone. After injury, animals were placed in a warm environment until they were fully awake and subcutaneous buprenorphine was administered once a day during the following 48 hours.

Spinal cord injury (SCI)

8 weeks old hIL-37tg and WT littermates mice were use in this experiment. Animals were anesthetized with a combination of Ketamine and Xylacine (90

mg/kg and 10 mg/kg respectively, intramuscular), laminectomy at thoracic level 11 was performed, and the exposed spinal cord was contused using the Infinite Horizon Impactor. The impact force used was 50 kdynes and tissue displacement was ranged between 400-600μm. After injury, animals were placed in a warm environment until they were fully awake and subcutaneous bupremorphine was administered once a day during the following 48 hours. Bladders were also emptied until reflex voiding was re-established.

Behavioral test

One week before injury, mice were habituated to experimental devices used for the nociceptive tests, and baseline measurements for mechanical and thermal stimuli were taken in both hind paws to compare later with measurements taken after SCI and SNI. Mechanical and thermal sensitivity was assessed at the following days:

- For SNI: at 4, 7, 10, 14, 21 days post-injury (dpi).
- For SCI: at 7,14,21,28 dpi. The first measurement was done at 7 dpi after SCI, to ensure that all the mice were able to perform plantar placement.

Mechanical stimuli were measured with an electronic Von Frey algesimeter (Bioseb, Chaville, France). Mice were placed on a wire net platform in plastic chamber 45 min before the experiment for habituation. Mechanical nociceptive threshold was taken as mean of three measurements of each paw, with 5 min interval between measurements, and expressed as the force (in grams) at which mice withdrew their paws in response to the stimulus.

Thermic stimuli were determined using a plantar algesimeter (Ugo Basile, Comerio, Italy). Mice were placed into a plastic box with an elevated glass floor 45 min before experiment for habituation. The beam of a projection lamp was focused onto the hind paw plantar surface. Thermal nociceptive threshold was taken as a mean of three trials per paw with 5 min resting between each trial, and expressed as a latency (in seconds) of paw response.

Histological analysis

Mice undergoing SNI were anesthetized with Dolethal at 4 and 7 dpi (sodium pentobarbital; Vetoquinol E. V. S. A. 0.01ml/10g, intraperitoneal) and then perfused with 4% paraformaldehyde (Sigma) in 0.1 M phosphate-buffered (PB). Lumbar spinal cord containing L4-L5 segments, ipsilateral L4-L5 dorsal root ganglia (DRG), as well as the sciatic nerve containing the spared sural branch were removed and post-fixed with 4% paraformaldehyde 0.1 PB for 1 hour and then cryoprotected in 30% sucrose solution for at least 48 hours. Spinal cords, DRG and sciatic nerves were frozen at -60ºC in cryoembedding compound (Tissue-Tek, OCT, Sakura). Cryostat (Leica) sections of 15 μm thickness of sciatic nerve, DRG and lumbar segments were serially cut and picked up on gelatin-coated glass. All sections were storage at -20ºC until its use.

For immunohistochemistry, frozen tissue sections were placed in a hotplate at 37ºC for 15 minutes and washed with Phosphate buffer Saline (PBS), then permeabilized with 0.3% triton-PBS and blocked with blocking buffer (BB: 5% Fetal Bovine Serum un 0.3%triton-PBS) for 1 hour and incubated with correspondent anti iba-1 primary antibodies overnight at 4ºC. Samples were incubated with secondary antibody bind to fluorocrom alexa-488 (1:500

Invitrogen) for 1 hour at room temperature and after washes with PBST, PBS and PB samples were mounted with Mowiol mounting media with DAPI (1μg/ml).

mRNA extraction and RT-PCR

To study cytokine expression was analyzed at 1 and 4 days after SNI in tgIL-37 and WT animals. Mice were anesthetized with Dolethal (sodium pentobarbital; Vétoquinol E. V. S. A. 0.01ml/10g, intraperitoneal) and were perfused with PBS and sciatic nerve containing the spared sural nerve, L4-L5 ipsilateral L4-L5 DRGs and L4-L5 spinal cords were collected. For mRNA extraction tissue was homogenized with Quiazol lysis reagent (Qiagen) and mRNA was 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.

To study gene expression, real time PCR (RT-PCR) was performed using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies). Data analysis were performed using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad laboratories). Expression of target genes was normalized with the GAPDH values as a housekeeping gene. The list of primers used are indicated in table 1 :

IL-37	Forward: … CTTAGAAGACCCGGCTGGAAG… (21 bp) Reverse: …TGTGATCCTGGTCATGAATGCT… (22 bp)
IL-1β	Forward: … TTCCCAATCCCTCAACAGTC… (20 bp) Reverse: … ATGTTCTGGAGCAGGCAGTG… (22 bp)
TNFα	Forward: …TCAGGTTGCCTCTGTCTCAG… (20 bp) Reverse: … GCTCTGTGAGGAAGGCTGTG… (20 bp)
IL-6	Forward: … GCTGGGATTTTCACCACTG … (20 bp) Reverse: …TGACTTGTCCTGAGACCTGATG … (22 bp)

CCL-2	Forward: … CCCCAAGAAGGAATGGGTCC … (20 bp) Reverse: … TGCTTGAGGTGGTTGTGGAA … (20 bp)
IL-10	Forward: …GCTGAGACTTTCGCTCCTCTC… (21 bp) Reverse: … AGCTCCAAGGCACCTGTTC… (19 bp)
GABA-ARα2	Forward: … AAGCCACTGGAGGAAAACATCT… (22 bp) Reverse: … CACACCAGAAGAACAAGCAGC… (21 bp)
GAPDH	Forward: …TCAACAGCAACTCCCACTCTTCCA… (24 bp) Reverse: … ACCCTGTTGCTGTAGCCGTATTCA… (24 bp)

Table 1. List of genes and respective primers used for the cytokine quantification

RESULTS

Assessment of pain response after SNI.

To examine pain-related behavior after SNI, we first examined the mechanical nociceptive and thermal nociceptive withdrawal threshold in both plantar paws before and after lesion. We found that mechanical stimulation in the injured paw was increased sensibility to stimuli in both experimental groups, starting at 4 dpi, the first day analyzed. This hypersensibility remained increased until the end of experiment at 21 days. Although tgIL-37 mice tended to show reduced mechanical hypersensitivity as compared to WT mice, no significant differences were observed between groups (*$P<0.05$) (Fig. 1A). The plantar paw contralateral to the SNI did not show mechanical hypersensitivity (Fig. 1B).

Paw withdrawal latency to thermal stimuli was also measured. At the injured paw, WT mice resulted in significant decreased thermal tolerance as compared to uninjured values. Thermal hyperalgesia was already observed at 4 dpi, and remained significant altered until the end of the follow up (21 dpi). Interestingly, and in contrast to mechanical stimuli, hIL-37tg did not show significant increased in thermal hypersensitivity after SNI at any of the time points analyzed (Fig. 1C)

Comparatively, differences in thermal hyperalgesia between experimental groups were observed as early as 4 days post-injury (Fig. 1C), although differences reached statistically significant from day 7 post-lesion. In the contralateral paw, WT mice also show thermal hypersensitivity starting at day 10 (Fig. 1D), although to lesser extend as compared to the ipsilateral paw. hIL-37tg mice did not show any significant changes in thermal tolerance in the contralateral paw after SNI (Fig. 1D). Therefore, our data demonstrate that IL-37 avoids thermal, but not mechanical, sensitivity after SNI.

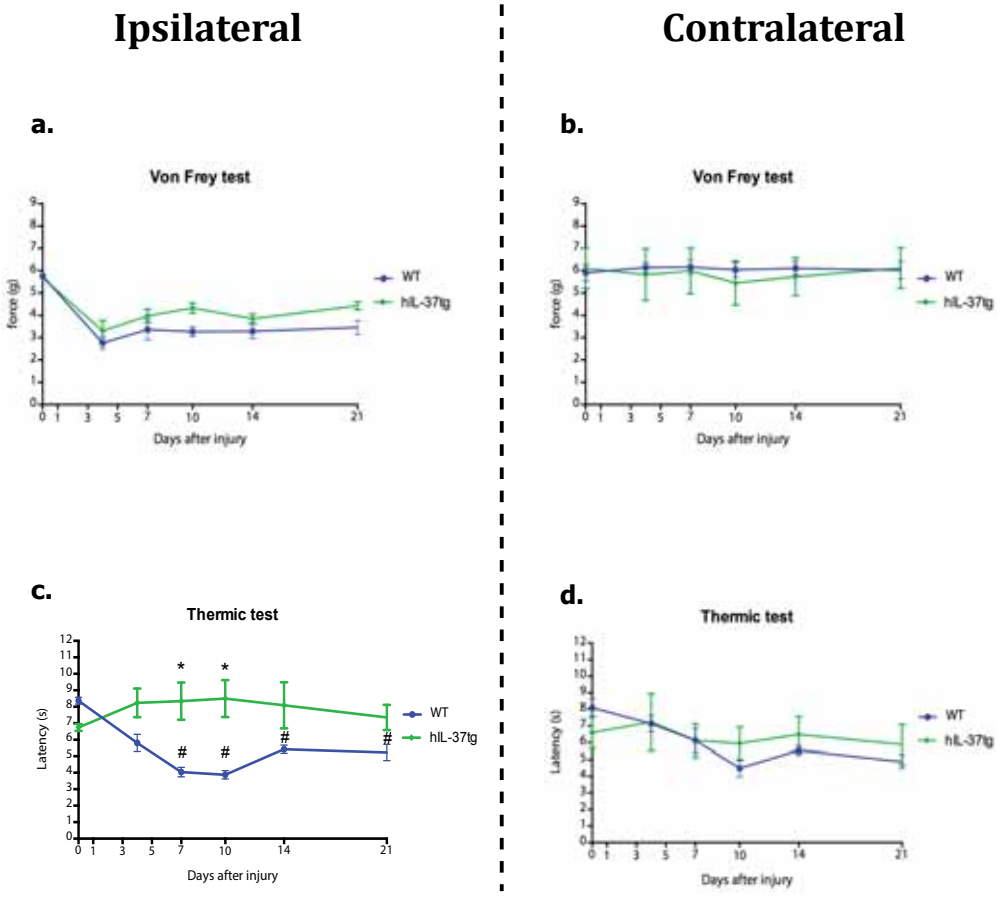

Figure 1. (A-B) Assessment of mechanical hypersensitivity in the ipsilateral (A) and contralateral (B) after SNI. Note that SNI triggers mechanical hyperalgesia in the ipsilateral paw. This is not prevented in hIL-37tg mice. (C-D) Evaluation of thermal sensitivity in the ipsilateral (C) and contralateral (D) plantar paw after SNI. Note that the hIL-37tg mice do not show thermal hyperalgesia after SNI. (n=7 per group). (*p<0.05) (#p<0.05 vs naïve). Error bars indicate SEM

Changes in cytokine expression after SNI.

We then assessed whether SNI resulted in augmented levels of IL-37 mRNA levels in hIL37tg mice after SNI. In uninjured mice, IL-37 was barely detected in the spinal cord, DRG and sciatic nerve. However, SNI led to significant up-regulation of IL-37 mRNA levels in the DRG and the sciatic nerve at 1 and 4 dpi, but not in the lumbar spinal cord (Fig. 2).

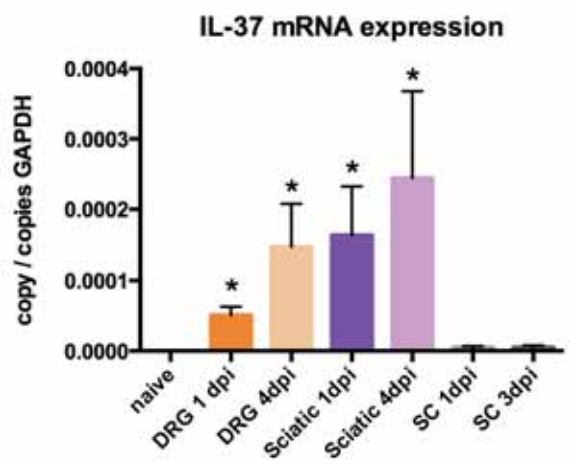

Figure 2. Expression of IL-37 in hIL37tg mice before and after SNI. IL-37 expression was assessed in the sciatic nerve, DRG and in the spinal cord (SC) at 1 and 4 days after injury. Significant expression of IL-37 is observed in the DRG and sciatic nerve after injury (n=5 per group) (*p<0.05). Error bars indicate SEM

We then studied the changes in cytokine profile in the sciatic nerve, DRG and spinal cord after SNI In the sciatic nerve of WT animals, we observed increased expression of IL-1β, IL-6, IL-10, TNFα and CCL-2 at 1 dpi (*p<0.05) (Fig 3a). Expression of IL-6, IL-10 and TNFα remained significantly increased in the sciatic nerve at 4 dpi. In the DRG we only found a significant increase of IL-6 at 1 dpi (Fig 3b). No changes in cytokine expression in WT were observed in the spinal cord (Fig 3c). We then analyze whether IL-37 expression resulted in significant changes in the expression of different cytokines.

In the sciatic nerve. IL-37 led to significant decrease of IL-6 and CCL-2 at 1dpi in tgIL-37 (*p<0.05) but not at 4 dpi (Figure 3b). Interestingly, IL-37 prevented the induction of cytokine expression after SNI (Figure 3a), whereas in the lumbar spinal cord did not lead to any significant change.

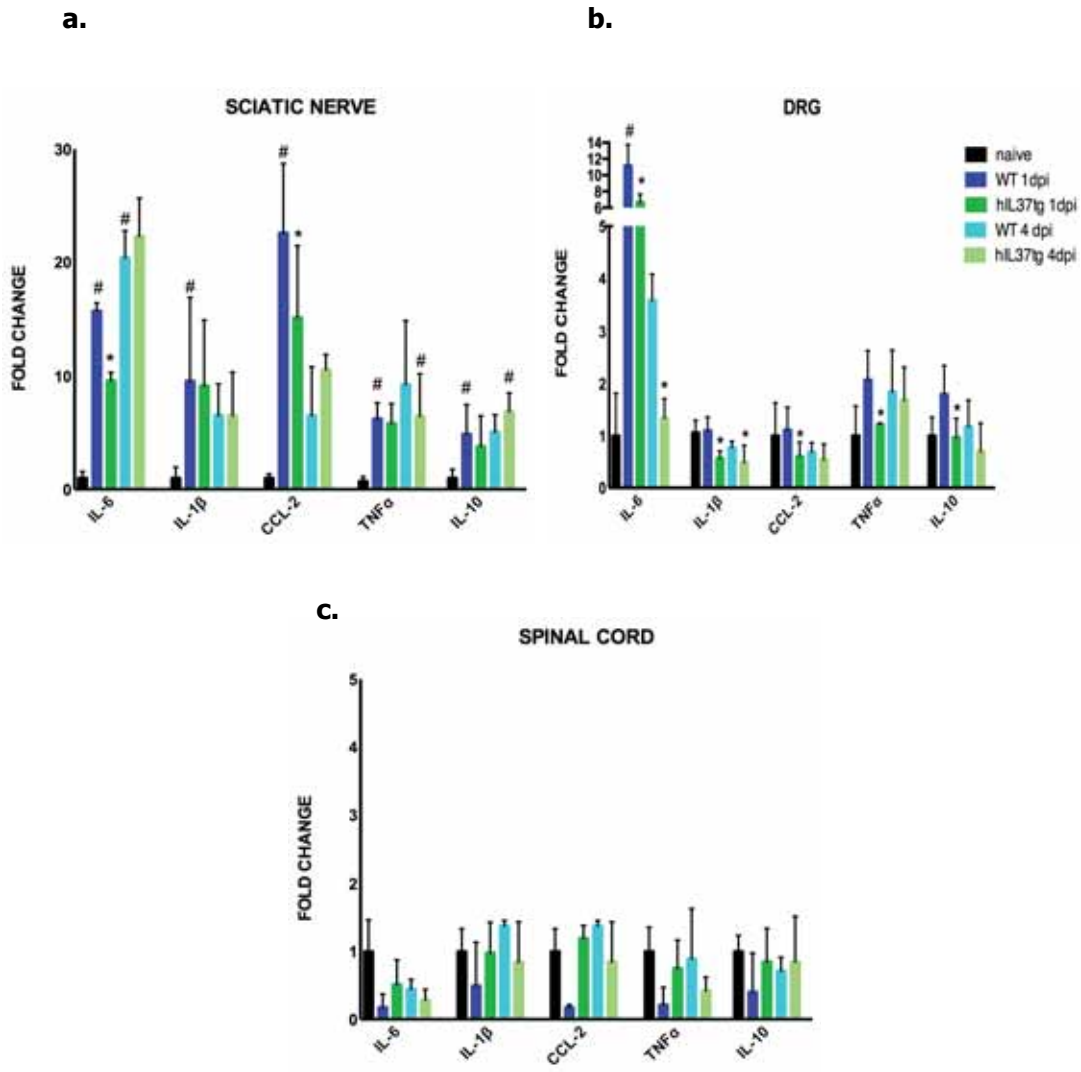

Figure 3. Expression of different cytokines and chemokines in the sciatic nerve (A), dorsal root ganglia (B) and lumbar spinal cord (C) after SNI in WT and hIL-37tg mice. Error bars indicate SEM). (*p<0.05 hIL37tg vs WT) (#<0.05 vs naïve).

Infiltration of *macrophages after SNI.*

We then assessed whether changes in cytokine expression observed in hIL-37tg mice resulted in decreased macrophage invasion in the sciatic nerve and DRG, as well as, attenuated microglia reactivity in the lumbar spinal cord.

In the WT mice, we found the presence of numerous macrophages at day 4 post-injury and 7 days in the sciatic nerve (Fig.4) and DRG after SNI (Fig.5).

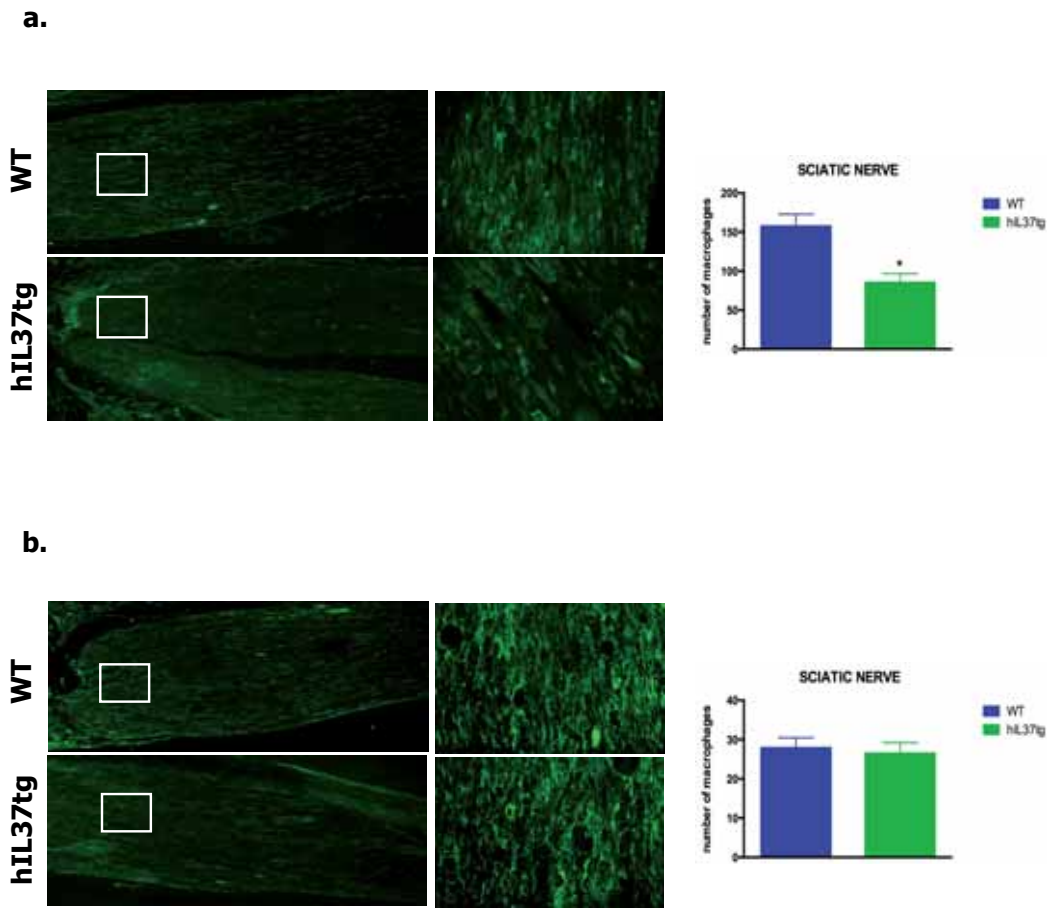

Figure 4. Quantification of macrophages in the sciatic nerve at 4 (A) and 7 (B) days after SNI. (Note that hIL-37tg mice showed reduced macrophages counts at 4 days after injury . (n=5 per group). (*p<0.05). Error bars indicate SEM.

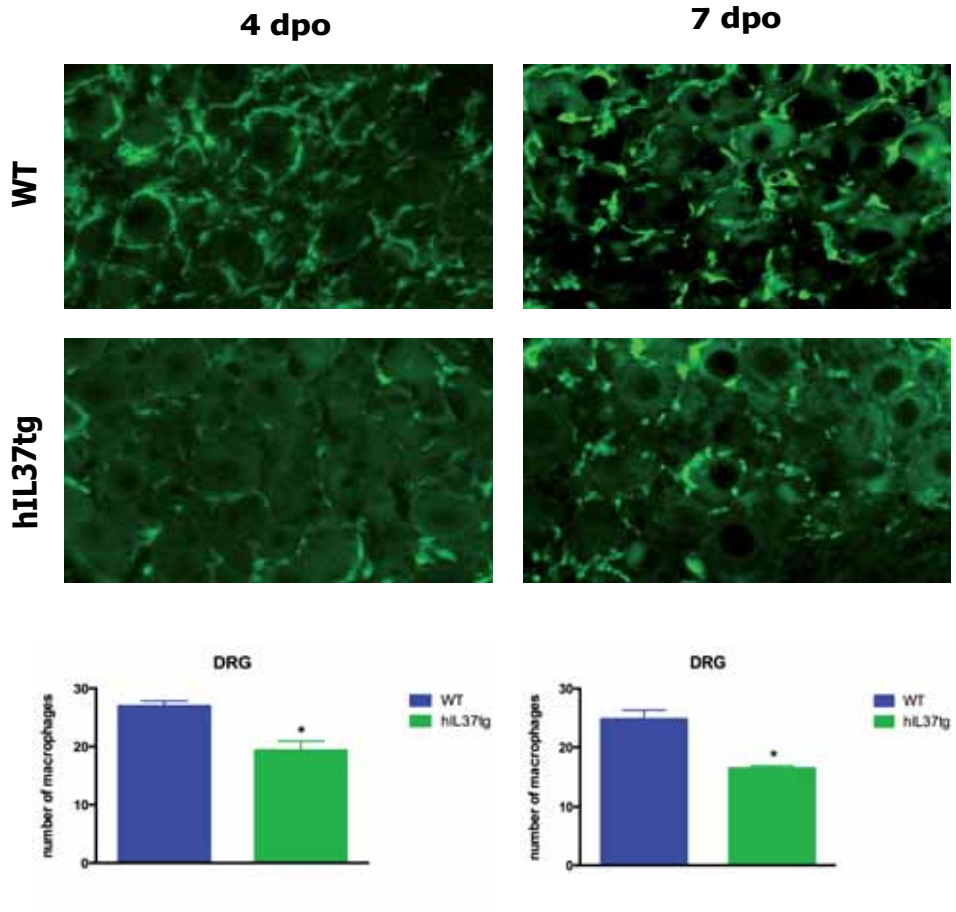

Figure 5. Quantification of macrophages in the DRG at 4 and 7 days after SNI. (Note that hIL-37tg mice showed reduced macrophages counts at both time points . (n=5 per group). (*p<0.05). Error bars indicate SEM.

Transgenic expression of IL-37 reduced the macrophages density counts in both tissues at 4 days after SNI, clearly indicating the anti-inflammatory effects of IL-37 in the injured sciatic nerve (Fig. 4). Macrophages density remained attenuated in the DRG of hIL-37tg mice at day 7 dpi, but not in the sciatic nerve (Fig 5).

No changes were observed in microglial counts and immunoreactivity in the spinal cord of hIL-37tg mice as compared to WT littermates after SNI.

Expression of $GABA_A$ receptor subunit α 2

In chapter 2, we demonstrated a high up regulation of the α2 subunit of the $GABA_A$ receptor in hIL37tg mice after SCI. Since reduction in GABAergic inhibition is associated with increased neuropathic pain, we studied whether IL-37 led to changes in the expression of the α2 subunit of the $GABA_A$ receptor in the lumbar spinal cord after SNI. No differences were observed in the expression of the α2 subunit of the $GABA_A$ injured WT mice after injury. However, the mRNA levels of the α2 subunit of the $GABA_A$ receptor was increased 3 times in the lumbar spinal cord of hIL-37tg mice as compared to WT littermates (*p<0.05) .

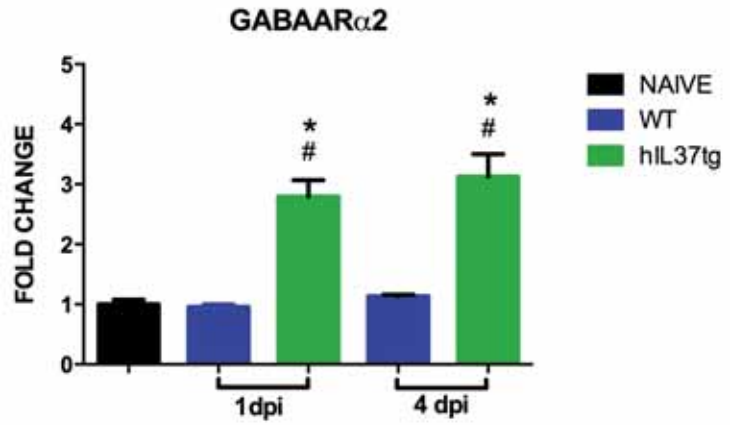

Figure 6. Expression of the subunit α 2 of the $GABA_A$ receptor after SNI. hIL37tg mice showed significant up-regulation of in the mRNA levels of subunit α2 at 1 and 4 days after injury (n=5 per group) (*p<0.05 vs WT) (#p<0.05 vs naïve). Error bars indicates SEM.

Assessment of neuropathic pain after SCI

Mechanical and thermal hypersensitivity was also measured after SCI. Since the contusion injury was centered into the spinal cord, changes in pain sensitivity affect both paws in a similar magnitude, and thus values were average.

Assessment of pain responses were initiated at day 7 after injury, time point when mice were able to perform plantar paw placement of both hindlimbs.

After SCI, response to mechanical stimuli was almost unaffected after injury and mice from both groups showed a slight reduced tolerance, although not significant, to the mechanical Von Frey filament (Fig. 6A). On the other hand, SCI resulted in increased hypersensitivity to hot stimuli in both experimental groups at 7 dpi. However, while thermal hyperalgesia remained in WT mice until the end of the follow up, this was normalized in hIL-37tg mice from day 14 (Fig. 6B). Significant difference between both groups were observed from day 14 after SCI. This data provides clear evidence that transgenic expression of IL-37 confers protection against thermal hyperalgesia after SCI.

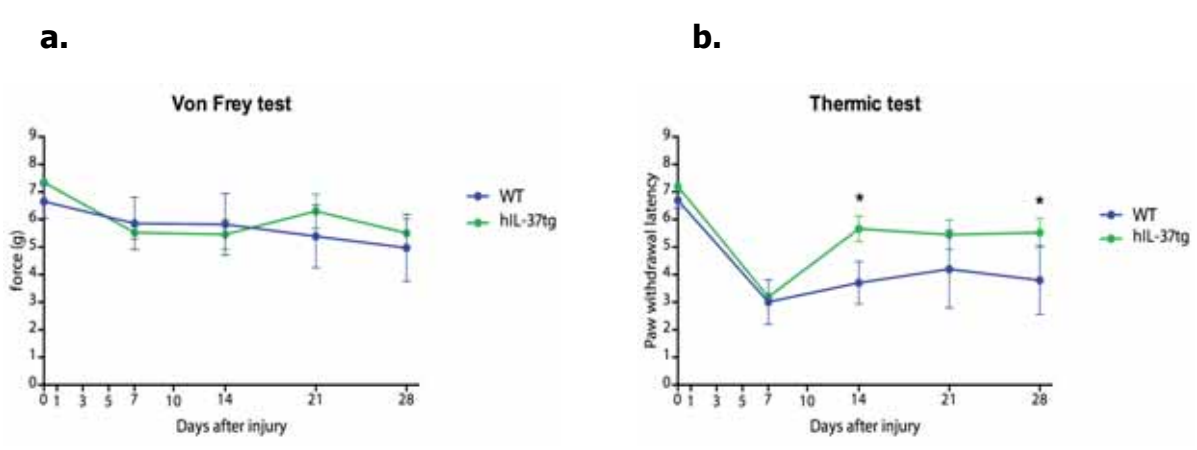

Figure 7. Effect of IL-37 in neuropathic pain after spinal cord injury. (A) SCI did not trigger mechanical hyperalgesia. (B) Assessment of thermal hyperalgesia triggered by SCI. Note that hIL-37tg mice were protected against thermal hyperalgesia. (n=7 per group). (*p<0.05). Error bars indicate SEM.

DISCUSSION

Development of neuropathic pain after injury into the peripheral and central nervous system is currently a major complication, which interferes with quality of life and rehabilitation. Several studies suggest a role of the inflammatory response in the development and maintenance of pain after injury (Marchand et al., 2005; Sommer and Kress, 2004; Zhang et al., 2015). In this study we therefore investigate whether il-37 leads to reduced neuropathic pain after SNI and SCI. We found that transgenic expression of IL-37 conferred protection against thermal hyperalgesia, but mechanical hypersensitivity in both models.

Similar to our results from chapter 1 and 2, here we demonstrate that IL-37 exerts suppressive actions on inflammation in the injured PNS. This was demonstrated by assessing the mRNA levels of several cytokines in the sciatic nerve and DRG and by quantifying macrophage infiltration in both regions of the PNS.

Previous studies revealed that inflammation is one of the main contributors to the development of neuropathic pain after SNI. This is evident in experiments where the use of knockout mice for some pro-inflammatory cytokines, such as IL-1β, TNFα, or IL-6 (Nadeau et al., 2011; Ramer et al., 1998), as well as, treatment with anti-inflammatory drugs results in reduced hyperalgesia. Moreover, depletion of macrophages by administration of clodronate led to decreased thermal hypersensitivity without affecting the development of mechanical hypersensitivity in a model of partial ligation of the sciatic nerve (Liu et al., 2000). These studies suggest that the reduction macrophages infiltration into the sciatic nerve and/or DRG results attenuates thermal hyperalgesia after nerve injury. Further support was also achieved by experiments using the Wld mutant mice (Myers et al., 1996). In this mouse model, where infiltration of macrophages is significantly delayed

after nerve injury (Myers et al., 1996), also resulted in reduction of thermal hyperalgesia. Our data, together with the works in the literature, indicates that there is a link between the anti-inflammatory actions of IL-37 and the reduced thermal hyperalgesia observed after SNI.

A part from the inflammatory response that take place in the PNS, glial cell activation also occurs in the CNS after SNI. Several studies also report that glial activation plays a key role in triggering pain response after PNS injury (Calvo and Bennett, 2012). Here, we also observed that microglia became activated in the lumbar spinal cord after SNI, which was not attenuated in the hIL-37tg mice. This might be due to the lack of IL-37 induction in the lumbar spinal cord observed in hIL-37tg mice, and consequently, the expression of pro-inflammatory cytokines was not attenuated.

A part from inflammation, there are other mechanisms involved in the development of neuropathic pain after neve injury. Transmission of nociceptive sensory information to the brain depends on the interplay between the inputs from nociceptive and non-nociceptive primary afferent fibers to the dorsal horn neurons in the spinal cord, which is known as "gate control theory" (Woolf and Salter, 2000). Contact with noxious stimuli sensitize peripheral nociceptors which leads to hyperalgesia (Moalem and Tracey, 2006; Pezet and McMahon, 2006; Scholz and Woolf, 2007; Sommer and Kress, 2004) . By the other side, inhibitory neurons acts as a balance and prevents pain sensation from innocuous stimuli. Synaptic inhibition is mediated by the activation of $GABA_A$ and $GABA_B$ receptors (Knabl et al., 2008; Munro et al., 2009; Woolf and Salter, 2000). GABA receptors are ionotropic receptors that by the entrance of Cl^- leads to a hyperpolarization of cell membrane (Zeilhofer, 2005). It is known that injury into the PNS and CNS

decreases the amount in the protein levels of some units of GABA receptors in dorsal horn interneurons resulting in hyperexcitability and the development of the neuropathic pain (Drew et al., 2004; Gwak et al., 2006; Janssen et al., 2011). In chapter 2, we found that in hIL37tg mice the most up-regulated gene is one of the subunits that forms the $GABA_A$ receptor, the subunit α2. Interestingly, it is demonstrated SNI triggers down-regulation in the protein levels of α2 subunit $GABA_A$ receptor in dorsal horn neurons, which leads to increased hyperalgesia (Witschi et al., 2011). Moreover, treatment with diazepam, promotes analgesia via the activation of this subunit (Knabl et al., 2008). The up-regulation of this α2 subunit in hIL37tg after SCI may promote contribute to attenuate thermal hyperalgesia in mice expressing IL-37 as compared with WT mice. Interestingly, we also found that the mRNA levels of the $GABA_A$ receptor α2 subunit were also increased in the lumbar spinal cord of hIL-37tg mice after SNI. In this line, we observed that transgenic expression of IL-37 prevented the thermal hyperalgesia after SNI and SCI. This data suggest that the effect of IL-37 in decreasing thermal hypersensitivity could be due through two different processes: reduction of the inflammatory response and enhanced inhibitory GABAergic tone. We do not know yet whether there is a link between these two processes, or by contrast, both events are regulated by IL-37 through independent mechanisms. This will be further studied in the laboratory.

Overall, our data demonstrates that IL-37 exert anti-inflammatory actions after injury into the PNS. In addition, our results suggest that IL-37 enhances GABAergic inhibition after SCI and PNI. Interestingly, our data also reveals that IL-37 mediates protection against thermal hyperalgesia after PNS and CNS injury, suggesting that the use of IL-37 could be a new approach to treat neurological

conditions were inflammation contribute to neurodegeneration and to development of neuropathic pain.

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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 (Velnar et al., 2009). Usually, inflammatory response undergoes resolution by switching the synthesis of cytokines, from pro-inflammatory to anti-inflammatory, and in the production of lipid mediators, from pro-inflammatory to pro-resolutory. After SCI, however, resolution of inflammation is hampered which leads to chronic inflammation (Donnelly and Popovich, 2008). This results bystander side effects, such as secondary tissue degeneration and development of neuropathic pain (Detloff et al., 2008; Hains and Waxman, 2006).

Secondary damage triggered by inflammation after SCI has important consequences on functional deficits due to the poor capacity of the CNS to regenerate damaged axons, and to replace dead neurons and glial cells, resulting in irreversible functional disabilities (Profyris et al., 2004; Rowland et al., 2008; Yiu and He, 2006). Therefore, experimental approaches aimed at attenuating the inflammatory response after SCI may lead to the development of new therapies for patients with acute SCI.

In the last years, IL-37 has been proposed as a modulator of the innate immune response. IL-37 (also known as IL-1F7) is an anti-inflammatory cytokine that belongs to the IL-1 family cytokines (Boraschi et al., 2011; Dinarello and Bufler, 2013). As most of other members of IL-1 family, IL-37 gene is located at chromosome 2 in humans, but the mouse homolog has not been identified yet. Therefore, the generation a transgenic mouse expressing the human form of IL-37

has been crucial to study the role of this cytokine upon different inflammatory challenges.

The anti-inflammatory effect of IL-37 has been reported in transgenic mice after induction of endotoxic shock by LPS (Nold et al., 2010). hIL-37tg mice showed decreased expression of pro-inflammatory cytokines such as IL-1α, IL-1β and IL-6, and the . Physiological effects of endotoxemia were also reduced (Nold et al., 2010). Transgenic expression of IL-37 also resulted in less hepatic damage, better respiratory compensation of the metabolic acidosis and less hypothermia (Nold et al., 2010). Moreover, in animal models of human diseases where inflammation is dysregulated, such as colitis or hepatitis mice, hIL-37tg mice were also showed clinical benefits (Bulau et al., 2011; McNamee et al., 2011). Moreover, in a model of cerebral ischemia, hIL37tg mice showed decreased expression of cytokines, such as IL-1α, IL-1β, TNFα and IL-6, and chemokines, such as CCL2 and CCL-3 (also known as MCP-1 and MIP-1 respectively) (Patel et al., 2014).

However, the effects of IL-37 after nervous system trauma and disease remains still unknown. In the present thesis, we studied whether IL-37 modulates the inflammatory response after SCI and PNS lesion, and how the effects of IL-37 affects tissue damage, locomotor recovery, and the development of neuropathic pain . We provide novel data demonstrating that IL-37 plays an important role in suppressing the inflammatory response after injury in the spinal cord and the peripheral nerve.

Our results reveal that hIL-37tg mice display barely detectable levels in the uninjured CNS and PNS, despite the expression of the gene is regulated by a constitutive promotor. This is due by the presence of an instability region in the untranslated region (UTR) of the IL-37 gene at 3' that provokes a rapid

degradation of the mRNA. However, after some inflammatory challenges, the instability region becomes stabilized (Bufler et al., 2004; Tierney and Medcalf, 2001), leading to increased mRNA levels, and consequently, the protein can is detected (Bufler et al., 2004; Nold et al., 2010). mRNA levels of IL-37 showed two peaks of expression in the contused spinal cord, at 12 hours and 3 days after injury. The first peak correlates with the peak of cytokine production, whereas the second peak coincides with the infiltration of circulating macrophages (David et al., 2012; Donnelly and Popovich, 2008).

Damage to the nervous system lead to a rapid reaction of microglia an astrocytes that start to secrete a repertory of pro-inflammatory cytokines, including IL-1β and TNFα or IL-6, among others (Davalos et al., 2005; David et al., 2012; Pineau and Lacroix, 2007; Pineau et al., 2010). These cytokines are detected in the damaged spinal cord for the first 3-24 hours where are involved in the triggering the infiltration of circulating immune cells (David and Kroner, 2011). The invasion of neutrophils peaks at 24 hours after injury to then, decrease and they are barely detected in the injured spinal cord at later times (Fleming et al., 2006; Neirinckx et al., 2014; Norenberg et al., 2004). Macrophage infiltration is detected at 1 day but reaches the maximum of infiltration around day 3 (Donnelly and Popovich, 2008; Popovich and Jones, 2003; Profyris et al., 2004). Macrophages remain in the spinal cord for several years (Popovich and Jones, 2003). Lymphocytes infiltrate at latter time points, peaking by 2-3 weeks after injury (David et al., 2012; Donnelly and Popovich, 2008; Sroga et al., 2003). IL-1β and TNFα along with other TLR ligands are able to induce IL-37 (Nold et al., 2010). Although the expression of IL-37 has been detected in macrophages, the fact we observed an early peak of IL-37 expression at 12 hours, when macrophages infiltration is minimal, suggest that

there are other sources of IL-37. However, the second peak of IL-37 expression coincides with the massive infiltrating macrophages into the damaged spinal cord, suggesting that macrophages are probably the source of this delayed expression of IL-37.

The early expression of IL-37 in hIL37tg mice correlates with reduced induction of pro-inflammatory cytokines after SCI, as well as with attenuation in neutrophil, macrophages an activated microglial cells counts. Lymphocyte recruitment, however, was unaltered for the first 7 days post-injury, when the influx of the immune cell subset is minimal. The reduced inflammatory observed after transgenic expression of IL-37 was linked to reduced functional deficits and tissue loss, suggesting that the anti-inflammatory action of IL-37 mediates beneficial effects in SCI.

The isoform b of IL-37 contains two known functional cleavage sites. The first cleavage site is located al position D20 and is recognized for Caspase-1, which processes IL-37 into a mature form (Bulau et al., 2014; Kumar et al., 2002). This mature form binds to SMAD-3 and is able to translocate to the nucleus to suppress the transcription of pro-inflammatory cytokines (Boraschi et al., 2011; Bulau et al., 2014; Nold et al., 2010). The second cleavage site is located at V46 and is recognized by an extracellular protease, likely an elastase, suggesting that IL-37 is a dual cytokine with an intra- and extra-cellular role (Boraschi et al., 2011). The confirmation of the extracellular role has been supported the demonstration that IL-37, although with a very low affinity, can bind IL-18BP, the natural inhibitor of IL-18, enhancing the ability to inhibit IL-18 (Bufler et al., 2002). In the same way, IL-37 binds IL-18Rα blocking the heterodimer formation with IL-18Rβ but without avoiding IL-18 signaling (Bufler et al., 2002; Kumar et al., 2002). Recently, it has

been discovered a specific extracellular receptor for IL-37: IL-1R8. The binding of IL-37 with IL-1R8 allows the formation of a heterodimer with IL-18Rα, which blocks the intracellular pro-inflammatory cascade (Li et al., 2015; Nold-Petry et al., 2015). Since we observed that the exogenous administration of IL-37 leads to beneficial effects on functional outcomes after SCI, it indicates the helpful effects of IL-37 are, in part, due to its binding to IL-8R. However, we cannot exclude the possibility that nuclear actions of IL-37 could be also involved in such helpful actions. This will be studied in the laboratory in the future with the generation of transgenic mouse that expresses a mutation in the caspase 1 site (D20A) of the IL-37b precursor, and thus, IL-37 will not translocate to the nucleus. The use of this transgenic mouse will resolve this fundamental issue.

We also found that administration of the full length recombinant IL-37 protein and the cleaved form at V46 ($IL\text{-}37_{46\text{-}218}$) exerted similar effects on functional recovery. Since previous studies demonstrate that the processed form of IL-37 has greater anti-inflammatory effects than the full length protein (Li et al., 2015; Pan et al., 2001), our data suggest that the full length IL-37 is processed into the cleaved form when administered in the injured spinal cord. Highlight that this result also corroborates the extracellular function of IL-37 in SCI, because the cleaved form does not have the signal required to be translocated to the nucleus.

To gain further information on the actions of IL-37 in SCI, we performed a genearray analysis of the contused spinal cord of hIL-37tg and WT mice. This was done at 7 dpi, since at this time point there are activated both, mechanisms involved in secondary damage, but also some processes that are important for tissue healing and repair (Bareyre and Schwab, 2003; Yunta et al., 2012). This data further confirmed the anti-inflammatory effects of IL-37, since transgenic mice

showed reduction in many genes related to the inflammatory response. In addition, IL-37 expression also led to down-regulation of genes functionally classified within the biological group of “stress response” and “response to injury”, suggesting that the environment of the contused spinal cord was less hostile in hIL-37tg mice.

On the other hand, the number of genes up-regulated in IL-37 transgenic mice was greater as compared to those down-regulated. Functional classification of up-regulated genes in hIL-37tg mice revealed that they were involved in “lipid and hormone metabolism and synthesis”, “transport”, “cell communication” and “development”. Among the top 10 up-regulated genes found in the hIL-37tg mice were present the protein myelin zero (PMZ or P0), claudin 19 (Cldn19) and peripheral myelin protein 22 (PMP22) that are exclusively found in PNS myelin. After SCI, massive death of oligodendrocytes (OL) occurs which leads to myelin loss. A part from speeding nerve conduction, myelin is also important to protect and give trophic support to axons (Fünfschilling et al., 2012; Maxwell, 1996; Nave, 2010; Papastefanaki and Matsas, 2015). The loss of myelin caused after SCI is partially compensated, although with major limitations, by OPC that form new oligodendrocytes and by the infiltration of Schwann cells from dorsal and ventral roots in an attempt to promote axonal remyelination (Keirstead et al., 1999; Zawadzka et al., 2010). The up-regulation of peripheral myelin genes in the injured spinal cord may indicate that remyelination by infiltrated Schwann cells is enhanced by transgenic expression of IL-37, and could explain, in part, the greater amount of myelin found in the hIL-37tg mice after contusion injury. Moreover, the fact that myocilin, a protein that play an important role in triggering myelination by Schwann cells in the PNS, was the second gene more up-regulated in the hIL-

37tg mouse (Kwon et al., 2013; Ohlmann et al., 2003), may support that remyelination by Schwann cells is enhanced by IL-37. Myocilin is induced by glucocorticoids (Clark et al., 2001; Shepard et al., 2001). Interestingly, spinal cords from hIL-37tg mice had enriched up-regulated genes related with the synthesis of steroids, linking IL-37,steroids, myocilin and PNS myelin.

Expansion of OPC after SCI is mediated, in part, by activation of WNT and Hedgehog signaling pathways. We found that contused spinal cord of hIL-37tg mice had up-regulated expression of some members of the WNT and Hedgehog family. However, we do not have any evidence yet that the formation of new oligodendrocytes is enhances by IL-37.

The different WNT and hedgehog family members are also important players in regulating regeneration and retraction of damage axons (Hollis and Zou, 2012; Onishi et al., 2014; Zou, 2004). Moreover, it is also known that macrophages inhibit axonal regeneration in SCI by cell contact interaction and through the release of unknown factors (Busch et al., 2009; Evans et al., 2014; Horn et al., 2008). Despite these observation may suggest that axonal regeneration could be induced in the hIL-37tg mice, we did not observe any sign of axonal outgrowth in the corticospinal tract after complete transection of the spinal cord. Howerver, we cannot discard that the elongation of other axonal populations could be enhanced upon transgenic expression of IL-37.

Another striking finding was the presence of the 4 genes that encodes for the 4 polypeptide chains of hemoglobin in the top 10 up-regulated genes in the hIL-37tg mice. We discard that the source of these genes are the erythrocytes that extravasates into the injured spinal cord after, since these cell type does not have nuclei. Recent works reveal there is synthesis of hemoglobin in some kidney cells

macrophages and neurons (Liu et al., 1999; Richter et al., 2009; Schelshorn et al., 2009). Although the role of hemoglobin in these cells is currently unknown, it is been suggested that it could bind ROS, and thus, confer protection against oxidative stress (Richter et al., 2009; Wilson and Reeder, 2008). Although to our knowledge it is currently unknown whether macrophages or neurons do express hemoglobin in the injured spinal cord, it may explain the protective effects of EPO in several models of CNS injury, including spinal cord contusion (Celik et al., 2002; Cetin et al., 2006; Utada et al., 2015). It is also known that the presence of hemoglobin in neurons enhances oxygenation, which could also provide protection against ischemic events (Schelshorn et al., 2009).

The gene most up-regulated in the spinal cord of hIL-37tg after SCI was the subunit $\alpha 2$ of the $GABA_A$ receptor. The $GABA_A$ receptor is a heteropentameric ligand-gated ion channel. In the spinal cord, $\alpha 2$-$GABA_A$ receptor is expressed in the superficial layers of the dorsal horn, as well as, in the terminal regions of primary nociceptive afferences, where it leads to neuronal hyperpolarization by increasing Cl^- conductance (Bohlhalter et al., 1996; Witschi et al., 2011). Activation $GABA_A$ receptors reduces nociceptive input to the spinal cord (Gwak et al., 2006). After SCI, there is reduction of GABAergic inhibition, which has important contribution to the generation of neuropathic pain (Drew et al., 2004; Gwak et al., 2006). Therefore, up-regulation of this subunit in the hIL-37 after SCI may confer protection against neuropathic pain.

Besides dysregulation of GABA receptors, development of pain after injury has also a strong inflammatory component, since the release of pro-inflammatory cytokines and other factors by macrophages (i.e. prostaglandins) increases axonal excitability (Kawasaki et al., 2008; Marchand et al., 2005; Scholz and Woolf, 2007;

Zhang et al., 2015). Indeed, several works demonstrate that the blockade of pro-inflammatory cytokines or delivery of anti-inflammatory drugs alleviates neuropathic pain in several models (Echeverry et al., 2009; Ledeboer et al., 2007). In this line, here we found that transgenic expression of IL-37 attenuated thermal, but not mechanical, hyperalgesia after SCI. This anti-nociceptive feature of IL-37 is not restricted to spinal cord trauma, since the transgenic expression of IL-37 also avoided the development of thermal hypersensitivity after SNL, a model of PNS lesion that that leads to long lasting neuropathic pain. Further experiments using this model revealed that hIL-37tg mice underwent similar features as occurred after SCI. For instance, IL-37 was barely detectable in the uninjured sciatic nerve and DRG, but it was up-regulated in both regions after lesion. Induction of IL-37 was linked to attenuation in the expression of pro-inflammatory cytokines and infiltration of macrophages, although this effect was more pronounced in the DRG. However, IL-37 was not induced in the lumbar spinal cord after SNL, and consequently, microglia reactivity was not impeded. This data provides novel evidence of the anti-inflammatory actions of IL-37 in the PNS. Our results are also in agreement with other studies in which a depletion or a delayed recruitment of macrophages impairs the development of thermal hyperalgesia (Echeverry et al., 2013; Liu et al., 2000; Myers et al., 1996) without affecting the mechanical hypersensibility (Barclay et al., 2007; Rutkowski et al., 2000). The more pronounced reduction in cytokine expression and macrophage infiltration in DRG as compared to the injured sciatic nerve, suggest that the inflammatory response that occurs in DRG after SNL may play a major role in the generation of neuropathic than the inflammation that occurs the nerve. Indeed, it is well known that several mediators released by immune cells, including cytokines, leads to

changes in gene expression in neurons that result in peripheral sensitization(von Hehn et al., 2012; Nickel et al., 2012; Scholz and Woolf, 2007). Moreover, the release of several molecules by primary afferents results in changes the expression of post-synaptic receptors that leads to central hypersensitivity (von Hehn et al., 2012; Latremoliere and Woolf, 2009; Scholz and Woolf, 2007). The loss, at the protein level, of $GABA_A$ receptor in the dorsal horn neurons after SNL are one of such changes that trigger neuropathic pain (Moore et al., 2002). Although we observed that mRNA levels of the α2 subunit of the $GABA_A$ receptor remained unaltered in the spinal cord of WT mice after SNL, this was 3 fold increased in the hIL-37tg mice, suggesting that GABAergic inhibition was enhanced upon IL-37 expression. Further studies are needed to determine whether the induction of this $GABA_A$ receptor subunit is directly related to the IL-37 expression, or in contrast, is a consequence of the reduced inflammatory that occurrs in the DRG of hIL-37tg mice. In addition, it is important to address whether the up-regulation of this subunit plays an important role in attenuating thermal hyperalgesia in the hIL-37tg mice. The last, but not the least, it needs to be elucidated until what extend the beneficial actions of IL-37 in the SNL are due to extracellular or intracellular function. Due to time constrains, I was unable to perform these studies, however, they will be addressed in the laboratory in the future.

Overall, the data presented in the present thesis provide strong evidence that IL-37 exerts potent anti-inflammatory actions in the CNS and PNS after injury. We also demonstrate that IL-37 confers protection against some undesirable side effects caused by inflammation, such as the development of secondary tissue damage and some aspects related with the appearance of neuropathic pain. We show also reveal that the beneficial effects of IL-37 are due, in part, to its binding to

extracellular receptors. Since inflammation contributes to neurodegeneration in most neurological disorders, our data may open new avenues for the treatment of different CNS and PNS conditions.

CONCLUSIONS

Chapter 1. Beneficial effects of IL-37 after spinal cord injury

- IL-37 is up-regulated after spinal cord injury. Showing two peaks of expression at 12 hours and 3 days after injury.
- The expression of IL-37 correlates with an attenuation of the inflammatory response spinal cord injury.
- IL-37 improves functional recovery and promotes myelin sparing and neuronal preservation.
- IL-37 does not promote axonal regeneration.
- Administration of recombinant IL-37 exerts beneficial actions in a model of SCI, suggesting that IL-37 could be a useful therapy in the acute phase after SCI.

Chapter 2. IL-37 modulates gene expression after spinal cord injury

- There is enrichment of many genes related to inflammation at seven days after spinal cord injury.
- Transgenic expression of IL-37 attenuates the expression of genes related to inflammation.
- The effects of IL-37 upregulates the expression of genes related to peripheral myelin, cellular transport, cellular communication and development.

- IL-37 induces the expression of the $GABA_A$ receptor α2 subunit, which is known to attenuate neuropathic pain.

Chapter 3. Anti-inflammatory role of IL-37 attenuates neuropathic pain after peripheral nerve injury.

- IL-37 expression is induced in the sciatic nerve and dorsal root ganglia after spared nerve injury
- Transgenic expression of IL-37 attenuate mRNA levels of pro-inflammatory cytokines in the sciatic nerve and dorsal root ganglia after spared nerve injury.
- The decrease of pro-inflammatory cytokines triggered by IL-37 is linked to decreased infiltration of macrophage in the sciatic nerve and dorsal root ganglia after spared nerve injury.
- IL-37 upregulates the expression of GABAA receptor α2 subunit after spared nerve injury.
- Injury to the spinal cord and the peripheral nerve results in development of neuropathic pain.
- IL-37 reduces neuropathic pain after spinal cord injury and peripheral nerve injury

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ABBREVIATIONS

Alas2	Delta-aminolevulinate synthase 2
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPK	AMP activated protein kinase
ANGPT	Angiopoietin
ANOVA	Analysis of variance
APC	Allophycocyanin
ASIA	American spinal injury association
ATP	Adenosine triphosphate
BB	Blocking buffer
BDA	Biotinylated dextran amine
BMP	Bone morphogenetic protein
BMS	Basso mouse scale
BSCB	Blood spinal cord barrier
CARD	Caspase recruitment domain
CCI	Chronic constriction injury
CCL	Chemokine CC motif ligand
Cldn19	Claudin 10
CMV	Citomegalovirus
CNS	Central nervous system
CSPG	Chondroitin sulfate proteoglycan
DAVID	Database for annotation, visualization and integrated discovery
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DPI	Days post injury
DRG	Dorsal root ganglia
ECM	Extracellular matrix
ES	Enrichment score
FACS	Fluorescence activated cell sorting

FADD	Fas-associated protein with death domain
FC	Fold change
FITC	Fluorescein isothiocyanate
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
Gr1	Myeloid differentiation antigen
GSA	General somatic afferent fibers
GVA	General visceral afferent fibers
Hba	Hemoglobin α chain
Hbb	Hemoglobin β chain
hIL37tg	Human IL-37 transgenic mouse
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
Iba-1	Ionized calcium binding adaptor molecule 1
IFNγ	Interferon γ
Ig	Immunoglobulin
IL	Interleukin
IL-37a	Interleukin 37 isoform a
IL-37b	Interleukin 37 isoform b
IL-37c	Interleukin 37 isoform c
IL-37d	Interleukin 37 isoform d
IL-37e	Interleukin 37 isoform e
IL-1F7	Interleukin 1 family member 7
IL-1H4	Interleukin 1 homolog 4
ILR	Interleukin 1 receptor family

IP-10	interferon γ induced protein 10
KC	Keratinocyte chemoattractant
KEGG	Kyoto encyclopedia of genes and genomes
LDL	Low density lipoprotein
LFB	Luxol fast blue
LIF	Leukemia inhibitory factor
LIX	Lipopolysaccharide induced CXC chemokine
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MCP	Monocyte chemoattractant protein
M-CSF	Macrophage colony-stimulating factor
MIG	Monokine induced by interferonγ
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MyoIF	Myosin IF
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
NMDA	N-methyl-D-aspartate
NP	Neuropathic pain
OL	Oligodendrocytes
OPC	Oligodendrocyte precursor cell
PB	Phosphate buffer
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline

PBST	Triton phosphate buffer saline
PCR	Polymerase chain reaction
PE	Phycoeritrin
PerCP	Peridinin chlorophyll
PFA	Paraformaldehyde
PMP22	Peripheral myelin protein 22
PMZ/P0	Protein myelin zero
PNI	Peripheral nerve injury
PSL	Partial sciatic ligation
RANTES	Regulated upon activation normal T cells expressed or secreted
rIL-37$_{1\text{-}218}$	Recombinant proIL-37
rIL-37$_{46\text{-}218}$	Recombinant mature IL-37
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
RT-qPCR	Real time quantitative PCR
Saa3	Serum amyloid 3
SC	Schwann cells
SCI	Spinal cord injury
SelP	Selectin P
SIS3	Smad3 inhibitor
SNI	Spared nerve injury
SNL	Sciatic nerve ligation
TGFβ	Transforming growth factor β
TIR	Toll/IL-1 receptor domain
TLR	Toll like receptor
TNC	Tenascin C
TNFα	Tumor necrosis factor α

VEGF	Vascular endothelial growth factor
WNT	wingless family proteins
WT	wild type