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Elevated plasma BDNF levels are correlated with NK cell activation in patients with traumatic spinal cord injury

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ABSTRACT

Background: The precise role of innate immune responses in the early stage of traumatic spinal cord injury (SCI), especially those mediated by natural killer (NK) cells, is poorly understood.
Methods: The frequency and phenotype of NK cells from traumatic SCI patients and healthy controls were assessed by flow cytometry. ELISA assay was used to detect the production of a series of cytokines, neurotrophins, and neurohormones in plasma samples. *In vitro* cell culture was performed to observe brain-derived neurotrophic factor (BDNF)-induced NK cell activation.
Results: A significant increase in the NK cell frequency and the presence of NK cells with the activated phenotype was observed, as reflected by the enhanced expression of CD69, HLA-DR, NKG2D, and NKp30 on the NK cells, in traumatic SCI patients within 24 h of injury, compared to case for the healthy controls. Meanwhile, a higher level of BDNF, a member of the neurotrophin family, was observed in the plasma samples of the SCI patients; the elevated level of BDNF was strongly and positively correlated with the percentage of NK cells during the early stage of traumatic SCI. Furthermore, the expression of CD69 and NKp30 on the NK cells increased following stimulation with BDNF for 24 h *in vitro*, which is consistent with the *in vivo* observation in SCI patients.
Conclusion: Collectively, our findings demonstrate the activation of NK cells within 24 h after traumatic SCI, and reveal a novel role of BDNF in regulating NK cell activation.

1. Introduction

Spinal cord injury (SCI), which usually results from vehicular accidents and accidental falls particularly in elderly people, is a life-altering illness leading to disorders in motor, sensory, and autonomic function. The annual incidence of SCI varies worldwide [1,2]. While the precise immunological events involved in the pathophysiology of SCI remain unclear, it has become increasingly clear that the inflammation caused by immune cells plays an important role in the progression of this disease [3–5].

Previous studies have shown that SCI initiates a potent immune response, partly characterized by the synthesis of cytokines and chemokines, as well as the synergistic infiltration of the injured area with peripheral immune cells including both innate and adaptive immune cells, such as macrophages, natural killer (NK) cells, neutrophils, B cells, and T cells [6,7]. NK cells not only play an important regulatory role by secreting cytokines [7], but also display altered cytotoxic activity in central nervous system (CNS) diseases [8,9]. However, the relationship between NK cells and SCI-induced inflammation has not been thoroughly studied. Particularly, there are few researches on the alteration of the phenotype and activation of NK cells in the early period (< 24 h) of traumatic SCI. Although their vital natural killing

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activity towards certain infected or transformed cells and modulatory functions, especially *via* the release of pro-inflammatory cytokines [6], are well known, how NK cells exert inflammatory functions in traumatic SCI is still unclear.

NK cells can be divided into two major phenotypic and functional subsets based on their CD56 expression levels [10,11]. NK cells from the $CD56^{bright}$ subset are relatively dominant in secondary lymphoid tissues (*e.g.*, tonsils) and have limited cytotoxicity, but produce large amounts of cytokines [12,13]. Whereas, the majority of circulating NK cells belong to the $CD56^{dim}$ subset; they exhibit a notably higher cytolytic capacity against infected or stressed target cells [14,15]. It is well known that NK cells express a multitude of activating and inhibitory receptors that interact with ligands expressed on the surface of target cells; the activation of NK cells is determined by the balance between these receptors [16,17].

In this study, we first characterized the NK cells in patients with traumatic SCI. We analyzed the frequency and phenotype of circulating NK cells and found that traumatic SCI patients showed a significant increase in the frequency of NK cells and the presence of NK cells with the activated phenotype. We also found that the level of BDNF was elevated significantly in the plasma of SCI patients, and that BDNF promotes the activation of NK cells *ex vivo* by upregulating the expression of CD69 and NKp30.

2. Materials and methods

2.1. Patients and samples

Peripheral blood samples were obtained from the inpatients at the First Affiliated Hospital of the Anhui Medical University. This study was approved by the Institutional Review Board for human experimentation, and all participants or their guardians signed informed consent forms before the experiments. All the enrolled patients met the criteria for the diagnosis of traumatic SCI (as defined by the American Spinal Injury Association, Atlanta, GA, USA), which was confirmed by two independent experienced spine surgeons. None of the SCI patients included as the experimental subjects in this study had comorbid neurodegenerative diseases.

In all SCI patients, neurological level of injury was present at or above the seventh cervical vertebra (C7). The distribution of age and sex among the SCI patients, as well as the healthy controls (HCs) is shown in Table 1. All blood samples were collected within 24 h after the SCI. The following patients, without limitations, were excluded: SCI patients with infections, SCI patients taking medicine in the short term or receiving intravenous antibiotic injections, and SCI patients that underwent other invasive operations. In short, patients that underwent any treatment that may possibly interfere with the immune response were excluded.

For comparison, HCs were recruited from the local community in the Hefei area at the same time. The HCs also had a similar socioeconomic status and dietary patterns as the SCI patients. Furthermore, we collected the comprehensive medical history of all the subjects, and they all underwent physical examinations. Then, subjects with blood-transmissible infections, medical abnormalities including CNS disorders, and traumatic or serious medical illnesses were excluded. Additionally, any pathology or treatment that could interfere with leukocyte parameters, such as immunosuppressive or immunomodulatory therapy, or the administration of a vaccine < 3 months ago was an additional exclusion criterion.

2.2. Flow cytometry analysis

Freshly isolated peripheral blood mononuclear cells (PBMCs) were incubated with the indicated fluorescently labeled antibodies (Table 2), and then analyzed on a BD FACSVerse™ Flow Cytometer (BD Biosciences, San Jose, CA, USA) using the FlowJo7.6.1 software (Tree Star, Ashland, OR).

Table 1
Subjects characteristics (age, gender, BMI, SCI injury levels, ASIA impairment Scale, Etiology and Associated injuries).

Characteristics	HC (n = 20)	SCI (n = 21)	p-Value	t-Value
Age (mean ± SEM)	53.45 ± 2.113	55.62 ± 2.153	0.4769	0.7182
Gender			0.275	
Male	11	15		
Female	9	6		
BMI (mean ± SEM)	21.4 ± 0.4387	21.73 ± 0.368	0.562	0.5849
Spinal cord Injury levels				
C2–C4	NA	7		
C5–C7		14		
ASIA impairment scale				
A	0	6		
B	0	5		
C	0	10		
D	0	0		
E	20	0		
Etiology				
Traffic	0	14		
Fall	0	6		
Other	0	1		
Associated injuries	NA	0		

Date shown as mean ± SEM. NA, not applicable.

Table 2
Monoclonal antibodies.

Antibody specificity	Clone	Conjugate	Cat.NO.	Manufacturer
CD3	SK7	APC-cy7	557832	BD Pharmingen™
CD4	RPA-T4	BV 510	300545	Biolegend
CD8	RPA-T8	Percp-cy5.5	560662	BD Pharmingen™
CD19	H1B19	V 500	561125	BD Pharmingen™
CD56	NCAM16.2	BV 421	562751	BD Horizon™
CD69	FN50	PE	310906	Biolegend
CD81	JS-81	FITC	561956	BD Pharmingen™
CD226	DX11	FITC	559788	BD Pharmingen™
CD244	2-69	PE	550816	BD Pharmingen™
HLA-DR	L243	BV 510	307646	Biolegend
NKG2D	1D11	Percp-cy5-5	562364	BD Pharmingen™
NKp30	P30-15	Alexa-647	325212	Biolegend
NKp44	44.189	APC	336942	eBioscience

2.3. Cytokine quantification

Quantification of the cytokine levels in plasma obtained from the HCs and SCI patients was performed using a quantitative sandwich ELISA kit (Dakewe Bio-engineering, Shenzhen, China), according to the manufacturer's recommendations. Plasma levels of the following cytokines were analyzed: IL-6, TNF-α, IFN-γ, IL-12p70, IL-17A, IL-12/23p40, IL-10, and TGF-β1. The detection limit for the measurement of all cytokines was 5 pg/ml; for TGF-β1, it was 15 pg/ml.

2.4. Neurotrophin and neurohormone quantification

Brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), Melatonin, and β-endorphin (β-EP) levels were determined using specific quantitative sandwich ELISA kits (R&D Systems, Minneapolis, MN for BDNF; Abcam, Cambridge, MA for NT-3; and LifeSpan BioSciences, Inc. Seattle, WA for melatonin and β-EP), in accordance with the manufacturer's instructions. The minimum detectable doses of BDNF, NT-3, melatonin, and β-EP are typically < 20, 4, 15.63, and 15.63 pg/ml, respectively.

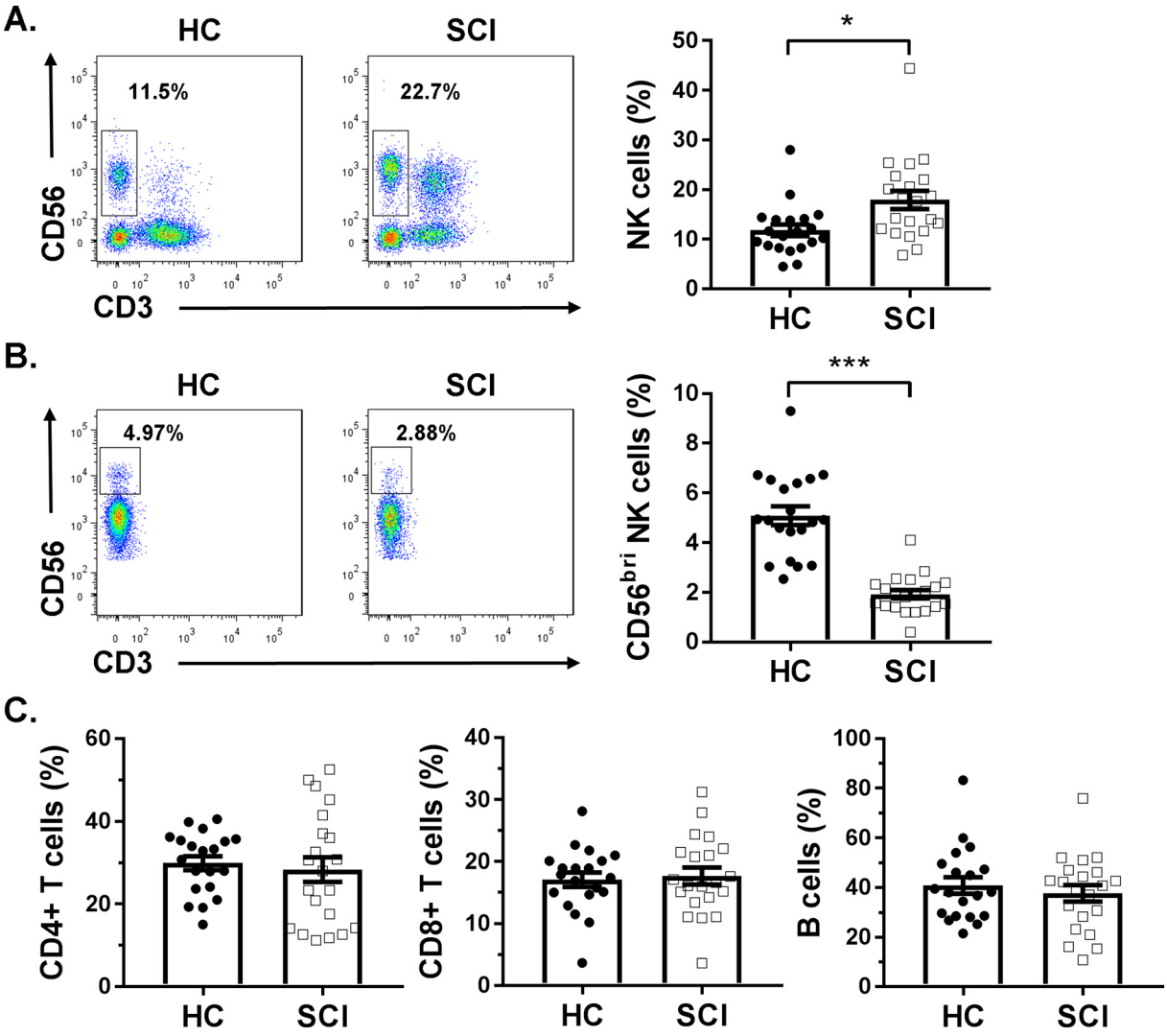

Fig. 1. The NK cell frequency increased notably in the peripheral blood of patients with SCI. The percentage of NK cells identified as the $CD3^{-}CD56^{+}$ population (A), the percentage of NK cells belonging to the $CD56^{bright}$ subset (B), and the percentage of CD4 + T cells, CD8 + T cells, and B cells (C) in the HCs and SCI patients are shown. Results are presented as the mean ± SEM. $*p < 0.05$; $***p < 0.001$.

2.5. BDNF-induced NK cell activation

Human PBMCs were resuspended in RPMI medium (HyClone Laboratories, Inc., Logan, UT, USA) containing 10% FBS (Gibco, Life Technologies, Grand Island, NY, USA), seeded at a density of 1.2×10^6 cells/ml into a 12-well cell culture plate (Trueline, USA), and treated with 15 ng/ml BDNF (PeproTech, Rocky Hill, NJ, USA) in the presence of 50 U/ml rhIL-2 for 24 h at 37 °C under conditions of 5% CO_2. BDNF-induced NK activation was assessed by detecting the expression of CD69, NKp30, and NKG2D.

2.6. Statistical analysis

Data of the experiments were expressed as the mean ± standard error of the mean (SEM) and analyzed using the GraphPad Prism version 7.00 software for Windows (GraphPad Software Inc., San Diego, CA, USA). Statistical analyses of the differences between the data for the SCI patients and HCs were performed using an unpaired two-tailed Student's test. The correlation between the NK cells and the levels of the indicated neurotrophins was explored using the Pearson's correlation test. The gender distribution was analyzed using chi-square tests. *p*-Values < 0.05 were considered statistically significant ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

3. Results

3.1. The frequency of NK cells increased significantly in patients with traumatic SCI

We enumerated the frequency of peripheral blood (PB) NK cells identified as $CD3^{-}CD56^{+}$ cells in 21 patients with traumatic SCI and 20 HCs. Phenotype analysis revealed that the frequency of NK cells in the PB from SCI patients (17.9 ± 1.8%) was significantly higher than that in the PB from the HCs (11.8 ± 1.2%) (Fig. 1A). Based on the CD56 expression, NK cells can be divided into the $CD56^{bright}$ and $CD56^{dim}$ subsets. The percentage of NK cells in the $CD56^{bright}$ subset was significantly lower in patients with SCI (Fig. 1B), indicating that the NK cells in SCI patients mainly belonged to the $CD56^{dim}$ subset. With regards to the other lymphocytes in PB, the proportions of $CD4^{+}$ T cells, $CD8^{+}$ T cells, and B cells between the patients with SCI and the HCs were comparable (Fig. 1C). Furthermore, we counted the total number of PBMCs in 2 ml of PB from the HCs and SCI patients; however, no significant difference between the subjects from these two groups was observed (data not shown).

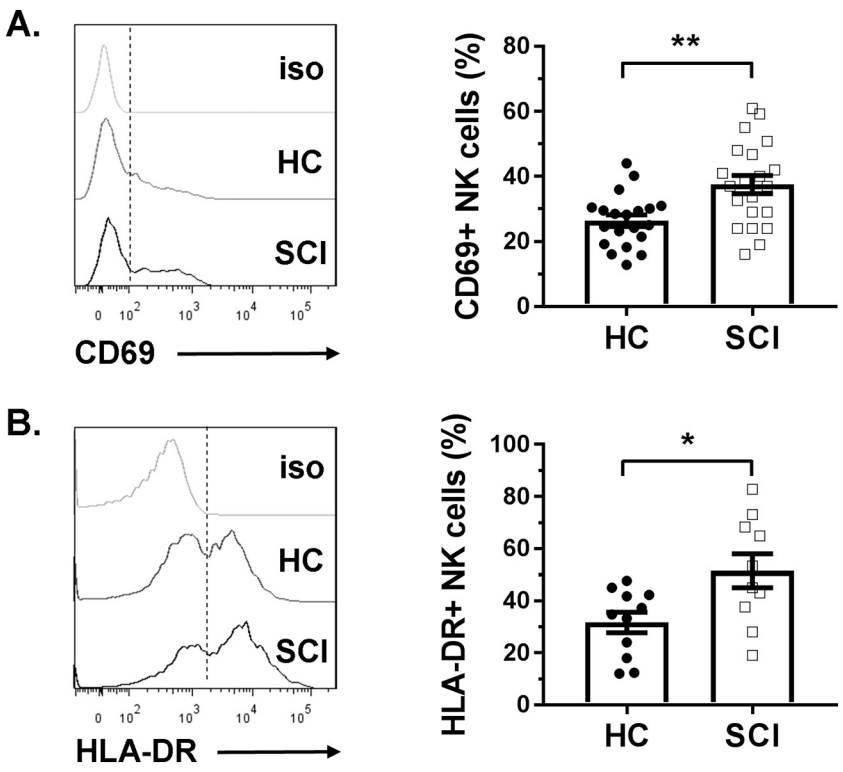

Fig. 2. The expression of the activation molecules CD69 and HLA-DR on NK cells is markedly upregulated in SCI patients. The cells were gated according to the $CD3^{-}CD56^{+}$ NK cell population, and the percentage of NK cells expressing CD69 (A) and HLA-DR (B, $n = 11$ for HCs, 10 for SCI) was determined. Results are presented as the mean ± SEM. $^{**}p < 0.01$.

3.2. NK cells derived from traumatic SCI patients display an activated phenotype

To compare the states of NK cell activation between the HCs and traumatic SCI patients, freshly isolated PBMCs from the HCs and SCI patients were analyzed to assess the surface expression of the activation molecules CD69 and HLA-DR. The proportion of NK cells expressing CD69 was observed to be 26.4 ± 1.8% in case of the HCs, and 37.5 ± 2.8% in case of the SCI patients (Fig. 2A). Similarly, 31.6 ± 3.9% of NK cells expressed HLA-DR in the HCs, compared to the case in the SCI patients (51.5 ± 6.5%) (Fig. 2B). The differences between the mean values of the proportion of NK cells expressing CD69 and HLA-DR in the HCs and SCI patients were highly significant.

Furthermore, the expression of a range of activation receptors expressed on the surface of NK cells, including NKG2D, NKp30, NKp44, CD81, CD226, and CD244, was detected. As shown in Fig. 3A and Fig. 3B, the proportions of NKs expressing both NKp30 and NKG2D were observed to be significantly higher in patients with traumatic SCI (38.4 ± 5.8% and 76.7 ± 3.7%, respectively, in SCI patients *vs* 18.1 ± 3.5% and 62.5 ± 3.0%, respectively, in HCs). Nevertheless, a similar profile for the expression of NKp44, CD81, CD226, and CD244 was observed in subjects from both the groups (Fig. 3C, data not shown). These data indicate that the NK cells in the traumatic SCI patients were in an activated state, compared to those in the HCs.

3.3. Expression of neurotrophins, neurohormones, and cytokines in plasma

To identify the involvement of neurotrophins, neurohormones, and cytokines within 24 h after traumatic SCI, we then measured the plasma levels of these molecules using quantitative ELISA. The level of BDNF increased significantly in the SCI patients, compared to the case in the HCs (Fig. 4A). In addition, the concentrations of NT-3 and β-EP in the HCs and SCI patients also showed a statistically significant difference; however, this difference was only slight (Fig. 4B, C). Comparable amounts of melatonin were detected in plasma samples from the subjects in both the groups (Supplementary Fig. 1A).

The potential association between the plasma levels of neurotrophins and the frequency of NK cells was assessed using Pearson's correlation test. A strong and positive correlation was found between the BDNF or β-EP levels and NK cell frequency in SCI patients (Fig. 5A, B). However, the plasma levels of NT-3 and melatonin failed to show a statistically significant correlation with the NK cell frequency (Fig. 5C and data not shown).

Assessment of cytokine levels in the plasma samples revealed that the levels of both pro-inflammatory cytokines (IL-6, and TNF-α) and Th1 cytokines (IL-12 and IFN-γ) in the plasma samples obtained from SCI patients were highly similar to those in the plasma samples obtained from the HCs (Supplementary Fig. 1B–E). However, the plasma levels of IL-17A and IL-12/23p40 decreased significantly in the traumatic SCI patients (Fig. 6A, B), indicating that the Th17 cell-mediated immune response may occur within 24 h after the SCI. Otherwise, the plasma levels of the anti-inflammatory cytokine TGF-β1 showed a dramatic increase in the traumatic SCI patients (Fig. 6C); however, the plasma levels of another anti-inflammatory cytokine, IL-10, were similar between the samples obtained from both the SCI patients and HCs (Supplementary Fig. 1F).

3.4. BDNF promotes NK cell activation in vitro

Since the expression of BDNF was the strongest among the indicated neurotrophins and neurohormones (Fig. 4A), and the highest positive correlation was observed between the plasma levels of BDNF and the frequency of NK cells in SCI patients (Fig. 5A), the effect of BDNF on NK cell activation was examined. PBMCs from the HCs were treated with BDNF *in vitro* for 24 h. Then, the expression of CD69, NKG2D, and NKp30 on NK cells was detected using flow cytometry.

As shown in Fig. 7, the expression of CD69 and NKp30 on NK cells increased significantly upon BDNF stimulation (Fig. 7A, B). However, the expression levels of NKG2D on NK cells were similarly high in samples obtained from the subjects in both the groups (data not shown). Collectively, these results indicate that BDNF alone promoted the NK cell activation.

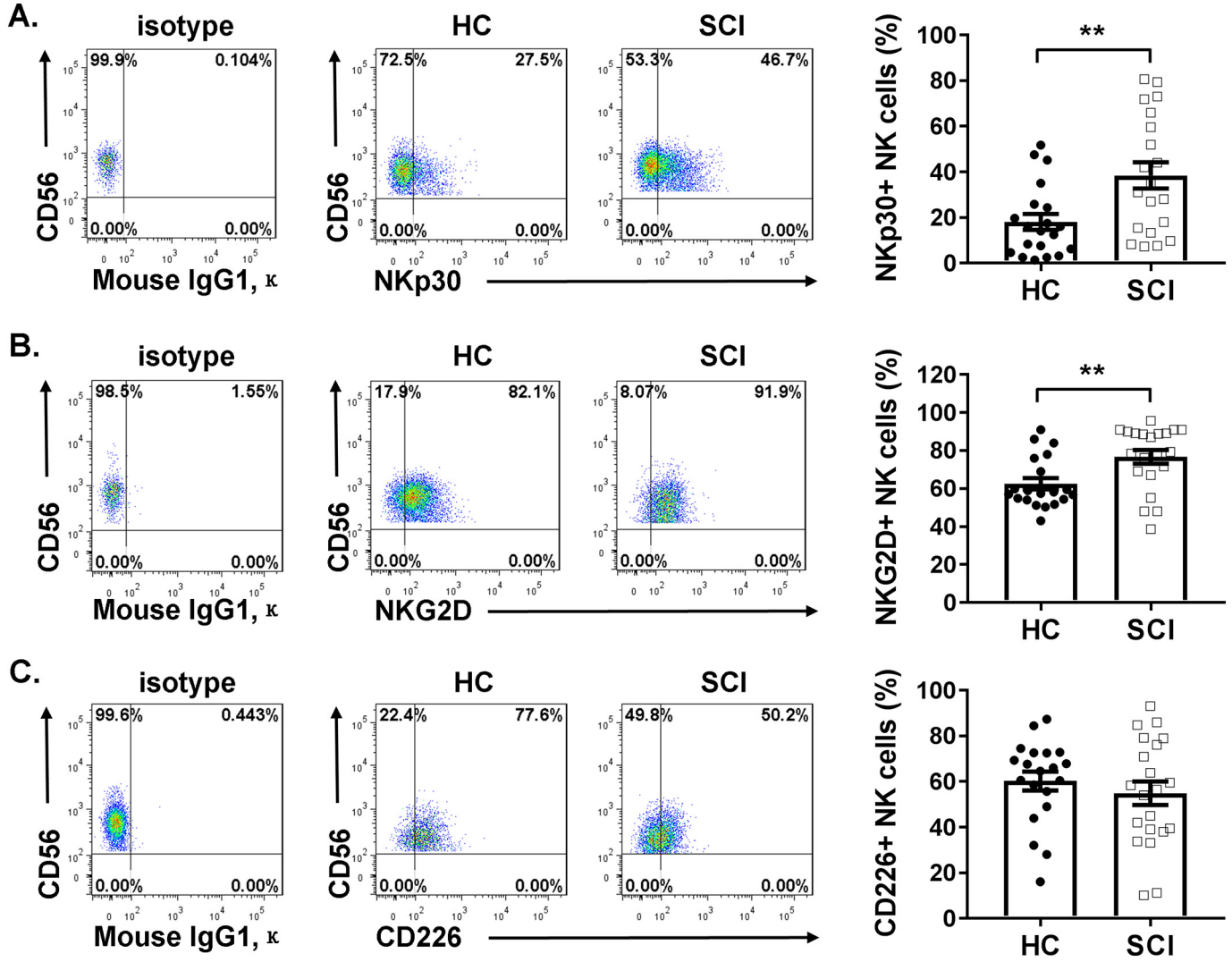

Fig. 3. Phenotypic characterization of NK cells from HCs and SCI patients. PBMCs from HCs and SCI patients were stained for the expression of CD3, CD56, and either NKG2D (A), NKp30 (B), CD226 (C), CD81, CD244, or NKp44 (data not shown). The percentage of $CD3^{-}CD56^{+}$ NK cells that expressed each marker is shown. Results are presented as the mean ± SEM. $^{**}p < 0.01$.

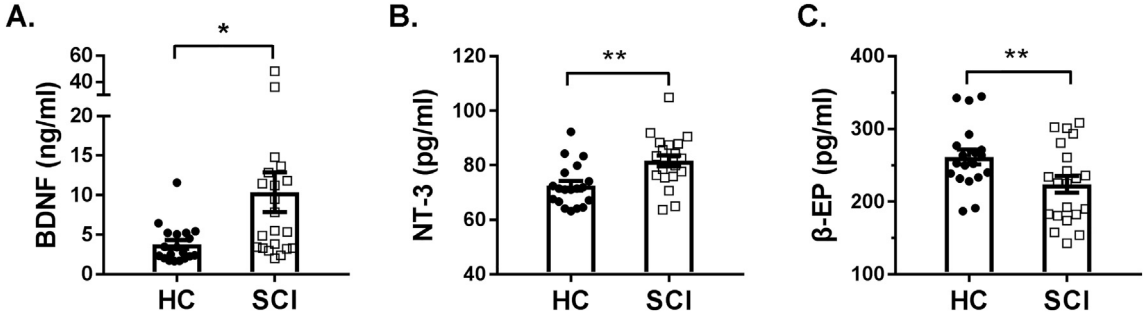

Fig. 4. Study of neurotrophins and neurohormones in plasma from HCs and SCI patients in the early stage of the injury. Quantification of the levels of the following neurotrophins and neurohormones was performed using ELISA: BDNF (A), neurotrophin-3 (NT-3, B), and β-Endorphin (β-EP, C).
Results are presented as the mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$.

4. Discussion

At least three notable highlights have emerged from our analyses of the PB obtained from patients with traumatic SCI. First, we observed an increased frequency and activated phenotype of NK cells from the traumatic SCI patients, which requires all the blood samples from the patients be collected within 24 h after SCI; this confirms that an innate immune response occurred in the early stage of SCI in these patients. Second, pro-inflammatory and Th1 cytokines are unlikely to act as critical messengers to regulate the immune response during traumatic SCI, since their plasma levels in the HCs and SCI patients were comparable. On the contrary, the production of IL-17A and IL-12/23p40 was reduced during the early period of SCI (Fig. 6). Finally, and most importantly, to the best of our knowledge, this is the first study to

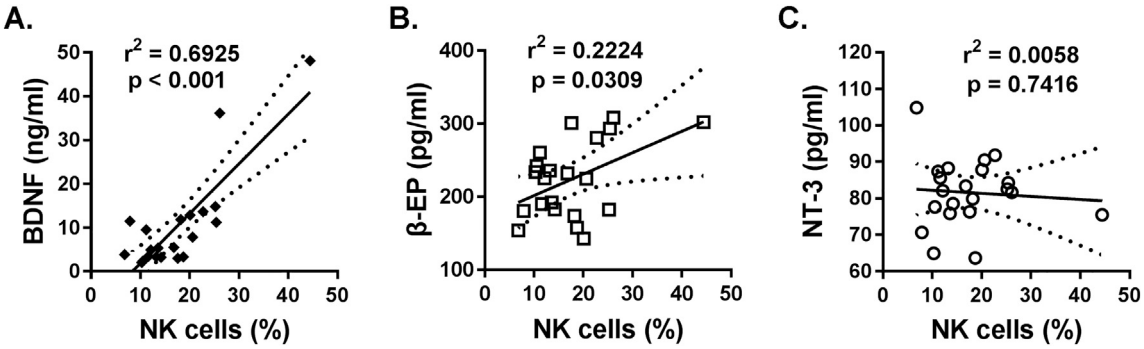

Fig. 5. Correlation between the plasma concentration of the indicated neurotrophins and the percentage of NK cells in SCI patients. A significant positive correlation was observed between the BDNF levels (A), as well as the β-EP levels (B), and the percentage of NK cells. Plasma concentration of NT-3 (C) failed to show a statistically significant correlation with the percentage of NK cells in SCI patients.

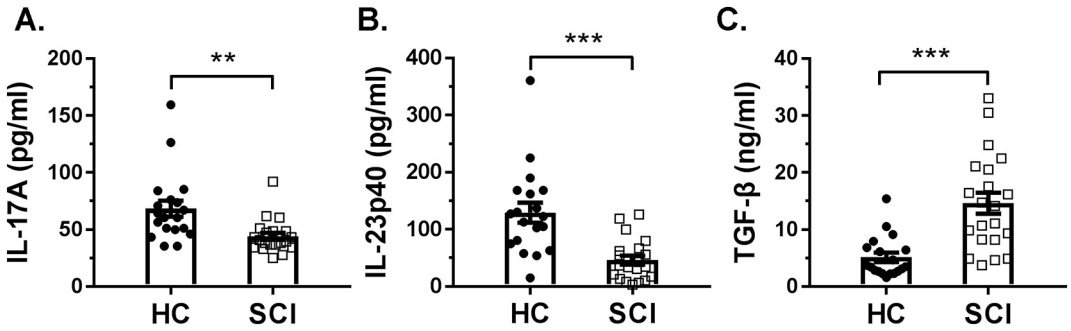

Fig. 6. Study of cytokines in plasma samples from HCs and SCI patients in the early stage of the injury. The plasma levels of IL-17A (A), IL-12/23p40 (B), and TGF-β1 (C) were detected using quantitative ELISA. Results are presented as the mean ± SEM. $^{**}p < 0.01$; $^{***}p < 0.001$.

investigate the association between increased BDNF levels and the elevation of NK cell frequency and activation (Figs. 1, 4, 5, and 7). The current results point to the probability that BDNF promotes the activation of NK cells *via* a potential receptor on these cells.

Accumulating evidences have revealed that the physiopathologic mechanism of SCI involves a series of complicated immune response and a variety of immune cells [18–22]. It has been reported that in SCI, an innate immune inflammatory response is triggered by damage-associated molecular patterns released from dead neural cells at the injury site [23]. However, despite the increase in inflammatory processes, SCI patients have generally been reported to show a state of immune suppression, followed by a susceptibility to infections [24,25]. A significant reduction of both the numbers and cytotoxic activity of NK cells was found in SCI patients > 3 months after they sustained the injury [26]. Another study has reported that deficits in NK cell function appeared at 2 weeks post SCI, and then, a nadir occurred at 2 months post SCI, followed by a deficiency of NK cell functions that lasted for more than a year [27,28]. On the other hand, we observed a more activated phenotype of NK cells in the PB obtained from patients < 24 h after they sustained an SCI. These conflicting observations may be mainly due to the differences in the time points of blood sample collection and diverse lesion levels after SCI in the respective studies, which is supported by the dynamics of the inflammatory responses observed in a mouse SCI model [29].

The key mediators in the complex cross-talk between the nervous and immune systems are neurotrophins or neurohormones, which regulate both the functions of neuronal cells and immune cell activity. The results shown in Fig. 4 indicate that the level of BDNF, among these indicated factors, demonstrated a robust increase after traumatic SCI. Furthermore, the level of BDNF was strongly and positively correlated with the NK cell frequency in traumatic SCI patients (Fig. 5A). Many studies have focused on the relationship between neurotrophins and immune cells, such as macrophages, monocytes, neutrophils, and adaptive T or B cells [30–34]; however, the relationship between BDNF and NK cells is poorly understood.

In a glioma-bearing mouse model, the levels of IL-15, a cytokine that favors NK cell accumulation, and the production of BDNF, which reduces the invasion of glioma cells into the cerebral parenchyma, increased in the brain tissues of mice housed in an enriched environment. The cooperation between the elevated BDNF levels and NK cell accumulation resulted in the reduction of glioma size and significantly prolonged the survival of the mice [35]. Although this report has demonstrated two independent mechanisms underlying the inhibition of the glioma growth, the potential direct link between BDNF production and NK cell activation was not explored. In our study, we found a significant increase in the expression of CD69 and NKp30 on NK cells after stimulation with 15 ng/ml BDNF *in vitro* for 24 h. The incubation time and concentration of BDNF were consistent with the *in vivo* conditions. These data support the existence of a direct link between BDNF and NK cells.

Along with the enrichment of the plasma BDNF level within 24 h after traumatic SCI (Fig. 4A), the levels of TGF-β1 also increased notably and showed a significant correlation with the BDNF levels (Fig. 6C and data not shown), which is consistent with the findings obtained from multiple sclerosis patients treated with testosterone [36]. It is well known that TGF-β1 is an inhibitory cytokine and plays a crucial role in immune suppression [37–39]. The increased production of TGF-β1 accounts for the induction of immunosuppressive responses after traumatic SCI, which might contribute to depressed immune function, as described in previous studies [27,28].

It is well-known that IL-17A is considered as a pro-inflammatory cytokine and can be produced by various cell types, such as Th17 cells, NK cells, NKT cells, and γδ-T cells. Also, the IL-23/IL-17 axis may play an important role in the pathogenesis of autoimmune diseases [40]. There are few reports showed that the expression levels of IL-17 and IL-23 were significantly increased and peaked at 24 h after SCI in rat

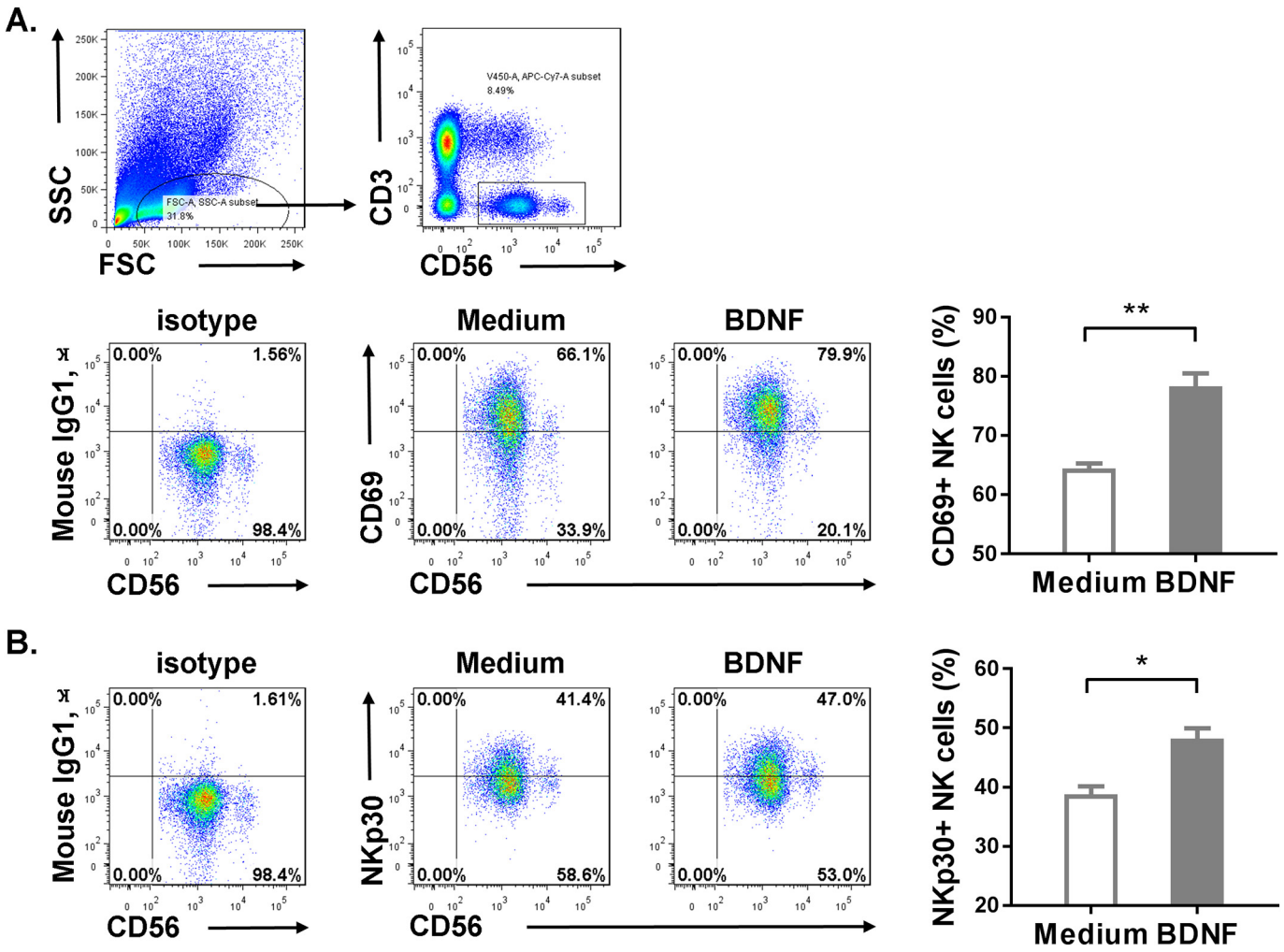

Fig. 7. Upregulation of the expression of NK cell activation receptors after treatment with BDNF for 24 h. PBMCs from the subjects were treated *in vitro* with 15 ng/ml BDNF in the presence of 50 U/mL rhIL-2 for 24 h or left untreated. The effect of this treatment on CD69 (A) and NKp30 expression (B) on the NK cells was assessed by flow cytometry analysis. Results are presented as the mean ± SEM, and represent values from at least 2 independent experiments. $*p < 0.05$; $**p < 0.01$.

model, which is likely consistent with their inflammatory role in immune response [41]. However, the obviously reduced expression levels of IL-17/23 were observed from patients within 24 h after traumatic SCI in our study, accompanied by a significant increase in anti-inflammatory cytokine TGF-β1. Moreover, we also showed that frequency of NK cells was increased significantly within 24 h after SCI. Although NK cells could suppress the production of IL-17 by Th17 cells *via* releasing the IFN-γ [42], it is unclear whether the reduced production of IL-17/23 was influenced by NK cells, or was regulated by other potential factors which might be induced after SCI.

In conclusion, our results show that the frequency of NK cells and the level of BDNF in the PB increased significantly in traumatic SCI patients within 24 h of the injury, compared to that in the HCs. The elevated level of BDNF was positively correlated with the frequency of NK cells in SCI patients. Furthermore, the NK cells displayed an activated phenotype, reflected by the enhanced expression of CD69 and NKp30 on NK cells after SCI *in vivo* or after stimulation with BDNF for 24 h *in vitro*. These findings suggest that NK cells and their activation participate in the pathophysiological processes of SCI. However, the cell types that are responsible for the elevated levels of BDNF and TGF-β1, and the mechanisms whereby BDNF and TGF-β1 function together in the regulation of immune responses need to be further investigated.

Abbreviations

SCI	Spinal cord injury
HC	Healthy control
NK cells	Natural killer cells
CNS	central nervous system
BDNF	Brain-derived neurotrophic factor
ELISA	enzyme-linked immunosorbent assay
IL	interleukin
IFN	interferon
TNF	tumor necrosis factor
TGF	transforming growth factor
NT-3	neurotrophin-3
PBS	phosphate-buffered saline
PBMCs	peripheral blood mononuclear cells
NCRs	natural cytotoxicity receptors
FITC	fluorescein isothiocyanate
PE	phycoerythrin
PerCP	peri-dinin-chlorophyll-protein complex
APC	allophycocyanin
FBS	fetal bovine serum

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105722.

Ethical approval and consent to participate

This study was approved by the Institutional Review Board for human experimentation of the Anhui Medical University and all participants or their guardians signed the informed consent before the experiments.

Consent for publication

Not applicable.

Availability of data and materials

The data and materials used and analyzed during the current work are available on reasonable request. Please contact the author for date requests.

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Authors' contributions

L.X. and Y.Z. designed the study, performed the analyses, and wrote the manuscript. R.Z., P.S., H.Z., T.M., Y.L., and X.W. analyzed the data. X.H., Q.L., and X.G. formulated the discussion of this manuscript. J.X. and C.S. supervised the analyses and wrote the manuscript. All the authors have read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgments

Not applicable.

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