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Impact of T, B and NK lymphocyte deletion on the motor recovery after spinal cord compression in mice

Dissertation

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1. Introduction

1.1 Epidemiology of spinal cord injury

Spinal cord injury (SCI) is a traumatic lesion of neural elements in the spinal cord, resulting in any degree of sensory and motor deficits, and autonomous nervous system dysfunctions including bladder and bowel dysfunction.

Worldwide about 2.5 million people live with spinal cord injury (SCI) and more than 0,13 million survive traumatic spinal cord injury each year, which means a change of life style, bound to a wheelchair for life (Thuret et al., 2006). The most common cause of traumatic spinal cord injury are traffic accidents, especially motor traffic crashes with nearly 50% of overall SCI, followed by falls, sports, community violence and workplace related traumas (Hulsebosch, 2002; Maharaj, 1996; O'Connor, 2002; Sekhon at al., 2001). Besides traumatic injury, the loss of spinal cord function can be caused by several diseases, for example: spinal tuberculosis, syphilis, spinal tumors, multiple sclerosis and poliomyelitis.

SCI most commonly happens to people in their mid-20s, with a median age of occurrence at 33.4 years it is triple as prevalent in males as in females and is severely impairing the quality of life (Bracken et al., 1981; Sekhon et al., 2001; Tator et al., 1993). Besides loss of voluntary motor function distal to the point of injury known as paralysis (plegia), patients suffer from lack of sensation and complications such as bladder and bowel dysfunctions, urinary infections, pressure sores, nutritional deficiency, sexual dysfunctions, osteoporosis and chronic pain (Rossignol et al., 2007).

Because SCI most commonly affects young and healthy individuals it is a significant financial burden to society. The Center for Disease Control estimated that the United States spends $9.7 billion ($US) on the treatment of SCI each year (2003). Overall costs per SCI patient are estimated to exceed $1 million (Mckinley et al., 1999).

1.2 Pathophysiology of spinal cord injury

The normal architecture of spinal cord is radically disrupted by injury. Depending on degree or kind of mechanical force applied to the spinal cord the

consequences are maceration, laceration, penetration, contusion, compression or demolition of the spinal cord tissue (Bunge et al., 1993; Kakulas et al., 1999; Thuret et al., 2006). Of these injury types, the contusion injury represents from 25 to 40% of the cases and is a progressive injury that enlarges over time (Hulsebosch, 2002).

There are three phases of the response after injury: the acute, secondary and chronic injury. A massive cell death of neurons, oligodendrocytes, astrocytes and their precursor cells at the lesion site characterises the acute phase, which encompasses the moment of injury and extends for the first few days after injury. A cascade of pathophysiological processes rapidly follows mechanical trauma to the neural and surrounding soft tissue. Over the few minutes after the lesion the injured nerve cells respond with a barrage of action potentials associated with significant shifts in electrolytes (Na^+, K^+ and Ca^{2+}), which contribute to termination of normal neural functions and spinal shock. Spinal shock lasts for about 24 hours and represents a generalized failure of circuitry in the spinal neural network. Vascular trauma caused by mechanical forces leads to the disruption of the blood brain barrier and disturbances in circulation. Loss of vascular autoregulation and reduction of blood flow, as well as vasospasms with formation of thrombi and bleeding in the grey matter, cause substantial ischemic necrosis and increase swelling, reducing blood supply further and exacerbating the neural injury (Hulsebosch, 2002; Thuret et al., 2006; Velardo et al., 2004).

In the secondary phase, which lasts over the time course of minutes to weeks, the ischemic cell death, electrolytic shifts and edema still persist from the acute phase. The increase of extracellular glutamate to toxic concentrations, as a result of cell lysis, as well as lipid peroxidation and free-radical production are further features of this phase. Further apoptosis - a secondary, programmed cell death, induces reactive gliosis including increased expression of the glial fibrillary acidic protein (GFAP) and astrocytic proliferation.

The damage caused by apoptosis of oligodendrocytes and loss of myelin, as well as activation of inflammatory and wound healing response, are additional components of the secondary phase. As a result of the invasion of inflammatory cells to the injured region the local concentrations of cytokines and chemokines increase. Additionally, inhibitory factors and/or barriers to regeneration are expressed around the lesion site. In summary secondary processes after spinal cord injury expand and

exacerbate the initial lesion considerably and lead to a further loss of function (Hulsebosch, 2002; Thuret et al., 2006).

Finally, in the chronic phase, which occurs over a time course of days to years, apoptosis continues in both anterograde and retrograde directions and the cell death causes formation of cavities and cysts which disrupt descending and ascending axonal tracts (Crowe et al., 1997). Further a variety of receptors and ion channels are altered with regard to their expression levels and activity. A characteristic feature of this phase of the repair process after SCI is also glial scarring, an intricate process that involves reactive astrocytes, glial progenitors, microglia and macrophages, fibroblasts and Schwann cells (Thuret et al., 2006). On the one hand this process prevents inflammation and neurodegeneration (Bush et al., 1999; Faulkner et al., 2004), but on the other hand it builds a barrier, a glial scar, which hinders axonal regeneration via numerous growth-inhibiting molecules (for review see Silver and Miller, 2004). Further demyelination results in conduction deficits and some neurons develop permanent hyperexcitability, which in turn leads to spastic paralysis and chronic pain syndromes in many SCI patients (Hulsebosch, 2002).

In contrast to described destructive processes, a degree of spontaneous repair after SCI is described (Beattie et al., 1997; Yang et al., 2006). Whereas there is little or no neurogenesis in the injured spinal cord, proliferation in the ependymal and periependymal canal generates new precusor cells which differentiate into glial cells (Azari et al., 2005; Thuret et al., 2006; Yamamoto et al., 2001). Limited axonal sprouting is present, but only a few axons regenerate over long distances to their original targets (Hill et al., 2001). New spinal circuits are built to bypass the lesion (Bareyre et al., 2004; Raineteau et al., 2002), cortical sensorimotor areas functionally rearrange (Bareyre et al., 2004; Raineteau et al., 2001) and the rubrospinal system reorganize and compensate for the loss of the motor function (Raineteau et al., 2001).

To conclude, there are multiple factors and events involved in the regeneration after spinal cord injury. The final functional impact of injury and the size of the lesion are dependent not only on the force of initial injury, but also on the secondary and chronic processes after SCI, which could require a period of days to years and represent a potential therapeutic window for improvement with various approaches (Hulsebosch, 2002).

1.3 Therapy of spinal cord injury

The spontaneous repair after SCI is not complete and interminable. There are no fully restorative therapies yet, but a variety of potentially useful therapies, ranging from cell transplantations to drug treatments, have been proposed and tested, or are in clinical trials (Thuret et al., 2006).

Various forms of molecular therapy are undergoing trials aimed to protect neurons from secondary cell death, promote axonal growth and enhance neural impulse conduction. Among these of special interest are growth promoting and neuroprotective agents, among which the beneficial cell adhesion molecules have a prominent role (Irintchev and Schachner, 2011).

Another possible therapeutic strategy is the cellular transplantation with the aims to bridge any cysts or cavities, to replace dead cells and to create a favorable environment for axonal regeneration. Among the cells commonly used in experimental animals are Schwann cells (Lavdas et al., 2010; Papastefanaki et al., 2007), neural stem cells, and embryonic stem cells, at various stages of differentiation (Chen et al., 2005; Cui et al., 2011; Schwerdtfeger et al., 2012; Xu et al., 2011).

However, up to date the only drug treatment for spinal cord injury patients is a corticosteroid called methylprednisolon, which, when administered within maximally 8 hours after injury, is thought to improve regenerative ability by inhibition of the inflammation and swelling and attenuation of the immune response (Thuret et al., 2006).

Nevertheless, the only efficient means up to date to improve the motor function after injury are exercise and physical rehabilitation therapy, commonly used as a palliative treatment in spinal cord injury patients (Engesser-Cesar et al., 2002).

1.4 Immune response after spinal cord injury

The role of immune system in recovery after spinal cord injury is still a subject of controversy. The importance of the immune response in processes of functional recovery after traumatic spinal cord injury has been emphasized under various conditions in different experimental model systems in adult mammals. The inflammatory reaction in the acutely injured cord is a fundamental defense

mechanism, which is aimed at elimination of invading pathogens and tissue debris. Recently it has become evident that the immune system can have both beneficial and adverse impact on regeneration after trauma (Beattie, 2004; Bechmann, 2005, 2007; Ibarra et al., 2003; Schwartz and Yoles, 2006; Thuret et al., 2006). On the one hand inflammatory cells promote wound-healing events that support recovery, and on the other they produce toxic molecules that damage neural tissue (Bethea, 2000; Chan, 2008; Trivedi et al., 2006).

The immune system consists of two types of defenses (Figure 1): the non-specific defense for any particular antigen (innate immunity) and the specific defense (adaptive/acquired immunity), whereas specificity is related to recognition and response to a specific antigen (Popovich et al., 2003; Trivedi et al., 2006).

Resident CNS microglia/macrophages, neutrophils and dendritic cells participate in the innate immune response typically shortly (minutes) after injury. The cells of the innate immune system perform a variety of functions in the injured spinal cord that include destruction of intruding microbes, phagocytosis of cellular debris, expression of chemokine receptors and presentation of antigens to T-lymphocytes, resulting in their activation (Cella et al.,1997; de Castro et al., 2004; Kigerl et al., 2006; Sallusto et al., 2000;).

Neutrophils accumulate within hours after spinal cord injury, their concentration in the injured region reaches a peak at 3 days post-injury and a second peak several weeks later (Carlson et al., 1998; Taoka et al., 1998) (Figure 2). Neutrophils support recovery processes through their ability to phagocytose cellular debris and summon macrophages into the damaged tissue (Kigerl et al., 2006). On their part, macrophages perform not only phagocytosis, but serve also as a reservoir for cholesterol, which is used by oligodendrocytes during remyelination of regenerating axons (Blight, 1985; Boyles, 1989). Inversely, neutrophils release reactive oxygen and nitrogen radicals as well as cytokines, chemokines and a variety of proteases. That way they contribute to the secondary tissue damage after spinal cord injury (Trivedi et al., 2006).

Resident microglia cells are also activated upon injury and reach the highest concentration at 7 day after injury (Trivedi et al., 2006). The protective functions of microglia cells are the participation in the removal of myelin debris, production of growth factors supporting neurite growth, as well as expression of transforming growth factor-beta1 (TGF-beta1). This is a cytokine/growth factor that inhibits the

release of cytotoxic molecules, reduces astrocyte proliferation, and promotes neuronal survival (McTigue et al., 2000; Schwartz, 2003). On the other hand, the activated microglia plays a role not only in repair, but also damage the spinal cord. They are involved in necrotic and apoptotic neuronal cell death increasing the initial spinal cord injury. Furthermore they produce tumor necrotic factor (TNF)-alpha, interleukin-1 (IL-1), reactive free radicals, and nitric oxide which all additionally damage the spinal cord (Popovich, 2000; Town et al., 2005; Yang et al., 2004).

Monocytes/macrophages are maximally activated in the second week post injury (Kigerl et al., 2006). The protective impact of the macrophages to the wound healing is their phagocytic function. In contrast, their destructive contribution includes production of pro-inflammatory cytokines and neurotoxins, including reactive oxygen radicals and inducible nitric oxide synthase (Blight, 1992, 1994; Popovich et al., 1994).

Dendritic cells are antigen presenting cells that express high levels of MHC II and pro-inflammatory cytokines. They support the ongoing inflammatory response, which may exacerbate secondary injury (Schwartz et al., 1999; Town et al., 2005; Trivedi et al., 2006).

Antigen presenting through antigen presenting cells is a functional bridge between innate immunity and cells that participate in adaptive immunity. The adaptive immune system is classified into humoral and cellular responses. The humoral response is mediated by B-lymphocytes, which produce antibodies. The cellular response is mediated by T-lymphocytes (cytotoxic, $CD8^+$ and helper cells, $CD4^+$) which are activated in response to specific antigens.

B-lymphocytes are present at the site of injury within hours after spinal cord injury and persist at least for up to 1 week post-injury (Popovich et al., 2001; Schnell et al., 1997). They produce antibodies and are responsible for memory in adaptive immunity. Up to date very little is known about the involvement of B-cells in spinal cord injury.

T-lymphocytes are present in low numbers in the non-injured spinal cord and upon injury their numbers progressively increase, in parallel with the activation of microglia and influx of peripheral macrophages, within the first week after injury (Popovich, 2000; Trivedi et al., 2006). They have a great potential for promoting tissue damage because of their ability to recognize specific antigens, such as myelin basic protein, and proliferate in response to those antigens. Activated T-cells kill

target cells and produce cytokines, and upon chronic activation they contribute to pathological fibrosis and scarring (Gonzalez et al., 2003; Jones et al., 2005; Popovich et al., 1996). On the other hand, several studies offer evidence in support of a neuroprotective role of T-cells, concluding that T-lymphocytes are most likely involved in both tissue damage and repair after spinal cord injury (Kipnis et al., 2002; Schwartz et al., 2001).

Spinal cord injury activates both innate and adaptive immune responses, functioning in an environment in which tissue damage and repair processes co-exist. The extent to which immune cells promote cellular injury or support recovery depends upon the timing and magnitude of their appearance and the nature of their interactions with one another as well as with both injured and intact cells.

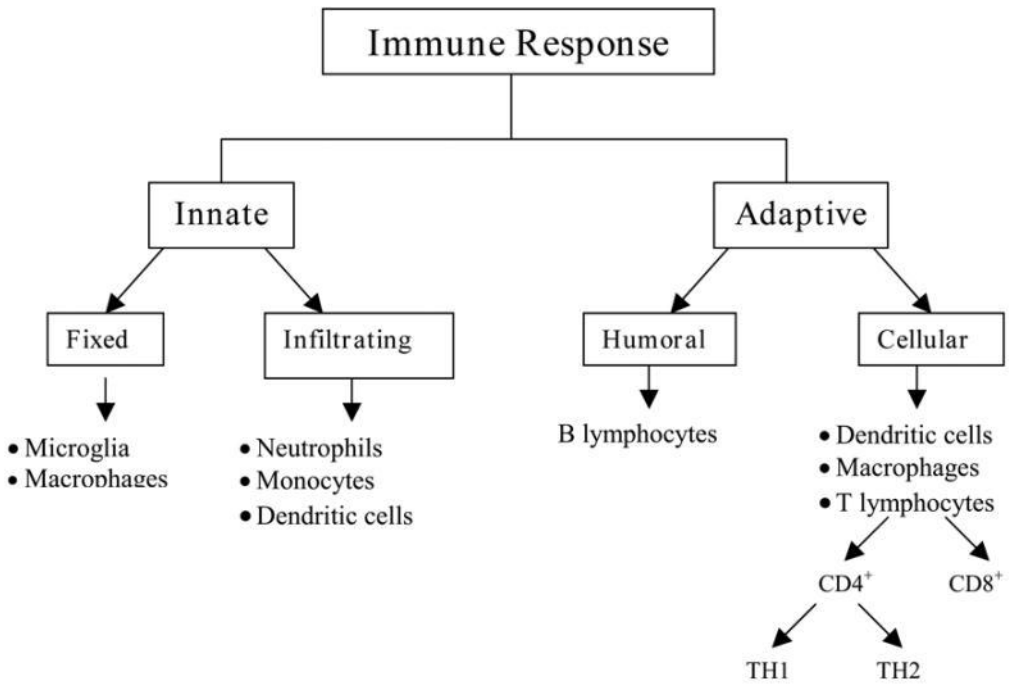


Figure 1: Innate and adaptive immune response. Modified from Trivedi et al., 2006.

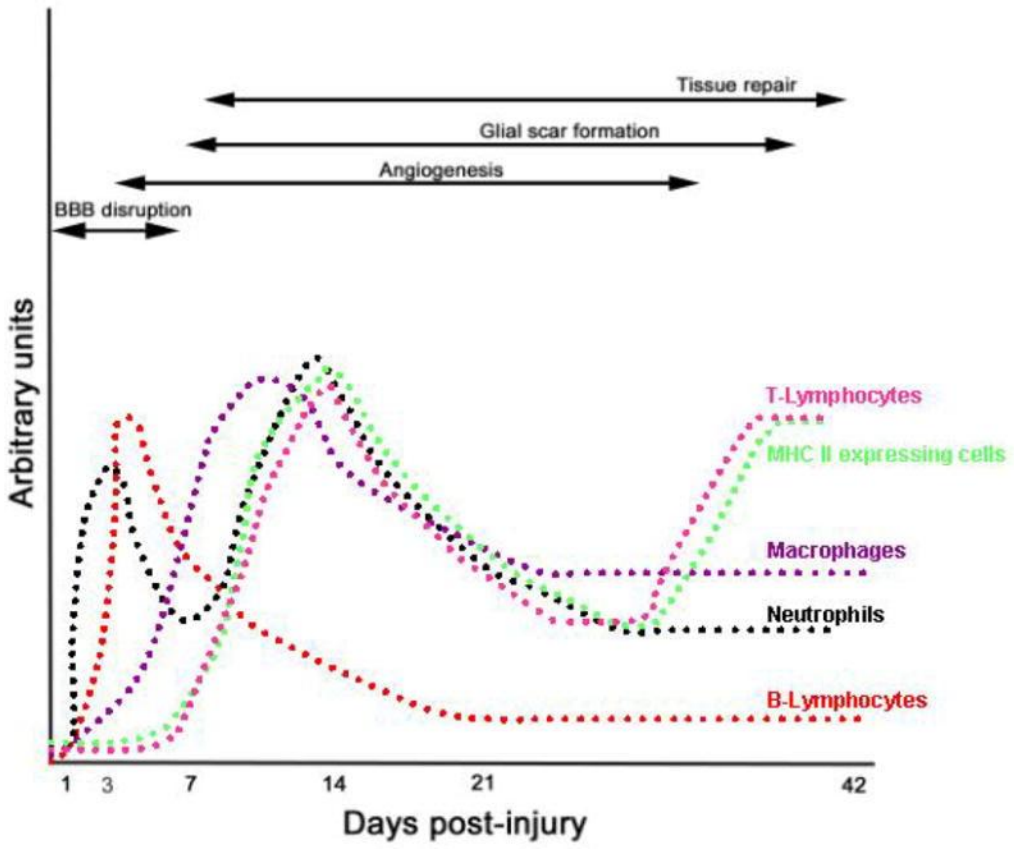


Figure 2: Time-line of the response of various immune cell types after spinal cord injury. Modified from Trivedi et al., 2006.

1.5 Perforin and perforin-deficient mice

1.5.1 Structure and function of perforin

Perforin, also called pore-forming protein (Pfp) or cytolysin, is a glycoprotein of 534 amino acids with sequence homology to the membrane attack component of complement C9 (Shinkai et al., 1988). Perforin has an N-terminal domain with lytic ability, an α-helix amphipathic domain in the middle, and a C-terminal domain. The α-helix regulates the transmembrane insertion and confers the stability required to form pores upon the targeted cell membrane. The C-terminal domain is responsible for calcium-dependent membrane binding (Chávez-Galán et al., 2009).

Perforin is primarily expressed in cytotoxic T-lymphocytes ($CD8^+$) and natural killer (NK) cells and is stored in cytoplasmatic granules of these cells. Its expression seems to be controlled at the transcription level through the interaction between killer cell-specific cis- and trans- acting factors. Perforin appears to be a major mediator

responsible for cytotoxic properties of these cells (Henkart, 1985; Liu et al., 1995; Podack et al., 1991). Therefore, perforin participates in the induction of the rapid, apoptosis-like cell death of cells recognized as aberrant or foreign (Tschopp, 1990; Young, 1989). Additionally, other studies suggested perforin involvement in immunoregulation and homeostasis (Shustov et al., 2000).

After the cytotoxic cell/ target-cell junction perforin is released by exocytosis (Peters et al., 1991) and begins the polymerization in the presence of Ca^{2+} to form cylindrical pores with an internal diameter of 5 to 20 nm (Chávez-Galán et al., 2009; Liu CC et al., 1995). The perforin pores enable the passive invasion of granzymes and granulysin through the target cell membrane, as well as ion exchange, which causes an osmotic imbalance and induces the cell death (Chávez-Galán et al., 2009) (Figure 3). Perforin is encoded by the Pfp gene (PRF1) which extends ca. 15kb along the chromosome 10 (Lu et al., 2003; Walch et al., 1994).

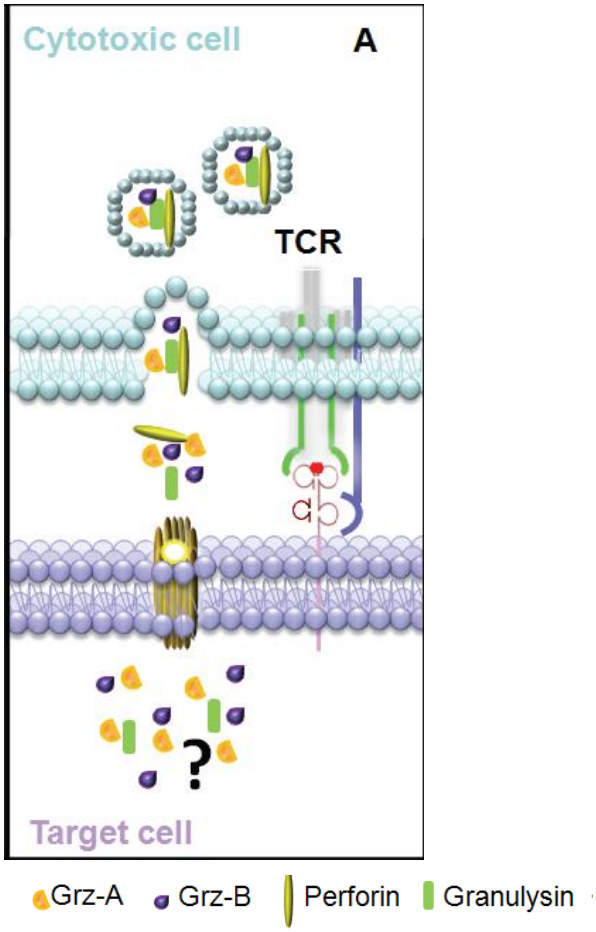


Figure 3: Perforin polymerizes on the target cell membrane forming a pore through which granzymes (Grz)-A, -B, and granulysin enter to induce apoptosis.
Modified from Chávez-Galán et al., 2009

1.5.2 Perforin- deficient mice

Mice completely lacking in perforin are generated by replacing the endogenous perforin genes with nonfunctional gene copies in embryonal stem cells described by Walsh et al. (1994). These mice are viable and fertile. After the infection with lymphocytic choriomeningitis virus (LCMV), there is substantial clonal expansion and activation of $CD8^+$ T-cells and NK cells, which fail to clear the LCMV. Additionally, a reduced efficacy of fibrosarcoma tumor cell elimination was observed in these mice. Perforin is also a key effector molecule for cytolysis of infected fibroblasts by virus-specific $CD8^+$ lymphocytes (Kägi et al., 1994; Walsh et al., 1994).

1.6 V(D)J recombination, RAG-2 gene and RAG-2-deficient mice

The adaptive vertebrate immune system performs its protective role largely through the recognition and elimination of target antigens. Antigen recognition of B- and T-lymphocytes is accomplished through the variable domains of immunoglobulin (Ig) or T-cell receptor (TCR) molecules near their N-termini (Figures 4 and 5). In order to achieve selective specificity for binding to a particular antigen, the immune system creates a large population of receptor molecules that differ in the primary sequence of their antigen-binding regions. Only the enormous diversity of the variable domains allows the immune system to protect the organism against variety of antigens. But this diversity could not be reached by conventional means by which the other gene products are produced in mammalian cells. With only approximately 10^5 genes in the entire genome, the diversity of around 10^{14} for Ig and about 10^{18} for TCR molecules cannot be achieved simply by inheriting genes for each antigen. Furthermore, the adoptive immune system needs an adequate level of plasticity, to meet newly generated treats from variant pathogens that are not recognized by an existing inherited repertoire. Much of this diversity, essential for the proper functioning of the immune system, is generated by the combinatorial recombination at the DNA level (Sadofsky, 2001).

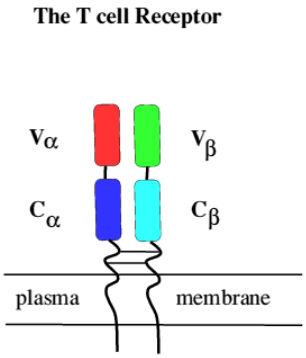


Figure4: The T-cell receptor with 2 paired polypeptide chains (α und β) both of which have constant (c_{α}, c_{β}) and variable portions (v_{α}, v_{β}). Available from http://www.cartage.org.lb/en/themes/Sciences/LifeScience/GeneralBiology/Immunology/Recognition/Tcell/Tcells/Tcells.htm.

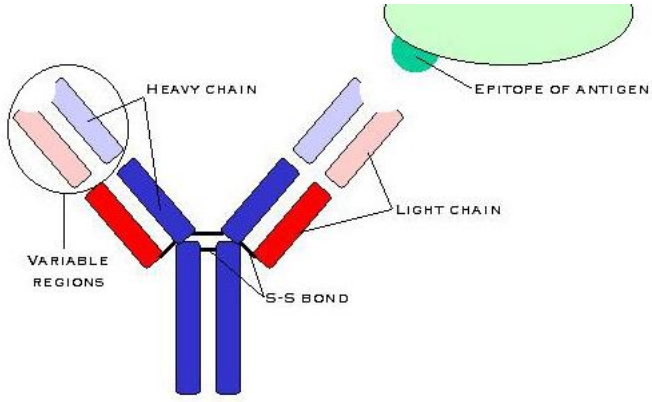


Figure 5: Schematic structure of an IgG immunoglobulin. It contains 2 heavy and 2 light chains bonded by S-S Bond. Each of four chains confirms of constant and variable regions. Available from http://www.pasteur.fr/recherche/unites/ImmStr/en/projects/recognition.html.

1.6.1. V(D)J recombination

V(D)J recombination is one of the main processes that contribute to the diversity among variable regions of T-cell receptors and immunoglobulins. Both TCR and Ig are heterodimers, and each polypeptide is comprised of a variable region and a constant region. The variable portion of one polypeptide in each heterodimer (Ig heavy chain and TCR β or δ chains) is assembled from three elements named 'V', 'D' and 'J' segments. The partner polypeptide (one of the Ig light chains κ or λ, or TCR α or γ) is assembled from V and J segments only. In mice and humans these segments are usually inherited as tandem clusters of related elements (Hansen, 2000).

The V(D)J recombination proceeds by assembling the encoding genes from germline variable (V), diversity (D) and joining (J) gene segments during the early stage of B- and T-lymphocyte differentiation. This mechanism includes cleavage of the DNA at segment boundaries followed by rejoining of particular pairs of the resulting termini. The cuts occur in two steps, first by nicking a particular DNA strand adjacent to the heptamer, followed by cleavage of the second strand to yield a terminal hairpin on the coding DNA. Broken coding ends are covalently sealed in a hairpin structure while signal ends are blunt, 5′-phosphorylated molecules. In the next step the broken molecules are processed and ligated to form signal and coding joints. Signal joints are formed by precise ligation of signal ends in a head-to-head fashion. Coding ends are ligated imprecisely to form coding joints. This process contributes to increasing the variability of the resulting functional genes. A large variability of segments to ligate accounts for a large diversity of the immune response. This DNA ligation step is dependent on proteins participating in the repair of other DNA double-strand breaks, the so-called NHEJ pathway (Akamatsu, 1998; Gellert, 2002; Sadofsky, 2001; Shinkai et al., 1992).

The cleavage is directed to a precise location by the presence of recombination signal sequences (RSSs). The RSS is composed of highly conserved heptamer (CACAGTG) and nonamer (ACAAAAACC) elements separated by an intervening relatively non-conserved spacer of a fixed length. There are two classes of RSSs, which are distinguished by the length of their spacer regions. Spacer length centers on 12 or 23 nt and the resulting signals are referred to as 12-RSS or 23-RSS. The existence of the two classes and their organizing allows avoiding potential pitfalls, for example, recombination of two V segments with each other. The mechanism of recombination incorporates a ‘12/23 rule’ (Tonegawa, 1983), which specifies that recombination is permitted only between two gene segments flanked by RSSs with different spacer lengths. V(D)J recombination is tightly regulated by limiting access to RSS sites within chromatin, so that particular sites are available only in certain cell types and developmental stages.

1.6.2 RAG-2 gene

Multiple processing steps of V(D)J recombination are initiated and mediated by the lymphoid-specific RAG1 and RAG2 (recombination activating gene) proteins,

which combine and form a nuclease. The functions of this nuclease (recombinase) are the recognition of the border of specific sequences (recombination signal sequences, RSSs) and the preparation of double-strand breaks. RAG1 and RAG2 bind specifically to recombination signal sequence, forming a stable protein-DNA complex. This complex requires the conserved heptamer and nonamer motifs of the recombination signal as well as both the RAG1 and RAG2 proteins and is able to either nick or form hairpins at the V(D)J signal sequence, depending on the divalent cation present (Hiom, 1997). The important role of RAG2 in building a stable complex with the RSS and in the regulatory role of binding to a methylated histone has recently been discussed (Schlissel et al., 2009). After cleavage, the complex is destabilized and the RAG proteins dissociate.

The mouse RAG2 protein is 527 residues in length and possesses a core region (1–382) that is sufficient to evoke the complete recombination of test substrates in cells. Sequence analysis has suggested that this active core region of the RAG2 protein is composed of a six-fold repeat of a 50-residue motif which is related to the kelch/mipp motif. This motif, which forms a four-stranded twisted antiparallel beta sheet, is arranged in a circular formation like blades of a propeller. This six-laded propeller structure of the RAG2 active core is supposed to play a crucial role in the tight complex formed by the RAG1 and RAG2 proteins and RSSs. Moreover, the presence of a plant homeodomain finger-like motif in the last quarter of the RAG2 sequence suggests a potential interaction of this domain with chromatin components (Callebaut et al., 1998; Gomez et al., 2000).

The evolutionary most conserved C-terminal quarter of the molecule includes an unusually acidic region and may contribute a regulatory role to the reaction (Gellert, 2002; Sadofsky, 2001). It is reported that the joining of heavy chain V-region to the already assembled DJ-segment in B-cell rearrangement, requires the C-terminal region of RAG2, although it is dispensable for the basic recombination reaction (Kirch et al., 1998).

Mutations in RAG2 as well as in RAG1 can lead to Omenn syndrome, a severe immunodeficiency characterized by the presence of activated, anergic, oligoclonal T-cells, hypereosinophilia and high IgE levels (Callebaut et al., 1998; Villa et al., 1998). RAG2 or RAG1 gene inactivation in mice leads to abortion of the V(D)J rearrangement process, early block in both T- and B-cell maturation and ultimately, to severe combined immune deficiency (SCID) (Corneo et al., 2000).

1.6.3 RAG-2-deficient mice

Mice lacking in large portion of the RAG2 coding region are generated and described by Shinkai et al. (1992). Mice homozygous for this mutation are incapable to generate mature T- or B-lymphocytes as a result of complete inability to initiate the V(D)J recombination. The RAG2 mutation leads to a complete block in B- and T-cells differentiation at an early stage (Shinkai et al., 1992).

RAG2-deficient mice are viable and don't show any detectable phenotype compared to wild-type mice, but because of their inability to develop functional B- and T-cells they have severe combined immune deficiency, and therefore their lifespan is reduced compared to control wild-type collective (Alt et al., 1992).

The thymus tissue of the RAG2 homozygote mice and the number of cells in it are severely reduced in comparison to wild-type mice or heterozygous mutants. The spleens of homozygote mutants are of comparable size to those of wild-type animals, but the number of cells in them is also reduced. The lymph nodes of RAG2-/- mice are hypoplastic. However, all other tissues and organs of RAG2-deficient mice are comparable in structure to those of wild-type animals. Most importantly, no detectable alterations were observed in a variety of tested organs including brain, and no obvious behaviorial differences between RAG2-/- and wild-type mice were observed (Shinkai et al., 1992).

2. Rationale and aims of the study

Numerous studies about the importance of the immune system in the processes of recovery after traumatic spinal cord injury have been inconclusive with regards if the immune system has overall beneficial or adverse impact on regeneration after injury (Beattie, 2004; Bechmann, 2005, 2007; Ibarra et al., 2003; Schwartz and Yoles, 2006; Thuret et al., 2006). To obtain more insights into the role of the immune system upon spinal cord injury, it is essential to compare the immune systems with normal and suppressed function.

The aim of this study was to evaluate the impact of severely compromised immune system on the functional consequences of traumatic spinal cord injury in adult mice. The analysis was performed on mice that are doubly and homozygously deficient in two genes (Pfp and Rag2 gene) in comparison with wild-type mice (n=12 per genotype). These doubly-transgenic mice (Pfp/Rag2-/- mice) on the C57BL/6 inbred background are deficient in T-, B- and NK-cell functions, and can thus be directly compared to the wild-type C57BL/6 mice. The Pfp/Rag2-/- mice have complete ablation of the adoptive immune response (lack of the competent T- and B-cells), and attenuation of the innate immune response (due to lack of the functional NK cells).

The aims of this study were to compare Pfp/Rag2-/- mice and C57BL/6 mice in the following parameters:

1. Recovery of motor functions after spinal cord injury [comparison was performed by single frame motion analysis described by Apostolova et al., (2006)];
2. The body weight loss and regain after spinal cord injury;
3. Lesion scar size, measured from the histological samples;
4. The numbers of immunohistochemically labeled microglia/macrophages in the spinal cord after injury.

3. Materials and Methods

3.1 Animals

Mice used in the experiments included control wild-type C57BL/6J mice obtained from the animal facility of the Universitätsklinikum Hamburg-Eppendorf, as well as Pfp and Rag2 doubly deficient (Pfp/Rag2-/-) mice obtained from the breeding at the animal facility of the Universitätsklinikum Hamburg-Eppendorf, originally founded from breeders obtained from Taconic Farms Inc, Denmark (12 backcrosses to C57BL/6NT background). All used mice were female and were used at the age of 12-15 weeks. Generation of the Pfp/RAG2-/- mice was performed via disruption of the relevant Pfp and RAG2 genes (Walsh *et al.*, 1994; Shinkai *et al.*, 1992). All animals were kept in the specific pathogen-free utility of the Universitätsklinikum Hamburg. Prior to injury all animals appeared healthy and without obvious behavioural abnormalities. Only a slight back fur reduction of the Pfp/Rag2-/- mice compared to wild type mice was observed.

All treatments of the animals were performed in accordance to the German laws on the protection of experimental animals, and the procedures were approved by the responsible committee of the State of Hamburg (Experimental animal project number G09/098). All experiments, collection of data and data analyses were preformed in a blinded fashion. Numbers of animals studied in different experimental groups and at different times after surgery are given in the Table 1.

	C57BL/6J	**Pfp/Rag2-/-**
Experiment 1	12	15
Experiment 2	12	12
Overall	24	27

Table1: Number of animals of different genotype and different experiments.
Experiment 1: locomotor analyses were performed before and 1, 3, 6 weeks after injury. Experiment 2: locomotor analyses were performed before and 1, 3, 6, 8, 10 weeks after injury.

3.2 Surgical procedures

For surgery, the mice were anesthetized by intaperitoneal injections of ketamine and xylazine [100 mg of Ketanest (Parke-Davis/Pfizer, Karlsruhe, Germany) and 5 mg of Rompun (Bayer, Leverkusen, Germany), per kilogram of bodyweight]. The back of the animal was shaved and cleaned with an antiseptic solution. Eye ointment was applied to the protect eyes from drying during anaesthesia. A dorsal incision was made through the skin and underlying tissue was removed to expose the vertebral bone plate. Laminectomy was performed at the T7-T9 level with mouse laminectomy forceps (Fine Science Tools, Heidelberg, Germany) to expose the spinal cord. A mouse spinal cord compression device was used to elicit compression injury (Curtis et al., 1993; Steward et al., 2003). The device consisted of a pair of forceps mounted in a stereotaxic frame. The spinal cord was maximally compressed (100%, according to the operational definition of Curtis et al. (1993) for 1 second by time-controlled current flow through the electromagnetic device. After injury the skin was surgically closed using 6-0 nylon stitches (Ethicon, Norderstedt, Germany).
Postoperatively the mice were kept warm for 24 hours to prevent hypothermia. After them the animals were provided with water and standard food *ad libitum*. During the postoperative period manual bladder voiding was performed once daily.

3.3 Analysis of motor function

3.3.1 Locomotor rating

The recovery of ground locomotion was evaluated using the Basso, Beattie, Bresnahan (BBB) rat rating scale (Basso et al., 1995) as modified for the mouse by Joshi and Fehlings (2002). The observations were performed by one and the same investigator. Motor ability of each animal was evaluated during open field locomotion and rated according to the scale (Figure 6). The rating was later confirmed by the analysis of video recordings of beam walking. Estimations were performed before and 1, 3, 6, 8 and 10 weeks after injury. Values for the left and right extremities were averaged.

TABLE 2. THE 21-POINT BASSO, BEATTIE, BRESNAHAN LOCOMOTOR RATING SCALE AND OPERATIONAL DEFINITIONS OF CATEGORIES AND ATTRIBUTES

0	No observable hindlimb (HL) movement
1	Slight movement of one or two joints, usually the hip and/or knee
2	Extensive movement of one joint or extensive movement of one joint *and* slight movement of one other joint
3	Extensive movement of two joints
4	Slight movement of all three joints of the HL
5	Slight movement of two joints *and* extensive movement of the third
6	Extensive movement of two joints *and* slight movement of the third
7	Extensive movement of all three joints of the HL
8	Sweeping with no weight support or plantar placement of the paw with no weight support
9	Plantar placement of the paw with weight support in stance only (i.e., when stationary) or occasional, frequent, or consistent weight supported dorsal stepping and no plantar stepping
10	Occasional weight supported plantar steps, no forelimb (FL)–HL coordination
11	Frequent to consistent weight supported plantar steps *and* no FL–HL coordination
12	Frequent to consistent weight supported plantar steps *and* occasional FL–HL coordination
13	Frequent to consistent weight supported plantar steps *and* frequent FL–HL coordination
14	Consistent weight supported plantar steps, consistent FL–HL coordination; *and* predominant paw position during locomotion is rotated (internally or externally) when it makes *initial contact* with the surface as well as just before it is *lifted off* at the end of stance or frequent plantar stepping, consistent FL–HL coordination, and occasional dorsal stepping
15	Consistent plantar stepping and consistent FL–HL coordination; *and* no toe clearance or occasional toe clearance during forward limb advancement; predominant paw position is parallel to the body at initial contact
16	Consistent plantar stepping and consistent FL–HL coordination during gait; *and* toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off
17	Consistent plantar stepping and consistent FL–HL coordination during gait; *and* toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact *and* lift off
18	Consistent plantar stepping and consistent FL–HL coordination during gait; *and* toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off
19	Consistent plantar stepping and consistent FL–HL coordination during gait; *and* toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact *and* lift off; and tail is down part or all of the time
20	Consistent plantar stepping and consistent coordinated gait; consistent toe clearance; predominant paw position is parallel at initial contact and lift off; tail consistently up; *and* trunk instability
21	Consistent plantar stepping and coordinated gait, consistent toe clearance, predominant paw position is parallel throughout stance, consistent trunk stability, tail consistently up

Definitions

Slight: partial joint movement through less than half the range of joint motion
Extensive: movement through more than half of the range of joint motion
Sweeping: rhythmic movement of HL in which all three joints are extended, then fully flex and extend again; animal is usually sidelying, the plantar surface of paw may or may not contact the ground; no weight support across the HL is evident
No Weight Support: no contraction of the extensor muscles of the HL during plantar placement of the paw; or no elevation of the hindquarter
Weight Support: contraction of the extensor muscles of the HL during plantar placement of the paw, or elevation of the hindquarter
Plantar Stepping: The paw is in *plantar* contact with weight support then the HL is advanced forward and *plantar* contact with weight support is reestablished
Dorsal Stepping: weight is supported through the dorsal surface of the paw at some point in the step cycle
FL–HL Coordination: for every FL step an HL step is taken and the HLs alternate
Occasional: less than or equal to half; ≤50%
Frequent: more than half but not always; 51–94%
Consistent: nearly always or always; 95–100%
Trunk Instability: lateral weight shifts that cause waddling from side to side or a partial collapse of the trunk

Figure 6: The BBB score, designed by Basso, Beattie and Bresnahan, for evaluation of the locomotor behavior of rats after SCI injury. It is a method based on detailed descriptive observation of the movements, mostly of the walking behavior of the rats. The animals are scored according there walking abilities with scores ranging from 0 to 21 (Basso et al., 1995).

3.3.2 Single frame motion analysis

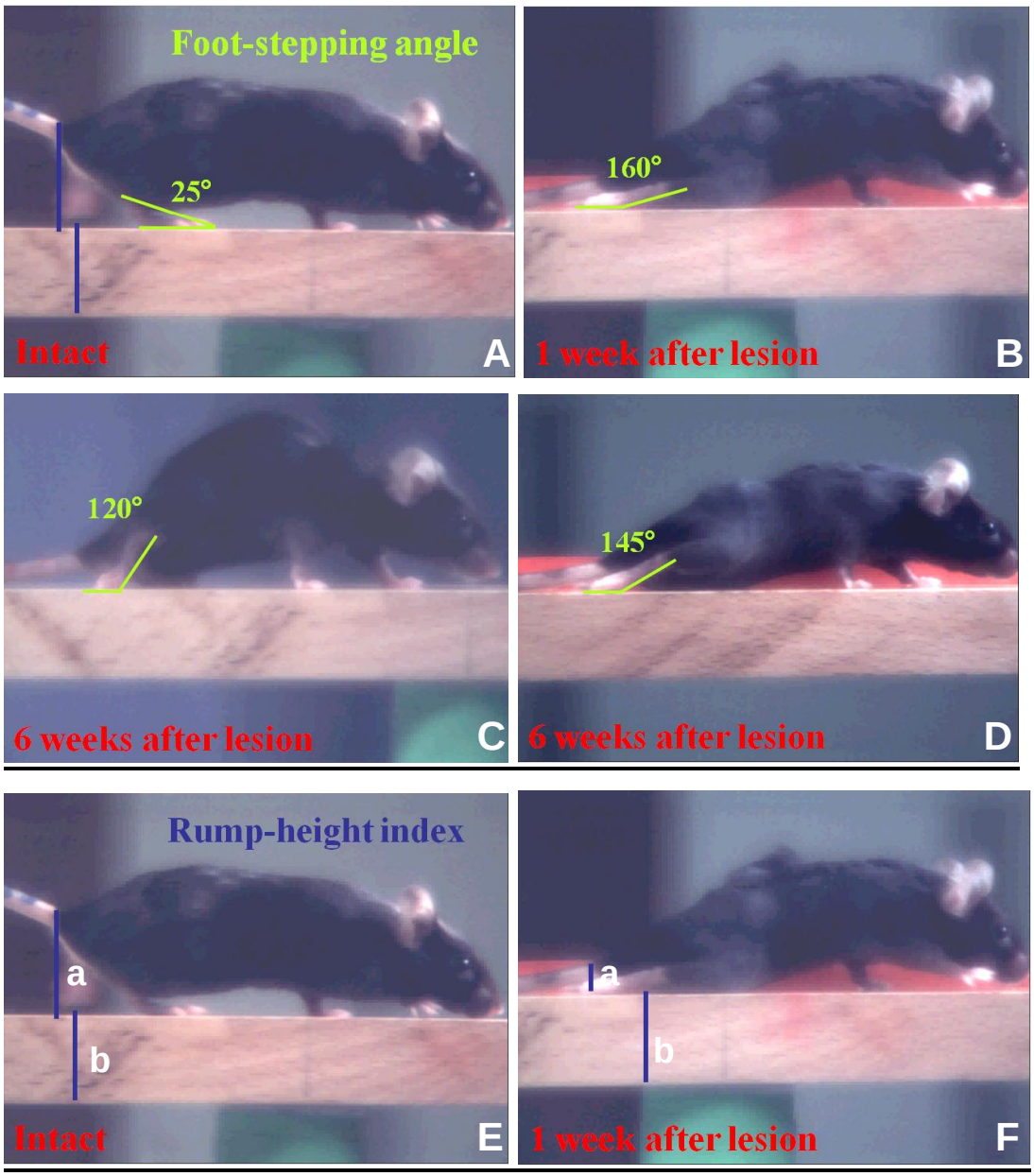
For the single frame motion analysis described by Apostolova et al. (2006) two different video tasks, beam walking and pencil grabbing, were recorded via a Panasonic VDR-D150EG DVD-Video-camera (Panasonic Deutschland, Hamburg, Germany). The recordings were performed before and 1, 3, 6, 8 and 10 weeks after spinal cord compression. The video sequences were converted into AVI format with Super© v2007.23 video converter (free software available at http://www.erightsoft.com) and analyzed with VirtualDub software, a video capture/processing utility written by Avery Lee (free software available at http://www.virtualdub.org). Selected frames at defined phase of locomotion were used for measurements performed with UTHSCSA Image Tool 2.0 software (University of Texas, San Antonio, TX, USA, http://ddsdx.uthscsa.edu/dig/). For estimates of two parameters, foot stepping angle (FSA) and rump-height index (RHI), beam-walking task was analyzed. Mice were trained to perform this test one day before recording. In the test the mouse runs spontaneously along a horizontal beam towards its home cage located at the end of the beam. The right and left side of each animal during two consecutive walking trials are filmed.

The first parameter, the foot stepping angle is a numerical estimate allowing objective assessment of the plantar stepping ability, one of the major behavioural aspects assessed by the BBB score and is defined by a line parallel to the dorsal surface of the hind paw and a horizontal line (Figure 7). The angle is measured at the beginning of the stance phase. In uncompressed mice this phase is well defined and the angle is smaller than 20°. After spinal cord compression the angle is increased to more than 150° and the animal drag behind their hind limbs with dorsal paw surfaces to beam surface. The values for the left and right side are averaged.

The second parameter, rump-height-index is defined as height of the rump, i.e. the vertical distance from the dorsal aspect of the animal's tail base to the beam, divided by the thickness of the beam measured along the same vertical (Figure 7). Three measurements per attempt and mouse are performed and averaged. This parameter is a numerical estimate of the ability to support the body weight.

The third parameter, the limb extension/flexion ratio was evaluated from the pencil grabbing recordings. An intact mouse, when held by its tail and allowed to grasp a pencil with its forepaws, tries to catch the object with its hind paws and

carries out cycling extension/flexion movements (Figure 7). Left and right side are filmed and three measurements for each animal and time point are averaged. The extension and flexion length is defined as the distance from the most distal mid-point of the paw to a fixed point of the animal's body, e.g. the tail base. The extension/flexion ratio is a numerical estimate of ability to initiate and carry out voluntary, non-weight-bearing movements.



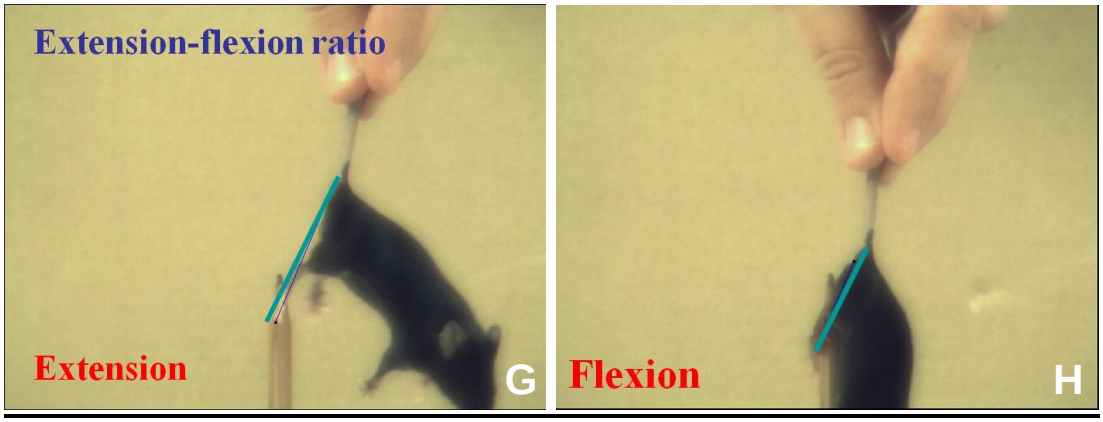


Figure 7: Single-frame motion analysis. Single frames of video sequences recorded during beam walking (*A-D*) and voluntary movements without body weight support ("pencil test", *G-H*) of mice subjected before (*A, B*) or after (C, D) spinal cord compression. The lengths of the distances a and b were used to calculate the rump-height index (E, F). Panels *G* and *H* show a phase of maximum flexion (*H*) immediately followed by an extension (*G*). The lengths of the lines drawn in the pictures were used to determine the extension-flexion ratio.

3.3.3 Recovery indices

The recovery index is an individual estimate for any given parameter described above and is calculated in percent as:

$$RI = [(X_{7+n} - X_7)/(X_0 - X_7)] \times 100,$$

where X_0, X_7 and X_{7+n} are values before, 7 days and a time point of n days after the surgery, respectively.

In other words, these assessments reveal size of a regain-of-function as a fraction of a loss-of-function induced by the surgery. These estimates are meaningful and comprehensive parameters allowing better comparisons within one investigation and between the results of different laboratories (Apostolova et al., 2006). Besides calculation of the recovery index is absolutely necessary if a parameter is prone to variability as a result of individual variability in body constitution and behavioural traits.

3.4 Preparation of tissue

Ten weeks after spinal cord injury mice were anesthetized with sodium pentobarbital (Narcoren®, Merial, Hallbermoos, Germany, 5 µl g^{-1} body weight, i.p.) and transcardially perfused with physiologic saline for 1 min followed by 4% formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, for 15 min. The cervical spinal cords were removed, post-fixed overnight in the same fixative at 4°C and cryoprotected by immersion in 15% sucrose solution in 0.1M cacodylate buffer, pH 7.3, for 2 days at 4°C. The tissue was frozen for 2 minutes in 2-methyl-butane (isopentane) pre-cooled to -30°C in the cryostat. Serial sagittal or coronal sections of 25 µm thickness were obtained from the cervical spinal cord on a cryostat (Leica CM3050, Nussloch, Germany). Sections were collected on SuperFrost®Plus glass slides (Roth, Karlsruhe, Germany) so that 6 sections 250 µm apart from each other were present on each slide.

3.5 Histology and immunohistochemistry

3.5.1 Immunohistochemical stainings

Immunohistochemical stainings were performed as described by Irintchev et al. (2005) on 25 µm thick sections. Sections, stored at -20°C, were air-dried for 30 minutes at 37°C. A 10 mM sodium citrate solution (pH 9.0, adjusted with 0.1 M NaOH) was prepared and preheated in a jar to 80°C in a water bath. The sections were incubated at 80°C for 30 minutes and afterwards the jar was taken out and left to cool down at room temperature. Afterwards, blocking of the unspecific binding sites for the secondary antibody was performed. The sections were incubated at room temperature (RT) for one hour in PBS containing 0.2% Triton X-100 (Fluka, Buchs, Germany), 0.02% sodium azide (Merck, Darmstadt, Germany) and 5% normal goat or donkey serum (Jackson Immuno Research Laboratories, Dianova, Hamburg, Germany). The selection of normal serum for blocking was determined by the species in which the secondary antibody was produced (see below). After one hour the blocking solution was aspired and the slices were incubated with the primary antibody, rabbit anti-ionized calcium binding adaptor molecule 1 (Iba-1), diluted in PBS containing 0.5% lambda-carrageenan and 0.02% w/v sodium azide in PBS. The

slides were incubated for 3 days at 4°C in a screw-cap staining jar (30 ml capacity, Roth). Following this, the sections were washed 3 times in PBS (15 minutes each) before an appropriate secondary antibody was applied. The sections were incubated with the second antibody diluted (1:200) in PBS-carrageenan at RT for 2 hours. Goat anti-rabbit IgG conjugated with Cy3 (Jackson Immuno Research Laboratories, Dianova, Hamburg, Germany) was used as a secondary antibody. After a subsequent wash in PBS, cell nuclei were stained for 10 minutes at RT with bis-benzidine solution (Hoechst 33258 dye, 5 µg/ml in PBS, Sigma). Finally, the sections were washed again, mounted with Fluoromount G (Southern Biotechnology Associates, Biozol, Eching, Germany) and stored in the dark at 4°C.

We used anti-Iba-1 (rabbit polyclonal, affinity purified, Wako Chemicals, Neuss, Germany, 1:1500 0.3) as a macrophage/microglia-specific cell-marker. Iba1 is a macrophage/microglia-specific calcium-binding protein involved in the activation of quiescent microglial cells, and thus expressed in both activated and quiescent cells, but to a higher degree upon activation (Imai and Kohsaka 2002).

3.5.2. Estimation of lesion scar volume

Estimation of lesion scar volume was performed using 25µm thick sections stained with cresyl violet. Cresyl violet (Nissl stain) shows cell bodies and proximal dendrites of neurons. Sections were dehydrated in an ascending series of ethanol (70%, 95% and 100%, 2 minutes each) and stained in 0.5% w/v cresyl violet acetate (Sigma, Deisenhofen, Germany) solution in 0.1 M acetate buffer, pH 4.0, for 20 minutes followed by washing in distilled water and 70% ethanol (1–2 minutes each). The differentiation was done in cresyl violet differentiator (90ml 95% ethanol, 10ml chloroform, 3 drops glacial acetic acid) for 1-2 seconds. The slides were then immersed in 95% ethanol and absolute ethanol for 2 minutes each and finally cleared in Roticlear (Roth, Karlsruhe, Germany). The differentiation was checked under the microscope to ensure that only nuclei and Nissl substance were stained and the differentiation was repeated, if necessary. Finally the sections were mounted under coverslips with Entellan neu (Merck, Darmstadt, Germany).

Estimations of the scar volume were performed using the Cavalieri's principle (Howard and Reed 1998). Areas of the scar required for volume estimation were measured directly under the microscope using the Neurolucida software (see above).

3.6 Photographic documentation

Photographic documentation was made on an Axiophot 2 microscope equipped with a digital camera AxioCam HRC and AxioVision software (Zeiss). The digital images were additionally processed using Adobe Photoshop 8.0 software (Adobe Systems Inc., San Jose, CA).

3.7 Statistical analysis

Statistical analysis of the motor recovery and recovery of weight loss over time was done by the one-way analysis of variance (ANOVA), followed by the honest Tukey's *post hoc* test. Statistical analysis of the histological and immunohistological studies was restricted to simple comparison of mean values of same genotypes, using the two-sided *t* test for independent groups. By two or more measurements per parameter and animal, the mean value was used as a representative value. Thus, for all comparisons the degree of freedom was determined by the number of animals. The accepted level of differences between groups was 5%. All statistical analyses were performed using the IBM SPSS Statistics 11.5 software (IBM Corporation, Somer, NY).

4. Results

4.1 Changes in the body weight of Pfp/Rag2-/- and wide-type mice after spinal cord injury

The body weight of the both mice groups was estimated as a parameter of the general conditions before and at various time points after the injury. Before spinal cord injury, there was no difference in the average body weight between Pfp/Rag2-/- mice and wild-type controls. Upon injury, there was a prominent, approximate 20% weight loss in both experimental groups, with Pfp/Rag2-/- mice losing slightly, but significantly more weight compared with the wild-type mice. During the course of recovery both groups gained some weight, but the difference in the body weight between the groups increased, to reach approximately 10% at 10 weeks after injury (Figure 8).

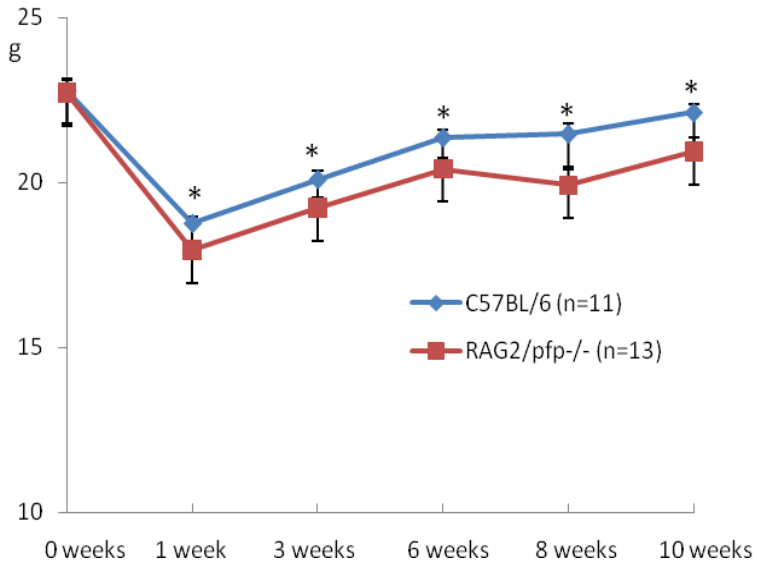


Figure 8: *Changes in the body weight of Pfp/Rag2-/- mice and wild-type controls after spinal cord injury*. Average body weight in Pfp/Rag2-/- and C57BL/6 mice before (0 weeks) and at various time-points after spinal cord compression. Asterisks indicate significant difference between the groups ($p < 0.05$; Two-way ANOVA with Tukey's *post hoc*).

4.2 Functional recovery after spinal cord injury

For evaluation of the functional recovery after spinal cord injury in wild-type and Pfp/Rag2-/- mice the measurement of BBB score as well as the single frame motion analysis were used. The investigations were performed before and 1, 3, 6, 8 and 10 weeks after spinal cord compression in both experimental groups.

4.2.1. BBB score

The Basso, Beattie, Bresnahan (BBB) rating scale (Basso et al., 1995) was used for estimation of ground locomotion recovery. This scale evaluates both simple and more complex aspects of the ground locomotion, like plantar stepping abilities, limb coordination and trunk stability. The observations were performed by one and the same investigator before and 1, 3, 6, 8 and 10 weeks after spinal cord injury. The pre-operative averaged values for the left and right extremities were similar in the both wild-type as well as double knock out mouse groups. After the induced spinal cord trauma the differences between the group mean values were found at all five time-points at which the mice were studied (1, 3, 6, 8 and 10 weeks, Fig. 9A), although the significant difference was found only at the four time points: 1, 3, 8 and 10 weeks after the compression. The degree of functional recovery as assessed by this parameter was significantly higher in Pfp/Rag2-/- mice than in controls both early (7 days upon injury), and to a higher degree at later time points, including the last time-point 10 weeks after injury, when the average BBB score value was 9.1 ± 0.8 in Pfp/Rag2-/- mice and 5.6 ± 0.7 in wild-type controls.

4.2.2 Foot-stepping angle

Foot–stepping angle is one of the parameters of single frame motion analysis. This angle is a numerical measure of the plantar stepping ability, and with this respect its values closely follow the BBB score values (Apostolova et al., 2006; Jakovcevski et al., 2007). The observations were performed by one and the same investigator before and 1, 3, 6, 8 and 10 weeks after spinal cord injury. Ten animals per group were analyzed. Three measurements per animal from each side were performed. The values for the left and right side were averaged.

Preoperative values in the Pfp/RAG2-deficient as well as wide type mice corresponded to those previously reported for uncompressed mice (Apostolova et al., 2006) and were around 20° (Figure 9). The measurement one week after the compression injury revealed rapid increase of the angle in the both experimental groups. The values reduced in following measurements at 3, 6, 8 and 10 weeks after the surgery, and lower values were measured in Pfp/Rag2-/- mice than in controls. However, the significant difference for the degree of functional recovery between the genotypes was found only for the measurement after one week.

Rump-height index

The next parameter of single frame analysis is rump-height-index. This index is a numerical estimate for ability to support the body weight during the ground locomotion. Rump-height index is defined as the vertical distance from the dorsal aspect of the animal's tail base to the beam, divided by the thickness of the beam measured along the same vertical. Two or three measurements per attempt and mouse were performed and averaged. No significant difference was found between the values of both mice groups prior to compression. Evaluations of values after spinal cord injury revealed differences between the groups (higher values in Pfp/Rag2-/- mice) at all time points (1, 3, 6, 8 and 10 weeks after injury), but only differences at 8 and 10 weeks after the injury were statistically significant (one-way ANOVA for repeated measurements, with Tukey's *post hoc* correction).

Extension/flexion ratio

The last parameter observed during the single frame analyze was extension/flexion ratio. Extension/flexion ratio is a measure of the ability to initiate and perform voluntary movements without body weight support. The estimation of the extension/flexion ratio showed no significant difference between the wild-type and Pfp/Rag2-deficient mice.

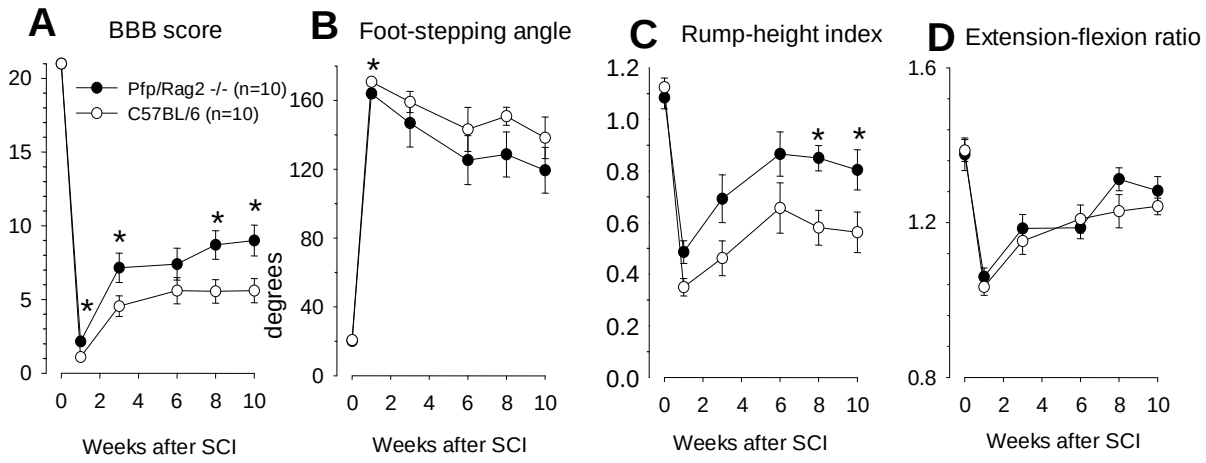


Figure 9: Time course and degree of functional recovery in Pfp/Rag2-/- mice and wild-type (C57BL/6) mice after severe spinal cord compression (SCI). Shown are mean values ± SEM of open-field locomotion (BBB) scores (A), foot-stepping angles (B), rump-height indices (C), and extension/flexion ratios (D) before surgery (day 0) and at 1, 3, 6, 8 and 10 weeks after injury. Numbers of mice studied per group are given in A. Asterisks indicate significant differences between group mean values at a given time period ($p < 0.05$, one-way ANOVA for repeated measurements with Tukey's *post hoc* test).

Relative measurements of functional recovery

The recovery index is an individual estimate of motor recovery and is calculated as:

$$RI = [(X_{7+n} - X_7)/(X_0 - X_7)] \times 100,$$

where X_0, X_7 and X_{7+n} are values for the given parameter before, 7 days, and at a time-point 7+n days after the surgery, respectively. This parameter allows better comparisons within one investigation and between the results of different laboratories (Apostolova et al., 2006).

Figures 10 A-D show recovery indices of double deficient and wild-type mice for BBB score, foot-stepping angle, rump-height index and extension/flexion ratio at 3, 6, 8 and 10 weeks after spinal cord compression. A significant difference of recovery degree was observed by BMS, foot-stepping angle and rump-height-index at 8 and 10 weeks after the injury (Figure 10A-C). During evaluation of extension/flexion ratio recovery index afforded no significant evidence (Figure 10D).

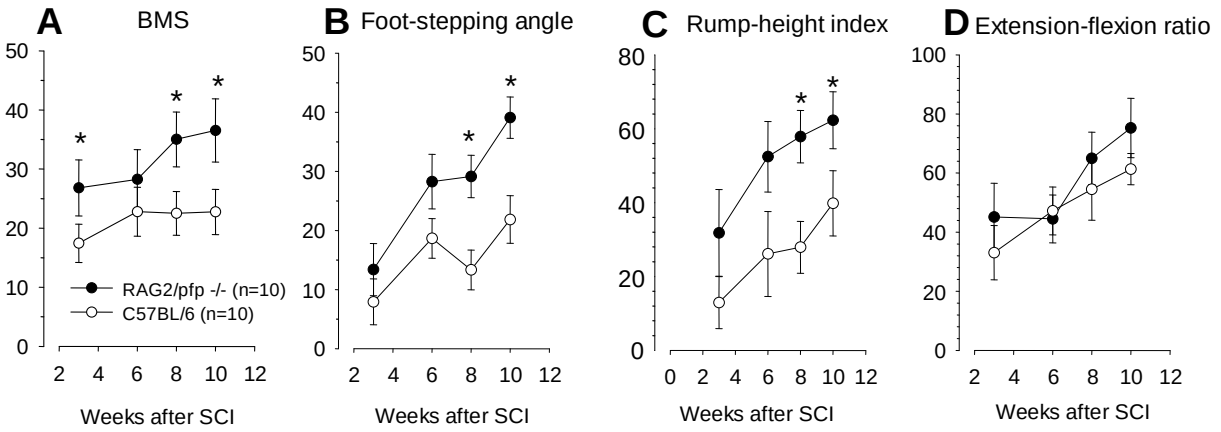


Figure 10: Recovery indices in Pfp/Rag2-/- mice and wild-type (C57BL/6tac) mice after severe spinal cord compression injury (SCI). Shown are mean values ± SEM of open-field locomotion (BMS) scores (A), foot-stepping angles (B), rump-height indices (C), and extension/flexion ratios ("pencil grab test") (D) recovery indices at 3, 6, 8 and 10 weeks after injury. Recovery indices are relative measurements of functional recovery that allow better comparison between groups. They are calculated for each parameter from the values before injury (V_0), values at 7 days after injury (V_7) and values at any later time-point (n) after injury (V_{7+n}) according to formula: $RI = [(V_{7+n} - V_7)/(V_0 - V_7)] \times 100$. Numbers of mice studied per group are given in A. Asterisks indicate significant differences between group mean values at a given time period ($p < 0.05$, one-way ANOVA for repeated measurements with Tukey's *post hoc* test).

4.3 Steriological analysis of spinal cord

4.3.1 Microglial cells after spinal cord injury

Iba-1 is a macrophage/microglia-specific calcium-binding protein involved in the activation of quiescent microglial cells (Imai and Kohsaka, 2002). As a result of this activation, the protein expression is enhanced and is observed at high levels in activated microglia. This allows reliable evaluation of both resting and activated microglial cells (Imai and Kohsaka, 2002).

The densities of $Iba1^+$ microglia cells were evaluated in Pfp/Rag2-C57BL/6tac doubly deficient mice and wild-type (C57BL/6tac) mice 10 week after spinal cord injury. No significant difference was found between the experimental groups, although there was a tendency towards lower numbers in Pfp/Rag2-/- mice in all regions studied (Figure 11).

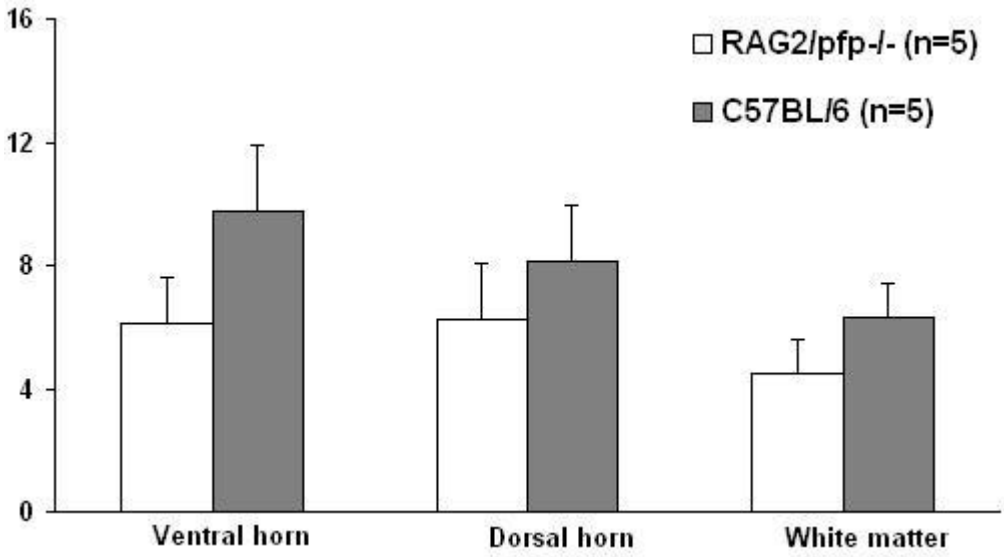


Figure 11: Densities of Iba-1^{+} microglia cells in Pfp/Rag2-/- mice and wild-type (C57BL/6tac) mice 1 week after spinal cord injury. Shown are mean values + SEM. There were no significant differences between group mean values ($p > 0.05$, two-tailed *t*-test).

Lesion scar volume after spinal cord injury

Lesion scar volume was measured based on the Cavalieri's principle (Howard and Reed, 1998) and using 25 µm thick sections stained with cresyl violet. The investigation was performed 10 weeks after spinal cord compression. In comparison with the wild-type animals the lesion scar volume was in Pfp/Rag2-/- mice reduced approximately to the half wild-type values (Figure 12).

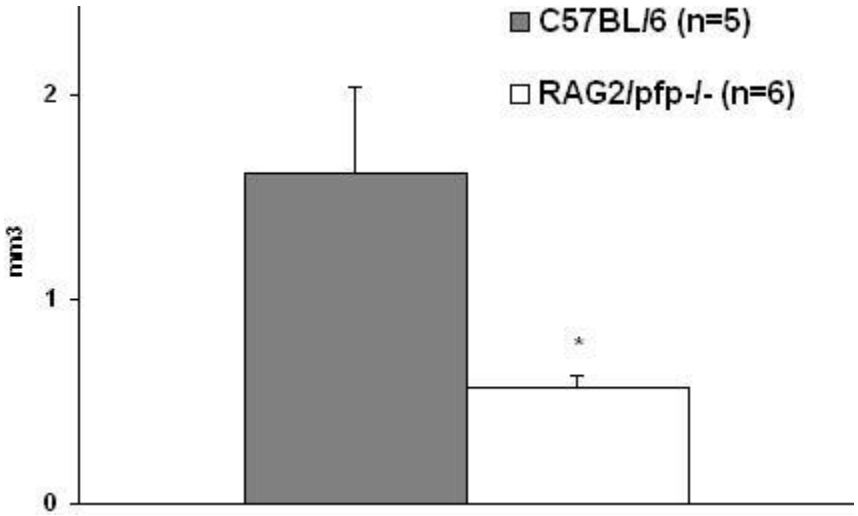


Figure 12: Lesion scar volume in Pfp/Rag2-/- mice and wild-type (C57BL/6tac) mice 10 weeks after spinal cord injury. Shown are mean values + SEM. Asterisk indicates significant differences between group mean values ($p < 0.05$, two-tailed t-test).

5. Discussion

Spinal cord injury is a severe neurological disorder with complex pathophysiology, involving primary damage due to the impact of injury, and secondary, remote damage, due to cellular and molecular responses of the tissue to injury (Fehlings and Nguyen, 2010). The involvement of various components of the immune system in the regeneration after spinal cord injury has been studied in various experimental paradigms, often with different outcomes (for recent reviews see, Ankeny and Popovich, 2009; David and Kroner, 2011). Our study was designed to assess the overall effect of a complete lack of the adaptive immune response on regeneration after spinal cord injury. We demonstrate that the mice lacking functional T-, B- and NK-lymphocytes show better recovery of motor function after spinal cord injury, decreased scaring, higher weight loss and not significantly lower number of microglia compared to the control mice.

5.1 Enhanced recovery of motor function in Pfp/Rag2-/- mice

The improved overall outcome after spinal cord injury in Pfp/Rag2-/- mice is reflected by improvement of motor functions, in particular plantar stepping function essential for ground locomotion. Plantar stepping, evaluated by the BBB score and the foot-stepping angle, requires low levels of supraspinal control. The rump-height index, a parameter estimating the abilities for coordinated and rhythmic activation of muscles working at different joints as well as the extension–flexion ratio, a parameter estimating the ability to initiate and perform voluntary movements without body weight support, were similar between the Pfp/Rag2-/- and control mice. These observations suggest that axonal regrowth/sprouting across the lesion site as well as reestablishment of synaptic connections, direct or indirect, with higher motor control centers, happen to a similar degree in wild-type and Pfp/Rag2-/- mice.

Previous studies on mice deficient for various components of the immune system resulted in observations that, at first sight, were not easily compatible with each other. Most likely depending on different experimental settings, immune-deficient mice would recover better or worse than their wild-type controls. Even within the same study, in mice lacking CD74 (a membrane protein functioning as a MHC II chaperone) which are arrested in development of B-lymphocytes, recovery after

spinal cord injury was background strain-dependent. CD74-deficient mice on a C57BL/6J background recovered better than their wild-type controls, whereas the opposite was true for the same mice on the BALB/c strain (Schori et al., 2007). The latter study demonstrated that B-cells play an important role in damaged spinal cord. In our study we used mice on the C57BL/6 background and confirmed that simultaneous B-, T- and NK-cell ablation is beneficial for recovery. Thus, it is likely and not unexpected that the genetic repertoire plays an important role in the influence of the immune system on recovery. Of note is that the impact of Pfp/Rag2 deficiency on motor recovery in our study was relatively modest, especially in the view of comparatively more robust decrease in tissue scaring in Pfp/Rag2-/- mice. It is thus possible to speculate that motor recovery in these mice reflects combined effects of various mechanisms, some of which might have beneficial and some detrimental influence to the overall outcome.

Thorough investigation of B cell responses to spinal cord injury has previously demonstrated that upon injury B-lymphocytes proliferate and form germinal centers in the spleen, as well as clusters of cells in the injured spinal cord tissue reminiscent of lymphoid follicles (Ankeny et al., 2006). Further study of the same group has shown that mice lacking mature B-lymphocytes regenerate better after spinal cord injury, emphasizing the damaging effect of the autoantibodies produced upon spinal cord injury in wild-type mice (Ankeny et al., 2009). It is noteworthy that in another model of CNS injury, aseptic cerebral injury, Rag1-deficient mice lacking T and B lymphocytes showed an attenuated response to acute injury compared to controls (Fee et al., 2003).

5.2 Altered cellular response to injury in the lumbar spinal cord of Pfp/Rag2-/- mice

A recent study has indicated that there is a multi-phasic response of immune cells at the site of spinal cord injury in rats: the early phase of T-cell infiltration peaking at 9 days post-injury and the late phase, peaking at about 2 months after injury (Beck et al., 2010). Notably, the first phase of the cellular infiltration correlates with the active spontaneous recovery of motor function, whereas during the second peak of T-lymphocyte infiltration a plateau in recovery is already reached (Beck et al., 2010). As both adaptive and innate components of the immune system are activated upon

injury, we investigated if the components of the innate immune response were affected by the Pfp/Rag2 mutations. In the injured central nervous system, microglia/macrophages play a prominent role not only at the site of injury, but also remote to the injury site (Popovich et al., 1997; Watanabe et al., 1999; Windelborn and Mitchell, 2012). Among the possible roles of microglia/macrophages in the lumbar spinal cord after lower thoracic injury is the stripping and remodeling of synapsis (Popovich et al., 1997). The functional interplay between T-lymphocytes and microglia/macrophages is well-documented (David and Kroner, 2011; Hausmann, 2003). Activated T-lymphocytes secrete cytokine signals that activate macrophages in two possible directions: interferon-γ, secreted by T-helper 1 lymphocytes, induces macrophages to produce pro-inflammatory cytokines (interleukin-1β, tumor necrotic factor α), whereas interleukin-4, secreted by T-helper 2 lymphocytes induces macrophages to express MHCII molecules and reduce reactive oxygen and nitrogen species production (David and Kroner, 2011). It is thus conceivable that a complete ablation of T-cell function would lead to a reduced microglia/macrophage response to injury. Previous studies on the influence of microglia/macrophages on recovery after injury has been inconclusive, revealing both pro-inflammatory and neuroprotective roles of microglia. While some view microglia/macrophages as integral components of the regenerative response, others emphasize their contribution to delayed neuronal apoptosis and demyelination in secondary damage response after injury (Ankeny and Popovich 2010; David and Kroner, 2011; Streit, 2002). Activated microglia/macrophages also secrete extracellular matrix components, such as keratin, dermatan and chondroitin sulfate proteoglycans which have an overall negative impact on axonal regeneration (Grimpe and Silver, 2002; Jones and Tuszynski, 2002; own unpublished data). Mice deficient in osteopontin, which is expressed by microglia at the site of injury, show less inflammation, but greater tissue damage and impaired motor recovery (Hashimoto et al., 2007). Recent studies demonstrated a contribution of dorsal horn microglia/macrophages to neuropathic pain following injuries to the central and peripheral nervous systems (Gwak et al., 2012; Tsuda et al., 2011). It is likely that absence of functional T-lymphocytes in our study modifies the microglia/macrophages phenotype from a damage-inducing towards a neuroprotective one.

5.3 Possible mechanisms behind better functional recovery of Pfp/Rag2-/- mice

Because spinal cord injury is associated with considerable demyelination and given that myelin reactive antibodies are elevated following injury of the central nervous system (Mizrachi et al., 1983; Palladini et al., 1987), it is tempting to hypothesize that the beneficial effect of the Pfp/Rag2 ablation on motor recovery could be mediated by the promotion of re-myelination and/or increased sparing of myelin. Given the fact that anti-myelin antibodies and myelin-activated T-lymphocytes, including those activated by myelin basic protein (MBP; Popovich et al., 1996), are elevated after spinal cord injury, it is plausible to hypothesize that they contribute to myelin damage. When T-cells are manipulated into a bias towards MBP-reactivity in transgenic mice, the outcome of the spinal cord injury was worse, thus indicating that this particular subpopulation of T-cells causes a neuroinflammatory response to myelin (Jones et al., 2005). Additionally, vaccination against neural antigens of the central and peripheral nervous systems exacerbated symptoms after facial nerve injury in mice (Ankeny and Popovich, 2007). Conversely, passive or active immunizations against MBP also have led to an improvement after central nervous system injuries in mice (Hauben et al., 2000; Moalem et al., 1999). These results have coined the term “protective autoimmunity” with the view that vaccination also in neurodegenerative disorders is neuroprotective (Schwartz and Kipnis, 2004). It is noteworthy in this context that injury-induced myelin debris, containing myelin-associated growth-inhibitory proteins, is a target of therapeutic strategies, including anti-NOGO antibodies, which improved regeneration after spinal cord injury in mammals (Freund et al., 2006; Schwab, 2004). However, an endogenous immune response to these proteins does not appear to play a significant role in recovery after injury.

In conclusion, this is, to my knowledge, the first study to demonstrate improved locomotor recovery after spinal cord injury in mice completely lacking functional T-, B- and NK-lymphocytes. Pfp/Rag2-/- mice thus present a new model to study how the supplementation of defined subpopulations of immune system cells, such as T-cell subtypes, or addition of myelin specific antibodies influence regeneration. Overall, the results of my study support the view that acute immune suppression is beneficial for regeneration after spinal cord injury.

6. Summary

The limited regeneration potential of the mammalian spinal cord is a serious challenge for medicine and science. The loss of the regenerative ability is explained with injury-induced changes in neurons, on one side, and, on the other, with changes in the environment at the site of injury. Elucidation of the complex pathophysiology of the spinal cord injury and experimental manipulations of this environment attract research interest because of the opportunity to create conditions beneficial for neuronal regeneration. The importance of the immune system in processes of functional recovery after traumatic spinal cord injury has been debated under various aspects in different experimental model systems in adult mammals. Recently it has been evident that the immune system can have both beneficial and adverse impact in regeneration after trauma. To obtain more insights into the contribution of the immune system under conditions of trauma it is essential to compare immune system with normal and suppressed function. This study evaluated the impact of a radically ablated immune system on the outcome of traumatic spinal cord injury in an adult mouse model. The analysis was performed on mice that are doubly and homozygously deficient in two genes (perforin and RAG-2 gen) in comparison with wild-type mice (n=12 pro genotype). These double-mutant mice (Pfp/Rag2-/- mice) on the C57BL/6 inbred background deficient in T-, B- and NK-cell functions, were compared to wild-type C57BL/6 mice. The comparison was performed by means of motor behavioral scoring (BBB score), single frame motion analysis and histological and immunohistochemical analyses. According to both the BBB score and single frame motion analysis the mice lacking functional T-, B- and NK-lymphocytes showed improved recovery of motor function in comparison to wide-type mice after spinal cord injury. They also displayed decreased scaring, higher weight loss and insignificantly lower number of microglia/macrophages in the lumbar spinal cord compared to the control mice. In summary, the results of this study support the view that acute immune suppression is beneficial for regeneration after spinal cord injury.

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9. Curriculum vitae

Der Lebenslauf entfällt aus datenschutzrechtlichen Gründen.

10. Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und
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Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer
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