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Sirt1 in the Regulation of Interferon Gamma in Severe Aplastic Anemia

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Keywords
Severe aplastic anemia · Cytotoxic lymphocytes · Sirt1 · Interferon gamma

Abstract
Recent studies have indicated that Sirt1 plays critical roles in the suppression of inflammation, T cell activation, and differentiation of hematopoietic progenitor cells. Severe aplastic anemia (SAA) is an immune-mediated disease that is characterized by elevated cytotoxic lymphocytes and type 1 cytokines. As a negative effector cytokine, interferon gamma (IFNγ) takes part in aplastic anemia through its inhibitory effect on hematopoiesis. In this study, we investigated the role of Sirt1 in the regulation of IFNγ in patients with SAA. A significant decrease in relative SIRT1 ($p < 0.05$) and increase in IFNG ($p < 0.05$) expression levels was observed in the sorted CD8+T cells of SAA patients compared to the controls. There was a significant negative correlation ($r = –0.53$, $p < 0.05$) between SIRT1 and IFNG expression in SAA patients. SRT3025, a Sirt1 activator, was shown to significantly reduce IFNγ ($p < 0.01$) and elevate Sirt1 ($p < 0.05$) expression in the CD8+T cells of SAA patients, and also showed a therapeutic role in an aplastic anemia mouse model. In conclusion, the defective Sirt1 may be correlated to the abnormal IFNγ expression in SAA patients, and activation of Sirt1 signaling may help improve the inflammatory status of SAA.

Introduction

Severe aplastic anemia (SAA) is an immune disease characterized by severe bone marrow failure and pancytopenia. Patients may suffer from severe anemia, life-threatening infection, and bleeding with rapid onset. The pathogenesis of SAA is complicated and needs to be clarified. Recent studies have indicated that the cytotoxic lymphocytes (CTLs) and interferon gamma (IFNγ) play a key role in SAA [1–3]. IFNγ plays a role in aplastic anemia due to its inhibitory effect on hematopoietic progenitor cells. IFNγ exposure can disrupt the generation and differentiation of myeloid progenitors, beyond that, IFNγ may also impair the self-renewal function of the hematopoietic stem cells (HSCs) in the presence of inflammation [4, 5].

Sirt1, as an NAD+-dependent protein deacetylase, is involved in many vital activities, such as stress, metabolism, inflammation, and cancer. In inflammation, Sirt1 antagonizes the NF-κB signal pathway in regulating oxidative and energy metabolism. Inhibiting Sirt1 may activate the inflammatory responses induced by NF-κB [6, 7]. A study by Caruso et al. [8] demonstrated that the Sirt1 expression level was decreased in inflammatory bowel disease patients, and Sirt1 activation could inhibit the NF-κB signal and attenuate the inflammation signals in a mouse colitis model. IFNγ as a sign of inflammatory and

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Table 1. Primers for real-time quantitative PCR detection

Primer	Sequence (5′ to 3′)
Sirt1	
Forward	TAGCCTTGTCAGATAAGGAAGGA
Reverse	ACAGCTTCACAGTCAACTTTGT
IFNG	
Forward	TCGGTAACTGACTTGAATGTCCA
Reverse	TCGCTTCCCTGTTTTAGCTGC
β-Actin	
Forward	TGGACATCCGCAAAGACCTGT
Reverse	CACACGGAGTACTTGCGCTCA

immune responses could be stimulated by NF-κB signal [9]. A mouse model study on aplastic anemia reported that the NF-κB signal was involved in the bone marrow infiltration of T cells [10]. In particular, Sirt1 has been demonstrated to be important in the differentiation and functioning of hematopoietic stem/progenitor cells [11, 12]. Based on these reports, we hypothesized that the aberrant Sirt1 may participate in the pathogenesis of SAA.

Materials and Methods

Study Subjects

A total of 18 newly diagnosed SAA patients who visited the Hematology Department of the General Hospital of Tianjin Medical University (Tianjin, China) were enrolled in our study, including 11 males and 7 females, with the median age of 49 years (range 25–65). The SAA diagnosis in these patients was carried out according to the International AA Study Group criteria: marrow cellularity <25% (or 25–50% with <30% residual hematopoietic cells), and two of three of the following parameters: neutrophil count $<0.5 \times 10^9$/L, platelet count $<20 \times 10^9$/L, and reticulocyte count $<20 \times 10^9$/L. Besides congenital aplastic anemia, paroxysmal nocturnal hemoglobinuria, myelodysplastic syndrome, and other clonal disorders needed to be excluded [13]. A total of 18 healthy volunteers were also enrolled as normal controls, including 8 males and 10 females, with a median age of 31 years (range 20–61). This study was approved by the ethics committee of the Tianjin Medical University. All patients and healthy controls signed an informed consent in accordance with the Helsinki Declaration.

Isolation of CD8+T Lymphocytes

Peripheral blood mononuclear cells (PBMCs) from SAA patients and healthy controls were isolated by density gradient centrifugation with Ficoll solution (Solabio, Beijing, China). Purified CD8+T cells were then obtained from PBMCs using human CD8 microbeads (Miltenyi Biotec, German), according to the manufacturer's instructions. Purity of CD8+T cells was detected by the CytoFLEX flow cytometry (Beckman Coulter, Indianapolis, IN, USA) with anti-CD8-FITC antibody (BD Biosciences, San Jose, CA, USA) and analyzed using the Kaluza analysis software (Beckman Coulter, Indianapolis, IN, USA).

Evaluation of Gene Expression by PCR

SIRT1 and IFNG gene expression was analyzed by real-time quantitative PCR. Total RNA was extracted from the purified CD8+T cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The extracted total RNA was reverse transcribed using FastQuant RT Kit With gDNase (Tiangen, Beijing, China). Real-time quantitative PCR was performed using SYBR Green (Tiangen, Beijing, China). β-Actin was used as the house-keeping gene for standardizing the expression of target mRNA. The relative expression levels of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method. The primers used for this analysis are listed in Table 1.

Flow Cytometric Detection of IFNγ and Sirt1 in CD8+T Cells

PMMNCs samples from healthy controls and SAA patients were stimulated with Phorbol 12-myristate 13-acetate (PMA; 10 ng/mL), ionomycin (1 μg/mL), and brefeldin A (BFA; 10 μg/mL), in a 37 °C carbon dioxide incubator for 4 h [14, 15]. IFNγ expression in each sample was detected by flow cytometry. To explore the role of Sirt1 in SAA, we analyzed the effect of SRT3025, a Sirt1 activator, on the expression of IFNγ in patients with SAA. We collected five SAA samples, each of which was divided into SRT3025-treated group and untreated group. In the SRT3025-treated group, 10 μm of SRT3025 (purchased from Selleck Chemicals) was added on the basis of PMA, ionomycin, and BFA stimulation, and the corresponding control groups were the same SAA samples treated with equivalent amounts of DMSO and basic stimulants. The expression of IFNγ and Sirt1 was then detected by flow cytometry. IFNγ and Sirt1 expression in the CTLs was detected using the following antibodies: anti-CD3-APC, anti-CD8-APC-CY7, anti-IFNγ-FITC (BD Biosciences, San Jose, CA, USA), rabbit anti-human Sirt1 antibody (1:200; Abcam, Cambridge, UK), and Alexa Fluor 488 Goat Anti-Rabbit IgG (H+L) (1:2,000; Proteintech Group, Inc., Chicago, IL, USA).

Experimental Animal Model Establishment and Grouping

Female C57BL/6 mice (8–10 weeks old) and CByB6F1 mice (8–10 weeks old) (Vital River Company, Beijing, China) were used for the establishment of the aplastic mouse model. The mice were bred under appropriate conditions (22 ± 2 °C and 50 ± 5% humidity) at the Animals Center of the Institute of Radiation Medicine, Chinese Academy of Medical Science. All animal experimental procedures were approved by the Institute of Radiation Medicine, Chinese Academy of Medical Science.

Establishment of aplastic anemia in mouse models: lymphocytes isolated from the lymph nodes of C57BL/6 donors and CByB6F1 recipients received total body irradiation at a dose of 4 Gy using an exposure instrument (Gammacell-40 137Cs irradiator; Atomic Energy of Canada Ltd). Then, lymphocytes (5×10^7) were injected through the angular vein, and the mice were harvested on day +17.

In this study, mice were divided into three groups: (1) normal control ($n = 5$), normal CByB6F1 mice; (2) AA mice group ($n = 5$); and (3) SRT3025-treated AA mice group ($n = 5$). In the SRT3025-treated AA mice group, the AA model mice were treated with SRT3025 (25 mg/kg/every other day) via i.p. injection, beginning 1 h after disease induction and continuing until day +16 post-disease induction. On day +17, blood was collected from the three

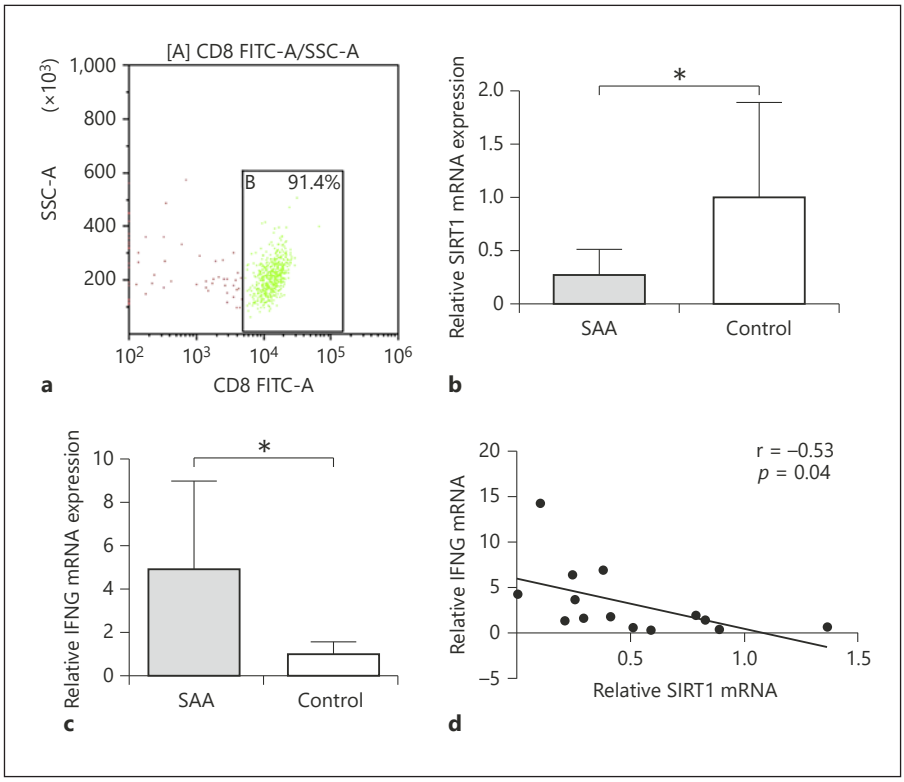

Fig. 1. CD8+T cells isolation and mRNA level determination. **a** CD8+T cells isolated from PBMCs of SAA patients and controls; the purity was more than 90%. **b** The relative SIRT1 mRNA level in CD8+T cells of SAA patients was significantly decreased compared to controls ($p < 0.05$). **c** The relative IFNG mRNA level in CD8+T cells of SAA patients was significantly elevated compared to controls ($p < 0.05$). **d** The relative SIRT1 mRNA level was negatively correlated to the IFNG mRNA level in CD8+T cells of SAA patients ($r = -0.53$, $p < 0.05$).

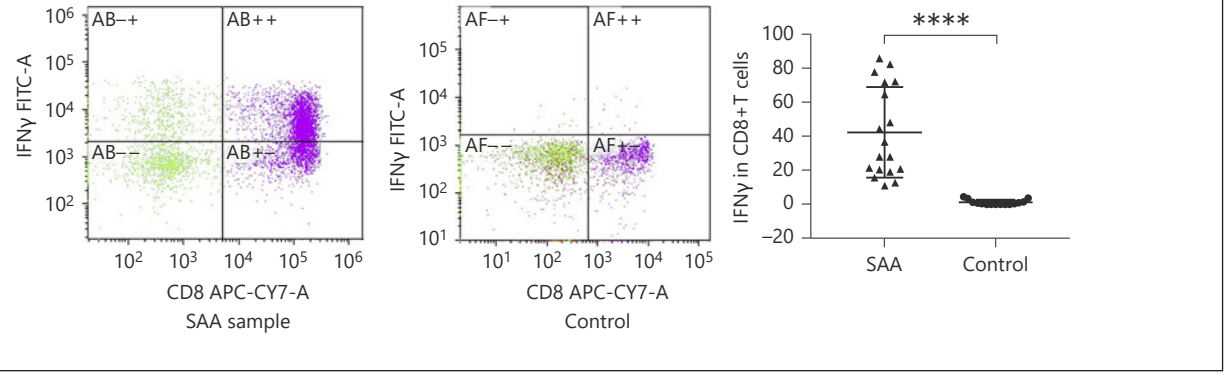

Fig. 2. IFNγ expression tested by flow cytometry. The IFNγ level was significantly higher in the CD8+T cells of SAA patients ($n = 18$) than in controls ($n = 18$) ($p < 0.01$).

mice groups for routine testing. IFNγ expression in each mouse was tested using flow cytometry, the same detection method described above for human samples.

Statistical Analysis

Data analyses were performed with GraphPad Prism 7.0 software. Data were presented as mean ± standard deviation. p values were analyzed by t test and Pearson's correlation test. p values of ≤ 0.05 were considered statistically significant.

Results

SIRT1 Expression Is Decreased in CD8+T Cells of SAA Patients

CD8+T cells were isolated from peripheral blood of the untreated SAA patients and healthy controls, and the purity of sorted CD8+T cells was more than 90% (Fig. 1a). Relative SIRT1 expression levels in the CD8+T cells were

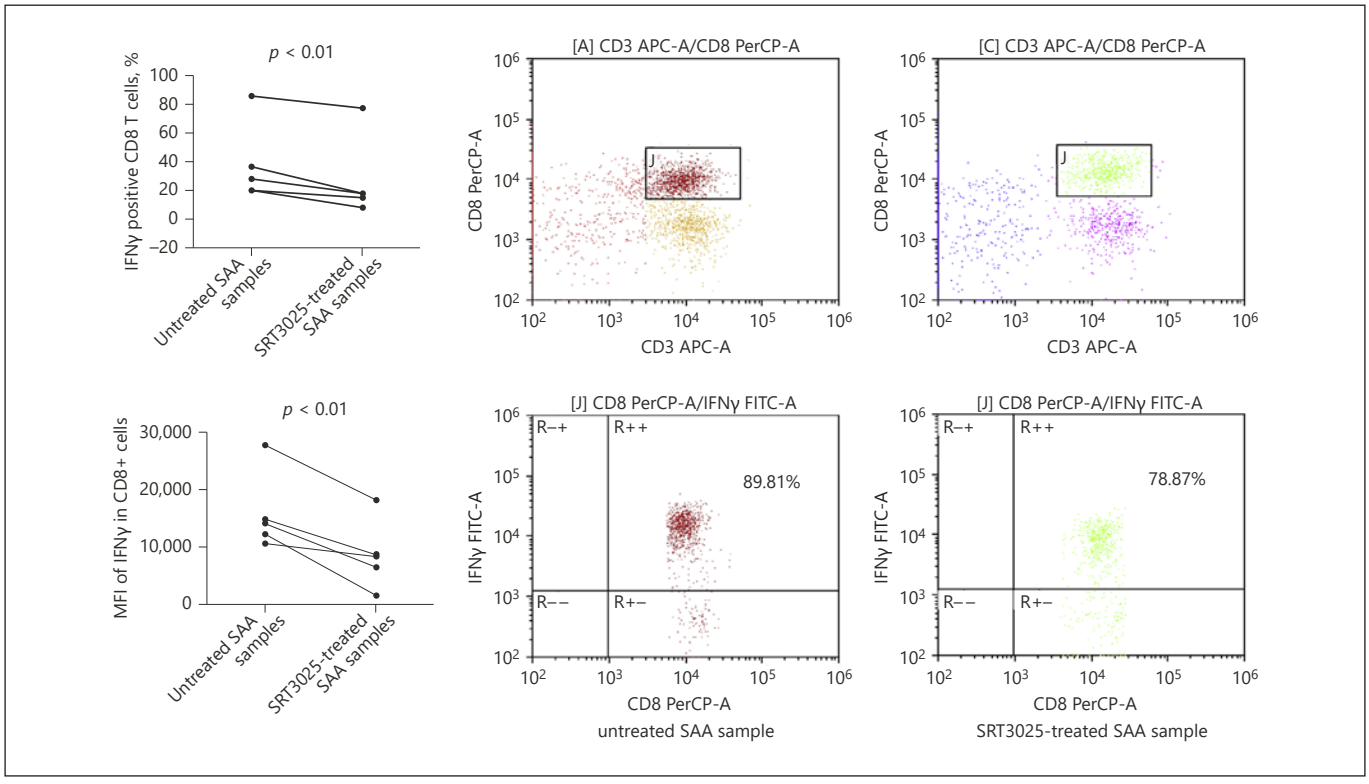

Fig. 3. Paired *t* test showed that the IFNγ expression in CD8+T cells of SAA patients ($n = 5$) decreased when treated with 10 μm of SRT3025 ($p < 0.01$).

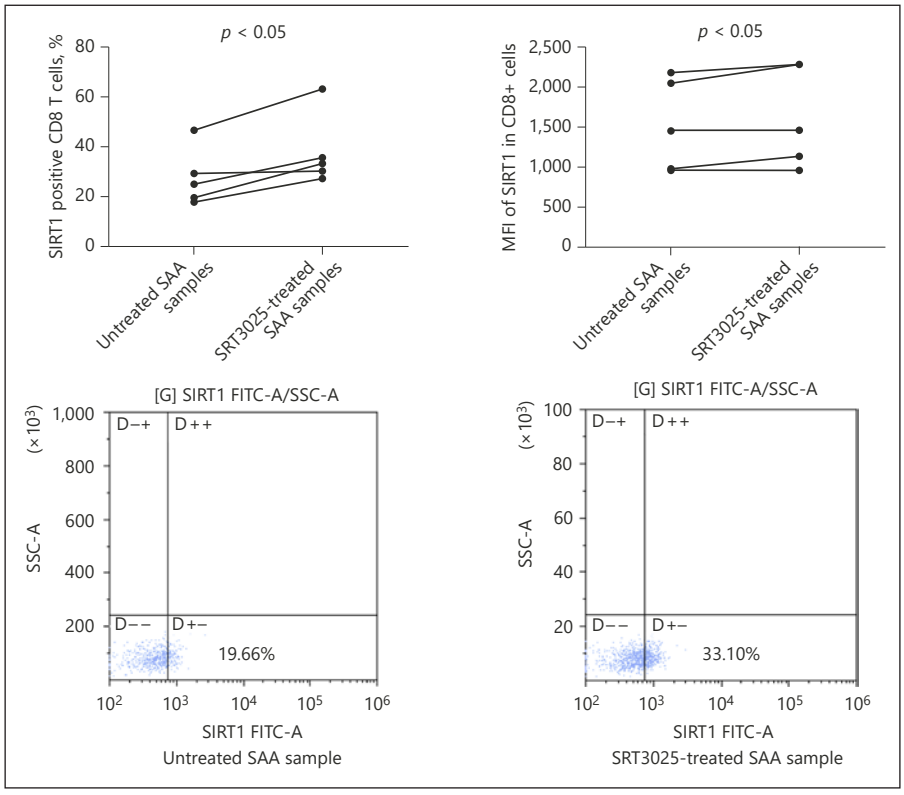

Fig. 4. Paired *t* test showed that the Sirt1 expression in CD8+T cells of SAA patients ($n = 5$) increased when treated with 10 μm of SRT3025 ($p < 0.05$).

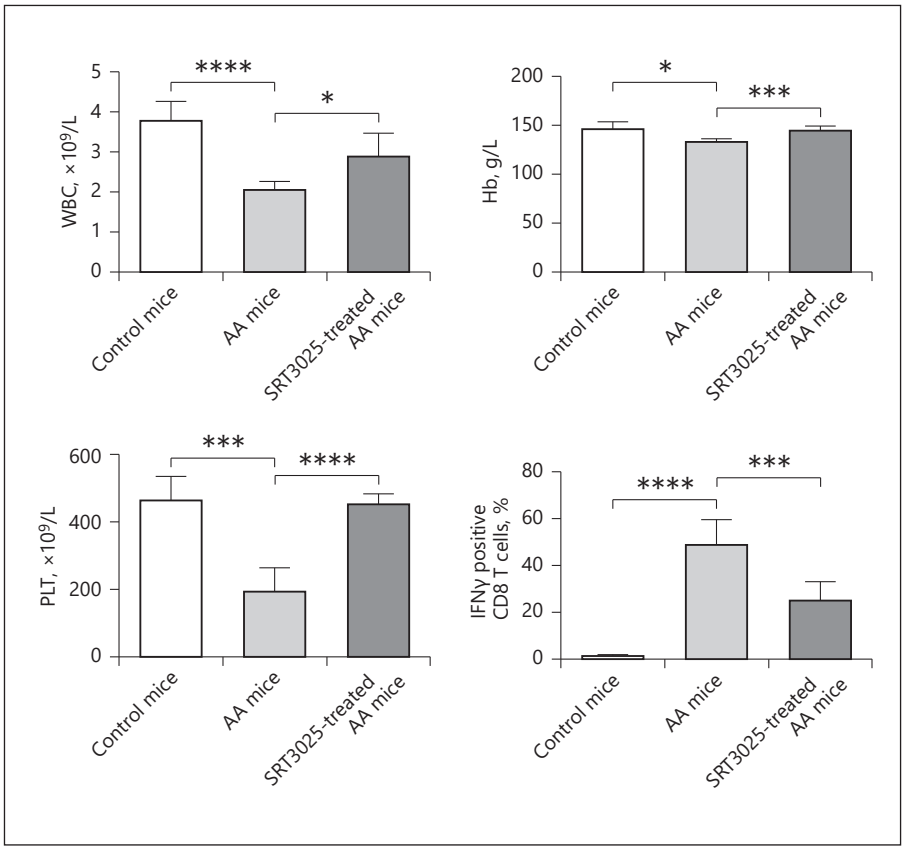

Fig. 5. Routine blood tests and IFNγ expression level of mice tested on day +17. After disease induction, the level of WBC, Hb, and PLT of the AA mice was significantly decreased compared to the normal controls ($p < 0.05$), and the IFNγ expression in CD8+T cells was significantly higher than in controls ($p < 0.05$). When comparing SRT3025-treated AA mice with nontreated AA mice, the levels of WBC, Hb, and PLT were significantly increased ($p < 0.05$), and the expression level of IFNγ was also significantly decreased ($p < 0.05$).

analyzed by real-time PCR. The SIRT1 expression in the SAA group (0.35 ± 0.09) was significantly lower compared to the healthy control group (1.00 ± 0.22; $p < 0.05$) (Fig. 1b).

IFNγ Expression Is Elevated in CD8+T Cells of SAA Patients

IFNγ levels in SAA patients and healthy controls were estimated by flow cytometry, after 4 h of stimulation with PMA, ionomycin, and BFA. The IFNγ expression in CD8+T cells of SAA patients (42.36 ± 6.24) was significantly higher compared to the healthy controls (1.32 ± 0.35; $p < 0.01$) (Fig. 2). Relative IFNG mRNA was determined by RT-PCR, and significantly elevated levels of IFNG expression were observed in the SAA group (4.90 ± 1.36) compared to the healthy control group (1.00 ± 0.23; $p < 0.05$) (Fig. 1c).

IFNG Was Negatively Regulated by Sirt1 in SAA

We analyzed the correlation between Sirt1 and IFNG expression and found that there was a significant negative correlation between Sirt1 and the IFNG level in CD8+T cells ($r = -0.53$; $p < 0.05$) (Fig. 1d). SRT3025-treated SAA samples showed a decrease of IFNγ expression ($p < 0.01$) and median fluorescence intensity (MFI) ($p < 0.01$) (Fig. 3). SRT3025 treatment increased Sirt1 expression in SAA samples ($p < 0.05$), with an increasing trend of MFI ($p > 0.05$) (Fig. 4).

SRT3025 Treatment Promoted Recovery in AA Mice

As mentioned above, we found that SRT3025 could reduce IFNγ expression in SAA patients in vitro. Hence, we tried to explore whether SRT3025 had the same effect in vivo. AA mice models were established using the CByB6F1 mice, and then AA mice were treated with SRT3025 (25 mg/kg/every other day, i.p. injection). We found that the SRT3025 treatment significantly alleviated the condition of AA mice. On day +17 of disease induction, blood and IFNγ expression were routinely tested. The results showed that SRT3025 treatment significantly increased the number of WBC, Hb, and PLT in AA mice ($p < 0.05$) (Fig. 5; Table 2), and IFNγ expression in the SRT3025-treated AA mice group was significantly lower than that in AA mice (Fig. 5; Table 2).

Table 2. Routine blood tests and IFNγ expression level of mice

	Control mice ($n = 5$)	AA mice ($n = 5$)	SRT3025-treated AA mice ($n = 5$)
WBC, $\times 10^9$/L	3.80±0.21[b]	2.08±0.08[a, b]	2.92±0.26[a]
Hb, g/L	146±3.48	133.4±1.43[a, b]	145.2±1.77
PLT, $\times 10^9$/L	464.6±31.54	192±31.72[a, b]	455±12.82
IFNγ$^+$CD8$^{+\ T}$ cell, %	1.51±0.21[b]	48.98±4.88[a, b]	25.26±3.55[a]

[a]The difference is statistically significant when compared to control mice ($p < 0.05$). [b]The difference is statistically significant when compared to SRT3025-treated AA mice ($p < 0.05$).

Discussion

SAA is an immune-mediated bone marrow failure syndrome that is characterized by aberrant immune responses and is mediated by CTLs, Th1, Th17, mDCs (myeloid dendritic cells), and the hypofunctional Tregs [1–3, 16–18]. Recent studies have demonstrated that the overactivated CTLs play critical roles in the pathogenesis of SAA. IFNγ is one of the negative effector cytokines, and it is highly expressed in CTLs of SAA patients. Thus, a sustained IFNγ exposure could harm the HSCs and hemopoietic progenitors by direct and indirect impact. IFNγ signal could induce proliferation and reduce self-renewal of the HSCs, and as a result, limit the function and promote exhaustion of the HSCs. IFNγ could also limit the responses of the HSC to thrombopoietin by increasing the suppressor of cytokine signal (SOCS1). The increased expression of Fas induced by IFNγ contributed to the apoptosis of HSCs in aplastic anemia [4, 5].

Sirt1 is a member of the sirtuin family, which has been demonstrated to play a critical role in cellular metabolism, inflammation, and cancer. Several reports have indicated that a decrease in Sirt1 expression could trigger and aggravate the inflammatory status. Sirt1 and NF-κB signaling show antagonistic effects in regulating inflammation and immune responses. Activation of Sirt1 could attenuate the inflammatory responses and cytokine storm driven by NF-κB signaling. Sirt1 deficiency stimulated the activation of NF-κB and histone hyperacetylation, which may be related to the activation of inflammatory genes [6, 7, 19–21]. Caruso et al. [8] reported that SIRT1 expression deficiency promoted the persistent inflammatory state in patients with inflammatory bowel disease, and inhibited the expression of SIRT1 in the lamina propria mononuclear cells of the control group, leading to increased IFNγ. Arieta Kuksin et al. [10] found that NF-κB signaling was involved in bone marrow infiltration of CD8+T cells in the mouse model of aplastic anemia. Qi et al. [22] demonstrated that the acetylation level of histone H3 was higher in CD8+T cells in SAA. Additionally, studies have also pointed out that Sirt1 may be dispensable for the differentiation of hematopoietic progenitor cells, and loss of Sirt1 prevented embryonic stem cells from differentiating into hematopoietic progenitor cells [11, 12].

In this study, we investigated the role of Sirt1 in SAA patients. CD8+T cells were chosen to be the focus of our research based on their role in SAA pathogenesis. We analyzed the Sirt1 mRNA and protein expression levels in SAA patients and healthy controls. Our results indicated that Sirt1 expression was downregulated significantly in CD8+T cells of SAA patients compared to the healthy controls, at both the gene and protein level. We measured the IFNγ expression level using RT-PCR and flow cytometry, and our results revealed high IFNγ expression levels in SAA patients, in accordance with the earlier reports [13, 23]. IFNγ expression was elevated significantly at both the gene and protein level in CD8+T cells of SAA patients compared to the healthy controls. We also evaluated the correlation between Sirt1 and IFNγ, and found that the relative Sirt1 expression was negatively correlated to IFNγ at the mRNA level. SRT3025, a Sirt1 activator, was proven to be beneficial in the treatment of Fanconi anemia. SRT3025 was able to increase the number of HSCs and improve the hemopoiesis in Fanconi anemia mice [24]. In order to estimate the effects of Sirt1 in SAA patients, we incubated the PBMCs with stimulants and 10 μm of SRT3025. Our flow cytometry results indicated that SRT3025 treatment could elevate Sirt1 and downregulate IFNγ expression in CD8+T cells of SAA patients significantly. Consistent with the above results, in vivo experiments showed that SRT3025 promoted the recovery of AA mice and significantly inhibited IFNγ expression.

In this study, we confirmed the role of Sirt1 in the pathogenesis of SAA, a T cell-mediated immune disease. Sirt1 was demonstrated to regulate immune responses and T cell activation. Overactivated CD8+T cells and IFNγ play a critical role in SAA. Our study revealed a defective Sirt1 expression in the CD8+T cells of SAA patients, which correlated with the high expression of IFNγ in SAA. Sirt1 activation could downregulate IFNγ in CD8+T cells of SAA patients. Moreover, the Sirt1 activator SRT3025 showed a therapeutic role in AA mice. Based on these results, we hypothesized that the Sirt1 signal may play a key role in regulating the inflammation status of SAA. In order to figure out the mechanism of Sirt1 in SAA, including the relationship between the low expres-

sion of Sirt1 and the bone marrow failure status of SAA, further investigations in SAA patients and mouse models are need.

Acknowledgements

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Statement of Ethics

This study was approved by the ethics committee of the Tianjin Medical University. All patients and healthy controls signed an informed consent in accordance with the Helsinki Declaration.

Disclosure Statement

The authors have no competing financial interests to declare.

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