Aus der Klinik für Neurologie, Abteilung für Experimentelle Neurologie
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DISSERTATION

Spinal Cord Injury-induced Immune Deficiency Syndrome and therapeutic implications for pharmacological inhibitors of the RhoA/ROCK-pathway

–

a translational approach

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Zusammenfassung

Abstract (english)

Introduction: Secondary complications such as infections are the leading cause of death after acute spinal cord injury (SCI). Impaired motor function and poor clinical condition do not sufficiently explain the increased infection susceptibility after SCI suggesting the presence of a neurogenic immune deficiency (SCI-induced immune deficiency syndrome, SCI-IDS). The publications summarized here investigate the functional relevance of the SCI-IDS, aim at further characterizing it with immunophenotyping and haematological profiling and provide a systematic identification of possible therapeutic interventions for SCI-IDS triggered consequences such as increased secondary axonal damage or impaired functional recovery.

Methods: Neurogenic implications of the SCI-IDS were investigated in a mouse model of experimental pneumonia related to SCI at different thoracic lesion levels. Additionally, the relevance of an intact sympathetic innervation of secondary immune organs was investigated by selective spleen denervation before SCI induction. A level dependency of pneumonia was then evaluated in an observational multicenter human study using multiple logistic regression models. Moreover, SCI-IDS was characterized in a longitudinal study (SCIentinel) including an interim-analysis to evaluate the feasibility. To systematically identify experimental interventions for SCI and SCI-IDS related consequences, electronic databases were searched for studies reporting neurobehavioral outcome after treatment with RhoA/ROCK-inhibitors. An overall effect size was calculated and study characteristics significantly associated with different observed effect sizes were identified. Publication bias was addressed using funnel plots, Egger regression and the trim and fill method.

Results: Susceptibility for experimental pneumonia was is increased for T3 spinal cord lesions compared to T9 lesions indicated by elevated bacterial load in the lungs and pronounced spleen atrophy. The transected spinal cord below T3 SCI was then identified to propagate SCI-IDS through decentralized sympathetic innervation of the spleen as a main immune organ. The lesion dependent susceptibility for pneumonia

was confirmed in human SCI by multiple regression analysis incorporating 1221 patients. The administrative interim-analysis of the SCIentinel study confirmed its feasibility after inclusion of 60 patients (51% of calculated sample size). Systematic review and meta-analysis of Rho/ROCK-inhibition calculated an overall neurobehavioral efficacy of 21% including data from 725 animals. This was reduced by 5.6% after trim and fill analysis indicating underlying publication bias.

Conclusions: SCI-IDS was identified to increase the susceptibility for pneumonia in a lesion level dependent manner. Early detection of SCI-IDS and development of prognostic markers could improve clinical care and reduce mortality. Evaluation of preclinical candidates targeting SCI-IDS supports a translational approach to the clinics.

Abstract (deutsch)

Einleitung: Sekundäre Komplikationen wie Infektionen sind die häufigste Todesursache nach traumatischer Rückenmarkverletzung. Die erhöhte Infektanfälligkeit nach einer Querschnittlähmung erklärt sich nicht vollständig durch verschlechterte motorische Funktion und den klinischen Zustand, was auf ein vorliegendes neurogenes Immundefizienz-Syndrom (SCI-IDS) hinweist. Die hier zusammengefassten Publikationen untersuchen die funktionelle Relevanz des SCI-IDS, zielen auf eine weitere immunphänotypische und hämatologische Charakterisierung ab und verschaffen einen systematischen Überblick über potentielle therapeutische Interventionen für durch das SCI-IDS verstärkte Komplikationen, wie ein gesteigerter axonaler Sekundärschaden oder eine verschlechterte funktionelle Erholung.

Methodik: Neurogene Auswirkungen des SCI-IDS wurden im Mausmodell einer experimentellen Pneumonie in Abhängigkeit von Rückenmarkverletzungen unterschiedlicher thorakaler Läsionshöhen untersucht. Ferner wurde nach einer solchen Verletzung durch selektive Milzdenervierung die Relevanz einer intakten sympathischen Innervation sekundärer Immunorgane geprüft. Eine Läsionshöhenabhängigkeit wurde mittels multipler Regressionsmodelle zusätzlich in einer multizentrischen humanen

Observationsstudie beurteilt. Eine weitere Charakterisierung des SCI-IDS fand in einer longitudinalen Patientenstudie statt (SCIentinel). Die Interimsanalyse zur Machbarkeit ist Teil dieser Dissertation. Um systematisch experimentelle Studien zu identifizieren, die auf die Behandlung von SCI oder SCI-IDS bedingten Konsequenzen abzielen, wurden elektronische Datenbanken nach Studien durchsucht, die den Wiedergewinn neurologischer Funktionen nach Behandlung mit RhoA/ROCK-Inhibitoren untersuchen. Für diese wurde eine Gesamteffektgröße berechnet und diejenigen Studiendetails bestimmt, die signifikant mit unterschiedlichen Effektgrößen assoziiert waren. Folgende Methoden wurden benutzt, um Publikations-Bias (Verzerrung durch nichtveröffentlichte Studien) zu bestimmen: Funnel-Plots, Egger-Regression und Trim-and-Fill.

Ergebnisse: Die Infektionsanfälligkeit nach experimenteller Pneumonie zeigte sich im Rahmen von T3 Rückenmarkquerschnittverletzungen erhöht, was sich im Vergleich zu T9 Verletzungen durch eine vermehrte Bakterienanzahl in der Lunge und eine ausgeprägtere Milzatrophie darstellte. Das vom Gehirn isolierte Rückenmark unterhalb der Transektionsverletzung auf Höhe T3 wurde als eine Ursache für das SCI-IDS identifiziert, bedingt durch eine sympathische Fehlinnvervation der Milz als immunologisches Effektororgan. Die Läsionshöhenabhängigkeit wurde mittels multipler logistischer Regression bei 1221 Patienten nach Rückenmarkverletzung bestätigt. Die administrative Zwischenanalyse der SCIentinel-Studie bestätigte die Machbarkeit nach Einschluss von 60 Patienten, was 51% gemäß Fallzahlberechnung darstellt. Die Meta-Analyse ergab für 725 eingeschlossene Tiere eine Gesamteffektgröße von 21% und die Adjustierung mittels Trim-and-Fill reduzierte diese um 5.6%, was einen vorliegenden Publikations-Bias impliziert.

Schlussfolgerung: Das SCI-IDS in Abhängigkeit von der Läsionshöhe wurde als ursächlich für eine erhöhte Anfälligkeit für Pneumonien identifiziert. Die frühe Erkennung eines SCI-IDS und die Bestimmung von prognostischen Markern könnten die klinische Behandlung verbessern und die Mortalität senken. Die Evaluation von präklinischen Therapeutika zur Behandlung einer Rückenmarkverletzung und derer Konsequenzen unterstützen Bemühungen, die auf eine Translation in die klinische Anwendung abzielen.

Background

The consequences for patients after traumatic spinal cord injury (SCI) differ from a sudden complete loss of motor and sensory function (tetraplegia) to diverse graduations of partially impaired motor and/or sensory function (paraplegia). This deleterious syndrome is currently affecting approximately 1.5 – 5.2 million people worldwide and in the United States the nation could save as much as $400 billion on future direct and indirect lifetime costs by preventing new injuries and developing therapies for chronic stages of the syndrome [1]. However, the lack of therapeutic strategies is a common feature of human SCI and no clinical study has yet reported successful treatment of SCI patients. Animal experiments have led to exponentially increasing pathophysiological knowledge during the last two decades and identified apparently promising interventions for use in the clinics. These strategies can be classified due to their underlying mechanisms into three Rs: (1) *rescue* of the initial damage at the lesion site with early interventions, (2) *reactivation* of spared axonal structures and (3) *rewiring* of injured axons or re-purposing of spared ones [2]. However, most of these treatment strategies failed to reproduce preclinical effects and encouraged translational efforts to assess laboratory results for their clinical application.

In the clinics, infections (i.e. pneumonia and urinary tract infections) are a leading cause of morbidity and mortality after SCI [3]. It has been shown that injuries to the central nervous system (CNS) disrupt the well-balanced interactions between the immune system and the CNS and lead to an abrupt and drastic systemic decrease of immune functions [4]. This also occurs after injuries to the spinal cord representing a part of the CNS, which results in a spinal cord injury-induced immune depression syndrome (SCI-IDS). In an experimental model SCI-IDS was demonstrated to be most pronounced between day one and three after SCI caused by a depletation of monocytes, T- and B-lymphocytes, MHC class II molecules and dendritic cells [5]. However, the underlying mechanisms remain unclear and result in an increased risk for developing infections after SCI. Furthermore, infections such as pneumonia and/or postoperative wound infections have been identified as independent risk factors for poor neurological outcome after motor complete spinal cord injury [6].

First publication: functional SCI-IDS propagates the susceptibility for bacterial infection

To elucidate underlying mechanisms of SCI-IDS, we investigated how the loss of supra-spinal sympathetic innervation influences the function of immune relevant organs (e.g. spleen, liver, adrenal gland) in a spinal cord level based approach [7]. The neuroanatomical structure of the spinal cord is considered to be unique compared to other CNS injury paradigms (traumatic brain injury, stroke) since relevant immune organs are sympathetically innervated from high thoracic spinal cord levels (T5-T9) whereas low thoracic do not affect their innervation.

In an animal model of SCI associated experimental pneumonia (SCI-AEP) we tested whether SCI-IDS increases bacterial load in lungs comparing high and low thoracic spinal cord lesions. Furthermore, the impact of preganglionic or postganglionic injury (Celiac ganglia) was evaluated by denervation of the spleen. We hypothesized a lesion dependent susceptibility for SCI associated pneumonia, which was further investigated in a human observational study. Together, this first publication characterizes underlying mechanisms of SCI-IDS as a consequence of SCI increasing the risk for infections in a lesion level dependent manner.

Second publication: Prospective multicenter study protocol to define the SCI-IDS

Here, we published a study protocol and interim feasibility analysis of the “SCIentinel” patient study which aims at finding prognostic biochemical surrogate parameter to identify SCI patients at risk to develop clinical relevant infections [8]. Early detection of SCI-IDS would help to prevent infections and subsequently decrease mortality and impaired neurological outcome [6]. Relevant biochemical markers were selected analogous to established laboratory analysis where increased infectious risk was detected in other paradigms. The expression of human leukocyte antigen (HLA-) DR on monocytes was therefore selected as primary outcome parameter and further blood parameters as secondary endpoints: leukocyte cell counts, Concanavalin A (ConA)-induced cytokine expression, immune modulators and indirect infection parameters. These prognostic parameters for the innate and adaptive immune system is correlated with SCI-lesion height and severity. Early detection of developing SCI-IDS would aim at *rescuing* the spinal cord by preventing secondary damage beyond the initial SCI through inflammation [2].

Third publication: Therapeutic value of RhoA/ROCK-inhibitors after SCI

To practically reduce the consequences of infections (such as an increased secondary axonal damage after SCI [2] and an impaired neurological recovery [6]) in the clinical setting, anti-inflammatory drugs might represent a valuable treatment strategy. Namely, the non-steroid anti-inflammatory drug (NSAID) Ibuprofen is a frequently used clinical medication and is furthermore known to inhibit a molecular pathway impairing neurological recovery after SCI. Inhibitory molecular molecules surrounding the environment of the injured axon within the scar tissue and myelin [9,10] are main reasons for the incapacity of axons to re-grow after SCI. Namely, chondroitin sulfate proteoglycans (CSPGs), Nogo-A, myelin-associated glycoprotein (MAG), oligodendrocyte-myelin glycoprotein (OMgp), Ephrins, and repulsive guidance molecules a (RGMa) are up-regulated and interact with receptors on the axon membrane [9,11]. Signaling from most of these receptors activates the intracellular small Ras homology gene family member (Rho) A GTPase, which interacts with the Rho-associated coiled-coil containing protein kinase (ROCK). Inhibition of the RhoA/ROCK-pathway has been shown to foster axonal sprouting or plasticity, to have neuroprotective effects, and to enhance neurological recovery after acute SCI [12]. Together with its anti-inflammatory effects likely to reduce neurodegeneration [12], this identifies the NSAID Ibuprofen as an ideal candidate targeting the *rescue* and *rewiring* of the injured spinal cord [2]. To evaluate the preclinical efficacy of Ibuprofen and more general RhoA/ROCK-inhibitors, we performed a systematic review and meta-analysis of the existing preclinical animal data in traumatic SCI [13]. We use stratified meta-analysis to determine the influence of study characteristics and to assess estimates of efficacy. Where experiments have been conducted but are not available for data assessment and in case the results of these experiments are different from the results of published experiments, the observed effect size from meta-analysis would be biased ('Publication bias'). Therefore, Funnel plotting, Egger-regression and the trim-and-fill method were used to determine the occurrence and impact of publication bias.

Methods

The methods are described in greater detail in the original publications, the following highlights the key points.

Characterization of SCI-IDS in an animal model and human study

In the first part of this study [7], we investigated whether SCI-lesion level enhances the susceptibility to infection after SCI in a mouse model. In the second part we intended to assess the impact of sympathetic signaling originating from below the injury site by denervation of the splenic nerves including sympathetic projections 12 days before SCI.

For the first part of this study male C57Bl6/J mice, 8-11 weeks old were randomly assigned to three groups undergoing a four-fifths transection injury of the spinal cord at thoracic level 3 (T3), thoracic level 9 (T9) or a sham surgery without damage to the spinal cord. Mice received pain medication one hour prior to surgery (Buprenorphine) and were anesthetized using isoflurane. The skin was incised, muscles detached from the vertebra and a single-level bilaminectomy was performed to expose the spinal cord. After opening the dura mater the dorsal part was lesioned with fine iridectomy scissors. The wound was rinsed with saline and closed in layers. Sham-injured (control) animals received a bilaminectomy without injury of the spinal cord. Antibiotics treatment (Enrofloxacin, twice daily) was started one day prior to the SCI surgery and terminated on the second day after SCI. Analgesic treatment, manual bladder compression and bathing daily in hand-warm water was applied as postoperative care.

Subsequent MRI-imaging (7-Tesla rodent scanner) two days after SCI was performed to ensure the lesion level and four-fifth dorsal transection. Mice were excluded if remaining tissue within the transection or incomplete 4/5 lesions were detected.

On day three after SCI the mice were anaesthetized for the second time (midazolam and medetomidin, i.p.), suspended by the two front upper teeth and intubated with a 22G peripheral venous catheter. 30-50 μl bacterial suspension containing 500 colony forming units (cfu) of Streptococcus pneumonia was administered into the lung to induce a clinically relevant and reliable infection. After antagonisation (Flumazenil and Atipamezole) mice were placed in a heated cage supplemented with moistened oxygen to support breathing after extubation.

Animals were sacrificed 24h after infection and lungs and spleen were removed. The spleen length was measured and normalized according to the weight of a 20g mouse. After homogenization in 0.5 ml C+Y media, plating on blood agar in serial dilutions (1:1, 1:10, 1:100 and 1:1000) and 24h incubation, the total number of cfu per lung was calculated. For histology, lungs were fixated in paraformaldehyde and stained using hematoxyilin/eosin.

For the second part of the study, additional mice received splenic nerve injury 12 days before SCI to shield the spleen from spinally generated nerve activity (SNA) originating from below the lesion site. Mice received pain medication one hour prior to surgery (Burprenorphine) and were anesthetized using isoflurane. After incision of the skin, muscles and peritoneum were dissected, and the spleen was exposed. All surrounding tissue down to the supplying blood vessels wall (Lienal artery and vein) was removed and the wound was closed in layers applying local pain relief (bupivacaine). Control animals received laparotomy only (lap group). Mice received antibiotics (Marbofloxacin) for four days after SCI.

All mice were cared for in accordance with the published International Health Guidelines under a protocol approved by the Animal Care and Use Committee and the local animal care committee, and treated in accordance with the European directive on the protection of animals used for scientific purposes and the respective German legislation. All mice were euthanized if their weight loss exceeded 20% of the preoperative weight. The number of animals in each group and the number of excluded animals are reported in the method section of the original publication.

The National Spinal Cord Injury Database (NSCID) based in Birmingham, Alabama (USA) was assessed for datasets of SCI patients recruited in 20 national centers. We selected datasets from patients (1) suffering from SCI of acute traumatic etiology, (2) completely assessed with the International Standards for Neurological Classification of SCI resulting in the American Spinal Cord Injury Association Impairment Scale (AIS), (3) with neurological level thoracic T1- T12 and (4) with complete records regarding the occurrence of pneumonia during acute care and inpatient rehabilitation. To analyze the role of disturbed innervation of the major splanchnic nerve (T5-T9), patients were stratified in three groups regarding their neurological level (i) T1-T4, (ii) T5-T8 and (iii) T9-T12. In addition, the patients were stratified into motor complete (AIS A and B) and

motor incomplete SCI (AIS C and D) to evaluate the impact of disrupted motor function as surrogate parameters for the degree of autonomic dysfunction.

SCIentinel study

This prospective international multicenter study incorporates patients with traumatic spinal cord injury and neurological impairment, and control patients with acute vertebral fracture without neurological deficits. To investigate the role of disturbed innervation of the major splanchnic nerve (T5-T9) as linked to alterations of the immune system function, SCI patients are stratified into three groups: (i) injury level T4 or above, n=48; (ii) injury level T5 or below, n=31 and iii) control group, n=39. The primary endpoint is a difference in HLA-DR expression on monocytes. The sample size calculation was performed on the basis of a pilot study [14]. Major exclusion criteria are severe multiple trauma, moderate or severe traumatic brain injury, preexisting infections, neoplasia, autoimmune diseases or medication with methylprednisolone. Patients are assessed at five time points after SCI: <31h, 31-55h, 7d, 14d and 10 weeks. At each assessment point potential occurring infections are evaluated and blood withdrawals are taken for immune-characterization: HLA-DR expression on monocytes as the primary endpoint, leukocyte and lymphocyte cell counts (B- cells, NK-cells, T-cells and T-cell-subpopulations [CD3, CD4, CD8]), cytokine levels after ConA-induced T-cell stimulation (IFNγ, TNFα, IL-2, IL-4, IL-5, IL-10, IL-17), circulating immune modulators (IL-6, epinephrine) and indirect infection parameters (CRP, procalcitonin) as secondary endpoints. Patients' samples are labelled with pseudonym identification numbers and processed in specialized laboratory within 6 h for immediate measurement or stored on -80 °C for further analysis, respectively. Patients' data and blood results are stored in the study center at the Departement of Experimental Neurology (Charité Berlin) in an electronic database (Microsoft Access 2013). The administrative interim-analysis is part of the study protocol publication where the feasibility and completeness of the study population is evaluated.

Systematic review and meta-analysis

For systematic reviews and meta-analyses assessing the therapeutical value of „Rho/ROCK-inhibitors" a study protocol was finalized in advance of any data collection and is accessible online. In order to identify animal studies describing the effect of Rho/ROCK-inhibitors on neurobehavioral recovery after SCI, precise search terms were

defined for PubMed, EMBASE and ISI Web of science. Search results were limited to animals. Two investigators independently assessed the search results.

Studies were included if they reported the effects of Rho/ROCK-inhibitors in animal models of acute traumatic spinal cord injury. We included experiments comparing functional outcome in a group of animals receiving Rho/ROCK-inhibitors (treatment group) with a control group receiving no treatment. Studies reporting only combined treatments were excluded. Studies had to report the number of animals for each group, the mean effect size and its standard deviation or standard error of the mean.

We extracted details of individual study characteristics from each publication and functional outcome was assessed for each experiment. Where the outcome was expressed graphically only, Universal Desktop Ruler was used to visually extract the data points. Where data was expressed serially, numerical values were extracted. Only the final time point of the assessment of functional recovery was included.

As an assessment for 'risk of bias' the methodological quality of each study was assessed using a 9-point item quality checklist, adapted from the CAMARADES (Collaborative Approach to Meta Analysis and Review of Animal Data from Experimental Studies) quality checklist[15]. This quality checklist overlaps substantially with the ARRIVE (Animal Research: Reporting In Vivo Experiments) guideline for reporting animal research [13].

A normalized effect size (ES) for each comparison was calculated, which is defined as the improvement of outcome in the treatment group compared to the control group relative to the outcome of a untreated, unlesioned "sham" animal. The size of the control group was adjusted if a single control group was compared to more than one treatment group. We used DerSimonian and Laird random effects model meta-analysis to calculate an overall estimate of effect size and stratified meta-analysis with partitioning of heterogeneity to investigate the impact of different study characteristics. Significant differences between *n* groups were assessed by partitioning heterogeneity and by using the χ^2 distribution with n-1 degrees of freedom. To allow for multiple comparisons, the significance level was adjusted respectively to the Bonferroni correction. For detection of publication bias the *metatrim* function of STATA (Stata Corp.) was used. Graphs were drawn using SigmaPlot (Systat Software Inc).

Results

Functional SCI-IDS propagates the susceptibility for bacterial infection

The lesion level dependency of SCI-IDS was evaluated in a model of experimental pneumonia after thoracic T3 and T9 lesions. Comparing the amount of cfu per lung 24h after induction of pneumonia high thoracic T3 injures revealed a significantly higher bacterial load than lower T9 injuries (effect size=1.5, $p \leq 0.05$). This was comfirmed in histological lung tissue staining, showing typical signs of pneumonia with massive leukocyte infiltration and hyper-cellularity. Sham operated animals showed comparable bacterial load to T9 SCI. Correspondingly, a significantly pronounced spleen atrophy was detected at day 4 post-SCI. After low thoracic SCI the innervation of immunologically relevant organs from sympathetic efferents mainly located at level T5-T9 (e.g. to the spleen) remains intact, whereas the supraspinal innervation gets disrupted after high thoracic lesions. To evaluate the loss of supra-spinal sympathetic control of immune organs after T3 SCI the innervation of the major lymphatic organ was disrupted in a spleen denervation model.

First, the influence of sympathetic spleen denervation after SCI was investigated. Comparing Th9 injured animals either receiving a laparotomy without sympathetic denervation as a control group or Th9 SCI and spleen denervation showed comparable levels of cfu in the lungs and spleen lengths. In case of massive supra-spinal signaling through sympathetic efferents to avoid spleen atrophy and dysfunction, the group of animals with sympathetically disconnected spleens should have differed from the control laparotomy group. Thus, peripheral post-ganglionic sympathetic injury was ruled out as the sole explanation for enhanced infection susceptibility.

Second, we therefore combined high thoracic T3 SCI with denervation of the spleen. The bacterial load in the lung was reduced after T3 SCI and sympathetic denervation of the spleen compared to T3 SCI with control laparotomy (effect size=0.9, $p=0.06$). Additionally, an increased spleen atrophy was observed in the control group. This identified the spinal cord isolated from supra-spinal signaling as the origin of sympathetic signaling disturbing the spleen function and thereby enhancing susceptibility to pneumonia.

As a translational approach, we investigated whether the observed lesion level dependent susceptibility to develop pneumonia can be confirmed in human SCI data.

1221 datasets from patients with AIS A-D spinal cord injuries were stratified into three groups (T1-T4, T5-T8 and T9-12) comparing patients with and without pneumonia occurring between admission to acute care within 24h and discharge from inpatient rehabilitation following SCI (median [interquartile range] post SCI: 55 days [39-78]). The groups showed similar clinical and baseline characteristics (gender, race, number of SCI due to penetrating injury, spinal surgery and pulmonary embolism) but revealed differences in age, use of mechanical ventilation, injury severity and neurological level. Using the occurrence of SCI-associated pneumonia as dependent variable the neurological level (T1-4, T5-8 or T9-12, respectively) was identified as a significant factor in motor complete SCI (AIS A and B) after the exclusion of 93 patients due to missing data on the dependent variable. This was confirmed in a multiple logistic regression analysis affirming that the neurological level is associated with an enhanced susceptibility for pneumonia (odds ratio: 1.35, 95% confidence interval 1.14-1.60, $p<0.001$) independently from mechanical ventilation, the occurrence of pulmonary embolism and other outcome relevant factors. Hence, the lesion height dependent risk to develop pneumonia observed in experimental SCI was verified in human SCI.

SCIentinel-study for characterization of human SCI-IDS

The administrative interim-analysis of the SCIentinel study to characterize human SCI-IDS with haematological profiling and immunophenotyping was performed when almost 50% of the calculated sample size was reached in April 2013, 21 months after the beginning of patient recruitment. At this stage, 64 patients have been enrolled for the study confirming the expected range of recruitment. 28 of 48 (58%) patients have been recruited in the first patient group with neurological level T4 or above, 11 of 31 (36%) in the second group with spinal cord injuries at thoracic level T5 or below and 17 of 39 (44%) in the third group with vertebral fractures without neurological impairment. Four patients were excluded within the observation period because they did not fulfil eligibility criteria. Additionally, four patients treated with high-dose methylprednisolone were excluded from the ‘per protocol’ analysis but investigated in a separate analysis. For the remaining 56 patients we observed a generally satisfying data completeness with regards to the primary outcome and further main laboratory parameter, although an insufficient completeness was registered for the last observational time point 10 weeks after SCI.

RhoA/ROCK-inhibitors in spinal cord injury

The initial systematic search identified 2817 studies and after application of the inclusion and exclusion criteria 14 studies were selected for the final 'analysis-in' dataset. All studies used rodent models (725 animals) of thoracic SCI (level T3 – T10) and reported neurobehavioral outcome assessment using the Basso, Beattie, and Bresnahan [BBB] score or the Basso Mouse Scale [BMS] for Locomotion.

The random effect meta-analysis calculated an overall treatment effect of 21% (95% CI, 16.0-26.6) for RhoA/ROCK-inhibitors. Subsequent stratified meta-analysis identified five study characteristics that accounted for a significant proportion of between-study heterogeneity using the χ^2 distribution and Bonferroni post-hoc correction as mentioned above.

(A) Comparing RhoA- and ROCK-inhibitors the different applied molecules showed a broad range of reported efficacy. Among RhoA-inhibitors C3-ADP-ribosyltransferase was the most effective whereas Y-27632 showed most beneficial effects among ROCK-inhibitors which act downstream the RhoA-GTPase. The NSAID Ibuprofen showed the least improvement among RhoA-inhibitors but Fasudil reported the smallest overall improvement.

(B) Animals undergoing hemisection as a SCI model revealed best neurobehavioral improvement, whereas transection of the spinal cord indicated worst recovery.

(C) The volatile anesthetic halothane showed least improvement in locomotor outcome. The combination of fentanyl, fluanisone and diazepam revealed highest effect sizes.

(D) Where experiments only used male animals the reported effect size was noticeably lower compared to studies not exclusively using male rodents. Animals with unknown sex, females or both genders showed comparable estimates of efficacy.

(E) The stratification for study quality revealed an inverse relationship between the study quality and effect size. Experiments from studies reporting one or two quality items showed distinctly increased effect sizes compared to higher quality studies.

We used Egger-regression, visual inspection of the funnel-plot and the trim-and-fill method to account for possible underlying publication bias. The regression line did not intersect the origin in the Egger-regression indicating present publication bias. Based on the funnel plots asymmetry the trim-and-fill method detected nine missing studies with zero or detrimental effects. This imputation reduced the overall effect sizes down to 15.6% (95% CI, 10.2-21.2) which implies an absolute overestimation of efficacy 5.4% lower than the initial estimate.

Discussion

Functional SCI-IDS occurring within days after acute SCI and the subsequently increased risk for developing pneumonia has been demonstrated to be lesion level dependent in an experimental model relating to a preserved function of secondary immune organs such as the spleen. As a translational approach this was verified in an observational clinical study of spinal cord injured patients. To further characterize SCI-IDS in the clinical setting, a prospective patient study protocol is included in this thesis, providing laboratory analysis after the initial SCI with immunophenotyping and haematological profiling. Early detection of a SCI-IDS onset might be a possible consequence of the "SCIentinel" study and imply the development of therapeutic clinical strategies. A systematic review and meta-analysis of animal literature therefore assessed the preclinical efficacy of RhoA/ROCK inhibitors after SCI as they contain anti-inflammatory substrates and candidates to improve neurobehavioral outcome. This might lead to the identification of pharmacological strategies that account for SCI-IDS related consequences and improve clinical care for these patients.

In an experimental SCI model the susceptibility for pneumonia was elevated in high thoracic T3 SCI compared to T9 SCI [7]. The application of S. pneumonia by lung intubation to induce experimental pneumonia and subsequent homogenization of lung tissue to assess the amount of cfu on blood agar plates is currently regarded as the gold standard in diagnosing experimental pneumonia, although no symptoms of pneumonia are assessed in the experimental model. Respiratory muscle function is provided by cervical innervation of the diaphragm at spinal level C3-5 accounting for up to 80% of breathing [7]. Further accessory muscles (Scalenes, Pectoralis Minor, Serratus Anterior, Sternocleidomastoid, etc.) innervated from the cervical spinal cord are responsible for respiratory function and remain unaffected at thoracic spinal cord injuries. Besides intercostal muscle function partly affected by thoracic SCI, the paralysis of major respiratory muscles could be ruled out as a cause for pneumonia in the used injury model.

The isolated spinal cord distal from the lesion was detected to propagate functional SCI-IDS and increased the susceptibility for pneumonia in a lesion dependent manner. After thoracic T3 SCI the spinal cord gets disconnected from inhibiting supra-spinal signaling and spinally generated sympathetic nerve activity from below the lesion site interfere

with the spleen function propagating functional SCI-IDS. The intact innervation of the spleen from thoracic spinal cord level T5-T9 delineates a crucial requirement to develop a functional SCI-IDS since the splanchnic denervation after T9 SCI was not able to increase the risk for infections itself. The alternative hypothesis based on the absence of supra-spinal signaling was refuted: T9 SCI that would leave the spleen innervation intact was compared with T9 SCI and spleen denervation showing comparable bacterial load in the lung and spleen size. This means, the role of the spleen as a main SCI-IDS effector organ was confirmed and the susceptibility for pneumonia demonstrated in a lesion level dependent manner. The denervation of the spleen ('Spleen Shielding') before T3 SCI showed a beneficial effect decreasing the bacterial load and spleen atrophy, although no statistical significance was detected. The disruption of parasympathetic efferents from the vagal nerve as a collateral damage during the splanchnic denervation might hamper compensatory mechanisms affecting the spleen function. Moreover, lesion independent effects of the hypothalamic-adreanal-pituitary gland (HPA)-axis are likely to further exacerbate functional SCI-IDS and might conceal the impact of spleen shielding. And possible consequences of spleen shielding in the context of SCI needs to be further clarified since syndromes similar to overwhelming post-splenectomy infections (OPSI) increase the susceptibility for fatal infections and might be a possible consequence after spleen denervation. Despite these limitations, 'spleen shielding' might represent a valuable clinical strategy to prevent developing SCI-IDS.

In a translational approach the SCI lesion level dependent risk for infections was confirmed in an observational clinical study. However, the study cohort is not population based and only represents patients treated in the USA [6,7]. Since no population based large data-registry is available worldwide, this constitutes the best available data. Country specific treatment strategies might represent possible bias and a limitation of this study. But the data was gained in a multicenter based approach and it has been demonstrated that data from the used database are in line with those from other national and international studies [7].

The qualitative characterization of SCI-IDS to develop clinical laboratory surrogate parameter is the aim of the SCIentinel study [8]. State of the art haematological profiling and immunophenotyping is used to determine immune cell function within the innate and adaptive immune system. Changes of HLA-DR expression on monocytes occur

within one day after initial trauma, can be measured in well-established laboratory methods and were therefore selected as primary outcome parameter to detect patients at risk to develop an infection. Furthermore, a large amount of laboratory parameters is assessed as secondary outcome parameters to extend the monocyte approach and to develop putative additional tools to detect SCI-IDS.

By minimizing the amount of concomitant injuries and preexisting diseases or medication we tried to reduce variation within the study population to better describe the SCI-associated 'neurogenic' impact on the immune system. Injury severity and lesion level could decisively influence the immune system's function in a 'neurogenic' manner. To rule out further 'non-neurogenic' effects of the surgical procedure during the initial stabilization after SCI (post-aggression syndrome, activation of HPA-axis) a control group was included receiving surgery after vertebral fracture without neurological impairment [8]. By implementation of two SCI groups below and above thoracic level T5 we followed the same rationale as described above: the innervation of the major secondary immune organs (spleen, liver, adrenal gland) from T5-T9 should remain intact in the group with lesions below T5 and disrupted in the group with lesions above T5. The injury severity is of essential significance in this stratification since incomplete lesions (AIS B-D) could leave partial innervation of secondary immune organs intact. Therefore, the initial neurological baseline assessment of the single neurological level and AIS representing current international standards in SCI care is critical to compare patients with complete (AIS A) and incomplete (AIS B-D) spinal cord lesions. The assessment of occurring infections is focused on urinary tract infections and pneumonia which are the most frequent infections after SCI. Both are assessed using established thresholds and diagnostic tools, assuring the generation of reliable and reproducible data. The interim-feasibility analysis revealed data completeness between 24% and 91% depending on the observation time point and level of injury. Especially at late time points the control group indicated a poor completeness rate and as a consequence the recruiting study centers were informed, patients were contacted regarding their appointment in the outpatient clinics and visited in rehabilitation hospitals. However, the study's sample size calculation is based on 50% missing values and one-way ANOVA. Taking into account that repeated measures ANOVA might be applicable for statistical analysis and requires generally less patients, the interim analysis demonstrated feasibility of the SCIentinel study within the established data assessment.

The early identification of SCI patients at risk to develop infections might improve clinical care, rehabilitation and mortality after SCI and enables for risk stratification/adjustment in clinical trials. However, therapeutic interventions are necessary to treat consequences of inflammation, such as impaired neuronal regeneration, and to foster neurological recovery [12]. Our 'bedside to bench' approach is based on using a systematic approach to identify, select and appraise relevant preclinical data. A systematic review is a thorough analysis of earlier conducted and published experiments. Then, meta-analysis methodology is used to evaluate animal data, e.g. the overall efficacy, the effect of study quality, the influence of biological factors such as timing of treatment and the presence of co-morbidities, and the presence of publication bias. In fact, systematic meta-analyses of individual clinical trials have greatly improved the predictive value of clinical research. They form the basis of evidence-based medicine and are already standard practice for clinical studies. The use of systematic reviews for the optimization of animal testing is however still rare, especially among the SCI research community.

We therefore conducted a systematic review and meta-analysis assessing the effect of RhoA/ROCK-inhibitors on locomotion after SCI. The drug group of RhoA/ROCK-inhibitors contains well-known clinical drugs, namely Ibuprofen and Indomethacin, which might be also of relevance for SCI-IDS related consequences [12] due to their anti-inflammatory effects. The reported overall efficacy was 21% including data from 725 animals, whereas stratified meta-analysis identified several aspects of study design that were significantly associated with different observed efficacies: the used drug, type of injury, anesthetics and sex of animal. The success of this approach is influenced by the quality and availability of data. We addressed the insufficient quality of the included animal studies using a nine-point item quality checklist for each publication. Publications with low study quality were associated with higher effect sizes compared to high quality studies reporting low effect sizes. This underlines the need to further improve the quality of preclinical research, since low quality studies tend to overestimate effect sizes. Several guidelines to improve the quality of animal research have been published (e.g. ARRIVE guidelines [13]) and their use needs to be further endorsed by scientists and scientific journals.

Classical methods such as non-systematic or even 'classical' systematic reviews including Cochrane reviews are potentially confounded by neglected data or by lacking access to unpublished data, respectively. Drawing erroneous conclusions that may be

skewed by missing data of performed experiments (publication bias) would be troubling if it is involved in the prioritization and validation of preclinical trials. Therefore, methods to detect and correct for publication bias were applied to the underlying dataset and identified a substantial amount of publication bias: nine theoretically missing experiments were imputed, adjusting the overall from 21% to 15.6%, which is a relative reduction of efficacy of 26%. It is unlikely that the presence of publication bias is limited to SCI animal research and should be considered a general problem for animal research hampering translation. Despite this limitations, we consider the improvement in neurobehavioral outcome after application of RhoA/ROCK-inhibitors a valuable approach to improve regeneration after SCI. Additional anti-inflammatory effects of certain RhoA/ROCK-inhibitors might prevent the consequences of SCI related complications such as SCI-IDS and thereby contribute to improved clinical care. We therefore consider this a potential therapeutic strategy for clinical application after careful implementation and monitoring in clinical trials.

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Eidesstattliche Versicherung

„Ich, Ralf Watzlawick, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: "*Spinal Cord Injury-induced Immune Deficiency Syndrome and therapeutic implications for pharmacological inhibitors of the RhoA/ROCK-pathway –a translational approach*" selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
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Anteilserklärung an den erfolgten Publikationen

Ralf Watzlawick hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1: Brommer B*, Engel O*, Kopp MA*, **Watzlawick R**, Muller S, Pruss H, Chen Y, DeVivo MJ, Finkenstaedt FW, Dirnagl U, Liebscher T, Meisel A, Schwab JM. Spinal cord injury-induced immune deficiency syndrome enhances infection susceptibility dependent on lesion level. ***Brain*** 2016. [Epub ahead of print]

Beitrag im Einzelnen: Mitarbeit bei der Durchführung der Tierexperimente. Mitarbeit bei Entwurf und Revision des Manuscripts, insbesondere im Teil der humanen Studiendaten.

Publikation 2: Kopp MA*, Druschel C*, Meisel C*, Liebscher T*, Prilipp E*, **Watzlawick R***, Cinelli P*, Niedeggen A, Schaser K-D, Wanner GA, Curt A, Lindemann G, Nugeva N, Fehlings MG, Vajkoczy P, Cabraja M, Dengler J, Ertel W, Ekkernkamp A, Martus P, Volk H-D, Unterwalder N, Kölsch U, Brommer B, Hellmann RC, Ossami Saidi RR., Laginha I, Prüss H, Failli V, Dirnagl U, Schwab JM. The SCIentinel study - Prospective multicenter study to define Spinal Cord Injury- Induced Immune Depression Syndrome (SCI-IDS) - Study protocol and interim feasibility data. ***BMC Neurol.*** 2013;13:168.
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Beitrag im Einzelnen: Wesentliche Mitarbeit bei der Planung und Durchführung der Literaturrecherche und statistischen Analyse, bei Entwurf und Revision des Manuskripts und bei der Erstellung der Abbildungen und Legenden.

Unterschrift, Datum und Stempel des betreuenden Hochschullehrers/der betreuenden Hochschullehrerin

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Unterschrift des Doktoranden/der Doktorandin

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Druckexemplare der ausgewählten Publikationen

Publikation 1:

Brommer B*., Engel O*, Kopp MA*, **Watzlawick R**, Muller S, Pruss H, Chen Y, DeVivo MJ, Finkenstaedt FW, Dirnagl U, Liebscher T, Meisel A, Schwab JM. Spinal cord injury-induced immune deficiency syndrome enhances infection susceptibility dependent on lesion level. ***Brain*** 2016. [Epub ahead of print] – IF 9.2

Publikation 2:

Kopp MA*, Druschel C*, Meisel C*, Liebscher T*, Prilipp E*, **Watzlawick R***, Cinelli P*, Niedeggen A, Schaser K-D, Wanner GA, Curt A, Lindemann G, Nugeva N, Fehlings MG, Vajkoczy P, Cabraja M, Dengler J, Ertel W, Ekkernkamp A, Martus P, Volk H-D, Unterwalder N, Kölsch U, Brommer B, Hellmann RC, Ossami Saidi RR., Laginha I, Prüss H, Failli V, Dirnagl U, Schwab JM. The SCIentinel study - Prospective multicenter study to define Spinal Cord Injury- Induced Immune Depression Syndrome (SCI-IDS) - Study protocol and interim feasibility data. ***BMC Neurol.*** 2013;13:168. – IF 2.5

Publikation 3:

Watzlawick R, Sena ES, Dirnagl U, Brommer B, Kopp MA, Macleod MR, Howells DW, Schwab JM. *Effect and reporting bias of RhoA/ROCK-blockade intervention on locomotor recovery after spinal cord injury: a systematic review and meta-analysis.* ***JAMA Neurology***. 2014; 71(1):91-9. – IF 7.3

* contributed equally as first author to this work.

Publikation 1 – Brommer et al. 2016

Aufgrund von Bestimmungen des Verlages, die eine Veröffentlichung des Volltextartikels ohne Zugangsbeschränkung untersagen, kann die Druckversion des Artikels hier nicht gezeigt werden.

Der Artikel

Brommer B*, Engel O*, Kopp MA*, Watzlawick R, Muller S, Pruss H, Chen Y, DeVivo MJ, Finkenstaedt FW, Dirnagl U, Liebscher T, Meisel A, Schwab JM. Spinal cord injury-induced immune deficiency syndrome enhances infection susceptibility dependent on lesion level. *Brain* 2016. [Epub ahead of print]

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BMC Neurology

STUDY PROTOCOL Open Access

The SCIentinel study - prospective multicenter study to define the spinal cord injury-induced immune depression syndrome (SCI-IDS) - study protocol and interim feasibility data

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Abstract

Background: Infections are the leading cause of death in the acute phase following spinal cord injury and qualify as independent risk factor for poor neurological outcome ("disease modifying factor"). The enhanced susceptibility for infections is not stringently explained by the increased risk of aspiration in tetraplegic patients, neurogenic bladder dysfunction, or by high-dose methylprednisolone treatment. Experimental and clinical pilot data suggest that spinal cord injury disrupts the balanced interplay between the central nervous system and the immune system. The primary hypothesis is that the **S**pinal **C**ord **I**njury-induced **I**mmune **D**epression **S**yndrome (SCI-IDS) is 'neurogenic' including deactivation of adaptive and innate immunity with decreased HLA-DR expression on monocytes as a key surrogate parameter. Secondary hypotheses are that the Immune Depression Syndrome is i) injury level- and ii) severity-dependent, iii) triggers transient lymphopenia, and iv) causes qualitative functional leukocyte deficits, which may endure the post-acute phase after spinal cord injury.

Methods/Design: SCIentinel is a prospective, international, multicenter study aiming to recruit about 118 patients with acute spinal cord injury or control patients with acute vertebral fracture without neurological deficits scheduled for spinal surgery. The assessment points are: i) <31 hours, ii) 31–55 hours, iii) 7 days, iv) 14 days, and v) 10 weeks post-trauma. Assessment includes infections, concomitant injury, medication and neurological classification using **A**merican Spinal Injury Association **i**mpairment **s**cale (AIS) and neurological level. Laboratory analyses comprise haematological profiling, immunophenotyping, including HLA-DR expression on monocytes, cytokines and gene expression of immune modulators. We provide an administrative interim analysis of the recruitment schedule of the trial.

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Discussion: The objectives are to characterize the dysfunction of the innate and adaptive immune system after spinal cord injury and to explore its proposed 'neurogenic' origin by analyzing its correlation with lesion height and severity. The trial protocol considers difficulties of enrolment in an acute setting, and loss to follow up. The administrative interim analysis confirmed the feasibility of the protocol. Better understanding of the SCI-IDS is crucial to reduce co-morbidities and thereby to attenuate the impact of disease modifying factors to protect neurological "outcome at risk". This putatively results in improved spinal cord injury medical care.

Trial registration: DRKS-ID: DRKS00000122 (German Clinical Trials Registry)

Keywords: Spinal cord injury, Immune paralysis, Lesion height dependency, High-dose methylprednisolone treatment, Infections

Background

The effective treatment of worldwide 2.5 million paralyzed, spinal cord injured patients represents an unmet medical need to date [1]. In addition, the numbers of non-traumatic cases (e.g. tumor, cervical spondylotic myelopathy) of spinal cord injury (SCI) are increasing. Infections, i.e. pneumonia and urinary tract infections are a leading cause of morbidity and mortality in patients with acute SCI [2,3]. However, attributing infections to motor-paralysis related dysfunction alone does not sufficiently explain the increased susceptibility to develop infections after SCI. Among others, dysphagia occurs also in healthy patients over night without causing pneumonia. Thus, higher rates of dysphagia in SCI patients with cervical lesions do not solely explain increased rates of pneumonia.

It has been elucidated recently that SCI might increase predisposition to infections by Central Nervous System (CNS)-specific mechanisms: CNS-injury induces a disturbance of the normally well-balanced interaction between the immune system and the CNS resulting in a Spinal Cord Injury-induced Immune Depression Syndrome (SCI-IDS) [4-9]. Presence of SCI-IDS has been verified independently after experimental [10] and human SCI [11]. In brief, SCI 'neurogenically' ablates the immune system and enhances susceptibility to develop infections, which in turn might cause a generalized wound healing impairment – also affecting wound-healing/repair of the spinal cord lesion itself. Of note, besides increasing the mortality, infections represent an independent risk factor for impaired functional neurological recovery e.g. by i) reducing the conversion rate from being 'completely' to 'incompletely' paralyzed and ii) impairing gain of American Spinal Injury Association (ASIA) motor scores [12].

Based on preclinical and first clinical observations within a pilot trial [5,6] we aim to develop prognostic surrogate parameters in order to predict and selectively identify patients 'at risk' to develop infections. Here, we propose to establish parameters after human SCI, which have been tested in other clinical paradigms of elevated infectious risk such as status post cardiopulmonary bypass [13] and after ischemic CNS injury [14,15]. Therefore, we apply methods established in humans, such as human leukocyte antigen (HLA)-DR expression on monocytes [14] and Concanavalin A (ConA)-induced cytokine expression in whole blood samples, as surrogate markers of SCI-IDS. HLA-DR expression on monocytes serves as primary outcome measure. Moreover, a peripheral blood leukocyte mRNA expression profile encoding for immune modulatory proteins will be investigated to sense and therefore predict an evolving immune suppression as early as possible. Furthermore, we will measure the presence of individual predisposition for infections by assessing polymorphisms in coding areas of MHC-proteins, toll-like pattern-recognition receptors and selected cytokines [16].

The prevention of infections facilitated by the 'neurogenic' immune suppression syndrome (with its pronounced penetrance referred to as 'immune paralysis') aims to (i) decrease mortality, (ii) reduce expensive hospitalization time and, (iii) improve the functional outcome, since SCI patients suffering infections are prone to develop inferior neurological recovery [12]. Molecular treatment strategies aiming to foster axonal plasticity and repair might be complemented by approaches that intend to protect the intrinsic neurological recovery potential. Given a realistic therapeutic timeframe of opportunity after SCI, the early identification of SCI-patients 'at risk' would offer a 'preventive' option to treat infections earlier and thereby avoid their detrimental consequences. This protective strategy might save neurological function 'at risk' and thereby improve quality of life. In addition, it would be possible to unmask putative confounding factors that influence neurological outcome measures of relevance for interventional or rehabilitative trials [12].

A solid body of evidence demonstrates that SCI is associated with an early onset of immune suppression (SCI-IDS). Identification of SCI patients as immune compromised is a clinically relevant finding, yet widely underappreciated and warranting further analysis. In order to decipher the underlying mechanisms of SCI-IDS we are following-up on experimental results [5,7,10,17], clinical pilot trials

[6,8], and cohort studies [9,18,19] with the international SCIentinel trial. We outline the study protocol and design, define the main outcome parameters, and provide the results of an administrative interim analysis.

Methods/Design

Study design, study coordination, and participating centers

The "SCIentinel" trial is designed as a prospective multicenter study for the detailed evaluation of the SCI-IDS (Figure 1). The overall coordination is performed by the Department of Experimental Neurology, Clinical and Experimental Spinal Cord Injury Research (Neuroparaplegiology) at the Charité University Hospital, Campus Mitte, Berlin, Germany.

The study comprises seven recruiting trial centers with a specialization in SCI treatment: Treatment Center for Spinal Cord Injuries, Trauma Hospital Berlin, Germany; Center for Musculoskeletal Surgery (Campus Virchow Clinic); Department of Trauma and Reconstructive Surgery (Campus Benjamin Franklin); Department of Neurosurgery (Campus Virchow Clinic and Campus Benjamin Franklin), Charité University Hospital Berlin, Germany; Division of Trauma Surgery, University Hospital Zurich; Spinal Cord Injury Center, University Hospital Balgrist, Zurich, Switzerland, and the Department of Neurosurgery, Toronto Western Hospital, Toronto, Canada. The study investigators are physicians specialized and experienced in the treatment and rehabilitation of patients with SCI.

Duration

A recruitment period of 30 months is scheduled. Each patient will be followed-up to three months post-trauma. After completion of recruitment and follow-up, a further six months period is planned for the database clearing, the statistical evaluation and the preparation of the trial report. Enrolment has started in August 2011. Expected study completion date is the first quartile of 2014. Publication of the trial report is scheduled for the end of the year 2014.

Ethics

The protocol received approval by the local Ethics Committees: Ethical Committee of the Charite – Universitätsmedizin Berlin (EA1/001/09), University Health Network Research Ethics Board, Toronto (REB10-0384-AE), Cantonal Ethics Commission, Zurich (KEK-ZH-Nr. 2011–0059). Participants will be informed about the trial, orally and in written form, using patient's information sheets and written informed consent will be obtained. This study complies with the Helsinki Declaration, the principles of Good Clinical Practice (GCP) and the Personal Data Protection Act. The study will also be carried out in keeping with local legal and regulatory requirements. The study has been registered in the German Clinical Trials Registry (DRKS-ID: DRKS00000122).

Participants

A number of 118 patients is planned to be allocated to the study for examination of the primary endpoint. In order to

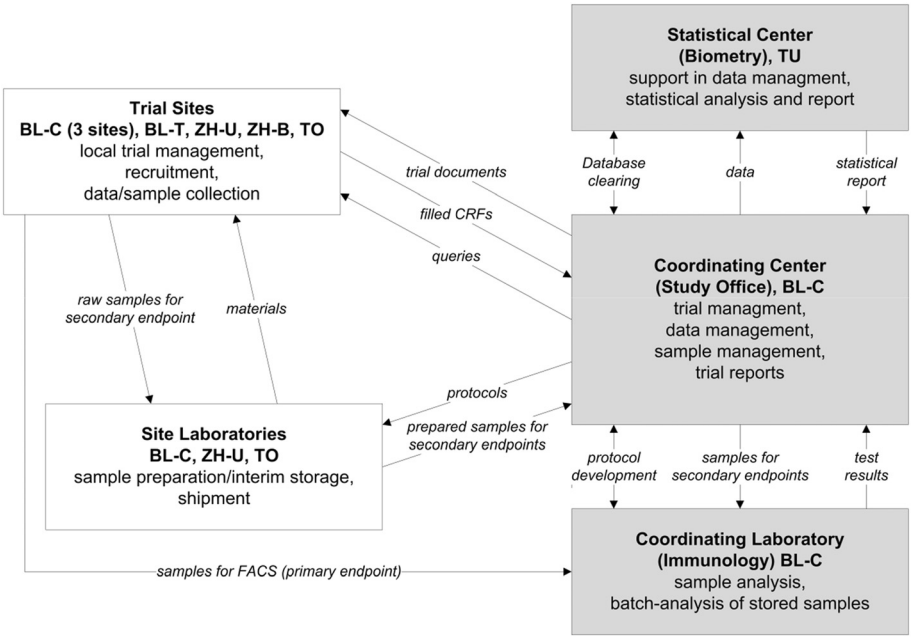

Figure 1 Multicenter structure and trial management. Legend: Scheme of the trial workflow. BL-C = Berlin-Charité, BL-T = Berlin-Trauma-Hospital; TU = Biometry Tübingen; ZH-U = Zurich-University Hospital; ZH-B = Zurich-Balgrist; TO = University Hospital Toronto.

allow for investigation of the role of disturbed innervation of the major splanchnic nerve (T5-T9) as linked to alterations of the immune system function, we categorize SCI patients into lesions with the neurological level T4 or above versus lesions neurological level T5 or below. Therefore, the patients will be assigned to 3 groups: i) 48 SCI-patients with a neurological level T4 or above; ii) 31 patients with a neurological level T5 or below; iii) 39 patients with an acute vertebral fracture, but without SCI as control group (Figure 2).

The study will not interfere with methylprednisolone treatment per site regimens and patients will be also enrolled, but are excluded from the analysis of the primary endpoint. Based on experiences from the clinical practice in the recruiting trial centers the rate of cases treated with methylprednisolone is estimated to be 5-10%. Thus, up to 12 patients will additionally be included for explorative examination of methylprednisolone-related alterations of the immune system after SCI (Figure 2).

Sample size calculation

The primary endpoint is a difference in HLA-DR expression on monocytes exactly quantified by analysing antibody-binding molecules/cell within the first week (day 3–4) post-trauma. Numbers of patients to be enrolled were calculated on the basis of a pilot study (n = 26) [6]. The sample size calculation was performed for an effect size of 0.18. The type one error was be set to 0.05 (two-sided); type two error was set to 0.2. The number of patients eligible for evaluation (for testing the hypothesis by the one way anova) is 56 in total (software nquery, version 7.0). Due to an expected rate of drop-outs/missing data of 50% and non-parametric testing, where applicable, the number of patients to be recruited increases to 118 in total. The expected rate of drop-outs/missing data relates to the structural conditions of acute SCI care in terms of i) delayed transfer to specialized SCI trial centers after primary care in external non-specialized centers and ii) loss to follow up due to discharge from inpatient rehabilitation,

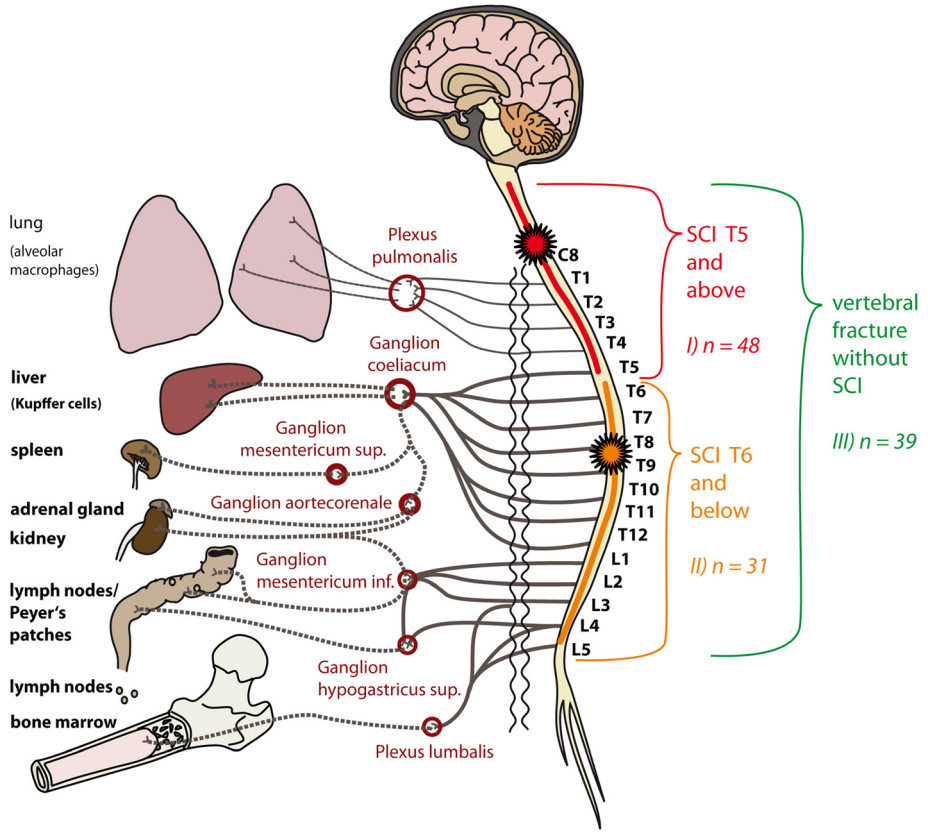

Figure 2 Relay-interaction of the immune system and the central nervous system. Legend: Groups for primary comparison in relation to the major sympathetic outflow (vegetative innervation). In Group I (SCI of the neurological level T4 or above) the lesion is localized within the spinal segments C2-T5, resulting in a disturbance of the sympathetic innervation of immunologically relevant organs through the coeliac ganglion and further ganglia connected through lower segments of the spinal cord. Of note, the neurological level is defined by the ISNCSCI as the most cranial segment with normal sensory function and a muscle grade of at least 3/5 with normal function in the segments above on both sides of the body, i.e. in case of a neurological level T4, the lesion begins in the segment T5. In Group II (SCI of the neurological level T5 or below) the lesion is located in the spinal segment T6 or below. Thus, the sympathetic outflow to the coeliac ganglion is expected being only partially disrupted or completely preserved. Group III consists of patients with vertebral fracture alone without injury to the spinal cord (control). Here, the sympathetic innervation is intact.

particularly in the control group or in cases of mild incomplete SCI. Consequently, to allow for a completion of the study within a feasible time period consideration of missing values is unavoidable.

The sample size calculation was performed taking the asymmetric natural distribution of the neurological lesion level into account [20]. Injury to the cervical and high thoracic spinal cord has the highest incidence and represents the largest group of SCI patients. Thus, the relation of the 3 groups to each other was determined as follows based on the pilot trial data i) 41% (n = 48); ii) 26% (n = 31); iii) 33% (n = 39).

Enrolment and eligibility criteria

With the seven study centers treating 20 to 60 acute SCI patients admitted to the emergency ward of each center per year we estimate to screen about 200 SCI patients per year and about 40 of them are expected to meet eligibility criteria (Table 1).

Investigators will evaluate the patients after admission to the emergency ward for eligibility. Before final inclusion into the study the investigator will conduct an interview with the patients to verify the inclusion- and exclusion-criteria and obtain written informed consent.

Table 1 Eligibility criteria

Inclusion criteria	
(1)	Patients with acute isolated spinal cord injury (AIS A-D) planned for surgical stabilization and decompression, lesion may include more than 1 segment
(2)	Patients with acute isolated spinal fracture planned for surgical stabilization, lesion may include more than 1 segment
(3)	≥ 2 spinal cord or vertebral lesions definable one from another
(4)	Legal age of the patient
(5)	Documented informed consent of the patient
Exclusion criteria	
(1)	Non-traumatic spinal cord injury
(2)	2 or more spinal cord or vertebral lesions definable one from another
(3)	Severe polytrauma *(definition: patients with severe injuries of life-sustaining organ systems, which per se and in the acute phase are life-threatening (e.g., severe pelvic trauma, severe body cavity injuries)*
(4)	Concomitant traumatic brain injury (TBI) *(definition: i) Patients with persisting neurological deficit in consequence of the TBI, ii) patient with severe TBI (Glasgow Coma Scale ≤ 8), and iii) patients with intracranial pressure monitoring sensors.)*
(5)	Neoplasia and/or antineoplastic therapy
(6)	Rheumatic disease, collagenosis, vasculitis or other autoimmune disease
(7)	Preexisting chronic infectious disease (before the injury)
(8)	Preexisting systemic steroid treatment
(9)	Severe alcohol or drug addiction
(10)	Pregnancy, lactation

Assessment time points

Measurements and observations are scheduled in all groups in the same manner (Figure 3). An inclusion assessment will be performed at the study entry and is documented in a case report form (CRF). The baseline CRF includes the neurological classification, assessment of injury date and time, medical history, concomitant injury, acute SCI therapy concerning high-dose methylprednisolone treatment as well as surgical intervention.

At the 5 study visits, equal blood samples and clinical data will be obtained. The clinical data comprise infections, medication, surgery, and blood transfusions. The first assessment should be completed as early as feasible after the injury, preferably before surgery, otherwise at least within 31 hours post-injury. The second assessment point refers to a time window of 31–55 hours post-injury. To ensure a sufficient time interval to the first assessment the second one should be performed on the following day at the earliest. This allows a better intra-individual analysis. The visits 3, 4 and 5 refer to day 7, 14 and to 10 weeks after injury, respectively. Blood withdrawals, except at time point 1 should be performed between 7:00 and 11:00 am. This setting determines the influence of the circadian rhythm to a minimum.

In the case, if it is unavoidable that a patient may be assigned to the study later as 31 hours (delayed recruitment) e.g., due to later admission or transfer to a study center (see also sample size calculation), study inclusion is tolerated at any later time point. In individual cases of delayed recruitment or patient's absence, documentation of visits 2–5 may be scheduled in extended time windows (Figure 3).

Definition of infections

The most prevalent infections after SCI, in detail chest infections and urinary tract infections, are diagnosed and documented in the study according to established definitions of disease to ensure a biometric comparability [15,21,22] (Table 2). Additionally, a distinction is made between non-symptomatic and symptomatic urinary tract infections, i.e. the latter are classified with regard to evidence of fever, increased spasticity, bladder spasm, suprapubic or flank pain, autonomic hyperreflexia, malaise, or lethargy. All other infections are documented according to the usual diagnostic criteria of the participating centers.

Neurological classification

The neurological evaluation is performed according to the International Standards for Neurological Classification of Spinal Cord Injury (ISNCSCI), a revision of ASIA classification [23,24]. Within the ISNCSCI-regime the ASIA impairment scale (AIS) as a measure for completeness of the injury, and the single neurological level of the lesion are assessed.

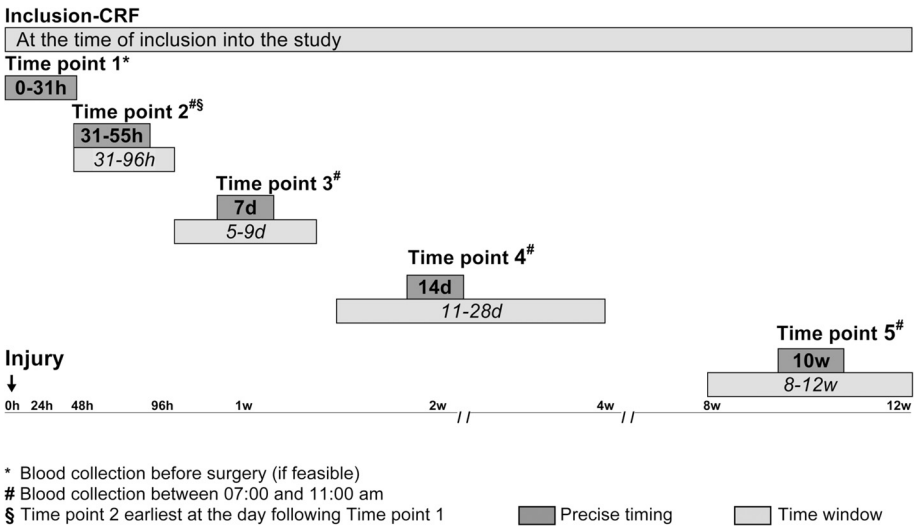

Figure 3 Longitudinal trial design. Legend: Assessment points and time frames. The starting point of the time line is the time of injury not the time of surgery. In the case of difficulties in scheduling the visits 2 to 5 may be conducted within the indicated time windows (*italic*). The circadian rhythmics are respected.

Blood sample handling

Peripheral blood is collected under sterile conditions from each participant at each visit. All samples are labeled with a six-digit pseudonym and any personal information of the participants is removed. Overall, one 2 ml and one 6 ml Ethylenediaminetetraacetic acid (EDTA) BD Vacutainer®, two 3 ml Heparin BD Vacutainer® and one 8.5 ml Serum BD Vacutainer® are needed. Furthermore one 5 ml Cyto-Chex® blood collection tube (BCT) and two 2.5 ml PAXgene™ RNA tubes are collected (Figure 4). Blood samples for flow cytometric analysis are analyzed as soon as possible, preferably within 24 hours latest within 48 hours. Preservation of intracellular and extracellular surface markers is ensured by using Cyto-Chex® BCT [25,26].

Supernatants from whole blood stimulation and further blood samples for secondary outcome measures are stored at −80°C for subsequent batch analysis (Figure 4). In the trial centers at Berlin and Zurich, one additional 8 ml BD Vacutainer® CPT™/Ficoll™ tube is collected for immediate preparation of Peripheral Blood Mononuclear Cells (PBMCs) for storage in liquid nitrogen. The handling of samples follows identical standard operating procedures and checklists at all trial centers.

Immune profiling of SCI-IDS

The following investigations are performed to determine the immune status: i) immunophenotyping with lymphocyte subpopulations (B, T, NK cells), T cell subpopulation (CD3, CD4, CD8) and monocytic HLA-DR expression; ii) ex vivo T lymphocyte cytokine secretion such as ConA-induced IFN-g, TNF-α, IL-2, IL-4, IL-5, IL-10 secretion and staphylococcal enterotoxin B (SEB)-induced IL-17 production; iii) gene expression analysis for immune

Table 2 Diagnostic criteria for chest infection and urinary tract infection

Type of infection	Criteria
Chest infection, if ≥ 3 criteria apply	Temperature < 36.0°C or ≥ 37.5°C
	Putrid secretion
	Pathological respiration (rales, bronchial breathing, tachypnea > 22/min)
	Opacities in chest x ray (required for the diagnosis of pneumonia)
	Detection of pathogenic germs in sputum
	pO2 < 70 mmHg/O2 saturation < 93%
Urinary tract infection, if ≥ 1 criteria apply	Bacteriuria with bacterial count > 10^5 cells/ml
	Leukocyturia ≥ 100 WBC/mm^3, respectively 100.000 WBC/ml
Symptomatic urinary tract infection, if ≥ 1 criteria apply	≥ 1 criteria for urinary tract infection
	≥ 1 of the following criteria: fever, suprapubic or flank discomfort, bladder spasm, increased spasticity, worsening dysreflexia

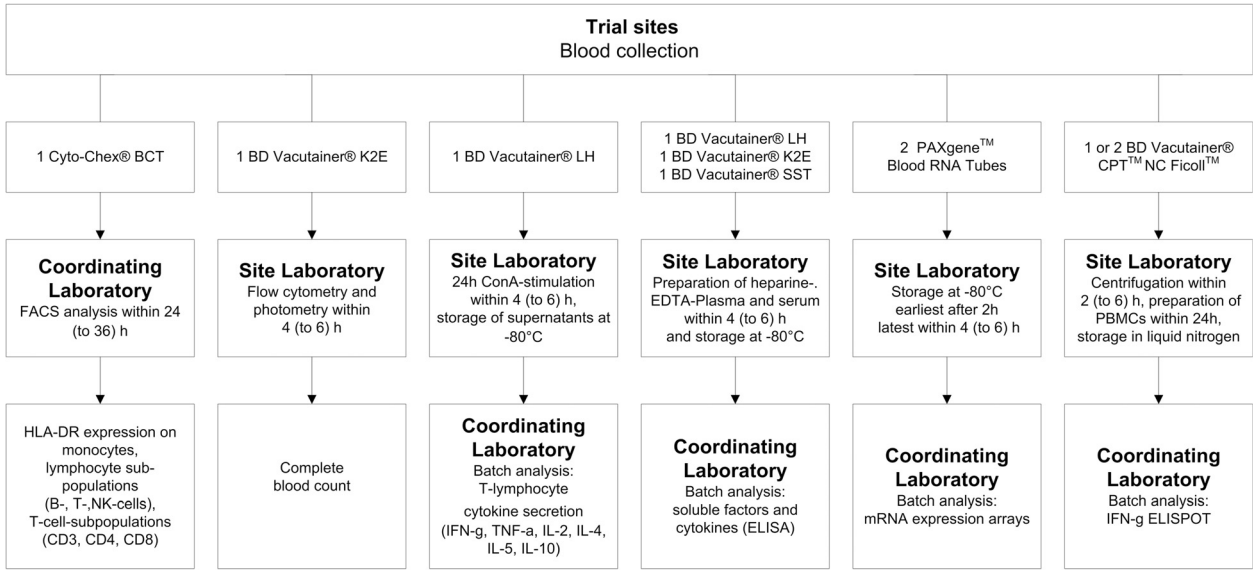

Figure 4 SCI-IDS Immunephenotyping – qualitative and quantitative measures. Legend: Scheme of blood collection, sample preparation and analysis procedures. ConA = Concanavalin A, ELISA = Enzyme Linked Immunosorbent Assay, ELISPOT = Enzyme Linked Immunosorbent Spot, FACS = Fluorescence-activated cell sorting, IFN-g = Interferon-gamma, TNF-g = Tumor necrosis factor – gamma.

activation/deactivation related genes (mRNA expression); iv) circulating immune modulators: such as IL-6, epinephrine; v) indirect infection parameters: procalcitonin, C-reactive protein (CRP), LBP (lipopolysaccharide-binding protein).

Immunophenotyping using fluorescence-activated cell sorting (FACS) including quantitative measurement of monocytic HLA-DR expression as the primary outcome measure will be conducted according to standardized operation procedures established at the Institute of Medical Immunology (Charité - Universitätsmedizin Berlin, Germany) for clinical diagnostics of an immune suppression state [14]. HLA-DR belongs to the MHC class II molecules that are responsible for antigen presentation to CD4+ T-cells. Monocytic HLA-DR expression reflects the state of the adoptive immune competence as it is positively regulated by Th1 cytokines and negatively regulated by anti-inflammatory cytokines, stress hormones and some exogenous agents. Furthermore, monocytic HLA-DR expression is associated with the ability to produce pro-inflammatory mediators upon challenge by bacterial products such as lipopolysaccharides (LPS). Reduced monocytic HLA-DR expression has been established to correlate with an increased risk for infections in critically ill patients previously [14,27,28].

Quantitative measures of HLA-DR expression

In contrast to the pilot study that measured HLA-DR expression on different cell types [6], the SCIentinel study measures quantitatively the amount of HLA-DR molecules per monocyte (in estimated numbers per cell). The reliability of the method has been previously approved in an inter-laboratory study [14].

The principle of the test is as follows. A mixture of Phycoerythrin (PE) beads conjugated with defined amounts of PE molecules is measured at the same instrument settings as the cells incubated with a mix consisting of anti-human HLA-DR-PE, anti-human CD14-PerCP-Cy5.5, and an inhibitor of HLA-DR turnover (BD Quantibrite™ HLA-DR/Monocyte reagent, BD Biosciences). The PE beads facilitate conversion of the FL2 axis into PE molecules bound per cell. The known ratio of PE to anti-HLA-DR antibody is used to convert the PE molecules per cell into antibodies per cell (AB/c). The anti-HLA-DR antibody, clone L243, reacts with a non-polymorphic HLA-DR epitope and is conjugated with PE molecules in a ratio of 1:1. The anti-CD14 antibody, clone MoP9, is conjugated with PerCP-Cy5.5. CD14 is expressed by the majority of monocytes. In addition, since the cyan dye component of PerCP-Cy5.5 binds to CD64, the anti-CD14 PerCP-Cy5.5 antibody detects all monocytes (CD14 brightly positive and weakly positive). The immunodiagnostical thresholds delineating a graded SCI-IDS penetrance are defined as indicated in Table 3 and were developed based on previous clinical trials after CNS injury [27].

Data management

Data are collected on a paper CRF (pCRF) basis. All patient's data are managed with a six-digit pseudonym. At the coordinating center personnel familiar with the trial protocol check the filled pCRFs for completeness and consistency. Implausible or missing data may be corrected

Table 3 Thresholds for the interpretation of the analysis of HLA-DR expression on monocytes

Antibodies/cell	Interpretation
> 15 000	Normal diagnostic findings
10 000 – 15 000	Immunodepression
5 000–10 000	Borderline immunoparalysis
< 5 000	Immunoparalysis

or added after consulting the investigator at the trial site through the study management (Queries). The corrected documents will be archived together with the completed CRFs. Data are entered and stored electronically in a database (Access) and are independently double-checked for correctness. The database has secured and restricted access centralized at IT-Center of Charité-Universitätsmedizin Berlin, Germany. Data backups are performed on a daily basis.

Interim evaluation of recruitment status

An administrative interim evaluation for control of the estimated recruitment schedule after enrolment and follow up of approximately 50% of the calculated sample size has been performed. Patients recruited until April 2013 and followed up to July 2013 were included. The groups for comparison were analyzed for their concordance with the estimated sample size (Table 4). Furthermore the completeness of data for the main laboratory outcome parameters (flow cytometry) at each time point was assessed within the 'per protocol' population (Table 5).

Statistical analysis

Data analysis of the primary endpoint consists of a comparison, between the 3 groups at the early and subsequent follow-up assessment points using one-way ANOVA or nonparametric tests, if appropriate. The analysis will be performed using the full dataset comprising all patients included according to the criteria as defined in the study protocol. In addition, in patients with complete intra-individual datasets also changes over time within the groups will be assessed with descriptive methods. Furthermore, in the case of a relevant amount of missing values, multiple imputation will be taken into consideration for verification of the analysis of the original data. Data obtained from patients treated with methylprednisolone will be primarily analyzed in a casuistic manner.

The statistical analysis of the administrative interim evaluation has been performed using SPSS for Macintosh, Version 19.0.

Discussion

The SCIentinel trial aims to define SCI-IDS characteristics. We investigate the hypothesis that SCI-IDS is not restricted to quantitative immune suppression (cell depletion) [5-8], but also affects qualitative immune function of 'spared' immune cells. Qualitative dysfunction of immune cells translates into suppressed innate and adaptive immune cell function that finally leads to impaired host defense [29]. Qualitative effects may extend into chronic SCI [29] and therefore the identification of patients prone to develop infections is also relevant during long-term follow up. Moreover, according to the proposed 'neurogenic' origin of the SCI-IDS we suggest rostral and complete spinal cord lesions to be associated with a more severe immune depression syndrome compared to more caudal or incomplete lesions. Furthermore we intend to investigate a putative sustained extension into chronic disease phases after SCI. Together, this is embedded the longitudinal prospective trial design and in a power calculation that takes differences between rostral and caudal neurological levels into account. In addition, the impact of facultative co-treatment with high dose methylprednisolone, which may further aggravate SCI-IDS is accessible for descriptive analysis.

Spine trauma

In order to differentiate the SCI-related 'neurogenic' effect on the immune system from the 'non-neurogenic' effect of the surgical intervention/trauma itself (activation of the stress axis/post-aggression syndrome) the study protocol incorporates a control group of patients with isolated vertebral fracture without neurological deficit. Of note, this refinement of the control group definition by the SCIentinel trial overcomes a weakness of the pilot study where patients after smaller surgical interventions such as arthroscopy served as control group [6], which only insufficiently mimics the post-aggression syndrome caused by surgical stabilization of the spine.

Table 4 Administrative interim analysis of the recruitment status for patients without high-dose methylprednisolone treatment

Groups	Overall n (%)	SCI T4 and above n (%)	SCI T5 and below n (%)	Control group n (%)	Drop out n
Estimated sample size	118 (100)	48 (100)	31 (100)	39 (100)	n.a.
Enrolled partients	60 (51)	28 (58)	11 (35)	17 (44)	4

Legend: Sixty patients without high-dose methylprednisolone treatment were included into the SCIentinel trial from August 2011 to April 2013. The number of patients recruited into the group of SCI patients of the neurological level T5 or below was slightly smaller than expected. This does not match the natural distribution of the lesion level and seems to be random. In total, four patients (7%) dropped out of the interim analysis due to occurrence of exclusion criteria.

Table 5 Administrative interim analysis of the completeness of laboratory data (flow cytometry) for each visit relative to the number of enrolled patients feasible for 'per protocol' analysis

Groups		Visit 1	Visit 2	Visit 3	Visit 4	Visit 5
Overall n = 56	Complete data n	32	38	44	44	25
	(% of 'per protocol' population)	(57)	(68)	(79)	(79)	(45)
SCI T4 and above n = 28	Complete data n	17	20	22	22	16
	(% of 'per protocol' population)	(61)	(71)	(79)	(79)	(57)
SCI T5 and below n = 11	Complete data n	6	6	8	10	5
	(% of 'per protocol' population)	(55)	(55)	(73)	(91)	(45)
Control group n = 17	Complete data n	9	12	14	12	4
	(% of 'per protocol' population)	(53)	(71)	(82)	(71)	(24)

Legend: The completeness of the main laboratory data was calculated for the three groups relative to the number of enrolled patients feasible for 'per protocol' analysis. The sample size calculation takes a 50% rate of drop out or missing values into account. Considering the drop out rate < 10% at the current stage of the trial, a 60% rate of data completeness is acceptable. Critical rates of overall completeness are observed at visit 1 and visit 5 and relate mainly to the control group and to patients with neurological level T5 or below.

In order to evaluate the impact of the lesion height we assess differences between rostral and caudal spinal cord lesions. Consequently, SCI patients were divided into two groups with/without expected impairment of the major sympathetic outflow and compared with the controls applying a three-group strategy for analysis (Figure 1). To assess the effect of lesion severity, we compare complete (AIS A) with incomplete lesions (AIS B-D) in a descriptive sub-group analysis.

Eligibility criteria

We defined the eligibility criteria (Table 1) in order to minimize inclusion bias and to obtain 'representative' data. The definitions stipulated in the SCIentinel protocol are consistent with previously published studies [6,8,18,30]. It was necessary to exclude patients with severe, life threatening polytrauma or severe TBI in order to limit variability caused by concomitant injury. For detection of immune system functionality, it is furthermore essential to exclude all circumstances combined with pre-existing alterations in the immunological profile such as chronic autoimmune diseases and pre-existing systemic treatment with corticosteroids.

The administrative interim analysis of the SCIentinel trial has confirmed the feasibility of the eligibility criteria. From August 2011 to April 2013 n = 64 patients have been enrolled for the study which represent 37 patients per year. Thus, the enrolment status is within the expected range. Four of the 64 patients (6%) were treated with high-dose methylprednisolone. Therefore, those patients are to be excluded from the 'per protocol' analysis. However, casuistic studies on immunosuppressive effects of methylprednisolone treatment in the context of the SCI-IDS are enabled. The remaining 60 patients represent 51% of the estimated sample size (Table 4).

Observation period

Observational time points (Figure 2) range from acute to sub-acute stages after SCI comparable to the pilot study [6]. Here, the time schedule corresponded to the hallmarks of parenchymal inflammation following SCI. The implementation of sub-acute assessments is in line with previous trials that demonstrated mid- and long-term alterations of the immune system after SCI [8,18,19].

The setting of defined time corridors instead of time points provides a more complete tracking of the participants. In order to prevent a high amount of missing datasets due to loss to follow-up, the protocol allows an inclusion at each documentation time point.

The administrative interim analysis of data completeness for the main laboratory parameters indicates - depending on the group or visit – a completeness of data between 24% and 91% with critical rates of completeness at visit 1 and visit 5, particularly in the control group (Table 5). As a consequence, the recruiting centers have been requested to enroll the patients as soon as possible after injury. Additionally, we intend to address the challenge of loss to follow up by travelling to external rehabilitation centers or by phone calls to remind patients of their study visit at the outpatient clinic. The rate of complete longitudinal datasets ranges between 18% and 36% but is expected to increase during the further trial course since trial routines are better established. Noteworthy, the sample size calculation takes 50% of missing values into account and was performed for one-way ANOVA. Repeated measure analysis requires lower sample sizes. Therefore, it seems feasible that a relevant amount of complete longitudinal datasets can be achieved also for the description of the intra-individual course of the SCI-IDS. Pursuing this study concept, serial investigations on short- and mid-term changes of the immunological function are provided.

Clinical observations

For the clinical research, it is imperative to use reliable reproducible clinical tests. The ISNCSCI standards revised over the years to provide better, more specific definitions [23,24]. We incorporated the AIS and the single neurological level into the trial protocol for neurological baseline characterization of SCI patients.

The determination of infections is of crucial importance in this study. Prevailing infectious complications of SCI patients are pulmonary and urinary tract infections [4]. Cameron and colleagues recently provided a review consisting of 12 articles of urinary tract infection (UTI) screening for SCI [31]. Noteworthy, there is no universal definition of UTI used in the literature allowing substantial heterogeneity. The American Paraplegia Society (APS) recommends to base the diagnosis of UTI solely on bacteriuria, with a threshold of 10^2 colony-forming units (cfu)/mL for SCI patients [32]. However, up to 75% of samples taken from asymptomatic SCI male patients under intermittent catheterization contain ≥ 10^2 cfu/mL [32]. Thus, a threshold of 10^2 cfu/mL is associated with low specificity, leading to an excess of treatment, with the risk of the emergence of resistant bacteria, adverse events, and unnecessary expense. In consequence, we defined according to the National group on urologic rehabilitation of paraplegics urinary tract infection as bacteriuria greater than 10^5 cells/ml or WBC count of more than 100/mm^3 [21].

Regarding the development of chest infection criteria defined by Mann et al. were incorporated [22]. For the diagnosis of pneumonia the finding of infiltrates or opacity in chest X-ray will be required consistent with Haeusler et al. who used similar criteria in the analysis of cellular immunodepression preceding infectious complications after acute ischemic stroke [15].

In summary, the clinical assessments and definitions used in the SCIentinel study allow for collection of reliable and reproducible data.

Primary outcome measure

For meeting the purpose of a more comprehensive characterization of the SCI-IDS and establishing prognostic surrogate parameters, it is essential to implement specific parameters, readily validated in clinical immunology, robust and reproducible [14]. Quantification of immune cell populations in the blood using FACS is a clinically established and precise method that qualifies as primary outcome measure. Our primary measure is the determination of HLA-DR-molecules per monocyte. Alterations in HLA-DR expression on monocytes occur early enough to identify patients at high risk for relevant immune depression and consecutive development of infections. Significantly decreased monocytic HLA-DR levels have been observed as early as day one after acute cerebral ischemia in patients who developed infectious complications in comparison to patients with an uncomplicated clinical course [27]. Furthermore, the method is already established and available in centralized immunological laboratories. It has sufficient diagnostic specificity as a basis for clinical decision-making and therefore is clinically highly relevant. Moreover, we implemented a large setting of secondary endpoints for the development of very early predictive parameters, for example on the level of mRNA expression.

Possible consequences

The objective of this study is the longitudinal characterization of immunological markers of the innate and acquired immune system after traumatic SCI. Furthermore, the aim is to detect diagnostic surrogate parameters for the clinical application after SCI. The characterization of a causative relationship between the lesion level and/or SCI severity and the type and/or extent of the immune response after SCI - including a critical characterization of the methylprednisolone effect - is essential for the implementation of therapeutic studies. Understanding and recognition of the immunological dysfunction and the altered susceptibility to infections may then assist in consecutive decision-making. This is relevant for the stratification of patients who are at high or low risk for infectious complications [33]. In addition, these findings may also hold the key for early therapeutic immunomodulation aiming to improve overall survival by anticipation and prevention of life-threatening infectious complications.

Abbreviations

ASIA: American Spinal Injury Association; AIS: ASIA impairment scale; BCT: Blood collection tube; CON A: Concanavalin A; CRF: Case report form; CRP: C-reactive protein; FACS: Fluorescence-activated cell sorting; EDTA: Ethylenediaminetetraacetic acid; GCP: Good clinical practice; HLA: Human leukocyte antigen; ISNCSCI: International standards for neurological classification of spinal cord injury; LPS: Lipopolysaccharides; PBMC: Peripheral blood mononuclear cell; PE: Phycoerythrin; SCI: Spinal cord injury; SCI-IDS: Spinal cord injury – induced immune depression syndrome; SEB: Staphylococcal enterotoxin B; TBI: Traumatic brain injury; UTI: Urinary tract infection.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MAK, CD, CM, JMS designed the trial protocol and wrote the first draft of the manuscript. TL, PC, AN, GAW, AC, GL, NN, MGF, PM, HDV, UD reviewed the trial protocol. All authors critically revised the manuscript draft for important intellectual content. MAK, CM, PC, HDV, BB, HP, VF, JMS contributed to the establishment of analytical methods. MAK, CD, TL, EP, RW, PC, AN, KDS, GAW, AC, GL, NN, MGF, PV, MC, JD, WE, AE, NU, UK, RH, RROS, IL, VF JMS substantially contributed to the sample and data acquisition. MAK, CD, HP, VF, JMS were responsible for overall trial coordination and supervision. MAK, CD, RW, PC, GL, NN, MC, BB, RCH, RROS, IL, VF were responsible for local trial management. CM, PC, MGF, PM, HDV, UD provided technical support. MAK, RW were responsible for data management. MK performed the interim feasibility analysis. JMS obtained funding. All authors have read and approved the final version of the manuscript.

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