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Peripheral Immune Remodeling After Spinal Cord Injury

Marked by Expansion of CD38$^+$, CTLA-4$^+$, and PD-1$^+$ T and NK Cells

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Abstract

Background and Objectives

Spinal cord injury (SCI) is associated with severe immunologic changes, such as SCI-induced immune deficiency syndrome, which heightens susceptibility to infections. However, the immune components underlying this immune reorganization remain poorly defined. This study aimed to characterize immune remodeling in patients with SCI across different time points postinjury.

Methods

High-dimensional flow cytometric profiling was performed on peripheral blood samples from patients with SCI in a cross-sectional observational study to assess immune changes at different postinjury time points. Patients in the subacute phase (22–67 days of postinjury [dpi]) and chronic phase (≥365 dpi) were compared with healthy, sex-matched, and age-matched controls.

Results

Alterations in the T-cell and natural killer (NK) cell compartments were observed, particularly in the subacute phase postinjury. Memory T cells and NK cells showed elevated expression of the NAD$^+$ metabolizing enzyme CD38 and immune checkpoint molecules, cytotoxic T-lymphocyte–associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1), indicating immune activation and possible exhaustion. Coexpression of CD38 and CTLA-4 on T cells was rare, suggesting distinct activation and inhibitory states. In chronic patients, we observed decreased frequencies of NK cells with no substantial changes in T cells and B cells. Notably, changes in CD38, CTLA-4, and PD-1 were no longer found in patients in the chronic phase.

Discussion

These findings reveal noteworthy changes in immune cell activation and exhaustion markers that may contribute to immune vulnerability after SCI, offering novel insights into potential therapeutic targets, such as NAD$^+$ metabolism and immune checkpoint modulation.

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Supplementary Material

Introduction

Spinal cord injury (SCI) induces severe damage to the nervous tissue of the spinal cord, causing either partial or complete paralysis. The neuronal damage not only results in motor and/or sensory deficits, it may also lead to SCI-induced immune deficiency syndrome (SCI-IDS),

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Glossary

AIS = American Spinal Injury Association Impairment Scale; **ANOVA** = analysis of variance; **ASIA** = American Spinal Injury Association; **CM** = central memory; **cSCI** = chronic SCI; **CTLA-4** = cytotoxic T-lymphocyte–associated protein 4; **DMSO** = dimethyl sulfoxide; **DPI** = days of postinjury; **EM** = effector memory; **EMRA** = effector memory T cells re-expressing CD45RA; **FBS** = fetal bovine serum; **HBG** = human beta globin; **HC** = healthy control; **HLA-DR** = human leukocyte antigen - DR isotype; **ICC** = interclass coefficient; **KLRG1** = killer cell lectin-like receptor G1; **NAD$^+$** = Nicotinamide adenine dinucleotide (oxidized form); **NIR** = Near-infrared; **NK** = natural killer; **NKT** = natural killer T cells; **NRQ** = normalized relative quantity; **PBMC** = peripheral blood mononuclear cell; **PBS** = phosphate-buffered saline; **PD-1** = programmed cell death protein 1; **qPCR** = quantitative PCR; **RPMI** = Roswell Park Memorial Institute medium; **RQ** = relative quantity; **S** = single-copy gene; **SA-β-gal** = senescence-associated β-galactosidase; **SCI** = spinal cord injury; **SCI-IDS** = spinal cord injury–induced immune deficiency syndrome; **SDS** = sodium dodecyl sulphate; **SEM** = standard error of the mean; **sSCI** = subacute spinal cord injury; **T** = telomere; **Th4** = thoracic level 4; **TL** = telomere length; **TLA** = telomere length analysis; **Tregs** = regulatory T cells; **UMAP** = Uniform Manifold Approximation and Projection.

a condition in which people suffer from a suppressed immune system accompanied by an increased susceptibility to infections.[1-4] Both pulmonary infections and urinary tract infections as well as infected pressure sores contribute substantially to the mortality in patients with SCI. Studies demonstrate that the heightened vulnerability to infections in patients with SCI cannot be fully explained by factors such as increased aspiration risk, bladder dysfunction, or high-dose methylprednisolone treatment. It is also influenced by immune dysfunction after SCI.[2,5-9]

This immune dysfunction is thought to emerge from the dysregulation of the autonomic nervous system. In particular, the sympathetic control of lymphoid organs and the simultaneous activation of neuroendocrine stress pathways can suppress the immune response. The degree of immune dysfunction depends on the location of the lesion.[1,9,10] To be specific, the T4 level is the most rostral innervation level of the celiac ganglion which innervates the spleen, and therefore, lesions above this level affect the splenic function.[11] Studies investigating the immune function have reported a decrease in natural killer (NK) cell counts following SCI, along with functional deficits such as reduced cytotoxicity.[2,12] By contrast, the effects on T cells are more variable,[2,12-14] and there is evidence suggesting that patients with SCI have a premature onset of immunosenescence.[15,16]

Immunosenescence is characterized by a decline in the number of naïve T cells, reduced vaccination responses, and increased baseline levels of proinflammatory cytokines.[17-20] Senescent T cells have reduced expression of costimulatory molecules CD27 and CD28, increased killer cell lectin-like receptor G1 (KLRG1) and CD57, and display shortened telomeres. In addition, senescent cells are characterized by increased activity of senescence-associated β-galactosidase (SA-β-gal). These T cells are still able to proliferate and become highly inflammatory and cytotoxic. By contrast, in the context of chronic antigen stimulation, T cells can become exhausted and are no longer able to exert their effector functions. Exhausted T cells are marked by a high expression of immune checkpoint molecules, such as the inhibitory receptor cytotoxic T-lymphocyte–associated protein 4 (CTLA-4), and the apoptosis-inducing receptor programmed cell death protein 1 (PD-1).[21] Although some studies suggest immune deficiency after SCI, a detailed understanding of the molecules involved is lacking.

This study provides a comprehensive characterization of the peripheral immune profiles of patients with SCI and age-matched healthy controls, focusing on biomarkers related to immunosenescence and immune exhaustion, because they could lead to increased susceptibility to infections. We aim to investigate both the short-term and long-term effects of SCI on these immunologic parameters. Since these biomarkers are affected by aging, both young and aged individuals were included. We further compared patients with SCI at subacute (22–67 days of postinjury (DPI), dpi) and chronic (≥365 dpi) time points with healthy age-matched and sex-matched controls, and detected increased frequencies of memory CD8$^+$ T cells in the subacute phase after SCI. In addition, substantially more of these memory T cells but also immunoregulatory NK cells expressed CD38 in patients in the subacute phase after SCI compared with healthy controls and patients in the chronic phase. CD38 is a surface enzyme that metabolizes NAD$^+$ (nicotinamide adenine dinucleotide) and mediates immune cell activation and diapedesis.[22,23] In addition, we found that patients with SCI in the subacute phase have higher frequencies of memory CD4$^+$ and CD8$^+$ T cells expressing CTLA-4 and cytotoxic NK cells expressing PD-1 compared with controls, suggesting immune cell exhaustion in the first months after injury. These levels returned to baseline in patients with chronic SCI (cSCI). Taken together, our results point toward an important role for CD38, CTLA-4, and PD-1 in the immune suppression seen after SCI.

Methods

Study Design and Reporting

This study follows a cross-sectional design, with peripheral immune profiles analyzed at a single time point for most

participants, including patients with SCI and matched healthy controls. A subset of patients was sampled twice. This study has been prepared in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology Cross-sectional checklist to ensure transparent and comprehensive reporting of our methods and findings.

Human Study Participants

Patients with SCI were included from the following centers: Noorderhart Hospital (Pelt, Belgium), Hospital Oost-Limburg (Genk, Belgium), University Hospitals Leuven (Leuven, Belgium), Algemeen Ziekenhuis Turnhout (Turnhout, Belgium), Adelante Centre of Expertise in Rehabilitation (Hoensbroek, The Netherlands), and Maastricht University Medical Center Plus (Maastricht, The Netherlands). To minimize selection bias, patients with SCI were recruited from multiple centers and healthy controls were matched by age and sex. The small subset of nontraumatic patients with SCI was inspected for outliers, and no outliers were identified. The sample size was determined primarily by patient availability at participating centers. HC participants were recruited at Hasselt University (Hasselt, Belgium). All biological samples were cryopreserved at the University Biobank Limburg.

Peripheral blood was collected from patients with SCI, who were not treated with corticosteroids, and HC (eTable 1). The HC group did not have any allergies, autoimmune disorders, or infections at the time of sampling and were matched to the patients with SCI in age and sex.

In total, 37 SCI patient samples were included at different time points: subacute SCI (sSCI) samples were taken at 22–67 days of post-SCI (n = 18) and cSCI samples were taken at ≥365 days of post-SCI (n = 19). A detailed overview of the individual patients with SCI is given in eTable 2. Participants were considered young when younger than 49 years of age and aged when 50 years of age and older.[18]

The American Spinal Injury Association Impairment Scale (AIS) is a standardized examination consisting of a dermatomal-based sensory examination, a myotomal-based motor examination, and an anorectal examination. The scoring system classifies SCI severity as follows: score A indicates a complete injury, score B indicates a sensory incomplete injury, score C indicates a motor incomplete injury with less than half of the key muscles below the neurologic level having a muscle grade of 3 or higher, score D indicates a motor incomplete injury with at least half of the key muscles below the neurologic level having a muscle grade of 3 or higher, and score E indicates normal function.[24]

Flow Cytometry

Peripheral blood mononuclear cells (PBMCs) were obtained via ficoll density gradient centrifugation (Lympholyte; Cedarlane Laboratories, SanBio B.V., Uden, The Netherlands) and were frozen in liquid nitrogen for storage (10% dimethyl sulfoxide [DMSO] in fetal bovine serum [FBS]; Thermo Fisher Scientific). For flow cytometry, cryopreserved PBMCs were handled as described previously.[13]

Shortly, the cryopreserved PBMCs were thawed and recovered in 20% FBS (Gibco, Thermo Fisher Scientific, Waltham, MA) in Roswell Park Memorial Institute medium (RPMI) 1640 (Lonza, Basel, Switzerland) supplemented with 0.5 mg/mL DNAse (Merck Life Science BV, Hoeilaart, Belgium).[13] Stainings for immune cell subsets were performed using 1 × 10^6 PBMCs per donor. First, PBMCs were stained with SPiDER-β Gal (SGO3, DOJINDO) for SA-β-gal detection, according to the manufacturer's description. Following this, PBMCs were washed in phosphate-buffered saline and stained with the fixable viability dye Zombie near-infrared (NIR) (Biolegend) for 15 minutes. After live/dead staining, the cells were stained for 20 minutes using anti-human antibodies (eTable 3) against CD8, CD28, KLRG1, CCR7 (CD197), CD27, CD56, CD45RA, CD4, PD-1 (CD279), CD3, CD16, CD57, CD38, human leukocyte antigen-DR isotype (HLA-DR), NKG2D, NKG2A, and CD19 (all from BioLegend). Cells were permeabilized using the FOXP3/Transcription factor staining buffer kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Cells were then intracellularly stained for Ki67 and CTLA-4 (CD152) for 20 minutes (all from BioLegend).

Samples were acquired using the Aurora (Cytek) and were analyzed using FlowJo 10.10.0 (BD Biosciences) for manual gating. FlowSOM and Uniform Manifold Approximation and Projection (UMAP) analysis of flow cytometry data was performed using the OMIQ software (Dotmatics).

Relative Telomere Length Measurement

Average relative telomere length (TL) was quantified using a modified single plex quantitative PCR (qPCR) method adapted from Cawthon, 2002 and 2009.[25,26] The method used for DNA quantity, DNA purity assessment, and gDNA quantification is described before,[27] and shortly outlined below.

PBMC gDNA was extracted by overnight lysis at 37°C in lysis buffer (10 mM Tris, 0.4 M NaCl, 2.4 mM EDTA), 20% sodium dodecyl sulphate (SDS), and 0.2% proteinase K solution (diluted in 35 mM SDS, 2.4 mM EDTA). The gDNA was further purified by a chloroform-based extraction process, precipitated by ethanol, and resuspended in sterilized Milli-Q. DNA quantity and purity were assessed by a Nanodrop 1000 spectrophotometer (Isogen, Life Science, Belgium). DNA was considered pure when the A260/280 was greater than 1.80 and A260/230 greater than 2.0. Gel electrophoresis was used to assess DNA integrity. To confirm a DNA input of 5 ng for each qPCR reaction, samples were diluted and checked using the Qubit dsDNA High-Sensitivity Assay Kit (Life Technologies, Europe) and measured with the Qubit Flex Fluorometer (Life Technologies, Europe). All samples were measured in triplicate on the QuantStudio 5 real-time PCR system (Applied Biosystems) in a 384-well format. First,

a single-copy gene (human β globin) reaction was performed, and this reaction mixture contained 5 ng DNA template, ×1 KAPA SYBR FAST, Low ROXTM master mix (Kapa Biosystems, Merck), and 450 nM human β globin primer 1 (GCTTCTGACACAACTGTGTTCACTAGC) and 450 nM human β globin primer 2 (CACCAACTTCA TCCACGTTCACC). Cycling conditions were as follows: 1 cycle at 95°C for 3 minutes, 40 cycles at 95°C for 3 seconds, and 58°C for 15 seconds. Second, a telomere-specific reaction was performed, containing 5 ng DNA template, ×1 KAPA SYBR FAST, Low ROXTM master mix (Kapa Biosystems, Merck), 2 mM dithiothreitol, and 100 nM TelG primer (ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT) and 100 nM TelC primer (TGTTAGGTATCCCTATC CCTATCCCTATCCCTATCCCTAACA). Cycling conditions were as follows: 1 cycle at 95°C for 3 minutes, 2 cycles at 94°C for 3 seconds and 49°C for 15 seconds, and 30 cycles at 94°C for 3 seconds, 62°C for 5 seconds, and 74°C for 10 seconds. After each qPCR, a melting curve analysis was performed. On each run, PCR efficiency was evaluated using a standard 6-point serial diluted standard curve (efficiency was 99% for TelC/G primer set, and 102% for human beta globin (HBG) primer set with an $R^2 > 0.99$). The average relative TL (T/S ratio) was calculated using the qBase software (Biogazelle, Zwijnaarde, Belgium). In qBase analysis, TL is expressed as a normalized relative quantity (NRQ). To obtain this value, the RQ is first calculated using the delta-Cq method for both the telomere (T) and the single-copy gene (S), incorporating their specific amplification efficiencies. Instead of using a single-calibrator sample, which can introduce additional error into the final relative quantities due to variability in the calibrator measurement, the data are normalized to the arithmetic mean quantification value of all samples. RQ and NRQ were calculated according to published mathematical models.[27,28] The method precision is shown by an intra-assay interclass coefficient for TelG/C run of 0.997 (95% CI 0.995–0.998; $p < 0.0001$), HBG run of 0.947 (95% CI 0.920–0.963; $p < 0.0001$), and for T/S ratio replicates 0.957 (95% CI 0.931–0.972; $p < 0.0001$).[27]

Statistics

Data were statistically analyzed using GraphPad Prism version 10.4.1 (GraphPad Software, Dotmatics, San Diego, CA), SAS 9.4 (SAS Institute Inc., Cary, NC), and JMP Pro version 17.2.0 (SAS Institute Inc., Cary, NC). For comparing HC, sSCI, cSCI, and both young and aged groups, a 2-way analysis of variance (ANOVA) was applied, followed by Sidak post hoc correction for multiple comparisons. When only 1 independent variable was compared across 3 groups (e.g., HC, sSCI, and cSCI), a 1-way ANOVA was performed. Data were reported as mean ± standard error of the mean (SEM).

To assess a possible interaction between age and days of postinjury (dpi) within the SCI patient group, linear mixed-effects models were performed and were applied using PROC MIXED in SAS 9.4. The model included age and dpi and their interaction as fixed effects, with sex, lesion level above and below thoracic level 4 (Th4), and AIS grouped (A/B; C/D) covariates. Least-squares means and pairwise comparisons were obtained with Tukey post hoc adjustment. Differences with p values < 0.05 were considered significant ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$).

Standard Protocol Approvals, Registrations, and Patient Consents

The study was approved by the Hasselt University medical ethics committee and the local hospitals' ethical committees. Informed consent, in accordance with all local ethical committees, was obtained from all donors.

Data Availability

The data sets used and analyzed during this study are available from the corresponding author on request.

Results

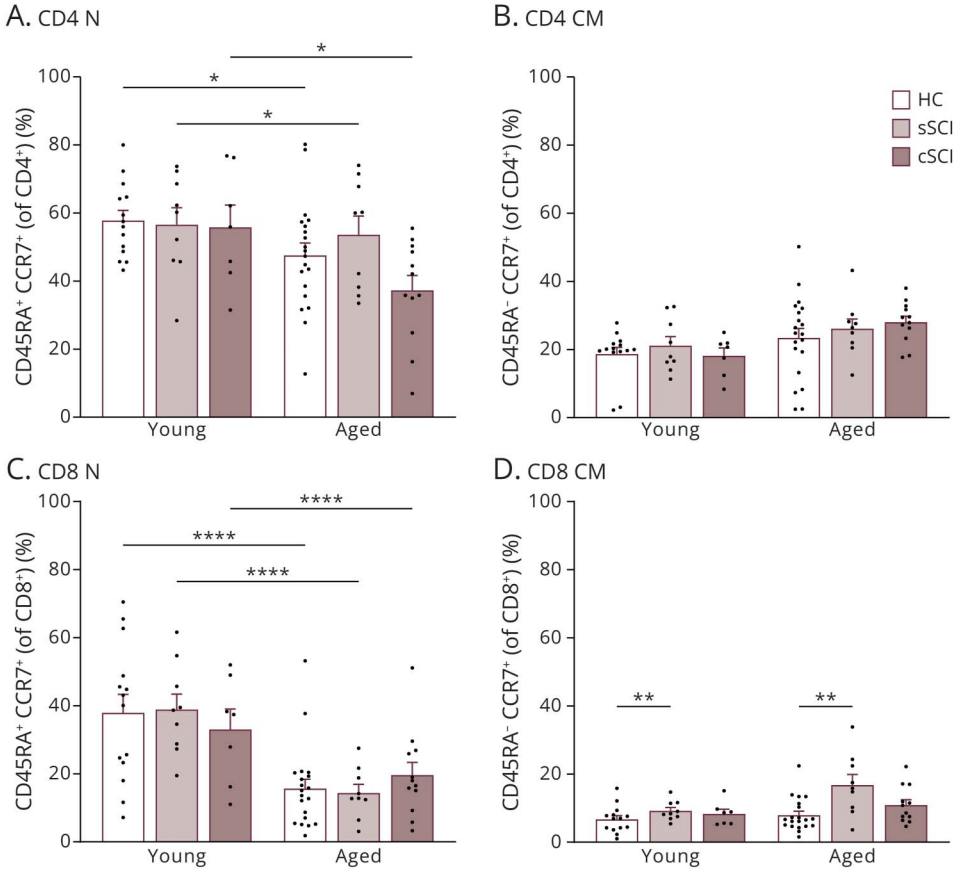
SCI Alters the Peripheral T-Cell Compartment

To assess the effect of SCI on peripheral T cells, we performed high-dimensional flow cytometry of PBMCs from HC, patients with sSCI (sSCI, 22–67 dpi), and those with cSCI (≥365 dpi).

Broad immune profiling of different T-cell subsets in both $CD4^+$ and $CD8^+$ T populations was executed, namely, naive (T naive, $CD45RA^+$ $CCR7^+$), central memory (CM) (T CM, $CD45RA^-$ $CCR7^+$), effector memory (EM) (T EM, $CD45RA^-$ $CCR7^-$), and EM T cells re-expressing CD45RA (TEMRA, $CD45RA^+$ $CCR7^-$). UMAP dimensionality reduction displayed clustering of $CD4^+$ and $CD8^+$ T cells in each of these subsets and revealed similar clustering patterns among HC, sSCI, and cSCI (eFigure 1A). Surface marker expression projected onto the UMAP plots depicts the phenotypic different T-cell populations in HC, sSCI, and cSCI individuals (eFigure 1B). FlowSOM-based clustering identified unique subset signatures, with the optimal number of clusters determined using the elbow method (eFigure 1C). Cluster names were assigned according to the above mentioned markers. Manual gating confirmed a similar distribution of $CD3^+$, natural killer T (NKT) cell, and $CD4^+$ and $CD8^+$ T-cell subsets across conditions (eFigure 2, A–D).

As the frequency of naïve and memory T-cell subsets changes with aging, we analyzed these subsets in young (22–49 years old) and aged (50–84 years old) individuals (naïve/CM, Figure 1; EM/EMRA, eFigure 2, E–H). As expected, in aged individuals, a decrease in naïve T cells was detected, most pronounced in the $CD8^+$ T-cell subset ($p < 0.0001$), but also significant within the $CD4^+$ T cells ($p = 0.0470$). In addition, in the first months (22–67 days) after injury, a significant increase in $CD8^+$ CM T cells ($p = 0.0031$) is detected in both young and old individuals compared with HC, but this was no longer apparent in the chronic disease stage (Figure 1D). In conclusion, while the percentages of the major T-cell subsets remain stable after SCI, increased frequencies of $CD8^+$ memory T cells are present in the subacute phase after injury, independent of age.

Figure 1 Flow-Cytometric Analysis Reveals Age-Dependent Decline in Naïve T Cells and Subacute Increase in Central Memory CD8$^+$ T Cells in Patients With SCI

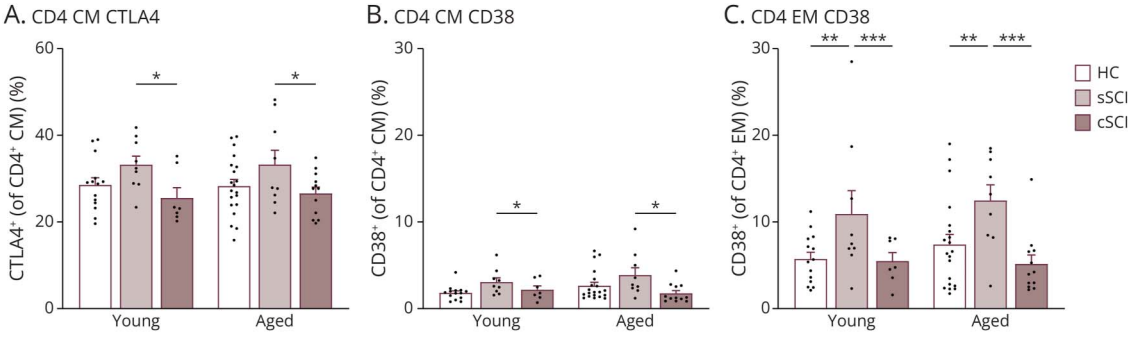


Frequencies of (A) naive (N) CD4 T helper, (B) central memory (CM) CD4$^+$ T cells, (C) naive CD8 cytotoxic T cells, and (D) central memory (CM) CD8$^+$ T cells within the CD4$^+$ and CD8$^+$ populations. These cell populations are separated by age group; young (22–49 years of age) and aged (50–84 years of age) because these cell population frequencies can be affected by aging. Data are shown as mean ± SEM. Two-way ANOVA with Sidak correction *: p value <0.05, **: p value <0.01, ****: p value <0.0001. cSCI = chronic spinal cord injury; HC = healthy control; sSCI = subacute spinal cord injury.

Alterations in CD4$^+$ T-Cell Subsets Following SCI

The percentages of CD4$^+$ T cells and the differentiation stages from naïve to memory did not change after SCI, but several functional markers demonstrate remarkable changes in frequency. There were an increase in CTLA-4$^+$ CD4$^+$ naive T cells with age ($p = 0.0013$) (eFigure 3A) and an increase in CTLA-4$^+$ CD4$^+$ CM T cells ($p = 0.0180$) (Figure 2A) in patients with sSCI compared with patients with cSCI. CTLA-4 is an inhibitory immune checkpoint receptor that downregulates T-cell responses. Furthermore, activation marker

Figure 2 Spinal Cord Injury Induces Expression of CTLA4 and CD38 on CD4$^+$ T-Cell Subsets in the Subacute Phase, Which Declines Again to HC Levels in the Chronic Phase in Both Young and Aged Individuals



Expression of (A) CTLA-4 on CD4$^+$ central memory (CM) T cells, (B) CD38 on CD4$^+$ CM T cells, and (C) CD38 on CD4$^+$ effector memory (EM) T cells. Data are shown as mean ± SEM. Two-way ANOVA with Sidak correction *: p value <0.05, **: p value <0.01, ***: p value <0.001. cSCI = chronic spinal cord injury; HC = healthy control; sSCI = subacute spinal cord injury.

CD38 was significantly higher within $CD4^+$ CM T cells in patients with sSCI compared with patients with cSCI (p = 0.0215) (Figure 2B). The frequency of $CD38^+$ $CD4^+$ EM T cells was increased in subacute patients and returned to baseline levels in chronic patients (p = 0.0052 and p = 0.0009, respectively) (Figure 2C). While both CTLA-4 and CD38 were increased on specific $CD4^+$ T-cell subsets in sSCI, only a minority of $CD4^+$ T cells coexpressed both molecules (Figure 3, A and B; eFigure 3F). The frequencies of CTLA-4 and CD38 on $CD4^+$ T-cell subsets were stratified by AIS classification (A/B vs C/D) and injury level (above vs below Th4) and revealed no differences. Frequencies of CTLA-4 and CD38 on other $CD4^+$ T-cell subtypes are presented in eFigure 3, B–E, and the frequencies of CTLA-4, PD-1, CD38, and Ki67 on $CD4^+$ and $CD8^+$ T cells can be found in eFigure 4. SCI-driven changes in the $CD4^+$ T-cell subsets were conserved across age groups. Overall, we found that specific $CTLA\text{-}4^+$ and $CD38^+$ $CD4^+$ T-cell subsets are increased in the subacute phase after SCI.

Changes in $CD8^+$ T-Cell Subsets After SCI

In line with our observations in $CD4^+$ T cells, 22–67 days after SCI, patients displayed increased frequencies of $CD8^+$ T-cell subsets with CD38 or CTLA-4 expression (Figure 4). In addition to this, the frequencies of $CTLA\text{-}4^+$ $CD8^+$ naive T cells and $CD38^+$ $CD8^+$ naive T cells were increased with age (p = 0.0020 and p = 0.0348, respectively) (eFigure 5, A and D). After SCI, CTLA-4 expression was significantly increased within CM $CD8^+$ T cells of patients in the subacute phase compared with HC (p = 0.0482), a change that returns to baseline levels in chronic patients (p = 0.0021) (Figure 4A). Similarly, CD38-positive EM and EMRA $CD8^+$ T cells were significantly increased in the subacute patients and returned to normal levels in chronic patients ($CD38^+$ EM: p = 0.0018 and p = 0.0248, respectively; $CD38^+$ EMRA: p = 0.0018 and p = 0.0300, respectively) (Figure 4, B and C). CTLA-4 and CD38 were similarly assessed on CM, EM and EMRA $CD8^+$ T-cell subsets, and these frequencies can be found in eFigure 5. Furthermore, all displayed cell frequencies were stratified by AIS classification (A/B vs C/D) and injury level (above vs below Th4). Stratification revealed that $CD38^+$ EMRA T cells were more abundant in patients with AIS A/B compared with those with AIS C/D (p = 0.0276) (data not shown). Since Th4-Th9 are important for spleen innervation, all reported cell frequencies of persons with injuries above and below the Th4 level were compared, but revealed no significant differences.

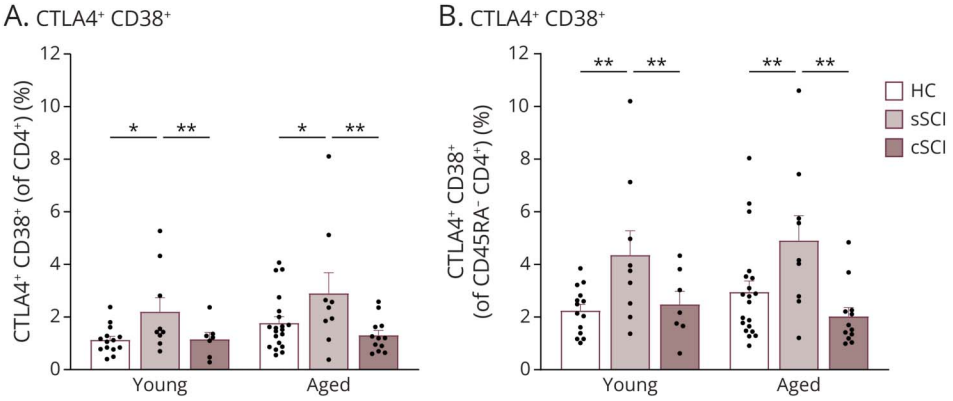
Immunosenescence Markers Following SCI

To assess whether SCI induces premature aging of the immune system in patients with SCI, we analyzed the frequency of CD27null, CD28null, $KLRG1^+$, and $CD57^+$ T cells in both $CD4^+$ and $CD8^+$ populations and found no significant differences between patients with SCI and HC (eFigure 6, A–H). Age-related increases were found for $CD8^+$ CD27null and $CD8^+$ $KLRG1^+$ T cells (p = 0.0065 and p = 0.0071, respectively). In addition, an overall age-associated increase for $CD8^+$ CD28null and $CD8^+$ $CD57^+$ T cells was noted (p = 0.0134 and p = 0.0497, respectively), although a post hoc test did not identify specific group effects. Another marker of senescence, SA-β-gal, was assessed using the SPiDER assay. The activity of this enzyme was increased with age in $CD8^+$ T cells specifically (p < 0.0001), but no change in activity was observed after SCI in subacute and chronic patients (eFigure 6, I and J). To further examine aging-related changes in the immune system, the TL of PBMC-derived DNA was measured. As expected, TL decreased with age in both HC and patients with SCI (p = 0.0461). However, SCI did not lead to significantly greater telomere shortening compared with age-matched HC (eFigure 6K). In summary, our study cohort demonstrated no evidence of premature aging of the immune system following SCI.

SCI Reshapes the NK Cell Landscape

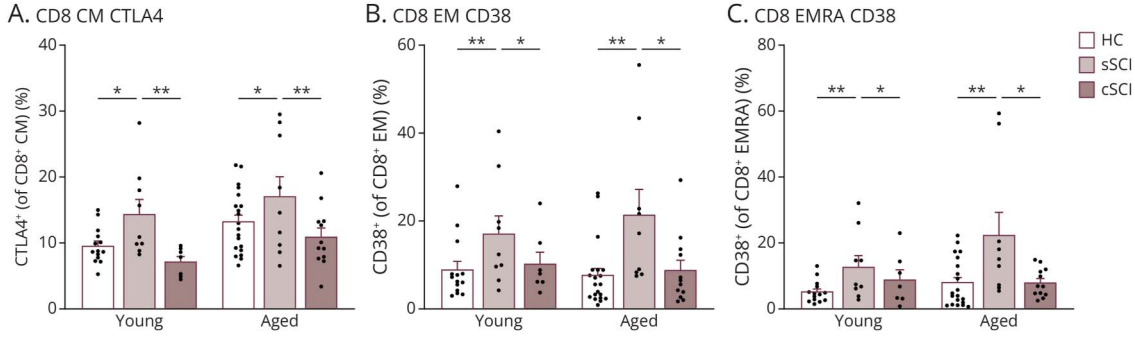
Next, we analyzed B-cell and NK cell populations within the $CD3^-$ fraction using UMAP and FlowSOM clustering. Distinct clustering patterns were observed among HC, sSCI, and cSCI groups, suggesting remodeling of these populations in patients (eFigure 7A). Surface marker expression maps

Figure 3 Subacute Spinal Cord Injury Increases Coexpression of CTLA-4+ $CD38^+$ on $CD4^+$ T Cells



The frequencies of $CTLA\text{-}4^+$ $CD38^+$ coexpressing cells among (A) total $CD4^+$ T cells and (B) $CD4^+$ CD45RA memory T cells. Data are shown as mean ± SEM. Two-way ANOVA with Sidak correction *: p value <0.05, **: p value <0.01. cSCI = chronic spinal cord injury; HC = healthy control; sSCI = subacute spinal cord injury.

Figure 4 Spinal Cord Injury Induces a Comparable Upregulation of CTLA-4 and CD38 on Specific CD8$^+$ T-Cell Subsets During the Subacute Phase, Which Returns to HC Levels in the Chronic Phase in Both Young and Aged Individuals



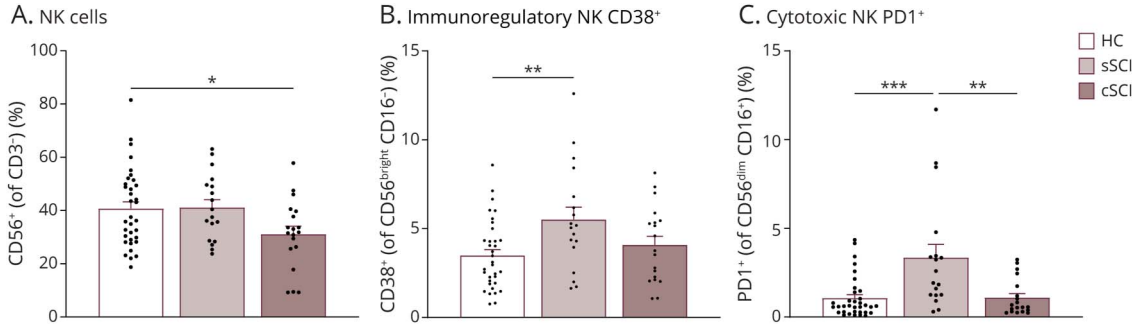
Percentages of the expression of (A) central memory (CM) CD8$^+$ CTLA-4, (B) effector memory (EM) CD8$^+$ CD38, and (C) EMRA CD8$^+$ CD38. Data are shown as mean ± SEM. Two-way ANOVA with Sidak correction *: p value <0.05, **: p value <0.01. cSCI = chronic spinal cord injury; HC = healthy control; sSCI = subacute spinal cord injury.

supported phenotypic divergence, and FlowSOM analysis identified multiple clusters differentially represented between groups (eFigure 7, B and C). While B-cell frequencies did not differ between patients with SCI and controls (eFigure 7D), significant alterations were found in the NK cell subsets (Figure 5). The percentage of NK cells significantly decreased in cSCI but not in sSCI patients compared with HC (p = 0.0439) (Figure 5A). Frequencies of specific subsets of NK cells remained unchanged after SCI (eFigure 7, E and F). However, patients with sSCI had increased frequencies of CD38$^+$ immunoregulatory NK cells, identified CD56^{bright} CD16$^-$ cells (p = 0.0092) (Figure 5B), suggesting increased activation similar to our observations in peripheral CD4$^+$ and CD8$^+$ T cells. By contrast, cytotoxic NK cells, identified CD56^{dim}CD16$^+$ cells, of patients with sSCI had an upregulation of PD1 (p = 0.0002 or p = 0.0012, compared with HC or cSCI, respectively) (Figure 5C). Like CTLA-4, PD-1 is an inhibitory receptor that limits cytotoxicity and cytokine production. These molecules can be upregulated in persistent inflammatory environments, which is the case in the first months after SCI. The frequency of several other markers such as CD57, CD38, KLRG1, and PD1 on CD56$^+$ NK cells is visualized in eFigure 8. Similarly, like the previously shown T-cell frequencies, the NK cell frequencies were stratified by AIS classification (A/B vs C/D) and injury level (above vs below Th4) and revealed no differences. The fact that we detect a return to baseline levels in chronic years after injury suggests that the immune system of patients with SCI is not permanently exhausted.

Discussion

Immunodeficiency syndrome is a major concern after SCI because it increases the risk of life-threatening infections. Despite the crucial role of the immune system in recovery and overall health, the immune response after SCI remains poorly understood. Our study demonstrates immunologic remodeling in the peripheral blood following SCI, particularly within the T-cell and NK-cell compartments. High-dimensional flow cytometry and subset analysis uncovered increased frequencies of memory CD8$^+$ T cells in the subacute phase after

Figure 5 Spinal Cord Injury Enhances CD38 and PD-1 Expression on Distinct NK Cell Subsets



Depicted are the frequencies of (A) CD56$^+$ NK cells, (B) CD38$^+$ immunoregulatory (CD56^{bright}CD16$^-$) NK cells, and (C) PD-1$^+$ cytotoxic (CD56^{dim}CD16$^+$) NK cells. Data are shown as mean ± SEM. One-way ANOVA *: p value <0.05, **: p value <0.01, ***: p value <0.001. cSCI = chronic spinal cord injury; HC = healthy control; sSCI = subacute spinal cord injury.

injury and decreased frequencies of NK cells in chronic patients. Other immune cell subsets such as B cells and $CD4^+$ T cells remained quantitatively unchanged in our study population. Other studies confirm that there are no major shifts in these immune cell subsets, although conflicting results have been reported. For example, increases in $CD3^+$ T cells and $CD4^+$ T cells were found 1-month postinjury.[13] By contrast, reduced frequencies of $CD3^+$ and $CD4^+$ T cells have also been reported.[14] No changes in $CD3^+$ or $CD19^+$ cells were observed around 100–135 dpi.[2] Initial analyses of cSCI (≥1 year) patients showed no change in $CD3^+$ T cells; however, a succeeding larger cohort demonstrated increased levels.[12,29] This indicates that differences across studies likely originate from variations in patient cohorts, time postinjury, injury severity, and analytical approach. Notably, the larger cohort also demonstrated reduced NK cell numbers and cytotoxic function,[12] in line with our findings. Furthermore, transcriptomic data from another study did not detect shifts in lymphocyte frequencies in cSCI (≥13 years), but revealed downregulation of killer inhibitory receptor genes typically expressed by NK cells, suggesting reduced NK cell functioning.[30] Moreover, research further demonstrates that lower NK cell numbers and function increase the risk and severity of viral and bacterial infections.[31,32] Our data demonstrate a decrease in NK cell frequency in chronic patients and an upregulation of PD-1^+ and $CD38^+$ NK cells in subacute patients. These findings, together with those of other studies, suggest that immune protection against infections mediated by cytotoxic NK cells is also affected in patients with SCI. Altogether, our findings support the notion that broad lymphocyte populations remain stable in patients with SCI, but more subtle or delayed alterations, particularly in NK and memory T cells, may arise over time.

Comprehensive immune profiling in our study indicated no evidence of immunosenescence after SCI. CD28null T cells, described to be elevated in patients with cSCI, remained unchanged in our cohort.[15,16] While we detected increases of several immunosenescence markers in aged compared with young individuals, no differences were seen after SCI. For example, we found decreased frequencies of naïve $CD4^+$ and $CD8^+$ T cells in aged individuals, as well as an increase in SA-β-gal activity in aged $CD8^+$ T cells. SCI did not alter the expression of KLRG1 and CD57 on NK and T cells. Telomere length from PBMCs was shorter in older individuals compared with young, but SCI did not elicit telomere shortening. Collectively, these findings underline that SCI does not induce a premature peripheral immunosenescence phenotype in our study cohort.

Beyond the overall changes in the frequencies of memory T cells and NK cells, this study reports that substantially more of these memory T cells and immunoregulatory NK cells expressed CD38 in patients in the subacute phase following SCI. CD38 is commonly upregulated on activated T cells, thus indicating an activation state of the immune system in the subacute phase after injury, which goes back to baseline levels in chronic patients. NK cells are also known to express CD38. By contrast, when $CD56^{bright}CD16^-$ NK cells express CD38, this leads to the production of adenosine, which is known to inhibit CD4 T-cell proliferation.[33] Thus, on $CD56^{bright}CD16^-$ NK cells, CD38 executes an immune regulatory function. As CD38 metabolizes NAD^+, its upregulation on immune cells may contribute to the depletion of NAD^+ seen after SCI.[34] NAD^+ is involved in several key processes, including cellular energy metabolism and DNA repair.[34] This depletion of NAD^+ after SCI is associated with secondary tissue damage, and strategies that replenish NAD^+ levels could potentially demonstrate therapeutic value in preclinical studies.

In addition to higher frequencies of $CD38^+$ T cells and NK cells, we found that patients with SCI in the subacute phase have higher frequencies of memory $CD4^+$ and $CD8^+$ T cells expressing CTLA-4 and cytotoxic NK cells expressing PD-1, suggesting immune exhaustion. Our study demonstrates changes in CTLA-4 expression in the subacute phase after SCI. CTLA-4 is an important immune checkpoint inhibitor. It may prevent activation of naive T cells due to the lack of CD80/CD86 costimulation and actively inhibits effector T cells via enhanced PD-1/PD-L1 signaling.[35] This dual effect of CTLA-4 may contribute to the SCI-IDS phenotype seen in patients with SCI and the increased incidence of infections. CTLA-4 is a target in cancer treatment, where anti-CTLA4 and anti-PD1 antibodies are commonly used to disable immune suppression and stimulate cancer elimination. Our results suggest these treatments may also have therapeutic potential after SCI.

Furthermore, our data indicate that T cells can be double positive for CD38 and CTLA-4. CD38 and exhaustion markers can be coexpressed on the same T cells in settings such as chronic viral infection or cancer, where they may reflect a dysfunctional or inhibitory phenotype.[22,36-40] In addition, coexpression can also be found on regulatory T cells, where CTLA-4 is high, and CD38 may also be elevated.[41-43] In the context of SCI, upregulation of these markers on peripheral T cells in the subacute phase may reflect a persistent T-cell activation and exhaustion during the first months after injury. This immune activation and exhaustion may be driven by dysregulation of the sympathetic nervous system (in higher-level injuries), persistent inflammation, and/or recurring infections.[9,44] Of interest, in chronic patients, these markers are downregulated again, suggesting the immune system returns to homeostasis.

Taken together, our study identifies a role for CD38, CTLA-4, and PD-1 in the T-cell and NK-cell response after SCI. Postinjury infections continue to adversely affect neurologic outcome and increase mortality.[44] Therefore, a better understanding of the mechanisms driving the immune response after SCI is crucial to finding novel avenues for therapeutic interventions.

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Author Contributions

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