

Original Article

Abnormality of lymphotoxin α in CD8⁺ T cells of patients with severe aplastic anemia

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Abstract: Severe aplastic anemia (SAA) is a serious disease that features bone marrow failure with high mortality rates. Cytotoxic T lymphocyte is considered to be the main cell that caused the destruction of hematopoietic cells. However, the mechanism of destruction was unclear. Herein, we investigated the expression level of CD8⁺ LT- α cells in peripheral blood of SAA patients and healthy controls. Furtherly, the CTL and bone marrow hematopoietic cells from SAA patients were co cultured with different concentrations of LT- α in vitro. The effect of CsA and IL-2 on LTA is detected simultaneously. Result demonstrated that both the protein and mRNA expression levels of LT- α in CD8⁺ cells in SAA patients were significantly increased. In addition, LT- α could obviously enhance the apoptosis of bone marrow hematopoietic cells induced by CTL. The enhancement effect was concentration-dependent. Furthermore, the LT- α protein expression of CTL was increased in SAA patients after the stimulation of IL-2, while the expression was inhibited after adding cyclosporin. Our findings indicated that as an important cytokine, IL-2 may play an important role in the process of CTL cell damage to hematopoietic cells. of SAA patients. What's more, this kind of effect was possibly regulated by the IL-2 pathway.

Keywords: Aplastic anemia, CD8⁺ T cells, lymphotoxin α

Introduction

Severe aplastic anemia (SAA) is a serious disease that features bone marrow failure with high mortality rates. SAA is recognized as an autoimmune disease. Whose pathogenesis is that hematopoietic stem/progenitor cells were impaired by overfunctional autoimmune CD8⁺ T lymphocytes [1]. CD8⁺ effector T cells, which also called cytotoxic T lymphocytes (CTL), are the main effect cells in the progress of immune response. The number and function of cytotoxic T lymphocytes were increased in SAA patients. CTLs usually damage target cells using three mechanisms: Cytokines, perforin-granzyme B and the Fas-FasL pathway [2]. Previous studies on the pathways of CTL damage to hematopoietic cells were mainly focused on the FAS and TNF- α . However, the disease progression were still out of control after blocking the pathways of FAS and TNF- α , which suggested the existence possibility of other pathways that CTL attacked target cells in patients with aplastic anemia. Lymphotoxin- α (LT- α) is important in

cellular apoptosis, inflammatory response and the modulation of the immune system [3]. TNF molecules can activate the caspase cascade, leading to damage of the mitochondrial membrane and apoptosis of target cells. LT- α shows a high degree of homology to TNF- α at amino acid sequences, but there were lots of differences in molecular structure and biological characteristics between LT- α and TNF- α [4]. LT- α is expressed by a variety of cells, including T cells, B cells and natural killer (NK) cells [5]. Recently, evidence suggests that LT- α seems to play a crucial role in the rheumatoid arthritis (RA), multiple sclerosis (MS) and many other autoimmune disease [6-8]. Until now, the mechanisms underlying how CTLs and LT- α in SAA patients attack bone marrow cells have not been elucidated. Herein, we tried to explain the specific mechanism of LT- α in CD8⁺ T cells from patients with SAA and furtherly explore the reasons which cause the over-function of T lymphocytes and thus lead to hematopoiesis failure in SAA.

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Table 1. Characteristics of untreated SAA patients

Case	Age/sex	Abnormal chromosome	Therapy
1	54/M	Absence	None
2	22/M	Absence	None
3	57/F	Absence	None
4	22/F	Absence	None
5	21/F	Absence	None
6	14/M	Absence	None
7	16/F	Absence	None
8	15/F	Absence	None
9	24/F	Absence	None
10	21/F	Absence	None
11	46/M	Absence	None
12	12/M	Absence	None
13	24/F	Absence	None

Table 2. The primers sequences

Target	Sense and anti-sense sequences	Bp
LT- α	F: 5'-AGGCATGAGGGATCACAG-3' R: 5'-AAAGAGGTTTATTGGGCTTC-3'	115 bp
GAPDH	F: 5'-TTCCACCCATGGCAAATCC-3' R: 5'-AGGCCATGCCAGTGAGCTTC-3'	500 bp

Materials and methods

Study subjects

A total of 28 patients with SAA were studied according to international AA Study Group Criteria, including 13 newly diagnosed cases and 15 cases in remission after immunosuppressive therapy (IST). All of these patients were hospitalized in the Hematology Department of General Hospital Tianjin Medical University from January 2010 to December 2011. SAA was diagnosed if at least two of the following parameters were met: neutrophil count less than $0.5 \times 10^9/L$, platelet count less than $20 \times 10^9/L$, and a reticulocyte count less than $20 \times 10^9/L$ with hypocellular bone marrow. If the neutrophil count was less than $0.2 \times 10^9/L$, AA was considered very severe (vSAA). All incoming cases were excluded if they had congenital AA or other autoimmune diseases. Patients were screened for paroxysmal nocturnal hemoglobinuria (PNH) by flow cytometry using anti-CD55 and anti-CD59 antibodies, and no PNH clones had been found. The Patients' features were listed in **Table 1**.

Fifteen cases in remission after immunosuppressive therapy were also identified. All of them left from the infusion of blood products, and their leukocyte counts were returned to normal (including the proportion of neutrophile granulocytes).

There were 15 healthy volunteers as normal control with a median age of 27 years (range 23 to 60). The study was approved by the Ethics Committee of the Tianjin Medical University. Informed written consent was obtained from all patients or their parents in accordance with the Declaration of Helsinki.

Monoclonal antibodies

CD8, LT- α and the relevant mouse isotype controls were purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Annexin V-FITC was also from Becton Dickinson.

Measurement of quantities of CD8+ LT- α + cells from peripheral blood

Objective cells (CD8+ LT- α + cells) were identified with a single-platform four-color flow cytometric analysis. 100 μ l whole peripheral blood was immunostained in TruCount tubes (BD Franklin Lakes, NJ, USA) for 30 mins, followed by lysis in 1.0 ml of FACS RBC lysing solution (BD Franklin Lakes, NJ, USA). Data acquisition and analysis were acquired on a FACS-Calibur and analyzed using the CellQuest 3.1 software (Franklin Lakes, NJ, USA).

Analysis of mRNA expression by semi-quantitative RT-PCR

Total RNA was prepared from purified CD8+ cells of SAA patients and healthy controls using Trizol-Reagent according to the manufacturer's instructions (Invitrogen, USA). Reverse-transcription (RT) reactions were performed using iScript cDNA Synthesis kit (Bio-Rad, Hercules). Primers for amplifying LT- α are shown in **Table 2**, and PCR was performed by the amplification instrument (GeneAmp 9700 PCR system, Applied Biosystems, USA). The amplified products were electrophoresed on an agarose gel. A negative control without cDNA template was run with every assay. GAPDH was used as a house-keeping gene for standardizing the expression of targeted mRNA. Transcript copy number was calculated by normalization to GAPDH expression.

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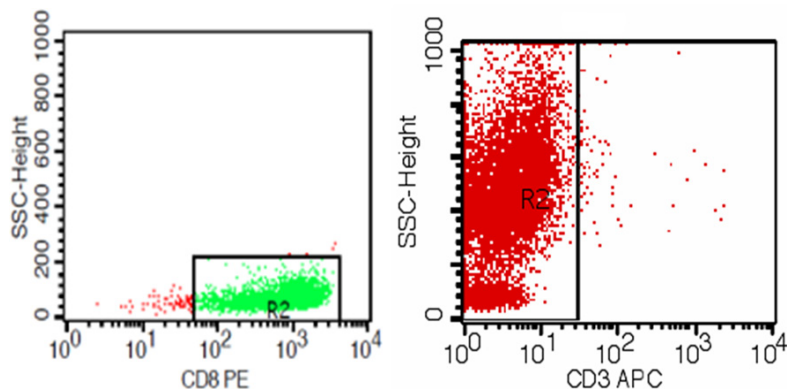


Figure 1. The purity of isolated CD8+ lymphocytes and CD3- cells using a FACS Aria flow cytometer.

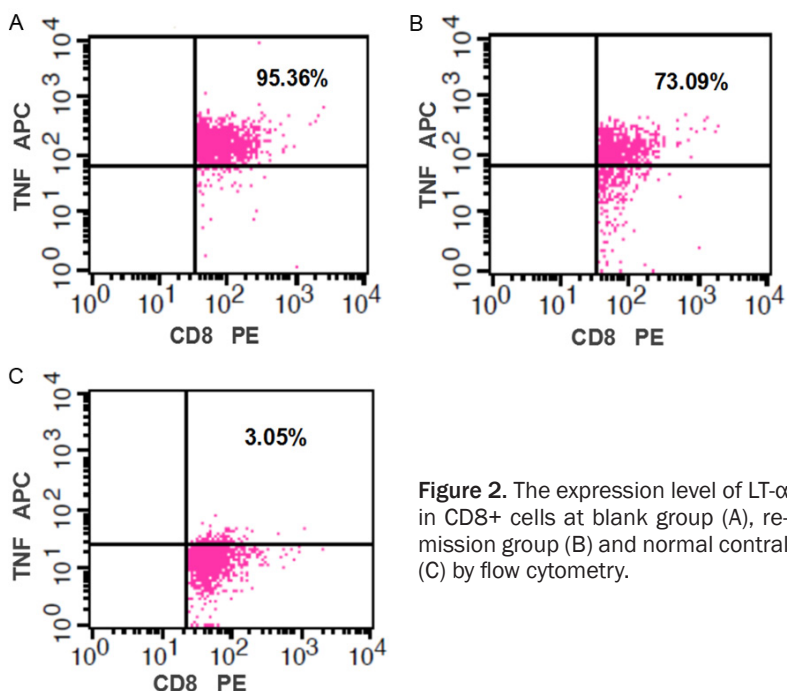


Figure 2. The expression level of LT- α in CD8+ cells at blank group (A), remission group (B) and normal control (C) by flow cytometry.

Isolation and purification of CD8+ T lymphocytes and CD3- cells

Bone marrow mononuclear cells (BMMNC) were isolated as mentioned above. The purified CD8+ T cells and CD3- cells were subsequently obtained using human CD8 and CD3 microbeads as recommended by the manufacturer (Miltenyi Biotech, German) respectively. After detected by the multiparameter flow cytometry (BD Biosciences) and analyzed using the Cell Quest software program (Version 3.1, Becton Dickinson), the purity of CD8+ T cells and CD3- cells was 90-95% (**Figure 1**).

Coculture of CD8+ and CD3- cells with different concentration of LT- α

To explore the effects of LT- α on the damage of hematopoietic cells induced by CD8+ T cells of SAA patients, CD8+ T cells (as effector cells) were sorted from BMMNC of SAA patients by magnetic activated cell sorting system (MACS). CD3+ cells MACS depleted BMMNCs from normal controls as target cells were co-cultured with the effector cells (1:1) in different concentrations of LT- α (0 ng/ml, 15 ng/ml, 25 ng/ml and 50 ng/ml) for 72 hours. The percentage of Annexin V positive cells were measured through flow cytometry.

The expression levels of LT- α in CD8+ T cells of blank control group, IL-2 group and the CsA group

Ten milliliters fresh human bone marrow were obtained from SAA patients and normal controls. BMMNCs were isolated by density gradient centrifugation using Ficoll-Paque Plus solution (Amersham Bioscience, Uppsala, Sweden) within 4 hours. BMMNCs were plated

in RPMI 1640 culture medium (containing 10% FBS and 1% mycillin; Gibco BRL, Grand Island, NY, USA) with a cell concentration of 5×10^5 per well. The experimental groups were divided into blank control group, IL-2 group and CsA group. Each groups were activated with IL-2 (100 U/ml, Novartis, China), CsA (400 ng/ml, Novartis, China) and blank control respectively and cultured at 37°C with 5% CO₂. After incubation for 72 h, PMA (20 ng/ml, Sigma, USA), ionomycin (1 μ g/ml, Sigma, USA) BFA (10 μ g/ml, Sigma, USA) per well were added to stimulate the cells for 4 h. Cells were harvested for flow cytometric analysis.

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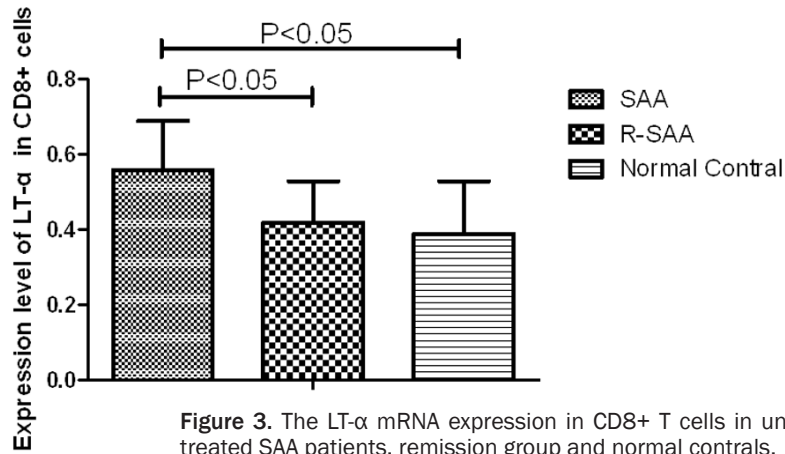


Figure 3. The LT- α mRNA expression in CD8+ T cells in untreated SAA patients, remission group and normal controls.

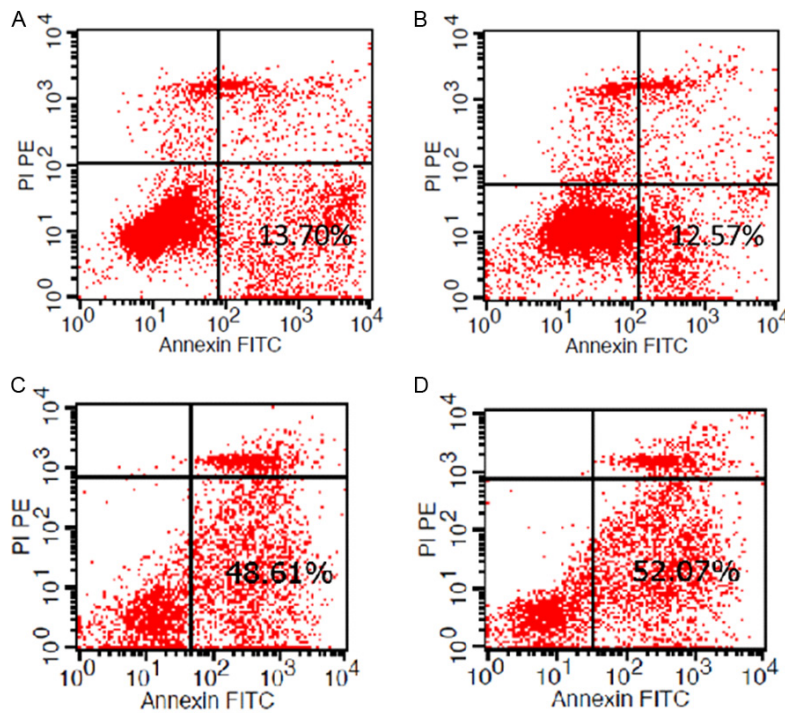


Figure 4. Analysis of apoptosis in target cells by flow cytometry after coculture of CD8+ and CD3- cells T cells with different concentration of LT- α . A. Blank group; B. 15 ng/ml group; C. 25 ng/ml group; D. 50 ng/ml group.

Statistical analysis

The statistical analysis software SPSS 21.0 was used. Normally distributed numerical variables are presented as mean \pm standard deviation (SD). The difference between 2 groups was compared using the Student's t test. Skewed distributed data are reported as the median and were analyzed using the Wilcoxon test. Differences were considered statistically significant with a P value of <0.05 .

Results

Increased expression of LT- α in CD8+ T cells of SAA patients

The expression of LT- α in CD8+ T cells of untreated group was (92.15 \pm 15.28)% which was significantly higher than that of remission group (63.57 \pm 18.65%) and control group (18.25 \pm 7.55%) ($P < 0.05$). However, there was no statistical significance between remission and control group ($P > 0.05$, **Figure 2**).

The normalized mRNA expression values for LT- α in CD8+ T cells in the untreated SAA group was 0.56 \pm 0.13, which was significantly higher than that in the control group (0.39 \pm 0.14; **Figure 3**). The LT- α mRNA expression in remission group was 0.42 \pm 0.11.

Coculture of CD8+ T cells and CD3- cells with different concentration of LT- α

The apoptosis rate in 25 ng/ml group (48.19 \pm 20.33%) and 50 ng/ml group (52.18 \pm 20.45%) were significantly higher than the blank group (13.64 \pm 8.18%) and 15 ng/ml group (12.49 \pm 4.43%) ($P < 0.05$). In addition, no statistically significant difference was seen between the 15

ng/ml group and the blank group ($P > 0.05$). The apoptosis rate of 25 ng/ml group had no significant difference compared with the 50 ng/ml group either ($P > 0.05$, **Figure 4**).

The intracellular LT- α expression levels detected by flow cytometry after combining with IL-2 or CsA

The quantity of LT- α in CD8+ cells at IL-2 group was (88.45 \pm 24.59)%, which was significantly

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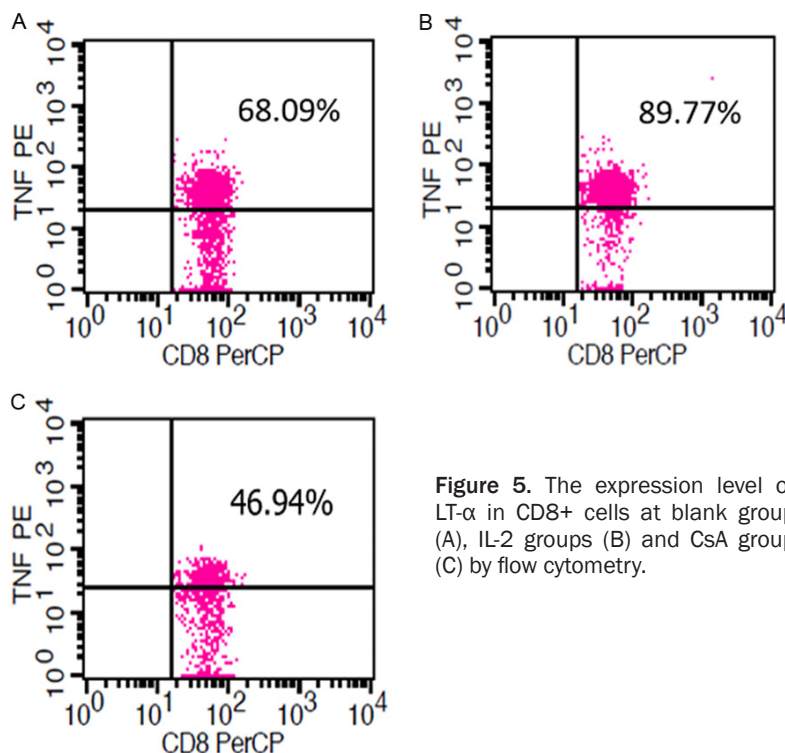


Figure 5. The expression level of LT- α in CD8+ cells at blank group (A), IL-2 groups (B) and CsA group (C) by flow cytometry.

higher than the blank control group ($64.15 \pm 19.25\%$) and CsA group ($42.53 \pm 19.42\%$) ($P < 0.05$), while the expression level of CsA group was significantly lower than the normal control ($P < 0.05$, as shown in **Figure 5**).

Discussion

Nowadays, an increasing number of studies have shown that SAA is an autoimmune disease with a disorder in cellular immunity [9]. In previous studies of our research group, we found an imbalance in the subset of dendritic cells (DC). The proportion of DC1 was increased and a hyperfunctional. Meanwhile, there was an imbalance in the ratio of Th1 and Th2 cells as well as the relative deficiency of regulatory T cells, which led to an increase of type I lymphokines (IL-2, IFN- γ). Thus the over activated CD8+ effector T cells and the elevating number of hematopoietic stem cell apoptosis-inducing ligand eventually resulted in the excessive apoptosis of bone marrow hematopoietic stem cells [10].

CD8+ cytotoxic T cells killed the target cells mainly through the three following ways: initiating the target cell apoptosis by secreting TNF- α , IFN- γ and other hematopoietic negative regula-

tory factors, which could bind to the corresponding receptors on the surface of target cells and then start the apoptosis of target cells; releasing perforins by exocytosis and damaging the membrane of target cell to kill target cells directly or releasing granzyme B into the target cells to start apoptosis; upregulating the expression of Fas on the surface of target cell to induce the apoptosis of target cells by caspase cascade [11, 12]. The activated cytotoxic T lymphocytes (mainly CD8+ T cells) had obvious inhibitory effect on bone marrow cells in vitro experiment. Cytotoxic T lymphocytes were the major cells to perform killing function in SAA patients [13].

Previous studies on the pathways of CTL damage to hematopoietic cells were mainly focused on the FAS and TNF- α . However, the disease progression were still out of control after blocking the pathways of FAS and TNF- α , which suggested the existence possibility of other pathways that CTL attacked target cells in patients with SAA.

LT- α , another member of the TNF superfamily, played an important part in autoimmune diseases. LT- α had the common feature of TNF family, but there were lots of differences in molecular structure and biological characteristics between LT- α and TNF- α . LT- α was produced and secreted by T and B cells. LT- α could regulate cell proliferation, differentiation as well as apoptosis and participated in the immune inflammatory response through combining with TNF receptor [14]. Studies on LT- α genetic defect mice revealed that LT- α played an important role in the update of dendritic cells [15]. In patients with rheumatoid arthritis, LT- α functioned through reducing joint inflammation and synovial hyperplasia degree after blocking the effect of LT- α [16]. In patients with multiple sclerosis, LT- α had an significant effect on the demyelinating lesions since blocking LT- α pathway in animal models could obviously

relieve the clinical symptoms and could even prevent the development of EAE [17]. Therefore, our preliminary study was aimed to elucidate the exact effect of LT- α in patients with SAA.

We detected the level of LT- α in CD8+ cells of SAA patients and found that both the protein and the mRNA expression levels of LT- α were significantly increased, which prompted that LT- α might be the important effect factor in the process of CTL damage to bone marrow hematopoietic cells. We furtherly cultured the CTL and bone marrow hematopoietic cells from SAA patients in vitro with different concentrations of LT- α . Results showed that LT- α could obviously enhance the apoptosis of bone marrow hematopoietic cells induced by CTL. Additionally, the enhancement effect was concentration-dependent. The apoptosis rate in 25 ng/ml group and 50 ng/ml group were significantly higher than the blank group and 15 ng/ml group. In addition, no statistically significant difference was seen between the 15 ng/ml group and the blank group. The apoptosis rate of 25 ng/ml group had no significant difference compared with the 50 ng/ml group either. Thus, LT- α might play an important role in the process of hematopoietic injury in SAA patients.

IL-2, also known as T cell growth factor (TCRF), was a kind of cytokines mainly produced by activated CD4+ T cells and CD8+ T cells. It had extensive biological activities and was the growth factor of all T cells subgroups. Therefore, it was considered as an important factor to regulate immune responses [18]. Our previous studies found that the secretion of IL-2, a type I lymphatic factor, was increased in the SAA patients and played an important role in the pathogenesis of SAA [19]. There were studies expounded that IL-2 could promote expression of LT- α gene through Jak - STAT pathway [20]. In recent years, cyclosporin had been a widely used drugs in the treatment of SAA, it worked mainly by inhibiting the production of IL-2 receptor and preventing the combination of IL-2 to IL-2R. Our study found the expression of LT- α in CTL was increased in SAA patients following the stimulation of IL-2, while the expression was inhibited after adding cyclosporin. Naturally we speculated that LT- α played a role in the hematopoietic injury of patients with SAA, and the action was regulated and influenced by IL-2, nevertheless the conclusion still needed a further study to confirm.

Conclusions

CTL of SAA patients attacked target cells through various pathways. However, our previous studies found that blocking those pathways could not completely inhibit the effects of CTL, so there might be another pathway which could lead to the hematopoietic damage. This study mainly focused on the role of LT- α in the pathogenesis of SAA. Results showed that LT- α was probably an important cytokine in the process of CTL cell damage to bone marrow hematopoietic cells of SAA patients. What's more, this kind of effect was possibly regulated by the IL-2 pathway. We would explore the action mechanism of LT- α ulteriorly and provide a foundation for the targeted therapy of SAA.

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Disclosure of conflict of interest

None.

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