The shortening telomere length of T lymphocytes maybe associated with hyper-function in servere aplastic anemia

CHAOMENG WANG*, TIAN ZHANG*, YIHAO WANG*, YANG LI, CHUNYAN LIU, HUI LIU, LIJUAN LI, KAI DING, TING WANG, HONGLEI WANG, ZONGHONG SHAO and RONG FU

Department of Hematology, Tianjin Medical University General Hospital, Tianjin 300052, P.R. China

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Abstract. Severe aplastic anemia (SAA) is a primary disorder of severe bone marrow failure characterizing with extreme pancytopenia and a profound diminution of bone marrow progenitor cells, which is associated with T cell hyper-function. Abnormal telomere shortening of bone marrow mononuclear cell has been reported in AA, which may lead to genomic instability, and result in cell senescence or apoptosis. Notably, certain studies identfieid that lymphocytes of shortening telomere length have undergone apoptosis escape in autoimmune diseases. In order to investigate the association between telomere lengths and function of T lymphocytes in SAA, the relative telomere lengths (RTLs) of different subtypes of T lymphocytes were investigated by flow-fluorescent *in situ* hybridization in 30 patients with SAA and 25 healthy controls. Then the levels of expression of cluster of differentiation 28 (CD28), CD158 and CD70 were measured, which represent the function of T lymphocytes. The apoptosis rate and the cell cycle progression of $CD8^+$T lymphocytes, and the level of secretion interferon-γ and tumor necrosis factor-α were also measured. Finally, the correlation between telomere length and these functional events of $CD8^+$T lymphocytes was analyzed in patients with SAA. The results showed that RTLs of $CD8^+$T lymphocytes in SAA were significantly shorter compared with those in controls. Furthermore, in patients with SAA, $CD8^+$T lymphocytes are associated with T cell hyper-function, which is related to the RTL. Thus, the shorter RTLs of $CD8^+$T lymphocytes in SAA may be associated with hyper-function of these cells, which contribute to the pathogenesis of SAA.

Correspondence to: Dr Zonghong Shao or Dr Rong Fu, Department of Hematology, Tianjin Medical University General Hospital, 154 Anshan Street, Heping, Tianjin 300052, P.R. China
E-mail: shaozonghong2016@sina.com
E-mail: florai@sina.com

*Contributed equally

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Introduction

Aplastic anemia (AA) is defined as pancytopenia which hypocellular bone marrow, no abnormal infiltrate, and no increase in reticulin. The etiology of the disease remains unclear, but most experts have posited that 'AA is an immune-mediated disease with active destruction of hematopoietic cells by T lymphocytes' (1-3). Activated suppressor T lymphocytes producing interferon (IFN) participate in the pathogenesis of bone marrow failure (4,5). $CD4^+CD25^+FOXP3^+$ regulatory T cells decrease in patients with AA, which may explain the increased autoreactive T cells and the development of AA phenotype (6). The disequilibrium of T cells has an important function in the pathogenesis of AA.

Telomeres are heterochromatic structures with tandem DNA repeats of 5'-TTAGGG-3' at the chromosomal ends. With each cell division, telomeres became shorter and shorter because of 'end-replication problem'. Therefore, telomere shortening is an important suppressive mechanism by limiting cellular proliferative capacity through regulating senescence checkpoint activation. Critically short telomeres are likely to form telomeres fusions and lead to genomic instability (7-10). Abnormal telomere shortening has been reported in patients with acquired hematologic disorders; extremely short telomeres suggest of increased hematopoietic stress. Telomeres are also markers of replication and/or oxidative stress in many disease pathways.

Our team has detected decreased telomere length in SAA, which was associated with expression of sheltering component POT1 (11). In order to understand the abnormalities of telomere more deeply, we measured the relative telomere length (RTL) of different T lymphocyte subsets in SAA patients and the CD28, CD70, CD158 expression level on $CD8^+$T lymphocytes, apoptosis rate of primary $CD8^+$T lymphocytes, type 1 cytokines including IFN-γ, tumor necrosis factor-α (TNF-α) secretion and cell cycles of $CD8^+$T lymphocytes to explore that the relationship between the telomere and the T lymphocytes activate state in the pathogenesis of this disease.

Materials and methods

Patients and normal controls. All the patients with SAA were collected in the Hematology Department of General

Hospital Tianjin Medical University from July 2015 to July 2016 and were diagnosed according to International AA Study Group Criteria (2). SAA was defined as bone marrow cellularity of <25% and severe pancytopenia with at least two of the following peripheral blood count criteria: i) Absoulute neutrophil counts are $<0.5x10^9$/l; ii) absolute platelet counts are $<20x10^9$/l; iii) absoulute reticulocyte counts are $<15x10^9$/l. If the neutrophil count was $<0.2x10^9$/l, SAA was considered very severe. Patients were excluded if they had congenital AA or other diseases, such as paroxysmal noctunal hemoglobinuria (PNH), myelodysplastic syndrome (MDS), iron deficiency anemia (IDA), megaloblastic anemia (MA), anemia of chronic disease (ACD), autoimmune hemolytic anemia (AIHA). Thirty SAA patients (15 males, 15 females) were enrolled in our study with a median age of 29 years (range, 5-63 years), including 22 untreated SAA (10 males, 12 females), 8 recover SAA (5 males, 3 females), all recover SAA patients have received the treatment of cyclosporine (CsA; 3-5 $mg \cdot kg^{-1} \cdot day^{-1}$), and anti-human thymocyte globulin (ATG; 2.5-5 $mg \cdot kg^{-1} \cdot day^{-1}$, 5 days). Twenty-five healthy volunteer as health controls whose race, living area, sex and age were same as those of SAA patietns were also enrolled in this study with a median age of 25 years (range, 15-64 years). Sufficient samples such as peripheral blood which were taken from their peripheral veins were available for testing.

Cell separation. Peripheral blood mononuclear cells (PBMCs) were isolated from heparin anti-coagulant venous blood of SAA and normal controls using Ficoll-Hypaque density gradient centrifugation, $CD4^+$, $CD8^+$T lymphocytes were purified using the respective anti-CD4 and anti-CD8 mAb-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

Non-adherent cells culture of MOLT-4. As inner control cells, MOLT-4s were plated in RPMI-1640 culture medium (containing 10% FBS and 1% mycillin; Gibco-BRL, Grand Island, NY, USA), at 37˚C in an atmosphere containing 5% CO_2, 2-3 days in liquid. MOLT-4 was human acute lymphoblastic leukemia cell line, which has longer telomere length. Some scholars have veritied that there are no significant difference among the different passages, so we makes it be a feasible control cells in measurement of telomere length by Flow-FISH (12).

Telomere length measurement by Flow-FISH. According to Telomere PNA kit/FITC for Flow Cytometry (Dako, Carpinteria, CA, USA), $1\text{-}2x10^6$ sorted sample cells and control cells (MOLT-4) were diluted with PBS 3 ml, divided into A, B tubes, and centrifugated to get rid of supernatant. DNA is denatured at 82˚C for 10 min in an eppendorf tube in the presence of hybridization solution with or without fluorescein-conjugated PNA telomere probe. Then, hyvridization takes place in the dark at room temperature overnight. The hyvridization is followed by 2 washes in wash solution at 40˚C for 10 min each. Finally the cells are resuspended in DNA-staining solution and stored in the dark at 2-8˚C for 2 to 3 h before analysis by flow cytometry. The specific fluorescence from telomere staining will be observed in FL1, and fluorescence from DNA staining will be observed in FL3. Finally, at least 20,000 cells were acquired and analysed by fluorescence-activated cell sorting (FACS) Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). DNA index of the cells determined as the following: RTL = (mean FL1 sample cells with probe - mean FL1 sample cells without probe) x DNA index of control cells x 100/(mean FL1 control cells with probe - mean FL1 control cells without probe) x DNA index of sample cells.

CD28, CD158 and CD70 expression level on $CD8^+$T lymphocytes by flow cytometry. Heparin anticoagulant venous blood of SAA and normal controls were prepared. Firstly, 20 μl whole blood were incubated with for 15 min 5 μl PerCP-conjugated anti-human CD3, 5 μl antigen presenting cell (APC)-conjugated anti-human CD8, the separately mixing with 5 ul PE-conjugated anti human CD28, 20 μl PE-conjugated anti human CD158 and 20 μl PE-conjugated anti human CD70 (all antibody except for CD158 from BD Biosciences; PE-conjugated anti-human CD158 from R&D Systems, Minneapolis, MN, USA) in the dark at 4˚C. Subsequently, erythrocyte were lysed for 10 min by hemolysin (BD Biosciences) in the dark at 4˚C, and washed by PBS. Finally the CD28, CD158 and CD70 expression level on $CD8^+$T were detected using FACSC alibur flow cytometry (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

The apoptosis rate the primary $CD8^+$T lymphocytes by flow cytometry. Apoptosis is normal physiologic processes, which is characterized by certain morphologic features, including plasma membrane asymmetry and attachment, condensation of the cytoplasm and nucleus, and inter nucleosomal cleavage of DNA (13). In apoptotic cells, the membrane phosphorlipid phosphatedylserine (PS) is translocated from inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external celluar environment. Annexin V a has high affinity for PS in the earlier stages of apoptosis, so FITC Annexin V can stain cells from the earliest stages to necrotic processes. However, viable cells with intact membranes exclude propidium iodide (PI), wheras the membranes of dead and damaged cells are permeable to PI.

In our experiments, the $1x10^6$ sorted $CD8^+$T lymphocytes were stained using FITC Annexin V and PI (FITC Annexin V Apoptosis Detection kit I; BD Biosciences) for 15 min in the dark at room temperature, then added 400 μl 1X binding buffer and the stained cells were analyzed by FACSCalibur flow cytometry (Bio-Rad Laboratories, Inc.).

IFN-γ, TNF-α of the post-stimulate $CD8^+$T lymphocytes by ELISA. The sorted cells ($1x10^6$) were collected and cultured in RPMI-1640 culture medium (containing 10% FBS and 1% mycillin; Gibco-BRL), then fixed with 10 ng/ml PMA (Sigma) and 0.4 ug/ml ionomycin calcium salt (sigma) at 37˚C in an atmosphere containing 5% CO_2 for 6 h and then centrifuge supernate for 20 min to remove insoluble impurity and cell debris at 1,000 x g at 2-8˚C. Collect the clear supernate and IFN-γ, TNF-α were respectively measured in the method of sandwich-ELISA using human IFN-γ ELISA

kit and human TNF-α ELISA kit (Elabscience Biotechnology Co., Ltd., Wuhan China) by the microplate reader (Bio-Rad Laboratories, Inc.).

Cell cycle analyzed by flow cytometry. Cell cycle of lymphocytes in SAA was detected using DNA *analyzing* agent (BD Biosciences). The sorted cells ($1x10^6$) were collected and prepared in a suspended solution, then fixed with 125 μl solution A at room temperature for 10 min. Futhermore, the cells were incubated with 200 μl solution B at room temperature for 10 min, followed by solution C for 10 min at 4˚C in the dark. The cell cycle was analyzed using FACSC alibur flow cytometry (Bio-Rad Laboratories, Inc.).

Statistical analysis. Date were calculated and displayed as mean ± standard and analyzed with SPSS 16.0 statistical software. For comparison of disease parameters, a t-test and correlation analysis was used. P-values <0.05 were considered to indicate a statistically significant difference.

Results

The purity of lymphocytes. The purity of enriched $CD4^+$, $CD8^+$T lymphocytes were evaluated by flow cytometry and was generally >90% (Fig. 1).

RTLs of $CD4^+$ and $CD8^+$T lymphocytes. RTLs were measured by Flow-FISH (Fig. 2). RTLs of $CD4^+$T lymphocytes were (86.16±11.91%) in SAA, which was no statistical difference those of healthy controls (91.65±7.25%) (P=0.09) (Fig. 3A). RTLs of $CD8^+$T lymphocytes were (82.17±12.17%), which was significant shorter than the healthy controls (95.71±9.11%) (P<0.01) (Fig. 3B).

Persistent CD28, CD158, CD70 expression on $CD8^+$T lymphocytes. Flow cytometric analysis revealed the co-stimulatory signals CD28 expression level on $CD8^+$T lymphocytes in SAA was (56.56+20.89%), which were significantly lower than those in health controls (66.53+17.82%) [P=0.033, 95% CI (-19.11, -0.84)] (Fig. 4A). In SAA patients, expression of CD158 on $CD8^+$T lymphocytes was (14.0030+8.1719%), that was no significantly with the health controls (11.35+5.92%) [P=0.146, 95% CI (-0.95, 6.26)] (Fig. 4B), and expression of CD70 on $CD8^+$T lymphocytes was (9.82+8.80%), which were significantly higher than those in health controls (4.77+4.67%) [P=0.006, 95% CI (1.55,8.55)] (Fig. 4C). These data were verified at the protein level by multicolor flow cytometry using $CD8^+$T cell.

The apoptosis rate of the primary $CD8^+$T lymphocytes. Flow cytometry assay revealed that $CD8^+$T lymphocytes in SAA were vulnerable to apoptosis (13.20+7.48%), which were significantly higher than those in normal controls (6.75+3.50%) [P<0.01, 95% CI (3.35, 9.55)] (Fig. 5).

IFN-γ, TNF-α of the post-stimulate $CD8^+$T lymphocytes. The microplate reader show that $CD8^+$T lymphocytes secreted IFN-γ in SAA were apparently higher than the health controls (20.59+6.05 vs. 15.82+4.21 U/ml) [P=0.018, 95% CI (0.90, 8.65)]. The concentration of TNF-α increased in SAA (18.12+7.96 U/ml), which were significantly higher than those in normal controls (11.49+4.72 U/ml) [P=0.002, 95% CI (2.52, 10.74)] (Fig. 6).

Cell cycle progression of $CD8^+$T lymphocytes. Flow cytometry assay revealed that $CD8^+$T lymphocytes in SAA were stimulated to enter the S phase (0.21±0.08%), which were significantly higher than those in normal controls (0.05±0.03%) (P<0.01) (Fig. 7). $CD8^+$T lymphocytes of untreated AA were promoted to S phase, which were significantly higher than those in normal controls.

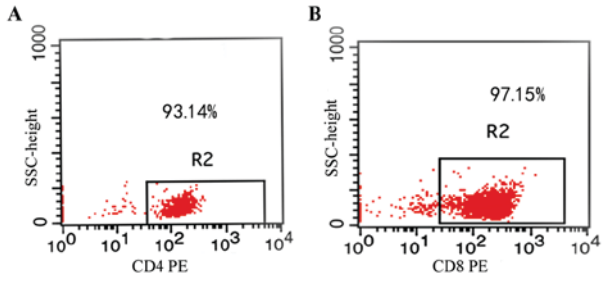

Figure 1. The purity of enriched $CD4^+$T, $CD8^+$T lymphocytes were evaluated by flow cytometry. (A) $CD4^+$T lymphocytes; and (B) $CD8^+$T lymphocytes.

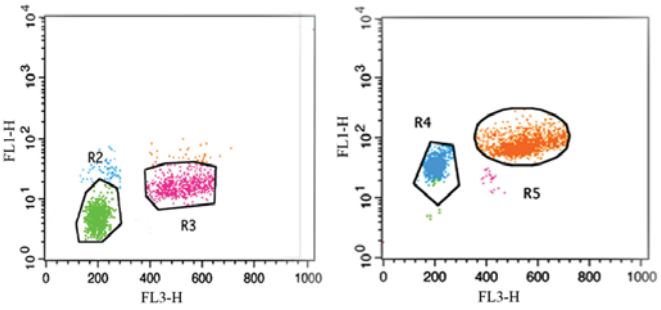

Figure 2. RTL was detected by flow-FISH. R2, sample cells without probe; R3, control cells without probe; R4, sample cells with probe; R5, control cells with probe; RTL, relative telomere length.

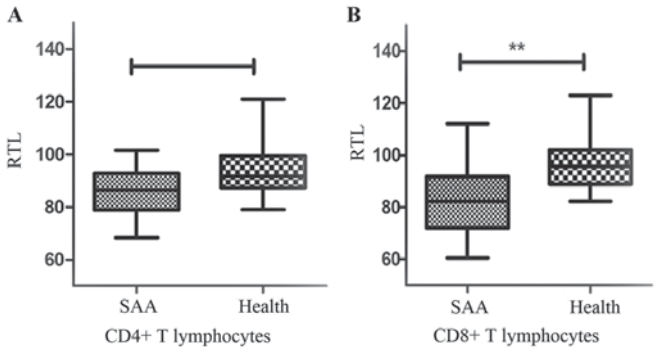

Figure 3. The comparison of RTL in $CD4^+$ and $CD8^+$T lymphocytes. (A) The bar chart of RTL about the comparison between SAA patients and health controls in $CD4^+$T lymphocytes. The RTL of $CD4^+$T lymphocytes of SAA was no significant difference with healthy controls. (B) The bar chart of RTL about the comparison between SAA patients and health controls in $CD8^+$T lymphocytes. The RTL of $CD8^+$T lymphocytes of SAA were significant shorter than healthy controls. Statistical significance is indicated as **P<0.01. RTL, relative telomere length.

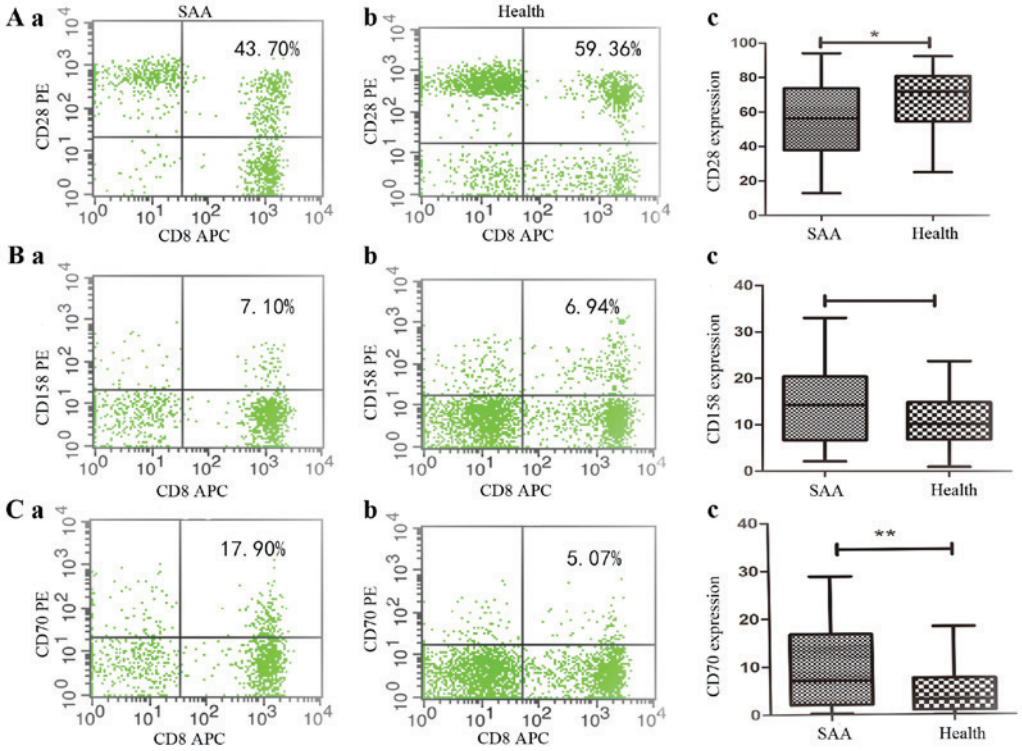

Figure 4. Persistent CD28, CD158, CD70 expression on CD8⁺T lymphocytes. (A) The CD28 expression on CD8⁺T lymphocytes. (a) SAA patients; (b) health people; (c) the percentage of CD28 in CD8⁺T cells. (B) The CD158 expression on CD8⁺T lymphocytes. (a) SAA patients; (b) health people; (c) the percentage of CD158 in CD8⁺T cells. (C) The CD70 expression on CD8⁺T lymphocytes. (a) SAA patients; (b) health people; (c) the percentage of CD70 in CD8⁺T cells. Statistical significance is indicated as $^{*}P<0.05$, $^{**}P<0.01$.

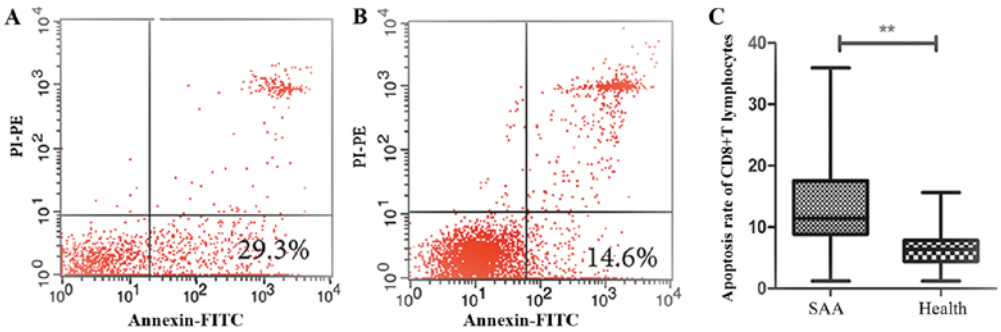

Figure 5. Flow cytometry results indicate the apoptosis of the CD8⁺T lymphocytes. The apoptosis of CD8⁺T lymphocytes was evaluated by Annexin V-FITC/PI double staining. The red dots in under left represent alive cells, the upper left represent necrotic cells, the under and upper right represent early and late apoptotic cells. (A) SAA patient; (B) health controls; (C) The bar chart of early apoptosis rate about the comparison between SAA patients and health controls. Results are presented as the mean ± standard deviation of experiments conducted in triplicate. Statistical significance is indicated as $^{*}P<0.05$, $^{**}P<0.01$. FITC, fluorescein isothiocyanate; PI, propidium iodide.

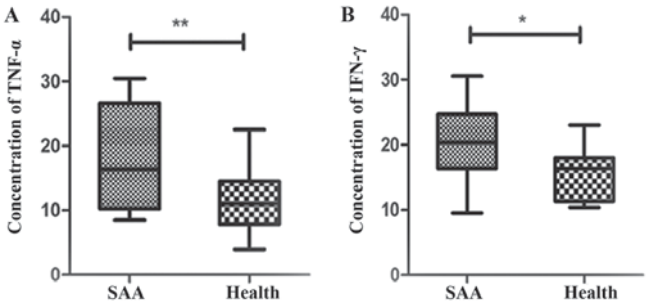

Figure 6. The concentration of IFN-γ, TNF-α in CD8⁺T lymphocytes *in vitro*. (A) The concentration of IFN-γ in CD8⁺T cells. (B) The concentration of TNF-α in CD8⁺T cells. Statistical significance is indicated as $^{*}P<0.05$, $^{**}P<0.01$.

Correlation RTL and the function of the CD8⁺T lymphocytes. We next addressed the question of whether the shorted telomere would affect the function of the CD8⁺T cell. Using linear regression analysis, we made the correlation between telomere length and those, such as expression of CD28, CD158 or CD70 on CD8⁺T lymphocytes, apoptosis rate of primary CD8⁺T lymphocytes, the level of secretion IFN-γ, TNF-α after stimulating CD8⁺T lymphocytes and cell cycle progression of CD8⁺T lymphocytes. We found significant positive correlations with RTL for CD28 (r=0.61, P=0.001) (Fig. 8A). However, there have been significant negative correlations with RTL for CD158 (r=-53, P=0.005) (Fig. 8B), CD70 (r=-0.51, P=0.016) (Fig. 8C), apoptosis

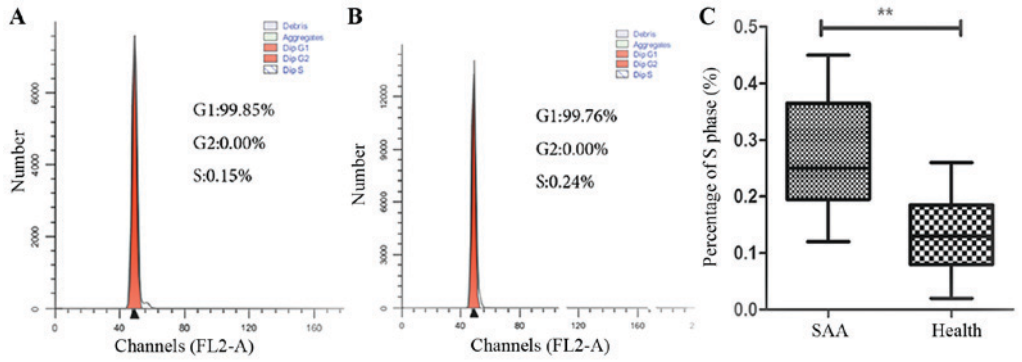

Figure 7. Cell cycle kinetics was detected by flow cytometry. $CD8^+$T lymphocytes of untreated AA were promoted to S phase, which were significantly higher than those in normal controls. (A) $CD8^+$T cells in normal controls; (B) $CD8^+$T cells in AA; (C) the percentage of S phase in $CD8^+$T cells. Statistical significance is indicated as $^{**}P<0.01$.

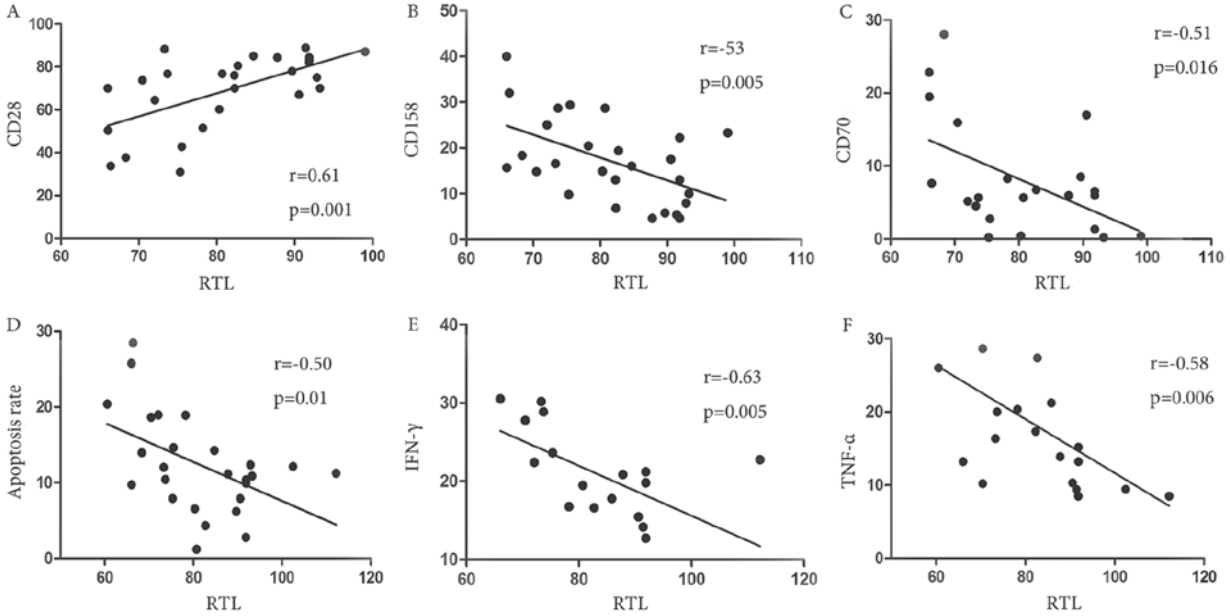

Figure 8. The correlation between RTL and the function index of $CD8^+$T lymphocytes. (A) RTL and CD28 (r=0.61, P=0.001); (B) RTL and CD158 (r=-53, P=0.005); (C) RTL and CD70 (r=-0.51, P=0.016); (D) RTL and apoptosis (r=-0.50, P=0.01); (E) RTL and IFN-γ (r=-0.63, P=0.005); (F) RTL and TNF-α (r=-0.58, P=0.006).

(r=-0.50, P=0.01) (Fig. 8D), IFN-γ (r=-0.63, P=0.005) (Fig. 8E), TNF-α (r=-0.58, P=0.006) (Fig. 8F) of $CD8^+$T lymphocytes (Fig. 8).

Discussion

Telomeres play an important role in maintaining chromosomes construction integrity and protecting genetic information integrity (14), the shortening of which mainly depends on antigen irritated and many stimulatory factors (15). On one hand, telomere shortening in lymphocytes is considered to present the immune system aging and may be relative to autoimmune responses (16,17). On the other hand, it presents a marker for replicative history of lymphocyte (18). Shortened telomere may ultimately trigger replicative senescence leading to cellular aging (19,20). Scheinberg *et al* (21) found that telomere length of mononuclear cells shortened, which had no response to drug reaction undergoing immunosuppressive therapy in AA patients. Also, disease relapse, clonal evolution, and overall survival rates were closely related with telomere length. So telomeres shorten generated chromosome instability, which was the important reason of bone marrow failure. Calado *et al* (22) found that the telomere lengths of AA were inversely correlated with the developing of cytogenetically abnormal clone. Wang *et al* (23) found shorter telomere length in $CD3^+$T lymphocytes of bone marrow with untreated AA patients, implying that telomere length change maybe the reason of bone marrow failure.

In most normal human somatic cells, telomere repeats are gradually lost with replication and age, owing to the inability of conventional DNA polymerase to fully replicate the 3'-end of DNA, then when it shorten to a certain degree, the cell will be tend to death (21). Several experimental findings suggest that the effectiveness of the immune response declines with age particular in the latter stages of life (24), organism will be lost CD28, which is a co-stimulatory signaling molecule and is believed to set the threshold for T cell activation. The co-stimulatory signaling molecule is found on T cells

must bind B7-1 and B7-2, which are expressed on APCs, to trigger T cell activation (25). In a word, CD28 has multiple roles during T cell activation, proliferation and survival (26). Simultaneously, these cells will acquire many new effector factors, such as killer-cell immunoglobulin-like receptors (KIRs), leukocyte function receptor (LFA-1), CD70, perforin and profound altered expression of several chemokines and cytokines (27). CD158 is KIR, which function in T cell activation is complicated. CD70 is the immunoglobulin superfamily member, which CD70 is similar with TNF family members and can regard as the co-stimulatory signaling molecule of T leukomonocytes regulating the activation of B leukomonocytes (28,29). In normal, T cells hardly express CD70 and CD158, but when the organism undergone a series of allergic reaction or autoimmune response, the activated T lymphocytes increased expression of CD70 and CD158, which indicates that they play a important role in pathological damage caused by immune disorders (30,31). The high level of effector molecules has the additional effect of lowering the T cell activation threshold, enhanced cytotoxicity and display suppressive functions, finally, it would progress into an autoimmune disease, e.g., rheumatoid arthritis (RA), multiple sclerosis, Wegener's granulomatosis, Graves' disease, or Ankylosing spondylitis (32,33).

SAA is a primary disorder of severe bone marrow failure, pathogenesis of which is known to be closely related to autoimmune T cell hyper-function, especially $CD8^+$T cells. Patients who suffered from SAA have a significant increase in $CD8^+$ suppressor T lymphocytes (34). Our study demonstrated for the first time that telomere length of $CD8^+$T lymphocytes shorted significantly in SAA patients, while telomere length of $CD4^+$T lymphocytes in SAA patients was no significant changes compared with normal controls, indicating that cellular immunity plays the dominant role, especially $CD8^+$T lymphocytes in AA. The results suggesting telomere length shortening is an important role in cellular immunity in the pathogenesis of AA. Our present data are consistent with findings in other autoimmune diseases, such as SLE, RA, systemic sclerosis and Type I diabetes, above all there are telomere shortening (35-38).

A further study indicated that $CD8^+$T lymphocytes was sustained activated in SAA. With sustained $CD8^+$T cell stimulation, CD28 expression decreases, CD70 expression increases, apoptosis rate increases, type 1 cytokines including IFN-γ, TNF-α secretion increases and the percentage of S phase will be high in $CD8^+$T lymphocytes. Above all results suggesting $CD8^+$T lymphocytes has lower activation threshold and hyper-function in SAA. Furthermore, we analyze the relationship between the RTLs and the function of $CD8^+$T lymphocytes. We found that there have been significant positive correlations with RTL for CD28. However, there have been significant negative correlations with RTL for CD158, CD70, apoptosis, IFN-γ, TNF-α. This phenomenon show that the shorten telomere of $CD8^+$T lymphocytes may be change their function in SAA.

In conclusion, we reported the short telomere length of different lymphocytes function subsets in SAA for the first time and primary explore the role of the shorten telomere cells. Telomere attrition is not only simply a biomarker; but also, a plausible mechanism for destabilization of the genome has been inferred from basic telomere biology. Furthermore, many studies should be done to explore the function of shortening telomere length of T cells in pathogenesis of AA.

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