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DISSERTATION

Spinal cord injury-induced immune depression syndrome: the function of Natural Killer (NK) cells

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Abstract

Spinal Cord Injury (SCI) leads to an interruption of the neural circuits impairing motor and sensory function below the lesion site. Additionally, SCI disrupts the immune system, resulting in the so-called Spinal cord injury-induced immune depression syndrome (SCI-IDS). The resulting immune suppression renders patients susceptible to infections in the postacute and chronic phases following SCI.

After SCI, NK cells manifest a long-lasting sub-optimal performance contributing to a decreased effectiveness in the response against pathogens. This correlates with an increase in infection incidence. The aim of our research work was to characterize the quantitative and qualitative aspects of SCI-IDS in rodents and humans using immune phenotyping and function assays focusing on Natural Killer (NK) cells, a crucial player of the first line defence system against infectious agents.

Using the experimental SCI contusion model at two different levels (T5 and T9), we tested whether lesion height and subsequent denervation of immune relevant organs would affect NK cell distribution, maturation, activation, migration and function in the potentially affected compartments.

In our clinical trial, we were able to establish for the first time a direct link between spinal cord injury and depressed NK cell function. Through a novel flow cytometric methodology, we provided evidence that not only NK cell cytotoxicity is impaired but also their capacity to produce proinflammatory cytokines throughout the postacute and chronic phases.

In conclusion, this work provides a deeper insight into the SCI-IDS. This includes a detailed analysis of cellular components of the syndrome, the examination of the role of lesion-height and contribution of lymphoid and hematopoietic tissue denervation on the performance of NK cells. These findings deliver both clinical and experimental evidence that SCI has a deep and long-lasting impact on NK cell distribution and function. This thesis provides further evidence for a strong link between the nervous and the immune system thereby advancing the existing knowledge in the field. Moreover, this work sheds light on the compromised state of the immune system defence arising following SCI, which creates a window for opportunistic pathogens. It ultimately paves the way for new research and therapeutic approaches targeting the immune system.

Abstrakt

Eine Rückenmarkverletzung [SCI] führt zu einer Unterbrechung von neuralen Verbindungen im Körper. Dies resultiert in einer Beeinträchtigung des Bewegungsaparats und Empfindungen unterhalb der Lesion. Eine weitere Begleiterscheinung, der SCI, ist eine Minderung der Immunfunktion, was ein Immundepressionssyndrom zufolge hat. Diese Immundepression macht Patienten während der post-akuten und chronischen Phase nach der SCI anfällig für Infektionen. In dieser Doktorarbeit quantifiziere und charakterisiere ich die Immundepression nach Rückenmarkverletzung in Nagern und Menschen durch FACS-Analyse, Immunphenotypisierung und Immunfunktionsassays, wobei ich mich auf die natürlichen Killerzellen (NK) fokussiere. Nach einer SCI zeigen NK eine dauerhafte, unzureichende Funktionalität, was die Effizient der Immunabwehr mindert und eine höhere Infektionsrate nach sich zieht. Wir haben anhand eines Kontursionsmodells auf zwei verschiedenen Wirbelhöhen getestet, ob die Lesionshöhe und folgende Denervierung verschiedener Immunorgane die Verteilung, Entwicklung, Aktivierung, Migration und Funktion von NK in den potentiell betroffenen Regionen beeinflusst.

In unserer klinischen Studie konnten wir zum ersten Mal eine Korrelation zwischen SCI und einer Funktionsbeeinträchtigung der NK nachweisen. Durch neue Methoden in der Durchflusszytometrie konnten wir beweisen, dass sowohl die Zytotxizität, als auch die Produktion von proinflammatorischen Zytokinen durch NK, während der postakuten und chronischen Phasen, eingeschränkt ist.

Zusammengefasst gibt diese These einen tieferen Einblick in die, durch Rückenmarkverletzung verursachte, Immundepression. Vorgestellt werden eine detaillierte Analyse der Zellkomponenten des Immundepressionssyndroms, der Einfluss der Lesionshöhe und der Anteil den lymphatisches und hämatopoietisches Gewebe auf die Funktionsfähigkeit von NK hat. Diese Ergebnisse belegen, dass SCI eine bedeutende und andauernde Wirkung auf die Verteilung und Funktion von NK hat. Es wird weiterhin geschlussfolgert, dass eine elementare Verbindung zwischen dem Nervensystem und dem Immunsystem besteht. Des Weiteren verdeutlicht diese These die entstehende Sicherheitslücke im Immunsystem, welche von opportunistischen Pathogenen ausgenutzt wird. Insgesamt glättet diese These damit

den Weg für weitere Forschungen und neue therapeutische Möglichkeiten für das angeschlagene Immunsystem.

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1 INTRODUCTION

1.1 Nervous system

The nervous system in vertebrates is an intricate network of neural connections. On the one hand, the nervous system is responsible for converting a sensory stimulus captured by peripheral receptors into a conscious perception. On the other hand, it is able to respond to an environmental stimulus by preparing and executing a purposeful motor program.

The sensory experience is captured by our visual, gustatory, olfactory, auditory, vestibular and somatosensory receptors and conveyed to the brain by peripheral nerves or afferents. The efferent information containing the motor responses flow back through the somatic nerves subsequently activating the skeletal muscles. Another type of efferent signal regulates visceral functions such as the cardiac muscle, exocrine glands and smooth muscle. In contrast to somatic (motoric) efferents, these functions are largely involuntary.

In vertebrates, the nervous system is anatomically divided into two main parts: the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS comprises the brain, spinal cord (SC) and optic nerve. The nerves connecting the CNS to all parts of the body constitute the PNS. The peripheral nerves can mediate either voluntary or involuntary functions. The nerves mediating voluntary functions are called somatic nerves and are either sensory or motor; the ones regulating involuntary functions are called autonomic.

The nervous system employs different methods to relay the necessary information to and from the organs. As already mentioned, the nerves are the direct extensions of the CNS into and from the periphery. Information is transmitted through axons, which is the main signalling unit of the nervous system. Nerve cells communicate between each other through synapses by the means of neurotransmitter release. However, this is not the sole way for the CNS to convey its message to the periphery: the endocrine system is modulated by “head ganglion” of the autonomic system, the hypothalamus[1].

1.2 The Spinal Cord

Like the brain, the spinal cord (SC) is also somatotopically organized and transports information into contiguous body parts. The sensory and motor information travel longitudinally within the white matter along ascending and descending pathways, respectively. In the spinal cord, the white matter surrounds the butterfly-shaped grey matter, which contains the neuronal cell bodies.

The sensory information conveyed from the peripheral organs into the CNS, enter the gray matter at the dorsal horn while motor neurons leave the grey matter through the ventral horn. At the level of each spinal cord segment, dorsal and ventral roots combine in the dorsal root (spinal) ganglion to form a spinal nerve, making the transition between the central and the peripheral nervous systems.

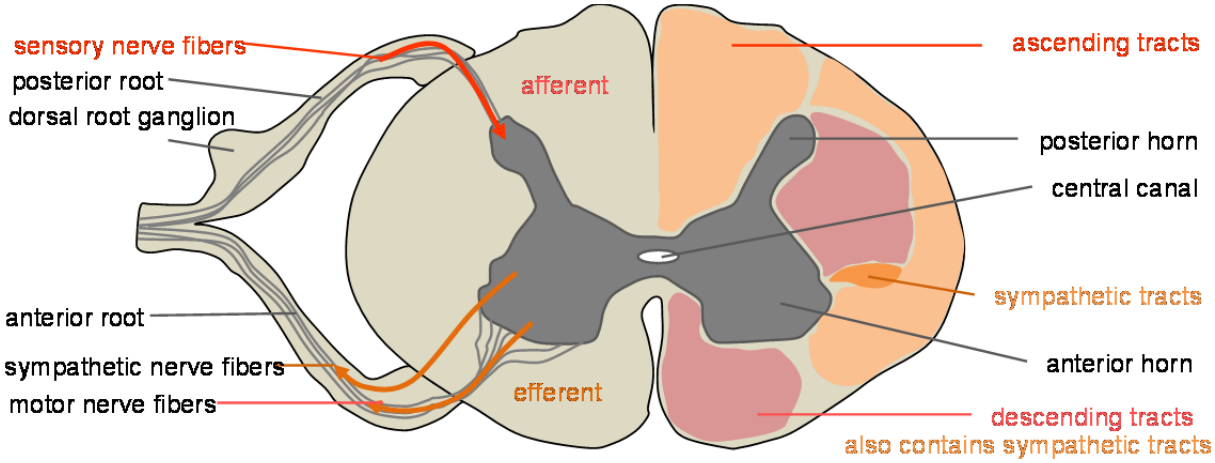

Fig. 1: Spinal cord segment. Spinal cord is composed of grey and white matter. White matter contains myelinated axons with ascending and descending nerve pathways, whereas the grey matter contains the nerve cell bodies

Humans have 33 vertebrae and 31 pairs of spinal nerves and segments: 8 cervical (C1-C8), 12 thoracic (T1-T12), 5 lumbar (L1-L5), 5 sacral (S1-S5) and 1 coccygeal (Co1).

Rats have 30 vertebrae (without the caudal) and 34 pairs of spinal nerves and segments: 8 cervical (C1-C8), 13 thoracic (T1-T13), 6 lumbar (L1-L6), 4 sacral (S1-S4), and 3 coccygeal (Co1-Co3).

Spinal nerves contain both somatic and visceral fibres. Both can be either afferent or efferent. Somatic afferents carry sensory information from the skin, skeletal muscles,

tendons and joints and somatic efferents carry motor impulses to skeletal muscles. Visceral afferents carry fibres responsible for visceral sensation and visceral efferents, are the so-called autonomic fibres (sympathetic and parasympathetic), carrying impulses to glands, cardiac and smooth muscles[2].

1.3 Spinal Cord Injury

1.3.1 Human Spinal Cord Injury

In 1928, Ramon y Cajal already noticed the inability of the neurons to proliferate[3], rendering the regeneration process impossible. The stimulation of axonal regrowth and plasticity is still nowadays the biggest challenge[4], not only in spinal cord injury research, but also in other types of CNS lesions. Multiple inhibitory molecules such as chondroitin sulfate proteoglycans, nogo, myelin-associated glycoprotein, semaphorin, etc.[5, 6] create a highly adverse environment, discouraging neuronal sprouting and plasticity after SCI.

Injury of the SC results in the interruption of ascending and descending circuits traversing the spinal cord. Functional loss is dependent on the extent to which the spinal cord has been damaged ranging from complete to incomplete (grades from A-E) according to ASIA (American Spinal Injury Association). “A” corresponds to a complete lesion with total loss of motor and sensory function on sacral S4-S5 segments; “B” to a loss of motor function but preservation of sensory function below injury level; “C” to a loss of sensory function but preservation of motor function below injury level and more than half of the key muscles have a muscle grade less than 3; “D” to a loss of sensory function but preservation of motor function below neurological level with more than half of the key muscles have a muscle grade more than 3; “E” corresponds to an injury to a vertebral column with no affection of the spinal cord and therefore no loss of neither motor nor sensory functions.

The incidence of traumatic spinal cord injury varies from 39 per million in North America, 16 per million in Western Europe, 15 per million in Australia, Central and South America 24-25 per million and Asia 21-25 per million[7]. Both in developed and in developing countries it affects mainly males between the ages of 18-32 due to

accidents or violence. Recently, due to increased life expectancy in developed world, males and females above the age of 65 are affected, mainly due to accidental falls[7].

1.4 The immune system

We live surrounded by microorganisms, which can potentially cause disease. The immune system has the function to recognize, attack and create an immunological memory of invading pathogens. At the same time it plays a self-regulatory role being able to recognize the organism own cells.

White blood cells or leucocytes can either belong to the innate or to adaptive immune system and all of them derive from hematopoietic stem cells of the bone marrow (BM) during adult life[8].

1.4.1 The innate immune system

If pathogens are able to break through anatomical barriers (e.g. skin) into the organism, most are eliminated within minutes to hours through innate immune defence mechanisms. Innate immunity relies on a limited number of receptors and secreted proteins that are encoded in the germline and that recognize features common to many pathogens.

Besides the complement system, the innate immune system comprises the natural killer (NK) cells and the phagocytes. The NK cells derive from a lymphoid progenitor (together with a fraction of dendritic cells) while phagocytes derive from a myeloid progenitor and comprise: 1) granulocytes (basophils, eosinophils and neutrophils); 2) macrophages, deriving from blood monocytes; 3) dendritic cells and 4) mast cells.

Cells of the innate immune system orchestrate a first line defence against the invading microorganisms. After recognition of an invader through pattern recognition receptors, phagocytes can engage the pathogen and ingest it. Another important outcome of the contact with pathogen-sensing receptors (e.g. Toll-like receptors) in macrophages and dendritic cells, is the production and secretion of cytokines and chemokines inducing inflammation. In turn, NK cells are stimulated both by these cytokines and directly by infected cells, activating its effector function in order to kill the infected cell[8].

1.4.2 Natural Killer (NK) cells

NK cells are lymphocytes, which are capable of killing tumor cells, virus-infected cells, bacteria, fungi and parasites without prior specific immunization. In humans NK cells are identified as being $CD3^-$, $CD56^+$ and in rats $CD3^-$, $CD161^+$.

Together with B- and T-lymphocytes, NK cells derive from a common lymphoid progenitor (CLP). The development of NK cells takes place in the BM where they develop a 'NK cell repertoire', and acquire 'self-tolerance' and 'effector machinery'. Mature NK cells then egress into the peripheral blood and secondary lymphoid organs (e.g. spleen)[9]. Upon an infection, NK cells can also undergo homeostatic proliferation and are recruited to the sites of infection[10-12]. Also a lymphopenic environment[13] or a tumor challenge[14] induces NK cells proliferation.

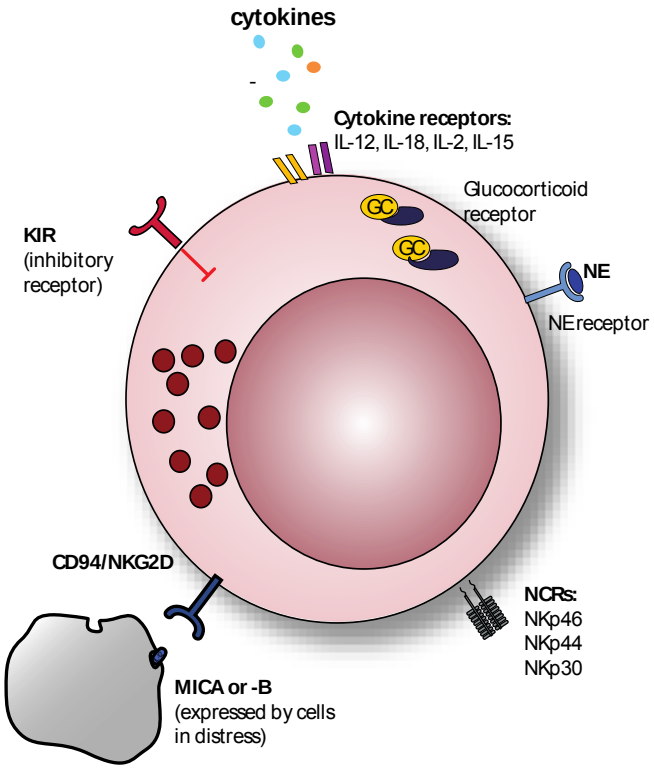

Fig. 2: Main receptor types expressed by human NK cells. The sum of ligations to activating and inhibitory receptors modulate NK cell activity. NK cells are also stimulated by cytokines resulting in the production of Immune modulatory cytokines e.g. (IFN-γ). Natural cytotoxicity receptors (NCRs) stimulation results in NK cells degranulation.

NK cells action is modulated by a repertoire of activating receptors, which upon activation induce NK cells killing and cytokine production; and inhibitory receptors, which prevent it. The balance between the "kill" and the "do not kill" signals determines whether NK cells will destroy the target cell. There are four main types of NK receptors (NKRs): 1) killer-cell immunoglobulin-like receptors (KIR) in humans and Ly49 in mice: bind to MHC I are crucial for distinguishing normal/self from

transformed/foreign cells; 2) CD94/NKG2D: bind to non-classical MHC I molecules and recognize stressed cells; 3) Natural Cytotoxicity receptors (NCRs): NKp46, NKp44, NKp30, which are activating receptors for the recognition of infected cells[8, 9]; and 4) cytokine receptors: IL-2, IL-12, IL-18, IL-15 secreted by other elements of the immune system, especially monocytes.

The maturation stages undergone by murine and human NK cells do not follow the same pathway. A 4-stage development model has been proposed in murine NK cells: 1) NK cell are negative for both CD11b andCD27; 2) $CD11b^{low}CD27^{+}$, giving rise to 3) double positive (DP) $CD11b^{high}CD27^{+}$ and finally 4) $CD11b^{high}CD27^{-}$ [15]. Each subset displays a different function: $CD11b^{low}CD27^{+}$ are potent cytokine producers but less cytotoxic than the intermediate DP subset and both subsets are more functional than the $CD11b^{high}CD27^{-}$ NK cells [16]. The least mature phenotype predominates in the BM and LNs, the double positive and the most mature phenotypes prevail in the blood, liver, spleen and lung [17].

Human NK cells develop from $CD56^{bright}CD16^{-}$ which lack perforin but have a higher proliferative potential, into $CD56^{+}CD16^{+}$ and finally into $CD56^{bright}CD16^{+}$, which have an increased perforin, content but diminished proliferative potential[18].

1.4.3 NK cell function

As immune effectors, NK cells exert two main functions: production of immunoregulatory cytokines in response to infected cells, cytokine or monokine receptor stimulation and cytotoxicity upon contact with target cells. NK cells produce cytokines such as Interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) in vast amounts. IFN-γ is able to “prime” macrophages, activate other members of the innate immune system, mediate antiviral and anti-bacterial immunity, promote autophagy, enhances antigen presentation, regulate Th1/Th2 balance and controls cellular proliferation and apoptosis[19]. TNF-α is capable of directly killing virus-infected cells[20]. NK cells also produce other cytokines such as granulocyte macrophage – colony stimulating factor (GM-CSF), necrosis factor-beta (TNF-β), IL-10 and IL-13[19, 21]. Cytotoxicity requires direct contact with target cells and the formation of an immunological synapse (IS). Upon fusion of the granular vesicles with the plasma membrane, pore-forming perforin and cytotoxic granules, namely granzymes are

released into the intercellular space inducing programmed cell death of the target cell[20, 22].

1.4.4 The adaptive immune system

An adaptive immune response is initiated when the antigen or the antigen-presenting cells (APCs) – neutrophils, macrophages, dendritic cells – reach the secondary lymphoid organs (e.g. spleen). There, the APCs activate lymphocytes by displaying the antigen and through co-stimulatory molecules. Then, lymphocytes proliferate and differentiate into its fully functional form.

B- and T- cells also form part of the adaptive immune system. The B-lymphocytes produce and release antibodies, which are capable of detecting extracellular antigens only. They are also able of neutralizing viruses and marking pathogens making them identifiable for destruction by phagocytes and complement. T-lymphocytes are divided into cytotoxic T-cells, expressing CD8, and helper T-cells, expressing CD4. $CD8^+$ T cells have a granzyme-mediated effector function, acting against intracellular viruses and bacteria, while $CD4^+$ T-cells are involved in activation of other cells. $CD4^+$ T-cells also contribute towards delimitation of infection by modulating macrophages, neutrophils and B-cells immune reaction[8].

1.4.5 Immune relevant organs

Lymphocytes circulate in the blood and lymph and accumulate in lymphoid organs. Primary lymphoid organs include the thymus and the bone marrow where lymphocytes are generated. Secondary lymphoid organs include the spleen, lymph nodes and mucosal lymphoid tissues of the gut, respiratory, urogenital tracts and other mucosa. It is in the secondary lymphoid organs where mature lymphocytes are maintained and where the adaptive immune responses are initiated.

1.5 The interplay between Nervous System and the Immune System

The nervous and the immune systems communicate in a bi-directional way. The nervous system plays an immunoregulatory role: 1) through the hypothalamic-pituitary-adrenal (HPA) axis, which coordinates the production and circulation of immunoregulatory hormones and neurotransmitters such as catecholamines and

glucocorticoids[23-26]; 2) hardwiring the immune-relevant and hematopoietic organs including sensory and autonomic innervation of spleen, lymph nodes, adrenal gland and bone marrow[25, 27, 28].

1.5.1 The Autonomic Nervous System (ANS)

There are two types of efferent information descending from the brain: 1) somatomotor signals, which activate skeletal muscles and are voluntary and 2) autonomic signals, which regulate innervated tissues and organs and are involuntary.

The autonomic nervous system (ANS) has two arms: Sympathetic and parasympathetic nervous systems. In the spinal cord, sympathetic nerve fibres arise in the thoracic and upper lumbar segments (T1-L3 in humans and T1-L2 in rats), while the parasympathetic nerve fibres innervating the pelvic organs arise in the lower lumbar and sacral segments (S2-4 in humans and L6-S1 in rats)[29].

Both sympathetic and parasympathetic signals are classically conveyed in a “two-neuron chain” fashion. Integrated information from central autonomic circuitry in the forebrain, limbic system, hypothalamic nuclei and brain stem is conveyed through the preganglionic neurons along the intermediolateral (IML) columns within the spinal cord. After exiting the SC via the ventral roots, preganglionic neurons synapse in the peripheral ganglia, giving rise to multiple postganglionic neurons [29, 30]. Preganglionic neurons are cholinergic, activating nicotinic acetylcholine (ACh) receptors at their first synapse. However, postganglionic neurons can be either cholinergic, in the case of parasympathetic transmission, or adrenergic, in the case of sympathetic transmission.

1.5.2 The hypothalamic-pituitary-adrenal (HPA) axis[29]

The HPA axis is a complex feedback system controlling hormone release. The HPA axis connects the hypothalamus with two major endocrine glands: the pituitary gland and the adrenal gland. Hypothalamus secretes corticotropin-releasing hormone (CRH), stimulating the pituitary gland to secrete adrenocorticotropic hormone (ACTH), which in turn controls hormone production in the gonads and in the adrenal gland. The latter is responsible for important immunoregulatory such as glucocorticoids (GC) from the adrenal cortex and catecholamines (CAs) including

epinephrine and norepinephrine (NE) from the adrenal medulla. In turn, GCs suppress the production of CRH and ACTH in a phenomenon called negative feedback loop. HPA axis intervenes in processes such as digestion, metabolism, thermoregulation, immunoregulation but also emotions and sexuality.

1.5.2.1 HPA and the neuroendocrine immune regulation

Like the somatosensory system, consisting of an afferent limb of sensory input and an efferent limb of motor output, the neural-immune interplay also co-exists in a dual chain: the afferent signalling is carried out by circulating cytokines and efferent signalling is carried out by autonomic nerves, hormones, neurotransmitters and soluble factors.

The sensory fibres of the vagus nerve contain cytokine receptors. One of the main tasks of the vagus nerve within the neural-immune interplay is to convey information to the brain about inflammatory events and the cytokine production of visceral organs[31].

The hypothalamus is the central organ of immunoregulation, enabling these two mega systems to work in concert in order to preserve homeostasis. Soluble mediators such as IL-1, TNF-α and IL-6 are perceived by peripheral receptors, which transduce its signal through ascending pathways, activating the paraventricular nucleus (PVN) in the hypothalamus. This phenomenon in turn, triggers the activation the HPA axis and the sympathetic nervous system[31-33]. This leads to release of NE from brain networks resulting centrally in increased arousal and vigilance. Peripherally, it results in increased sympathetic output, namely increase NE plasma levels, NE release from sympathetic nerve terminals and epinephrine from the adrenal medulla[32].

Hormonal and neurotransmitter fluctuations in the spleen, namely NE, have shown to be dependent on intact sympathetic innervation of the organ[34]. Changes in hormone and neurotransmitter content within the immune organs, are thought to influence immune function by triggering changes in the immune cells operating milieu such as cytokines and growth factor composition.

1.5.3 Immune cell receptors

1.5.3.1 Adrenergic receptors

Both immune and nerve cells express receptors and endocrine hormones. Cells of the innate immune system express both α- (α1- and α2-) and b-adrenergic receptors (AR) (β1-, β2- and β3-), which are able to detect catecholamines. Virtually every immune cell, with the exception of Th2 lymphocytes, express β-AR. Lymphocytes, especially NK cells, express the largest number of receptors (about 4000)[32]. Catecholamines maintain homeostasis by regulating fuel metabolism, heart rate, blood vessel tone and thermogenesis[32]. In immune cells, catecholamines regulate differentiation, trafficking/migration and effector functions. CAs exert contrary actions depending on the binding to α1- or β2-AR, the former augmenting and the latter suppressing effector functions. Catecholamines mainly drive the Th2-cell anti-inflammatory response (mediated by IL-4 and IL-10) through the inhibition of INF-γ production by the Th1 cells[30, 32, 35]. The Th1-effector-cell β2-AR stimulation results in suppressed IFN-γ production via cAMP-PKA pathway. β2-AR activation also reduces signalling through NF-ΚB, MAPK and ERK pathways[30].

Through β2-AR stimulation, catecholamines directly affect the function of B-cells, influencing antibody production. In vitro, the presence of catecholamines and Th2-cells drives B-cell antibody production towards IgG1 and IgE[30].

NK cells are particularly sensitive to the suppressive effects of stress and catecholamines [32]. In response to acute catecholamine release, the numbers of NK cell in circulation tend to increase along with granulocytes and in opposition to T- and B-lymphocytes. This phenomenon is completely abolished by the administration of propranolol, a β-AR antagonist[36]. β2-AR stimulation via catecholamines or agonists inhibits NK cell function 1) directly by suppressing the production of granzyme b, perforin and IFN-γ and 2) indirectly by suppressing the secretion of cytokines essential to NK cell activity such as IL-12, IFN-γ or IFN-α. In vitro epinephrine and isoproterenol application and in vivo metaprolol administration inhibit NK cell activity due to elevated cAMP levels[37, 38]. Also splenic nerve stimulation[34] and central administration of CRH, which increases sympathetic outflow, decreases NK cell activity in the periphery[32, 39].

Catecolamines have paradoxical effects on macrophages. Epinephrine and NE also have a suppressive influence on macrophages and monocytes by blocking their activation and tumor and viral killing through IFN-γ[30]. However, CAs boost macrophages anti-microbial activity mediated by α2-AR. CAs also inhibit their capacity to produce TNF-α, IL-12 and IL-1 and potentiate IL-10 production in a β2-AR mediated fashion.

Normal cellular functions are carried out through the activation of different intracellular signalling pathways by external stimuli, namely AR ligands.

Another important role of the ANS is in the regulation of hematopoiesis. β2-AR stimulation also plays a major role in the BM by promoting hematopoiesis. It also enhances acute phase response after infection or tissue damage, by mobilizing different immune cells. The enhancement of the central sympathetic outflow by known environmental stimuli like infections, increase the release of NE with the BM environment[40]. NE appears to have a positive effect on the numbers of granulocytic and macrophagic populations, however the opposite effect is apparent in the lymphocytic population, where α1-AR antagonism, leads to a decay in lymphocyte numbers.

1.5.3.2 Cholinergic receptors

Although lymphoid tissue is mostly innervated by sympathetic noradrenergic fibers, parasympathetic neural circuits have also been identified as playing an important role in mediating cytokine release in sepsis, endotoxemia and other inflammatory conditions[41]. This phenomenon was denominated the inflammatory reflex and is mediated by cholinergic neural transmission through the α7 nicotinic acetylcholine (α7nACh) receptors expressed on macrophages, monocytes, dendritic cells, T- and B-lymphocytes.

1.5.3.3 Glucocorticoid receptors

Glucocorticoids are known for having a depressive effect on the immune system. Glucocorticoid transmission has been reported to play an immunoregulatory role on lymphocytes namely apoptosis, repression of genes encoding for lymphokines and suppression of cytotoxic function[42, 43].

1.5.4 The 'Hardwiring' of immune relevant organs

The neurons innervating immune organs are mostly sympathetic, while there is no clear evidence of parasympathetic neurons[30]. Vagal afferents convey sensory information, but have an important role in controlling immunity, since they can activate splenic sympathetic nerve subsequently suppressing innate immunity [44]. In secondary lymphoid organs (spleen, lymph nodes (LNs) and mucosa-associated lymphoid tissue (MALT)), noradrenergic nerves travel with the vasculature and associated connective tissue, entering the surrounding lymphoid parenchyma, where they form neuro-effector junctions with immune cells[30].

The 'hardwiring' of the immune relevant organs enables the close contact of nerve ends and hence the direct effect of catecholamines on lymphocytes. Autonomic modulation of immune function may also occur upon contact of immune cells with hormones in circulation in the peripheral blood.

1.5.4.1 Innervation of the bone marrow

Bone marrow is the most important organ regarding hematopoiesis. All blood cells, including immune competent cells, derive from hematopoietic stem cells (HSC) present mainly in the bone marrow where they also undergo a process of proliferation and maturation. Hematopoiesis is regulated by local and systemic humoral factors. Locally, hematopoietic cells are exposed to cytokines, growth factors, membrane-bound adhesion molecules and stromal cells, which mediate leucocyte production, activation, migration and release[27]. However, there is also evidence that circulating steroid and peptide hormones modulate hematopoietic cell function through cognate receptors present in hematopoietic progenitor stem cells (HPSCs). There is evidence that the BM is supplied with an efferent autonomic innervation and afferent sensory innervation[45]. Nerve endings supply the BM with noradrenaline, whereas dopamine and adrenaline originate from the blood vessels supplying the BM. Nerve fibers innervating the BM enter the bone along the blood vessels branching into the parenchyma[25] ending among hemato- and lymphopoietic cells[32].

Some of the peptides involved in neuroimmune modulation in the BM are Neuropeptide Y (NPY), substance P (SP), calcitonin gene-related peptide (CGRP),

tyrosine hydroxylase (TH) and vasoactive intestinal peptide (VIP). Using a multisynaptic tract-tracing pseudorabies virus (PRV), Denés et al. unveiled neural connections from femoral BM back to the CNS. BM innervation was traced to the IML columns between the levels T8-L1 in the spinal cord, into structures responsible for autonomic innervation in the brainstem, and brain[27].

In normal conditions, BM cell release follows a circadian rhythmicity and is regulated by noradrenergic secretion from BM sympathetic local nerves[46]. Indeed, BM cells are also equipped with α1-, β2- and β3-ARs which mediate SNS neurotransmission and modulation over hematopoietic cell function including hibernation, differentiation and renewal[30].

Higher sympathetic activity associated with physical/emotional stress or injury/infection, as well as β2-AR stimulation, prompt hematopoiesis of all blood cell lines and release of HSCs[47] including in acute phase response following tissue damage and infection[30]. Disruption of sympathetic transmission, for example by chemical sympathectomy or β-AR blockade, disrupts HSC mobilization[30].

The high levels of norepinephrine and epinephrine observed in conditions like shock, polytrauma, burns or CNS injury might be beneficial for survival by counteracting anemia and lymphopenia[30, 48]. However, persistent sympathetic activation accompanied by a hypercatecholaminergic state is one of the mechanisms involved in BM failure (part of multiple organ failure syndrome), emerging from severe injury. This translates in a G-CSF-mediated HSC mobilization into circulation and injured tissues with a concomitant growth arrestment of HSCs within the BM, leading to a decrease in BM cellularity[49]. This has been shown in lung contusion and hemorrhagic shock to be mediated by β2- and β3-AR stimulation, which could be prevented by pretreatment with propranolol or β2- and β3- selective blockade[48].

1.5.4.2 Innervation of the spleen

Spleen is innervated by neurons from the superior mesenteric and celiac ganglia, which have been traced back to the sympathetic preganglionic neuron and finally the intermediolateral (IML) column T3-T12 within the spinal cord. Innervation of the spleen is predominantly sympathetic and enters the spleen accompanying the

splenic artery. Noradrenergic fibers penetrate the lymphoid parenchyma and form junctions with immune cells in the periarteriolar lymphatic sheath (PALS)[30, 50].

1.6 Trauma-induced immune suppression: acute and chronic stress reactions

Exposure to severe trauma such as hemorrhagic shock, blunt trauma of the chest, burns and surgery has been associated with increased rates of infectious complications. These conditions have also been shown to down-regulate the immune system in a similar fashion as CNS injury does and are broadly part of a syndrome called systemic inflammatory response syndrome (SIRS). Also psychological stressors ranging from pre-examination stress to bereavement or depression also have been shown to impair immune response by disturbing the neuro-endocrine-immune interplay[51].

Dhabhar et al. have studied the changes induced by acute and chronic stress in different leucocyte subpopulations. They suggested that short-term stress has a boosting effect on immunity, whilst long-term stress is detrimental. According to these studies, acute stress, mediated by adrenal hormones, leads to an initial increase followed by a rapid but reversible decrease in absolute numbers of T cells, B cells, NK cells and monocytes in blood. Acutely stressed mice showed an enhanced cell-mediated immune response when compared with non-stressed controls. In adrenelectomized animals exposed to restraint stress, administration of CORT (endogenous type I and type II receptor agonist) or RU28362 (a specific type II agonist), induced a decrease in leucocyte absolute numbers, when compared with animals to whom cyanoketone (CK), a steroid synthesis inhibitor has been administered. These results suggest that cortisol levels may mediate the changes in leukocyte numbers. According to the authors of this study, the observed leukopenia is due to selective leucocyte redistribution in different immune compartments and not due to leukocytic death and apoptosis[52].

A study in healthy volunteers showed an increased respiratory infection rate and clinical cold symptoms as the scores for psychological stress increased after inoculation of five strains of respiratory viruses. Acute stress lasting for less than 1 month was not associated with increased susceptibility to cold, whether severe chronic stress lasting more than 1 month was associated with an increase in risk of

disease [51, 53]. Psychological stress was also positively correlated with the reactivation of latent herpes simplex virus (HSV), Epstein-Barr Virus (EBV) and cytomegalovirus (CMV)[50, 54, 55]. Stress also interferes with wound repair, namely through the disruption of cytokine production essential for wound healing[51].

1.7 Immunodepression after central nervous system (CNS) injury

Infections are the leading cause of death not only after SCI, but also succeeding other kind of CNS injuries such as stroke and traumatic brain injury (TBI).

The disruption of the immune system function secondarily to a CNS injury was broadly named CNS injury-induced immunodepression syndrome (CIDS).

CNS injured patients suffer 4% to 9% more nosocomial infections than the general hospitalized patient population and 3% more than surgical patients. Infections tend to strike during the first days after injury, but the risk of contracting infections remains high beyond the acute phase[56]. Adverse factors such as polytrauma, invasive medical treatment (surgery, catheterization and mechanical ventilation), immobilization, impairment of swallowing ability, often leading to aspiration, as well as the systemic inflammatory response syndrome (SIRS) in response to CNS damage, all represent complications associated with CNS trauma. Either posed together or alone, these challenges seem to create the optimal alignment for the intrusion of infective agents. Aspiration per se has been often pointed out as the main explanatory factor for the development of pneumonia in CNS lesions. However, ventilator-associated pneumonia occurs in about 20% of patients without CNS injury[57] but rises to about 60% in cases of CNS trauma[58-60]. This indicates that other factors underlie the increased susceptibility to infectious diseases of patients subject of CNS injuries[61].

Spontaneous infection (pneumonia) has been shown in experimental models of CNS injury, particularly during the acute phase after the initial insult (1-3 days)[62]. Fluctuations of adrenal steroids and catecholamines abundantly released as a component of the stress reaction in response to trauma or injury have been suggested to underlie changes such as: 1) apoptotic loss of peripheral blood lymphocytes in lymphoid organs; 2) mobilize peripheral pools of leukocytes into infection foci; 3) downregulate the inflammatory reaction by suppressing the

secretion of pro-inflammatory mediators including IFN-γ, IL-6 and TNF-α and enhancing the secretion of anti-inflammatory mediators such as IL-4 and IL-10; 4) downregulate the expression of MHC II, impairing antigen presentation to T cells and 5) alter the Th1/Th2 ratio[23, 41, 62].

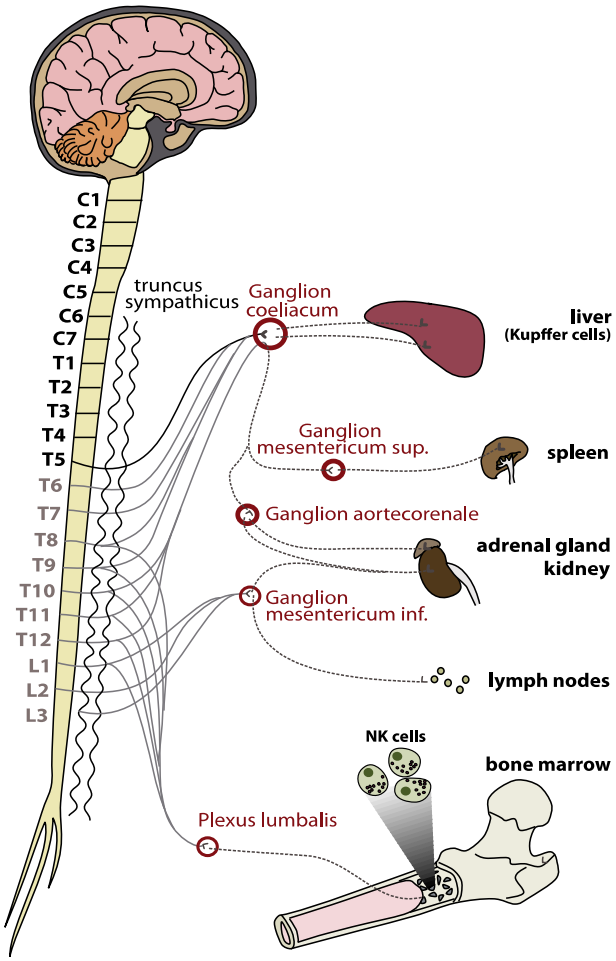

Fig. 3: Innervation of Immune relevant organs. Sympathetic innervation of abdominal immune relevant organs conveyed from the sympathetic trunk from level T5-T12 through the celiac ganglion. Bone marrow autonomic innervation is conveyed from level T8-L1 through the lumbar plexus

1.8 Spinal cord injury-induced immune depression syndrome (SCI-IDS)

The mechanisms involved the immune deficits observed in SCI patients are still poorly understood. The immune system down-regulation probably involves a complex combination of factors including: 1) maladaptive reaction of immune relevant organs to absent or aberrant supraspinal control; 2) abnormal pattern of hormone secretion potentially influencing immune system activation and inhibition of individual immune cellular processes; 3) trauma-related stress/shock reaction and exposure of

the organisms to various antigenic particles including pathogen-associated microbial patterns (PAMPs) and to deteriorative metabolic processes and inflammatory cascades triggered by the traumatic event.

1.8.1 Susceptibility to infectious complications in SCI patients: implication in morbidity and mortality

Spinal cord Injury leads to multiple changes in the organism. Beside the interruption of the motoric and sensory neural circuits resulting in paraplegia and sensory loss, other less apparent changes have been registered throughout times. It has also been observed an increased susceptibility to infectious diseases and altered autonomic responses among SCI patients. The infectious complications are predominantly respiratory, urinary tract and septicemia and are the major cause of death among SCI patients during the post-acute and chronic phases after injury. This has prompted further research into the mechanisms involved in this increased susceptibility to infectious diseases[63]. Pneumonia is the leading cause of death both short term (<1 year) and long term (>1 year) after the injury[64]. Lesion height and severity (completeness) are determining factors for both patient long-term survival and the incidence of infectious complications. The incidence of pneumonia is higher in patients with complete tetraplegia (38%) compared with complete paraplegia (15%)[23]. In this matter, factors such as dependency on assisted ventilation, which is higher among high-level cervical injury (C1-C4), immobility and the viability of innervation of the respiratory muscles enabling coughing, also are determining factors in the development of respiratory infections[64].

1.8.2 Neuroendocrine changes after SCI

Any CNS injury disrupts neural immunoregulatory pathways, namely the HPA axis, the SNS and the cholinergic anti-inflammatory pathway.

In addition, injury to the spinal cord can lead to the interruption of the circuitry and direct damage of structures directly involved in the sympathetic control such as the lateral column and the IML nucleus. Damage to the sympathetic centers within the spinal cord and the deafferentiation of the peripheral organs leads to autonomic dysfunction, a frequent but life-threatening condition among SCI patients. The initial

phase after SCI is characterized by a high sympathetic outflow. Besides that, patients also experience a period of “spinal shock”, characterized by muscles flaccidity. As this phase subsides, muscle tonus gradually increases culminating in a long-lasting phase of muscles hypertonicity or spasticity. Paralleling what happens in the somatosensory system, also the SNS undergoes a phase of diminished activity characterized by hypotension, reflex bradycardia/cardiac arrest, followed by a gradual increase in sympathetic activity – autonomic dysreflexia – especially in patients with a lesion higher than thoracic level 6 (T6) (above the major SNS splanchnic outflow)[65].

Autonomic dysreflexia (AD) or dysautonomia is characterized by hypertensive episodes, headache and flushing. It results from reduced sympathetic activity below the lesion, morphological changes in preganglionic sympathetic neurons and peripheral α-AR hyperresponsiveness[65]. Besides that, damage to spinal cord circuitry, deprives lymphoid organs of most of the supraspinal control altering its function, a state which was called decentralization. Concurrently, the abnormal flow of sympathetic mediators into lymphatic organs has a detrimental effect on adaptive and innate immunity, paving the ground for an immune deficit observed in SCI patients[61]. Indeed, sympathetic activation leads to a release of norepinephrine (NE) and glucocorticoids (GCs) from nerve terminals and the adrenal gland. Prolonged supra-optimal activation of GC-receptors or β2-AR has a suppressive effect on immune cells, which can be restored through selective GC-receptor and β2-AR blockade[66].

Zhang et al. suggested that immunoregulatory hormones released during AD in mice, induce nonselective mature and immature splenic leukocyte apoptosis, probably due to increased intrasplenic sympathetic nerve activation. The increasing frequency of spontaneous AD also correlates positively with the extent of immune suppression [66].

Studies with humans have revealed elevated levels of adrenocortical hormones – GCs in chronic tetraplegia compared with paraplegic patients and healthy controls. They detected no differences in the amount of ACTH, prolactin, which points to an adrenal rather than a pituitary deficit[67]. In line with these findings, Cruse et al. observed higher urine free cortisol levels in neurologically impaired patients (tetraplegics, paraplegics and stroke patients)[68]. Altogether these results suggest a

prolonged HPA dysregulation for months and years after the traumatic event, which can be detrimental to the health of the individual.

1.8.3 Cellular basis of SCI-IDS

Riegger and colleagues have characterized the phenomenon of immune depression after SCI during the acute phase following injury. They observed an abrupt decline in several leucocyte subpopulations namely monocytes, dendritic cells and especially T- and B-lymphocytes in the peripheral blood compartment, taking place during the first week after the injury. This quantitative immune suppression is only temporary as the cell counts return to baseline values at the end of the first week. This phenomenon has been observed both in the SCI experimental model in rats[69] and in humans[70].

The leucocyte depletion revolves within one week after the injury but the increased susceptibility to infectious diseases among these patients remains increased in the post-acute and chronic phases after the injury, suggesting a prolonged downregulation of the immune system in qualitative terms.

1.8.4 Immune functional changes after SCI

For the last two decades The following observation have been reported as a result of secondary immune deficits after SCI: 1) reduced $CD4^+/CD8^+$ cell ratio[71]; 2) reduced NK cell percentages[72]; 3) reduced B cell numbers and suppressed antibody production and response after high thoracic lesions[60]; 4) depressed NK cytotoxicity and *in vitro* lymphocyte transformation[45, 68, 72]; 5) Depressed T cell function/activation (IL-2R)[68, 73]; 6) impaired neutrophil phagocytosis[74]; 7) Impaired proliferation of hematopoietic progenitor cells[45]; 8) splenic atrophy and leukocyte apoptosis[60, 66]; 9) decreased APC activation within the spleen[75].

Cruse and colleagues hypothesized that the changes observed could be correlated with a chronic stress response characterized by a prolonged hypercholinergic and hypercortisolic state. Like SCI patients, stroke patients, also monitored in this study, displayed a similar downregulation of the immune system when compared with healthy controls[68].

1.9 NK cell function in stress, CNS injury and SCI

NK cells play a crucial role in the front line defense against infections as well as tumors. The lack of NK cells renders individuals susceptible to viral infections, incapable of an adequate response to bacterial endotoxin, impaired tumor surveillance and unable to control metastatic growth[76]. NK cells lyse viral- or bacterial-infected cells through the granzyme b- and perforin-mediated killing and cytokines secretion upon contact with infected cells or as a result of stimulation by cytokines (e.g., IL-2, IL-12 and IL-15) produced by other innate immune cells. NK cells can also be activated by ligation of CD16 (FcγRIII) with IgG activating CD69 mediating antibody-dependent cellular cytotoxicity (ADCC) and antiviral cytokine production[77]. In addition to activating and inhibitory receptors that modulate NK cells' activity, they also possess a high number of β-AR, which render NK cells more susceptible to fluctuations of adrenal hormones.

Normal NK cell function 1) relies on an intact nervous system including sympathetic innervation of lymphoid organs where NK cells are generated and stored and 2) is regulated by adrenal hormones.

Activation of the splenic nerve by electrical stimulation reduces splenic NK cell cytotoxicity in a β-AR-mediated mechanism. So does surgical stress/laparotomy. The reversal of laparotomy-induced decreased NK cytotoxicity by splenic denervation, confirms the reliance of NK cell function on intact innervation[34]. Nevertheless, laparotomy-induced decreased NK cytotoxicity is not entirely mediated by inputs conveyed by the splenic nerve. It is also the effect of increased GCs levels from the stimulation of the adrenal gland through the HPA axis, also as a result of surgery. GC-receptors blockade with mifepristone led to an improvement of NK cell cytotoxicity[78]. The proposed mechanisms for NK cell suppression by GCs have been the downregulation of NK cell IL-2 receptor expression[79] as well as the expression of natural cytotoxicity receptors (NCR) – NKp46 and NKp30 – which are involved in NK cells effector functions against NK-specific target cells K562[80].

Decreased NK cells activity induced by both swim and surgical stresses enabled the growth of otherwise NK cell-sensitive tumors and the dissemination of metastatic disease[81]. Adrenal demedullation and β-AR blockade abolished NK cells activity

suppression induced by swim stress[82], confirming the regulatory role of catecholamines over NK cell function.

Also NK cell mobilization from marginal pools is thought to be mediated by β-AR present in lymphatic smooth muscles. Catecholamines diminish leucocyte adhesion to blood vessels promoting leucocytes egress into circulation[83]. Likewise, denervation of the BM also induces leucocyte mobilization[84].

The large number of β-AR in NK cells' surface, especially β2, predisposes NK cells to the modulatory actions of epinephrine and NE. NE has been shown to downregulate[85] whilst epinephrine has been shown to enhance[86] NK cell cytotoxicity *in vitro* and *in vivo*[87, 88]. Upon B-AR binding, noradrenaline activates cAMP signaling cascade through PKA regulating transcription factors involved in granzyme b, perforin and IFN-γ gene transcription such as NF-ΚB, AP-1, SP-1, CBF[89].

Taken together, these findings suggest that a NK cell activity is held hostage by stress conditions involving trauma. A major stressor like SCI could modulate the long-lasting NK cell deficit allowing the outbreak of infectious diseases in these patients. For this reason, prophylactic measures should be taken in order to prevent debilitating infectious conditions in SCI patients, by targeting NK cells and controlling the HPA axis and the sympathetic nervous system, which probably play has a major role on the NK cells' suppression.

1.10 Aim of the thesis

The working hypothesis for this thesis concerns the observation that spinal cord injury leads to a downregulation of the immune system (chapter 1.8). The aim is to characterize further aspects of the spinal cord injury-induced immune depression syndrome.

The objectives of this thesis are to investigate NK cell function in a rodent model of SCI and in human patients affected by SCI.

We hypothesized that high lesion level would induce a deeper NK cell suppression than a lower lesion. This paradigm was tested in both humans and rats. The rodent study was designed with the aim to discern whether organ denervation induced by SCI, would directly alter organ cellular constitution, especially regarding NK cells. This study encompassed the analysis of different compartments regarding NK cells'

distribution pattern, function (immunoregulatory cytokine production), state of activation, priming for migration and maturation. These characteristics could potentially be jeopardized by a sub-optimal microenvironment prompted by an inadequate nerve supply to the organs where NK cells mature and operate.

The clinical trial with patients affected by SCI aimed to investigate about dependency of the extent of NK cell deficit on lesion height and severity. We were also able to demonstrate the neurogenic nature of NK cells deficit after SCI.

2 MATERIALS AND METHODS

2.1 Experimental Spinal Cord Injury

All procedures described in this thesis were approved by the relevant authority, Landesamt für Gesundheit und Soziales, Berlin, Germany (TVA G0258/12), and performed in accordance with the European directive on the protection of animals used for scientific purposes and the respective German legislation.

2.1.1 Spinal cord contusion model

50 12-week-old male Lewis rats (220-300 g, Charles River) were randomly assigned to undergo SCI either at T5 level, T9 or sham-operation (bi-laminectomy at T9 level). The animals were randomly assigned to be sacrificed 3 and 28 days later.

. Rats were anesthetized by intraperitoneal injection of ketamine hydrochloride (Ketanest, Parke Davis, Berlin, Germany; 100 mg/kg) and xylazine hydrochloride (Rompun, Bayer, Leverkusen, Germany; 10 mg/kg). They received pain medication one hour prior to surgery (Buprenorphine, Temgesic, Schering-Plough, 0.05 mg/kg). To prevent xerophthalmia during anesthesia, both eyes were covered with retinopalmitate (Retinopalmitol, Ciba). The skin overlying the vertebral column was incised, and the muscles were detached from the vertebra. The skin overlying the vertebral column was incised, and the muscles were detached from the vertebra. A single-level bilaminectomy was then performed to expose the spinal cord at level T5 or T9, respectively. After opening the dura mater, vertebrae were fixated to the NYU Impactor (New York University Neurosurgery Laboratory, New York, NY) and a contusion injury was evoked from a 50mm height. The wound was rinsed with normal saline and closed in layers. The sham-operation (control) consisted in the surgical removal of the dorsal arch of the vertebra – spinous process and lateral arches without dura or spinal cord injury. The muscular layer and the skin were sewed back.
The post-operative care comprised analgesic treatment (Buprenorphine, Temgesic, Schering-Plough, 0.05 mg/kg), manual bladder compression and bathing daily in hand-warm water to prevent urine burns. All rats were kept in controlled conditions of light and temperature, with food and water ad libitum. Weight and temperature were measured daily in order to monitor for health status and infection profile.

2.1.2 MRI studies: Measurement of lung infection

T5-, T9-lesioned and sham-operated rats were randomly assigned to undergo a transverse and longitudinal magnetic resonance imaging (MRI) scan of the lungs in order to determine the development spontaneous pneumonia. MRI was performed 5 to 7 days posterior to SCI- or sham-operation using a 7 Tesla rodent scanner (Pharmascan 70/16, Bruker BioSpin, Ettlingen, Germany) in the centre for small animal imaging in the Neuroscience Research Centre (NWFZ), Charité – Berlin. An image sequence of twenty axial slices comprising the 2 lungs was performed with a slice thickness of 0.5mm using a T1-weighted FLASH sequence. Bruker software Paravision 4.0 was used for data acquisition and image processing. Rats were anesthetized with 1.5-2.0% isoflorane using a nose cone. Temperature was maintained at 37°C by a heated water blanket. Respiration and ECG signal were monitored. ECG signal was filtered and data acquisition was synchronized to heart beating in order to minimize artefacts. Analysis and quantification of the volume of inflammatory lesions in the lungs was performed using ImageJ (ImageJ 1.43u software, Wayne Rasband, National Institut of Health, USA). According to previous studies[90, 91], we manually outlined the lung borders on all slices to calculate the lung areas. Area with physiologic appearance were also outlined in order to set background noise. A signal-to-noise ratio larger than 3.5 was considered as signal of lung inflammation and this was expressed as a fraction of whole lung area.

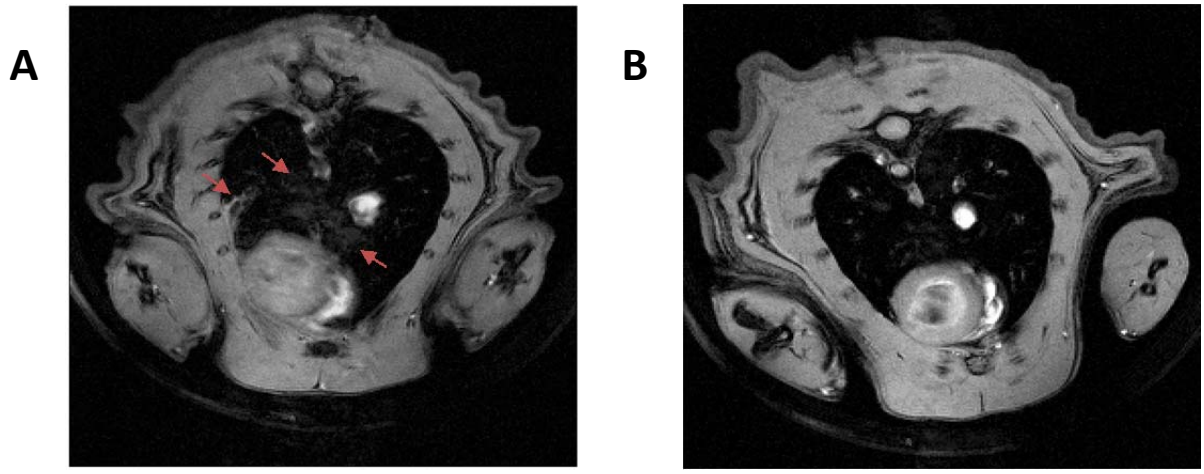

Fig. 4: Representative MRI showing spontaneous pneumonia in SCI but not in sham rats. MRI transverse section showing areas of increased intensity (inflammatory lesions) in a rat with a T5 spinal cord lesion (shown by red arrows on **A**) compared with an equivalent lung section in sham-operated control (**B**).

2.1.3 Multi-compartmental study of NK- cells in acute and chronic phases after SCI contusion

Animals were randomly assigned to be sacrificed at day 3 or day 28 after surgery (T5-, T9-SCI lesion or bilaminectomy).

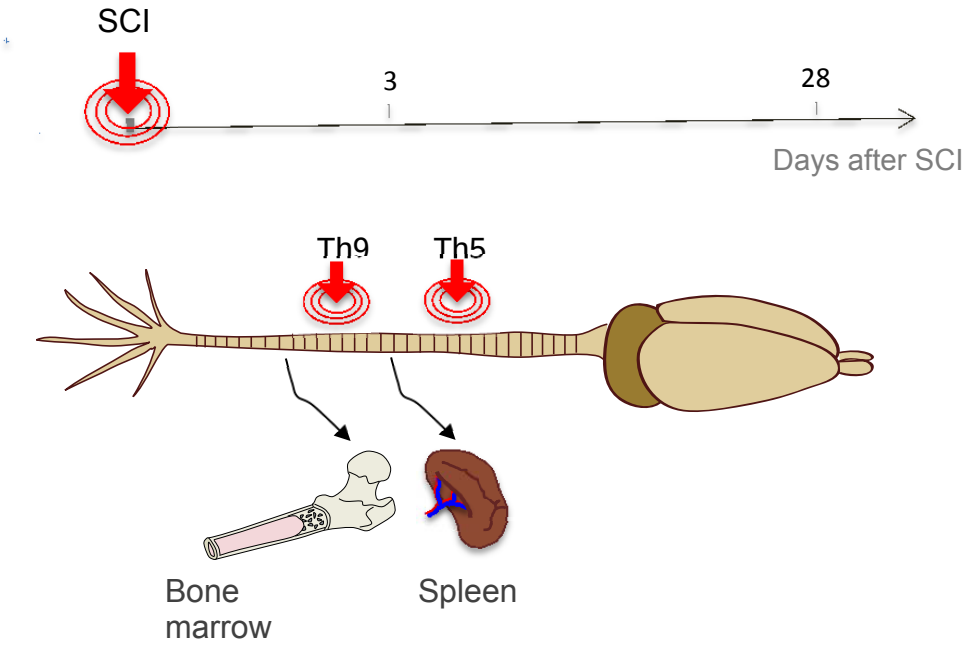

Fig. 5: Multi-compartmental assessment of NK cell function after SCI. Rats were sacrificed at day 3 and 28 after SCI or sham-operation. We evaluated NK cell function in the BM, spleen and blood using two lesions paradigm. We hypothesized that T5 contusion lesion would disrupt both spleen and BM innervation while T9 would only disrupt BM innervation, impairing NK cell function in the denervated organs.

2.1.3.1 Blood and tissue collection

Animals were sacrificed after anaesthesia induction with isoflorene and an overdose of i.p. Ketanest and Rompun. Blood was withdrawn by intracardiac puncture into sterile heparinized syringes. Spleen and bone marrow were acquired post-mortem and prepared. Bone marrow cells was obtained in sterile condition by flushing PBS+ 2% FCS with a 23G needle through the femur and tibia in one side of the body. Tissues were processed for FACS stainings and ex-vivo stimulation.

2.1.3.2 PBMCs preparation

Part of the acquired blood was left aside for white blood cell counting. Plasma was divided from cells through gradient separation using Lymphocyte separation medium

(PAA), plasma was immediately stored at -20°C, the layer of peripheral blood mononuclear cells (PBMCs) were isolated after the lysis of the remaining erythrocytes (Pharmlyse, BD). PBMCs were washed with RPMI-1640 (Gibco) supplemented with 10% fetal calf serum (FCS) (lonza) and resuspended in 0.5 ml RPMI-1640 supplemented with 10% FCS and Penicillin (100 U/ml, Biochrom) Streptavidin (100 µg/ml, Biochrom) (P/S). Cell concentration was calculated.

2.1.3.3 Bone marrow leukocytes preparation

Femur and tibia were broken proximally and washed with 15ml PBS+ 2% FCS. Cell solution was filtered through a 40µm filter (BD). Erythrocytes were lysed (Pharmlyse, BD), the leukocytes were washed with RPMI/10% FCS and resuspended in 1ml RPMI-1640 supplemented with 10% FCS and P/S.

2.1.3.4 Splenocytes preparation

The tissue was smashed through a 40µm filter (BD) and washed twice with RPMI/10% FCS and resuspended in 1ml RPMI-1640 supplemented with 10% FCS and P/S. Cell concentration was calculated.

2.1.3.5 Immune phenotyping of rat PBMCs and tissues cell solutions - FACS analyses

From the three different compartments (PBMCs, spleen and BM), we used 50µl of the cell solution for immune phenotypical stainings and posterior FACS analysis. An LD marker was used to exclude dead cells. FMOs were used as negative controls.

NK-cells ($CD3^-CD161^+$) were analyzed regarding their maturation– $CD11b^-CD27^+$ least mature, through $CD11b^+CD27^+$ to most mature $CD11^+CD27^-$[92, 93]. We also analysed the expression of activating receptor - NKG2D - a marker for cells in distress (expressing MICA). The downregulation of this marker leads to decreased NK cells activation. We further investigated the expression of CD62L – which enables NK cells to be recruited from lymph nodes mediating cell-endothelial interaction[94].

The antibodies used in NK cell immune phenotyping for FACS analysis are described in Table 1.

Table 1: NK cells immune phenotyping by FACS

Antigen	Fluorochrome	Catalog #	Vendor	Clone	Antibody class
CD161 (NKR-P1A)	PerCP eFluor 710	46-1610	ebioscience	10/78	IgG1
CD3	FITC	201403	biolegend	1F4	IgM
NKG2D	PE (NKR-P2)	12-3140	ebioscience	11D5F4	IgG1
CD62L	eFluor 660	50-0623-82	ebioscience	OX85	IgG1
Live/dead fixable dead cells (LD)	BV570	L-34959	invitrogen		
CD27	PE-cy7	25-0271	ebioscience	LG-7F9	IgG
CD11b	V450	562108	BD	WT.5	IgA

After staining, cell solution was washed in PBS/BSA and then fixed. The recording was made in FACS Fortessa (BD Beckton Dickinson – DRFZ) using Diva Software. We gated the lymphocyte population in forward (FSC) and sideward scatter (SSC) – different cell size and granularity. All the voltages were set for all the used antibodies. A number from 10.000 to 50.000 NK cells were recorded.

2.1.3.6 Functional assay: PMA and ionomycin stimulation

In a sterile environment, a concentration of 10.000 PBMCs/µl was added into FACS tubes. Two different conditions were prepared: 1) with phorbol-12-myristate-13-acetate (PMA) (20ng/ml) (Sigma Aldrich) and ionomycin (1µg/ml) (Sigma Aldrich); and 2) with medium alone to serve as the unstimulated control. Cells were incubated for 1h at 37°C in 5% CO_2 after which Brefeldin A (BFA) (10µg/ml) (Sigma Aldrich) was added to each tube and incubation continued for another 5h at 37°C in 0.5% CO_2. The stimulation procedure takes in total 6h while it is kept in sterile conditions in an incubator at 37°C, 0.5% CO_2.

2.1.3.7 FACS stainings and acquisition

After stimulation, cells were stained for CD3, CD161 and a dead cell marker (according to Table 2) for 20 min. Cells were then fixed and permeabilized according to manufacturers' instructions (Cytofix/Cytoperm, BD Biosciences) and stained for IFN-γ) for 30min. Cells were then resuspended in PBS/BSA and measured using

fluorescence-activated cell sorting (FACS) Fortessa (BD Bisciences) in Deutsches Rheuma Forschung Zentrum (DRFZ) FACS facility and acquired with DivaSoftware. A total of 20.000 to 50.000 NK cells per tube were recorded. Analysis is made using FlowJo software version 8.7 for Mac. NK cells were analyzed according to their capacity to produce IFN-γ. Single stainings were used for compensations and unstimulated samples as negative controls.

Table 2: Extra- and intracellular markers for NK cells functional analysis by FACS

antigen	fluorochrome	order #	vendor	clone	antibody class
CD161 (NKR-P1A)	PerCP-eFluor 710 or PE	46-1610	ebioscience	10/78	IgG1
CD3	FITC	201403	biolegend	1F4	IgM
Live/dead fixable dead cells (LD)	BV570	L-34959	invitrogen		
IFN-γ	Alexa Fluor 647	507809	bioledend	DB-1	IgG1

NK-cell concentrations (cells/µl) were calculated by normalizing the cell percentages (%) obtained in the FACS to the leukocytes numbers obtained by manual counting using the microscope. The concentrations (cells/µl) of NK-cells producing IFN-γ were calculated adjusting NK cell counts to 100%.

2.1.4 Statistics

Flow cytometric recordings were analyzed using Flow Jo software 8.7 for Mac (Tree Star, Ashland). Cell counts were calculated from the counted leukocyte numbers. Statistical analysis was carried out using Graphpad Prism 5 for Mac (Graph Pad Software, Inc.), by one-way ANOVA for 3-group comparison with Bonferroni's multiple comparison test when comparing 2 groups. Individual results are represented as dots and the mean as a bar. Means and standard deviation (SD) are given in figure captions. Values of $P<0.05$ were considered significant.

2.2 Human Spinal Cord Injury

2.2.1 Prospective study with human individuals suffering SCI

The study of NK cell function after human SCI was conducted in the frame of the SCIentinel – a multicentre prospective study of the immune system function following SCI.

2.2.1.1 Study design, study coordination, participating centres and duration

We evaluated in detail of NK cell activity up to 10 weeks after SCI as a prospective 2-centre study. The study was coordinated by the Department of Experimental Neurology, Clinical and Experimental Spinal Cord Injury Research (Neuroparaplegiology) at the Campus Mitte of the Charité University Hospital, Berlin Germany.

Patients were recruited for a period of 24 months from 2 specialized centres for SCI treatment. These included Treatment Centre for Spinal Cord Injuries, Trauma Hospital Berlin, Germany and Centre for Musculoskeletal Surgery (Campus Virchow Clinic) Berlin, Germany. Each patient is followed up for a period of 3 months after injury.

2.2.1.2 Ethics and informed consent

The Ethical Committee of the Charité – Universitätsmedizin Berlin approved the protocol (EA1/001/09). Clinicians informed the participants about the trial both orally and in written form through patient information sheets. Written informed consent was obtained prior to inclusion in the study. This study complied with the Helsinki Declaration in its recent German version, the Medical Association code of conduct, the principles of Good Clinical Practice (GCP) and the Federal Data Protection Act. The study was carried out according with local legal and regulatory requirements.

2.2.1.3 Participants

56 patients were recruited to participate in the study. Patients were allocated to 3 different groups in order to investigate if NK cell dysfunction has a lesion level dependency: 1) 23 SCI-patients with a lesion level T5 or above; 2) 17 patients with a lesion level T6 or below; 3) 16 patients with an acute vertebral fracture, but without SCI (control group). The number of patient to be allocated to each group respects the normal frequency of spinal cord injury events[95]. Analysis of the dependency of NK cell function on the lesion completeness was conducted post-hoc. Patients were divided into 3 different groups according ASIA impairment scale: ASIA A (complete lesion); ASIA B, C and D (incomplete lesion) and ASIA E (vertebral fracture with no neurological deficit). Patient drop out or being recruited at later phases of the study, gave rise to a high number of missing values. In order to increase statistical power, we recruited a higher patient number than initially calculated.

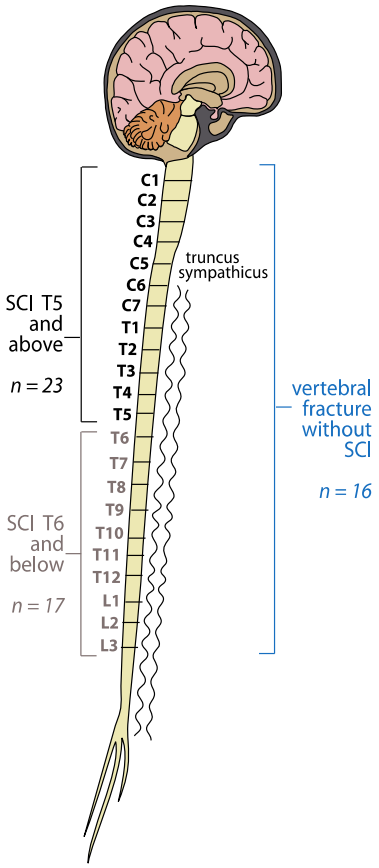

Fig. 6: **Allocations of participants into trial groups according to lesion height.** Patients with a SCI were allocated into two different groups according to the lesion height: patients with a neurologic level of lesion above the sympathetic outflow (T5 and above group) and patients with a neurologic level of injury below the sympathetic outflow level (T6 and below group). Patients with a vertebral fracture who were neurologically silent, were allocated to the control group. An additional group of age-matched healthy controls was added to the study in order to set a reference value for the outcome parameters obtained from the NK cell stimulation assays and to be able to compare to previously performed studies

2.2.1.4 Sample size calculation

The number of patients to be enrolled in the study was calculated based on a study (n=18) analysing growth and activity of leucocytes following SCI[45]. The primary end point is a difference of NK cell cytotoxicity during the chronic phase after SCI. The sample size calculation (software G Power, version 3.1.3) was performed with an

effect size of 0.54. Errors of the first type were to be set to 0.05 (two-sided); errors of the second type were set to 0.2. To test the hypothesis with a one-way ANOVA, a sample size of 39 patients would be required, due to possibility that a non-parametric test may be required for adequate statistical analysis, 5% more patients should be recruited, resulting in a number of 41 patients. The sample size calculation was performed based on epidemiological data taking an unequal distribution of the neurological level into account[95]. The group sizes are 1) T5 & above 40% (n=16); 2) T6 & below 30% (n=13) 3) and the size of the control group was set as 30% (n=12).

2.2.1.5 Enrolment and eligibility criteria

The study enrolment was carried out according to the criteria pre-established in the SCIentinel Study. In brief, patients admitted to this study must be 18 years or older, with acute isolated SCI (ASIA A-D) after decompression or stabilization surgery and patients with a neurologically silent vertebral fracture (ASIA E) after stabilization surgery. Furthermore, if patients have more than one lesion, they should be definable one from another. After admission to the hospital, the patients were subject to a clinical examination and an interview to establish eligibility for the study.

2.2.1.6 Documentation schedule

The study enrolment was documented in Case Report Forms (CRFs). These included information regarding neurological classification - including American Spinal Injury Association (ASIA) impairment scale (AIS), injury date and time, medical history, concomitant injuries, medication, acute SCI therapy concerning high-dose methylprednisolone treatment as well as surgical intervention.

Patients were recruited into the trial during predefined periods of time after the injury. Three time slots were elected for blood withdrawal and subsequent analysis: from day 5 to day 9 after the injury (which will be mentioned throughout the thesis as day 7), from day 11 to day 28 post-lesion (will be mentioned as day 14) and 8 to 12 weeks after the injury (will be called week 10). Each of the elected time slots provided an insight about late acute, post-acute and chronic phases post-trauma, respectively. Patients could be recruited for the study at any time point.

Blood withdrawals should take place between 7:00 and 11:00 a.m. to minimize the influence of circadian rhythm on the tested parameters.

2.2.1.7 Definition of infections

Pneumonia and urinary tract infections (UTIs), which are the most common infections among SCI patients, were diagnosed and documented according to definition of disease to ensure comparability[96],[97]. All other infections were diagnosed and documented according to the usual criteria used in the participating centres.

2.2.1.8 Neurological classification

Neurological evaluation is performed according to the International Standards for Neurological Classification of Spinal Cord Injury Patients (ISNCSCI), a revision of the American Spinal Injury Association (ASIA) classification. ISNCSCI comprises the assessment of completeness of lesion – ASIA Impairment Scale (AIS) – and the single neurological level of the lesion.

2.2.1.9 Target cell population

K562 is a human leukemic cell line devoid of MHC class I and are therefore specific targets of NK cells. They were obtained from Deutsches Rheuma-Forschungszentrum DRFZ (courtesy of the Romagnani group) and kept under sterile conditions in the Department of Experimental Neurology. We cultured K562 in RPMI-1640 (Gibco) supplemented with 10% fetal calf serum (FCS) (lonza) and Penicillin (100U/ml, Biochrom) Streptavidin (100µg/ml, Biochrom). The cell cultures were kept in sterile conditions at 37°, 5% CO_2.

2.2.1.10 Blood sample handling

Peripheral blood is drawn under sterile conditions from each participant at the specified time points. All samples are managed with a six-figure pseudonym and any personal information of the participants is removed.

One 8ml BD Vacutainer® CPT™/Ficoll™ tube is collected from each admitted patient who fulfils the inclusion criteria for immediate preparation of PBMCs.

2.2.1.11 NK cell stimulation procedure

Before the beginning of stimulation, we estimated by flow cytometry (FACS Canto, BD biosciences) frequency of NK cells from each patient, in order to establish effector: target cell ratio. In a sterile environment, we prepared four different conditions, each in the concentration of 10.000 PBMCs/µl in FACS tubes: i) with K562 cells in 5:1 effector: target cell ratio; ii) with phorbol-12-myristate-13-acetate (PMA) (20ng/ml) (Sigma Aldrich) and ionomycin (1µg/ml) (Sigma Aldrich); iii) with interleukin (IL)-12 (50µg/ml) and IL-18 (50µg/ml); and iv) with medium alone to serve as the unstimulated control. To all four conditions, we added CD107a FITC (2µg/ml) (BD Phamingen, BD Biosciences) and monensin (0.7µg/ml) (BD GolgiStop, BD Biosciences). After 1h incubation at 37°C in 5% CO_2, Brefeldin A (BFA) (10µg/ml) (Sigma Aldrich) was added to each tube. Cells were incubated for further 5h. Brefeldin A prevents exocytosis of cytokine-containing vesicles allowing later intracellular staining. Monensin prevents the acidification of the cytokines inside the vesicles and the degradation of CD107a when internalized.

Table 3: Extra- and intracellular markers for NK cells functional analysis by FACS

antigen	fluorochrome	order #	vendor	clone	antibody class
CD56	APC	341027	BD Biosciences	NCAM16.2	IgG2b
CD3	PerCP Cy5.5	300429	biolegend	UCHT1	IgG1
Live/dead fixable dead cells (LD)	BV570	L-34959	invitrogen		
IFNγ	PE-Cy7	557643	BD Biosciences	B27	IgG1
TNF-α	eFluor 450	48-7349-41	ebioscience	MAb11	IgG1
CD107a	FITC	555800	BD Biosciences	H4A3	IgG1

After 6 hours of stimulation, PBMCs were stained for NK cell markers CD3 PerCP cy5.5, CD56 APC and a fixable yellow dead cell marker for 20 min. Cells were then fixed and permeabilized according to manufacturers' instructions (Cytofix/Cytoperm, BD Biosciences) and stained for IFN-γ PE cy7 and TNF-α efluor 450 for 30min. Cells were resuspended in PBS/BSA and measured using fluorescence-activated cell sorting (FACS) Canto (BD Biosciences) in Deutsche Rheuma Forschung Zentrum

(DRFZ) FACS facility and acquired with Diva Software. A total of 20.000 to 50.000 NK cells per tube were recorded.

The antibodies used in these experiments are described in Table 3.

An additional 6 ml of blood was collected into an EDTA covered BD Vacutainer® in order to each patient's complete blood count, including lymphocytes absolute counts.

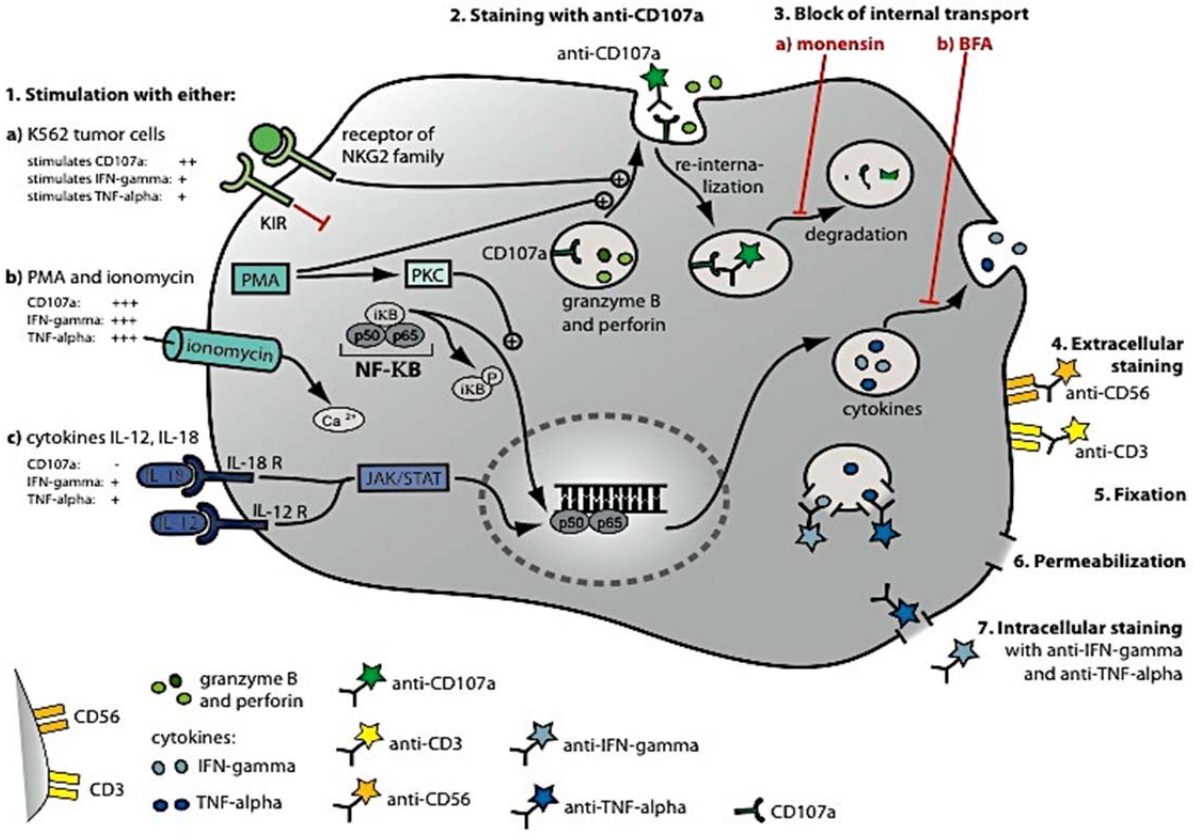

Fig. 7: NK cell stimulation paradigms and staining protocol. NK cell were subjected to 3 different stimulation paradigms: **a)** specific target cells (tumor cell line **K562**, which lack MHC I receptors, thereby activating NK cells cytolytic machinery (leading to CD107a expression); **b) PMA/ionomycin** which activate both effector functions and immunomodulatory cytokine (IFN-γ and TNF-α production pathways; **c) IL-12 and IL-18**, which lead to the production of IFN-γ. This type of stimulation did not attain optimal IFN-γ production levels and was dropped

2.2.1.12 Data and statistical analysis

Analysis was made using FlowJo software version 8.7 for Mac. The gating was performed as follows: gating on lymphocyte population, exclusion of dead cells, and selection of NK cells ($CD3^-$ $CD56^+$). After adequately compensated, the expression of $CD107a^+$, IFN-γ^+, TNF-α^+ is individually measured[98].

Data analysis of the primary endpoint (NK cell CD107a expression 8-12 weeks after the lesion) consists in the comparison between 3 groups at the 3 pre-established time points using repeated measurements - linear mixed models. The primary hypothesis of the differences between SCI and vertebral fracture patients NK cell cytotoxicity was tested using a t-test for unpaired samples. A regression analysis was used to adjust for age as a possible confounder. A two-sided significance level of $\alpha=0.05$ was considered. We tested all secondary hypotheses in an exploratory manner without adjustment for multiple testing.

Secondary endpoints were analysed comparing the 3 patient groups and a group of healthy controls during the 3 pre-defined time windows using linear mixed models (random intercept models).

The analysis was performed using the full dataset comprising all patients included according to the criteria as defined in the SCIentinel study protocol[99] and all models were age-adjusted. Interaction terms for group and time were included to test differences between groups over time. Skewed data distributions were log-transformed before analysis. In addition, in patients with complete intra-individual datasets changes over time within the groups will be assessed with descriptive methods. Group differences were tested using Model-based post hoc tests.

The statistical analysis was performed using IBM SPSS statistics version 22 for Macintosh.

3 RESULTS

3.1 Experimental spinal cord injury

3.1.1 SCI leads to an increased susceptibility to pulmonary infections during the acute phase

T5- and to a lesser extent T9-operated rats, showed a significant increase in signal intensity in series of 20 MRI transverse sections of the whole lung compared to sham-operated rats. This points to an increased lung inflammation volume, most probably caused by spontaneous pneumonia.

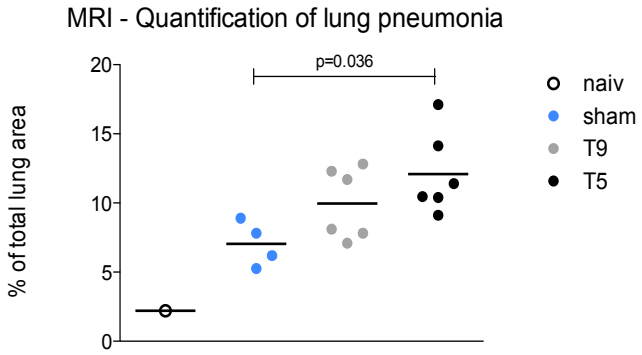

Fig. 8: Quantification of lung pneumonia by MRI scan. 15 lung transverse sections from each rat were analysed using image J (naïv: 2.2; sham: mean=7.04, SD=1.63; T9: mean=9.97, SD=2.56; T5: mean=12.10, SD=2.97)

3.1.2 Multi-compartmental study of T- and NK- cells in acute and chronic phases after SCI contusion

3.1.2.1 Spinal cord injury induces changes in the composition of lymphocytic population during the acute phase

Peripheral blood PBMCs, spleen and bone marrow of Lewis rats were analysed by flow cytometry after transection injury of the spinal cord. 16 rats underwent sham operation (bilaminectomy), 17 rats underwent SCI contusion at the level of T9 and 17 underwent an SCI contusion at the level of T5. The animals were randomly assigned to be sacrificed either 3 days or 28 days after SC contusion injury. We sought to access the impact of organ denervation caused by SCI on the organ-resident NK

cells by comparing two different lesion heights. NK cells tissue distribution and function are liable to neuroendocrine regulation[36, 88].

SCI results in a decrease of blood (Fig. 9A) and BM (Fig. 9) leukocyte numbers during the acute phase succeeding injury, which subsides during the chronic phase. Interestingly, both surgery- and SCI-induced stress appear to have a negative effect on splenic cellularity on day 3 (Fig. 9B), as all groups show a decreased splenocyte count. Splenocyte numbers significantly recover by day 28 (p=0.0002) in all 3 groups.

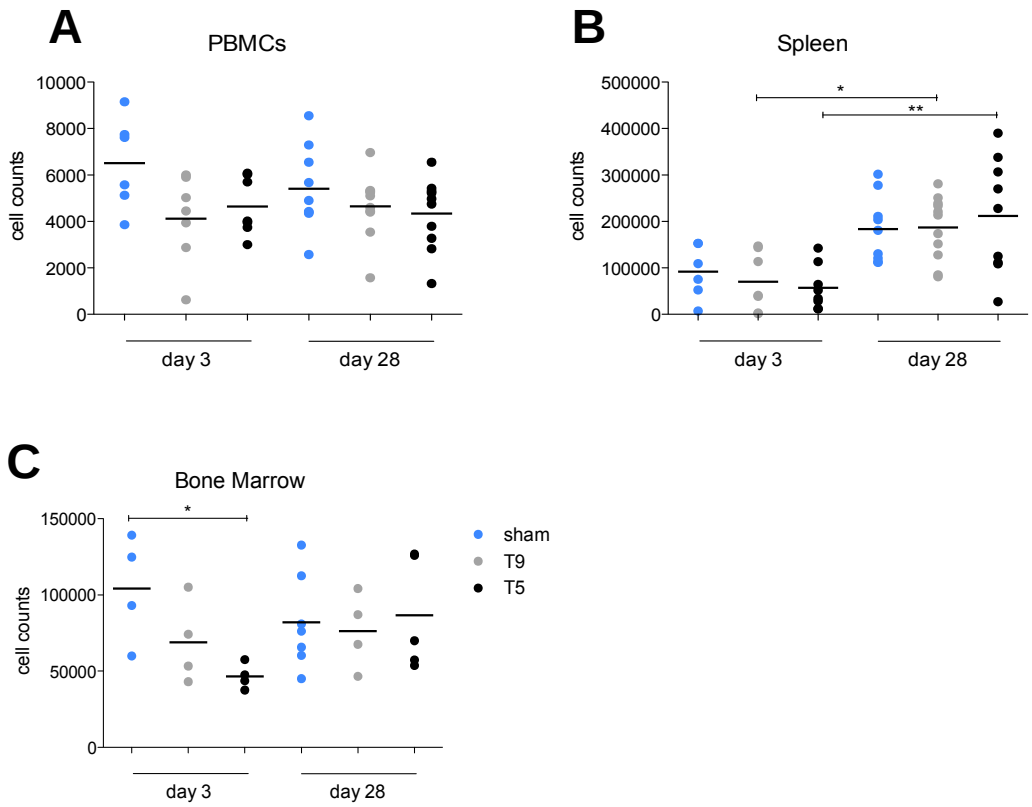

Fig. 9: Leukocyte counts (cells/µl) from a predefined volume of cell solution, obtained using a cell counting chamber under the microscope. At day 3, whole blood and BM leukocyte counts were decreased in SCI animals compared to sham. Both SCI groups recovered their whole blood and BM leukocyte counts until day 28. All groups revealed decreased splenocyte counts on day 3, which recovered on day 28 after surgery. **A. PBMCs** (cells/µl) (day 3: p=0.058; sham: mean=6515, SD=1978; T9: mean=4118, SD=1890; T5: mean=4648, SD= 1253; day 28: p=0.34; sham: mean=5414, SD= 1810; T9: mean=4654, SD=1400; T5: mean=4345, SD=1533). **B. Splenocytes** (cells/µl of cell solution) (day 3: p=0.538; sham: mean=91800, SD=57745; T9: mean=70032, SD=63618; T5: mean=57475, SD= 47793; day 28: p=0.767; sham: mean=183611, SD=71548; T9: mean=187273, SD=68003; T5: mean=211822, SD=123723). **C. Bone Marrow** cellularity (cells/µl of cell solution) (day 3: p=0.036; sham: mean=104225, SD=35295; T9: mean=68900, SD=27327; T5: mean=46575, SD= 8387; day 28: p=0.887; sham: mean=81914, SD=30679; T9: mean=76325, SD=24849; T5: mean=86720, SD=36675). Significances were calculated with Kruskal-Wallis test and Bonferroni's multiple comparison test or one-way ANOVA analysis of variance when samples are normally distributed; *p=0.05; **p=0.01; ***p=0.001

3.1.2.2 SCI influences NK cell counts and frequencies

Spinal cord injury induced changes in the leukocyte constituency of the blood, spleen and bone marrow. SCI, especially the highest lesion, induced an increase in the frequency of NK cells on day 3 after injury, which was more pronounced in blood (Fig. 10A, $p<0.05$) and in BM (Fig. 10C, $p<0.05$) than in the spleen (Fig. 10E). On day 28, blood and BM NK cell frequencies of SCI-operated groups were not significantly different from the sham-operated group (Fig. 10A, C and E).

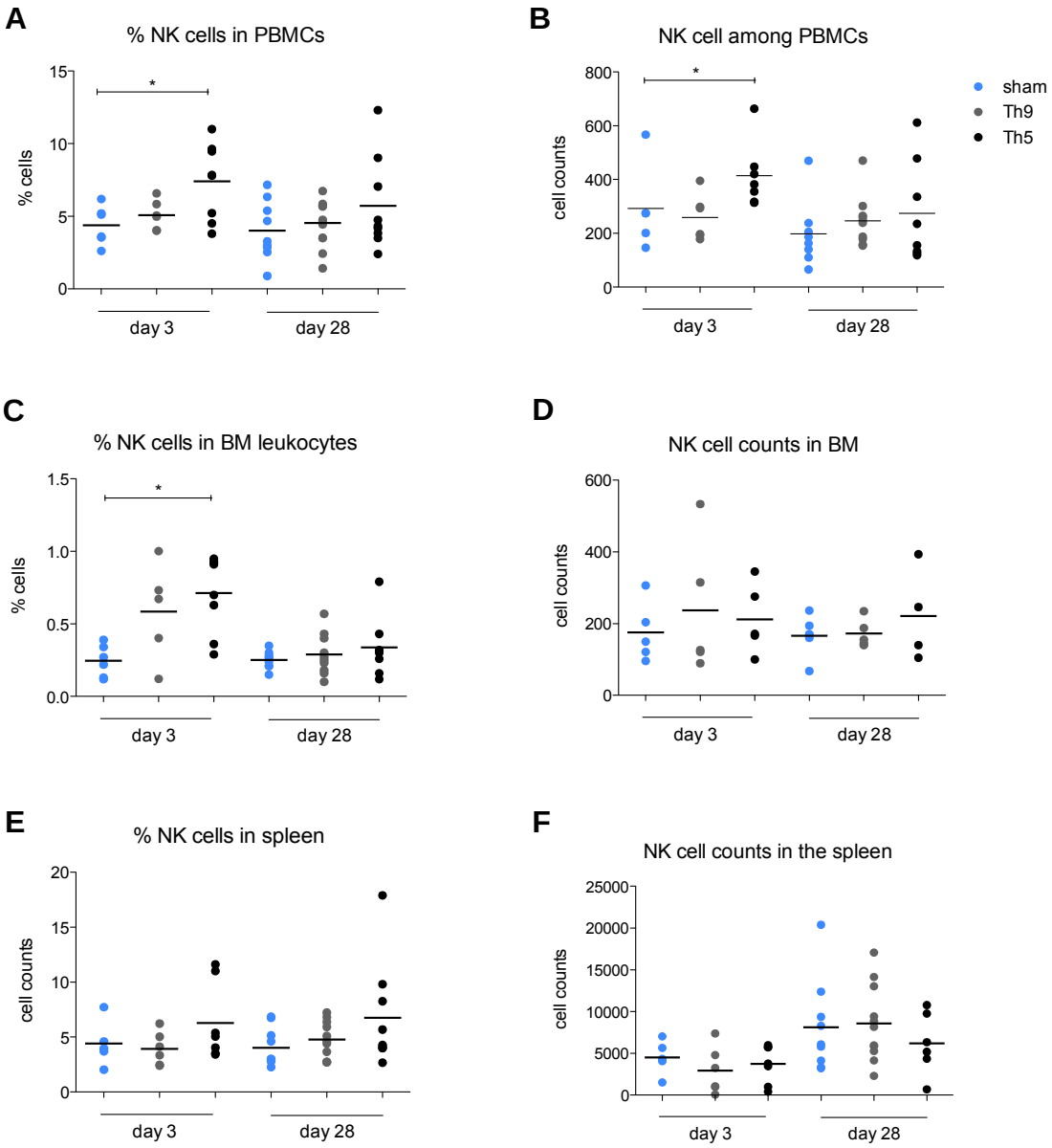

Fig. 10: NK cell frequencies (%) and numbers (cells/µl). NK cell frequencies (**A., C. and E.**) were obtained using flow cytometry, whereas NK cell numbers (**B., D. and F.**) were obtained by adjusting the frequencies obtained by FACS to the cell numbers obtained under the microscope (Fig. 9). in SCI rats, NK cell frequencies

were increased in blood (A.) and BM (C.). However, the adjustment of high percentages to low leukocyte numbers yielded a similar NK cell number in SCI rats compared to sham-operated rats (B. and D.). In the spleen, neither NK cell frequency nor numbers were significantly different in SCI compared with sham-operated rats. **A. % NK cells within peripheral blood mononuclear cells PBMCs** (day 3: p=0.0211; sham: mean=4.38, SD=1.34; T9: mean=5.09, SD=1.01; T5: mean=7.42, SD=2.64; day 28: p=0.54; sham: mean=4.02, SD=2.015; T9: mean=4.54, SD=1.57; T5: mean=5.71, SD=3.18). **B. NK cell counts (cells/µl).** (day 3: p=0.035; sham: mean=292.8, SD=162.7; T9: mean=259.1, SD=84.83; T5: mean=414.2, SD=120.3; day 28: p=0.53; sham: mean=197.5, SD=122.6; T9: mean=246.3, SD=93.13; T5: mean=274.2, SD=185.7). **C. % NK cells within bone marrow leukocytes** (day 3: p=0.026; sham: mean=0.22, SD=0.09; T9: mean=0.56, SD=0.38; T5: mean=0.71, SD=0.29; day 28: p=0.60; sham: mean=0.25, SD=0.06; T9: mean=0.29, SD=0.14; T5: mean=0.34, SD=0.21). **D. NK cells within bone marrow leukocytes** (day 3: p=0.77; sham: mean=195.1, SD=81.62; T9: mean=265.7, SD=203.8; T5: mean=239.6, SD=86.31; day 28: p=0.53; sham: mean=166.3, SD=55.7; T9: mean=172.6, SD=39.45; T5: mean=221.0, SD=129.6). **E.** % NK cells within splenocytes (day 3: p=0.36; sham: mean=4.41, SD=2.08; T9: mean=3.93, SD=1.51; T5: mean=6.28, SD=3.51; day 28: p=0.40; sham: mean=4.01, SD=1.71; T9: mean=4.75, SD=1.68; T5: mean=6.75, SD=4.77). **F.** NK cells within splenocytes (day 3: p=0.61; sham: mean=4533, SD=2047; T9: mean=2930, SD=2793; T5: mean=3731, SD=2793; day 28: p=0.40; sham: mean=8118, SD=5502; T9: mean=8570, SD=4561; T5: mean=6188, SD=3697).

Blood was the only immune compartment where an increase in absolute numbers of NK cells took place during the acute phase after SCI (Fig. 10B). In contrast to frequencies (%), absolute numbers of BM NK cells do not appear to be directly affected by SCI (Fig. 10D). The increase in BM NK cells frequency 3 days after SCI rather reflects a decrease in other immune players such as T- and B-cells. Splenic NK cells show a significant increase from the acute to the chronic phase, reflecting an increase in the overall splenic cellularity (Fig. 10F).

3.1.2.3 SCI does not seem to influence NK cell maturation, migration and activation

We hypothesised that a decrease in NK cell function would translate in a deficient maturation process, diminished activating status and diminished migratory potential into the affected tissues.

We tested this hypothesis by conducting series of stainings using extracellular markers CD27 and CD11b for maturation, CD62L for migration/homing and NKG2D for activation. In this study we did not observe any significant difference between the 3 groups (data not shown).

3.1.2.4 Spinal cord injury induced a functional deficit on individual NK cells, particularly during the chronic phase

We hypothesized that the increased susceptibility to infectious diseases during the chronic phase after spinal cord injury is due to a cell functional deficit disturbing the primary immune response to infectious agents.

We tested NK cells response to PMA/ionomycin stimulation 3 and 28 days posterior to either sham-operation or to SCI. PMA is a non-specific lymphocyte-activating stimulus bypassing membrane receptor interaction and reacting directly with intracellular protein kinase C pathway. Ionomycin is a calcium ionophore. PMA/iono represent the highest stimulus for NK cell weaponry activation[18]. As readout of NK cell activity, we used NK cell capacity to produce IFN-γ.

We were also interested whether higher lesions would more pronouncedly suppress NK cells function resident in organs denervated subsequently to the injury. In order to access the lesion level dependency and impact of organ denervation on NK cells suppression, we stimulated NK cells in different compartments.

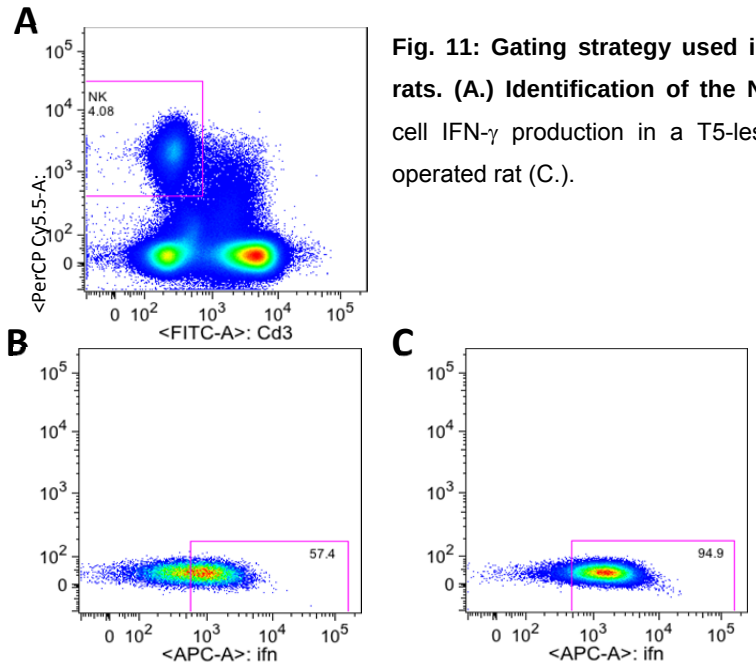

Fig. 11: Gating strategy used in the analysis of NK cell function in rats. (A.) Identification of the NK cell population (CD161+CD3-). NK cell IFN-γ production in a T5-lesioned rat. (B.) comparing to a sham-operated rat (C.).

Systemic NK cells isolated from PBMCs, displayed a diminished capacity of individual NK cells to produce IFN-γ. This functional deficit was observed both in the acute and the chronic phase ($p<0.05$). It affected both the animals with a higher and with a lower SCI injury (Fig. 12A). SCI did not appear to impact on splenic NK cells

during the acute phase after injury. However, during the chronic phase, animals with SCI display a decreased NK cell function, especially pronounced in the ones with a higher lesion ($p<0.05$) (Fig. 12B). BM NK cells from SCI animals showed a non-significant deficit on day 3 and a significant deficit on day 28 ($p<0.05$) (Fig. 12C).

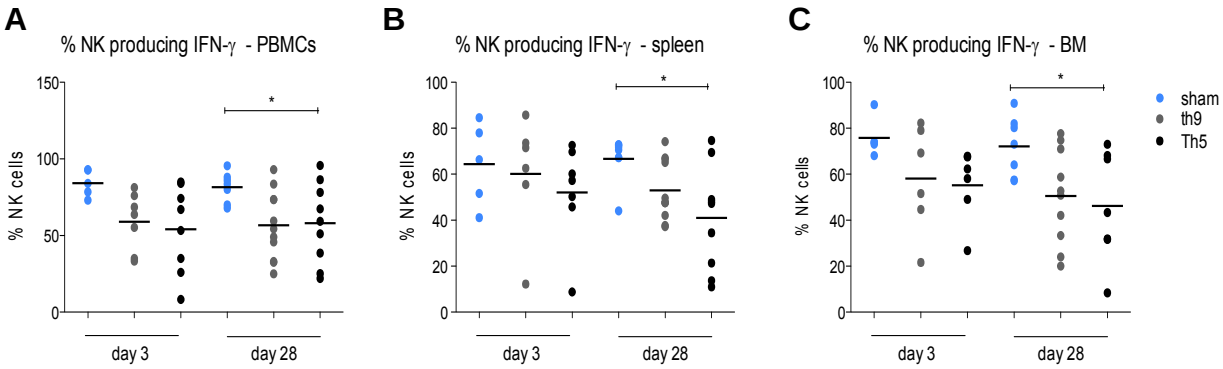

Fig. 12: Percentage (%) of NK cells producing IFN-γ. On day 28, we observed in SCI rats, in all three analysed compartments a significant decline in the percentage of NK cell producing IFN-γ compared with sham-operated rats. **A. Isolated PBMCs** (day 3: p=0.07; sham: mean=84.24, SD=8.78; T9: mean=59.04, SD=18.91; T5: mean=54.13, SD=28.49; day 28: p=0.05; sham: mean=81.63, SD=9.88; T9: mean=56.65, SD=22.23; T5: mean=58.23, SD=26.29). **B. Isolated splenocytes** (day 3: p=0.63; sham: mean=64.36, SD=18.04; T9: mean=60.2, SD=25.67; T5: mean=52.11, SD=21.40; day 28: p=0.04; sham: mean=66.69, SD=10.24; T9: mean=52.96, SD=12.97; T5: mean=41.03, SD=22.84). **C. Isolated BM leukocytes** (day 3: p=0.19; sham: mean=76.60, SD=9.55; T9: mean=55.86, SD=25.28; T5: mean=55.30, SD=15.60; day 28: p=0.05; sham: mean=72.16, SD=13.03; T9: mean=50.55, SD=20.60; T5: mean=46.19, SD=24.05).

When the frequency (%) of NK cells producing IFN-γ was normalized to NK cell counts, showing the concentration of NK cells producing IFN-γ, there were no significant differences in NK cells IFN-γ production between the different groups in both time points (Fig. 13A, B and C).

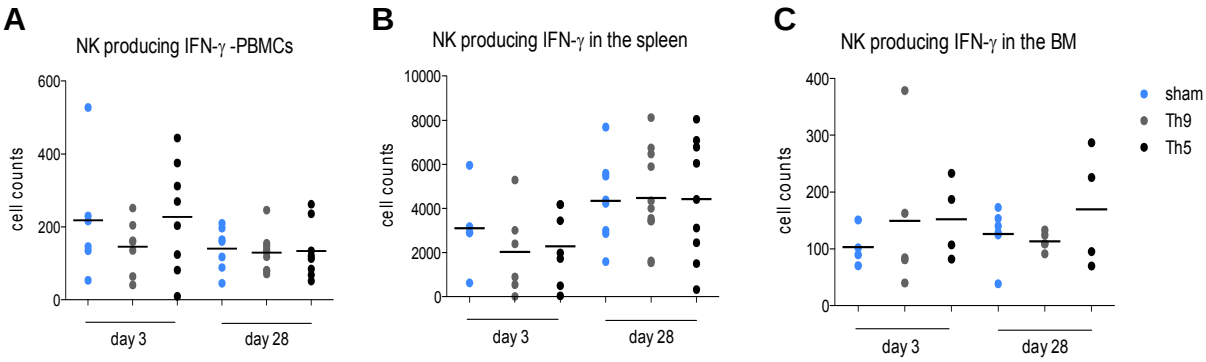

Fig. 13: NK cells producing IFN-γ. After calculating NK cells numbers (**Fig. 10**), we adjusted the percentage of NK cell producing IFN-γ to its absolute numbers. There are no significant differences between SCI- and sham-operated animals regarding absolute numbers of NK cells producing IFN-γ **A. NK cells/µl producing IFN-γ in isolated PBMCs** (day 3: p=0.47; sham: mean=218.3, SD=164.3; T9: mean=145.6, SD=74.14; T5: mean=227.7, SD=150.2; day 28: p=0.94; sham: mean=140.7, SD=59.41; T9: mean=129.9, SD=50.82; T5: mean=134.0, SD=76.57). **B. Numbers of NK cells producing IFN-γ in isolated splenocytes** (day 3: p=0.61; sham: mean=3117, SD=1892; T9: mean=2026, SD=1963; T5: mean=2293, SD=1691; day 28: p=0.99; sham: mean=4353, SD=1908; T9: mean=4472, SD=2107; T5: mean=4419, SD=2723). **C. Numbers of NK cells producing IFN-γ in isolated BM leukocytes** (day 3: p=0.81; sham: mean=103.1, SD=34.46; T9: mean=149.1, SD=135.7; T5: mean=152.2, SD=70.03; day 28: p=0.39; sham: mean=126.3, SD=46.46; T9: mean=113.2, SD=16.1; T5: mean=169.4, SD=103.9)

3.2 Human Spinal Cord Injury

3.2.1 Prospective study with human individuals suffering SCI

We hypothesized that NK cells function would be downregulated after SCI, extending into the chronic phase. We further hypothesized that high lesions would result in a deeper suppression in NK cell functions than lower lesions and that more severe lesions would result in a deeper suppression in NK cell functions than less severe lesions.

In order to test these hypothesis we submitted NK cells from each patient to the stimulatory paradigms described in chapter 2.2.1.11 NK cells activity was measured by FACS posteriorly to stimulation and staining procedures.

Besides testing NK cells for cytotoxicity, as it has previously been done[45, 68], we also tested whether SCI would impact on other NK relevant functions, including the production of proinflammatory cytokines. As a marker for NK cell cytotoxicity, we used CD107a[98], which is expressed both upon contact with K562 cells and by

PMA/ionomycin stimulation. Besides the expression of CD107a, PMA/ionomycin also leads to a robust production of IFN-γ and TNF-α. We tested whether such a high stimulus, would enable us to detect any fine NK cell deficits comparing the 3 study groups at 1, 2 and 10 weeks after injury.

We compared 3 groups of patients: 1) suffering a lesion at the level of T5 and above; 2) suffering a lesion at the level of T6 and below; 3) suffering a vertebral fracture without any injury to the spinal cord. Groups 1) and 2) included patients sustaining an SCI with a severity from ASIA A-D while group 3) entails patients with a lesion of ASIA E severity. The reference values were given by age-matched healthy controls. The latter group contributed to the setting of the baseline of the measured parameters. The healthy control groups have also been widely used as the sole control group when measuring NK cell activity after SCI. For this reason, it also provides a reference, enabling the comparison of our study to studies previously performed by other groups[24, 45, 72].

3.2.1.1 Both surgical stress and SCI induced lymphopenia during the acute and post-acute phases, but only SCI patients remain lymphopenic during the chronic phase

All groups registered a recovery in terms of lymphocytes numbers from the acute to the chronic phase. However, only the ASIA-E patients recover fully, nearly achieving the reference value of healthy controls. ASIA E patients (with a vertebral fracture) also display a significant recovery of lymphocyte counts from week 1 and 2 to week 10 ($p<0.01$) (Fig. 14). At 1 and 2 weeks post-injury all study groups present a significant decline in lymphocyte counts when compared with the reference group of age-matched healthy controls (on day 7: T5 & above: $p=0.0005$; T6 & below: $p=0.002$; ASIA-E: $p=0.013$; on day 14: T5 & above: $p=0.0005$; T6 & below $p=0.006$; ASIA-E: $p=0.003$; on the 10th week: T5 & above: $p=0.091$; T6 & below $p=0.029$; ASIA-E: $p=0.922$).

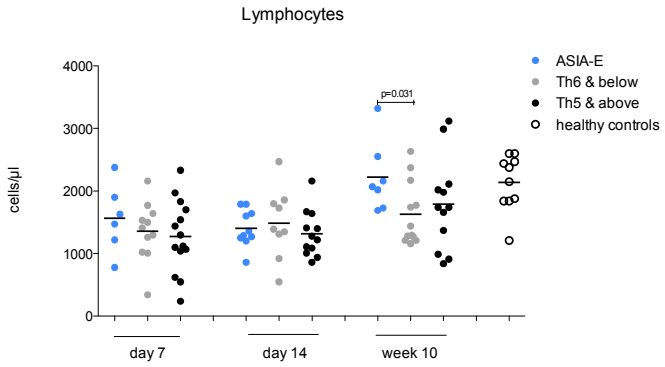

Fig. 14: Lymphocyte counts in healthy individuals, control patients, SCI patients T6 and below and SCI patients T5 and above on week 1, 2 and 10 after SCI. (day 7: ASIA-E: mean=1562, SD=552.3; T6 & below: mean=1358, SD=472.2; T5 & above: mean= 1275, SD=579.3; day 14: ASIA-E: mean=1405, SD=295.6; T6 & below: mean=1487, SD=561.4; T5 & above: mean= 1316, SD=369.9; week 10: ASIA-E: mean=2220, SD=563.6; T6 & below: mean=1628, SD=508.9; T5 & above: mean= 1789, SD=446.3; healthy controls: mean=2141, SD=446.3). Significances were calculated with repeated measurements linear mixed model. Shown are only significant differences between SCI and ASIA-E patients; significances between different time points and between patients and healthy controls are mentioned in the text

3.2.1.2 Both SCI and surgical stress influence NK cells counts and frequencies

Both NK cells counts (NK cells/µl) and frequencies (%) were analysed after defining a lymphocytic gate. Both SCI patient groups show decreased NK cell numbers one week post-injury. Patients with T5 & above recover NK cell numbers (NK cells/µl) only at the latest time point (from day 7 to week 10: p=0.016; from day 14 to week 10: p= 0.0005). Patients with T6 & below recover NK cell numbers already on week 2 (from day 7 to week 10: p=0.0005; from day 14 to week 10: p= 0.029). ASIA-E patients display a significant increase in NK cell counts from day 14 to week 10 (p=0.03) (Fig. 16A).

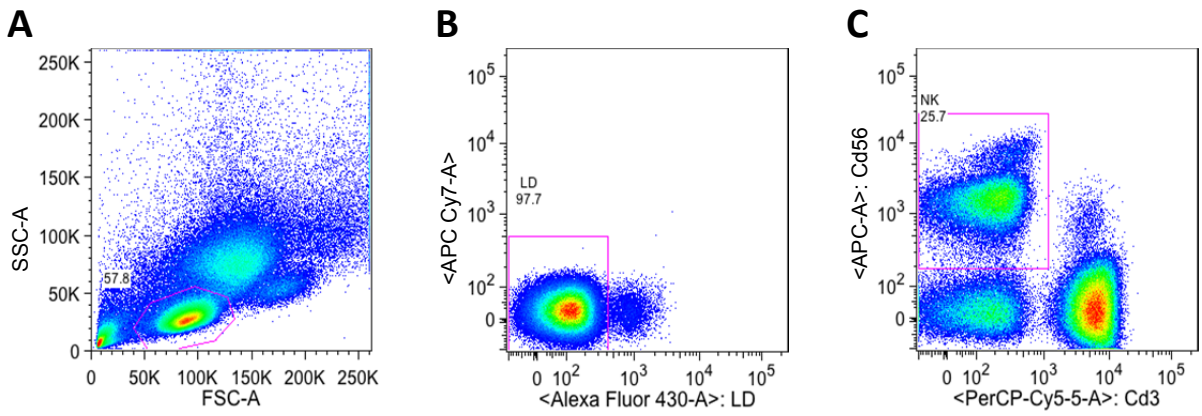

Fig. 15: Gating strategy used in the analysis of NK cell by FACS. A. Gating the lymphocyte population; **B**. Dead cell exclusion; **C.** NK cell gating ($CD56^{+}CD3^{-}$)

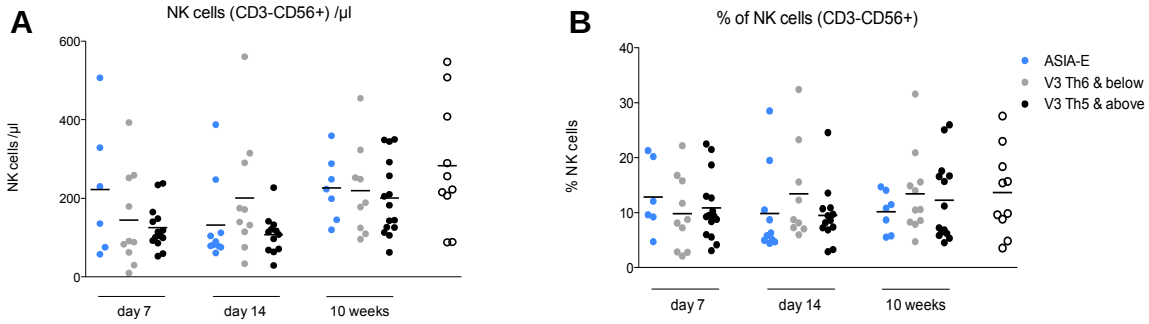

Fig. 16: Fluctuations of the NK cell population in the study groups along time. We did not observe any significant quantitative deficit regarding neither NK cell numbers nor frequencies in SCI compared with ASIA E patients during the studied period. **A.** Counts (NK cells/µl). On day 7, a significant decrease is seen between T6 & below (p=0.0005) and T5 & and above (p=0.011) comparing to healthy controls (ASIA-E: mean=222.4, SD=172.5; T6 & below: mean=144.7, SD=122.7; T5 & above: mean= 125.2, SD=56.2). On day 14 all groups are significantly decreased compared to the healthy controls: ASIA-E: p=0.015; T6 & below: p=0.022; T5 & above: p= 0.0005 (ASIA-E: mean=131.8, SD=104.4; T6 & below: mean=200.4, SD=153.5; T5 & above: mean= 107.5, SD=49.99). On the week 10 there are no significant differences between the studied groups, meaning all groups have recovered NK cell counts until normal (reference) values (ASIA-E: mean=226.3, SD=82.35; T6 & below: mean=219.6, SD=115.8; T5 & above: mean=200.7, SD=96.58. Healthy controls: mean=283.1, SD=158.7). **B.** Frequencies (%) within the lymphocytic population (day 7: ASIA-E: mean=12.87, SD=6.56; T6 & below: mean=9.84, SD=6.74; T5 & above: mean= 10.86, SD=5.93; day 14: ASIA-E: mean=9.85, SD=7.98; T6 & below: mean=13.43, SD=8.99; T5 & above: mean= 9.49, SD=5.40; week 10: ASIA-E: mean=10.16, SD=3.67; T6 & below: mean=13.41, SD=7.53; T5 & above: mean=12.25, SD=7.37; healthy controls: mean=13.68, SD=7.78)

3.2.1.3 Both SCI and surgical stress reduce K562-induced NK cell cytotoxicity

We investigated the different components of NK cell function. First we analysed NK cells' capacity to recognize NK-specific target cells – K562, and to activate its killing machinery by expressing CD107a (LAMP-1), a cytotoxicity marker. In line with previous studies evaluating NK cell function using a similar assay[24, 45], we showed decreased NK cells cytotoxicity in SCI patients compared with healthy controls (day 7: T6 & below: p=0,0005 and T5 & above: p=0.0005; day 14: T6 & below: p=0.001 and T5 & above: p=0.0005; week 10: T6 & below: p=0.025 and T5 & above: p=0.012). Surprisingly, also ASIA E patients demonstrated a suppressed NK cell cytotoxic response to K562 cells in comparison with healthy controls, all time points especially at day 7 (p=0.006) and at day 14 (p=0.0005), suggesting that other suppressive agents such as surgery-induced stress may be playing an inhibitory role upon NK cells, but are not as long-lasting as SCI-induced suppression (Fig. 17).

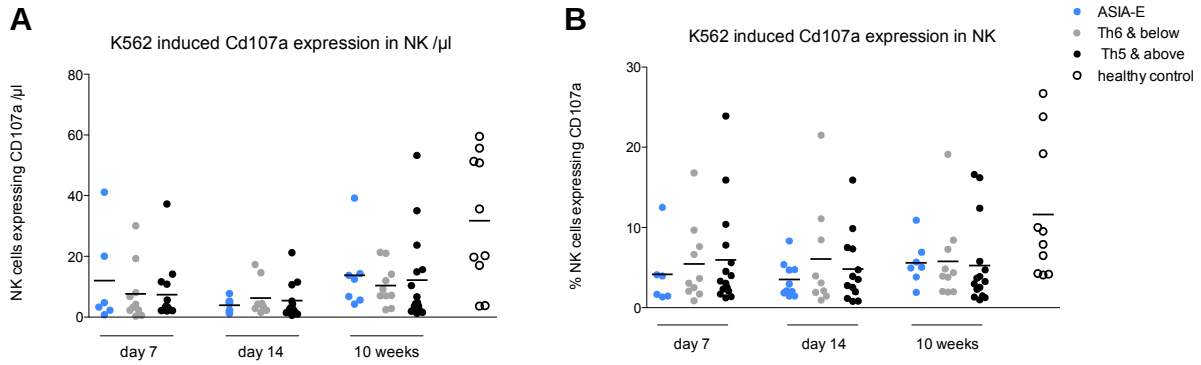

Fig. 17: K562-induced expression of CD107a in NK cells. NK cells cytotoxicity against K562-target cells was significantly declined in all 3 patient groups comparing with healthy controls at all time points. **A. CD107a-expressing NK cell counts (NK cells/µl)** (day 7: ASIA-E: mean=12.05, SD=15.90; T6 & below: mean=7.68, SD=9.6; T5 & above: mean= 7.35, SD=9.50; day 14: ASIA-E: mean=3.90, SD=2.12; T6 & below: mean=6.31, SD=6.07; T5 & above: mean= 5.43, SD=6.10; week 10: ASIA-E: mean=13.77, SD=11.93; T6 & below: mean=10.39, SD=6.69; T5 & above: mean=12.17, SD=14.87; healthy controls: mean=31.70, SD=21.51). **B. Frequencies (%) of NK cells expressing CD107a.** (day 7: ASIA-E: mean=4.17, SD=4.27; T6 & below: mean=5.46, SD=4.91; T5 & above: mean= 5.98, SD=5.93; day 14: ASIA-E: mean=3.50, SD=2.25; T6 & below: mean=6.05, SD=6.74; T5 & above: mean= 4.83, SD=4.36; week 10: ASIA-E: mean=5.60, SD=2.81; T6 & below: mean=5.77, SD=5.16; T5 & above: mean=5.29, SD=5.3; healthy controls: mean=11.61, SD=8.48)

3.2.1.4 PMA and ionomycin stimulation unravels neurogenic deficit encompassing multiple NK cell functions

We further analysed NK cells potential to express CD107a (Fig. 18) and to produce IFN-γ (Fig. 20) and TNF-α (Fig. 21) upon stimulation with the high stimulus – PMA and ionomycin . Although all studied groups displayed a diminished NK cell function on the 1st and 2nd week after injury, neurologically intact patients (ASIA-E) recovered at the latest measured time point on all tested functional parameters, while SCI patients remained consistently low throughout the studied period. On day 7, patients with a T6 lesion or below and with a T5 lesion or above have a significantly diminished NK cell cytotoxicity (CD107a expression) compared with healthy controls (T6 & below: p=0.002, and T5 & above: p=0.001) (Fig. 19A). On day 14, the same trend is followed (T6 & below: p=0.007; T5 & above: p=0.007) At the last time point only the patients with higher lesions display a significant reduction of NK cells cytotoxicity comparing with healthy controls (p=0.007) (Fig. 19A).

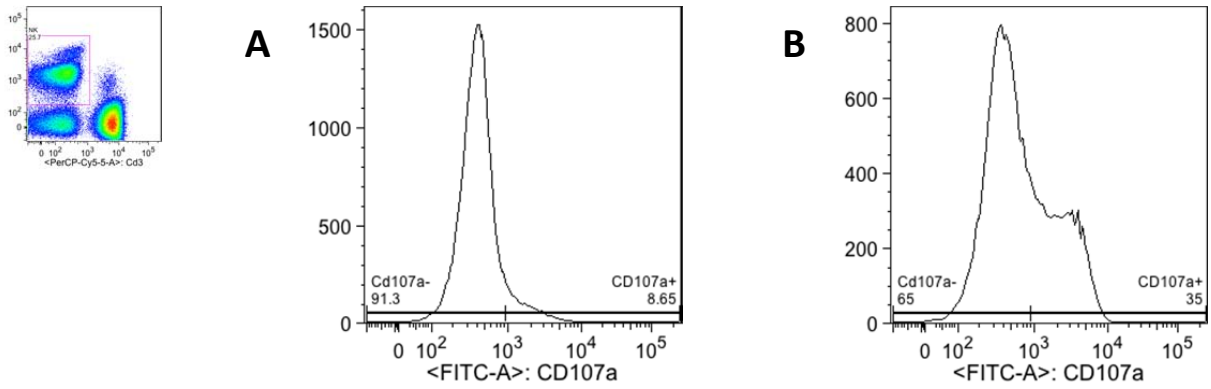

Fig. 18: NK cell expression of CD107a in a SCI (A) and a ASIA-E (B) patient

Regarding individual NK cell cytotoxicity, as given by the measurement of NK cell frequencies (%) of CD107a expression, none of the groups registers any significant increase along time. Individual NK cell degranulation of healthy controls outperformed the one from patients with higher lesions at all time points (on day 7: p=0.038; on day 14: p=0.007 and on the 10th week: p=0.007).

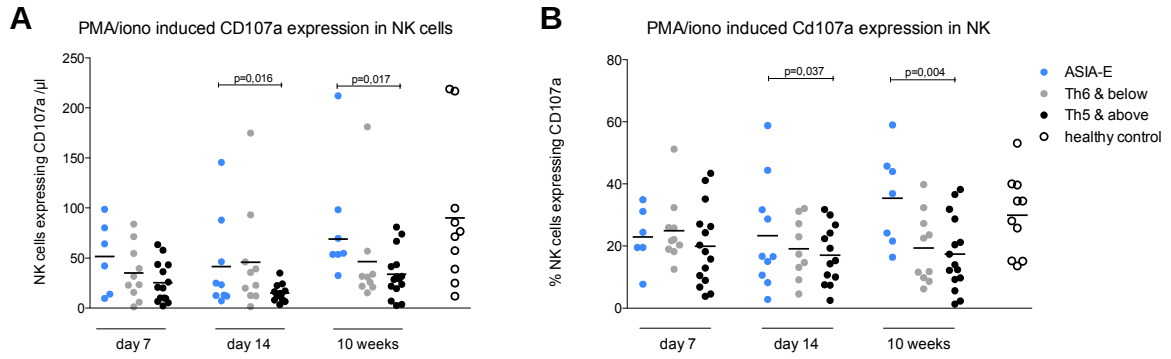

Fig. 19: NK cells' CD107a expression after PMA/ionomycin stimulation. SCI patients maintained a diminished NK cell CD107a expression (cytolytic function) throughout the 10 week study period, whereas ASIA-E patients recovered until reference values of healthy controls. **A. NK cell counts (NK cells/µl) expressing CD107a**; (day 7: ASIA-E: mean=51.55, SD=35.95; T6 & below: mean=35.10, SD=27.44; T5 & above: mean= 25.45, SD=20,21; day 14: ASIA-E: mean=41.49, SD=46.1; T6 & below: mean=45.81, SD=52,12; T5 & above: mean=15.22, SD=8.83; week 10: ASIA-E: mean=82.14, SD=60.74; T6 & below: mean=46.57, SD=51.82; T5 & above: mean=33.75, SD=24.15; healthy controls: mean=90.26, SD=72.51). **B. Frequencies (%) of NK cells expressing CD107a;** (day 7: ASIA-E: mean=22.85, SD=9.68; T6 & below: mean=24.91, SD=10.66; T5 & above: mean= 19.94, SD=12.78; day 14: ASIA-E: mean=23.35, SD=17.55; T6 & below: mean=19.16, SD=9.77; T5 & above: mean= 17.09, SD=9.41; week 10: ASIA-E: mean=35.40, SD=15.36; T6 & below: mean=19.37, SD=11.42; T5 & above: mean=17.41, SD=11.88; healthy controls: mean=11.61, SD=8.48). Significances were calculated with repeated measurements linear mixed model. Shown are only significant differences between SCI and ASIA-E patients; significances between different time points and between patients and healthy controls are mentioned in the text

SCI impairs the ability of NK cells to produce IFN-γ and TNF-α starting from the acute phase and continuing into the chronic phase. However, ASIA E patients' NK cells recover their ability to produce proinflammatory cytokines by the latest time point measured.

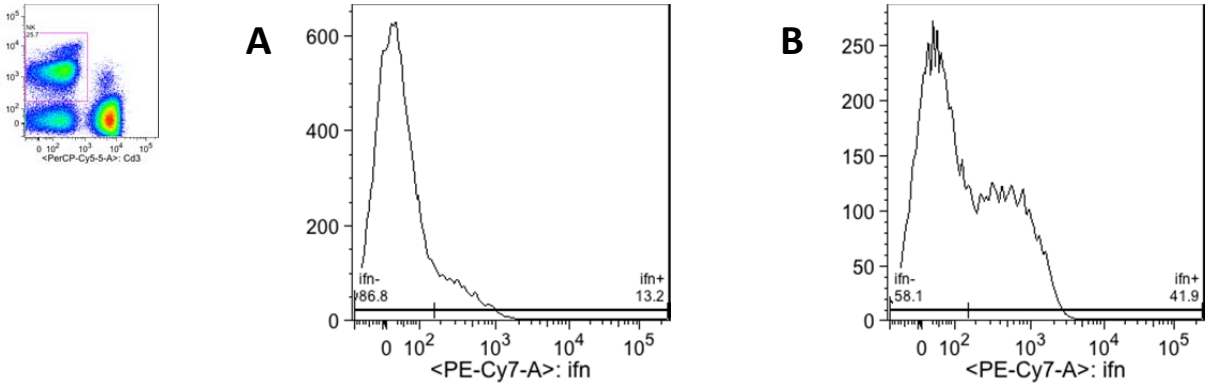

Fig. 20: Representative histograms: NK cell IFN-γ production in SCI (A) and ASIA-E (B)

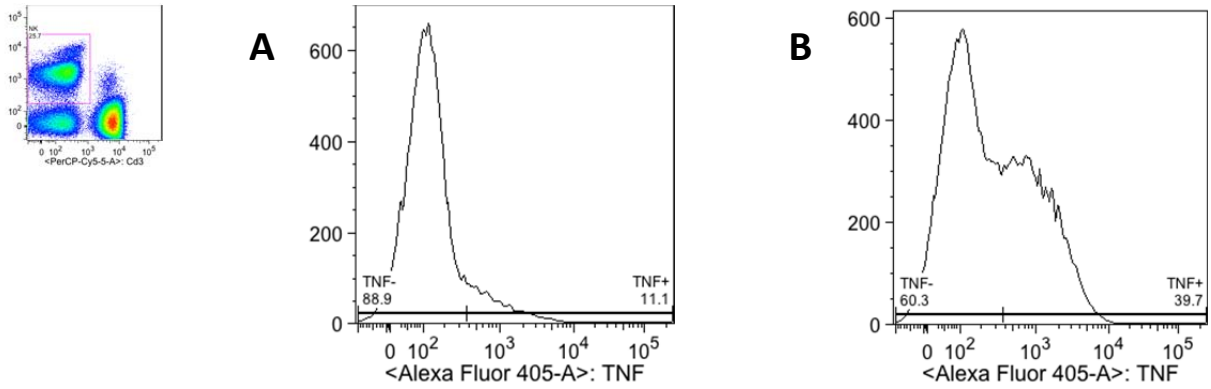

Fig. 21: Representative histograms: NK cell production of TNF-α in SCI (A) and ASIA-E (B)

The group of patients with a lower lesion, but not the group with a higher lesion, registers a significant increase in terms of concentration of NK cells producing IFN-γ (cells/µl) from day 7 to week 10 (p=0.009) (Fig. 22A). When frequencies (%) of NK cells producing IFN-γ are taken into account, patients with a lower lesion showed a significant recovery from the initial to the latest time point (p=0.012), as well as ASIA-E patients (from day 7 to week 10: p=0.35 and from day 14 to week 10: p=0.044) (Fig. 22B). Patients with a lower lesion recover NK cells TNF-α production earlier (day 7 to week 10: p=0.012) then patients with higher lesions (p=0.014 from day 14 to the 10th week) and ASIA-E patients (p=0.039 from day 14 to the 10th week). Taking % of NK cell TNF-α production into account, only ASIA-E patients display a significant recovery (from day 7 to week 10: p=0.009; and from day 14 to week 10: p=0.003) (Fig. 22D).

On day 7, both high and low lesion numbers of NK cells producing IFN-γ and TNF-α (cells/µl), were significantly decreased comparing with healthy control (NK cells/µl producing IFN-γ: p=0.001; TNF-α: p=0.001 for T6 & below; and IFN-γ: p=0.008; TNF-α 0.005 for T5 & above; % NK producing IFN-γ: p=0.001; TNF-α: p=0.013 for T6 & below; and IFN-γ: p=0.011; TNF-α 0.040 for T5 & above). Only patients with higher lesions maintained a suppressed NK cell IFN-γ production (cells/µl) on day 14 (p=0.0005) and on week 10 (p=0.012). The percentage (%) of NK cells producing IFN-γ was decreased in patients with a higher lesion compared to healthy controls on day 14 (p=0.002) as well as on week 10 (p=0.001). Regarding TNF-α production (cells/µl), the two SCI groups show a suppressed response on day 14 (T6 & below: p=0.036 and T5 & above: p=0.0005). On week 10 only the patients sustaining a high

lesion maintain a suppressed NK cell TNF-α production (p=0.03) (Fig. 22C). Individual NK cell TNF-α production (%) was decreased for patients with higher lesions at all measured time points (day 7: p=0.04; day 14: p=0.003; and week 10: p=0.028) compared with healthy controls. For patients with a lower lesion the suppression of NK cells TNF-α production was only significant on the initial time point (p=0.040).

Noticeably, in all NK cell functional parameters, ASIA E control group showed an improvement along the measured time period, overshooting the healthy control group (Fig. 22).

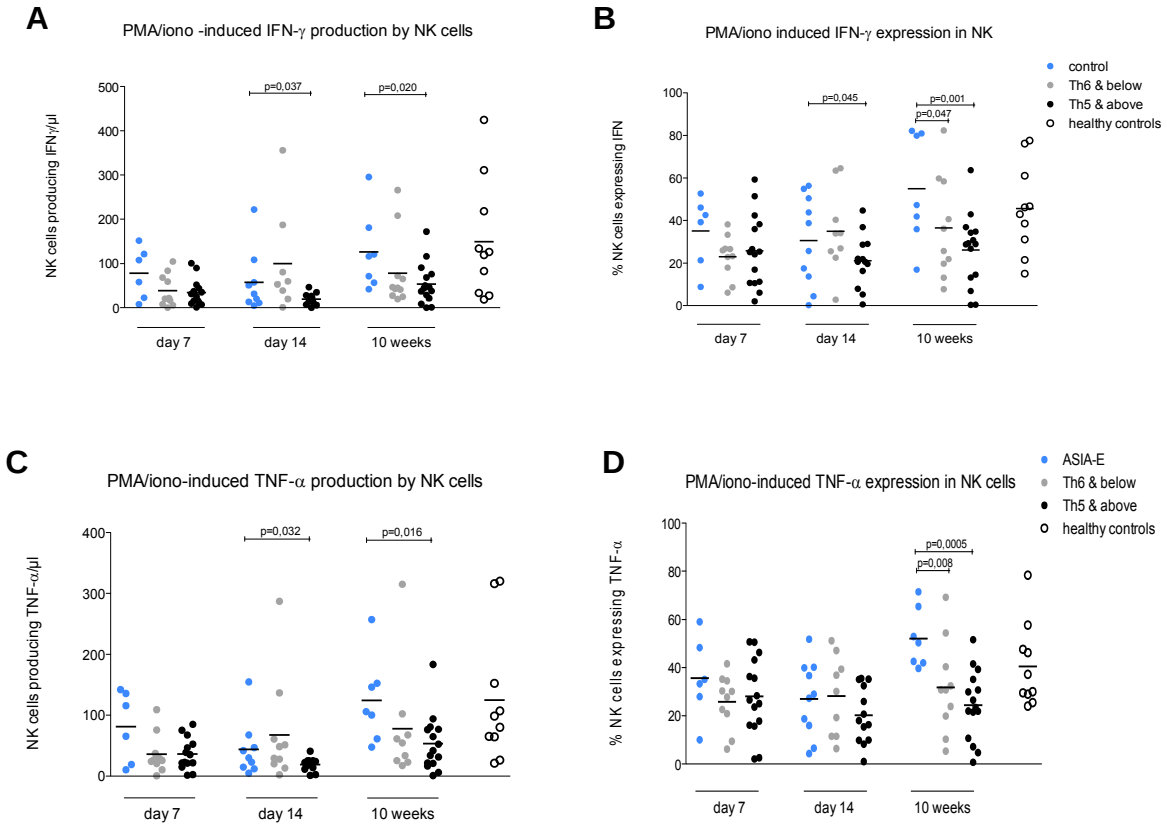

Fig. 22: NK cell cytokine production after PMA/ionomycin stimulation. SCI patients maintained a reduced NK cell cytokine production throughout the 10-week study period, whereas ASIA-E patients recovered until reference values of healthy controls. **A. IFN-γ-producing NK cell counts** (NK cells/µl). (day 7: ASIA-E: mean=78.25, SD=57.56; T6 & below: mean=38.87, SD=36.98; T5 & above: mean=34.11, SD=29.88; day 14: ASIA-E: mean=57.74, SD=69.54; T6 & below: mean=91.48, SD=112.9; T5 & above: mean=19.29, SD=13.38; week 10: ASIA-E: mean=126.2, SD=88.27; T6 & below: mean=83.07, SD=83.79; T5 & above: mean=53.31, SD=46.28; healthy controls: mean=149.70, SD=132.5). **B. Frequencies (%) of NK cells producing IFN-γ;** (day 7: ASIA-E: mean=35.12, SD=16.65; T6 & below: mean=22.96, SD=9.96; T5 & above: mean= 25.74, SD=16.95; day 14: ASIA-E: mean=30.59, SD=21.04; T6 & below: mean=34.93, SD=19.58; T5 & above: mean=21.08, SD=12.26; week 10: ASIA-E: mean=55.01, SD=26.09; T6 & below: mean=36.56, SD=23.88; T5 & above:

mean=26.16, SD=16.90; healthy controls: mean=45.70, SD=21,04). C**. TNF-α-producing NK cell counts (NK cells/µl);** (day 7: ASIA-E: mean=81.38, SD=58.20; T6 & below: mean=36.02, SD=32.45; T5 & above: mean=36.23, SD=26.13; day 14: ASIA-E: mean=43.94, SD=45.92; T6 & below: mean=67.67, SD=85.73; T5 & above: mean=19.12, SD=10.90; week 10: ASIA-E: mean=124.4, SD=70.36; T6 & below: mean=77.79, SD=92.91; T5 & above: mean=53.43, SD=46.23; healthy controls: mean=125.1, SD=108.5). **D. Frequencies (%) of NK cells producing TNF-α** (day 7: ASIA-E: mean=35.62, SD=16.90; T6 & below: mean=25.86, SD=11.28; T5 & above: mean=28.04, SD=15.72; day 14: ASIA-E: mean=27.04, SD=15.56; T6 & below: mean=28.16, SD=16.59; T5 & above: mean=20.29, SD=11.66; week 10: ASIA-E: mean=52.07, SD=12.27; T6 & below: mean=31.74, SD=19.30; T5 & above: mean=24.46, SD=14.39; healthy controls: mean=40.51, SD=17.28). Significances were calculated with repeated measurements linear mixed model. Shown are only significant differences between SCI and ASIA-E patients; significances between different time points and between patients and healthy controls are mentioned in the text

3.2.1.5 Impact of lesion severity on NK cell function

We conducted a post-hoc analysis regarding the dependency of NK cell function on lesion severity. Therefore, we reassigned the patients into 3 different groups according to lesion severity (or completeness): 1) ASIA A (complete lesion); 2) ASIA B-D (incomplete lesion) and 3) ASIA E (vertebral fracture without neurological impairment). We tested whether lesion severity would have an impact on the major outcome parameters: NK cell cytotoxic function and capacity to produce immunomodulatory cytokines. Complete lesions failed to suppress NK cells function to a bigger extent than incomplete lesions.

We observed no impact of lesion severity on K562-induced NK cell cytotoxicity. As observed in studies comparing lesion height (see chapter 3.2.1.3), all 3 patient groups displayed a significantly declined NK cell cytotoxic function compared with healthy controls.

SCI patients consistently show a declined NK cell cytotoxicity (CD107a expression after PMA/ionomycin stimulation) compared with healthy controls throughout the studied period (at day 7: complete vs healthy controls: p=0.001; and incomplete vs healthy controls: p=0.001; at day 14: complete vs healthy controls: p=0.0005; and incomplete vs healthy controls: p=0.006; and at week 10: complete vs healthy controls: p=0.037; and incomplete vs healthy controls: p=0.025) (Fig. 23B).

Patients with a complete lesion display a declined NK cell capacity to produce IFN-γ (Fig. 23C) and TNF-α (Fig. 23D) compared with healthy controls at all time points (IFN-γ: day 7: p=0.002; at day 14: p=0.0005; and at week 10: p=0.026. TNF-α: day 7:

p=0.001 at day 14: p=0.0005; and at week 10: p=0.073). Patients with an incomplete lesion only show a significantly declined NK cytokine production capacity at the initial time point (IFN-γ: day 7: p=0.003; at day 14: p=0.236; and at week 10: p=0.207; TNF-α: day 7: p=0.005; at day 14: p=0.067; and at week 10: p=0.138).

We observed no significant differences in NK cell function between ASIA E patients and healthy controls throughout the studied period.

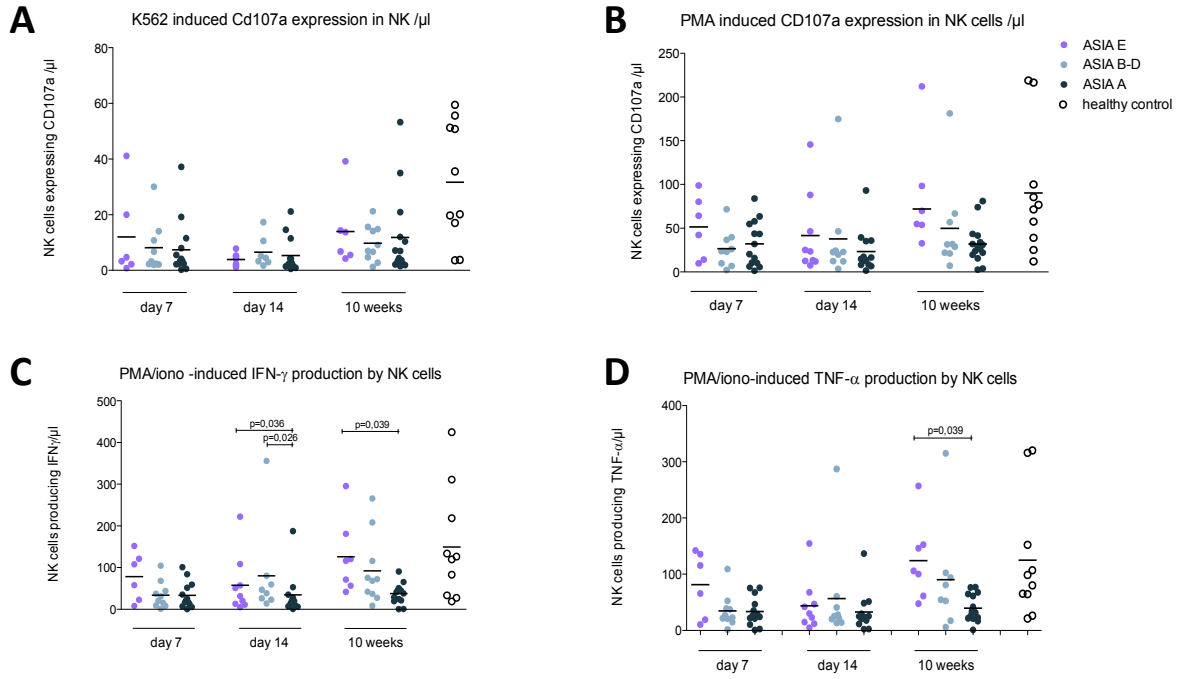

Fig. 23: NK cell function in patients with a complete (ASIA A), incomplete spinal cord lesion (ASIA B-D) and controls (ASIA E). Complete lesions do not have a significantly deeper suppressive impact on NK cell activity than incomplete lesions. However, neurologically intact patients recover NK cell function in all measured parameters to normal levels at the latest time point. **A. NK cell Cd107a expression after exposure to K562 target cells** (day 7: ASIA-E: mean=12.05, SD=15.90; T6 & below: mean=8.19, SD=9.29; T5 & above: mean=7.41, SD=9.93; day 14: ASIA-E: mean=3.90, SD=2.12; T6 & below: mean=6.53, SD=5.54; T5 & above: mean=5.38, SD=6.33; week 10: ASIA-E: mean=13.99, SD=13.06; T6 & below: mean=9.75, SD=6.46; T5 & above: mean=11.81, SD=15.09; healthy controls: mean=31.70, SD=21.51). **B. NK cell Cd107a expression after PMA/ionomycin stimulation** (day 7: ASIA-E: mean=51.55, SD=35.95; T6 & below: mean=26.54, SD=21.15; T5 & above: mean=31.97, SD=26.04; day 14: ASIA-E: mean=41.49, SD=46.51; T6 & below: mean=37.62, SD=52.81; T5 & above: mean=23.25, SD=24.16; week 10: ASIA-E: mean=86.89, SD=65.10; T6 & below: mean=49.63, SD=52.58; T5 & above: mean=31.92, SD=21.86; healthy controls: mean=90.26, SD=72.51). **C. NK cells/µl producing IFN-γ after PMA/ionomycin stimulation** (day 7: ASIA-E: mean=78.25, SD=57.56; T6 & below: mean=33.99, SD=31.91; T5 & above: mean=33.59, SD=31.47; day 14: ASIA-E: mean=57.74, SD=69.54; T6 & below: mean=80.59, SD=113.3; T5 & above: mean=34.73, SD=52.82; week 10: ASIA-E: mean=126.2, SD=88.27; T6 & below: mean=92.19, SD=83.09; T5 & above: mean=37.58, SD=23.26; healthy controls: mean=149.7, SD=132.5). **D. NK cells/µl producing TNF-α after PMA/ionomycin stimulation** (day 7: ASIA-E: mean=81.38, SD=58.20; T6 & below: mean=34.63, SD=29.76; T5 & above: mean=33.55, SD=25.64; day 14:

ASIA-E: mean=43.94, SD=45.92; T6 & below: mean=56.77, SD=87.60; T5 & above: mean=32.83, SD=36.05; week 10: ASIA-E: mean=124.4, SD=70.36; T6 & below: mean=90.23, SD=96.99; T5 & above: mean=39.77, SD=23.85; healthy controls: mean=125.1, SD=108.5)

4 DISCUSSION

Presented in this thesis is the first prospective longitudinal study investigating NK cell function following rodent and human SCI.

The aim of this thesis was to analyse the specific role of NK cells contributing to the SCI-IDS. Although the mechanisms underlying the heightened mortality and morbidity remain elusive, this work confirms and emphasizes that defects in cellular immunity are indeed present after SCI and might be responsible for the increased infection frequency observed among these patients. Studies using the experimental model enabled us to explore the impact of SCI and subsequent decentralization of immune relevant organs on cellular distribution and immune response.

The work with experimental animals was integrated in on-going studies characterizing different aspects of SCI-IDS. The project using human samples was developed in the frame of the SCIentinel prospective multicentre study[99]. This thesis supports the neurogenic nature of the immune depression. Our research substantiates the long suspected supraspinal control of the nervous system on cellular distribution and function. It addressed the function of NK cells after human SCI and a potential dependency on the injury height and severity. Furthermore, it describes the multi-organ involvement in the immune suppression, expanding the existing knowledge on the field.

The quantitative immune deficit occurring during the first week after SCI may explain the increased susceptibility to infections occurring during the acute phase. However, after this lymphocytosis has subsided, the incidence of infectious complications still remains high among SCI patients. Our work was prompted by the hypothesis that a cellular functional deficit might be the cause of the enduring increased susceptibility to infectious diseases. We focused on the role played by NK cells in the development of the immune deficit during the chronic phase following SCI. Here, we show a NK cell functional deficit occurring both in rats and in humans. This goes in line with previous studies[45, 68] showing a NK cell downregulation at late stages after SCI.

Collectively, our work not only expose an innate immune deficit after both experimental and human SCI, but also it provides a confirmation that SCI leads to a higher infection rate, namely pneumonias, as shown by our MRI studies. This further

emphasizes the detrimental effect of nervous system disruption on the immune system.

4.1 SCI suppresses immunity in the experimental model

In the course of this project we used a spinal cord contusion model inflicted by a 10g rod dropped from a height of 50mm (with a NYU Impactor) as described in chapter 2.1.1. The resulting lesion destroys most fibres at the contusion site and results in dense scarring and gliosis. Given the high degree of grey matter destruction generated by this model, we assume that the great majority of sympathetic fibres are also destroyed (Fig. 24). After conducting a pilot study employing an SC transection model, we chose a SC contusion model for its high clinical relevance, closely mimicking the type of spinal cord injury most frequently occurring in humans[100].

Fig. 24 MRI scan: 3-day experimental contusion lesion at the level T9

4.1.1 NK cells: a compartmental approach

During adult life, NK cells originate from precursor cells in the bone marrow where they undergo a complex maturation process acquiring a receptor repertoire and effector functions. From BM, NK cells are mobilized into the blood, LNs, spleen, liver and lungs where NK cells with a mature phenotype and higher effector functions predominate. Inflammatory processes induce NK cells recruitment[17]. Survival, proliferation and differentiation of BM hematopoietic pluripotent stem cells (HPSCs) are processes modulated by cytokines and growth factors. Egress of these cells from BM niches relies on an intact nervous system.

Here we show that BM and splenic NK cell function is indeed dependent on the intact innervation of these organs. Meaning, the amount of destroyed nerve fibres should have an impact on NK cell distribution and weaponry.

On day 3, the SCI animal group with a lesion at the level of T5 showed a significant elevation in NK cells. This increase was noted both in relative - percentages (%); and in absolute terms - concentration (cells/µL) calculated from the total leucocyte counts. This phenomenon did not occur in the group of animals with a lower lesion – NK cell frequencies (%) and counts (cells/µl) are at an identical level as the sham-operated control group.

According to Riegger et al.[69], B- and T-cells in peripheral blood registered a nadir at day 3 after SCI. This phenomenon could explain the increase in NK cells percentage (%) at this time point in the animal group with a higher lesion. However, the same phenomenon cannot explain the concurrent elevation of NK cells absolute numbers. The reason for the increase in NK cells numbers in circulation could be either: 1) an increase in proliferation rate and egress from the BM or 2) mobilization from marginal pools or from peripheral organs such as liver, lung and spleen.

Various studies have reported a lymphocytosis following either adrenaline administration or acute physiological stress, mainly NK cells, which appear to be more influenced by adrenaline than B- and T- lymphocytes[36, 101, 102]. Indeed, our acute phase studies post-SCI, are in line with these results[60, 103]: the animal group with a T5 lesion shows a 30% increase in the numbers of NK cells compared with sham-operated animals. During the chronic phase, most of the SCI-operated animals have NK cell numbers identical to the sham group. Paradoxically, despite the higher numbers of circulating NK cells in the T5-operated group, only fewer NK cells produce IFN-γ.

Although immunological transformations after SCI may have some parallels with the ones occurring after stress or catecholamine administration, a reaction to stress alone cannot explain the observed immuneparesis.

It only makes sense to examine immune cell function both in the context of the absolute numbers and in relative proportions of leucocyte subpopulations. In the frame of an immune suppression, it is not enough to report on the function of a whole cell group, neglecting the absolute cell counts of that group. Reports on stress-induced decrease of NK cell activity have been pointed out as actually being the consequence of decreased NK cell numbers in that compartment[38].

The compartmental approach is essential in order to monitor the NK cell distribution, as this could underlie changes in blood leukocyte and NK cell numbers. The changes observed in our animal model regarding NK cell absolute numbers, proportion, function and kinetics induced by SCI, may have significant consequences for the ability of the immune system to respond to an antigenic challenge. According to Dhabhar and colleagues, stress-induced release of catecholamines and glucocorticoids leads to a redistribution of immune cells throughout different immune compartments rather that an increase in cell turnover[104].

4.1.2 SCI affects NK cells distribution during the acute phase

Catecholamine and ß$_2$-agonist administration increased outflow of leucocytes, especially lymphocytes from the spleen[105-107] and granulocytes from the BM[108]. Indeed, the increased release of catecholamine both into circulation (from adrenal glands) and tissue NE (from nerve fibres) characterizing the acute phase after SCI, could explain the mobilization of NK cells from the spleen and BM into circulation, elucidating the increase of peripheral blood absolute NK cell numbers observed in our study[60, 62, 106, 109, 110]. The impact of SCI on the immune cells seems to be rather selective: priming B- and T-cells to undergo apoptosis in the spleen[60, 66, 103] resulting in decreased cell counts and simultaneously generating a hostile local tissue microenvironment capable of suppressing NK cell effector function during the chronic phase.

A decrease in splenic weight and cellularity (namely B- and T- lymphocytes) has been shown in mice after T3 transection, but neither after T9 transection, contusion nor sham-operation[60]. The drop in splenic weight and cellularity in the T3 transection group was associated with increased concentrations of splenic NE and serum CORT, which were not observed in the remaining groups. In our study we failed to observe significant differences concerning spleen cellularity in any of the groups 3 days after the injury. All groups showed significantly lower splenocyte numbers on day 3 compared to day 28 after injury. Our findings indicate that the decrease in splenic cellularity is driven by surgical stress and therefore does not have a specific neurogenic nature. SCI transection[60] and crush[75] models induce a deeper drop of splenic cellularity than the SCI contusion model. The degree of immune suppression induced by transection lesion compared to the contusion one, presumably correlates

to the wider extent of spinal cord tissue damage, especially in the IML horn, where sympathetic fibres traverse.

The immune changes observed after SCI are likely to increase susceptibility to bacterial (Brommer et al. unpublished data) and viral infections as a result of disrupted central autonomic pathways. A decline in $CD4^+$ effector function (IFN-γ production) at this same lesion height has been correlated with failure to clear viral infection in the liver [75]. The widespread decrease in NK cell IFN-γ production demonstrated in the current study, further support the notion that a multi-sided immune deficit leads to an increased incidence of infection in subjects with SCI.

Besides inducing splenic immune cell apoptosis[36, 38, 60], having a direct detrimental effect on NK cell function, high catecholamine levels have also been associated with disruption of normal BM function. This translates in elevation of plasma granulocyte colony-stimulating factor (G-CSF) and prolonged mobilization of hematopoietic progenitor cells (HPCs). High G-CSF levels are also known to decrease NK cells activity[111], which might be involved in the drop in NK cells activity observed in our experiments.

The increase in BM NK cell percentages (%) within the lymphocyte population suggests that the BM is rather depleted of B- and/or T-cells, resembling the changes observed in peripheral blood[69]. However, in terms of absolute numbers, NK cells are only slightly increased in the SCI group. An increase in BM NK cell counts and frequencies have also been described 3 days after MCAo[112].

We analysed the expression of the maturation markers CD11b and CD27 in NK cells in order to determine whether the increase of the NK cell population both in peripheral blood and in BM during the acute phase after SCI was due to an enhanced proliferation. At day 3, no difference was observed in terms of NK cells maturation stages between the studied groups in any of the analysed compartments. This finding suggests that the increase in NK cell population in SCI animals is not caused by an increase in BM proliferation rate.

The increase in NK cells percentages (of both leucocytes and lymphocytes) but only mild increase in terms of absolute numbers shows that NK cell numbers are rather resilient to the depressive effect of SCI. Numerically, the T- and B-lymphocytes,

appear to be the most affected cells during the acute phase after SCI (probably due to apoptosis in the spleen).

Noticeably, splenic NK cell numbers and percentages of SCI animals were not affected in the same manner as observed in the peripheral blood and BM. The importance of this finding is still unclear.

4.1.3 SCI suppresses NK cells' function during the chronic phase

IFN-γ has already been used as a parameter for accessing T cell function after experimental stroke and SCI[75], especially regarding the shifts towards Th1 or Th2 phenotypes[62]. IFN-γ is a paramount player within the immune defence system. Among its numerous effector functions, it increases B cell IgG production, is associated with the control of viral replication and upregulates cell expression of MHC I, thereby rendering infected cells prone to be easily identified and targeted by T-cells. Within the cell-cell interplay it is able to promote macrophage, dendritic and NK cell activation, growth and differentiation [77]. NK cells are among the major IFN-γ producers and therefore crucial anti-viral defenders. In order to assess NK cell function, we checked IFN-γ production as a functional parameter after PMA/ionomycin stimulation of PBMCs at the above mentioned time points. In these experiments, we tested whether the PMA/ionomycin stimulation paradigm would adequately activate cytokine transcription machinery and therefore potentially expose subtle deficits on NK cells' activity if present. Like T-cells, NK-cells' function could also be assessed by measuring their IFN-γ production capacity.

The downregulation observed in SCI rats is at a single-cell level: there is a decreased IFN-γ production per NK cell. However, when the adjustment to NK cell counts was performed, the decline in IFN-γ production was no longer observed, suggesting that a compensatory mechanism is taking place, balancing reduced IFN-γ production by an increase in NK cell counts. This phenomenon occurs on all 3 compartments on day 28.

4.1.4 Declined NK cell function does not translate in inadequate NK cells maturation, migration and activation status

Using the experimental SCI model, we showed a decrease in individual NK cell function affecting all analysed compartments, especially during the chronic phase after SCI. FACS analysis of NK cell population showed a decrease in IFN-γ production not confined to the peripheral blood compartment, but it is also verifiable in the spleen and in the bone marrow. Effector functions are stronger in mature NK cells with a higher activation status. They are triggered by interaction of inhibitory, activation receptors and adhesion molecules. We tested whether indeed the observed NK cell fluctuations and functional deficit would translate in membrane receptor expression changes pointing to an inadequate maturation process, sub-optimal activation status and diminished migratory ability.

Changes in NK cell numbers, proportions and function, contrary to our original hypothesis, were not accompanied by changes in the expression of membrane markers for neither maturation (CD27, CD11b), homing (CD62L), nor activation (NKG2D). Alterations in adhesive properties of NK cells to endothelium are thought to be due to high catecholamine levels upon stress causing mobilization of NK cell from peripheral reservoirs[36, 113].

4.1.5 Potential mechanisms

SCI results in a set of microenvironmental alterations that have a profound effect on peripheral immunity. Immune system, including NK cells is likely to be affected by alterations on the hormonal level resulting from the disruption of the autonomic nervous system provoked by spinal cord trauma. Besides SCI, high corticosterone and catecholamine levels have been reported in infection[114-116], tissue damage[117] and administration of cytokines[118]. In addition to these, both the SCI experimental model and human SCI superimpose a surgical challenge upon the affected individuals, which are likely to further raise the chronic stress hormone-levels.

In the course of this project we sampled NK cells from immune compartments potentially denervated due to SCI – spleen in case of T5 lesion, but not T9 lesion and BM, in case of T9 lesion. Spleen is an important NK cell reservoir, whereas BM is

place of origin for NK cells. For this reason, this study offers a more complete picture how SCI affects the NK cell kinetics.

On day 3 after SCI, animals with a T5- and to a lesser extent the T9-lesion exhibit a BM cellular depletion – 55,3% and 44% respectively, compared with the sham-operated group. This BM stunning is of crucial clinical relevance, as it is probably the cause of the quantitative deficit occurring during the acute phase after SCI [69]. The proportions of this cellular depletion will undoubtedly compromises immune system response to infectious agents. T- and most probably B- cells are among the most affected cell types although similar impact in other leukocyte subclasses cannot be excluded by this study (granulocytes, monocytes, etc.). These findings suggest a selective control of cellular mobilization by the nervous system. An increase in NK cells proportions within the BM point to a selective deployment of T- and B-lymphocytes rather than an increase in NK cells proliferation, as no difference was found in NK cells expression of maturation markers: CD11b, CD27 between SCI injured animals and the sham-operated group. In our studies, it is not possible to exclude that the increase in NK cell numbers observed in peripheral blood originates from the BM, however the influx of NK cells into peripheral blood is more likely to emerge from the spleen where the decrease in cellularity does not follow a selective fashion like the one observed in the BM.

Our data not only identifies a functional deficit in circulating NK cells, but also in BM and splenic NK cells. BM NK cells function is affected at a similar extent both in T5 and in T9 lesion (both disrupt BM innervation). This suggests that changes within the BM microenvironment superimposed either by direct BM denervation or by a systemic inflammatory challenge, jeopardizes NK cells capability to mount adequate immune responses against infectious stimuli after SCI.

Changes within the BM microenvironment are likely to be responsible for the deployment of functionally defective NK cells into the circulation. However, our results are not able to show whether the suppressive challenge was inflicted early in the life of a NK cell, within the BM, or it suppresses NK cells in a continuous fashion, including within the blood stream.

In the rat, spleen innervation originates from the thoracic segments T5-12[119]. T5 lesion purportedly destroys the nerve fibres supplying the spleen, whereas the T9 lesion keeps most of the spleen innervation intact. Splenic NK cell function in the T9-

contusion group does not decline as much as in the T5-contusion group, suggesting that innervation is likely to play a role in the regulation of NK cell function. However, it is not possible to exclude the influence of suppressive factors such as adrenal hormones on splenic NK cells.

Importantly, the course of the immune suppression did not seem to be equal in all animals: not all animals subjected to SCI lesion displayed a decreased NK cell function at the investigated time points. Some animals display recovery of NK cell function comparable to sham-level, while others remain decreased during the chronic phase.

4.2 Human SCI: NK cell suppression during chronic phase post-SCI encompasses multiple NK cell immune functions and is neurogenic in nature

Although the phenomenon SCI-induced decline of NK cell activity has been previously recognized[24, 45, 68, 73, 120], the present study is the first to systematically characterize NK cell function after SCI and establish a neurogenic link between the nervous system traumatic event and the cellular dysfunction. In line with previous studies[45, 72], our findings show that a decreased NK cell function is neither dependent on the level nor on the severity of the injury.

Cr^{51} releasing assay was the standard method to study NK cells activity. It measures the amount of Cr^{51} released by the target cells into the medium after being exposed to NK cells' killing machinery. It provides information concerning the end stage lysis of target cells. In our studies, we assed NK cells function employing flow cytometry to determine NK cells CD107a expression, a marker for NK cell degranulation, as well as production of IFN-γ and TNF-α, crucial immunomodulatory cytokines. Our study was to our knowledge, the first to deliver such a comprehensive approach regarding NK cell function, and to present all three parameters as quantifiable measures of NK cell activity.

We were able to replicate findings of impaired NK cell activity in SCI patients using both 1) K562 target-cell line and 2) PMA/ionomycin stimulation measured by FACS. In line with previous studies, we were able to demonstrate a significant reduction in NK cell activity of SCI patients compared with healthy controls using the K562 target

cell line [45, 64, 68]. Nonetheless, also the NK cell cytotoxicity of patients with a vertebral fracture with no neurological damage (ASIA-E) was significantly depressed compared with the healthy controls. Indeed, a NK cell functional deficit has also been reported in case of psychological stress [77, 81, 82], burns and also surgery-induced stress [78]. In order to be able to distinguish the impact of surgical stress from the one caused by the nervous system trauma on NK cells activity, we decided to include a control group of patients solely subjected to surgical stress and no neurological impairment (ASIA E patients).

The measurement of NK CD107a expression after contact with K562 by FACS may not be sensitive enough to perceive small differences between the stress-induced cellular dysfunction and the SCI-induced cellular dysfunction. Therefore, we used PMA/ionomycin, a high stimulus for NK cells. This stimulation not only induces NK cell degranulation, but also it enables the measurement of proinflammatory cytokines – IFN-γ and TNF-α. At day 7 and 14 succeeding both SCI and only vertebral fracture, patients showed a decreased NK cell activity in any of the measured parameters. This phenomenon suggests that during the acute and post-acute phases after the traumatic event, NK cells are susceptible to the suppressive effects of both surgery and nervous system trauma. Only at 10 weeks after the traumatic event, the suppressive effect of SCI outweighed the one of surgery: the group with only vertebral fracture, recover NK cells activity to the reference levels of healthy controls, while NK cells of both groups of SCI patients remain underperforming.

PMA/ionomycin stimulation up-regulated CD107a expression in the surface of NK cells, which highly correlates with NK cells degranulation and cytotoxic capacity[98, 121]. PMA/ionomycin also prompted the NK cell production of proinflammatory cytokines IFN-γ and TNF-α. Multi-parameter flow cytometric analysis enables the analysis of different NK activity features simultaneously, and not only cytotoxicity as in the Cr^{51} releasing assay.

4.3 Conclusion and outlook

In this thesis, I performed a detailed analysis of the role played by NK cells in the development and maintenance of a state of diminished immunological alert affecting both rodents and humans after traumatic spinal cord injury.

Here, we describe NK cells dynamics in the period following SCI, encompassing the study of the origin of NK cells within the BM, the proliferation, deployment into circulation, state of activation and pooling in peripheral organs. This systemic approach provided a deeper insight into the plausible causes to the functional down-regulation of NK cells succeeding spinal cord trauma.

We were able to show that the immune deficit striking after SCI prevails into the chronic phase after the injury and it is during this phase qualitative rather than quantitative. Furthermore, our study shows that also within the lymphoid and hematopoietic organs, NK cells show a suppressed response to stimulation. In our study we were not able to show any defect in NK cells maturation process that potentially would underlie the functional deficit observed in the studied immune compartments. This suggests that NK cells and probably other immune cells are continuously exposed to suppressive agents. These suppressive agents (e.g. adrenal hormones) appear to be rather ubiquitous, affecting all three analysed compartments. NK cell in the spleen of rats with a T5 injury failed to display a significantly inferior function to the rats operated at the level T9. This finding suggests that other factor besides organ innervation might play a lesser role in the immune cell function. Indeed this study confirms for the first time that SCI-IDS affects different immune compartments.

Since NK cells are important players in the stage of immune cell interplay, decreased secretion of immunomodulating agents such as IFN-γ and TNF-α are likely to have a profound effect on other immune system players such as monocytes and dendritic cells. These interactions go beyond the spectrum of our work but certainly warrant further research.

Our study also point out some differences between rodents and humans regarding NK cell dynamics. Unlike ASIA-E patients, sham-operated rats display a superior NK cell performance than the SCI-operated rats throughout the measured time spectrum. ASIA-E patients sustain a declined NK cell function at the early phase following

trauma, substantially recovering only at the latest phase. This phenomenon points to possible differences between human and rodent NK cells in a biological perspective.

Collectively these findings pave the way for immune therapeutically approaches targeting NK cells. Our study underlines the necessity to tackle this tangible immune deficit at an early stage succeeding SCI, in order to prevent potentially deadly infectious complications.

5 APPENDIX

5.1 Abbreviations

AC	Adenilate Cyclase
ACTH	Adrenocorticotropic Hormone
AD	Autonomic Dysreflexia
ANS	Autonomic Nervous System
APC	Antigen-Presenting Cell
AR	Adrenergic Receptor
ASIA	American Spinal Injury Association
BM	Bone Marrow
CA	Catecholamine
cAMP-PKA	cyclic adenosine monophosphate - Protein Kinase A
CD	cluster of differntiation
CGRP	Calcitonin Gene-Related Peptide
CK	Cyanoketone
CLP	Common Lymphoid Progenitor
CMV	Cytomegalovirus
CNS	Central Nervous System
CORT	corticosterone (cortisol analogue in rodents)
CRH	Corticotropin-Releasing Hormone
DAG	Diacylglycerol
DC	Dendritic Cell
DHEA	Dehydroepiandrosterone
DS	Dehydroepiandrosterone Sulfate
EBV	Epstein-Barr Virus
ERK	Extracellular-signal-regulated Kinases
FACS	Fluorescence-activated cell sorting
GC	Glucocorticoid
G-CSF	Granulocyte Colony-Stimulating Factor
GM-CSF	Granulocyte macrophage Colony-Stimulating Factor
HLA	Human Leukocyte Antigen
HPA	Hypothalamic-Pituitary-Adrenal (axis)
HPSC	Hematopoietic Progenitor Stem Cell
HSC	Hematopoietic Stem Cell
HSV	Herpes Simplex Virus
IFN	Interferon
IL	Interleukin
IML	Intermediolateral (column)
KIR	Killer-cell Immunoglobulin-like Receptor
LC-NE	Locus Coeruleus - Norepinephrine
LN	Lymph Node
MALT	Mucosa-Associated Lymphoid Tissue
MAPK	Mitogen-Activated Protein Kinase
MHC	Major Histocompatibility complex
MHV	Mouse Hepatitis Virus
NCR	Natural Cytotoxicity Receptors

NE	Norepinephrine
NF-KB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer (cells)
NKP	Natural Killer (cell) Percursor
NKR	Natural Killer (cell) Receptor
NPY	Neuropeptide Y
PALS	Periarteriolar Lymphatic Sheath
PI	Propidium Iodide
PAMP	Pathogen-Associated Microbial Pattern
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PNS	Peripheral Nervous System
PRV	Pseudo-Rabies Virus
PVN	Paraventricular Nucleus
SC	Spinal Cord
SCI	Spinal Cord Injury
SCI-IDS	Spinal Cord Injury-induced Immune Depression Syndrome
SIR	Systemic Inflamatory Response Syndrome
SNS	Sympathetic Nervous System
SP	Substance P
TBI	Traumatic Brain Injury
TH	Tyrosine Hydroxilase
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
VIP	Vasoactive Intestinal Peptide

5.2 Publications

5.2.1 Publications included in this thesis

Inês Laginha,, Marcel A. Kopp, Claudia Druschel, Benedikt Brommer, Rick C. Hellmann, Ralf Watzlawick, Ramin-Raul Ossami-Saidi, Harald Prüss, Vieri Failli, Ulrich Dirnagl, Sebastian Voigt, Monica Killig, Chiara Romagnani and Jan M. Schwab "Natural Killer (NK) Cell Function after Spinal Cord Injury (SCI): a prospective study protocol" (in preparation).

Inês Laginha,, Marcel A. Kopp, Claudia Druschel, Benedikt Brommer, Rick C. Hellmann, Ralf Watzlawick, Ramin-Raul Ossami-Saidi, Harald Prüss, Ulrich Dirnagl, Monica Killig, Chiara Romagnani and Jan M. Schwab "Spinal Cord injury-induced Immune depression Syndrome (SCI-IDS): Natural Killer (NK) Cell Function - a prospective study" (in preparation).

Inês Laginha,, Benedikt Brommer, Rick C. Hellmann, Marcel A. Kopp, Claudia Druschel, Ralf Watzlawick, Ramin-Raul Ossami-Saidi, Ulrich Dirnagl, Sebastian Voigt, Monica Killig, Chiara Romagnani and Jan M. Schwab "Spinal Cord injury-induced Immune Depression Syndrome (SCI-IDS) – Characterization of Natural Killer (NK) cell function in Rats" (in preparation).

5.2.2 Additional work that was published during the time of this PhD-research

Harald Prüss, Marcel Kopp, Benedikt Brommer, Nicole Gatzemeier, **Inês Laginha**, Jan M. Schwab, "Non-resolving aspects of acute inflammation after spinal cord injury (SCI): indices and resolution plateau." Brain pathology 21.6 (2011) pp. 652-60.

Failli V, Kopp MA, Gericke C, Martus P, Klingbeil S, Brommer B, **Laginha I**, Chen Y, DeVivo MJ, Dirnagl U, Schwab JM. „Functional neurological recovery after spinal cord injury is impaired in patients with infections." Brain, 135 (2012) pp. 3238-50.

Kopp, M. A., Druschel, C., Meisel, C., Liebscher, T., Prilipp, E., Watzlawick, R., Cinelli, P., Niedeggen, A., Schaser, K. D., Wanner, G. A., Curt, A., Lindemann, G., Nugaeva, N., Fehlings, M. G., Vajkoczy, P., Cabraja, M., Dengler, J., Ertel, W., Ekkernkamp, A., Martus, P., Volk, H. D., Unterwalder, N., Kolsch, U., Brommer, B., Hellmann, R. C., Saidy, R. R., **Laginha, I.,** Pruss, H., Failli, V., Dirnagl, U., Schwab,

J. M. „The SCIentinel study-prospective multicenter study to define the spinal cord injury-induced immune depression syndrome (SCI-IDS)-study protocol and interim feasibility data“ BMC Neurol, 13 (2013) pp. 168.

5.2.3 Review articles

Benedikt Brommer, Marcel A. Kopp, **Inês Laginha** and Jan M. Schwab, “Sekundäre Immunodefizienz (Immunoparalyse) nach Rückenmarkverletzung” (Secondary Immunodeficiency (Immunoparalysis) after Spinal Cord Injury), Neuroforum, 3.10 (2010) pp. 208-217.

5.3 Affidavit

I, Maria Inês Duarte Laginha certify under penalty of perjury by my own signature that I have submitted the thesis on the topic "Spinal cord injury-induced immune depression syndrome: the function of Natural Killer (NK) cells", I wrote this thesis independently and without assistance from third parties and I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My contributions in the selected publications for this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author of correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date

Signature

5.4 Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

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