Int J Clin Exp Pathol 2016;9(9):8836-8846
www.ijcep.com /ISSN:1936-2625/IJCEP0026246

Original Article

Ex-vivo expansion and function identification of $CD4^+$ $CD25^+$ $CD127^{dim}$ regulatory T cells in severe aplastic anemia

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Received February 19, 2016; Accepted July 12, 2016; Epub September 1, 2016; Published September 15, 2016

Abstract: Background: Regulatory T (Treg) cells play an important role in regulating immune responses. Successful ex-vivo expansion of Tregs has been achieved after T cell receptor (TCR) stimulation in the presence of T cell growth factors. In this study, we evaluated the proliferation ability and ex-vivo suppressive function of Tregs in severe aplastic anemia (SAA). Methods: To define the optimum population for Treg cell therapy in SAA, $CD4^+$ $CD25^+$ $CD127^{dim}$ Treg subsets were sorted from the blood and bone marrow of SAA patients and normal controls, and expanded in vitro in the presence of anti-CD3/CD28 beads with high concentration of recombinant human interleukin 2 (rhIL-2). Their phenotype was determined by flow cytometry. Mixed lymphocytes culture with Teff was performed to assess the function of ex-vivo expanded Treg cells. Results: The Treg cells were expanded to approximately 10-fold in 1-2 weeks and retained the Treg cell phenotypic characteristics. Tregs from SAA patients even have more powerful proliferation ability than that from normal control. Moreover, the ex-vivo expanded Tregs have a high expression of CTLA-4. In ex-vivo immunosuppression experiment, the expansion rate of Teff from the four co-culture groups, including (control Teff: control Treg), (control Teff: SAA Treg), (SAA Teff: control Treg) and (SAA Teff: SAA Treg), did not show any significant difference. The IFN-γ secretion by Teff was suppressible by expanded Tregs from SAA patients. Conclusion: Tregs from SAA patient can be sorted and expanded successfully in vitro, and used efficiently to suppress effective T cells proliferation, paving the way for future clinical trials.

Keywords: Aplastic anemia, regulatory T cells, in vitro, expansion

Introduction

Acquired severe aplastic anemia (SAA) is a rare disease characterized by severe pancytopenia and bone marrow failure. Although the exact etiology is still unknown, previous studies have shown that it is an immune-mediated disease with destruction of hematopoietic cells by activated T cells [1, 2]. In recent years, many studies have demonstrated dysregulation of T cell function, especially that cytotoxic T lymphocytes (CTLs) leads to bone marrow failure in SAA [3, 4]. However, it remains unclear that how T cell become activated. Regulatory T cells (Tregs) is a special subset of T cells reported first by Sakaguchi in 1995 [5]. One of the major characteristics of Tregs is immunosuppression, which performed as inhibiting the activation and proliferation of $CD4^+$ and $CD8^+$ T cells, preventing the antigen presenting process of antigen presenting cells (APC), and mediating target cell death directly. Studies have shown that Tregs played a pivotal role in controlling adaptive immune responses and maintenance of self-tolerance whether in mice or humans. Tregs express a variety of cell surface molecules, while some of them have a relatively close relationship with its function, such as CD25 (IL-2Rα chain), FoxP3 (forkhead box protein 3), CTLA-4 (Cytotoxic T lymphocyte antigen, CD152), GITR (glucocorticoid-induced tumor necrosis factor receptor). Previous studies have shown that FoxP3 is required for Tregs to exert their suppressive ability and the ectopic expression of this transcription factor by lymphoid and non-lymphoid cells is able to confer suppressive function, making FoxP3 a critical regulator of Tregs function [6-8]. These $FoxP3^+$ cells express high levels of CD25 and low levels of CD127 ($FoxP3^+$ $CD25^{high}$ $CD127^{low}$ $CD4^+$ T cells)

[9]. CD127 expression is associated with acquisition of Tregs function [10]. It inversely correlates with FoxP3 expression and Tregs suppressive activity [11]. With the gradual understanding of Tregs, now more and more researchers use $CD4^+$ $CD25^+$ $CD127^{dim}$ as the molecular markers of Tregs.

There is accumulating evidence that impaired function of Tregs has been implicated in the development of several common autoimmune diseases [12, 13], including SAA [14, 15]. Until now, most reports of Treg abnormality in aplastic anemia have shown that the numbers of circulating Tregs decreased in most patients. Tregs can be expanded from patient blood and were safely used in recent phase 1 studies in graft versus host disease (GVHD) [16, 17] and type 1 diabetes [18]. Treg cell therapy is also conceptually attractive for SAA. However, barriers exist to this approach. The stability of Tregs expanded from SAA patient is unknown. In this study, we intended to investigate the in vitro proliferation ability and function of expanded $CD4^+$ $CD25^+$ $CD127^{dim}$ Tregs in SAA patients, and then provide a theoretical basis for cell targeted therapy of SAA.

Materials and methods

Study subjects

A total of thirty (seventeen males, thirteen females) patients with a median age of 25 years (range 7-65 years) were included in the study. This cohort of patients included eleven (three males, eight females) SAA cases who were previously untreated and nineteen cases (fourteen males, five females) with recovering-SAA (R-SAA).

The diagnosis of SAA was established by bone marrow biopsy, bone marrow smear, and peripheral blood cell count according to International AA Study Group Criteria [2]. SAA was defined by following criteria: (1) bone marrow cellularity <25% or 25-50% with <30% residual (hematopoietic) cells; (2) two of the following three: neutrophils $<0.5 \times 10^9$/L, platelets $<20 \times 10^9$/L, and reticulocytes $<20 \times 10^9$/L [19, 20]; (3) no evidence of malignant disease, no extensive iron deposition, storage disease, myelofibrosis, and no other autoimmune diseases. Bone marrow cytogenetic studies were performed in all patients, and the clinical characteristics of patients are listed in **Table 1**.

Recovering SAA patients were defined as substantial improvement after therapy with rabbit antithymocyte globulin (rATG) (Fresenius, Germany), decreasing dose of cyclosporine (CsA), glucocorticoid and hematopoietic stimulating factors (recombinant human erythropoietin, granulocyte colony- stimulating factor, recombinant human thrombopoietin, and/or IL-11 in combination), and all the patients became completely transfusion independent. The duration of erythrocytes/platelets transfusion independency in 25%, 50% and 75% patients was 86/87, 140/126 and 196/183 days after ATG therapy, respectively. The median duration that neutrophils, erythrocytes and platelets returned to normal level was 35 days, 11 months and 12.5 months after ATG therapy, respectively. Among all the R-SAA patients, eleven have achieved complete response (CR), while the other eight were partial response (PR) according to Camitta criteria (1976).

Normal values of regulatory T cells counts were determined by fifteen age-matched healthy volunteers aged 18-71 years old. 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.

Flow cytometry

PB and BM mononuclear cells were stained with the following antibodies: CD4-PerCP, CD25-FITC, CD127-APC, CTLA-4-PE, and isotypic control mouse IgG_1-PE (all from BD Biosciences) according to the manufacturer's instructions. Data acquisitions were performed on a FACS-Calibur flow cytometer (BD Biosciences) and analyzed by FlowJo software (Version 7.6.1; TreeStar).

Purification of lymphocyte subpopulations

PB and BM mononuclear cells were separated by density-gradient centrifugation, respectively. Two T-cell subpopulations, including $CD4^+$ $CD25^+$ $CD127^{dim}$ Tregs and $CD4^+$ $CD25^-$ effector T cells (Teff) were purified using a commercial human $CD4^+$ $CD25^+$ $CD127^{dim}$ regulatory T cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions.

Table 1. Clinical and demographic parameters of patients participating in the study

Diagnosis	Number	Age (years)	ANC ($\times 10^9$/L)	Hb (g/L)	PLT ($\times 10^9$/L)	Ret %	Bone marrow karyotype	Therapy	Duration (months)
Untreated SAA	11	16 (7-65)	0.62±0.82	64.38±13.68	15.05±13.33	0.39±0.30	Male 46, XY [20] Female 46, XX [20]	Not previously treated except for transfusions	2 (1-3)
Recovering SAA	19	26 (8-59)	2.95±1.87	126.21±33.58	128.96±62.93	2.45±1.85	Male 46, XY [20] Female 46, XX [20]	Treated with combination indicated	32 (6-116)

Values are expressed as mean ± SD. Age and duration values are mid (min, max). ANC: Absolute neutrophil count; Hb: Hemoglobin; PLT: Platelet; Ret: Reticulocyte. Duration of "untreated SAA": from the onset of disease to time of Tregs measurement; duration of "recovering SAA": from the ATG therapy to time of Tregs measurement.

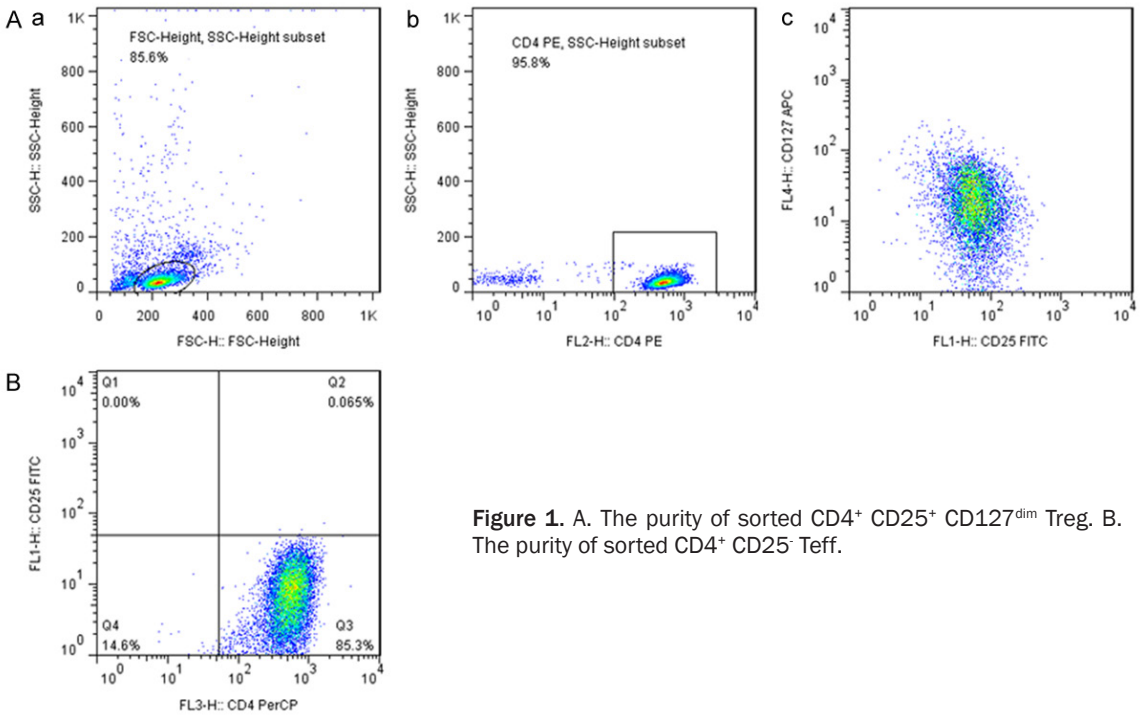

Figure 1. A. The purity of sorted $CD4^+$ $CD25^+$ $CD127^{dim}$ Treg. B. The purity of sorted $CD4^+$ $CD25^-$ Teff.

Treg proliferation

Once purified, Tregs were collected and cultured for 7-14 days at 37°C with 5% CO_2 in TexMACS™ Medium (Miltenyi Biotec) (100 μl/10^5 cells) supplemented with 1% penicillin/streptomycin, 1% 2-mercaptoethanol, 1% non-essential amino acids (NEAA), 1% HEPES, 1% sodium pyruvate, 10% fetal calf serum (FBS), and 1000 U/mL rIL-2. MACSiBead Particles pre-loaded with CD3 and CD28 antibodies (Miltenyi Biotec) was added at a bead-to-cell ratio of 4:1. Tregs were cultured in a 96-well round-bottom plate (1 × 10^5 cells/well). Change the culture medium, count the cells and save the supernatants one or two days. On day 7, the cells were re-stimulated with anti-CD3/C28 beads.

Suppression assays

After cell proliferation, Tregs were mixed at a ratio of 1:4, and then added to autologous or allogeneic Teff cells seeded at 1 × 10^5 cells/well in a 96-well round-bottom plate. To ensure accurate results, the experiment was repeated three times. Cells were cocultured for 2-3 days at 37°C with 5% CO_2 in TexMACS™ Medium (Miltenyi Biotec) (100 μl/10^5 cells) supplemented with 1% penicillin/ streptomycin, 1% 2-mercaptoethanol, 1% NEAA, 1% HEPES, 1% sodium pyruvate and 10% FBS. MACSiBead Particles pre-loaded with CD3 and CD28 antibodies (Miltenyi Biotec) was added at 5 × 10^4/well. CCK-8 reagent (Dojindo) was added at 10 μl/well (final concentration) during the last 4 hours of culture. Evaluation of cell proliferation was assayed by microplate reader and absorbance (A450 nm) was taken as the ability of cell proliferation. The cell proliferation rate was calculated using the formula: (absorbance of co-culture well/absorbance of blank well) × 100. For cross-culture experiments, Tregs from AA patients were mixed with autologous and healthy Teff at a ratio of 1:4, and vice versa.

Detection of levels of IL-10 and IFN-γ

Culture supernatants obtained from Tregs proliferation were analyzed for IL-10 by ELISA based on the manufacturer's instructions (USCNK). Supernatants obtained from the suppression assays were analyzed for IFN-γ based on the manufacturer's instructions (USCNK).

Statistical analysis

All analyses were performed using SPSS 21.0 software (SPSS Science). Data were presented as mean ± standard deviation (SD). The significance of the differences was assessed by One-way ANOVA. A value of $P<0.05$ was considered statistically significant.

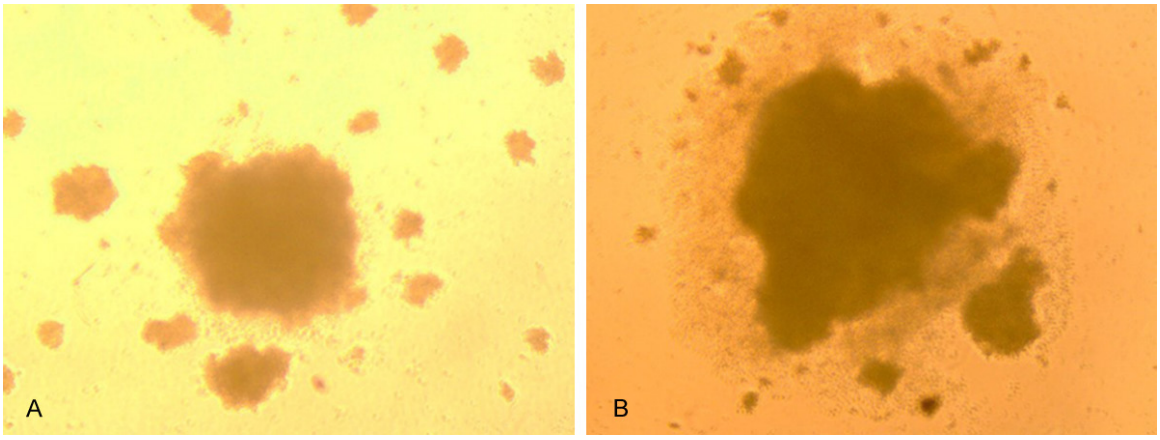

Figure 2. A. At low magnification, Treg cells in culture at day 1. B. At low magnification, Treg cells in culture at day 8.

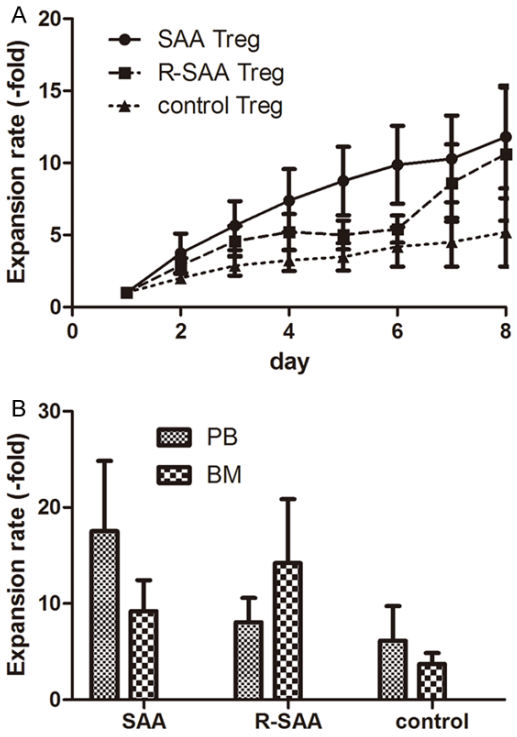

Figure 3. A. The growth curves of viable cell counts. B. The expansion rate of Treg cells from PB/BM of SAA (n=3/8), R-SAA (n=6/13) and normal control groups (n=7/7).

Results

Purity of CD4$^+$ CD25$^+$ CD127^{dim} Treg

CD4$^+$ CD25$^+$ CD127^{dim} Treg cells comprised approximately 5-10% of all the CD4$^+$ T cells in the peripheral blood of human, while SAA patients had significantly decreased frequencies of Tregs [21]. After sorting, the purity of CD4$^+$ CD25$^+$ CD127^{dim} Treg and CD4$^+$ CD25$^-$ Teff were both more than 80% (**Figure 1**).

Treg proliferation

Treg cells before and after culture is showed in **Figure 2**, and cell proliferation is obviously observed. **Figure 3A** shows the growth curves of viable cell counts at various time points, demonstrating approximately 10-fold expansion of the Treg cells. Tregs from SAA patients may have more powerful proliferation ability than that from normal control. The expansion rate of Treg cells from peripheral blood/bone marrow of SAA, R-SAA and normal control group were (17.54±10.34)/(9.21±8.54), (8.05±5.67)/(11.29±18.28) and (6.14±8.80)/(3.71±2.84), respectively (**Figure 3B**).

Cells were harvested according to their growth condition, and the purity was tested again by flow cytometry which still remain about 60% (**Figure 4A**). Based on the gate of CD4$^+$ CD25$^+$ CD127^{dim} Tregs and isotype control used by mouse anti-human IgG antibody, we analyzed the expression of the functional molecular marker CTLA-4 (**Figure 4B**). The result showed that the CTLA-4 expression of expanded Treg cells from SAA patients was more than 60%, substantially and significantly increasing when compared with Tregs before expansion [21].

IL-10, one of the most important cytokine secreted by Tregs, is a key inhibitor of effector T cell activation and a mediator of intestinal homeostasis [22]. The concentration of IL-10 in supernatants of cultured Tregs from

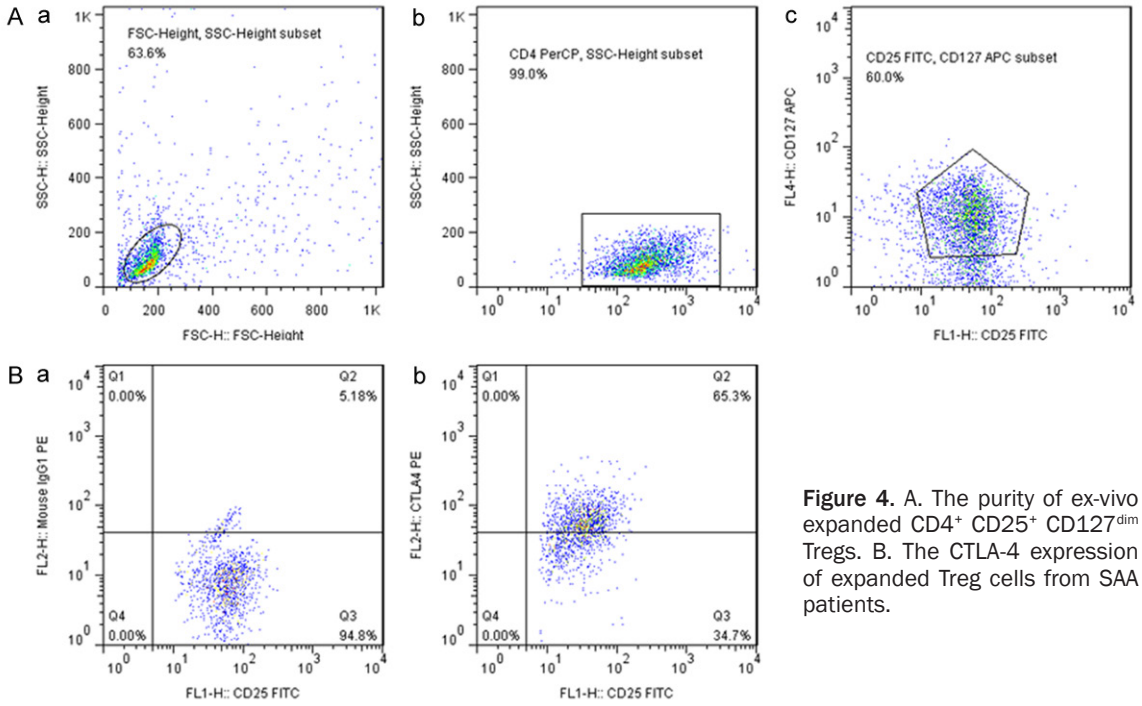

Figure 4. A. The purity of ex-vivo expanded $CD4^+$ $CD25^+$ $CD127^{dim}$ Tregs. B. The CTLA-4 expression of expanded Treg cells from SAA patients.

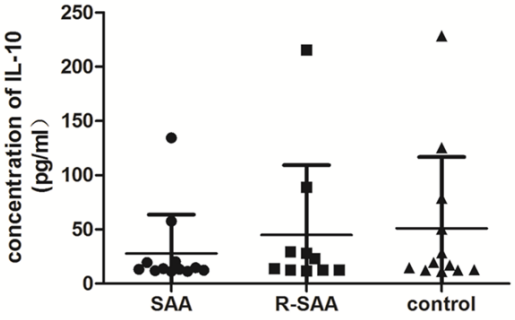

Figure 5. The concentration of IL-10 in supernatants of cultured Tregs from patients with SAA (n=12), R-SAA (n=10) and normal control (n=12).

SAA, R-SAA and normal control group were (27.82±35.92) pg/ml, (44.98±64.30) pg/ml and (50.94±65.91) pg/ml, without any significant difference (**Figure 5**).

Suppression assays

To address the efficiency of Treg-mediated immunosuppression for effector T cells, we carried out co-cultures of Treg/Teff and analyzed the cell proliferation of Teff at different condition, i.e. with or without autologous and allogeneic Tregs. **Figure 6** shows the growth curves of viable Teff cell counts at continuous time points of three days, demonstrating that Teff from both normal control and SAA group cultured with Tregs from SAA patients showed at least equal to or even significantly worse proliferative abilities compared with those cultured with Tregs from normal control. Furthermore, the expansion rate of Teff inthe cross-culture groups, including (control Teff: control Treg), (control Teff: SAA Treg), (SAA Teff: control Treg) and (SAA Teff: SAA Treg) were (1.89±0.49), (1.86±0.61), (1.94±0.64), and (1.90±0.64). The results did not show any significant difference within the four groups (**Figure 7**).

The concentration of IFN-γ in supernatants of the four cross-culture groups were (25.87±24.07) pg/ml, (21.89±18.65) pg/ml, (15.63±9.35) pg/ml, and (10.16±5.52) pg/ml, respectively (**Figure 8**). The control Teff: control Treg group was significantly higher than SAA Teff: SAA Treg, demonstrating that expanded Tregs from SAA patients may successfully suppress the IFN-γ secretion by Teff.

Discussion

As one of the key lymphocyte subpopulation to induce immune tolerance, Tregs are believed to control development and progression of autoimmunity by suppressing autoreactive T cells. Accumulating data provided evidence

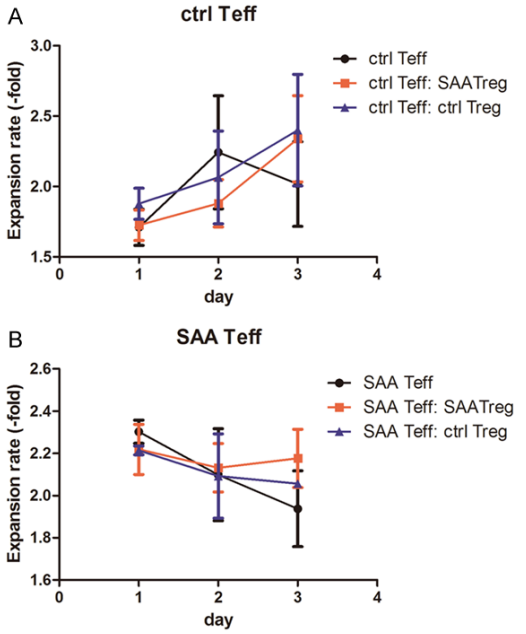

Figure 6. The growth curves of viable Teff cell counts at different condition. A. Teff cells from normal control. B. Teff cells from SAA patient.

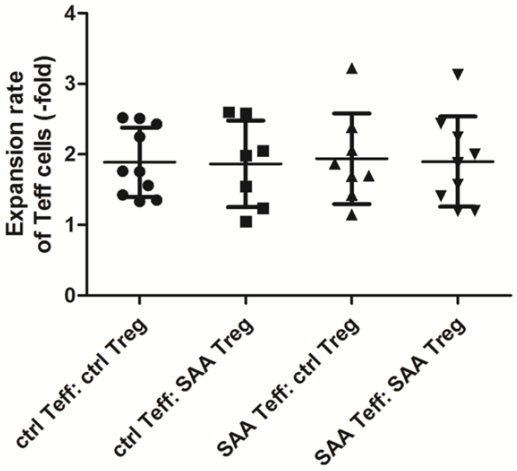

Figure 7. The expansion rate of Teff in the four cross-culture groups, including (control Teff: control Treg) (n=10), (control Teff: SAA Treg) (n=7), (SAA Teff: control Treg) (n=87) and (SAA Teff: SAA Treg) (n=9).

that impaired function of Tregs contributed to the development of autoimmunity diseases, such as autoimmune hepatitis [23, 24], type 1 diabetes [25], multiple sclerosis [26], rheumatoid arthritis [27], and systemic lupus erythematosus. With the discovery that FoxP3 plays a central role in the differentiation and maintenance of Treg cells [6, 7], the use of flow cytometry-based analysis of FoxP3 expression in T cells became the gold standard for defining Treg cells. However, it then became evident that FoxP3 can also be expressed by effector T cells following activation [28], raising the possibility that any assessment of Treg cell number or function may include recently activated effector T cells in the Treg cell population. Furthermore, as FoxP3 is a nuclear protein, assessment of its expression in T cells requires fixation and permeabilization of the cells, resulting in an inability to obtain viable cells for further functional analysis. In the past few years, additional markers, such as CD127 (also known as IL-7Rα) [11], have been identified that assist in the distinction of effector T cells from Treg cells and facilitate the experimental purification of Treg cells [29].

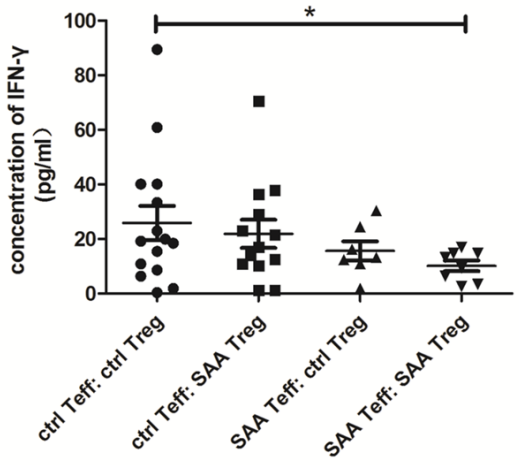

Figure 8. The concentration of IFN-γ in supernatants of the four cross-culture groups, including (control Teff: control Treg) (n=15), (control Teff: SAA Treg) (n=13), (SAA Teff: control Treg) (n=7) and (SAA Teff: SAA Treg) (n=8). *P<0.05.

An increasing number of studies have shown that SAA is an autoimmune disease caused by dysregulation of immune cell subsets, especially T lymphocytes [1, 30, 31]. There are several immune abnormalities associated with pathogenesis of SAA, including imbalance of DC subsets (elevated DC1), enhancement of DC function, insufficiency of regulatory T cell, imbalance of Th1/Th2 subsets (enhanced Th1), increase of type I lymphoid factors (IL-2, IFN-γ), and decrease of NK cell proportion [32]. Previous data have shown inadequate numbers of PB Tregs in patients with AA [14], and the quantities of Tregs in AA patients could be

improved substantially after IST. Nevertheless, little is known regarding the function of Tregs in AA because of the attack of autologous T cells on BM hematopoietic progenitors. Recent studies have revealed several impairments of Tregs in AA. Kordasti and his colleagues have shown that sorted Tregs from AA patients were unable to suppress cytokine secretion (both IL-2 and IFN-γ) by autologous effector T cells [15]. Another study also reported the reversed ratio of Treg frequencies of BM versus PB, the abnormality of migratory potential, and defective immunosuppression on Teff cells in vitro [33].

Physiologically, Tregs probably have multiple suppressive mechanisms, and the importance of each one may vary depending on the environment and the context of immuneresponses [34]. Tregs have powerful ability to suppress proliferation of auto-reactive T cells through contact-dependent mechanisms or inhibitory cytokine production [13]. It is recognized that the fine immune homeostasis between Tregs and effector T cells, regulating immune homeostasis, is often disrupted in autoimmune disorders [12, 35-37], including aplastic anemia [38].

As has been shown in animal models and human studies, such low-Treg numbers in relation to effectors will not suppress immune responses [39]. Therefore, it is of particular importance to expand these cells ex vivo prior to administration to the patient, ensuring their stability and function is maintained in vitro. Fortunately, a few efficient strategies for the large-scale expansion of Tregs have been developed recently. Tregs are relatively refractory to T cell receptor (TCR) stimulation in vitro [40]; however, they can be driven to proliferate by combining TCR stimulation with high concentration of IL-2 [41] or IL-2 combined with IL-15 [42]. For example, large-scale ex-vivo expansion of human Tregs by stimulation with anti-CD3 and anti-CD28 monoclonal antibody-coated beads and high-dose IL-2 has successfully been demonstrated [43]. Importantly, these expanded cells retain their suppressor function both in vitro [44] and in vivo in animal models [45].

In this setting, we investigated the in vitro proliferation ability and function of expanded $CD4^+$ $CD25^+$ $CD127^{dim}$ Tregs in SAA patients. The results suggest that Treg cells from SAA patient can be sorted and expanded successfully with anti-CD3 and anti-CD28 monoclonal antibody-coated beads and high-dose IL-2 in vitro. Also it can be used efficiently to suppress effective T cells proliferation, paving the way for future clinical trials. Interestingly, the CTLA-4 expression of expanded Treg cells from SAA patients was substantially and significantly increasing when compared with Tregs before expansion. CTLA-4 is crucial for the suppressive function of $Foxp3^+$ in vitro and in vivo [46]. It is expressed by Tregs which can modulate CD80 and CD86 expression by dendritic cells and there by inhibit the activation of effector T cells [47]. This provides us a hypothesis that there may be some kind of mechanism or abnormal microenvironment in SAA patient, which may somehow inhibit the function of Tregs in vivo. While once away from the morbid environment, Treg cells can be expanded and perform regular suppressive function in vitro. The further mechanism is under study and deserves to be investigated.

It has been hypothesized that an increased number of fully active Tregs could revert the imbalance of suppressor/effector cell ratio and may change the natural history of autoimmune diseases. Indeed, therapies that increase Treg numbers and activity have been shown to be effective at reversing autoimmune diseases in animal models such as experimental autoimmune encephalitis [48] and diabetes [49]. Of note, ex vivo expanded regulatory T cells, adoptively transferred in lupus-prone mice, reduced the rate of renal disease development, and a second transfer, in animals that already developed proteinuria, further delayed the progression of renal disease and significantly improved survival [50]. Moreover, a dose-escalation trial using autologous ex vivo expanded polyclonal Tregs ($CD4^+$ $CD25^+$ $CD127^{dim/-}$) in diabetes patients is currently ongoing [51].

We believe that in vitro expanded autologous/heterologous Tregs may help treating autoimmune diseases, including SAA, and that $CD4^+$ $CD25^+$ $CD127^{dim}$ Treg cells are interesting from this point of view, because they appear to be a homeostatic attempt to counteract, at least in part, the disease. Nonetheless, like all therapies, clinical use of ex-vivo-expanded Treg cells is associated with potential risks. The most concerning is the possible instability of these

ex-vivo-generated cells. There is at least a theoretical risk that these Tregs have the potential to revert to T effector cells in vivo, especially if antigen-specific Tregs are infused. In support of this, it has been shown that a minority of adoptively transferred Tregs loses FoxP3 expression and can differentiate into conventional T cells [52]. Understanding why and how Tregs lose their suppressive phenotype, the relevance of this in the clinical setting and in particular how this can be prevented would improve both the safety and efficacy of Treg cell therapy strategies.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (81400085, 81400088, 81570106, 81570111), Tianjin Municipal Natural Science Foundation (14JCYBJC25400, 15JCYBJC24300), Health Industry Research and Special Projects (201202017), Tianjin Cancer Research of Major Projects (12ZCDZSY17900, 12ZCDZSY18000), Tianjin Science and Technology support key project plan (20140109).

Disclosure of conflict of interest

None.

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