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Role of IL-12 in overcoming the low responsiveness of NK cells to missing self after traumatic brain injury

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ABSTRACT

Blood samples from 32 patients with severe Traumatic brain injury (TBI) were studied and compared with 11 cardiac surgery patients, and 29 healthy controls. A dramatic decreased expression of HLA class I molecules on monocytes was associated with increased KIR+ NK cell frequency in TBI patients. Overall, the phenotype of TBI NK cells marked by KIR and CD57 expression and lower level of NKp46 and DNAM-1 reflected a differentiated state. The NK-cell response to missing self was marked by lower degranulation and lower IFN-γ production after stimulation with HLA class I deficient cell line. In contrast, the NK-cell ADCC was not altered. IL-12 was able to restore both IFN-γ production and the cytotoxicity capacities of NK cells. This study provides the first extensive description of the phenotype and functions of NK cells in TBI patients. Further evaluation of IL-12 treatment to overcome immunosuppression-induced nosocomial infections is warranted.

1. Introduction

Traumatic brain injury (TBI) is a leading cause of death and prolonged disability worldwide [1–3]. After brain injury, severe acquired immunosuppression with impairment of both innate and adaptive immunity is observed [4]. Immunosuppression is critically involved in the occurrence of nosocomial pneumonia which is the main complication recorded in the ICU following TBI [1,5]. Pneumonia alters outcome and neurological recovery [4,6]. Improving nosocomial pneumonia prevention by overcoming immunosuppression therefore remains a critical issue. In the setting of immunosupression, we showed that circulating dendritic cell functions are severely impaired in patients with aneurysmal subarachnoid hemorrhage [5,7]. Recent data from other groups have indicated that innate lymphoid cells have crucial roles in regulating immune response after BI [6,8]. Accordingly, we recently demonstrated that BI patients exhibit a maturation defect in the ex vivo granulomatous response involving innate lymphocytes: γδ T cells as well as natural killer (NK) lymphocytes. Interestingly, a severely decreased recruitment of NK cells into the granulomatous structure was correlated with the occurrence of secondary pneumonia [7,8].

NK cells represent 4% to 15% of blood lymphocytes and do not express the antigen-specific-receptor expressed by B and T cells. NK-cell functions are regulated by a broad panel of activating and inhibitory receptors [8–11]. These cells are naturally cytotoxic by granule polarization and exocytosis of various proteins including perforin or granzymes and by producing high amount of pro-inflammatory cytokines (IFN-γ, TNF-α). NK cells also express Fcψ receptor III3 known as CD16. This receptor recognizes antibody-coated target cells through their Fc region. Fc-CD16 binding mediates antibody-dependent cytotoxicity (ADCC) and IFN-γ production. An absence of HLA class I expression on monocytes (target cells) leads to NK cell activation. This phenomenon is called "missing self" and is marked by the absence of inhibitory NK receptor engagement with self HLA class I molecules allowing NK cells to eliminate cells with low or absent expression of HLA class I molecules [7,8]. This sophisticated pattern provides a robust line of defense against cancer and infections.

Several lines of evidence suggest that NK cells play a role in the defense of the immunocompromised host in the ICU [6,9–11]. In

Abbreviations: TBI, Traumatic brain injury; CP, Cardiac surgery patients; HC, Healthy control; GCS, Glasgow Coma Score.

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particular, data from our lab [7,12,13] and from other groups [6,14–16] suggest that NK cells are critically involved in brain-injured host defense. These cells are also important players in the setting of bacterial pneumonia [4,12,13], probably through the production of IFN-γ, a cytokine that is able to enhance immune response against bacterial infections and improve patient outcome [14–17].

To date, the phenotype of NK cells in ICU-acquired immunosuppression has not been thoroughly described. Moreover, the mechanisms leading to these alterations are still not understood. NK cells engage in crosstalk with other immune cells such as monocytes. We hypothesized that HLA class I molecules can be downregulated on monocytes, therefore participating in alterations of NK cells. We simultaneously investigated monocytes and NK cells as potential crosstalkers in TBI patients. Since TBI significantly increases susceptibility to infections by brain specific mechanisms [2,4], we used samples from cardiac surgery patients as positive controls.

2. Materials and methods

2.1. Ethics

This work belongs to a global study on immune dysfunctions in ICU. An institutional review board for human experimentation approved the protocol (Comité de Protection des Personnes de Nantes, authorization number AC-2008-433/French). Written informed consent from next-of-kin was required for enrollment. When possible, retrospective consent was obtained from patients. Critically ill patients were enrolled from January 2013 to December 2013 in two French surgical ICUs at a university hospital.

2.2. Study population

The brain-injured cohort was made up of traumatic brain-injured patients (Glasgow Coma Scale (GCS) below or equal to 12 aged 18 years or older, hospitalized in ICU and requiring mechanical ventilation. The major surgery cohort was composed of patients aged 18 years or older who were scheduled for elective coronary artery bypass or aortic valve replacement with cardiopulmonary bypass. Exclusion criteria for the two cohorts were previous immunosuppression, cancer in the previous 5 years, treatment with corticosteroids before hospitalization for brain injury, and pregnancy. Control samples were collected from matched healthy blood donors (age ± 10 years, sex, race). All blood donors were recruited at the Blood Transfusion Center (Etablissement Français du Sang, Nantes, France) and informed consent was obtained from all individuals.

2.3. Sample collection

EDTA-anticoagulated blood samples were withdrawn after ICU admission (<48 h after admission, day 1) and on day 7 after TBI and immediately sent to the laboratory. For cardiac surgery, samples were collected immediately before and 6 h after the end of surgery. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation, frozen at −80 °C and stored until investigation of NK cell phenotype and functions.

2.4. Follow-up

The following data were recorded: general characteristics including demographics, medical history, severity of traumatic brain injury assessed according to the Glasgow Coma Scale, infections, duration of ventilator support and ICU hospitalization and death at day 90. During the 28-day follow-up period, clinical assessments were performed twice daily in the ICU.

2.5. Cells

Peripheral blood mononuclear cells (PBMC) were isolated from citrate-phosphate-dextrose blood from healthy adult volunteers by gradient centrifugation on Ficoll-Hypaque (Lymphoprep, Axis-Shield, PoC AS, Oslo, Norway). P815 murine cell line and HLA class I deficient 7221.221 (221) cell line were cultured in RPMI 1640 medium (Life Technologies, Paisley, U.K.) containing glutamine (Life Technologies) and penicillin-streptomycin (Life Technologies) and supplemented with 10% human serum (EFS, Nantes) for P815 cell line and 10% FBS (Life Technologies) for 221 cell line.

2.5.1. Phenotype and functional assays by flow cytometry

PBMCs were preincubated with anti-CD107a (H4A3; BD Biosciences, San Jose, CA). NK-cell degranulation was assessed after incubation with media for 5 h (negative control), with 721.221(221) (E: T ratio of 1:1) or with P185 cell line after a preincubation with CD16 specific mAb or IgG control at 10 μg/mL. For the last 4 h of incubation, the cells were treated with brefeldin A (Sigma) at 10 mg/mL to block trans-Golgi transport and allow the intracellular accumulation of IFN-γ. The cells were cell surface stained and then permeabilized before intracellular IFN-γ staining with PE-conjugated anti-human IFN-γ (B27, BD Biosciences). For some experiments, PBMC were cultured overnight with IL-12 10 ng/mL before the functional assays.

PBMC were stained with Abs against CD3(SK7), CD56 (NCAM16.2), CD16 (NKP15), CD8 (HIT8a), CD161 (DX12), ILT2 (GH1/75), CD57 (HNK-1), DNAM-1 (DX11), NKp46 (9E2), Granzyme A (CB9), perforin (γG9) (BD Biosciences), NKG2C (134,591) (R&D Systems), NKp44 (Z231), NKp30 (Z25), NKG2A (Z199), KIR2DL1/S1 (EB6), KIR2DL2/3/2DS2 (GL183), KIR3DL1/S1 (Z27), HLA-DR (Immu357) (Beckman Coulter, Fullerton, CA) and KIR2D (1A6) [3] CD14 (RMO-52) (EFS, Rennes), HLA-A, −B, −C (F41-IE3) (EFS, Nantes). Flow cytometry was performed using a FACSCalibur apparatus with CellQuest software (BD Biosciences) and analyzed using FlowJo 7.6.1 software (Tree Star, Ashland, OR) [18].

2.6. Statistical analysis

All statistical analyses were performed with Prism-6 software (GraphPad Software). The one-way analysis of variance (ANOVA) test was used for comparisons of multiple groups. Dunnett's multiple comparisons test was used as a *post hoc* test for intergroup comparisons. Continuous nonparametric variables are expressed as medians (interquartile range). Significance was defined as P less than 0.05. * $P \ll 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

3. Results

3.1. Population

Healthy donors, traumatic brain-injured (TBI) and major surgery patients are described in Table 1. The median Glasgow Coma Scale score was 7 (6–9) in the brain-injured patients. All TBI patients were mechanically ventilated. During ICU hospitalization, 19 (59%) patients developed hospital-acquired pneumonia. Four (13%) brain-injured patients died in the ICU. In the major surgery population, no patient developed hospital-acquired infection. We used samples from cardiac surgery patients as positive control because this condition represents a reproducible acute condition in which severe systemic inflammatory response syndrome is followed by IS [17,19].

3.2. Monocytes and T cells are altered after TBI

The proportion of monocytes ($CD14^+$ cells in the monocyte gate) was significantly higher in the TBI patients on days 1 and 7 compared with the healthy controls (HC) and major surgery control patients

Table 1
Demographic characteristics.

	Healthy controls (n = 29)	Traumatic brain-injured patients(n = 32)	Major surgery (n = 11)
Age, years, *median (25–75th percentiles)*	35 (23–57)	52 (25–60)	78 (66–80)
Male, *number* (%)	18 (62)	24 (75)	6 (55)
Medical history, *number* (%)	NA		
Immunosuppression		0 (0)	0 (0)
Diabetes mellitus		0 (0)	2 (18)
Chronic heart failure		0 (0)	0 (0)
Chronic renal failure		0 (0)	0 (0)
Alcoholism		6 (19)	0 (0)
Nicotine addiction		10 (31)	2 (18)
Severity on admission, *median (25–75th percentiles)*	NA		
Apache-II		40 (34–53)	NA
SOFA score		8 (7–10)	NA
Glasgow Coma Scale		7 (6–9)	NA
ASA score		NA	3 (3–3)
Trauma severity			
Injury Severity Score		20 (15–39)	NA
Abbreviated Injury Score			
Head–neck		4 (4–5)	NA
Face		1 (0–3)	NA
Thorax		0 (0–1)	NA
Abdomen		0 (0–1)	NA
Extremities		0 (0–1)	NA
Skin		0 (0–1)	
Duration of extracorporeal circulation, min, *median (25–75th percentile)*	NA	NA	58 (53–78)
Hospital acquired infection, yes, *number* (%)	NA	21 (66)	0 (0)
Site of hospital acquired infection, *number* (%)			
Pneumonia		19 (59)	0 (0)
Meningitis		0 (0)	0 (0)
Bacteremia		1 (3)	0 (0)
Urinary tract infection		7 (21)	0 (0)
Invasive fungus infection		1 (3)	0 (0)
Time on mechanical ventilation, days, *median (25–75th percentiles)*	NA	12 (7–17)	1 (0–1)
ICU length of stay, days, *median (25–75th percentiles)*	NA	13 (10–22)	1 (1–2)
Death in ICU, *number* (%)	NA	4 (13)	0 (0)
Death at day 90, *number* (%)		4 (13)	0 (0)

ASA: American Society of Anesthesiology, ICU: intensive Care Unit, NA: non-applicable, ND: not done, SOFA: sequential organ failure assessment.

(CP) postoperatively (Fig. 1A). In contrast, the mean fluorescent intensity (MFI) of CD14 was significantly lower on monocytes from TBI patients on days 1 and 7 compared with HC and CP pre- and postoperatively (Fig. 1B–D). We found a significant down-expression of HLA-DR molecules on TBI monocytes on days 1 and 7 compared with HC and CP preoperatively (Fig. 1C–D). In parallel, the frequency of NK cells determined as $CD3^-$ $CD56^+$ cells in lymphocyte gate (Fig. 1E) was similar in TBI patients and HC regardless of the post-BI kinetic. Moreover, T lymphocyte ($CD3^+$ $CD56^-$) frequency was significantly lower in TBI patients on days 1 and 7 compared with HC (Fig. 1E). The expression of granzyme A (Fig. 1F–H) and perforin (Fig. 1G–H) in T lymphocytes was significantly lower in TBI patients compared with HC on days 1 and 7. These results demonstrate that both monocytes and T cells compartments are severely impaired in TBI patients.

3.3. The expression of HLA class I molecules on monocytes was dramatically decreased in TBI patients

In this immune-depressed context marked by HLA-DR down-regulation and T cell alterations, we investigated the expression of HLA class I molecules. These molecules are not only essential to trigger T cell lymphocytes to defend the organism against pathogens but also crucial to NK cell activation which recognize altered or absent HLA class I molecules in different contexts (stress, viral infection, tumoral process and allogeneic cell/organ transplantation). Interestingly, HLA class I molecules, which are highly expressed on HC monocytes, were dramatically down-expressed on TBI monocytes on days 1 and 7 (Fig. 2A). In contrast, the expression of HLA class I molecules (Fig. 2A) was not altered in CP before and after surgery and the down-expression of HLA class I was specific to TBI (Fig. 2A–B). As expected, NK cells were activated, and the frequency of $CD69^+$ NK cells (19.5 ± 4.1, $n = 16$) was significantly increased in TBI patients compared to the control counterpart (7 ± 1.1, $n = 14$, $p = 0.01$). However, no correlation between $CD69^+$ NK cell frequency and HLA class I MFI on monocytes was observed in our study.

3.4. HLA class I deficiency on monocytes is associated with an increased KIR^+ NK cells frequency in TBI patients

Specific inhibitory receptors interact with HLA class I molecules to prevent attack of normal cells by NK cells whereas cells with altered HLA class I molecule expression will be destroyed. Different types of inhibitory receptors are described: the KIR family of receptors and the CD94/NKG2A or ILT2 molecules. In an attempt to determine whether HLA downregulation impacts the immunobiology of TBI NK cells, we evaluated the expression of the different HLA specific inhibitory NK cell receptors by flow cytometry. Interestingly, $KIR2D^+$ (KIR2DL1/2/3/S1/2) NK cell frequency was inversely correlated with HLA class I expression on monocytes (Fig. 2C) whereas no correlation was apparent in CP patients (Fig. 2D). We observed a trend toward an increase of $KIR2D^+$ NK cell frequency in TBI patients compared with HC (Fig. 2E–F). However, a significantly higher frequency of NK cells co-expressing KIR and NKG2A (Fig. 2E–G), two HLA specific inhibitory receptors, was observed in TBI patients without increased frequency of $NKG2A^+$ NK cells in TBI patients (data not shown). In contrast, no significant differences were observed for ILT2 expression in TBI patients (data not shown).

3.5. The NK cell repertoire is characterized by a higher frequency of late differentiated NK cell subset

The next step was to investigate different markers of differentiation leading to better characterization of the NK cell repertoire in TBI patients. NK cells in TBI patients express CD57 with a significantly higher frequency on day 1 than HC subjects (Fig. 3A). Concerning the activating NK receptors, we highlighted a significantly decreased frequency of $CD16^+$ NK cells (Fig. 3B), a lower expression of NKp46 (Fig. 3C) and finally a decreased expression of DNAM-1 on TBI NK cells compared with HC (Fig. 3D) as illustrated in Fig. 3E–F for representative individuals. No significant differences were noted in the studied groups concerning other studied NK receptors such as CD161, NKG2C, NKp30, NKp44 and NKG2D (data not shown). Overall, the phenotype of TBI NK cells marked by KIR and CD57 expression and lower level of activating NKp46 and DNAM-1 reflects a differentiated state [2,20].

3.6. The cytotoxic capacities of NK cells are impaired in TBI patients

In order to follow up the phenotypic study of the functional abilities of TBI patient NK cells, we evaluated ex vivo cytotoxic enzyme (perforin and granzyme A) expression by flow cytometry. Perforin expression in NK cells was significantly lower not only in TBI on days 1 and 7 but also in CP compared with HC (Fig. 4A). However, we observed a significantly decreased expression of granzyme A in the NK cells which was specific to TBI patients (Fig. 4B). It should be noted that in the TBI patients, granzyme A level expression in the NK cells was correlated

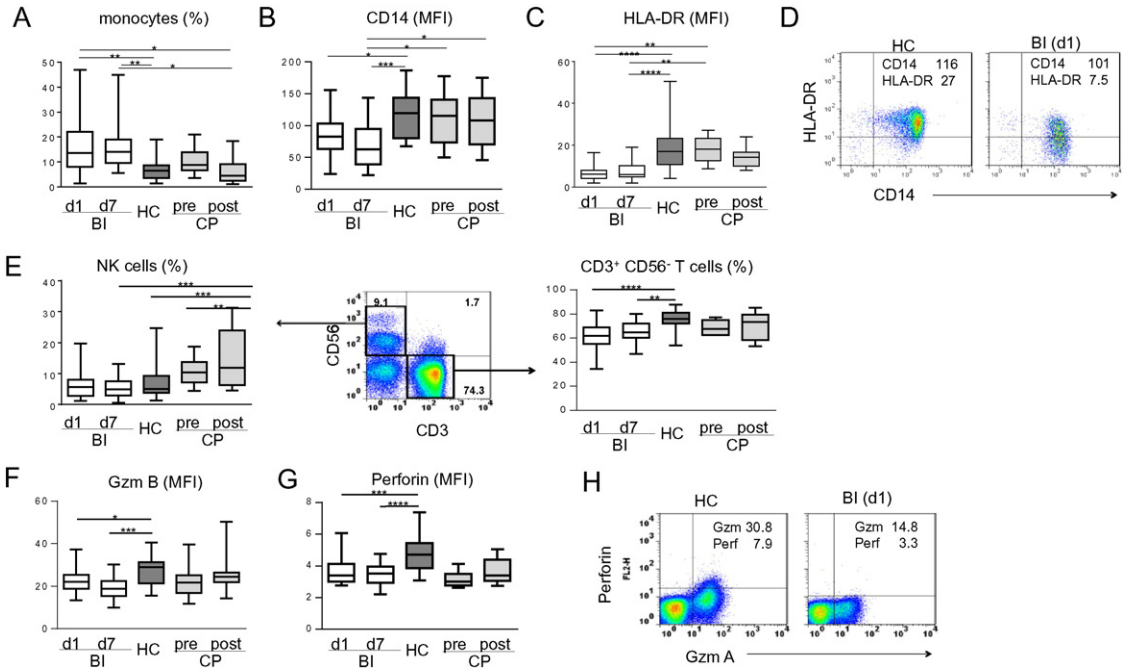

Fig. 1. The immune profile of TBI patients resembles sepsis-induced immunosuppression. Summary box and whisker plot summarizing (A) the percentages of monocytes, (B) the CD14 MFI and (C) HLA-DR-MFI on monocytes in TBI on days 1 (n = 17–23) and 7 (n = 17–23), HC (n = 21–23) and CP pre- (n = 9) and postoperatively (n = 9). Top and bottom whiskers represent the values of the top and bottom 25% of cases, respectively; boxed area, interquartile range. (D) Representative density plots illustrating CD14 and HLA-DR expression on monocytes from HC and BI patient at day 1. The MFI for each marker is indicated on the density plots. (E) Representative density plot illustrating both $CD56^+$ $CD3^-$ NK cells and $CD3^+$ $CD56^-$ T lymphocytes within lymphocyte population gated following FSC/SSC characteristics. Summary box and whisker plot summarizing the percentages of NK cells and T lymphocytes in TBI on days 1 (n = 23) and 7 (n = 22–23), HC (n = 23) and CP pre- (n = 10–11) and postoperatively (n = 10–11). Summary box and whisker plot summarizing (F) granzyme A and (G) perforin MFI in T lymphocytes ($CD3^+$ $CD56^-$) from TBI on days 1 (n = 23) and 7 (n = 21), HC (n = 21) and CP pre- (n = 10) and postoperatively (n = 10). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$. (H) Representative density plots illustrating granzyme A and perforin expression on $CD3^+$ T lymphocytes from HC and BI patient at day 1. The MFI for each marker is indicated on the density plots.

with $CD16^+$ NK cell frequency (Fig. 4C) but it was inversely correlated with KIR^+ NK cell frequency on days 1 and 7 (Fig. 4E); no correlation was apparent in CP patients (Fig. 4D and F). These data suggest a different impact of the impaired granzyme A phenotype on antibody-dependent cytotoxicity (ADCC) or on spontaneous lysis.

3.7. NK cells from TBI patients are hyporesponsive in response to missing self

Based on our phenotypic study of NK cells, we hypothesized that the shaping of the NK-cell repertoire in TBI could lead to hyporesponsiveness

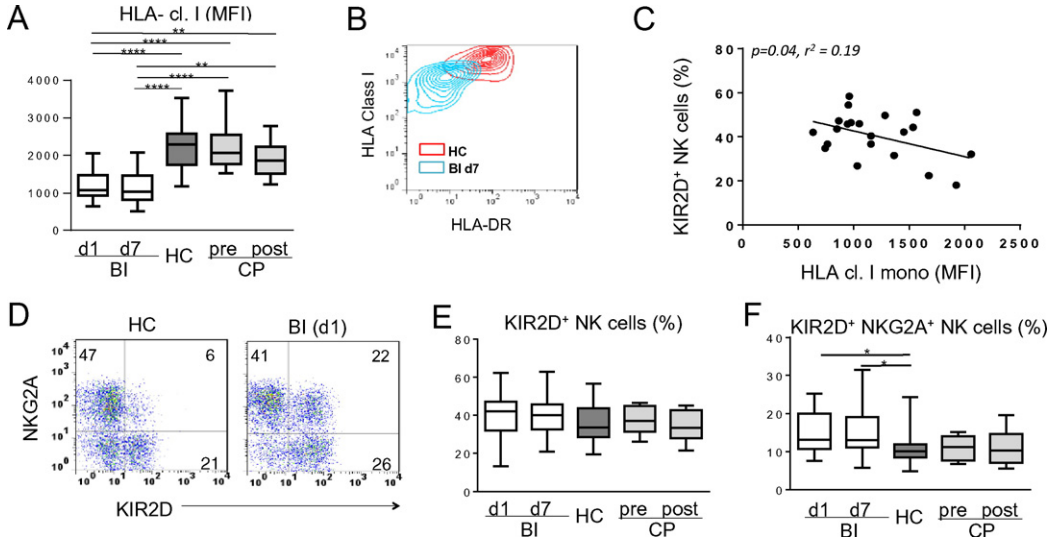

Fig. 2. HLA class I deficiency on monocytes is associated with an increased KIR^+ NK-cell frequency in TBI patients. Summary box and whisker plot summarizing (A) HLA class I MFI on monocytes in TBI on days 1 (n = 22) and 7 (n = 22), HC (n = 21) and CP pre- (n = 9) and postoperatively (n = 9). Top and bottom whiskers represent values of the top and bottom 25% of cases, respectively; boxed area, interquartile range. (B) Representative staining of HLA class I and HLA-DR molecules in NK cells from HC and TBI patients on day 7. (C) Correlation between expression of KIR^+ (KIR2D) NK cells (%) in NK cells and HLA class I expression (MFI) on monocytes from 18 TBI patients on day 1 and (D) from CP pre- (n = 11) and postoperatively (n = 11). (E) Representative density plots illustrating KIR2D and NKG2A expression on NK cells from HC and BI patient at day 1. Summary box and whisker plot summarizing (F) the percentages of $KIR2D^+$ NK cells and (G) $KIR2D^+$ $NKG2A^+$ NK cells in NK cells in TBI on days 1 (n = 22–23) and 7 (n = 22), HC (n = 22–23) and CP pre- (n = 10) and postoperatively (n = 10). $^*P < 0.05$, $^{**}P < 0.01$ and $^{****}P < 0.0001$.

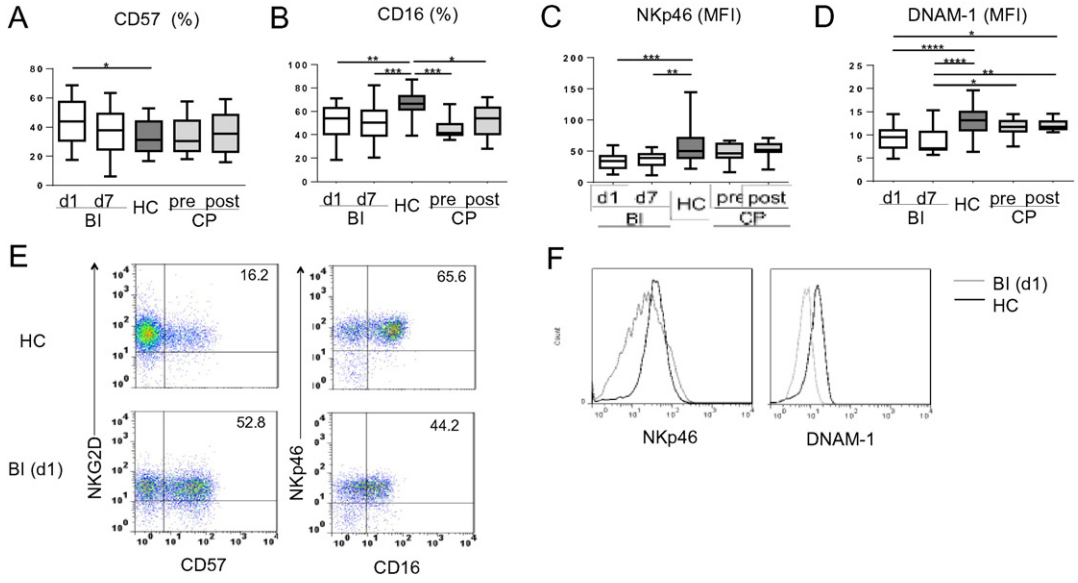

Fig. 3. The NK-cell repertoire is characterized by a higher frequency of late differentiated NK cell subset. Summary box and whisker plot summarizing the percentages (A) of $CD57^+$ NK cells (B) $CD16^+$ NK cells, (C) NKp46 MFI and (D) DNAM-1 MFI in NK cells from TBI on days 1 (n = 21–23) and 7 (n = 21–23), HC (n = 22–23) and CP pre- (n = 10–11) and postoperatively (n = 11). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$. (E) Representative density plots illustrating CD57, NKG2D, CD16 and NKp46 expression and (F) representative histograms illustrating NKp46 and DNAM-1 expression on NK cells from HC and BI patient at day 1.

of TBI NK cells to HLA class I deficient cells. To evaluate the functional ability of NK cells, we determined their (i) cytotoxic capacities (assessed by the CD107 expression, a surrogate marker of degranulation) and (ii) IFN-γ production after activation of spontaneous lysis (Fig. 5A–C) and reverse ADCC (Fig. 5D–F). For this purpose, we stimulated NK cells with 2 cell lines deficient in HLA class I expression (221 and Fc^+ P815 cell lines). Degranulation was significantly decreased in the TBI patients on days 1 and 7 compared with HC (Fig. 5C) in contrast to IFN-γ production (Fig. 5B) which was only decreased in TBI patients on day 7 compared with HC and CP postoperatively. In contrast, the functional abilities to produce IFN-γ (Fig. 5E) and to degranulate (Fig. 5F) after stimulation with specific CD16 antibody (ADCC) were not impaired in the TBI patients. Overall, the hyporesponsiveness of TBI NK cells was only associated with spontaneous lysis and not with reverse ADCC.

3.8. IL-12 restored IFN-γ production and degranulation of TBI NK cells

In NK crosstalk with monocytes and dendritic cells (DCs), the IL-12 produced by both cells triggers IFN-γ production and NK cell functions [19,21,22]. It has been previously observed that depressed IL-12 production by monocytes correlates with altered lymphocyte functions in trauma patients [4,20]. Thus, since IL-12 treatment restores cytokine production and cytotoxicity by NK cells [21–23], we evaluated the impact of IL-12 preincubation on TBI and HC NK cell function cells after activation of spontaneous lysis (Fig. 6A) and reverse ADCC (Fig. 6B) using the HLA class I deficient 221 and Fc^+ P815 cell lines respectively. IL-12 significantly triggered the IFN-γ and degranulation of TBI NK cells against HLA deficient 221 cells, spontaneously (via inhibitory receptors) (Fig. 6A) and via the ADCC pathway (Fig. 6B). The impact of IL-12 in

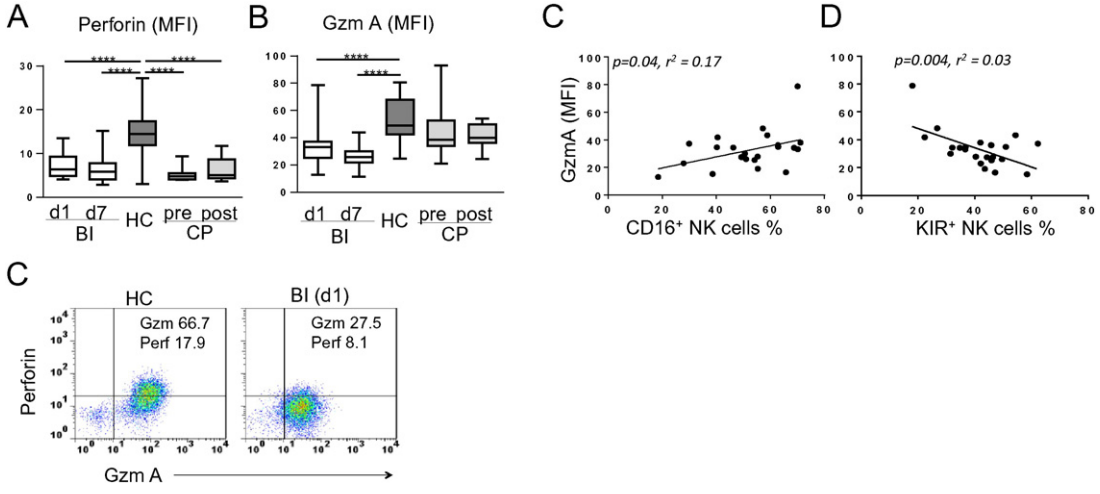

Fig. 4. Cytotoxic capacities of NK cells are impaired in TBI patients. Summary box and whisker plot summarizing (A) perforin and (B) Granzyme A MFI in NK cells from TBI on days 1 (n = 23) and 7 (n = 21), HC (n = 21) and CP pre- (n = 10) and postoperatively (n = 10). Top and bottom whiskers represent the values of the top and bottom 25% of cases, respectively; boxed area, interquartile range. (C) Representative density plots illustrating granzyme A and perforin expression on NK cells from HC and BI patient at day 1. The MFI for each marker is indicated on the density plots. Dot representation of granzyme A MFI in NK cells **(D)** as a function of $CD16^+$ NK cell frequencies NK cells in TBI patients (n = 23) and **(E)** in CP pre- (n = 11) and postoperatively (n = 11); (F) as a function of KIR^+ ($KIR2D^+$) NK-cell frequencies NK cells in TBI patients (n = 22) and (G) in CP pre- (n = 11) and postoperatively (n = 11). $^{****}P < 0.0001$.

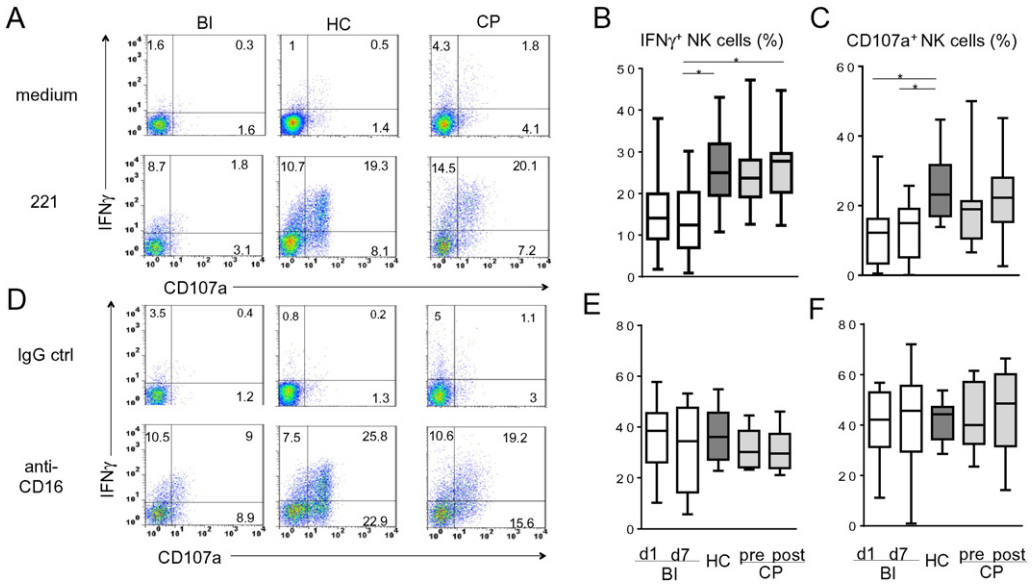

Fig. 5. TBI patient NK cells are hyporesponsive in response to missing self. (A) Representative density plots illustrating CD107a expression and IFN-γ production by NK cells from one representative TBI, HC and CP individual in a 5-h functional assay following the PBMC: target ratio of 1:1 with medium and 221 target cell line to determine spontaneous lysis. NK cells were gated as $CD56^+$ $CD3^-$ cells in the lymphocyte gate. Summary box and whisker plot summarizing the percentages of (B) $IFN\gamma^+$ NK cells and (C) $CD107a^+$ NK cells following spontaneous lysis from TBI on days 1 (n = 13) and 7 (n = 12), HC (n = 14) and CP pre- (n = 11) and postoperatively (n = 11). (D) Representative density plots illustrating CD107a expression and IFN-γ production by NK cells from one representative TBI, HC and CP individual in a 5-h functional assay following the PBMC: target ratio of 1:1 with medium and Fc^+ P815 cell line with IgG control or CD16 specific mAb to determine the reverse ADCC from TBI on days 1 (n = 13) and 7 (n = 12), HC (n = 16) and CP at pre- (n = 11) and postoperatively (n = 11). Summary box and whisker plot summarizing the percentages of (E) $IFN\gamma^+$ NK cells and (F) $CD107a^+$ NK cells following the reverse ADCC pathway. $^*P < 0.05$.

triggering NK-cell functions was evaluated by comparison of the fold increased $IFN\gamma^+$ $CD107a^+$ NK cell frequency determined for TBI on days 1 and 7 versus HC via the spontaneous lysis and reverse ADCC pathways (Fig. 6C). Interestingly, IL-12 treatment was particularly efficient on hyporesponsive TBI NK cells via spontaneous lysis when compared with HC NK cells which is consistent with the impaired missing-self recognition of TBI NK cells previously highlighted in our study.

4. Discussion

In the present study performed on severe TBI patients, we observed for the first time a significantly decreased expression of HLA class I on monocytes as well as severe impairment of NK-cell functions. Most of these alterations lasted 7 days. Pre-incubation with IL-12 was able to restore IFN-γ production and the cytotoxicity capacities of NK cells. This cytokine may therefore be considered as a potential treatment candidate in TBI patients with IS.

We first monitored HLA-DR expression on monocytes, the landmark of immunosuppression after BI [4,23–25]. HLA-DR is probably the most studied and accurate biomarker of IS currently available in ICU patients [24,26]. Despite an increased number of circulating monocytes, their membrane expression of CD14 and HLA-DR were severely impaired after TBI compared with healthy volunteers and with cardiac surgery patients.

During their development, NK cells acquire functional capacities via the engagement of their inhibitory KIR with cognate HLA ligands [25,27]. This specific interaction allows NK cells to be "licensed" to become functionally competent and to acquire effector functions [2,26]. This functional education is also essential to maintain self-tolerance. In the present experiment, the drastically decreased expression of HLA class I molecules observed on monocytes was correlated with an increased expression of $KIR2D^+$ NK-cell frequency in TBI patients, and NKG2D may play a role in inflammatory diseases [28]. It has been previously shown that the absence of HLA ligand favors the outgrowth of KIR NK cells [27,29] and it is conceivable that the NK repertoire is skewed by the decreased expression of HLA class I ligands in a TBI context. Even though the frequency of KIR2D + NK cells was not significantly increased in the TBI patients compared with the controls, the NK-cell subset co-expressing KIR2D and NKG2A (HLA specific inhibitory receptors) was preferentially represented in the TBI patients. Of note, NK cells from TBI patients present a late state of differentiation marked by CD57 and KIR expression and a lower expression of activating receptors such as

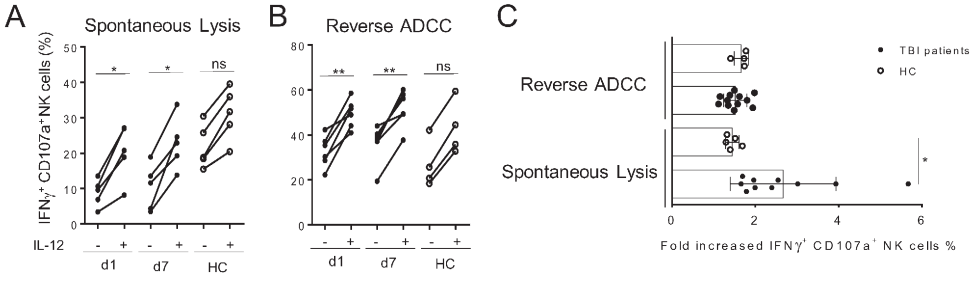

Fig. 6. IL-12 restored IFN-γ production and degranulation of TBI NK cells. Scatter plots representing $IFN\gamma^+$ $CD107a^+$ NK cells in a 5-h functional assay following the PBMC: target ratio of 1:1 to determine (A) spontaneous lysis with and without O/N treatment with IL-12 of PBMC of TBI on days 1 (n = 5) and 7 (n = 5) and HC (n = 5) and (B) reverse ADCC with and without O/N treatment with IL-12 of PBMC of TBI on days 1 (n = 6) and 7 (n = 6) and HC (n = 4). (C) Bars indicate the mean of the fold increased $IFN\gamma^+$ $CD107a^+$ NK cell frequencies (with IL-12/without IL-12 ratio) for spontaneous lysis (10 TBI and 5 HC) and reverse ADCC (12 TBI and 4 HC). $^*P < 0.05$.

NKp46 and DNAM-1 [2,30]. Interestingly, a higher frequency of this CD57 + KIR + NKG2A + NK cell subset was described in TAP (Transporter associated with Antigen Processing) deficient patients who express a low level of HLA class I molecules [29,31]. Overall, the phenotypic alterations described in TBI patients suggest an impaired functional activity of NK cells from TBI patients.

Since TBI NK cells show alterations in the expression of both inhibitory and activating receptors, we evaluated the two major effector functions of NK cells which are: (i) cytotoxicity associated with degranulation and extracellular release of cytolytic enzymes (granzyme and perforine) and, (ii) production of cytokines. The functional profile of TBI NK cells is characterized by poor IFN-γ response and reduced degranulation in response to HLA deficient target cells. These results are line with those of Souza-Fonseca-Guimaraes et al. [2,30] demonstrating that the ex vivo production of IFN-γ by NK cells is impaired in septic patients. However, the low level of cytotoxic granule molecules cannot completely explain the hyporesponsiveness of TBI NK cells since they had good ADCC against the cell target P815. Indeed, although there was a decreased frequency of CD16 + NK cells in TBI patients, degranulation and IFN-γ production following stimulation with anti-CD16 were similar to those observed in control individuals. This observation could be explained by a consistent level of CD16 on differentiated CD57 + KIR + NK cells which confer enhanced responsiveness [31]. Another explanation for the lower responsiveness against HLA deficient target could be associated with the higher frequency of late differentiated NK cells in TBI patients. Indeed, it has been previously shown that the differentiated status of NK cells is associated with a loss of functionality [2,10,11]. It was proposed that the more differentiated (CD57 + KIR +) NK-cell subset expresses lower levels of major signaling unit for IL-2, IL-15 and IL-18 [2–4] linked to hyporesponsiveness after cytokine stimulation. On the contrary, in other acute conditions, patients displayed a high ADCC of their NK cells [9–11] underlining the fact that the NK-cell functional impairment observed in TBI patients is somehow specific compared with other ICU patients.

Antigens presenting cells positively interact with NK cells through the production of cytokines such as IL-12, IL-15, and IL-18 [9,22]. In the present experiments, the strong response to ex vivo IL-12 therapy shows that the IL-12 pathway remains functional in circulating NK cells. IL-12 increases the production of IFN-γ by NK cells, stimulates cytotoxicity of activated NK cells, and enhances ADCC against abnormal cells [22,32]. In the setting of bacterial infections, it was demonstrated that NK cells naturally internalize the bacterial pathogen-associated molecular pattern muramyl dipeptide and the adjunction of IL-12 stimulates the production of IFN-γ [32,33] Moreover, in conjunction with IL-15, IL-12 is responsible for the non-antigen-specific IFN-γ production in CD8 T cells in response to *Listeria monocytogenes* infection [33,34]. It was recently demonstrated that human NK cells exhibit memory-like functions (see reference (34,35) for review). Interestingly, memory-like NK cells are able to produce more IFN-γ than naive NK cells [5,35] and this phenomenon appears to be highly dependent on IL-12 signaling. This critical feature provides a new rationale for using reactivation with IL-12 in NK-cell immunotherapy protocols. DCs, particularly conventional DC, are the main producers of IL-12. We previously found that the number and functions of DC were impaired in BI patients with subarachnoid hemorrhage [5,36–39]. In particular, the production of IL-12 by conventional DC through TLR3/4 stimulation was dramatically decreased compared with that of healthy donors. These data strengthen the need for an exogenous administration of IL-12 since endogenous production is impaired.

Our study has several strengths including a global characterization of both the phenotype and effector functions (cytotoxicity and IFN-γ production) of NK cells. In addition, when a single early sampling time was reported in other studies, our kinetic suggested that NK-cell impairment lasts during the period in which nosocomial infections occur (within the first week of ICU hospitalization). Moreover, to underscore the specific features of TBI-induced immunosuppression, we used samples from cardiac surgery patients as positive controls. Finally, we propose IL-12 as a new potential treatment available to overcome NK cell alterations.

Some limitations should be mentioned. First, we studied a limited number of patients, and the clinical consequences of the immunological impairment described here could not be assessed. In particular, our preliminary results cannot support a direct correlation between a decreased class I expression and the neurological evolution after traumatic brain injury. Furthermore, the analyses were performed on frozen samples and not on fresh cells. However, considering the large number of analyses and the validated methods used [36–40], technical biases should be limited. We did not explore patients for genetic NK-cell immunodeficiency but the patients included had no history of severe viral infections, notably in childhood. Treatment with exogenous cytokine may induce side effects or immune deregulation, and further studies are needed before proposing IL-12 treatment in TBI patients. Finally, we explored circulating NK cells but the status of NK cells from tissues could be different [40].

In conclusion, this study provides the first extensive description of the phenotype and functions of NK cells in TBI patients. We found that TBI-induced immune suppression is characterized by a terminally differentiated phenotype of NK cells (high expression of inhibitor receptors and dampened cytotoxic ability). Finally, our results advocate for a further evaluation of IL-12 treatment to overcome IS-induced nosocomial infections in TBI patients.

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Authors' contributions

Contribution: A.R. and G.D. designed and performed the majority of experiments, analyzed data, and wrote the manuscript. R.C. and M.V. analyzed data, and wrote the manuscript. HM, JBP, and BR provided patient materials and analyzed data. C.R. and K.A. developed and supervised the entire project, designed experiments, interpreted data, and wrote the manuscript.

Conflict of interest disclosure

The authors declare no competing financial interests.

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