Natural Killer T Cells Are Numerically and Functionally Deficient in Patients with Trauma

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Keywords

α-Galactosylceramide · Cytokine · Natural killer T cells · Proliferation · Trauma

Abstract

Natural killer T (NKT) cells rapidly produce Th1 and Th2 cytokines such as interferon-γ (IFN-γ) and interleukin (IL)-4. This study examined the frequency and function of NKT cells in trauma patients. Frequencies, proliferative responses to α-galactosylceramide (α-GalCer), and Th1/Th2 cytokine secretion levels of NKT cells in peripheral blood mononuclear cells from trauma patients and healthy controls (HC) were measured by flow cytometry. Circulating NKT cell levels were significantly reduced in trauma patients. Proliferation and IFN-γ production of circulating NKT cells in response to α-GalCer were markedly decreased in trauma patients. CD69 expression levels produced by NKT cells were significantly upregulated in trauma patients compared to those in HC. In addition, annexin V+ NKT cells were profoundly increased in trauma patients after α-GalCer stimulation. Trauma patients

had higher plasma levels of IL-6, IL-8, and TNF-α compared to HC. In particular, the proliferative response of NKT cells to α-GalCer was significantly decreased in the presence of these cytokines. Such decrease was partially recovered after treatment with blocking antibodies against these cytokines. This study demonstrates that circulating NKT cells are numerically deficient and functionally impaired in IFN-γ production in trauma patients. These findings provide an important insight into the trauma-related innate immune response.

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Introduction

Trauma refers to sudden physical injury caused by an external force. It is one of the leading causes of death worldwide. According to the Global Burden of Disease Study 2017, injuries account for 4.48 million deaths annually or an age-standardized death rate of 57.9 per

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100,000 individuals [1]. The injury severity score (ISS) is regarded as the gold standard to assess trauma severity. It is correlated with mortality, morbidity, and hospitalization time after trauma [2]. According to the ISS, traumatic injuries are classified into mild (ISS <9), moderate (9 ≤ ISS ≤ 15), and severe or major trauma (ISS >15) [2]. In the past, most people with major trauma died from initial blood loss without medical care. However, after major trauma many people are now able to survive due to advances in treating hemorrhage and coagulopathy [3]. In most survivors, traumatic injury often evokes two opposing immune responses: (1) systemic inflammatory response syndrome (SIRS) characterized by increased levels of inflammatory cytokines and immune cell activation, and (2) compensatory anti-inflammatory response syndrome (CARS) characterized by elevated levels of anti-inflammatory cytokines and immune paresis [4]. A study by Hazeldine et al. [4] reported that traumatic injury resulted in immediate immune dysfunction, supported by evidence of concomitant immune activation and suppression detected within minutes of injury. Although the purpose of the inflammatory response was once protective against infection, paradoxically, these marked immune alterations (i.e., CARS immediately following SIRS) now reduce the survivors' resistance to infection. These marked immune alterations involve both innate and adaptive immune systems. Furthermore, survivors still remain at risk for developing multiple organ dysfunction syndrome and sepsis [5].

Human natural killer T (NKT) cells are a distinct subset of T cells that express an invariant Vα24-Jα18 T cell receptor (TCR) chain paired with the Vβ11 TCR chain. TCR αβ pairs can recognize self or foreign glycolipids such as α-galactosylceramide (α-GalCer) presented by CD1d, a major histocompatibility complex class I-like molecule as cognate antigen [6]. NKT cells play a bridging role between innate and adaptive immune cells, including dendritic cells, monocytes, NK cells, T cells, and B cells by rapidly producing large amounts of Th1 and Th2 cytokines such as interferon-γ (IFN-γ) and interleukin (IL)-4 [7, 8]. Furthermore, NKT cells play either a protective or harmful role in a broad range of diseases, including autoimmunity, cancer, infection, sepsis, and ischemia/reperfusion-related tissue injury [8–10].

Several recent studies have shown that trauma-induced immune alterations, such as elevated proinflammatory cytokines [11, 12], impaired leukocyte function [13, 14], and altered monocyte phenotype [15], are associated with or predictive of mortality, multiple organ failure, and sepsis [4]. Thus, monitoring alterations of the

immune system in trauma patients may play a potential role in assessing future poor outcomes. However, these studies have been limited to monocytes and neutrophils among innate immune cells. Our previous study has shown that circulating NKT cells are deficient in trauma patients and that their deficiency is associated with the severity of trauma [16]. These findings suggest that NKT cells may play an important role in the innate immune response to traumatic injury. However, little is known about immune alterations in NKT cells in response to trauma. Accordingly, the aims of this study were: (i) to examine numbers and proliferative responses of NKT cells, (ii) to examine Th1/Th2 cytokine levels produced by NKT cells, and (iii) to determine the mechanism responsible for NKT cell dysfunction in trauma patients.

Patients and Methods

Patients

The study cohort was composed of 32 patients with trauma (11 females and 21 males; mean age ± standard deviation [SD]: 60.1 ± 15.6 years) who visited Chonnam National University Hospital Regional Trauma Center and 32 non-injured healthy controls (HC; 16 females and 16 males; mean age ± SD: 50.0 ± 10.8 years). Blood samples were usually obtained from patients within 12–36 h after traumatic injury. The subjects were enrolled among residents living in Jeollanam-do province, South Korea from October 2016 to September 2018. Patients were included in this study if they visited the emergency room within 36 h of traumatic injury and if they were older than 18 years. Patients were excluded if they died, were discharged within 36 h of hospitalization, were under the age of 18 years, or were pregnant. No subject in the control group had a documented history of respiratory disorders such as chronic obstructive pulmonary disease and pulmonary embolism, autoimmune disease, pregnancy, infectious diseases, recent surgery, malignancies, chronic liver, renal, or endocrine diseases. HC did not experience fever during 72 h prior to enrollment either. The clinical and laboratory characteristics of patients with trauma are summarized in Table 1.

Monoclonal Antibodies and Flow Cytometry

The following monoclonal antibodies (mAbs) and reagents were used in this study: fluorescein isothiocyanate (FITC)-conjugated anti-CD3, FITC-conjugated annexin V, phycoerythrin (PE)-conjugated anti-6B11, PE-conjugated anti-CD3, PE-conjugated anti-IFN-γ, PE-conjugated anti-IL-4, PE-conjugated anti-CD69, PerCP-conjugated anti-CD45, and PE-conjugated mouse IgG isotype control (all from BD Biosciences, San Diego, CA, USA); PE-conjugated anti-programmed death-1 (anti-PD-1; eBioscience, San Diego, CA, USA), and allophycocyanin (APC)-conjugated anti-6B11 mAbs (BioLegend, San Diego, CA, USA). Cells were stained with combinations of appropriate mAb for 20 min at 4 °C. Stained cells were analyzed on a Navios flow cytometer (Beckman Coulter, Brea, CA, USA) using Kaluza software (version 1.5a; Beckman Coulter).

Isolation of Peripheral Blood Mononuclear Cells and Identification of NKT Cells

Peripheral venous blood samples were collected into heparin-containing tubes and peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation using Ficoll-Paque Plus solution (Amersham Biosciences, Uppsala, Sweden). NKT cells were identified phenotypically as CD3+6B11+ cells by flow cytometry as described previously [17].

NKT Cell Proliferation Assay

Proliferative abilities of NKT cells were assayed by flow cytometry as described previously [17]. Briefly, freshly isolated PBMCs were suspended in complete media supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA), seeded into a 24-well plate at a density of 1×10^6 /well, and then cultured at 37 °C in a 5% CO₂ humidified incubator for 7 days in the presence of IL-2 (100 IU/mL; BD PharMingen, San Jose, CA, USA) and α -GalCer (100 ng/mL; Alexis Biochemicals, Lausen, Switzerland) or 0.1% DMSO as a control. Cells were harvested and stained with FITC-conjugated anti-CD3, PE-conjugated anti-6B11, and PerCP-conjugated anti-CD45 mAbs. Percentages of CD3+6B11+ NKT cells were determined by flow cytometry using a CD45/SSC gate. The proliferation index was defined as the percentage of NKT cells (100 ng/mL α -GalCer) minus the percentage of NKT cells (0 ng/mL α -GalCer) on day 7 divided by the percentage of NKT cells on day 0. It was expressed as the fold increase.

To determine the effect of a proinflammatory cytokine cocktail and its blocking antibody, freshly isolated PBMCs were stimulated with a cytokine cocktail consisting of IL-6 (50 ng/mL; PeproTech, London, UK), IL-8 (10 ng/mL; PeproTech), and TNF- α (5 ng/mL; PeproTech) for 3 days in the presence or absence of cytokine inhibitors (i.e., blocking antibodies) and then cultured for 7 days in the presence of IL-2 (100 IU/mL) and α -GalCer (100 ng/mL) or DMSO as a control. Blocking antibodies against cytokines included anti-IL-6 (5 μ g/mL), anti-IL-8 (5 μ g/mL), and anti-TNF- α (5 μ g/mL; all from BD Biosciences).

Intracellular Cytokine Staining

IFN- γ and IL-4 expression levels in NKT cells were detected by intracellular cytokine flow cytometry as described previously [18]. Briefly, freshly isolated PBMCs (1×10^6 /well) were incubated at 37 °C in 1 mL of complete media consisting of RPMI 1640, 2 mM L-glutamine, 100 units/mL of penicillin, and 100 μ g/mL of streptomycin supplemented with 10% FBS for 2 h in the presence of α -GalCer (100 ng/mL) or 0.1% DMSO as a control. For intracellular cytokine staining, 1 μ L of brefeldin A (GolgiPlug; BD Biosciences) for 1 mL of cell culture was added. After incubation at 37 °C in a 5% CO₂ humidified incubator for an additional 4 h, cells were stained with FITC-conjugated anti-CD3 and APC-conjugated anti-6B11 mAbs for 20 min at 4 °C, fixed in 4% paraformaldehyde for 15 min at room temperature, and permeabilized with Perm/Wash solution (BD Biosciences) for 10 min. The cells were then stained with PE-conjugated anti-IFN- γ and PE-conjugated anti-IL-4 mAbs for 30 min at 4 °C and analyzed by flow cytometry.

Enzyme-Linked Immunosorbent Assay

Plasma levels of IL-6, IL-8, and TNF- α were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D systems Inc., Minneapolis, MN, USA) according to instructions of the manufacturer.

Table 1. Clinical and laboratory characteristics of 32 trauma patients

Sex (male/female), <i>n</i>	21/11
Age, years	60.1 $\pm$ 15.6
<i>Clinical variables</i>	
APACHE II score	9.6 $\pm$ 8.1
SAPS II score	25.8 $\pm$ 16.2
ISS score	16.3 $\pm$ 8.5
ISS categories	
Mild (<9)	6 (18.8)
Moderate (9–15)	10 (31.3)
Severe (>15)	16 (50.0)
SIRS	19 (59.4)
Infectious complications ¹	10 (31.3)
Mortality ²	2 (6.25)
<i>Vital signs</i>	
MAP, mm Hg	89 $\pm$ 19
HR, beats/min	94 $\pm$ 15
Body temperature, °C	37.0 $\pm$ 0.6
<i>Laboratory variables</i>	
Leukocytes, cells/ μ L	9,653 $\pm$ 4,012
Lymphocytes, cells/ μ L	1,114 $\pm$ 745
Neutrophils, cells/ μ L	7,712 $\pm$ 3,270
Monocyte, cells/ μ L	747 $\pm$ 416
Hemoglobin, g/dL	10.2 $\pm$ 1.7
Platelets, $\times 10^3$ / μ L	139 $\pm$ 39
Bilirubin, mg/dL	0.9 $\pm$ 0.4
BUN, mg/dL	17.4 $\pm$ 11.8
Creatinine, mg/dL	1.1 $\pm$ 1.4
CRP, mg/dL	3.5 $\pm$ 3.4
PaO ₂ , mm Hg	111.6 $\pm$ 38.2
Lactate, mmol/L	2.6 $\pm$ 2.3
Bicarbonate, mmol/L	23.9 $\pm$ 4.10
Prothrombin time, INR	1.18 $\pm$ 0.24

Data are presented as *n* (%) or the mean $\pm$ SD

APACHE, acute physiology and chronic health evaluation; SAPS, simplified acute physiology score; ISS, injury severity score; SIRS, systemic inflammatory response syndrome; MAP, mean arterial pressure; HR, heart rate; BUN, blood urea nitrogen; CRP, C-reactive protein; PaO₂, partial pressure of oxygen in arterial blood; INR, international normalized ratio.

¹ Includes pneumonia (*n* = 5), urinary tract infection (*n* = 2), wound infection (*n* = 2), and bacteremia (*n* = 1).

² Refers to 28-day mortality.

Statistical Analysis

Numbers and proliferation indices of NKT cells and expression levels of IFN- γ , IL-4, CD69, PD-1, and annexin V in NKT cells were compared between HC and patients by analysis of covariance after adjusting for age and sex using the Bonferroni correction for multiple comparisons. The Mann-Whitney U test was used to compare plasma levels of cytokines between HC and patients. The Wilcoxon matched-pairs signed-rank test was used to compare changes in proliferation indices of NKT cells after cytokine stimulation. Relationships between proliferation indices of NKT cells

Fig. 1. Reduced circulating NKT cell numbers in the peripheral blood from trauma patients. **a** NKT cell percentages among peripheral blood lymphocytes. **b** Absolute NKT cell numbers (per microliter of blood). Data in **a** and **b** were obtained from 32 HC and 32 patients with trauma. Symbols represent individual subjects and horizontal lines are median values. * $p < 0.01$, ** $p < 0.0001$ by the ANCOVA test. **c** Representative NKT cell percentages as determined by flow cytometry.

and clinical or laboratory parameters were examined using Spearman's correlation analysis. Statistical significance was considered for $p < 0.05$. Statistical analysis and graphic works were performed using SPSS version 18.0 software (SPSS, Chicago, IL, USA) and GraphPad Prism version 5.03 software (GraphPad Software, San Diego, CA, USA), respectively.

Results

Patient Characteristics

A total of 32 patients with trauma were included in this study (Table 1). The severity of injury was categorized as mild (<9), moderate (9–15), and severe (>15) according to the ISS scoring system, the gold standard for evaluating injury severity. Of these patients, 6 (18.8%) with mild injury, 10 (31.3%) with moderate injury, and 16 (50.0%) with severe injury were found. Two (6.25%) patients died, including 1 with mild injury and 1 with severe injury.

Reduced Numbers of Circulating NKT Cells and Their Clinical Relevance in Patients with Trauma

Frequencies of CD3+6B11+ NKT cells represent the percentages among peripheral blood lymphocytes. Percentages of circulating NKT cells were significantly lower in patients than in HC (median 0.04 vs. 0.12%, $p < 0.01$; Fig. 1a). Absolute NKT cell numbers were calculated by multiplying NKT cell fractions by total lymphocyte numbers (per microliter of peripheral blood). Patients with trauma had significantly lower absolute NKT cell numbers than HC (median 0.46 vs. 1.60, $p < 0.0001$; Fig. 1b). Representative NKT cell percentages as determined by flow cytometry are shown (Fig. 1c). In addition, linear regression analysis showed that log-transformed NKT cell numbers were correlated with the lymphocyte count ($p < 0.0001$) and C-reactive protein level ($p = 0.030$; online suppl. Table 1; see www.karger.com/doi/10.1159/000504324 for all online suppl. material).

Fig. 2. Decreased proliferative responses of NKT cells to α -GalCer in trauma patients. **a** Representative NKT cell percentages determined by flow cytometry. **b** Proliferation indices of NKT cells. Data in **b** were obtained from 32 HC and 32 patients with trauma. Symbols represent individual subjects. Horizontal lines are median values. * $p < 0.0001$ by ANCOVA test.

Fig. 3. Reduced IFN- γ expression in α -GalCer-stimulated NKT cells from trauma patients. **a** Representative cytokine-producing NKT cell percentages determined by flow cytometry. **b** Data obtained from 14 HC and 12 patients with trauma. The ratio of IFN- γ /IL-4 from each subject is also illustrated. Symbols represent individual subjects. Horizontal lines are median values. * $p < 0.05$ by ANCOVA test.

Impaired Proliferative Response of NKT Cells to α -GalCer in Patients with Trauma

The representative percentage of NKT cells among peripheral blood lymphocytes from an HC subject was markedly increased from 0.37% on day 0 to 12.9% on day 7 in response to α -GalCer, resulting in a proliferation index of 34.0. In contrast, a representative patient with trauma displayed a weak proliferative response of NKT cells (Fig. 2a). Overall proliferation indices were significantly lower in patients with trauma than those in controls (median 1.1 vs. 20.8, $p < 0.0001$; Fig. 2b). Furthermore, CFSE dilution assay showed that NKT cells from HC profoundly expanded and extensively divided 7 days after stimulation with α -GalCer. However, few NKT cells from patients with trauma expanded and divided (online suppl. Fig. 1).

Relationship between Proliferative Response of NKT Cells and Clinical Parameters in Trauma Patients

To evaluate the clinical relevance of NKT cell function in 32 patients with trauma, we investigated the correlation between NKT cell proliferation indices and clinical parameters by Spearman's correlation analysis. NKT cell proliferation indices were positively correlated with lymphocyte count ($p = 0.022$) but negatively correlated with creatinine levels ($p = 0.032$). However, NKT cell proliferation indices showed no significant correlation with gender, age, mortality, SIRS, infectious complications, APACHE score, SAPS score, ISS score, leukocyte count, neutrophil count, hemoglobin level, platelet count, bilirubin level, blood urea nitrogen level, C-reactive protein level, PaO_2 level, lactate level, bicarbonate level, prothrombin time, mean arterial pressure level, heart rate, or body temperature (Table 2).

Impaired IFN- γ Production by NKT Cells of Trauma Patients

We next measured levels of representative Th1/Th2 cytokine secretion by NKT cells of trauma patients. Percentages of IFN- γ + NKT cells were found to be significantly lower in trauma patients than those in HC (median 3.13 vs. 11.0%, $p < 0.05$). However, percentages of IL-4+ NKT cells were comparable between patients and HC. In addition, patients had a significantly lower ratio of IFN- γ /IL-4 than HC (median ratio 0.66 vs. 1.61, $p < 0.05$; Fig. 3).

Activation of NKT Cells in Trauma Patients

Our previous study showed deficiencies of circulating NKT cell numbers in patients with trauma [16]. To deter-

Table 2. Spearman's correlation coefficients for the proliferation indices of NKT cells with respect to clinical and laboratory findings in trauma patients

	Correlation coefficient (ρ)	p value
Sex (male)	-0.193	0.291
Age (years)	-0.229	0.209
APACHE II (score)	-0.276	0.127
SAPS II (score)	-0.022	0.907
ISS (score)	-0.103	0.576
ISS (categories)	-0.117	0.524
SIRS	-0.325	0.070
Infectious complications	0.033	0.858
Mortality	0.028	0.879
MAP (mm Hg)	-0.163	0.374
HR (beat/min)	-0.132	0.472
Body temperature ($^{\circ}\text{C}$)	-0.098	0.592
Lymphocytes (cells/ μL)	0.405	0.022
Hemoglobin (g/dL)	0.103	0.576
Platelets (cells/ μL)	-0.018	0.923
Bilirubin (mg/dL)	-0.234	0.198
BUN (mg/dL)	-0.069	0.710
Creatinine (mg/dL)	-0.381	0.032
CRP (mg/dL)	-0.087	0.637
PaO_2 (mm Hg)	0.020	0.915
Lactate (mmol/L)	-0.297	0.098
Bicarbonate (mmol/L)	0.006	0.974
Prothrombin time (INR)	-0.106	0.565

APACHE, acute physiology and chronic health evaluation; SAPS, simplified acute physiology score; ISS, injury severity score; SIRS, systemic inflammatory response syndrome; MAP, mean arterial pressure; HR, heart rate; BUN, blood urea nitrogen; CRP, C-reactive protein; PaO_2 , partial pressure of oxygen in arterial blood; INR, international normalized ratio.

mine whether circulating NKT cell deficiency was associated with activation-induced cell death, we investigated the activation and apoptosis indicated by CD69 upregulation and annexin V staining, respectively, in circulating NKT cells. CD69+ and annexin V+ NKT cells were examined by flow cytometry. Percentages of CD69+ NKT cells were found to be significantly higher in trauma patients than those in HC (median 10.0 vs. 5.98%, $p < 0.05$; Fig. 4a, b). However, no significant difference was observed in annexin V+ NKT cell levels between patients and HC (Fig. 4c, d). To determine whether dysfunction of NKT cells was related to anergy or exhaustion, we examined expression levels of PD-1, a representative inhibitory receptor. Percentages of PD-1+ NKT cells were comparable between patients and HC (Fig. 4e, f).

Fig. 4. Expression of CD69, PD-1, and annexin V in circulating NKT cells obtained from trauma patients. Representative percentages of CD69-expressing cells (a), annexin V-positive cells (c), and PD-1-expressing cells (e) among NKT cells determined by flow cytometry. b, d, f Data obtained from 16 HC and 25 patients with trauma. Symbols represent individual subjects. Horizontal lines are median values. * $p < 0.05$ by ANCOVA test.

Fig. 5. Increased apoptosis of α -GalCer-stimulated NKT cells in trauma patients. **a** Representative percentages of apoptotic (annexin V-positive) NKT cells on days 0 and 7. **b** Data obtained from 8 HC and 16 patients with trauma. Values are expressed as the mean $\pm$ SEM. * $p < 0.05$ by ANCOVA test.

Increased NKT Cell Death in Trauma Patients

Both percentages of annexin V+ NKT cells on day 0 and day 7 without α -GalCer were similar between trauma patients and HC. However, after stimulation with α -GalCer for 7 days, NKT cell apoptosis was higher in trauma patients than in HC (mean $\pm$ SEM: 50.2 ± 5.4 vs. $23.0 \pm 4.7\%$, $p < 0.05$; Fig. 5). These findings suggest that increased NKT cell apoptosis in culture is related to the impaired proliferative response of NKT cells observed in trauma patients.

Increased Plasma Levels of IL-6, IL-8, and TNF- α in Trauma Patients Can Affect NKT Cell Proliferation

A variety of proinflammatory cytokines can affect NKT cell proliferation [17]. Thus, we measured plasma levels of proinflammatory cytokines such as IL-6, IL-8, and TNF- α using ELISA. Patients with trauma showed significantly higher plasma levels of IL-6, IL-8, and TNF- α than HC (median: IL-6, 92.3 vs. 0.7 pg/mL, $p < 0.05$; IL-8, 24.0 vs. 1.5 pg/mL, $p < 0.005$; TNF- α , 2.1 vs. 2.1 pg/mL, $p < 0.05$; Fig. 6a).

We next determined whether these proinflammatory cytokines could affect the NKT proliferative capacity (Fig. 6b). Proliferation indices were found to be significantly lower in cytokine-treated cultures than those in untreated cultures (median 6.3 vs. 30.2, $p < 0.01$). They were then significantly increased after treatment with

blocking antibodies compared to cytokine-treated cultures (median 6.3 vs. 11.0, $p < 0.05$; Fig. 6c). However, these blocking antibodies partially reversed the dysfunction of NKT cells. Taken together, these results suggest that the proliferative dysfunction of NKT cells may be partly due to proinflammatory cytokines in trauma patients.

Discussion

The present study showed that proliferation and IFN- γ production by circulating NKT cells in response to α -GalCer were reduced in trauma patients, although their capacity for IL-4 production was preserved. Suppression of proliferation and IFN- γ production in NKT cells has also been observed in other inflammatory or infectious diseases, such as systemic lupus erythematosus, tuberculosis, human immunodeficiency virus type 1 infection, and scrub typhus [18–21]. Suppression of cell proliferation and IFN- γ production has also been reported in adaptive T cells following severe injury or trauma-hemorrhage [22, 23]. One possible explanation for adaptive T cell suppression is that a specific subset of neutrophils in acute systemic inflammation can inhibit T cell responses via Mac-1 and the reactive oxygen species signaling pathway [22]. Another possibility is that myeloid-derived sup-

Fig. 6. Effect of stimulation with a proinflammatory cytokine cocktail on proliferative responses of NKT cells to α -GalCer. **a** Plasma levels of IL-6, IL-8, and TNF- α in trauma patients. Plasma samples of patients were collected before specific treatment on admission. Plasma levels were determined by ELISA. Data were obtained from 15 HC and 30 trauma patients. * $p < 0.05$, ** $p < 0.005$ by Mann-

Whitney U test. **b, c** Effect of a proinflammatory cytokine cocktail and its blocking antibody on proliferative responses of NKT cells to α -GalCer. Data in **c** were obtained from 8 HC. Symbols represent individual subjects. Horizontal lines are median values. * $p < 0.05$, ** $p < 0.01$ by Wilcoxon matched-pairs signed-rank test.

pressor cells are induced after inflammation and suppress adaptive T cell responses [24]. Likewise, these two mechanisms can explain defective innate T cell responses, including NKT cells in trauma patients. Further studies are needed to elucidate these mechanisms in suppressed innate T cell responses in trauma patients.

Our previous data have shown that a numerical deficiency of circulating NKT cells is associated with severity grades of traumatic injury (i.e., APACHE score, SAPS score, and ISS score) [16]. In the present study, however, no significant correlation was observed between NKT cell proliferative indices and severity grades. Nevertheless, our results revealed that NKT cell proliferation indices were negatively correlated with serum creatinine levels in trauma patients, suggesting that NKT cell dysfunction might reflect renal dysfunction. Similarly, our previous

study showed that numerical deficiency of circulating NKT cells is accompanied by lupus nephritis [19]. Collectively, these data suggest that the circulating NKT cell level and function may be used as a biomarker for predicting disease severity or organ dysfunction in trauma. However, the correlation with serum creatinine levels was weak. Moreover, SIRS tended to be negatively associated with NKT cell proliferation indices, although the p value did not reach statistical significance. This may be due to small numbers of enrolled subjects. Therefore, further analysis using a large sample size is required to determine the clinical relevance of NKT cell function.

CD69 is known to be an early activation marker, whereas PD-1 is considered a relative later activation marker [25, 26]. Our data demonstrated that trauma patients displayed higher levels of CD69+ NKT cells with-

out upregulation of PD-1, indicating that NKT cells were activated early following trauma. In addition, our previous study reported the same phenomenon (i.e., early activation) that occurs to mucosal-associated invariant T cells, another subset of innate T cells, after traumatic injury [27]. Collectively, these findings suggest that trauma may induce early activation of innate T cells irrespective of their cell types. Interestingly, our additional data showed that CD69 expression levels were much higher in NKT cells compared to those in conventional T cells, suggesting that NKT cells might be activated much earlier than conventional T cells after trauma (online suppl. Fig. 2). Furthermore, previous studies have demonstrated that NKT cells are activated much more rapidly than conventional T cells in response to CD1d-dependent stimulations, such as α -GalCer or a variety of infectious agents [28–31]. Taken together, these results indicate that NKT cells are activated much earlier than conventional T cells, irrespective of CD1d-dependent or CD1d-independent stimulations.

Our data showed that NKT cells were more susceptible to apoptosis when they were stimulated with α -GalCer in trauma patients. This observation has also been reported in other inflammatory or infectious conditions, such as adult-onset Still's disease (AOSD) and tuberculosis [17, 20]. In particular, AOSD and trauma patients have high levels of IL-6, IL-8, and TNF- α in sera [32]. A previous study using in vitro experiments showed that TNF- α can induce NKT cell death by blocking downstream of NF- κ B activation during thymic development [33]. In addition, Tang et al. [34] reported that proinflammatory cytokine-activated Kupffer cells by lipids can promote hepatic NKT cell deficiency through activation-induced cell death. A previous study using a mouse model reported that IL-6 treatment can inhibit NKT cells in CD4⁺ T cell- and STAT3-dependent manners [35]. Collectively, these findings lead us to speculate that proinflammatory cytokine-activated antigen-presenting cells may induce circulating NKT cell deficiency through activation-induced cell death. Proinflammatory cytokines such as IL-6, IL-8, and TNF- α were also found to be able to suppress the proliferative function of NKT cells in response to α -GalCer.

Conclusions

This is the first study to assess the function of NKT cells in trauma patients. The present study demonstrates that circulating NKT cells are early activated and func-

tionally impaired in IFN- γ production and proliferation following trauma via increased proinflammatory cytokines. In addition, NKT cell dysfunction might reflect renal dysfunction. These findings provide important insight into the trauma-related early innate immune response.

Acknowledgments

This study was supported by the National Research Foundation of Korea funded by the Korean Government (Grants 2019R1A-2C1003238, 2017R1D1A1B03029239, 2018R1A6A3A11042850 and 2019R1I1A1A01040762), and the Chonnam National University Hospital Biomedical Research Institute (Grant CRI16036-22 and CRI18092-1).

Statement of Ethics

The study protocol was approved by the Institutional Review Board of Chonnam National University Hospital. Written informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

Disclosure Statement

The authors declare that they have no competing interests.

Author Contributions

Y.-G.J., H.-M.J., Y.-N.C., J.-C.K., S.-J.K., and Y.-W.P. designed this study, collected clinical information, analyzed raw data, performed statistical analysis, and contributed to writing of the paper. Y.-G.J., H.-M.J., and Y.-N.C. performed the experiments. All authors read and approved the final version of the manuscript.

References

- 1 Roth GA, Abate D, Abate KH, Abay SM, Ababafati C, Abbasi N, et al.; GBD 2017 Causes of Death Collaborators. Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet*. 2018 Nov;392(10159):1736–88.
- 2 Palmer C. Major trauma and the injury severity score — where should we set the bar? *Annu Proc Assoc Adv Automot Med*. 2007;51:13–29.
- 3 Gruen RL, Brohi K, Schreiber M, Balogh ZJ, Pitt V, Narayan M, et al. Haemorrhage control in severely injured patients. *Lancet*. 2012 Sep;380(9847):1099–108.

- 4 Hazeldine J, Naumann DN, Toman E, Davies D, Bishop JR, Su Z, et al. Prehospital immune responses and development of multiple organ dysfunction syndrome following traumatic injury: a prospective cohort study. *PLoS Med*. 2017 Jul;14(7):e1002338.
- 5 Wafaisade A, Lefering R, Bouillon B, Sakka SG, Thamm OC, Paffrath T, et al.; Trauma Registry of the German Society for Trauma Surgery. Epidemiology and risk factors of sepsis after multiple trauma: an analysis of 29,829 patients from the Trauma Registry of the German Society for Trauma Surgery. *Crit Care Med*. 2011 Apr;39(4):621–8.
- 6 Brennan PJ, Brigl M, Brenner MB. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol*. 2013 Feb;13(2):101–17.
- 7 Van Kaer L. alpha-Galactosylceramide therapy for autoimmune diseases: prospects and obstacles. *Nat Rev Immunol*. 2005 Jan;5(1):31–42.
- 8 Godfrey DI, Kronenberg M. Going both ways: immune regulation via CD1d-dependent NKT cells. *J Clin Invest*. 2004 Nov;114(10):1379–88.
- 9 Szabo PA, Anantha RV, Shaler CR, McCormick JK, Haeryfar SM. CD1d- and MR1-restricted T cells in sepsis. *Front Immunol*. 2015 Aug;6:401.
- 10 Hu C, Zhang C, Yang C. The role of natural killer T cells in acute kidney injury: angel or evil? *Curr Protein Pept Sci*. 2017;18(12):1200–4.
- 11 Maier B, Lefering R, Lehnert M, Laurer HL, Steudel WI, Neugebauer EA, et al. Early versus late onset of multiple organ failure is associated with differing patterns of plasma cytokine biomarker expression and outcome after severe trauma. *Shock*. 2007 Dec;28(6):668–74.
- 12 Jeschke MG, Gauglitz GG, Finnerty CC, Kraft R, Mlcak RP, Herndon DN. Survivors versus nonsurvivors postburn: differences in inflammatory and hypermetabolic trajectories. *Ann Surg*. 2014 Apr;259(4):814–23.
- 13 Hietbrink F, Koenderman L, Althuisen M, Pillay J, Kamp V, Leenen LP. Kinetics of the innate immune response after trauma: implications for the development of late onset sepsis. *Shock*. 2013 Jul;40(1):21–7.
- 14 Muszynski JA, Nofziger R, Greathouse K, Nateri J, Hanson-Huber L, Steele L, et al. Innate immune function predicts the development of nosocomial infection in critically injured children. *Shock*. 2014 Oct;42(4):313–21.
- 15 Cheron A, Floccard B, Allaouchiche B, Guignant C, Poitevin F, Malcus C, et al. Lack of recovery in monocyte human leukocyte antigen-DR expression is independently associated with the development of sepsis after major trauma. *Crit Care*. 2010;14(6):R208.
- 16 Jo YG, Choi HJ, Kim JC, Cho YN, Kang JH, Jin HM, et al. Deficiencies of circulating mucosal-associated invariant T cells and natural killer T cells in patients with multiple trauma. *J Korean Med Sci*. 2017 May;32(5):750–6.
- 17 Lee SJ, Cho YN, Kim TJ, Park SC, Park DJ, Jin HM, et al. Natural killer T cell deficiency in active adult-onset Still's Disease: correlation of deficiency of natural killer T cells with dysfunction of natural killer cells. *Arthritis Rheum*. 2012 Sep;64(9):2868–77.
- 18 Kang SJ, Jin HM, Cho YN, Oh TH, Kim SE, Kim UJ, et al. Dysfunction of circulating natural killer T cells in patients with scrub typhus. *J Infect Dis*. 2018 Oct;218(11):1813–21.
- 19 Cho YN, Kee SJ, Lee SJ, Seo SR, Kim TJ, Lee SS, et al. Numerical and functional deficiencies of natural killer T cells in systemic lupus erythematosus: their deficiency related to disease activity. *Rheumatology (Oxford)*. 2011 Jun;50(6):1054–63.
- 20 Kee SJ, Kwon YS, Park YW, Cho YN, Lee SJ, Kim TJ, et al. Dysfunction of natural killer T cells in patients with active Mycobacterium tuberculosis infection. *Infect Immun*. 2012 Jun;80(6):2100–8.
- 21 Moll M, Kuylenstierna C, Gonzalez VD, Andersson SK, Bosnjak L, Sönnnerborg A, et al. Severe functional impairment and elevated PD-1 expression in CD1d-restricted NKT cells retained during chronic HIV-1 infection. *Eur J Immunol*. 2009 Mar;39(3):902–11.
- 22 Pillay J, Kamp VM, van Hoffen E, Visser T, Tak T, Lammers JW, et al. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *J Clin Invest*. 2012 Jan;122(1):327–36.
- 23 Walz CR, Zedler S, Schneider CP, Mayr S, Loehe F, Bruns CJ, et al. Depressed T cell-derived IFN-gamma following trauma-hemorrhage: a potential mechanism for diminished APC responses. *Langenbecks Arch Surg*. 2007 May;392(3):339–43.
- 24 Hüsecken Y, Muche S, Kustermann M, Kling-sporn M, Palmer A, Braumüller S, et al. MDSCs are induced after experimental blunt chest trauma and subsequently alter antigen-specific T cell responses. *Sci Rep*. 2017 Oct;7(1):12808.
- 25 Simms PE, Ellis TM. Utility of flow cytometric detection of CD69 expression as a rapid method for determining poly- and oligoclonal lymphocyte activation. *Clin Diagn Lab Immunol*. 1996 May;3(3):301–4.
- 26 Brown KE, Freeman GJ, Wherry EJ, Sharpe AH. Role of PD-1 in regulating acute infections. *Curr Opin Immunol*. 2010 Jun;22(3):397–401.
- 27 Jo YG, Jin HM, Cho YN, Kim JC, Kee SJ, Park YW. Activation and impaired tumor necrosis factor-α production of circulating mucosal-associated invariant T cells in patients with trauma. *J Innate Immun*. 2019;11(6):506–15.
- 28 Chiba A, Dascher CC, Besra GS, Brenner MB. Rapid NKT cell responses are self-terminating during the course of microbial infection. *J Immunol*. 2008 Aug;181(4):2292–302.
- 29 Choi HJ, Xu H, Geng Y, Colmone A, Cho H, Wang CR. Bacterial infection alters the kinetics and function of iNKT cell responses. *J Leukoc Biol*. 2008 Dec;84(6):1462–71.
- 30 Kim S, Lalani S, Parekh VV, Vincent TL, Wu L, Van Kaer L. Impact of bacteria on the phenotype, functions, and therapeutic activities of invariant NKT cells in mice. *J Clin Invest*. 2008 Jun;118(6):2301–15.
- 31 Tupin E, Kinjo Y, Kronenberg M. The unique role of natural killer T cells in the response to microorganisms. *Nat Rev Microbiol*. 2007 Jun;5(6):405–17.
- 32 Chen DY, Lan JL, Lin FJ, Hsieh TY. Pro-inflammatory cytokine profiles in sera and pathological tissues of patients with active untreated adult onset Still's disease. *J Rheumatol*. 2004 Nov;31(11):2189–98.
- 33 Kumar A, Gordy LE, Bezbradica JS, Stanic AK, Hill TM, Boothby MR, et al. NF-κB Protects NKT Cells from Tumor Necrosis Factor Receptor 1-induced Death. *Sci Rep*. 2017 Nov;7(1):15594.
- 34 Tang T, Sui Y, Lian M, Li Z, Hua J. Pro-inflammatory activated Kupffer cells by lipids induce hepatic NKT cells deficiency through activation-induced cell death. *PLoS One*. 2013 Dec;8(12):e81949.
- 35 Sun R, Tian Z, Kulkarni S, Gao B. IL-6 prevents T cell-mediated hepatitis via inhibition of NKT cells in CD4+ T cell- and STAT3-dependent manners. *J Immunol*. 2004 May;172(9):5648–55.