Int J Clin Exp Med 2017;10(2):2939-2945
www.ijcem.com /ISSN:1940-5901/IJCEM0045535

Original Article

SOCS1/3 regulates the differentiation of T cells by inhibiting stat3 and maybe related to Hashimoto's thyroiditis

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Received July 20, 2016; Accepted December 23, 2016; Epub February 15, 2017; Published February 28, 2017

Abstract: Objective: This study aims to explore the regulation of suppressor of cytokine signaling (SOCS1/3) on the differentiation of T cell and the role in Hashimoto's thyroiditis (HT). Methods: The $CD4^+$ T lymphocytes and B lymphocytes were separated from HT patients. The expression levels of SOCS1 and SOCS3 in $CD4^+$ T cells were up-regulated using gene transfection technique, signal transducer and activator of transcription 3 (STAT3) was silenced simultaneously. They were co-cultured with B cells and their effects on the differentiation of TH17, Treg and Tfh cells were detected by flow cytometry. The $CD4^+$ T cell related transcription factors such as retinoic acid related orphan receptor γt (ROR γt), Fork head box protein P3 (FOXP3) and B-cell lymphoma 6 (BCL 6) were detected by fluorescence quantitative PCR. The levels of cytokines in cell culture supernatant were detected by enzyme linked immunosorbent assay. The protein levels of SOCS3, SOCS1 and STAT3 were detected by western blotting method. Results: The expression of IL-17 was significantly reduced while the expression of Foxp3 was significantly increased after $CD4^+$ T cells were transfected with SOCS1/3 and polarized in vitro for 72 h. Silencing stat3 inhibited the TH17 and Tfh cells while promoted Treg cells ($P<0.01$). SOCS1/3 and stat3 inhibited the ROR γt and BCL6 while promoted Foxp3. The inhibition of IL-6, L-17, IL-23 and IL-21 and the promotion of TGF-β1 were more obvious in SOCS1/3 + stat3 silence group than that of SOCS1/3 group and stat3 silence group ($P<0.01$). STAT3 protein levels significantly decreased and SOCS1/3 protein levels significantly increased in SOCS1/3 + stat3 silence group than that of control group ($P<0.05$). Conclusions: SOCS1/3 regulates the differentiation of T cells by inhibiting stat3, which may be related to the pathogenesis of HT.

Keywords: Suppressor of cytokine signaling (SOCS1/3), Th17, regulatory T cell, Hashimoto's thyroiditis (HT)

Introduction

Hashimoto's thyroiditis (HT) is an autoimmune disease, its specific immunological mechanism is not very clear. HT causes the thyroid tissue damage and low thyroid function with the infiltration of many lymphocytes and the production of specific autoantibodies in the thyroid gland.

Studies showed that the invasive cells in the thyroid gland of HT patients were mainly $CD4^+$ T cells [1]. Imbalance of Th17 cells and Treg cells in microenvironment may be one of the main mechanisms of HT pathogenesis [2]. Studies also found that the number of Tfh cells increased in peripheral blood and thyroid tissue of HT patients, which indicated that Tfh cells were related to the HT process [3, 4]. The suppressor of cytokine signaling (SOCS1/3) is an important member of the SOCS family and a class of molecules that regulate cytokine signaling pathways, it plays a negative role in T cell mediated immune response and immune regulation [5]. The negative regulation of SOCS protein is mainly through the two domains of "SOCS box" and "SH2". SH2 can combine with the phosphorylation of tyrosine residues. SOCS box has the characteristic of ubiquitin E3 ligase and mediate the degradation of substrate proteins [6, 7]. Studies showed that T cells in HT patients with low expression of SOCS1/3, which may affect the polarization and immune function of T cells [8].

It was reported that SOCS regulated the differentiation of Treg, Th2, Th17 and Th1 [8]. It is not reported whether SOCS affect the differentia-

Table 1. Primers used in this study

Primer	Sequence
GAPDH	F: 5'-ACC ACA GTC CAT GCC ATC AC-3'
	R: 5'-TCC ACC ACC CTG TTG CTG TA-3'
Foxp3	F: 5'-CAC CCA GGA AAG ACA GCA ACC-3'
	R: 5'-GCA AGA GCT CTT GTC CAT TGA-3'
RORγt	F: 5'-ACC TCC ACT GCC AGC TGT GTG CTG TC-3'
	R: 5'-TCA TTT CTG CAC TTC TGC ATG TAG ACT GTC CC-3'
BCL6	F: 5'-GACGTTGTCATCGTGGTGAG-3'
	R: 5'-GGTTGCATTTCAACTGGTCA-3'

tion of Tfh cells. STAT3 signaling pathway plays an important role in the differentiation of $CD4^+$ T cells [9]. Therefore, in this study we explored the regulation of SOCS1/3 on the differentiation of Tfh cells, which could improve the understanding about the mechanism of SOCS affecting the differentiation of T cells and was helpful to understand the role of SOCS1/3 in the pathogenesis of HT.

Material and methods

Isolation of $CD4^+$ T cells and $CD19^+$ B cells from peripheral blood of HT patients

A total of 50 HT patients (the levels of TGAb and TPOAb increased, pathologically confirmed) were recruited in this study. 5 ml venous blood was collected and the PBMC was separated by Ficoll density gradient centrifugation method. The $CD4^+$ T cells and $CD19^+$ B cells were separated using immunomagnetic beads. The cells were counted and their activity was detected by Trypan blue staining method.

Construction of SOCS1/3 slow virus vector and transfection of $CD4^+$ T cells

The pEF-FLAG-I/mSOCS1 vector and pEF-FLAG-I/mSOCS3 over-expression vector were constructed. Package the virus: Briefly, 293T at confluence of 70%~80% were used for transfection. The recombinant plasmid was transfected into 293T cells, Then after 8 hours, the medium containing transfection mixture residues was discarded and fresh medium was added, the cell supernatant was collected after 48 hours and removal of cell debris was carried out by centrifuge, then the 0.45 μm PVDF film was used to filter and harvest the packaged virus particles. The isolated peripheral blood $CD4^+$ T cells were stimulated with CD3/CD28 antibody for 24 h, and then the culture medium was replaced with the supernatant containing virus particles. The cells were divided into pEF-FLAG-I empty vector control group, SOCS1 vector group, SOCS3 vector group, stat3 silence group, SOCS1 vector + stat3 silence group and SOCS3 vector + stat3 silence group.

The polarization of TH17, Treg, Tfh in vitro and flow cytometry detection

After transfection, the cytokines of IL-2 (10 U/mL), IL-1β (10 ng/mL), TGF-β (30 ng/mL), anti-IL-4 antibody (1 μg/ml) and anti IFN-γ antibodies (1 μg/ml) were added for Th17 polarization, the cytokines of IL-2 (10 U/mL) and TGF-β (30 ng/mL) were added for Treg polarization, the cytokines of IL-6 (10 U/mL) and IL-21 (10 U/mL) were added for Tfh polarization. They were co-cultured with $CD19^+$ B cells for 3 days. The cells of TH17 and Tfh group were stimulated with PMA (50 ng/ml), ionomycin (500 ng/ml) and 1X Golgi Stop for 4 h. All cells were collected for flow cytometry detection. Intracellular IL-17A was stained with BD Perm/Wash buffer, nuclear Foxp3 was stained with Biolegend foxp3 fix/perm agents. $CD4^+$ $CXCR5^+$ $ICOS^{high}$ was the characteristic mark of Tfh cell surface.

Detection of RORγt, FoxP3 and Bcl6 mRNA expression by RT-PCR

The harvested cells were washed with RNase free PBS. Total RNA was extracted using RNeasy Mini Kit according to the manufacturer's protocol. Their concentration and purity were detected with Qubit Fluorometer. 1 μg RNA was subjected to reverse transcription using reverse transcription kit (Promega). Real-time PCR were performed using SYNBR Green PCR Master Mix (Qigen). Primers used in this study were shown in **Table 1**. Reaction parameters were pre-degeneration at 95°C for 5 min, degeneration at 95°C for 30 sec, annealing at 60°C for 45 sec and extension at 72°C for 30 sec with 40 cycles. The relative mRNA expression levels were calculated with $2^{-\Delta\Delta Ct}$ method.

Detection of IL-6, IL-17, IL-21, IL-23 and TGF-β with ELISA

The levels of IL-6, IL-17, IL-21, IL-23 and TGF-β were detected with ELISA kit according to the manual. OD values at 450 nm were determined by ELISA detector.

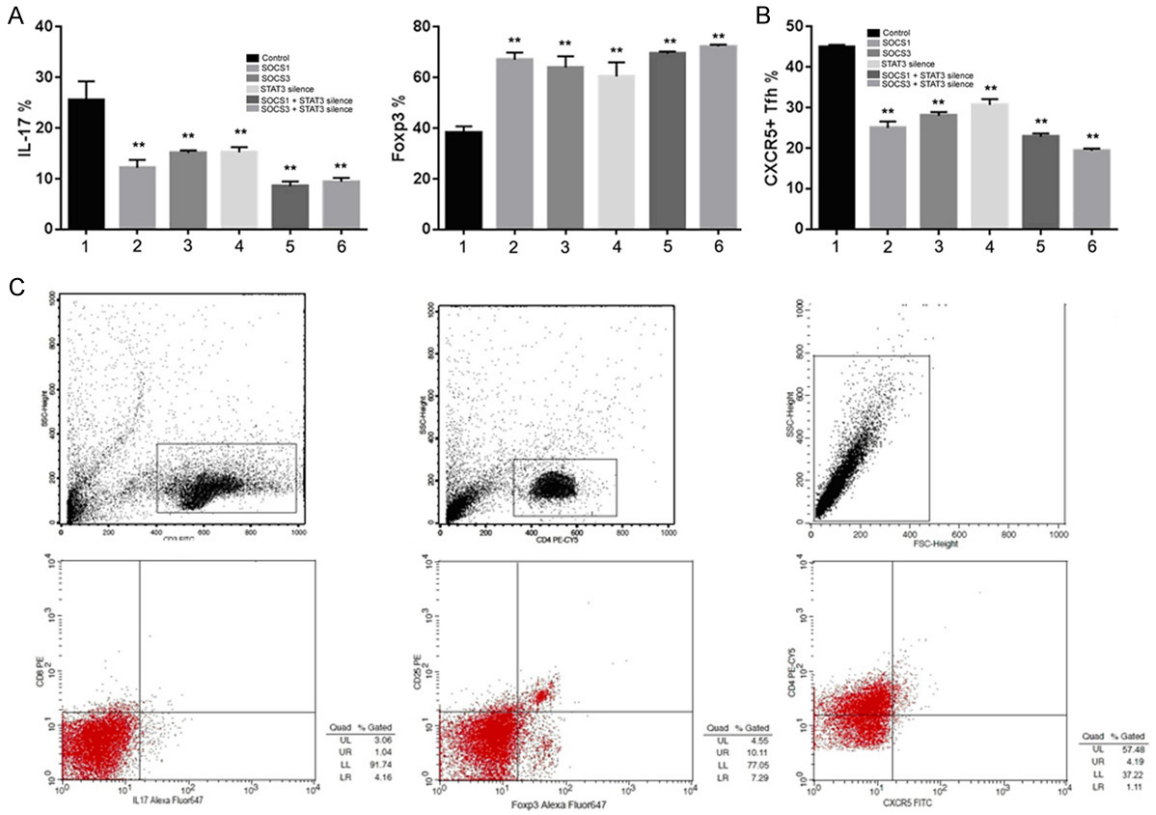

Figure 1. Effects of SOCS1/3 on the differentiation of Th17, Treg and Tfh cells. 1: Control group; 2: SOCS1 group; 3: SOCS3 group; 4: STAT3 silencing group; 5: SOCS1 + STAT3 silencing group; 6: SOCS3 + STAT3 silencing group; A: IL-17 and Foxp3; B: CXCR5$^+$; C: flow cytometry images. $^{**}P<0.01$ vs. control group.

Western blotting detection

The cells were lysed with RIPA lysis buffer and total proteins were extracted and analyzed with SDS-PAGE electrophoresis. Then it was electro-transferred to the PVDF membrane. After the transmembrane, PVDF membrane was rinsed with TBS for 10 to 15 min, placed in TBS/T blocking buffer containing 5% (w/v) skimmed milk powder and shook at room temperature for one hour. It was incubated at room temperature for two hours after added with appropriate dilution degree of primary antibodies (diluted with TBST containing 1% (w/v) skimmed milk powder, 1:5000). Then the membrane was rinsed with TBST for three times (5 to 10 minutes one time). The membrane was incubated at room temperature for one hour with HRP labeled secondary antibody (1:10000) diluted with TBST containing 0.05% (w/v) skimmed milk powder and rinsed for three times with TBST (5 to 10 minutes at a time). The protein bands were scanned and quantified as a ratio to β-actin.

Statistical analysis

The results are expressed as mean ± SD and analyzed with SPSS 17.0 software, t-test and ANOVA were used to evaluate the differences between groups. A value of $P<0.05$ was taken to denote statistical significance.

Results

There was a positive correlation between Tfh cell differentiation and SOCS1/3 and STAT3

SOCS1-Tg-CD4$^+$ T cells and SOCS3-Tg-CD4$^+$ T cells were constructed with gene transfection technique. As shown in **Figure 1A**, the expression of IL-17 was significantly reduced while the expression of Foxp3 was significantly increased after CD4$^+$ T cells were transfected with SOCS1/3 and polarized in vitro for 72 h ($P<0.01$), which suggested that SOCS1/3 can inhibit the expression of Th17 and promote the differentiation of Treg. It was found that silencing STAT3 could do better in inhibiting Th17 and

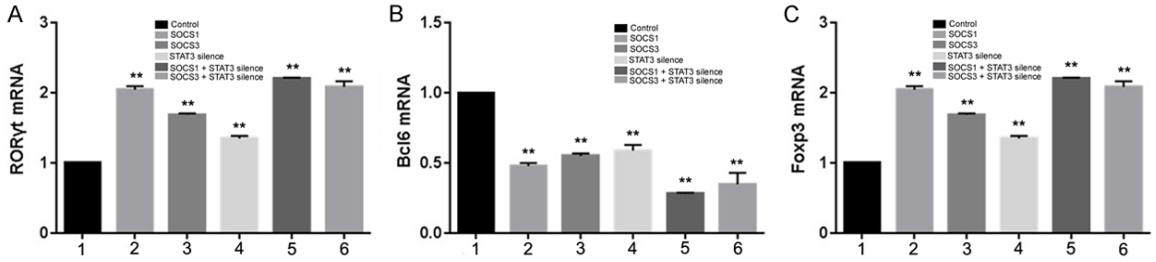

Figure 2. Real-time PCR results of RORγt, Bcl6 and FoxP3 mRNA expression. 1: Control group; 2: SOCS1 group; 3: SOCS3 group; 4: STAT3 silencing group; 5: SOCS1 + STAT3 silencing group; 6: SOCS3 + STAT3 silencing group; A: RORγt mRNA expression; B: Bcl6 mRNA expression; C: FoxP3 mRNA expression. $^{**}P<0.01$ vs. control group.

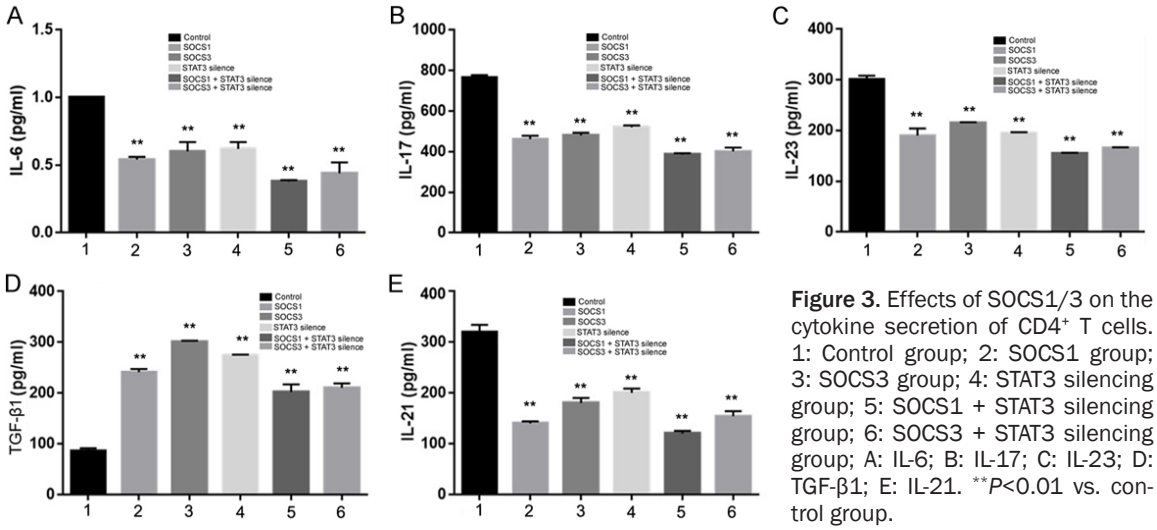

Figure 3. Effects of SOCS1/3 on the cytokine secretion of $CD4^+$ T cells. 1: Control group; 2: SOCS1 group; 3: SOCS3 group; 4: STAT3 silencing group; 5: SOCS1 + STAT3 silencing group; 6: SOCS3 + STAT3 silencing group; A: IL-6; B: IL-17; C: IL-23; D: TGF-β1; E: IL-21. $^{**}P<0.01$ vs. control group.

promoting the differentiation of Treg when compared with SOCS1/3 transfection alone ($P<0.01$), which suggested that the effect of SOCS1/3 on the differentiation of Th17 and Treg cells may be by the negative modulation of Stat3. As shown in **Figure 1B**, the proportion of Tfh cells was significantly decreased in SOCS1/3 transfection group and silencing STAT3 group when compared with control group ($P<0.01$), and the proportion of Tfh cells was significantly decreased in SOCS1/3 + stat3 silence group than that of SOCS1/3 group and stat3 silence group ($P<0.01$). These results suggested that there was a positive correlation between Tfh cell differentiation and SOCS1/3 and STAT3. Some flow cytometry images were shown in **Figure 1C**.

RT-PCR results were shown in **Figure 2**. It showed that the expression of RORγt and Bcl6 were inhibited while the expression of FoxP3 was promoted in SOCS1/3 transfection group and silencing STAT3 group compared with control group ($P<0.01$), the inhibition of RORγt and Bcl6 and the promotion of FoxP3 were more obvious in SOCS1/3 + stat3 silence group than that of SOCS1/3 group and stat3 silence group ($P<0.01$).

The levels of IL-6, L-17, IL-23 and IL-21 significantly decreased while the level of TGF-β1 significantly increased in SOCS1/3 transfection group

The ELISA results were shown in **Figure 3**. It showed that the levels of IL-6, L-17, IL-23 and IL-21 significantly decreased while the level of TGF-β1 significantly increased in SOCS1/3 transfection group and silencing STAT3 group compared with control group ($P<0.05$), the inhibition of IL-6, L-17, IL-23 and IL-21 and the promotion of TGF-β1 were more obvious in SOCS1/3 + stat3 silence group than that of SOCS1/3 group and stat3 silence group ($P<0.01$).

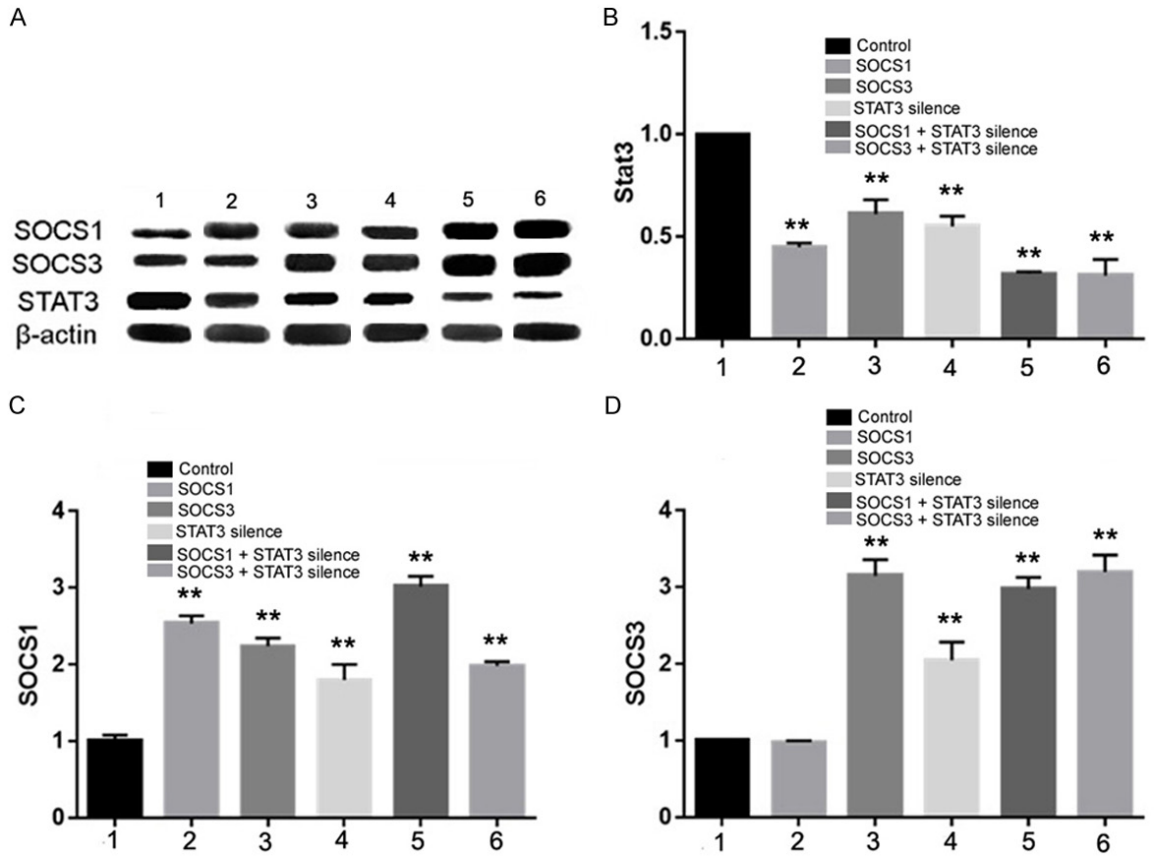

Figure 4. Western blotting results of SOCS1, SOCS3, and STAT3 protein levels in $CD4^+$ T cells. 1: Control group; 2: SOCS1 group; 3: SOCS3 group; 4: STAT3 silencing group; 5: SOCS1 + STAT3 silencing group; 6: SOCS3 + STAT3 silencing group; A: Western blotting image; B: STAT3 relative levels; C: SOCS1 relative levels; D: SOCS3 relative levels. $^{**}P<0.01$ vs. control group.

STAT3 protein levels significantly decreased in SOCS1/3 transfection group

The expression changes of STAT3 protein in the JAK/STAT3 pathway was shown in **Figure 4**. It showed that STAT3 protein levels significantly decreased in SOCS1/3 transfection group than that of control group ($P<0.05$), SOCS1/3 protein levels significantly increased in silencing STAT3 group compared with control group ($P<0.05$). STAT3 protein levels significantly decreased and SOCS1/3 protein levels significantly increased in SOCS1/3 + stat3 silence group than that of control group ($P<0.05$).

Discussion

Previous study showed that the number and function of T cells were abnormal in the HT patients [10], but the molecular mechanisms that lead to abnormal immune function remained unclear. In this study we observed the effects of negative regulatory factor SOCS1/3 on the differentiation of CD4T cells in patients with HT and explored the molecular pathogenesis of HT.

T cells play an important role in the immune response of the organism, the imbalance of T cell differentiation is the cause of a variety of diseases such as autoimmune disease. Therefore, cytokines secreted by different T cell subsets often play an important role, SOCS1/3 as an important negative regulation molecule is also involved in it. We found that over-expression of SOCS1/3 could inhibit the differentiation of Th17 and Tfh cells and promote the differentiation of Treg cells, and the effect of SOCS1/3 on the differentiation of T cells was more significant after silencing stat3. These results suggested that there was negative correlation between SOCS1/3 and STAT3, SOCS1/3 may inhibit the differentiation of Th17 and Tfh cells and induce the expression of Treg

by inhibiting Stat3. Previous studies have found that SOCS can participate in the regulation of the dynamic balance of Th17 and Treg in the body by inhibiting the IL-6/STAT3 signaling pathway [11, 12].

Th17 has pro-inflammatory effects in a variety of diseases, while Treg has a role of inhibiting inflammation. Previous study showed that Th17 increased and Treg decreased in HT patients, these changes are closely related to the thyroid function and pathogenesis process of HT patients [13, 14]. We found that SOCS1/3 overexpression or stat3 silence could inhibit the differentiation of Th17 and promote the differentiation of Treg, which suggested that specific intervention of SOCS1/3 or STAT3 expression may improve thyroiditis.

There are many kinds of cytokines involved in the process of T cell differentiation, the differentiated cell subsets could secrete special cytokines to play functions. IL-6 is an important cytokine to induce the production of TH17 and Tfh cells. We found that the level of IL-6 was significantly decreased while the level of TGF-β increased after SOCS1/3 overexpression. TGF-β was a kind of immunosuppressive factors mainly secreted by Treg cells. Studies showed that the proportion of Treg cells in HT patients was lower than that of normal healthy population, and its function was impaired [13, 14]. We found that the level of IL-21 was significantly decreased after SOCS1/3 overexpression. IL-21 is mainly secreted by Tfh cells and play an important role in the differentiation and development of Tfh cells. IL-21 also can induce type conversion of B cells and affect antibody secretion to regulate the function of B cells [15, 16]. These results suggested that SOCS1/3 could maintain the immune homeostasis and prevent the occurrence of autoimmune diseases by regulating the expression profile of cytokines.

In a word, we found that SOCS1/3 was the key factor regulating the differentiation and function of T cells, which maybe through inhibiting stat3. SOCS1/3 could inhibit the secretion of inflammatory factor IL-17 and promote the differentiation of Treg cells. Inhibition of SOCS1/3 on STAT3 also resulted in the reduction of Tfh cells, which inhibiting B cells to produce antibodies and reduce the occurrence of inflammation. Therefore, the specific intervention of SOCS1/3 expression or silencing STAT3 has a certain effect on the treatment of HT.

Acknowledgements

This work was supported by the National Natural Science Fund of China (Grants 8126-0125) and Project from Education Department of Jiangxi Province (GJJ10056).

Disclosure of conflict of interest

None.

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References

[1] Suciu-Foca N, Manavalan JS, Scotto L, Kim-Schulze S, Galluzzo S, Naiyer AJ, Fan J, Vlad G and Cortesini R. Molecular characterization of allospecific T suppressor and tolerogenic dendritic cells: review. Int Immunopharmacol 2005; 5: 7-11.

[2] Littman DR and Rudensky AY. Th17 and regulatory T cells in mediating and restraining inflammation. Cell 2010; 140: 845-858.

[3] Wang S, Baidoo SE, Liu Y, Zhu C, Tian J, Ma J, Tong J, Chen J, Tang X, Xu H and Lu L. T cell-derived leptin contributes to increased frequency of T helper type 17 cells in female patients with Hashimoto's thyroiditis. Clin Exp Immunol 2013; 171: 63-68.

[4] Qin Q, Liu P, Liu L, Wang R, Yan N, Yang J, Wang X, Pandey M and Zhang JA. The increased but non-predominant expression of Th17- and Th1-specific cytokines in Hashimoto's thyroiditis but not in graves' disease. Braz J Med Biol Res 2012; 45: 1202-1208.

[5] Mahony R, Ahmed S, Diskin C and Stevenson NJ. SOCS3 revisited: a broad regulator of disease, now ready for therapeutic use? Cell Mol Life Sci 2016; 73: 3323-36.

[6] McCormick SM and Heller NM. Regulation of macrophage, dendritic cell, and microglial phenotype and function by the SOCS proteins. Front Immunol 2015; 6: 549.

[7] Ahmed CM, Larkin J 3rd and Johnson HM. SOCS1 mimetics and antagonists: a complementary approach to positive and negative regulation of immune function. Front Immunol 2015; 6: 183.

[8] Eschler DC, Hasham A and Tomer Y. Cutting edge: the etiology of autoimmune thyroid diseases. Clin Rev Allergy Immunol 2011; 41: 190-197.

[9] Egwuagu CE. STAT3 in CD4+ T helper cell differentiation and inflammatory diseases. Cytokine 2009; 47: 149-156.
[10] Morris GP, Brown NK and Kong YC. Naturally-existing CD4 (+) CD25 (+) Foxp3 (+) regulatory T cells are required for tolerance to experimental autoimmune thyroiditis induced by either exogenous or endogenous autoantigen. J Autoimmun 2009; 33: 68-76.
[11] Korn T, Oukka M, Kuchroo V and Bettelli E. Th17 cells: effector T cells with inflammatory properties. Semin Immunol 2007; 19: 362-371.
[12] Figueroa-Vega N, Alfonso-Perez M, Benedicto I, Sanchez-Madrid F, Gonzalez-Amaro R and Marazuela M. Increased circulating pro-inflammatory cytokines and Th17 lymphocytes in Hashimoto's thyroiditis. J Clin Endocrinol Metab 2010; 95: 953-962.
[13] Montagna G, Imperiali M, Agazzi P, D'Aurizio F, Tozzoli R, Feldt-Rasmussen U and Giovanella L. Hashimoto's encephalopathy: a rare proteiform disorder. Autoimmun Rev 2016; 15: 466-476.
[14] Bossowski A, Moniuszko M, Dabrowska M, Sawicka B, Rusak M, Jeznach M, Wojtowicz J, Bodzenta-Lukaszyk A and Bossowska A. Lower proportions of CD4+ CD25 (high) and CD4+ FoxP3, but not CD4+ CD25 + CD127 (low) FoxP3 + T cell levels in children with autoimmune thyroid diseases. Autoimmunity 2013; 46: 222-230.
[15] Zhu C, Ma J, Liu Y, Tong J, Tian J, Chen J, Tang X, Xu H, Lu L and Wang S. Increased frequency of follicular helper T cells in patients with autoimmune thyroid disease. J Clin Endocrinol Metab 2012; 97: 943-950.
[16] Okada T, Moriyama S and Kitano M. Differentiation of germinal center B cells and follicular helper T cells as viewed by tracking Bcl6 expression dynamics. Immunol Rev 2012; 247: 120-132.