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·Original Article·

Alterations of natural killer cells in traumatic brain injury

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ABSTRACT

To investigate the relationship between natural killer (NK) cells and traumatic brain injury (TBI), we tracked an established phenotype of circulating NK cells at several time points in patients with different grades of TBI. In serial peripheral blood samples, NK cells were prospectively measured by flow cytometry of $CD3^-$ $CD56^+$ lymphocytes. Compared to healthy controls, TBI patients had reductions in both the percentage and the absolute number of NK cells. Furthermore, the magnitude of NK cell reduction correlated with the degree of TBI severity at several time points. That is, NK cell population size was independently associated with lower Glasgow Coma Scale scores. In addition, at some time points, a positive correlation was found between the NK cell counts and Glasgow Outcome Scale scores. Our results indicate that TBI induces a reduction in the number of NK cells, and the magnitude of the reduction appears to parallel the severity of TBI.

Keywords: natural killer cells; traumatic brain injury; central nervous system; immunity

INTRODUCTION

Traumatic brain injury (TBI), a major cause of neurological disability and death, is a public health problem and an enormous financial burden world-wide[1-5]. In addition to the adverse effects on the central nervous system (CNS), unfavorable outcomes of TBI often stem from extracranial complications. That is, injury-induced damage to the CNS can suppress the immune system and result, most commonly, in fever, pneumonia, and urinary tract infections[6, 7]. This immune-deficiency syndrome increases these patients' susceptibility to infections, although the underlying mechanism has not been identified[8,9].

Natural killer (NK) cells are the first line of defense to combat infections, and they are one of the earliest cell types to arrive at target organs of inflammation. NK cells can affect the initiation of autoimmunity and regulate inflammation[10-12]. Under certain pathological circumstances, NK cells can home readily to the CNS[10]. Therefore, the functions of NK cells have become a growing focus of interest, particularly with regard to their roles as regulators of autoimmunity and inducers of inflammatory responses in animal models and in humans[13]. However, the extent to which NK cells modulate inflammatory responses in specific organs has not been characterized. Whether NK cells are simply passive migrants or active participants in the pathogenesis of CNS injury is a complex and sometimes even paradoxical issue[11, 14].

Previous studies have revealed an early decrease of NK-cell function in patients with septic or severe TBI[15, 16]. However, those assessments were limited to the evaluation of dynamic changes of NK cells in blood samples collected during the early course of intensive treatment (within 72 h or 7 days), or included only severely-injured patients[16, 17]. Here, we investigated the quantitative changes of

circulating NK cells in patients with TBI varying from mild and moderate to severe. In addition, we explored the relationship between NK cell numbers and neurological outcomes. Finally, as we attempted the next step toward understanding the pathogenesis of immunodepression after TBI, we envisioned providing a foundation for therapeutic intervention in CNS injury.

MATERIALS AND METHODS

Patients and Controls

During the experimental period from March 2012 to April 2013, 30 patients who were treated consecutively in the Neurotrauma Intensive Care Unit at the Department of Neurosurgery of the General Hospital, Tianjin Medical University, China were enrolled. Patients (between 18 and 60 years) having nonpenetrating TBI were screened for eligibility according to the inclusion criteria on admission within 6 h after TBI. We randomly assigned 10 adults in each group with different grades of TBI. All patients presented with closed head injury and scored from 5 to 15 on the Glasgow Coma Scale (GCS)[18]. TBI was graded as mild, moderate, or severe on the basis of GCS score on admission. Mild TBI with a GCS score of 13–15 is considered a concussion, and full neurological recovery is likely in most cases. A patient with moderate TBI (GCS 9–12) is lethargic or stuporous, whereas severe TBI signifies a GCS ranging from 3 to 8 and a patient who is comatose[1]. According to the exclusion criteria and GCS assessments, the enrolled patients were divided into mild (n = 10; age range 18–54 years), moderate (n = 10; 21–56 years), and severe TBI groups (n = 10; 20–59 years). Unmatched healthy controls consisted of 20 volunteers ranging in an age from 18 to 50 years. The patients were provided with standard supportive care to meet all ethical standards and avoid technical bias. The data from each group were summarized according to baseline characteristics, including medical history, blood screening, and evaluation of peripheral NK cells. Samples of peripheral blood from moderate and severe TBI patients were examined on days 1, 3, 7, 14, and 21, and those from mild TBI patients on days 1, 3, 7, and 14 after TBI diagnosis, and evaluated for immunological factors.

Exclusion criteria were malignant hypertension, diabetes mellitus, severe coronary artery disease, myocardial infarction, heart failure, renal disease, and other diseases that may severely affect immune function. Other exclusion criteria were an undetermined time of injury, spinal cord injury, recipients of blood transfusion or steroid treatment, massive abdominal and/or chest injuries requiring surgery, death during hospitalization, inability to perform follow-up, or any immunodeficiency on admission.

All enrolled patients or their caregivers provided written informed consent before enrollment in the study. The inclusion criteria for each patient were approved, as designated in a standardized trial protocol designed by the Ethics Committee of Tianjin Neurological Institute. As recommended by the *Guidelines for the Management of Severe Traumatic Brain Injury* (3rd edition)[19], all patients received standard care from the time of enrollment onward. To avoid the confounding effects of aging and related diseases, patients older than 60 years of age were excluded. No patient used any drug affecting hypothalamic-pituitary function, and none was treated with steroids, as suggested in the *Guidelines for Management* (XV)[19].

Classification of TBI

Structural damage was rated by the Marshall Classification on computed tomography (CT) scans[20]. Class I indicates no visible pathology; class II, cisterns present, with a midline shift of ≤5 mm and no lesions >25 mL; class III, cisterns compressed or absent, with a midline shift of ≤5 mm and no lesions >25 mL; class IV, a midline shift of >5 mm; and class V, a lesion requiring surgical evacuation[4, 21, 22].

All patients with closed head injuries who had a GCS score of 3 to 15 were admitted to our trauma center, and the locations of brain injury were recorded at that time.

NK Cell Isolation and Analysis

To quantify NK cells in the circulation, peripheral blood mononuclear cells (PBMCs) were separated from fresh blood samples as described[14, 23]. Then 2 mL of PBMCs was subjected to density gradient centrifugation at 300 g for 20 min at room temperature. The NK cells thus purified were washed three times with phosphate-buffered saline (PBS; pH 7.2) and re-suspended in 200 μL PBS supplemented with 0.5% bovine serum albumin. The samples were then labeled with R-phycoerythrin-conjugated monoclonal CD3 antibody and fluorescein isothiocyanate-conjugated CD56 antibody for 20 min at room temperature. Both of the anti-

human monoclonal antibodies were from BD Biosciences PharMingen (San Diego, CA). Human NK cells were gated as $CD3^-CD56^+$ lymphocytes for further analysis[15, 24]. A FACSCalibur cytometer (Becton Dickinson, Mountain View, CA) was used, and the data were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR).

Assessment of Neurological Outcome

Primary neurological outcomes were assessed with the Glasgow Outcome Scale (GOS)[25]. GOS scores are: 1, death; 2, vegetative state; 3, severe disability (unable to live independently); 4, moderate disability (capable of living independently); and 5, mild or no disability[21]. Outcome measures were assessed at 3 and 6 months after injury by a specialist trained in physical medicine and rehabilitation who was unaware of group assignments. No patients were lost to follow-up.

Serum Cortisol Assay

Hormone assays were performed at increasing intervals after the diagnosis of TBI in the groups scored as moderately and severely disabled. The serum cortisol levels were measured using an enzymatic immunoassay under a protocol approved by the Tianjin Medical University General Hospital Clinical Laboratory. Normal morning serum cortisol concentrations are 5 to 21 μg/dl[26].

Statistical Analysis

The statistical package SPSS 17.0 for Windows (SPSS Inc., Chicago, IL) was used for analysis. Normally-distributed continuous variables are presented as mean ± SEM. Continuous variables with skewed distributions are presented as the median (range). The categorical data are presented as counts and percentages. We applied the Fisher's exact test for categorical variables, the Kruskal-Wallis test for non-parametric data, and the Mann-Whitney U test (Z) for two-group comparisons as appropriate. Correlations between NK cells and GCS/GOS were evaluated using Spearman correlation coefficients (r). $P \leq 0.05$ was considered to be statistically significant.

RESULTS

Clinical Characteristics of Patients with TBI

The clinical baseline features of patients with TBI are summarized in Table 1. Among the mild, moderate, and severe TBI groups, no significant difference was found in the initial data for age, gender, medical history, and smoking or drinking history. Most patients were male, and a motor-vehicle accident was the most common cause of injury. However, these groups had significant differences in leucocyte counts and body temperatures on admission. In the group with mild TBI, the initial GCS score was 13 to 15. The group with moderate TBI had an initial GCS score from 9 to 12, and the initial GCS score of those with severe TBI was 5 to 8. The average length of stay in the trauma center for each group was at least 21 days. No patients were lost to follow-up.

A neuroradiologist reviewed all CT scans, and classification was based on the initial CT findings (Table 2). Our study excluded the recording of penetrating head injuries and the severity of extracranial injuries. Mild TBI could be isolated, but complex traumatic intracranial lesions occurred frequently in the groups with moderate and severe TBI (Table 2)[4]. All individuals with normal findings on CT scans (class I) and patients who required surgery (class V) were excluded on admission.

Changes of Peripheral NK Cells after TBI

The percentages of $CD3^-CD56^+$ NK cells (% of total lymphocytes) first decreased and then increased after injury compared with the levels in healthy controls (Fig. 1A–C). A similar pattern was found for the absolute numbers of NK cells (Fig. 1a–c). Marked decreases in the percentages and absolute numbers of NK cells were found in patients with mild TBI on day 1 ($Z = -2.002$, $P = 0.045$ and $Z = -4.026$, $P < 0.001$) and day 3 ($Z = -2.772$, $P = 0.006$ and $Z = -3.915$, $P < 0.001$). Within 7 days of mild TBI, both values returned to the normal level ($Z = -1.144$, $P = 0.253$ and $Z = -0.440$, $P = 0.660$) (Fig. 1A and 1a). In the moderate TBI group, the percentages and absolute numbers of NK cells were lower than those in controls on day 7 after injury ($Z = -3.740$, $P = 0.000$ and $Z = -4.334$, $P < 0.001$). The percentage of NK cells remained below the normal level on post-injury day 21 ($Z = -1.980$, $P = 0.048$) (Fig. 1B), but the absolute numbers of these cells normalized at this time point ($Z = -1.078$, $P = 0.281$) (Fig. 1b). In patients with severe TBI, the lowest percentages and absolute numbers of $CD3^-CD56^+$ NK cells were found on days 3 and 7 in comparison with other days and controls ($Z = -3.608$, $P < 0.001$ and $Z = -4.399$, $P < 0.001$ on day 3; $Z = -3.872$, $P < 0.001$ and $Z = -4.399$, $P < 0.001$ on day 7) (Fig. 1C and 1c). In addition, the percentages and

Table 1. Demographic and clinical characteristics of patients with mild, moderate, or severe TBI, and the control group

Characteristics	Mild TBI	Moderate TBI	Severe TBI	Control	Statistical analysis (χ^2)	*P* Values
Patients (*n*)	10	10	10	20	-	-
Age (years)	28.00 (19.50–38.75)	29.00 (23.50–48.75)	28.50 (21.00–49.25)	30.00 (24.25–43.00)	0.808	0.847
Male (*n* (%))	8 (80%)	8 (80%)	7 (70%)	14 (70%)	0.624	0.891
Medical history (*n* (%))						
Cardiovascular disease	0 (%)	0 (%)	1 (10%)	0 (%)	4.082	0.253
Diabetes mellitus	1 (10%)	1 (10%)	0 (0%)	1 (0.5%)	1.241	0.743
Hypertension	1 (10%)	0 (%)	0 (%)	1 (0.5%)	1.823	0.610
Current smoking	4 (40%)	2 (20%)	2 (20%)	3 (15%)	2.506	0.474
Current drinking	2 (20%)	1 (10%)	3 (30%)	3 (15%)	1.558	0.669
Cause of injury						
Motor vehicle accident	7 (70%)	8 (80%)	8 (80%)	-	0.373	0.830
Fall	3 (30%)	2 (20%)	1 (10%)	-	1.250	0.535
Assault	0 (%)	0 (%)	1 (10%)	-	2.069	0.355
GCS on admission	15.00 (14.00–15.00)	10.50 (9.75–11.25)	7.00 (5.75–8.00)	-	-	-
Leucocytes on admission	12.30 (9.65–13.86)	14.45 (11.77–15.91)	16.05 (9.45–21.16)	5.75 (5.40–6.65)	29.670	<0.001
Temperature on admission	36.95 (36.63–37.30)	37.5 (37.13–37.93)	37.70 (37.58–38.05)	36.30 (36.20–36.38)	35.978	<0.001

Data are expressed as median (25th–75th percentile). The categorical data are presented as counts and percentages. Fisher's exact test and the Kruskal-Wallis test were used for comparison. *P* values are for comparisons of all four outcomes in the TBI and control groups.

Table 2. Baseline characteristics of patients with TBI

Characteristics	Mild TBI	Moderate TBI	Severe TBI
Patients (*n*)	10	10	10
CT classification			
I	0	0	0
II	10	5	2
III	0	3	4
IV	0	2	4
V	0	0	0
Focal injuries			
Epidural hematoma	3	2	1
Subdural hematoma	2	2	2
Contusion	3	4	3
Intracerebral hematoma	2	4	5
Diffuse axonal injury	0	0	3

absolute numbers of $CD3^-CD56^+$ NK cells among PBMCs did not return to normal by follow-up day 21 (Z = -2.860, P = 0.004 and Z = -3.959, P <0.001) (Figure 1C and 1c).

Neurological Outcome

The neurological outcome (unfavorable functional status and survival) for each participant was assessed at 3 and 6 months after TBI diagnosis using the GOS (Table 3). At 3 months, 9 (90%) of the mild TBI patients had a GOS score of 4 or 5, in contrast to the 7 (70%) in the moderate TBI group and 6 (60%) with severe TBI. At 6 months, all of the mild TBI patients had a GOS score of 4 or 5, whereas those in the moderate and severe TBI groups retained their 3-month scores (Table 3).

Correlation between NK and GCS

Bivariate correlation analysis showed that lower percentages and absolute numbers of $CD3^-CD56^+$ NK cells both aligned with a low GCS score on days 7, 14, and 21 but not at any other time point (Fig. 2).

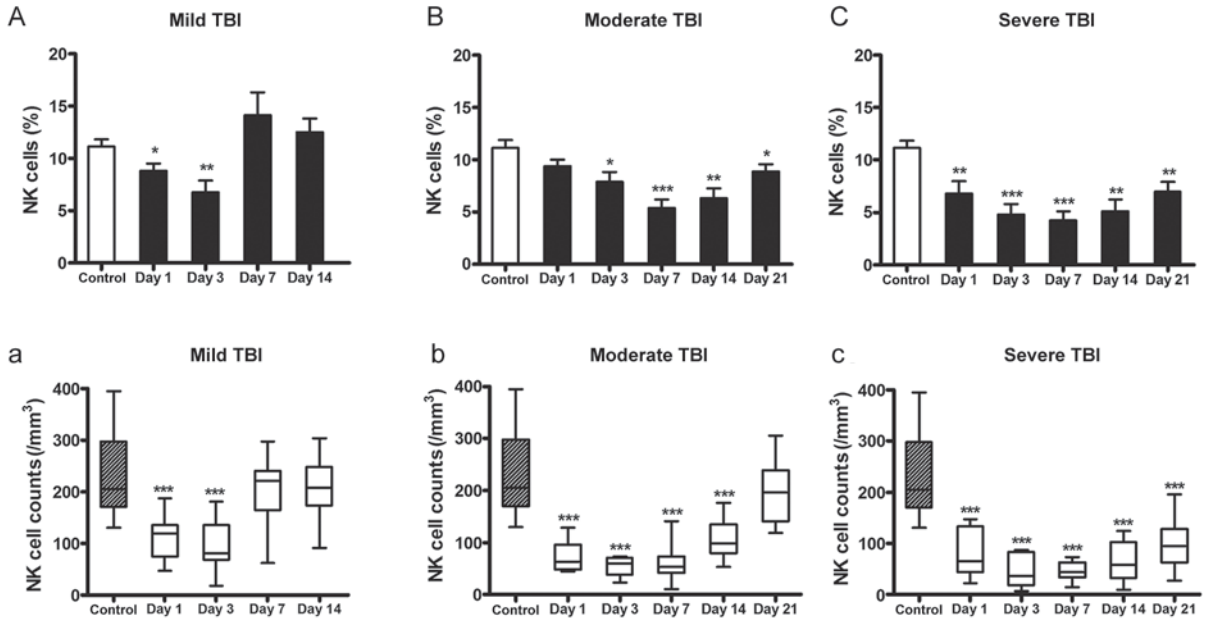

Fig. 1. Percentages and absolute numbers of NK cells among PBMCs of patients with traumatic brain injury (TBI) and healthy volunteers (control) analyzed by flow cytometry. (A–C) Percentages of $CD3^-CD56^+$ NK cells at different time points in TBI (*n* = 10 for each group) and control (*n* = 20) groups. Mean and SEM (error bars). The percentage of $CD3^-CD56^+$ NK cells in healthy volunteers (blank bars) was 11.00% (8.41–14.00). (a–c) Absolute numbers of $CD3^-CD56^+$ NK cells among PBMCs at different time points in TBI groups and controls. Data are expressed as the median (25th–75th percentile). The absolute number of $CD3^-CD56^+$ NK cells in healthy volunteers (cross-hatched boxes) was 205/mm^3 (170–298). ****P* <0.001, ***P* <0.01, **P* <0.05 compared with control, Mann-Whitney U test. *P* values express differences between values in the TBI groups and control group at each time point.

Table 3. Effect of TBI on Glasgow Outcome Scores at 3 and 6 months

Glasgow Outcome Scores	At 3 months			At 6 months		
	Mild TBI	Moderate TBI	Severe TBI	Mild TBI	Moderate TBI	Severe TBI
1 (Death)	-	-	-	-	-	-
2 (Vegetative state)	-	-	1	-	-	1
3 (Severe disability)	1	3	3		3	3
4 (Moderate disability)	-	2	6	1	2	6
5 (Mild or no disability)	9	5	-	9	5	-
Total	10	10	10	10	10	10

Correlation between NK and GOS

In PBMC samples collected on days 1, 7, and 14 post-TBI, the percentages as well as the absolute numbers of NK cells were both associated with low GOS scores at 3 months after TBI (Fig. 3A, B). At 6 months after injury, the lower percentages of NK cells concurred with lower GOS scores on days 7 and 14 (Fig. 3C). Clearly, the absolute numbers of NK cells correlated well with the GOS on days 1, 7, and 14 after the injury (Fig. 3D).

Changes of Serum Cortisol

The serum cortisol concentrations did not fall below the 95% confidence limit in any patient following moderate or

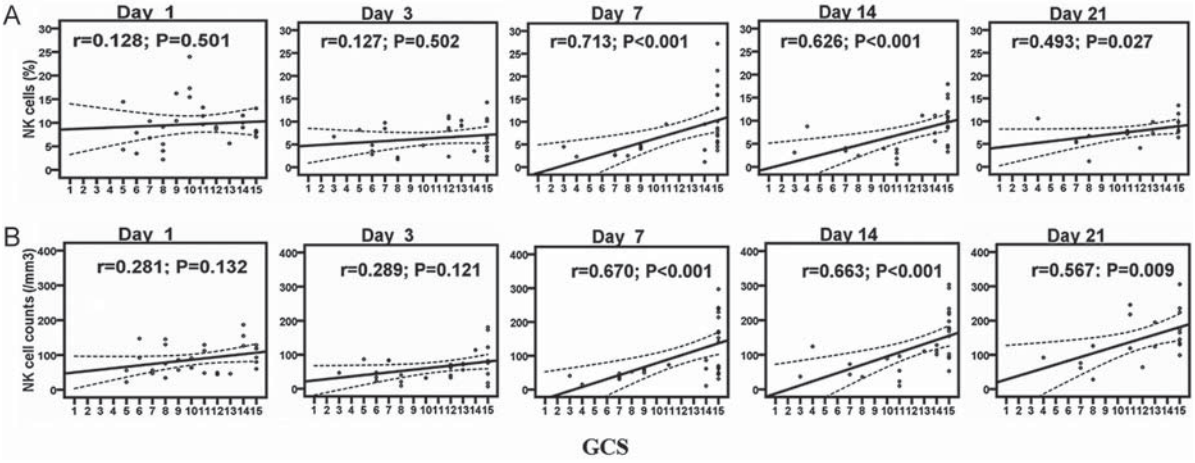

Fig. 2. Correlation of the percentages (A) and the absolute numbers (B) of $CD3^-CD56^+$ NK cells with the Glasgow Coma Scale (GCS) in patients with TBI on days 1, 3, 7, 14 and 21 (*n* = 30). GCS was correlated with NK cells as assessed by Spearman correlation coefficients. Dashed lines represent 95% confidence intervals. Statistical significance is shown as *P* and *r* values.

severe TBI. However, patients with moderate or severe TBI had a borderline response of cortisol values, which peaked on day 1 and fell close to the 95% confidence limits on day 3 (Fig. 4).

DISCUSSION

Although immune dysfunction associated with CNS injury has been reported[27], and NK cells are known to occupy the CNS following brain trauma, the critical issues of how NK cells recruit the agents of infection and function during immunodeficiency are not well understood[8, 28]. Accordingly, we investigated the characteristics of NK cells during the course of closed head injury and recorded dramatic alterations of these innate immune cells in peripheral blood.

Having the ability to lyse target cells without the need for prior antigen stimulation, NK cells are important effectors of innate immunity[24, 29, 30]. They can promote or inhibit adaptive immune responses[30, 31]. The average value for $CD3^-CD56^+$ NK cell content among PBMCs from healthy volunteers was ~11% in our study, in complete agreement with previous reports[15, 30], and the absolute number of $CD3^-CD56^+$ cells among PBMCs from healthy volunteers was 205/mm^3 (170–298), similar to that published by Forel *et al.*[15]. In the present study, we evaluated the relationship between NK cell content and different degrees of TBI. Flow cytometry revealed a significant reduction of the $CD3^-CD56^+$ cell population after brain injury. That is, in patients with mild, moderate, or severe TBI, the percentages of $CD3^-CD56^+$ NK cells among total lymphocytes first dropped and then increased compared to healthy controls. The absolute numbers of NK cells showed a similar pattern. Compared to the percentages, the absolute numbers of $CD3^-CD56^+$ NK cells provided smaller *P* values (<0.001). This may more realistically represent the decline in NK cell production in this situation. Following the onset of TBI, the more severe the brain injury, the greater the loss of NK cells.

In this study, on day 3 after TBI, all patients had a prominent decrease in the absolute numbers of $CD3^-CD56^+$ NK cells. It has been reported that severe CNS injury precipitates significant deficiencies of immune function within 72 h[17]. In accord with this, Piek *et al.* found that infections begin most frequently 2 to 4 days after TBI, peaking from days 5 to 11[7]. Immunosuppression may account for the high rate of infection in these patients. Our investigation clearly showed small numbers of $CD3^-CD56^+$ NK cells in PBMC around the peak time of infection, presumably representing the least effective immune function. Such a depletion of NK cells might have evolved to control excessive autoimmunity[32]. In addition, the decreased NK cells recovered on day 7 in patients with mild TBI, and on day 21 in those with moderate TBI. However, NK cells in patients with severe TBI did not return to the normal level.

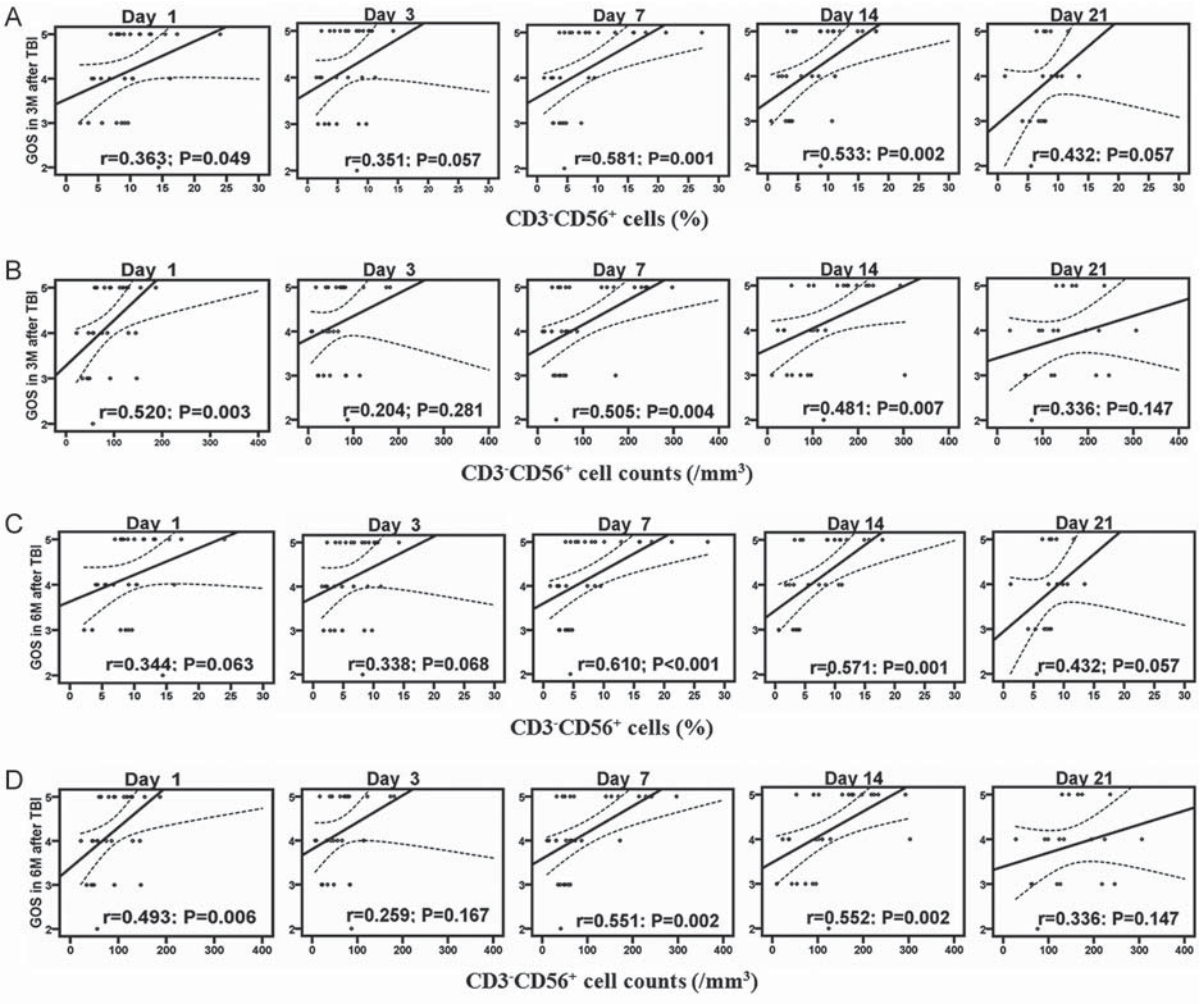

Fig. 3. Correlation of the percentages and the absolute numbers of $CD3^-CD56^+$ NK cells on days 1, 3, 7, 14, and 21 with Glasgow Outcome Scale (GOS) in patients 3 months (A, B) and 6 months (C, D) after TBI diagnosis (*n* = 30). GOS was correlated with NK cells using Spearman correlation coefficients. Dashed lines represent 95% confidence intervals. Statistical significance is shown as *P* and *r* values.

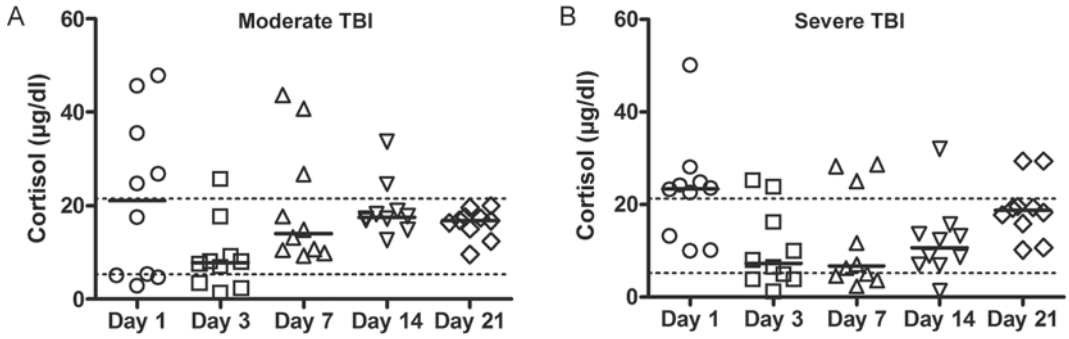

Fig. 4. Time-course of morning serum cortisol levels in patients with moderate and severe TBI. The range between the dashed lines shows confirmed morning serum cortisol levels of 5 to 21 μg/dl in healthy persons. In patients with moderate TBI, borderline low values appeared on day 3 post-TBI (A) and minimal cortisol values were present on day 3 in the severe TBI group (B).

The changes in NK cells began rapidly within 72 h of the lesion-causing event, after which recovery proceeded slowly, lasting for several weeks. Moreover, there was a statistically significant positive correlation between NK cells and GCS values. Elsewhere, assessments of the ultimate outcome after TBI were based on the last GOS score recorded, an evaluation charted at 3 or 6 months post-TBI[33]. Our evidence documented a positive correlation between NK cell changes and GOS scores at multiple time points, indicating a relationship between immune status and physiological recovery. Inadequate production of innate immune cells and the subsequent immune deficiency likely affects the prognosis of patients with TBI.

An intact hypothalamic-pituitary-adrenal (HPA) axis with effective anti-inflammatory activity is indispensable for host survival of critical illness[34]. Post-traumatic hypopituitarism and hypothalamo-pituitary dysfunction are common and potentially serious complications of TBI[2]. Dysfunction of the HPA axis is known to result in critical corticosteroid insufficiency[34]. However, most available evidence indicates that steroid therapy does not improve the outcome in patients with severe TBI. In fact, the administration of steroids has a deleterious effect; so, their use is not recommended for TBI[19]. In anticipation that steroid levels might impact our results, only patients who had not used steroids were enrolled. In all patients with moderate or severe TBI, morning serum cortisol concentrations were checked and were well within the normal range, in agreement with the preliminary study of Mrakovcic *et al.*[16]. The cortisol concentrations fell to borderline low values on day 3 after TBI, and the changes resembled those of NK cells. However, cortisol can suppress the immune system and directly inhibit NK cell activity, as described in some reports[35-37]. Therefore, we cannot yet eliminate the possibility that changes of NK cell number were the consequence of stress cortisol imbalance in our patients. Moreover, activation of the sympathetic and parasympathetic nervous systems can trigger the conditioned NK cell response[38, 39]. Further, damage or stimulation of some limbic structures can significantly affect the cytotoxic capacity of NK cells[40]. It has been shown that lesions of limbic structures in rats cause a gradual depression of the cytotoxicity of NK cells, which peaks 10 days after the initial event, followed by recovery to the baseline on day 21 or later[40]. This result is similar to that in our preliminary study. Evidently then, injury to the brain, regardless of the specific location or cause, can impact NK cells.

So far, the mechanisms underlying NK cell depletion and its biological significance are not well understood. Many causes might account for the abnormalities of NK cells in TBI. First, NK cells may be recruited to cross the damaged blood-brain barrier thereby entering the brain and reducing their presence in peripheral blood. Second, since the CNS harbors a unique spectrum of antigen-presenting cells (astrocytes and microglia), NK cells arriving there could become receptive to an array of cellular components that they had not encountered in the periphery[11]. NK cells in the inflamed CNS can directly lyse microglia to inhibit the activation of autoimmune T-cells[14, 23]. The depletion of NK cells might improve the survival of neighboring neurons by avoiding detrimental over-activation, which would amplify the inflammatory response. Alternatively, an increased susceptibility to infection might result from CNS injury-induced immunodepression in TBI patients. Nevertheless, our study documented that the absolute numbers of circulating NK cells changed far more than the percentage of NK cells in all groups of TBI patients. The importance of this outcome is uncertain but indicates that NK cells, among other cells (i.e., dendritic cells or monocytes), have a significant influence on innate immune function and play a role in patients' ability to survive TBI.

In summary, how NK cells act to modulate inflammatory responses in CNS injury is not completely known, partially because the CNS is uniquely complex as compared with other organ systems[41]. However, we found a significantly reduced expression of $CD3^-CD56^+$ cells associated with the degree of TBI severity. That reduction indicated that NK cells may contribute to the amplification of drastic immunodepression after TBI. Consequently, measuring NK cells expressing $CD3^-CD56^+$ may have novel prognostic and therapeutic implications for patients with TBI.

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