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Original Article

Activation profile of intracellular mitogen-activated protein kinases in CD8+ T lymphocytes of patients with severe aplastic anemia

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Abstract: Objective: Severe aplastic anemia (SAA) is a rare autoimmune disease characterized by severe pancytopenia and bone marrow failure, which is caused by activated T lymphocytes. Mitogen-activated protein kinases (MAPKs) are serine and threonine protein kinases that can be activated by phosphorylation in response to extracellular stimuli, such as mitogens, growth factors, cytokines, and osmotic stress. MAPKs signaling pathways are dynamic and sensitive regulators of T cell function and differentiation. This study aims at understanding the role of intracellular MAPKs in CD8+ T lymphocytes of patients with severe aplastic anemia. Methods: The activation of extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK) and p38 MAPK in CD8+ T lymphocytes were measured by flow cytometry. Results: There was no statistical difference in the expression of phospho-ERK in CD8+ lymphocytes between SAA patients and normal controls. The expression of phospho-JNK and phospho-p38 in CD8+ Lymphocytes in SAA patients was higher than that in normal controls. Moreover, the expression of phospho-JNK was positively correlated with the percentage of perforin in CD8+ lymphocytes between SAA patients and normal controls. Conclusion: The activation of JNK and p38 MAPK signaling pathways in CD8+ T lymphocytes can be the underlying intracellular mechanisms causing CD8+ T lymphocytes hyperactivity in SAA.

Keywords: Mitogen-activated protein kinases (MAPKs), severe aplastic anemia, CD8+ T lymphocytes

Introduction

Severe aplastic anemia (SAA) is a bone marrow failure syndrome characterized by bone marrow aplasia and severe peripheral blood pancytopenia. Immune-mediated destruction of hematopoietic stem/progenitor cells (HSPCs) by activated T lymphocytes plays a central role in the pathophysiology of SAA. Many studies have demonstrated that dysregulation of T-cell function, especially of CD8+ T lymphocytes, leads to bone marrow failure in SAA [1-3]. CD8+ T lymphocytes usually damage target cells using three mechanisms: cytokines, perforin-granzyme B and the Fas-FasL pathway. The mechanisms underlying how CD8+ T lymphocytes in SAA become activated have not been elucidated [4].

Mitogen-activated protein kinases (MAPKs), which include the extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK) and p38 MAPK, play a significant role in mediating signals triggered by cytokines, growth factors and environmental stress. Nuclear translocation of activated MAPKs can induce and trans-activate transcription factors including nuclear factor (NF)-κB and activator protein 1, which facilitate the modulation of gene transcription in cellular activation, proliferation, apoptosis and the expression of cytokines, chemokines, adhesion molecules, and metalloproteinases [5, 6].

However, the activation profile of intracellular ERK, JNK, and p38 MAPK for the abnormal effector functions of CD8+ T lymphocytes and their relationship with the immunopathogenesis in SAA have not been elucidated. This study aims at understanding the role of intracellular MAPKs in CD8+ T lymphocytes of patients with SAA.

Table 1. Characteristics of untreated patients

Case	Gender	Age	Neutrophil ($\times10^9$/L)	Hemoglobin (g/L)	Platelet ($\times10^9$/L)	Reticulocyte ($\times10^9$/L)	Abnormal chromosome
1	Male	11	0.89	68	6	0.31	Absense
2	Female	41	0.67	83	4	0.48	Absense
3	Male	63	0.31	59	26	0.59	Absense
4	Female	79	0.94	47	5	0.18	Absense
5	Female	27	0.25	78	3	0.76	Absense
6	Female	27	0.06	60	9	0.17	Absense
7	Female	58	0.72	64	1	0.83	Absense
8	Male	29	0.61	59	5	0.46	Absense
9	Male	72	0.06	74	17	0.15	Absense
10	Female	66	0.04	61	25	0.11	Absense
11	Female	53	0.06	65	41	0.2	Absense
12	Male	14	0.97	121	5	0.49	Absense
13	Male	8	0.45	71	4	0.99	Absense
14	Male	49	0.14	68	22	0.16	Absense
15	Female	23	0.68	111	3	0.24	Absense
16	Female	16	0.53	61	19	0.21	Absense
17	Female	50	0.28	71	36	0.2	Absense
18	Female	44	0.03	65	31	0.12	Absense
19	Male	20	1.56	75	7	0.28	Absense
20	Male	68	0.49	89	54	0.2	Absense

Materials and methods

Study subjects

Twenty patients (eleven females, nine males) with SAA were diagnosed in the Hematology Department of General Hospital Tianjin Medical University from November 2015 to June 2016 according to International AA Study Group Criteria [3]. The disease was considered SAA if pancytopenia with at least two of the following parameters were met: a neutrophil count $<0.5\times10^9$/L, a platelet count $<20\times10^9$/L, and a reticulocyte count $<20\times10^9$/L with hypocellular bone marrow. If the neutrophil count was $<0.2\times10^9$/L, AA was considered very severe. Patients were excluded if they had congenital AA or other autoimmune diseases. All patients and normal controls were screened for paroxysmal nocturnal hemoglobinuria (PNH) by flow cytometry using anti-CD55 and anti-CD59 antibodies, and no PNH clones had been found. Bone marrow cytogenetic studies were performed in all patients, and no chromosomal abnormalities were identified. Patients' features are listed in **Table 1**.

Sixteen cases (eight females, eight males) in remission SAA (R-SAA) are those who improved after immunosuppressive therapy (antithymocyte globulin, cyclosporine, glucocorticoid) and hematopoietic stimulating factors (granulocyte colony-stimulating factor, recombinant human erythropoietin, recombinant human thrombopoietin, and/or IL-11 in combination). All of the patients in R-SAA achieved bone marrow hematopoietic recovery and became transfusion independent, while some had normal peripheral blood cell counts but still required drug therapy. Patients' features are listed in **Table 2**.

There were nineteen healthy volunteers (ten females, nine males) in the normal controls. The blood of the normal controls was normal upon routine examination. This study was approved by the Ethics Committee of Tianjin Medical University. Informed written consent was obtained from all patients in accordance with the Declaration of Helsinki.

Monoclonal antibodies

The fluorophore-conjugated monoclonal antibodies: anti-CD3-PerCP, anti-CD8-FITC, anti-perforin-PE, anti-Granzyme B-APC, anti-phospho-ERK1/2 (T202/Y204)-PE, anti-phospho-JNK (pT183/pY185)-APC, anti-phospho-p38 (pT180/pY182)-PE, plus the relevant mouse

Table 2. Characteristics of remission patients

Case	Gender	Age	Neutrophil ($\times10^9$/L)	Hemoglobin (g/L)	Platelet ($\times10^9$/L)	Reticulocyte ($\times10^9$/L)	Abnormal chromosome	Therapy
1	Male	28	12.48	123	183	89.4	Absense	ATG+CsA
2	Male	41	1.54	160	143	97	Absense	ATG+CsA
3	Female	10	4.22	153	136	93.8	Absense	ATG+CsA
4	Male	20	2.65	128	101	91.8	Absense	ATG+CsA
5	Male	32	5.06	123	80	90.0	Absense	ATG+CsA
6	Female	14	2.05	127	159	45.9	Absense	ATG+CsA
7	Male	29	9.12	116	170	69	Absense	ATG+CsA
8	Male	7	5.45	121	85	46.7	Absense	ATG+CsA
9	Female	25	3.81	160	266	65.6	Absense	ATG+CsA
10	Male	27	3.84	128	98	125.2	Absense	ATG+CsA
11	Female	24	3.1	102	128	159.4	Absense	ATG+CsA
12	Male	23	1.27	158	134	102.3	Absense	ATG+CsA
13	Female	16	1.94	134	172	70.7	Absense	ATG+CsA
14	Female	47	2.82	91	171	62.8	Absense	ATG+CsA
15	Female	64	1.79	138	160	36.8	Absense	ATG+CsA
16	Female	28	2.16	113	162	135.9	Absense	ATG+CsA

isotype controls, were purchased from Becton Dickinson (BD, Franklin Lakes, NJ, USA).

Flow-cytometric analysis of intracellular signaling molecules in CD8+ lymphocytes

Human peripheral blood was collected in the presence of EDTA tubes from the patients and normal controls treated with or without 40 nM PMA at 37°C for 10 minutes. Dilute the required amount of BD Phosflow™ Lyse/Fix Buffer (5× concentrate) 1:5 with distilled water (at room temperature) and then pre-warm the solution to 37°C. Mix one volume of blood with 20 volumes of the pre-warmed BD Phosflow™ Lyse/Fix Buffer (1×). Mix well by vigorously inverting the tubes 8-10 times, and then incubate the tubes in a 37°C water bath for 10 min. Spin down the cells at 500×g for 8 min in a tabletop centrifuge, aspirate the supernatant, and wash the cells once with Hank's solution. Vortex to loosen the cells and permeablize the cells by adding with BD Phosflow™ Perm Buffer III for 30 minutes on ice. Wash the cells twice and resuspend with BD Pharmingen™ Staining Buffer. Stain the cells with anti-phospho-ERK1/2 (T202/Y204)-PE, anti-phospho-JNK (pT183/pY185)-APC, anti-phospho-p38 (pT180/pY182)-PE for 30-60 min at room temperature, wash and prepare for flow cytometric analysis.

Fluorochrome-conjugated antibodies specific for surface markers of CD8+ T lymphocyte were used in combination with antibodies that recognize intracellular phosphorylated ERK, JNK, or p38 MAPK for staining fixed and permeabilized peripheral blood. Mouse IgG isotypic antibodies were used to normalize the background signal (BD Pharmingen).

The expression of activated phosphorylated signaling molecules was analyzed by flow cytometry (BD FACSC alibur flow cytometer). The differential expression of phosphorylated MAPKs in CD8+ T lymphocyte was presented as mean fluorescence intensity (MFI). Their relative expression (%) was calculated based on the percentage change of phosphorylated MAPKs expression in resting and stimulated cells stimulation MFI/resting MFI×100%.

Measurement of effector proteins in CD8+ T cells

The effector proteins of CD3+ CD8+ T cells, perforin, and granzyme B were measured from peripheral blood samples. Anti-perforin-PE and anti-granzyme B-APC were stained with mAb at 4°C for 20 min after permeabilization of the cells following staining of extracellular markers anti-CD3-PerCP and anti-CD8-FITC. Stained cells were then analyzed by flow cytometry.

Statistical analysis

All statistical calculations were performed using SPSS Statistics 18.0. Statistical analysis

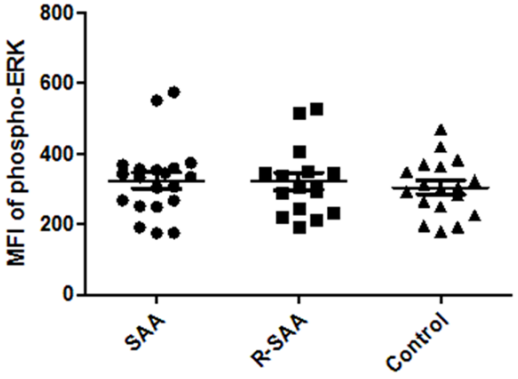

Figure 1. Expression of phosphor-erk in resting and activated peripheral blood. There was no statistical difference in the expression of phospho-ERK in CD8+ lymphocytes between SAA, R-SAA and normal controls (all P>0.05).

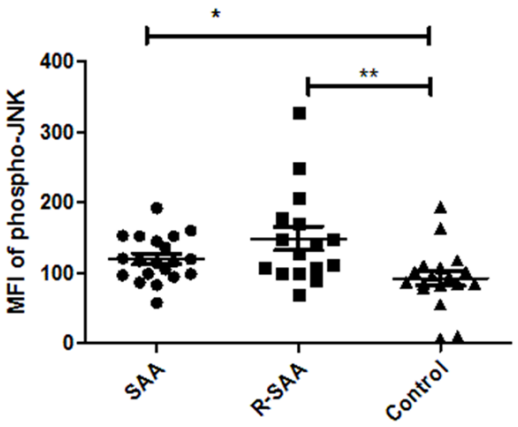

Figure 2. Expression of phosphor-jnk in resting and activated peripheral blood. The expression of phospho-JNK in CD8+ Lymphocytes in SAA was higher than that in normal controls (*P*<0.05). The expression of phospho-JNK in CD8+ Lymphocytes in R-SAA patients was higher than that in normal controls (*P*<0.01). *P<0.05, **P<0.01.

was performed with the parametric unpaired t-test for normally distribution data, the non-parametric test for skewed data, and a Spearman correlation analysis for correlated data. A *P* value of <0.05 was considered statistically significant.

Results

Expression of phospho-ERK in CD8+ T lymphocytes

The expression of phospho-ERK in CD8+ T Lymphocytes of SAA, R-SAA and normal controls was (324.1±103.6), (321.6±98.21) and (304.6±79.86), respectively. As shown in **Figure 1**, there was no statistical difference in the expression of phospho-ERK in CD8+ T lymphocytes between SAA, R-SAA and normal controls (all P>0.05). In addition, no correlation was observed between the expression of phospho-ERK and perforin, granzyme B (all P>0.05).

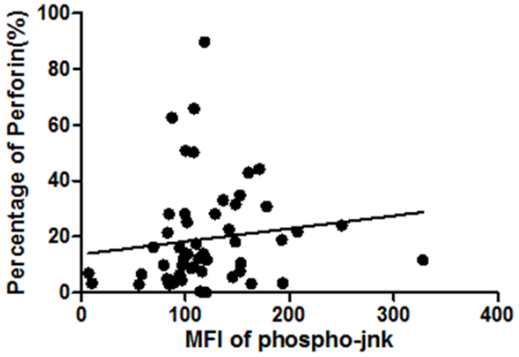

Figure 3. Correlation of phospho-jnk MFI and perforin. The expression of phospho-JNK with perforin (n=54, r=0.3080, P=0.0235).

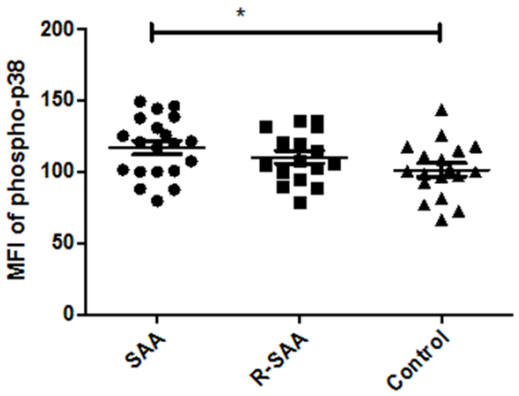

Figure 4. Expression of phosphor-p38 in resting and activated peripheral blood. The expression of phospho-p38 in CD8+ Lymphocytes in SAA was higher than that in normal controls (*P*<0.05). There was no statistical difference in the expression of phospho-p38 in CD8+ lymphocytes between R-SAA and normal controls (P>0.05). *P<0.05.

Expression of phospho-JNK in CD8+ T lymphocytes

The expression of phospho-JNK in CD8+ T lymphocytes was (120.1±32.19) in SAA, and (92.43±43.75) in normal controls. The expression of phospho-JNK in CD8+ T lymphocytes in SAA was higher than that in normal controls

Table 3. Correlation of MAPK expression with clinical parameters

		Neutrophil	Hemoglobin	Platelet	Reticulocyte
Phospho-erk (MFI)	Spearman r	-0.0375	-0.003477	0.06656	-0.1022
	P value	0.8277	0.9839	0.6997	0.5530
Phospho-jnk (MFI)	Spearman r	0.08702	0.08731	0.1893	0.1617
	P value	0.6138	0.6126	0.2689	0.3461
Phospho-p38 (MFI)	Spearman r	-0.3155	-0.3121	0.07835	-0.2768
	P value	0.0608	0.0639	0.6497	0.1022

(*P*<0.05). The expression of phospho-JNK in CD8+ Lymphocytes was (149.2±66.76) in R-SAA, and (92.43±43.75) in normal controls (*P*<0.01). The expression of phospho-JNK in CD8+ Lymphocytes in R-SAA was higher than that in normal controls (*P*<0.01) (**Figure 2**). In patients and normal controls, the expression of phospho-JNK was positively correlated with the percentage of perforin in CD8+ lymphocytes (n=54, r=0.3080, P=0.0235) (**Figure 3**). No correlation was observed between the expression of phospho-JNK and granzyme B (P> 0.05).

Expression of phospho-p38 in CD8+ T lymphocytes

The expression of phospho-p38 in CD8+ Lymphocytes of SAA, R-SAA and normal controls was (117.1±20.70), (110.3±17.91) and (101.3±19.43), respectively. As shown in **Figure 4**, the expression of phospho-p38 was significantly higher in SAA than that in normal controls (P<0.05). There was no statistical difference in the expression of phospho-p38 in CD8+ lymphocytes between R-SAA and normal controls (P>0.05). In addition, no correlation was observed between the expression of phospho-p38 and perforin, granzyme B (all P>0.05).

Correlation of MAPK expression with clinical parameters

There was no significant relationship between the expression of MAPK with clinical parameters (**Table 3**).

Discussion

SAA is an autoimmune disease caused by dysregulation of immune cell subsets, especially T lymphocytes [8, 9]. Several immune abnormalities associated with the pathogenesis of SAA including imbalance DC subsets (elevated DC1), enhanced DC function, regulatory T-cell insufficiency, imbalance of Th1/Th2 subsets (enhanced Th1), increased type I lymphoid factors (IL-2, IFN-c), and a decreased proportion of NK cells [10-12]. Our previous study showed that an imbalance of CD4+ T-cell subsets and increases in IFN-c production by Th1 cells, caused by antigenic stimulus or T-cell proliferation, leads to activation of CD8+ T cells and excessive expression of apoptosis ligands in stem cells [13]. Our previous study demonstrated that the number and function of CD8+ T cells was significantly increased in SAA patients compared with normal controls [14]. Moreover, we showed that overexpression of linker for activation of T cells (LAT), which recruits multiple signaling molecules important for T-cell activation once the T-cell receptor (TCR) is engaged, is responsible for T-cell proliferation, imbalance of Th1/Th2 subsets, increased activation of CD8+ T cells, and even bone marrow failure in SAA [15]. The MAPK pathway is crucial for T cell receptor (TCR) signaling in the development and function of T cells. Altered MAPK signaling has been associated with the inflammatory and autoimmune diseases lupus and arthritis and with some pathogenic viral infections. Therefore, we assume that MAPKs changed during T-cell activation, which may cause excess T-cell proliferation and even increases in the quantity and function of CD8+ T cells.

The Erks were the first members of the MAPK family to be identified [7, 16]. Erk1 (Mapk3) and Erk2 (Mapk1) are the two main isoforms of the prototypical MAPK Erk. Erk1 and Erk2 are activated in T cells following TCR triggering by a well-studied signaling cascade that involves the sequential activation of the small GTPase Ras, recruitment and activation of Raf-1, phosphorylation of Mek1 and Mek2, and dual phosphorylation of Erk1/2 [17]. The Erk pathway is also induced by growth-promoting or mitogenic signals [18] and is necessary for G1 to S phase progression in a variety of cell types [19]. Three members of the JNK family have been identified in eukaryotic cells (JNK1, JNK2 and JNK3) [20]. While Jnk1 and Jnk2 are more ubiquitously expressed, Jnk3 expression is limited to

brain, testis and heart. The JNK signaling pathway appears to act as a critical intermediate in the regulation of lymphocyte activation and proliferation. Four members of the p38 MAPK have been now identified: p38α, p38β, p38γ and p38δ [21]. The p38 MAPK gene was phosphorylated on tyrosine in response to hyperosmolarity and endotoxic lipopolysaccharide. Inhibition of p38 MAPK also inhibits IFN-γ production in CD8+ T lymphocytes, and persistent activation of p38 MAPK in CD8+ T lymphocytes promoted IFN-γ production [22].

Methods to detect intracellular kinase signaling intermediates by flow cytometry have been recently developed. Termed "phospho-flow", these methods employ fluorescence-conjugated monoclonal antibodies that recognize phosphorylated epitopes of intracellular kinases, and may be combined with surface phenotypic markers to observe changes in kinase pathways by cellular subset. Effector functions, like cytokine production, are processes intrinsically linked to intracellular signaling and kinase activity within each cell. In this study, we examined ERK, Jnk, p38 phosphorylation and perforin, granzyme B production by CD8+ T cells upon polyclonal stimulation with PMA. We present a method that allows observation of kinase phosphorylation and cytokine production within the same cell after stimuli, while maintaining a stable cellular phenotype. Monitoring of signaling and effector functions in distinct immune subsets provides a platform to investigate and relate intracellular kinase signaling activity to immune cell effector function and phenotype in disease states [23].

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease associated with aberrant activation of T lymphocytes. Abnormal activation of intracellular signaling molecules in lymphocytes by inflammatory cytokines can instigate the inflammation in SLE. The basal expression of phospho-p38 MAPK in CD8+ T lymphocytes was significantly higher in SLE patients than controls. The expression of phospho-p38 MAPK in CD8+ T lymphocytes and phospho-JNK in CD8+ T lymphocytes was also significantly elevated in SLE patients upon the activation by IL-18, exhibiting significant correlation with the plasma concentrations of Th1 chemokine CXCL10. The expression of phospho-JNK in IL-18 activated CD8+ T lymphocytes was significantly correlated with SLE disease activity index [24]. Rheumatoid arthritis (RA) is an autoimmune disease characterized by pronounced inflammation and leucocyte infiltration in affected joints. RA patients had a selective increase in ERK phosphorylation compared with demographically matched controls due to a mechanism distal of Ras activation. Increased ERK responses included CD8+ T lymphocytes and did not correlate with disease activity. Treatment with subtherapeutic doses of a MEK-1/2 inhibitor delayed arthritis onset and reduced severity, suggesting that increased ERK phosphorylation predisposes for autoimmunity and can be targeted to prevent disease [25]. Phospho-flow analysis identified phosphorylation-activation of specific signaling effectors in the PB from patients with ERA. Notably, phosphorylation of these signaling effectors did not distinguish ERA from late RA, suggesting that the activation status of discrete cell populations is already established early in disease. However, when the ratio of MFI values for p-AKT and p-p38 is >1.5, there is a high likelihood of having a diagnosis of RA. The results suggest that longitudinal sampling of patients undergoing therapy may result in phosphosignatures that are predictive of drug responsiveness [26]. Soejima K et al. wanted to determine via immunohistochemistry, whether or not c-Jun NH2-terminal kinase (JNK) cascade is activated in labial salivary infiltrating T cells in patients with Sjögren's syndrome (SS). Phosphorylated forms of MKK4, JNK, and c-Jun were detected in salivary infiltrating mononuclear cells. Expression of phosphorylated JNK was found in CD8+ T cells. Moreover, co-expression of phosphorylated JNK and c-Jun was demonstrated in the mirror sections. The JNK cascade is activated in salivary infiltrating CD8+ T cells in SS patients, which appears to contribute to the inflammatory salivary microenvironment of SS [27]. Autoimmune activation and deregulated apoptosis of T lymphocytes are involved in multiple sclerosis (MS). The immunomodulatory effect of AS602801, a JNK inhibitor, was firstly evaluated on activated peripheral blood mononuclear cells (PBMCs) from healthy volunteers (HVs) and secondly in unstimulated purified CD8+ cells from RRMS patients and HVs. In activated PBMCs from HVs, we showed that AS602801 blocked T-lymphocyte proliferation and induced apoptosis. In RRMS CD8+ cells, AS602801 induced apoptosis genes and ex-

pression of surface markers. Untreated cells from RRMS active-phase patients significantly released interleukin-23 (IL-23) and interferon-gamma (IFN-γ) and expressed less apoptosis markers compared to the cells of HVs. The analysis of the JNK-dependent apoptosis pathway can provide biomarkers for activated lymphocytes in the active phase of RRMS and a gene expression signature for disease status. The reported results might be useful to stratify patients, thereby supporting the development of novel therapies [28].

Compared to above mentioned autoimmune disease, we found similar results in SAA patients. The expression of phospho-JNK and phospho-p38 in CD8+ Lymphocytes in SAA patients was higher than that in normal controls. Moreover, the expression of phospho-JNK was positively correlated with the percentage of perforin in CD8+ lymphocytes between SAA patients and normal controls. But there was no statistical difference in the expression of phospho-ERK in CD8+ lymphocytes between SAA patients and normal controls. In conclusion, the increased expression of MAPK in peripheral blood T cells of patients with SAA may lead to hyperfunctional CD8+ T cells, contributing to the excessive apoptosis of hematopoietic cells in this disease. Therefore, MAPKs might play a major role in the immunopathogenesis of SAA and thus be a novel target for treatment in SAA.

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Disclosure of conflict of interest

None.

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