$CD8^+HLA\text{-}DR^+$ T cells are increased in patients with severe aplastic anemia

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Abstract. The aim of the present study was to investigate the number and function of $CD8^+HLA\text{-}DR^+$ cells, which are considered to be activated cytotoxic T lymphocytes (CTLs), in peripheral blood to further examine the pathogenesis of severe aplastic anemia (SAA). Thirty-eight patients with SAA were included in the present study. Patients were screened for paroxysmal nocturnal hemoglobinuria by flow cytometry using anti-CD55 and anti-CD59 antibodies. The number of $CD8^+HLA\text{-}DR^+$ T cells was measured by three-color flow cytometry using anti-CD8-peridinin chlorophyll, anti-CD3-fluorescein isothiocyanate (FITC) and anti-HLA-DR-FITC antibodies. The expression of perforin, granzyme B, tumor necrosis factor-β (TNF-β) and FasL in $CD8^+HLA\text{-}DR^+$ T cells was detected by flow cytometry with the appropriate monoclonal antibodies. Total RNA was prepared from purified $CD8^+HLA\text{-}DR^+$ cells of healthy controls and SAA patients, and then polymerase chain reaction (PCR) was performed. Apoptosis of $CD8^+HLA\text{-}DR^+$ cells was detected by flow cytometry following staining with Annexin V. The proportion of $CD8^+HLA\text{-}DR^+$ T cells was analyzed by flow cytometry in peripheral blood and was identified to be significantly higher in untreated SAA than in remission patients and in the controls. The expression of perforin, granzyme B, TNF-β and FasL in $CD8^+HLA\text{-}DR^+$ T cells was analyzed by flow cytometry and PCR, which revealed increased expression in the untreated SAA group compared with that in the control group. Furthermore, the apoptosis of $CD3^-$ bone marrow cells from normal individuals was enhanced following co-culture with $CD8^+HLA\text{-}DR^+$ T cells from untreated SAA patients. In conclusion, the present study demonstrated that $CD8^+HLA\text{-}DR^+$ T cells may contribute to bone marrow failure in SAA.

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Introduction

Severe aplastic anemia (SAA) is a serious disease that features bone marrow failure and is associated with high mortality rates. SAA has been recognized as an autoimmune disease caused by active destruction of hematopoietic cells by T lymphocytes (1). In recent years, a number of factors have been identified that contribute to the pathogenesis of SAA, including the activation of dendritic cells (DCs), imbalance of the Th1:Th2 ratio, excessive production of interferon-γ, expansion of $CD8^+$ T cells and heightened Fas antigen expression in hematopoietic stem/progenitor cells (2,3). Cytotoxic T lymphocytes (CTLs) usually damage target cells using three mechanisms: Cytokines, perforin-granzyme B and the Fas-FasL pathway (4). The mechanisms underlying how CTLs in SAA attack bone marrow cells have not been elucidated. Therefore, using HLA-DR as a molecular marker of activated CTL (5), the present study assessed the quantity and function of $CD8^+HLA\text{-}DR^+$ T cells in patients with SAA.

Flow cytometric analysis and polymerase chain reaction (PCR) were used to assess the $CD8^+HLA\text{-}DR^+$ T cell count in SAA patients and healthy controls. Furthermore, the expression levels of perforin, granzyme B, tumor necrosis factor-β (TNF-β) and FasL in CTLs were assessed. In order to confirm the cytotoxic effect of $CD8^+HLA\text{-}DR^+$ T cells on hematopoietic cells, CTLs from SAA and $CD3^-$ bone marrow cells from the normal controls were co-cultured.

Materials and methods

Patients. Thirty-eight patients with SAA were diagnosed in the Hematology Department of the General Hospital, Tianjin Medical University (Tianjin, China) between June 2008 and February 2011 according to the International AA Study Group Criteria (3). The disease was considered severe if at least two of the following parameters were met: Neutrophil count $<0.5x10^9$/l, platelet count $<20x10^9$/l and reticulocyte count $<20x10^9$/l with hypocellular bone marrow. Very severe aplastic anemia (VSAA) was diagnosed in cases of SAA with a neutrophil count $<0.2x10^9$/l (3). Patients were excluded if they had congenital AA or other autoimmune diseases. Patients were screened for paroxysmal nocturnal hemoglobinuria by flow cytometry using anti-CD55 and anti-CD59 antibodies. The patient population comprised 21 males and 17 females. The median age was

Table I. Characteristics of untreated SAA patients.

Case	Age/gender	Granulocytes ($x10^9$/l)	Hemoglobin (g/l)	Platelets ($x10^9$/l)	Abnormal chromosomes	Therapy
1	15/M	0.23	58	9	Absence	ATG + CsA
2	32/M	0.45	34	5	Absence	ATG + CsA
3	30/M	0.43	56	11	Absence	ATG + CsA
4	25/F	0.26	73	14	Absence	ATG + CsA
5	28/F	0.37	52	7	Absence	ATG + CsA
6	20/F	0.41	47	9	Absence	ATG + CsA
7	48/M	0.35	62	13	Absence	ATG + CsA
8	32/F	0.25	59	5	Absence	ATG + CsA
9	40/M	0.29	61	12	Absence	ATG + CsA
10	25/M	0.20	50	9	Absence	ATG + CsA
11	18/M	0.38	56	13	Absence	ATG + CsA
12	28/M	0.26	53	8	Absence	ATG + CsA
13	45/F	0.49	49	6	Absence	ATG + CsA
14	29/M	0.02	63	9	Absence	ATG + CsA
15	10/M	0.31	56	10	Absence	ATG + CsA
16	25/F	0.06	60	13	Absence	ATG + CsA
17	31/F	0.39	55	9	Absence	ATG + CsA
18	42/M	0.20	64	11	Absence	ATG + CsA
19	30/M	0.33	43	7	Absence	ATG + CsA
20	17/F	0.29	68	12	Absence	ATG + CsA
21	43/F	0.23	54	9	Absence	ATG + CsA
22	39/M	0.05	70	15	Absence	ATG + CsA
23	19/M	0.19	67	11	Absence	ATG + CsA
24	27/F	0.22	54	7	Absence	ATG + CsA
25	18/M	0.36	65	10	Absence	ATG + CsA
26	22/M	0.44	57	6	Absence	ATG + CsA

SAA, severe aplastic anemia; M, male; F, female; ATG, antithymocyte globulin; CsA, cyclosporine.

28 years (range, 10-48). Table I lists the features of 26 cases who were untreated (untreated group) and 12 who were in remission following immunosuppressive therapy (IST; remission group). The remission criteria were as follows: Independent transfusion, reticulocytes ≥$20x10^9$/l, absolute neutrophil count (ANC) ≥$1.0x10^9$/l and platelet ≥$20x10^9$/l. There were 23 healthy volunteers in the normal control group, including 11 males and 12 females, with a median age of 27 years (range, 21-38). The study was approved by the Ethics Committee of Tianjin Medical University (Tianjin, China). Informed written consent was obtained from all patients in accordance with the Declaration of Helsinki.

Surface and intracellular phenotyping by flow cytometry. Prior to analysis, the peripheral blood samples were stimulated in a short-term culture. All cells were maintained in RPMI-1640 medium supplemented with 20 ng/ml phorbol myristate acetate (PMA), 1 μg/ml ionomycin and 10 μg/ml Brefeldin A (BFA) for 6 h (Abcam, Hong Kong, China).

The number of $CD8^+HLA\text{-}DR^+$ T cells was measured by flow cytometry using anti-CD8-peridinin chlorophyll (PerCP), anti-CD3-fluorescein isothiocyanate (FITC) and anti-HLA-DR-FITC antibodies. The expression of perforin, granzyme B, TNF-β and FasL in $CD8^+HLA\text{-}DR^+$ T cells was detected by flow cytometry with the following monoclonal antibodies, which were all obtained from BD Biosciences (Franklin Lakes, NJ, USA): Perforin-phycoerythrin (PE), granzyme B-PE, TNF-β-PE and FasL (CD178)-PE. Data acquisition and analysis were conducted on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) using the Cell Quest software program (version 3.1; BD Biosciences).

Analysis of mRNA expression by PCR. $CD8^+HLA\text{-}DR^+$ T cells were purified by a double-positive selection process using MACS separators (Miltenyi Biotec, Auburn, CA, USA), as recommended by the manufacturer. Total RNA was prepared from purified $CD8^+HLA\text{-}DR^+$ cells of healthy controls and SAA patients using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse-transcription (RT) reactions were performed using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). Primers for amplifying perforin, granzyme B, TNF-β and FasL are listed in Table II and PCR was performed under the conditions presented in Table III (GeneAmp 9700 PCR system;

Table II. Primers for amplifying perforin, granzyme B, TNF-β and FasL.

Gene	Primer	Molecular weight (bp)
Perforin	F: 5'-CAGGTCAACATAGGCATCCACG-3' R: 5'-GAACAGCAGGTCGTTAATGGAG-3'	160
Granzyme B	F: 5'-GAAACGCTACTAACTACAGG-3' R: 5'-CCACTCAGCTAAGAGGT-3'	126
FasL	F: 5'-TGTTTATGAGCCAGACAAATGG-3' R: 5'-AAGACAGTCCCCCTTGAGGT-3'	203
TNF-β	F: 5'-AGGCATGAGGGATCACAG-3' R: 5'-AAAGAGGTTTATTGGGCTTC-3'	115
GAPDH	F: 5'-TTCCACCCATGGCAAATTCC-3' R: 5'-AGGCCATGCCAGTGAGCTTC-3'	500

F, forward; R, reverse; TNF-β, tumor necrosis factor-β.

Applied Biosystems, Grand Island, NY, USA). The amplified products were electrophoresed on an agarose gel. A negative control without cDNA template was run with every assay. The transcript copy number per subject was calculated by normalization to GAPDH expression.

Co-culture of CD8⁺HLA-DR⁺ T cells with target cells and analysis of apoptosis by flow cytometry. $CD8^+HLA\text{-}DR^+$ T cells (effector cells) from untreated SAA patients, remission patients and normal controls were co-cultured with bone marrow mononuclear cells (with $CD3^+$ T cells depleted) from remission patients and normal controls (target cells). The effector T cells of untreated SAA patients were co-cultured with the target cells of remission patients and normal controls, which were designated as SAA groups 1 and 2, respectively. There were two control groups: One was the remission SAA group (effector cells from untreated SAA patients and target cells of remission patients) and the other was the normal control (effector cells from untreated SAA patients and target cells of normal controls). Apoptosis of target cells was detected by flow cytometry following staining with Annexin V (BD Biosciences). The levels of lactate dehydrogenase (LDH; Sigma, St. Louis, MO, USA) in the supernatant were determined by automatic biochemistry analyzer (Roche Diagnostics AG, Risch, Switzerland).

Statistical analysis. Statistical analysis was performed using the parametric unpaired t-test for normally distributed data, and the non-parametric unpaired t-test for skewed data. Data are presented as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference. All statistical computations were performed using SPSS statistical software version 19 (SPSS, Inc., Chicago, IL USA).

Results

Ratio of CD8⁺HLA-DR⁺ T cells increases in SAA. The ratios of $CD8^+HLA\text{-}DR^+$ T cells to $CD8^+$ T cells and to $CD3^+$ T cells were 39.3±8.1 and 27.8±7.1 in SAA patients, and were significantly higher than the corresponding ratios in the controls (18.3±6.7 and 8.5±2.3; $P<0.05$; Fig. 1).

Table III. Conditions of PCR.

Gene	Conditions
Perforin	Initial denaturation for 3 min at 94˚C, 30 sec at 94˚C, 30 sec at 57˚C and 1 min at 72˚C (40 cycles); a terminal extension for 5 min at 72˚C
Granzyme B	Initial denaturation for 3 min at 94˚C, 30 sec at 94˚C, 30 sec at 53˚C and 1 min at 72˚C (40 cycles); a terminal extension for 5 min at 72˚C
FasL	Initial denaturation for 3 min at 94˚C, 30 sec at 94˚C, 30 sec at 55˚C and 1 min at 72˚C (40 cycles); a terminal extension for 5 min at 72˚C
TNF-β	Initial denaturation for 3 min at 94˚C, 30 sec at 94˚C, 30 sec at 54˚C and 1 min at 72˚C (40 cycles); a terminal extension for 5 min at 72˚C

TNF-β, tumor necrosis factor-β.

Increased expression of perforin, granzyme B, TNF-β and FasL in CD8⁺HLA-DR⁺ T cells of SAA patients. The percentages of $CD8^+HLA\text{-}DR^+$ T cells expressing granzyme B, TNF-β and FasL in in the untreated SAA group were 8.5, 96.1, 94.3 and 72.1%, respectively, which were higher than the corresponding values in the control group (1.8, 82.0, 32.9, 15.6%; Fig. 2).

The normalized mRNA expression values for perforin, granzyme B, TNF-β and FasL in $CD8^+HLA\text{-}DR^+$ T cells in the untreated SAA group were 0.66±0.25, 0.56±0.26, 0.61±0.16 and 0.77±0.24, respectively, which were significantly higher than those in the control group (0.53±0.14, 0.40±0.13, 0.46±0.15 and 0.58±0.16, respectively; $P<0.05$; Fig. 3).

Increased in vitro cytotoxicity in CD8⁺HLA-DR⁺ T cells from untreated SAA patients. The rates of apoptosis in the SAA group 1 ($CD8^+HLA\text{-}DR^+$ T cells from untreated SAA patients

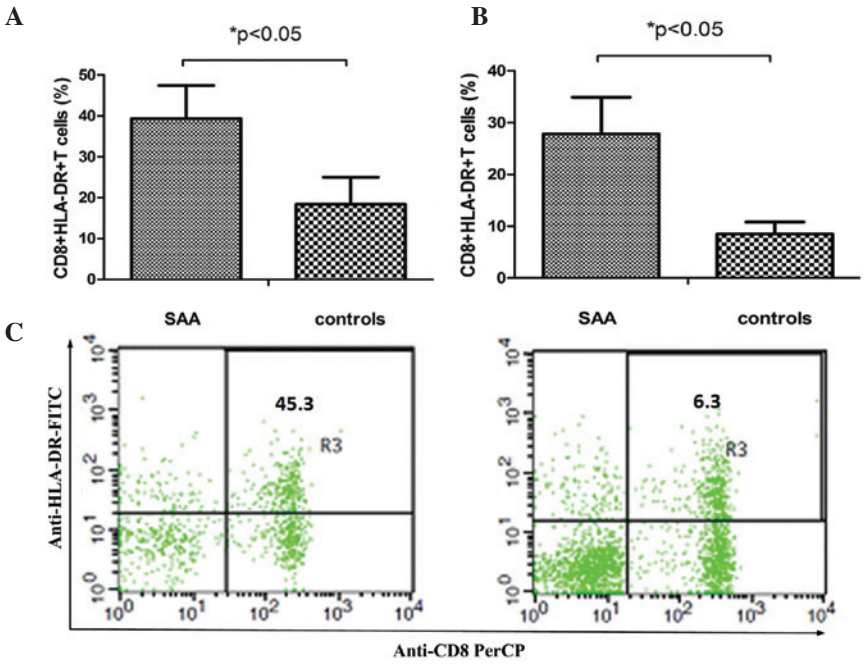

Figure 1. (A) Ratio of CD8+HLA-DR+ T cells in CD8+ cells compared between patients presenting with SAA and healthy controls. The mean of the ratio is presented. (B) Ratio of CD8+HLA-DR+ T cells in CD3+ cells compared between patients presenting with SAA and healthy controls. The mean of the ratio is presented. (C) Percentage of CD8+HLA-DR+ T cells determined by flow cytometric analysis in SAA and healthy controls following labeling with anti-CD8-PerCP, anti-CD3-FITC and anti-HLA-DR-FITC antibodies. SAA, severe aplastic anemia; FITC, fluorescein isothiocyanate; PerCP, peridinin chlorophyll.

and CD3- bone marrow mononuclear cells from remission patients), SAA group 2 (CD8+HLA-DR+ T cells from untreated SAA patients and CD3- bone marrow mononuclear cells from normal controls), the remission group (CD8+HLA-DR+ T cells and CD3- bone marrow mononuclear cells from remission patients) and the normal control (CD8+HLA-DR+ T cells and CD3- bone marrow mononuclear cells from normal controls) were 41.12±24.84, 45.81±20.47, 35.03±22.09 and 20.95±13.82%, respectively. There were no significant differences between the SAA 1, SAA 2 and remission groups (P>0.05). However, the rate of apoptosis in each of these groups was higher than that in the normal control (P<0.05; Fig. 4).

The LDH levels in the SAA 1, SAA 2, remission and normal control groups were 74.56±49.13, 62.61±31.76, 61.06±28.41 and 28.60±8.91 U/l, respectively. There were no significant differences between SAA 1, SAA 2 and remission groups (P>0.05). However, the levels of LDH in these groups were significantly higher than those in the normal control (P<0.05; Fig. 4).

Discussion

Numerous studies have established that SAA is an autoimmune disease caused by cellular immune abnormalities (6). Several immune abnormalities have been associated with the pathogenesis of SAA, including a DC subset imbalance (elevated DC1), enhanced DC function, regulatory T cell insufficiency, a Th1/Th2 imbalance (enhanced Th1), increased type I lymphoid factors (IL-2, IFN-γ) and a decreased proportion of natural killer cells. These abnormalities cause the activation of CD8+ T cells and excessive expression of apoptosis ligands in stem cells (7-12). ~70% of SAA patients respond to IST, including ATG/ALG and CSA (cyclosporine), which further proves that there must be an aberrant immune activation and tolerance in SAA (13,14). Although it is well established that the failure of bone marrow in SAA is due to attack by CD8+ T cells, the mechanisms underlying this process have not been elucidated (15). In the present study, it was demonstrated that the number of CD8+HLA-DR+ T cells and their expression of cytotoxic factors was increased in SAA. The *in vitro* cytotoxicity of the CD8+HLA-DR+ T cells was increased compared with healthy controls. Based on these results, it is concluded that CD8+HLA-DR+ T cells are, at least in part, responsible for bone marrow failure in SAA.

CD8+ T cells, also termed CTL, damage their target cells. The MHC II molecule HLA-DR is considered as a marker of antigen-activated T cells; therefore, CD8+HLA-DR+ T cells are a subset of activated effector T cells. Several studies have demonstrated that the proportion of these cells is significantly decreased in HIV infection and increased in common variable immunodeficiency disease (16,17). In the present study, it was demonstrated that the number of CD8+HLA-DR+ T cells in patients with SAA was higher than that in the healthy controls, and it was hypothesized that these cells contribute to bone marrow failure in SAA.

CD8+HLA-DR+ T cells were further enriched in SAA in order to analyze their expression of cytotoxic factors. CTL, which are mainly activated by antigens, induce apoptosis in target cells by three main pathways (4). Firstly, perforin is a calcium-dependent pore-forming protein synthesized in cytolytic lymphocytes and sequestered in secretory granules. Upon immunological reaction between a cytolytic lymphocyte and a target cell, perforin is released at the plasma membrane and polymerizes into transmembrane tubules (forming pores) which triggers the death of the target cell. Secondly, following binding to its receptors on target cells, TNF-β secreted from CTL stimulates signaling pathways leading to apoptosis. Finally, upregulated FasL on CTL binds to Fas on target cells, leading to activation of the

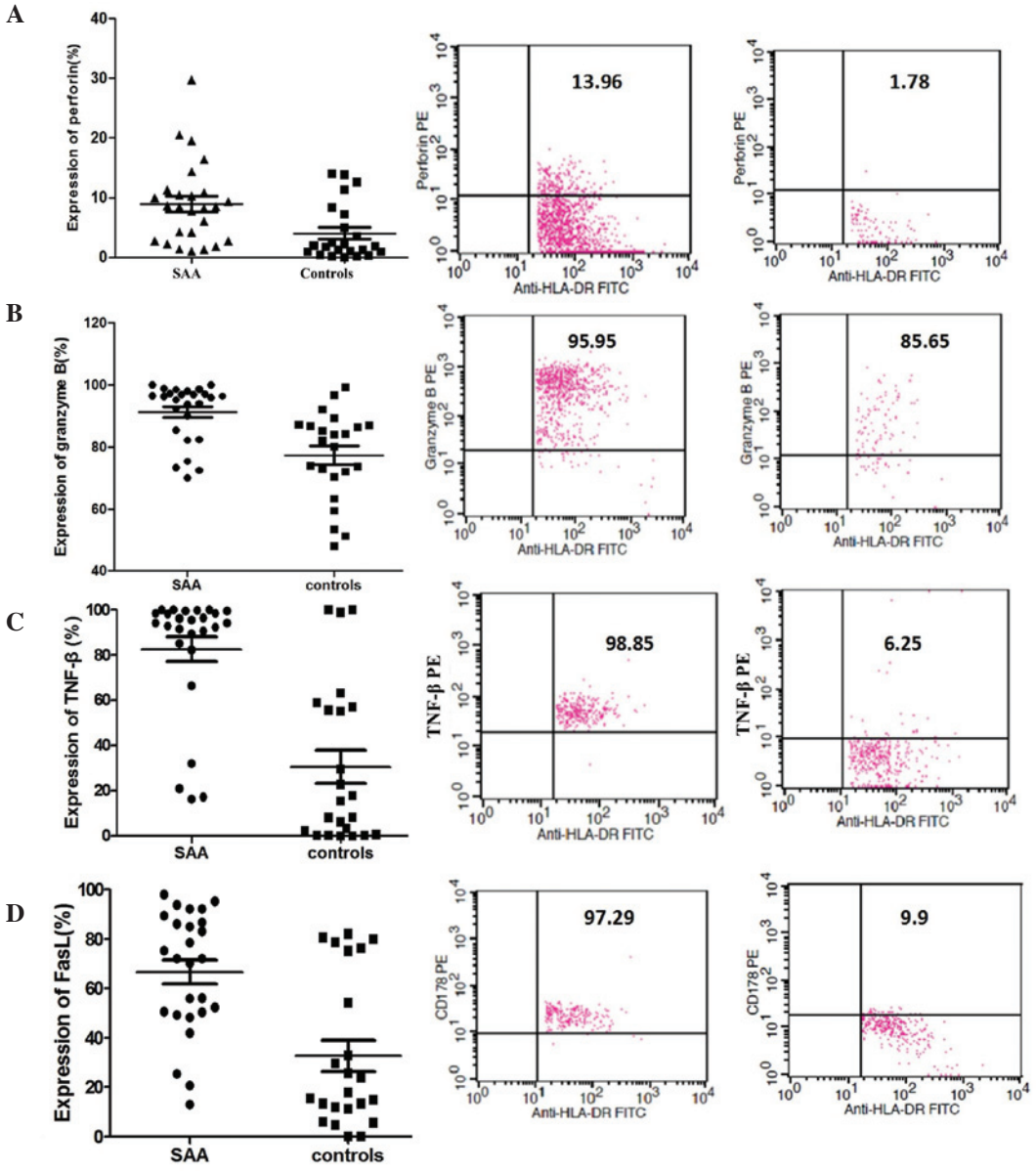

Figure 2. Expression of perforin, granzyme B, TNF-β and FasL in $CD8^+HLA\text{-}DR^+$ T cells was detected by four-color flow cytometry with the following monoclonal antibodies: Perforin-PE, TNF-β-PE, granzyme B-PE and FasL (CD178)-PE. Six hours prior to analysis, the peripheral blood samples were stimulated by short-term culture in RPMI-1640 medium supplemented with PMA, ionomycin and BFA. The median and the ratio of these factors are shown. Expression of (A) perforin, (B) granzyme B, (C) TNF-β and (D) FasL. PMA, phorbol myristate acetate; BFA, Brefeldin A; TNF-β, tumor necrosis factor-β; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

caspase cascade and target cell apoptosis. Therefore, in order to examine the function of $CD8^+HLA\text{-}DR^+$ T cells, their expression of perforin, granzyme B, FasL and TNF-β were assessed.

Perforin is stored in the cytoplasm of CTL, is the main cytotoxic protein and an important immune effector (18). Following release by CTLs, perforin combines with and inserts into the membrane of target cells. The pores in the membrane formed by perforin induce perturbation of osmotic pressure between the inside and outside of cells. Meanwhile, granzyme B enters the target cells through the pores and initiates apoptosis (19). The expression of perforin and granzyme B is increased in CTL in patients with systemic lupus erythematosus and is associated with disease activity (20). Similarly, the expression of perforin and granzyme B is also increased in the blood and local lesions of patients with lichen planus (21).

FasL is predominantly expressed on the surface of activated T and B lymphocytes, and binds to Fas on target cells to stimulate the signaling pathways of apoptosis. The interaction between FasL and Fas polymerizes Fas-associated death domain protein (FADD) in target cells, recruits caspase 8 and activates a series of enzyme reactions that ultimately lead to DNA degradation (22). In a study by Nadeau *et al* (23), the levels of FasL in the plasma and Fas on the neutrophils in pediatric patients with idiopathic neutropenia were significantly higher than those in healthy controls.

TNF-β is important in cellular apoptosis, the inflammatory response and the modulation of the immune system (24). Activated TNF molecules trigger TNF receptor polymerization, which subsequently enhances their affinity for their corresponding ligand. When TNF and TNFR interact, TNFR

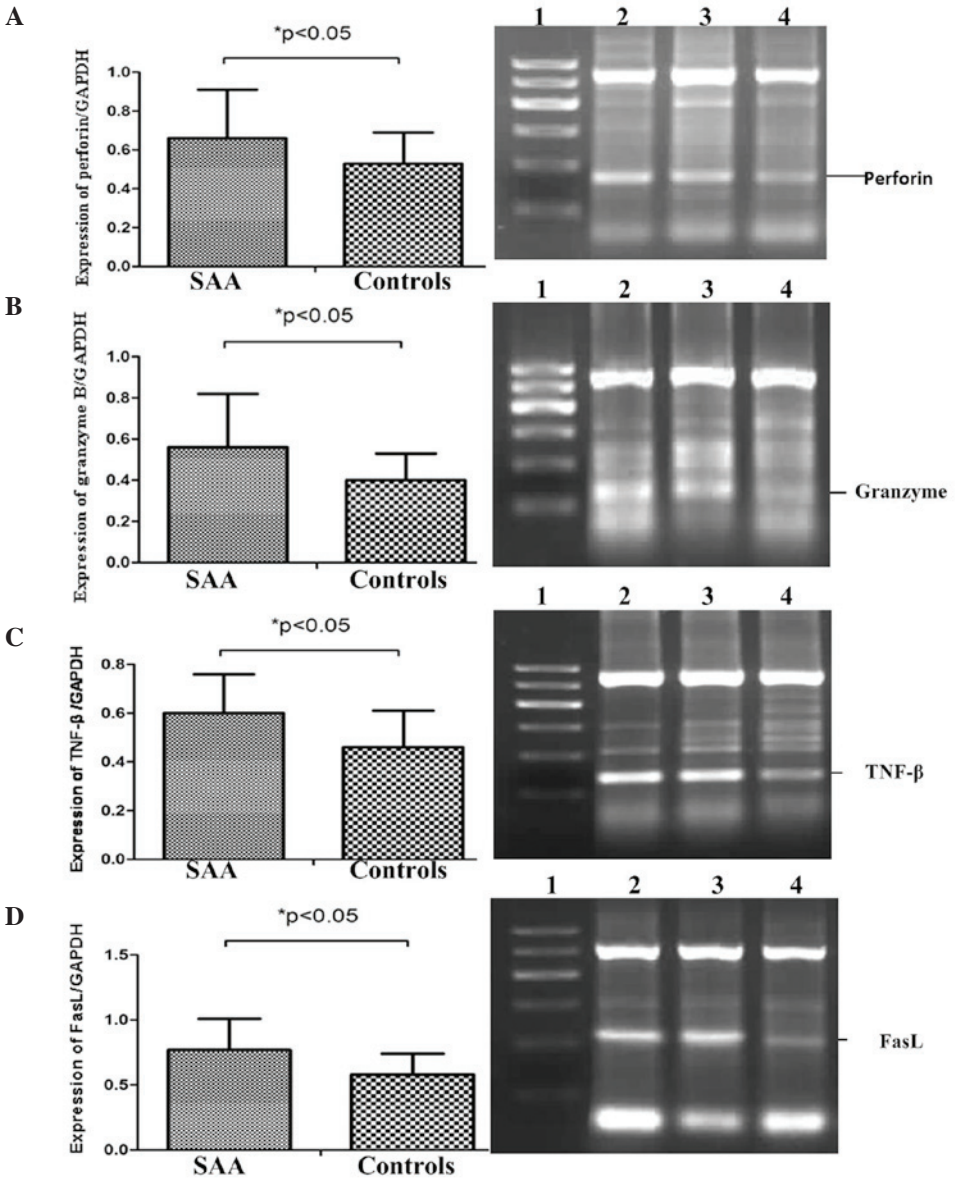

Figure 3. $CD8^+HLA\text{-}DR^+$ T cells from SAA patients and healthy controls were purified using a double positive selection process by MACS separators. The mRNA expression of perforin, granzyme B, TNF-β and FasL of $CD8^+HLA\text{-}DR^+$ T cells was analyzed by polymerase chain reaction. Transcript copy numbers per subject were calculated by normalization to GAPDH expression. The expression of perforin, granzyme B, TNF-β and FasL was significantly different between SAA patients and healthy controls ($^*P<0.05$). Expression of (A) perforin/GAPDH, (B) granzyme B/GAPDH, (C) TNF-β/GAPDH and (D) FasL/GAPDH. Lane 1, 600 bp molecular weight marker; lanes 2 and 3, SAA patients; lane 4, healthy control. SAA, severe aplastic anemia; TNF-β, tumor necrosis factor-β.

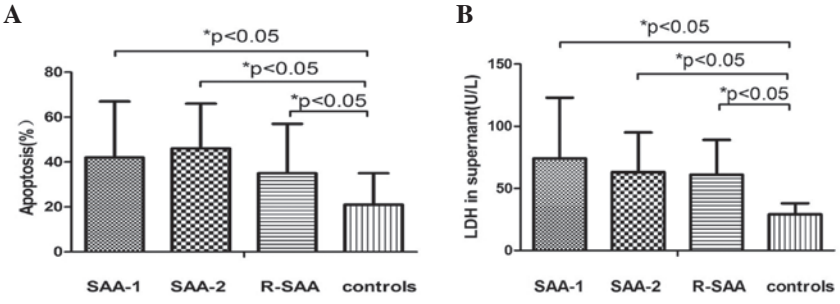

Figure 4. $CD8^+HLA\text{-}DR^+$ T cells (effector cells) from untreated SAA patients, remission patients and normal controls were co-cultured with ($CD3^+$ T cell-depleted) bone marrow mononuclear cells from remission patients or normal controls (target cells). (A) Cytotoxicity of $CD8^+HLA\text{-}DR^+$ T cells *in vitro*. (B) Levels of LDH in supernatant of co-cultured $CD8^+HLA\text{-}DR^+$ T cells and $CD3^-$ bone marrow mononuclear cells. $^*P<0.05$. SAA-1, $CD8^+HLA\text{-}DR^+$ T cells from untreated SAA patients and $CD3^-$ bone marrow mononuclear cells from remission patients; SAA-2, $CD8^+HLA\text{-}DR^+$ T cells from untreated SAA patients and $CD3^-$ bone marrow mononuclear cells from normal controls; R-SAA, $CD8^+HLA\text{-}DR^+$ T cells and $CD3^-$ bone marrow mononuclear cells from remission patients; Controls, $CD8^+HLA\text{-}DR^+$ T cells and $CD3^-$ bone marrow mononuclear cells from normal controls. SAA, severe aplastic anemia; LDH, lactate dehydrogenase.

activates the caspase cascade, leading to damage of the mitochondrial membrane and apoptosis of target cells.

Protein and mRNA expression levels of perforin, granzyme B, TNF-β and FasL were significantly increased in $CD8^+HLA\text{-}DR^+$ T cells from SAA patients compared with those from healthy controls. The increased activation status of $CD8^+HLA\text{-}DR^+$ T cells in SAA may impair hematopoiesis in bone marrow cells through the activity of perforin, granzyme B, TNF-β and FasL. These markers may also be useful in the diagnosis of SAA. In future studies, the expression of perforin, granzyme B, TNF-β and FasL at different stages in intensive suppressive therapy (IST) treatment will be assessed in order to determine the optimal regimen, evaluate the therapeutic effect and elucidate the prognosis for patients.

In the present study, $CD8^+HLA\text{-}DR^+$ T cells from untreated SAA patients, remission patients or healthy controls were co-cultured with bone marrow mononuclear cells (removing $CD3^+$ T cells) of the remission patients and healthy controls, and the cytotoxicity of these co-cultures was assessed. The severity of bone marrow failure in the patients with SAA prevented us from using bone marrow mononuclear cells from these patients in co-culture with $CD8^+HLA\text{-}DR^+$ T cells from healthy controls. The $CD8^+HLA\text{-}DR^+$ T cells from untreated SAA and remission patients were more cytotoxic than those from healthy controls; however, there was no significant difference in cytotoxicity between the cells from untreated SAA and remission patients. These observations suggested that the induction of apoptosis in hematopoietic cells by $CD8^+HLA\text{-}DR^+$ T cells may be critical in the bone marrow failure characteristic of SAA. It was previously reported that 30% of patients may relapse following IST (25). In the present study, the cytotoxicity of $CD8^+HLA\text{-}DR^+$ T cells was reduced following IST treatment, but it did not reach the level of the control subjects as the normal state. Thus, in order to decrease the risk of relapse, it is important for patients with SAA to maintain the IST treatment for a longer period of time and to taper cyclosporine gradually (26).

In conclusion, the number and cytotoxicity of $CD8^+HLA\text{-}DR^+$ T cells in the peripheral blood of patients with SAA was increased, which may contribute to the excessive apoptosis of hematopoietic cells in patients with SAA. $CD8^+HLA\text{-}DR^+$ T cells appear to have a prominent role in the immunopathogenesis of SAA and may be used as a new target for the treatment of SAA. The expression of perforin, granzyme B, TNF-β and FasL was increased in $CD8^+HLA\text{-}DR^+$ T cells. These proteins are important in the bone marrow failure of SAA and may be useful as novel diagnostic and therapeutic indexes for SAA.

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