Cellular Immunology 335 (2019) 6–14

Contents lists available at ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

NK cells suppress CD8$^+$ T cell immunity via NKG2D in severe aplastic anemia

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ARTICLE INFO

Keywords:
Natural killer cells
Cytotoxic T cells
Autoimmunity

ABSTRACT

The roles of natural killer (NK) cells in shaping the immune system had raised wide interests. Here we intended to explore the regulatory functions of NK cells on CD8$^+$ T cells in severe aplastic anemia (SAA) using human participants and lymphocyte infusion-induced bone marrow failure (BMF) mouse model. In SAA patients, NK cells had over-expressions of NKG2D and NKp46, under-expression of NKG2A and enhanced cytotoxicity. NK cells limited autologous CD8$^+$ T cell immunity in an effector/target ratio manner. The suppression was dependent on the existence of NKG2D. We also observed upregulated MICA expression on activated CD8$^+$ T cells, which were susceptible to NK cell mediated lysis in SAA. Animal model concurred with the data from patients. Infusion of NK cells suppressed the proliferation of CD8$^+$ T cells and decreased IFN-γ production. In conclusion, NK cells served NKG2D-dependent immunoregulatory roles by attenuating autologous CD8$^+$ T cell response in SAA.

1. Introduction

Severe aplastic anemia (SAA) is an autoimmune bone marrow failure disease featuring a profound diminution of bone marrow progenitor cells, causing extreme pancytopenia [1]. Suffering from SAA, which characterizes acute onset, rapid progression and high mortality, patients often died of severe bleeding or infection. The pathophysiology is immune mediated, with activated cytotoxic T cells implicated in most cases [2]. Standard immune suppressive therapy (IST) with anti-human thymocyte globulin (ATG) and Cyclosporine A (CsA) is effective in 60–70% of SAA patients [3], as ATG and CsA appear to eliminate and functionally suppress activation of expanded CD8$^+$ T cell clones [4]. However, patients may still develop relapse due to discontinuation of CsA [5] or the reactivation of CD8$^+$ T cell oligoclones. Up to date, the specific mechanism of self-regulation of T cell immunity in SAA has not been fully elucidated.

Natural killer (NK) cells are a group of heterogeneous lymphocytes, important in innate and adaptive immune system. It is gradually acknowledged that NK cells, as immune-modulators, interact with T cells and dramatically alter their immune responses [6] in a variety of diseases including viral infection and antoimmunity. On one hand, NK cells positively regulate CD8$^+$ T cell function against antigens [7], on the other, some reports have also shown that NK cells inhibit CD8$^+$ T cell responses in murine cytomegalovirus infection [8,9]. The influence was allegedly via interactions with NK receptors NKG2D or NKp46, perforin-dependent manner or cytokine secretions [10]. The roles of NK cells in SAA had been noticed [11]. However, there is limited attention to whether NK cells influence CD8$^+$ T cells in SAA. Previously, we have reported our preliminary studies on the abnormalities of quantities and functions of NK cells in SAA [12]. Here we focused the impact of NK cells on CD8$^+$ T cells and the mechanism responsible for it.

2. Materials and methods

2.1. Participants

Twenty patients with untreated SAA who showed complete response to immune suppressive therapy (IST) (SAA-CR) were enrolled in

Abbreviations: ATG, Anti-human Thymocyte Immunoglobulin; BMF, Bone Marrow Failure; CBC, Complete Blood Count; CsA, Cyclosporine A; ELISA, Enzyme Linked Immunosorbent Assay; FCM, Flow Cytometry; HC, Healthy Controls; IST, Immune Suppressive Therapy; LDH, Lactate Dehydrogenase; mAbs, Monoclonal Antibodies; MFI, Mean Fluorescence Intensity; MICA, MHC class I Chain-related protein A; NK, Natural Killer; NKG2DL, NKG2D ligand; PMA, Phorbol 12-Myristate 13-Acetate; qRT-PCR, Quantitative Real-Time PCR; SAA, Severe Aplastic Anemia; SAA-CR, SAA in Complete Response to IST

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https://doi.org/10.1016/j.cellimm.2018.10.004
Received 25 September 2018; Received in revised form 11 October 2018; Accepted 12 October 2018
Available online 12 October 2018

the Hematology Department of General Hospital Tianjin Medical University from Jan 2015 to Dec 2017. IST referred to the combination of rabbit anti-human thymocyte immunoglobulin (ATG) (Genzyme Polyclonals S.A.S., France) and Cyclosporine A (CsA) (R.P. Scherer GmbH & Co. KG, Germany). The diagnosis was according to international AA Study Group Criteria [13]. SAA-CR required transfusion independence. Another 20 healthy controls (HC) volunteered to participate in the research. Participants were excluded if they had cancers, infections, congenital or other autoimmune diseases such as paroxysmal noctunal hemoglobinuria or myelodysplastic syndrome. The research was ethically approval by Tianjin Medical University General Hospital. Written informed consents were obtained from all individuals or guardians of the minors.

2.2. Bone marrow failure (BMF) mice

CB6F1 mice (strain: 303, 8 weeks old, SPF grade) and C57BL/6 mice (HFK Bioscience Co. Ltd., China) were used to build the BMF mouse model. CB6F1 mice received a total body irradiation (TBI) dose of 4 Gy before infusion of 5×10^7 lymph node cells from C57BL/6 mice. Meanwhile, some CB6F1 mice received allogeneic 10^7 NK cells transfusion to establish BMF + NK mouse model. Control (CB6F1) mice received no TBI or lymphocytes, but same amount of normal saline. Peripheral blood and bone marrow from all mice in control group, BMF group and BMF + NK group were obtained on day 17 since irradiation. All animal experiments were approved by the Institutional Animal Care and Use Committee of the National Institute of State Scientific and Technological Commission.

2.3. Flow cytometry (FCM)

Fresh drawn peripheral blood from participants and mice was processed and stained with specific fluorochrome-conjugated monoantibodies (mAbs) to perform FCM analysis. Monoclonal antibodies (mAbs) for human including PerCP CD3 (SP34-2), APC CD56 (B159), FITC CD16 (3G8), PE NKp46 (9E2), PE NKp44 (p44-8), PE NKG2D (1D11), PE CD158a (HP-3E4), PE CD158b (CH-L), PE Perforin Reagent Set, PE GranzymeB (GB11), APC CD8 (RPA-T8), PE IFN-γ (4S.B3) and for murine including APC CD3 (17A2), FITC CD8 (53–6.7), PE CD4 (H129.19) were purchased from BD Pharmingen (Franklin Lakes, NJ, USA). Human PE NKG2A (FAB1059P) and anti-MICA (MAB1300) was purchased from R&D Systems (Minneapolis, USA).

NK cells were identified as $CD3^-CD56^+$ lymphocytes in humans and $NK1.1^+TCR\beta^-$ cells in mice. Intracellular IFN-γ was detected before cells were incubated with Brefeldin A (1 μg/mL) (R&D Systems) to inhibit cytokine exocytosis at 37 °C for 6 h. Multiparameter flow cytometry using BD FACS-Calibur Flow Cytometer and BD FACS-Canto II Flow Cytometer (BD Biosciences, USA) was performed, and data were analyzed using FlowJo software 7.6.1 (TreeStar).

2.4. Cell purification and activation

Peripheral NK cells were separated using NK Cell Isolation Kit (Miltenyi Biotec, Germany) and activated with rhIL-2 (1000 IU/mL) (Minneapolis, USA) for 72 h. Peripheral $CD8^+$ T cells were isolated using anti-CD8 mAb-conjugated microbeads (Miltenyi Biotec, Germany) and activated with anti-CD3/CD28 beads (1:1 ratio) (Life Technologies, USA) and phorbol 12-myristate 13-acetate (PMA) (25 ng/ml)/ionomycin (1 μg/ml) (R&D Systems) for 72 h. The harvested cells were used for subsequent co-culture experiments. In some experiments, to block NKG2D, immobilized human Fc-NKG2D/CD314 antibody (Clone 149810, R&D Systems) or isotype control were added to NK cells 30 min before co-culture.

Table 1
Characteristics of Patients and Healthy Controls.

characteristics	SAA (N = 20)	SAA-CR (N = 20)	HC (N = 20)
Male/Female	10/10	10/10	9/11
Age (year)	35.5 (9–69)	40 (11–70)	34 (18–64)
Chromosome	Male 46, XY [23] Female 46, XX [23]	Male 46, XY [23] Female 46, XX [23]	Male 46, XY [23] Female 46, XX [23]
Treatment	Untreated	IST	No
HB (g/L)	78.05 ± 12.36*,^	114.60 ± 30.77	124.80 ± 8.80
RBC (10^{12}/L)	2.45 ± 0.38*,^	3.56 ± 1.08	4.03 ± 0.27
PLT (10^9/L)	9 (1, 19)*,^	78 (14, 241)*	242 (139, 418)
WBC (10^9/L)	2.25 ± 1.52*,^	7.71 ± 3.65	6.36 ± 1.55
ANC (10^9/L)	0.97 ± 1.11*,^	4.85 ± 3.30	4.32 ± 1.39
Ret (10^9/L)	23.34 ± 18.19*,^	84.91 ± 32.82	66.74 ± 61.29
Ret (%)	0.99 ± 0.77*,^	2.59 ± 1.26*	1.86 ± 2.43
CD4/CD8	0.63 ± 0.36*,^	0.97 ± 0.44*	1.63 ± 0.60

Note: SAA: Untreated severe aplastic anemia, SAA-CR: SAA patients who showed complete response after immunosuppressive therapy (IST), HC: Healthy control, HB: Hemoglobin, RBC: Red blood cell, PLT: Platelet, WBC: White blood cell, ANC: Absolute neutrophil count, Ret: Reticulocyte, CD4/CD8: the ratio of $CD4^+$ T cells and $CD8^+$ T cells in peripheral $CD3^+$ T cells.
* $p < 0.05$, compared with HC.
^ $p < 0.05$, compared with SAA-CR.

2.5. NK cell cytotoxicity assay

NK cell cytotoxicity was evaluated using the standard lactate dehydrogenase (LDH) release assay. LDH Cytotoxicity Assay Kit was purchased from ThermoFisher. The target K562 cells (obtained from the Chinese Academy of Medical Sciences & Peking Union Medical College, China) were incubated with effector NK cells from patients with SAA, SAA-CR or HC for 4 h at 37 °C in an atmosphere containing 5% CO_2. NK cells at 1×10^6/mL, 3×10^6/mL, 1×10^7/mL, 3×10^7/mL and 9×10^7/mL were seeded into a 96-well plate, K562 cells (1×10^6/mL) were added to NK cells to provide effector/target ratios of 1:1, 3:1, 10:1, 30:1 and 90:1. NK cell cytotoxicity percentage was calculated as: (experimental LDH release − spontaneous LDH release − LDH from NK cells)/(maximal LDH release − spontaneous LDH release) × 100 (%).

2.6. Cell apoptosis assay

To quantify the apoptotic rates of $CD8^+$ T cells in cocultures, human APC CD8 (RPA-T8) and FITC Annexin V Apoptosis Detection Kit II (BD Pharmingen) were used according to the manufacturer's instructions. Cell apoptosis was assessed by BD Calibur cytometer.

2.7. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from $CD8^+$ T cells in human participants and converted to cDNA using PrimeScript™ RT reagent Kit (TaKaRa, Japan). The forward and reverse primers were as follows: 5′-TGCTTC TGGCTGGCATCTTCC-3′ and 5′-TAGTTCCTGCAGGCAGTCTGC-3′ for MICA, 5′-TGGACATCCGCAAAGACCTGT-3′ and 5′-CACACGGAGTACT TGCGCTCA-3′ for β-actin. Gene expressions were measured by qRT-PCR on BIO-RAD iQ5 Real-Time System (BIO-RAD, USA), using SYBR Premix Ex Taq (Takara, Japan). Relative expressions of mRNA were normalized to β-actin and presented using a modification of the $2-\Delta\Delta Ct$ method.

2.8. Blood counts and bone marrow biopsy histology in mice

Blood was collected from the retro-orbital sinus of mice to perform complete blood count (CBC) in a pocH-100*i* hematology analyzer (Sysmex, Japan). Femurs from mice were fixed with 4% paraformaldehyde overnight, and sectioned for paraffin embedding. Hematoxylin-Eosin (H&E) staining was performed, and slides were

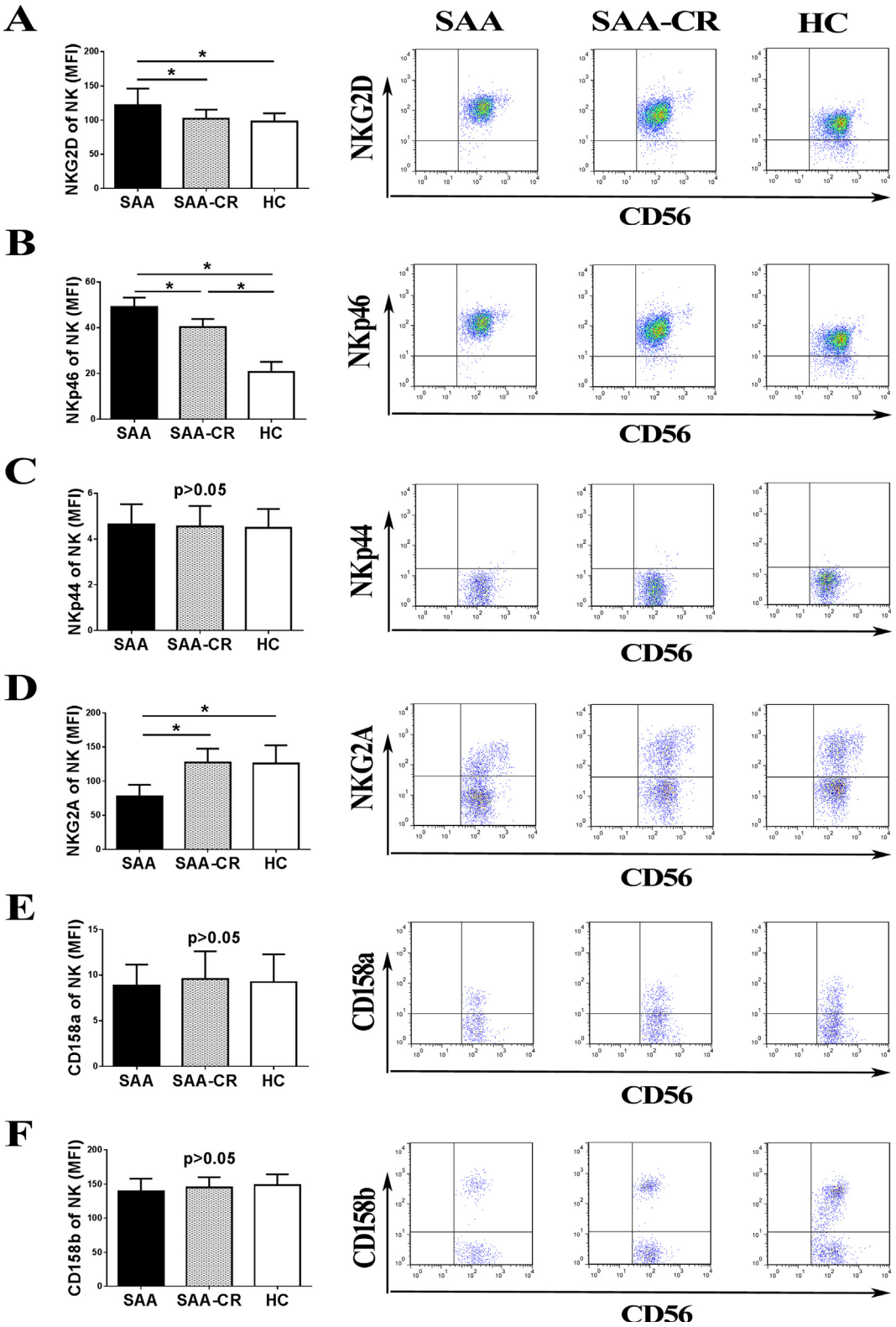

Fig. 1. NK cells are activated in severe aplastic anemia (SAA). $CD3^{-}CD56^{+}$ lymphocytes (NK cells) from patients with SAA, SAA in complete remission (SAA-CR) and healthy controls (HC) were marked by flow cytometry for the surface expression of NKG2D (A), NKp46 (B), NKp44 (C), NKG2A (D), CD158a (E) and CD158b (F). Mean fluorescence intensity (MFI) of the respective receptors are analyzed (n = 20 for each group, $^{*}p < 0.05$).

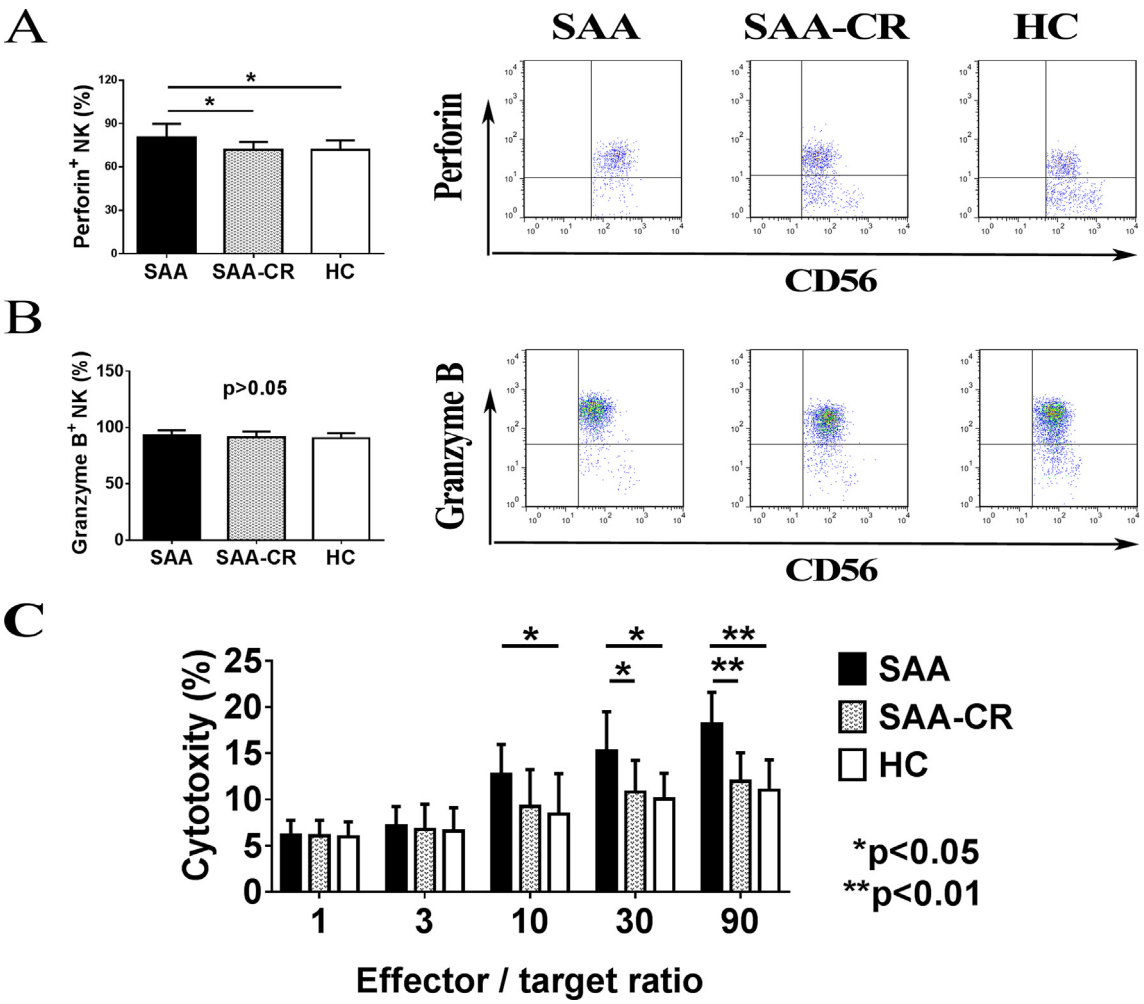

Fig. 2. Cytotoxity of peripheral NK cells is enhanced in SAA. Intracellular proteins including Perforin (A) and Granzyme B (B) were analyzed using flow cytometry in NK cells from patients with SAA, SAA-CR and healthy controls (HC). Positive (%) cells are analyzed. (C). NK cells from patients with SAA, SAA-CR and HC (n = 20 for each group) were incubated with K562 cells at the indicated effector/target ratios. LDH release (mean ± SD) is shown ($^{*}p < 0.05$).

observed with a light microscope (Nikon Diaphot, Tokyo, Japan) with the 10X objective lens.

2.9. Enzyme linked immunosorbent assay (ELISA)

Plasma IFN-γ from mice was measured by ELISA (Cloud-Clone Corp, USA) using a microplate reader (Bio-Rad).

2.10. Statistical analysis

Differences of quantitative parameters between groups were assessed using the *t* test or one-way ANOVA or nonparametric test. Differences of qualitative data were compared using χ^2 test. Significance was considered when $P < 0.05$. The quantitative data were expressed as mean ± SD. The SPSS 19.0 software (IBM, USA) was used for statistical analyses.

3. Results

3.1. NK cells from SAA exhibited over-expressed activating receptors and enhanced cytotoxicity

Patients' clinical characteristics were detailed in Table 1. In order to investigate the status of NK cells in SAA patients, we first examined the surface expression of activating and inhibiting receptors on NK cells. Compared to SAA-CR and HC, expressions of the activating receptors including NKG2D and NKp46 were over-expressed on NK cells from patients with SAA (Fig. 1A–B), while expression of the inhibiting receptor NKG2A was under-expressed (Fig. 1D). A slight increasing trend of activating receptor NKp44 expression (Fig. 1C) and a decreasing trend of inhibitory receptors including CD158a and CD158b expressions (Fig. 1E–F) on NK cells in SAA were also found.

Next we evaluated the cytotoxicity of NK cells in SAA by detecting the intracellular perforin and granzyme B. We found that NK cells from SAA patients produced more perforin than NK cells from HC or SAA-CR (Fig. 2A). No differences of granzyme B^+ NK cells were found among patients with SAA, SAA-CR and HC (Fig. 2B). Furthermore, we examined NK cell mediated lysis towards K562 target cells. As the effector/target ratios increased, NK cells exhibited enhanced cytotoxic activity in SAA compared with SAA-CR and HC (Fig. 2C).

3.2. NK cell mediated lysis towards autologous $CD8^+$ T cells from SAA was dependent on NKG2D

Since NK cells were strongly activated and showed increased cytotoxic function in SAA, we detected whether NK cells could influence $CD8^+$ T cell immunity in SAA. To examine the cytotoxicity of activated NK cells on autologous $CD8^+$ T cells, we coincubated rhIL-2 stimulated NK cells and anti-CD3/CD28 and PMA/ionomycin activated $CD8^+$ T cells from SAA and HC under different effector/target ratios. Seventy-

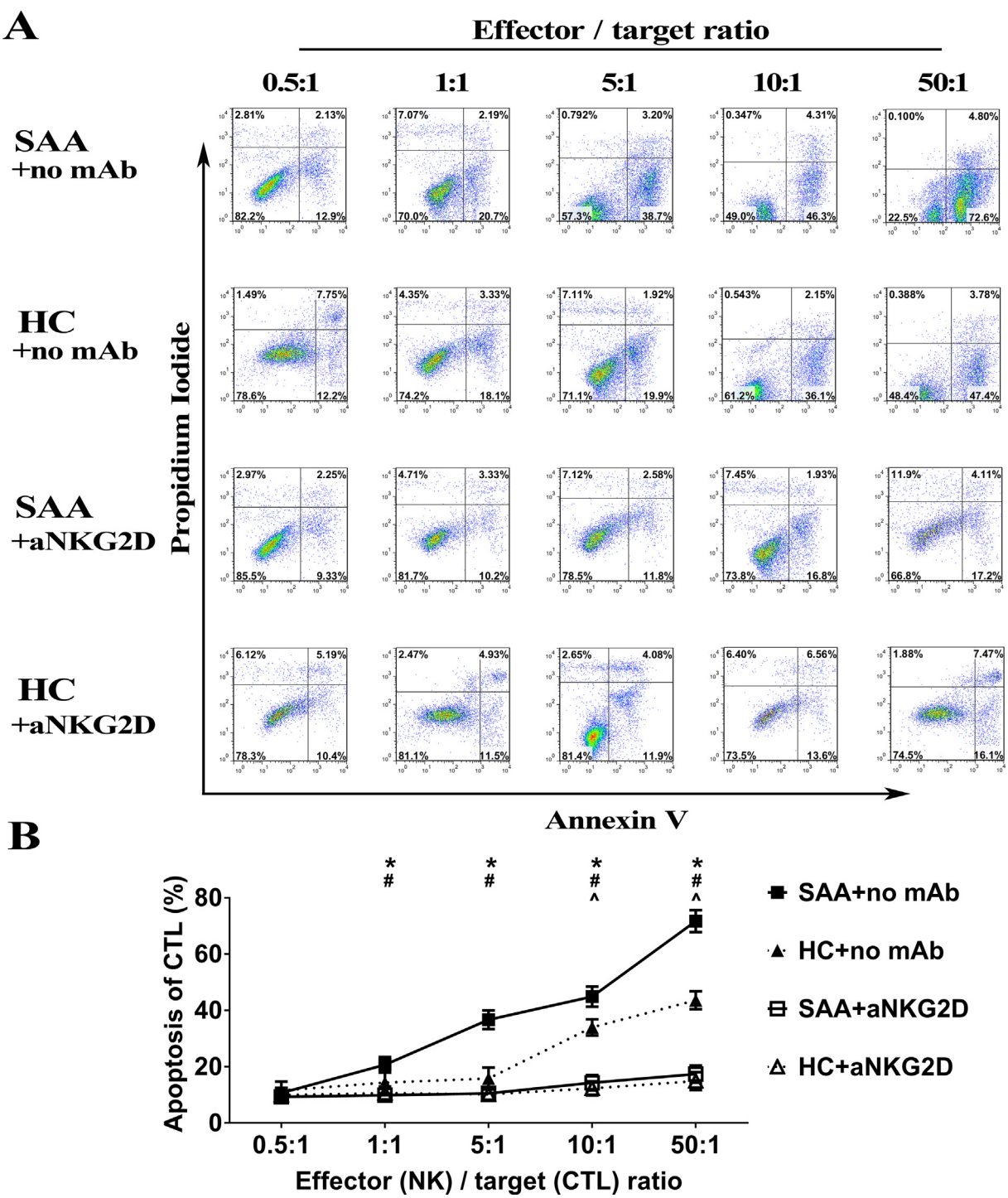

Fig. 3. Increased NK cell cytotoxicity towards autologous $CD8^+$ T cells is dependent on NKG2D. (A) Purified NK cells from patients with SAA or healthy control (HC) were stimulated with rhIL-2 as effector cells. Isolated autologous $CD8^+$ T cells were activated with anti-CD28 and PMA/Inomycin as target cells. Cells were cocultured with anti-NKG2D mono-antibody (aNKG2D) or isotype control at the indicated effector-target ratios for 72 h, and then stained with anti-CD8, Annexin V and Propidium Iodide for FCM detection. (B) Statistical analysis of (A). Apoptotic rates (%) of $CD8^+$ T cells (n = 10 for each group, mean ± SD) are presented (*SAA + no mAb vs. HC + no mAb, $p < 0.01$; $^\#$SAA + no mAb vs. SAA + aNKG2D, $p < 0.01$; $\hat{}$HC + no mAb vs. HC + aNKG2D, $p < 0.01$).

two hours later, early apoptosis rates of $CD8^+$ T cells were determined by FCM. Cytotoxicity of NK cells on $CD8^+$ T cells was enhanced as effector/target ratio was increased both in SAA and HC. Yet, NK cells isolated from SAA patients produced significant rising cytotoxicity compared with HC (Fig. 3A–B). Meanwhile, this lysis was mediated via NKG2D, as NKG2D-blocking antibody could reduce the apoptosis rates of $CD8^+$ T cells both in SAA and HC.

3.3. NK cells limited $CD8^+$ T cell immunity via NKG2D

Next, we aimed to address whether NK cells influenced $CD8^+$ T cell immune function and the influence relied on NKG2D. We cocultured rhIL-2 stimulated NK cells and autologous anti-CD3/CD28 and PMA/ionomycin activated $CD8^+$ T cells under different effector/target ratios, IFN-γ^+ $CD8^+$ T cells were analyzed 72 h later (Fig. 4A). IFN-γ production by $CD8^+$ T lymphocytes was down-regulated as effector cells

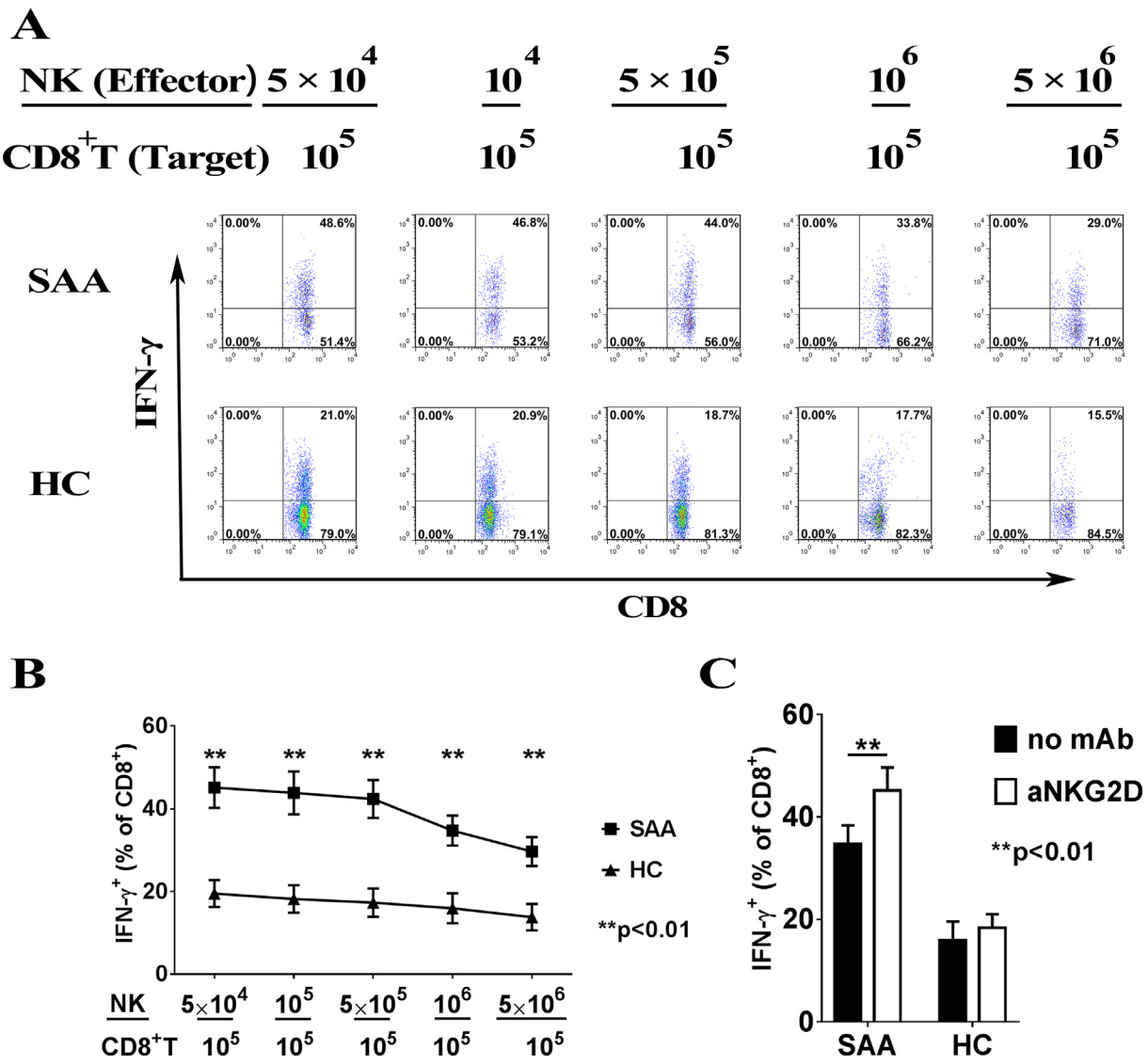

Fig. 4. NK cells limit $CD8^+$ T cell immunity in SAA. (A) Peripheral $CD8^+$ T cells isolated from patients with SAA and healthy controls were activated with anti-CD3/CD28 antibodies and coincubated with purified autologous NK cells stimulated with rhIL-2 at the indicated effector target quantities for 72 h. Intracellular IFN-γ production of $CD8^+$ T cells are determined using flow cytometry. (B) Statistical analysis of (A). IFN-γ^+ $CD8^+$ of all $CD8^+$ T cells (n = 10 for each group, mean ± SD) are shown ($^*p < 0.01$). (C) Activated 1×10^5 $CD8^+$ T cells and autologous stimulated 1×10^6 NK cells are coincubated with anti-NKG2D mAb or isotype control. IFN-γ production was monitored 72 h later (n = 10 for each group, $^{**}p < 0.01$).

(NK cells) increased in SAA (Fig. 4B). Interestingly, when we blocked NKG2D in the coculture system, IFN-γ production by $CD8^+$ T cells was significantly enhanced in SAA (Fig. 4C). These data indicated that NK cells could suppress $CD8^+$ T cell immunity in SAA and the suppression was mediated via NKG2D.

3.4. NKG2DL MICA was expressed on activated $CD8^+$ T cells from SAA

Since NK cell mediated lysis was dependent on NKG2D, we suspect $CD8^+$ T cells from SAA expressed NKG2DL and the NKG2D/NKG2DL interaction played a critical role in the negative regulation of $CD8^+$ T cell immunity mediated by NK cells. Previous report had demonstrated that NKG2DLs were induced on activated $CD8^+$ T cells [14]. In the current study, we observed the expressions of MHC class I chain-related protein A (MICA), one of NKG2DLs, on stimulated $CD8^+$ T cells on day 0, day 1, day 3, day 5 and day 7 using FCM and qPCR. Time-course analysis of MICA expression showed that MICA was significantly highly expressed on day 3 after activation in SAA, SAA-CR and HC, then the expression persisted on day 5 and dropped on day 7. MICA on $CD8^+$ T cells was higher in SAA compared with SAA-CR and HC (Fig. 5A and B). MICA mRNA expressions in $CD8^+$ T cells peaked on day 1, then declined thereafter (Fig. 5C). Consistent with FCM data, higher MICA mRNA was expressed in $CD8^+$ T cells from SAA compared with those from SAA-CR and HC.

3.5. NK transfusions inhibited $CD8^+$ T cell response and improved hematopoiesis in bone marrow failure (BMF) mouse model

Since $CD8^+$ T cells were susceptible to autologous NK cell lysis in patients with SAA, we sought to evaluate the potential effects of NK cells transfusions in BMF animal model. BMF mouse model was successfully established as the pancytopenia was achieved in our study (Fig. 6D). Compared with BMF mice, a decrease in $CD8^+$ T cells (Fig. 6A) and IFN-γ (Fig. 6B) was observed in ones receiving NK transfusions (BMF + NK). Meanwhile, BMF + NK group exhibited increasing bone marrow cells (Fig. 6C), hemoglobin and platelet (Fig. 6D). In addition, BMF + NK mice had significantly better survival outcomes (Fig. 6E). These results showed that treatment with NK cells

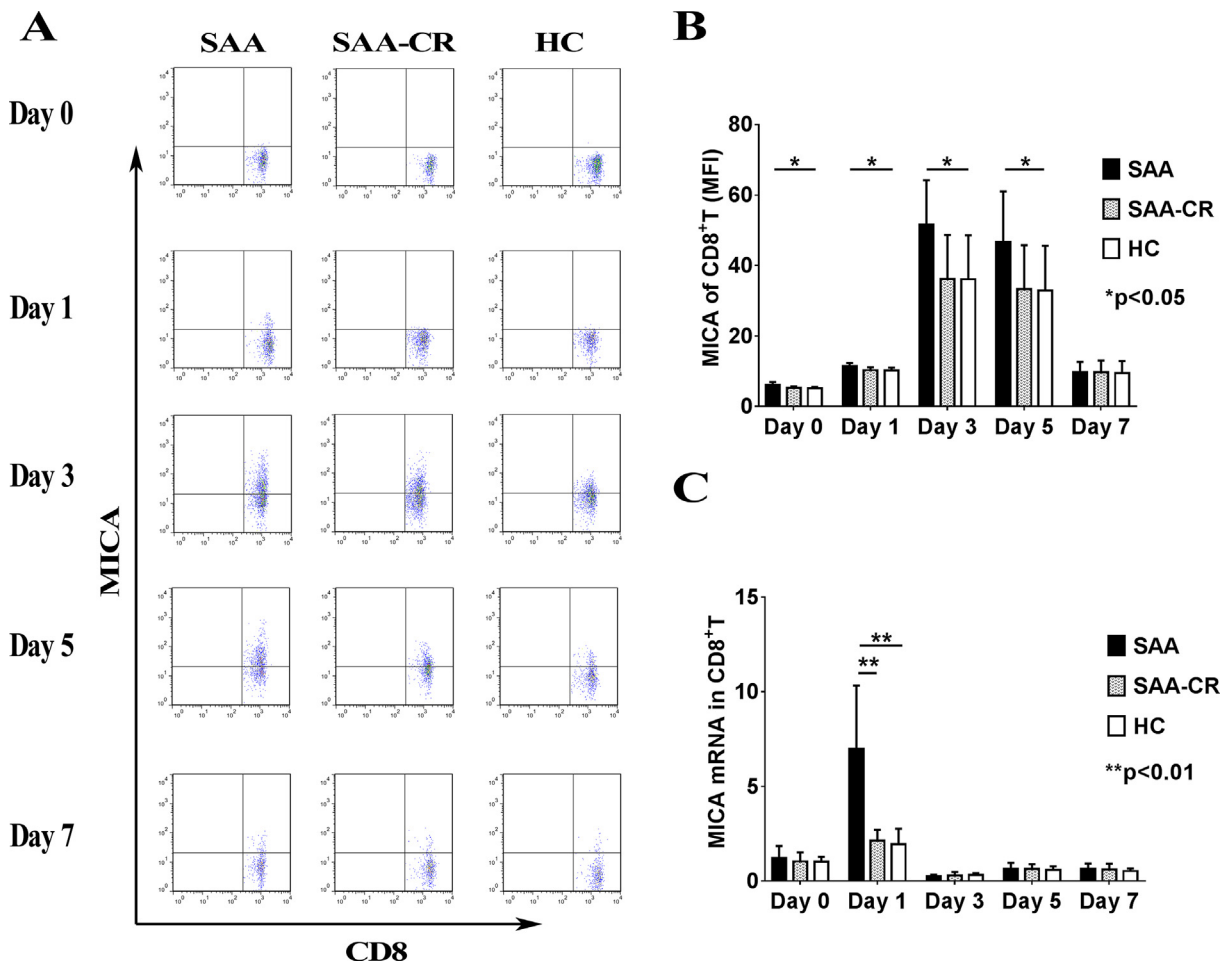

Fig. 5. $CD8^+$ T cells from SAA express elevated MICA on cell surface after anti-CD28 and PMA activation. PBMCs from patients with SAA, SAA-CR and HC were stimulated with anti-CD28 and PMA/Inomycin. (A) On day0, day1, day3, day5 and day7, $CD8^+$ T cells were identified with $CD3^+CD8^+$ and analyzed for the surface expression of MICA by flow cytometry. (B) Statistical analysis of (A). MFI is presented (n = 20 for each group). (C) MICA transcripts were analyzed by quantitative real-time PCR when $CD8^+$ T cells were harvested by immunomagnetic positive purification at different times. Data was presented using a modification of the $2-\Delta\Delta Ct$ method ($^*p < 0.05$, $^{**}p < 0.01$).

suppressed $CD8^+$ T cell immunity hence impacted the immunopathology in SAA.

4. Discussion

SAA is an autoimmune disease with complex mechanism. Both innate and acquired immune system participate in its pathogenesis. Our previous study has indicated the change of the amount of NK cells in SAA [12]. The current study further demonstrated the involvement of NK cells in the pathogenesis of SAA.

Activating and inhibitory receptors on NK cells perceive signals and determine whether NK cells are activated or restrained [15]. In our study, while NK cells acquired high expressions of activating receptors including NKp46 and NKG2D, the inhibitory receptor NKG2A was down-regulated. Theoretically, this paradigm makes NK cells more activated after binding to their specific ligands or MHC class I chain-related proteins [16–18], and offers NK cells a function of immunosurveillance to combat overfunctional T cells in SAA [19]. According to previous studies, NK functions are activated mainly by NKG2D, which may trigger perforin-mediated killing of T cells [20]. The cytotoxic effects of NK cells rely on the generation and releases of cytolytic proteins such as perforin and granzyme [21–23] in response to exogenous stimulation pertaining IL-2 or target cell interactions [24]. We observed that NK cells from SAA patients produced more perforins and stronger cytotoxicity towards K562 cells. Thus it is seemed that NK cell cytotoxicity in SAA was enhanced with involvement of NKG2D-mediated perforin production.

Hyper-functional $CD8^+$ T cells played a critical part in the pathopoiesis of SAA [25]. Previous reports have proved that NK cells limit $CD8^+$ T cell immunity in chronic infections [7,26]. In our setting, we demonstrated that NK cells induced apoptosis of autologous $CD8^+$ T cells in SAA as the effector/target ratios increased. Our data also showed that NK cell mediated $CD8^+$ T cell killing was dependent on NKG2D, because NKG2D blockade resumed $CD8^+$ T cell viability. Besides inducing $CD8^+$ T cell apoptosis, our results indicated that NK cells also regulated T cell response by abolishing IFN-γ production from $CD8^+$ T cells with a NKG2D-dependent manner in SAA. This result is consistent with the reports of many previous animal experiments which had proved that blockade of NKG2D inhibited NK cell activity towards T cells [27] and consequently increased IFN-γ production of T cells in chronic virus infection [26] and graft-versus-host disease [28].

Since NKG2D is crucial in the NK cell-mediated regulation of target $CD8^+$ T cells, whether NKG2D/NKG2DL interaction is necessary in this process should be investigated. NKG2DLs were once thought to be restrictedly expressed on infected or malignant cells [29]. Recently, more and more evidences have supported that hematopoietic cells, mature monocyte-derived dentritic cells [30,31] and T cells also express NKG2DLs. Human $CD4^+$ and $CD8^+$ T cells expressed NKG2DLs upon antigen activation [14]. MICA, which is the most important NKG2D ligand, is expressed early on activated $CD8^+$ T cells. In the current

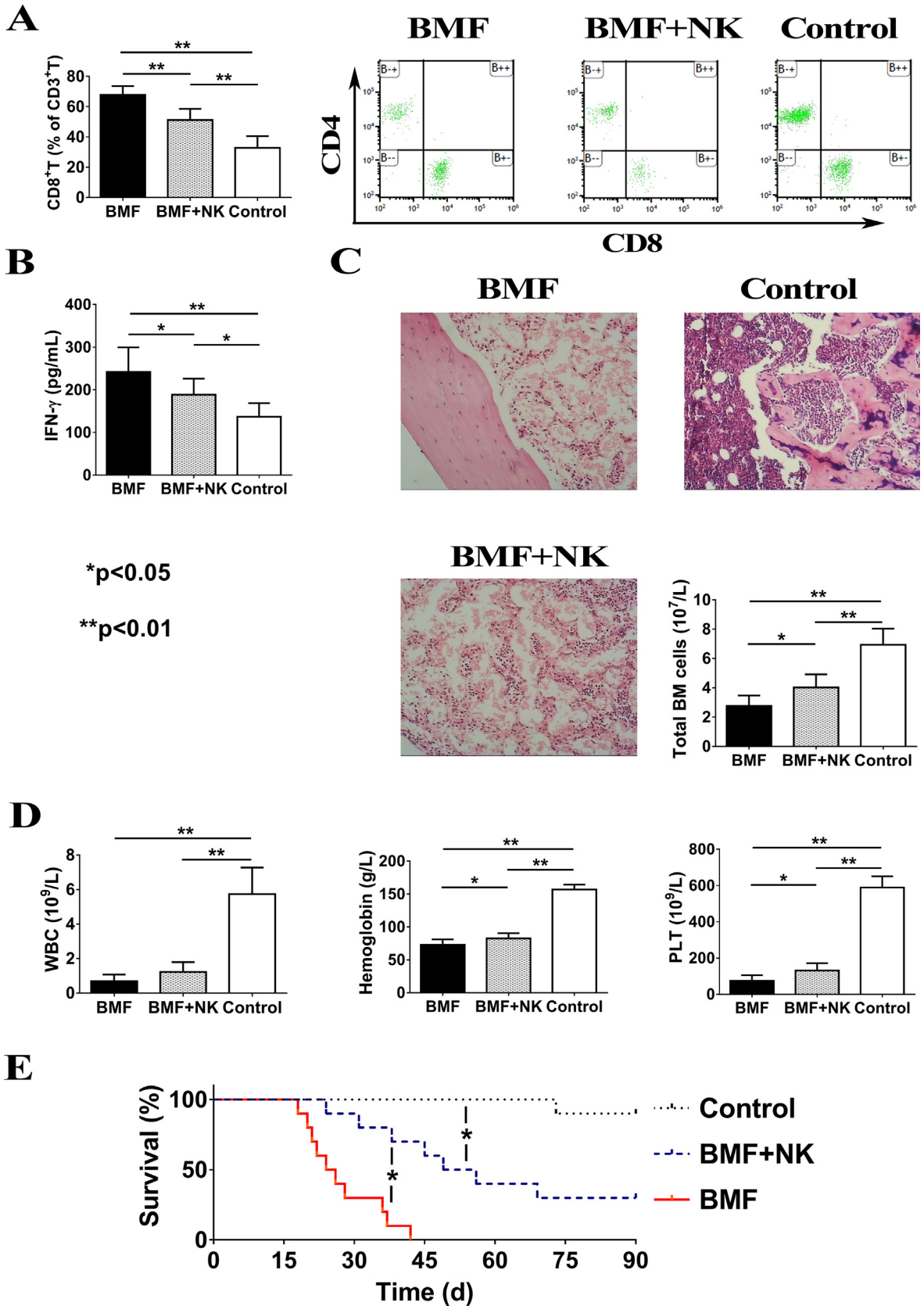

Fig. 6. NK cells suppress $CD8^+$ T cell immunity in bone marrow failure (BMF) mouse model. (A) CB6F1 mice received 4.0 Gy total body irradiation (TBI) and infusion of 5×10^7 lymph node cells from C57BL/6 mice to induce BMF. Some mice received 10^7 NK cells (BMF + NK) transfusion simultaneously. Control (CB6F1) mice received no TBI or lymphcytes, but same amount of normal saline. Seventeen days later, mice were bled for analysis. $CD8^+$ T of total $CD3^+$ T cells were determined by flow cytometry. (B) Serum IFN-γ was analyzed by ELISA. (C) Bone marrow histopathology (HE staining, 10×) and BM nucleated cell number are presented. (D) White blood cell (WBC), hemoglobin and platelet (PLT) counts are analyzed. (E) Survival outcomes were compared among three groups. N = 10 for each group ($^*p < 0.05$, $^{**}p < 0.01$).

study, MICA mRNA expressions in $CD8^+$ T cells peaked on day 1, while MICA protein was significantly highly expressed on day 3 after $CD8^+$ T cells were activated. So NK cells should only suppress the $CD8^+$ T cells which are in active or proliferative status. This can avoid the over-regulation of $CD8^+$ T cells by NK cells in SAA. However, our thesis still lacked intensive study on how NKG2D/MICA interactions took part in NK cell-mediated lysis.

According to the findings of our study, enough amount of NK cells might lead to an effective elimination of abnormally activated $CD8^+$ T cells which may be followed by hematopoietic recovery in SAA. However, since NK cells are deficient in SAA [11,12,32,33], this speculation can not be realized naturally in SAA patients. So trying to increase the NK cells should be beneficial for SAA patients. In the current study we successfully built a mouse model mimicking the lymphocyte infusion-induced bone marrow failure [34] to examine the impact of NK cells infusion on $CD8^+$ T cells. Consistent with what we found in patients, the suppressed proliferation of $CD8^+$ T cells and decreased IFN-γ production were found after NK cell transfusion. Moreover, increased bone marrow cells and peripheral hemoglobin and platelet also exhibited.

In conclusion, our study explored the regulatory roles of NK cells in SAA. By attenuating autologous $CD8^+$ T cell response, NK cells might play an immunoregulative role in SAA. We proposed NK cells might bring a novel aspect to SAA therapy. Our future studies would be more thorough on how NK cells contributed to the pathophysiology of SAA.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (81770110, 81600554, 81570106, 81370607, 81570111, 81400085, 81400088), Natural Science Foundation of Tianjin City (16ZXMJSY00180, 17JCQNJC10200, 14JCYBJC25400, 15JCYBJC24300), Tianjin Municipal University Science and Technology Development Fund Project (20140109).

Disclosure of conflicts of interest

The authors have no conflict of interests.

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