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Increased Expression of TIGIT on CD4$^+$ T Cells Ameliorates Immune-Mediated Bone Marrow Failure of Aplastic Anemia

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ABSTRACT

Aplastic anemia (AA) is an autoimmune disease in which T cell activation is suspected to play an important role. T cell immunoglobulin and ITIM (immunoreceptor tyrosine-based inhibition motif) domain (TIGIT) is an inhibitory receptor, which exhibits inhibitory functions on the immune response. However, its role in AA has not been clearly determined. In the current study, we showed that the frequency of TIGIT-positive CD4$^+$ T cells was reduced in the vast majority of AA patients (85%, 17/20). In TIGIT-silenced human CD4$^+$ T cells, stimulation of agonistic anti-TIGIT monoclonal antibody significantly facilitated cell proliferation, increased production of IL-2 and IFN-γ, and inhibited production of IL-10. However, in TIGIT-overexpressed human CD4$^+$ T cells, cell proliferation and the production of IL-2, IFN-γ, and TNF-α were significantly hindered; in contrast, the secretion of IL-10 was improved. RT-PCR and Western blotting showed that T-bet expression in human CD4$^+$ T cells was significantly decreased by TIGIT overexpression, but only slightly altered by TIGIT knockdown. In mouse models, lentivirus-mediated TIGIT-overexpressed CD4$^+$ T cell transfer significantly rescued the decreased red blood cell count, attenuated the increase in serum INF-γ and TNF-α levels, and lengthened the median survival time. The mRNA levels of CD34, stem cell factor (SCF), and granulocyte/macrophage-colony-stimulating factor (GM-CSF) in bone marrow mononuclear cells were also up-regulated. In conclusion, increased expression of TIGIT could inhibit the function of CD4$^+$ T cells in vitro and ameliorate immune-mediated bone marrow failure of AA in vivo providing a new potential strategy for the treatment of AA. J. Cell. Biochem. 115: 1918–1927, 2014. © 2014 Wiley Periodicals, Inc.

KEY WORDS: APLASTIC ANEMIA; AUTOIMMUNE DISEASE; CD4$^+$ T CELLS; TIGIT

Aplastic anemia (AA) is a life-threatening autoimmune disease, which is characterized by pancytopenia and bone marrow (BM) hypoplasia [Young et al., 2008; Dezern and Brodsky, 2011]. Although AA may be caused by drugs, viruses, or chemical exposure, the exact pathological mechanism is not yet fully clear [Young et al., 2008]. Hematopoietic stem cell transplantation is one of the optimal treatment of choices, but is not applicable to all AA patients due to the lack of histocompatible donors [Margolis and Casper, 2000]. Treatment with the immunosuppressive agents cyclosporine and anti-thymocyte globulin (ATG), a substitute therapy that is widely used in the clinic, has been found to be effective in prolonging survival time [Frickhofen and Rosenfeld, 2000; Kojima et al., 2000]. This emphasizes the critical role of the immune-mediated destruction of hematopoietic progenitor/stem cells in the pathology of AA. Although the auto-antigens and target cells involved in AA remain elusive, evidence indicates that AA is an autoimmune disease [Risitano et al., 2004; Young et al., 2008].

The achievement of immune tolerance is modulated by kinds of immune cells and serial co-stimulators. In AA, most attention has

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been given to T helper type 1 (Th1) cells, a subset of $CD4^+$ T cells, because Th1 cells are expanded in AA patients [Kordasti et al., 2012]. Furthermore, T-bet, a member of the T-box family of transcription factors that are particularly expressed on Th1 but not Th2 cells, is also elevated in peripheral blood T cells from patients with AA [Szabo et al., 2002; Solomou et al., 2006; Shan et al., 2013]. Th1 cells are recruited into the BM destroy hematopoietic stem cells by secreting cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor-α (TNF-α) [Sloand et al., 2002; Dubey et al., 2005; Kordasti et al., 2012]. The enhanced function of $CD4^+$ T cells is due to the imbalance of co-stimulators. T cell immunoglobulin and mucin domain-containing molecule 3 (Tim-3), a co-inhibitory type 1 transmembrane protein, has been identified to be expressed at low levels on $CD4^+$ T cells in AA patients compared to that of the healthy controls [Shan et al., 2013]. In addition to Tim-3, cytotoxic T-lymphocyte antigen 4 (CTLA-4), programmed cell death protein 1 (PD-1), lymphocyte activation gene-3 (LAG-3), CD160 and many other inhibitory receptors could also be co-expressed on $CD4^+$ T cells and deliver inhibitory signaling in many autoimmune diseases such as systemic lupus erythematosus, myocarditis, encephalomyelitis, rheumatoid arthritis, and inflammatory bowel diseases [Okazaki et al., 2011; Wherry, 2011; Gianchecchi et al., 2013; Romo-Tena et al., 2013].

The T cell immunoglobulin and ITIM (immunoreceptor tyrosine-based inhibition motif) domain (TIGIT), also known as WUCAM or VSTM3, is a newly identified inhibitory type 1 transmembrane protein. The gene encoding TIGIT is located on human chromosome 16 [Stengel et al., 2012]. Protein-structure analysis demonstrated that human TIGIT is a transmembrane glycoprotein containing a single extracellular Ig-like domain, a type I transmembrane region and a cytoplasmic tail bearing ITIMs [Yu et al., 2009; Stengel et al., 2012]. TIGIT is mainly expressed on NK cells, activated $CD4^+$ T cells and CD8 T cells [Boles et al., 2009; Seth et al., 2009 ; Yu et al., 2009; Levin et al., 2011]. The poliovirus receptor (PVR, also known as CD155) and PVRL2 (nectin2, also known as CD112) have been identified as the ligands for TIGIT [Stanietsky et al., 2009; Yu et al., 2009]. The interaction of TIGIT expressed on NK cells and T cells with PVR or PVRL2 expressed on dendritic cells could mediate an inhibitory effect [Stanietsky et al., 2009]. In a mouse model, TIGIT deficiency made them more susceptible to autoimmune diseases [Joller et al., 2012], indicating the immunosuppressive role of TIGIT. As CD155 and CD112 could bind both to the inhibitory receptor TIGIT and the activating receptor CD226 on T cells and NK cells, this group of interacting proteins forms a network of molecules similar to the well-characterized CD28-CTLA-4-CD80-CD86 network [Levin et al., 2011]. As expected, TIGIT can inhibit $CD4^+$ T cell functions by competing with CD226 and can also directly inhibit T cells in a T cell-intrinsic manner [Lozano et al., 2012]. These findings indicated an inhibitory role of TIGIT in immune response. However, the role of TIGIT in the pathology of AA remains unknown. We hypothesized that TIGIT expression was disordered in AA and functionally related to $CD4^+$ T cells.

In the present study, we explored the function of TIGIT in $CD4^+$ T cells in AA. It was found that the percentage of TIGIT-positive $CD4^+$ T cells decreased in AA. Overexpression of TIGIT could inhibit the function of $CD4^+$ T cells and alleviate AA in mouse models.

MATERIALS AND METHODS

PATIENTS IN THIS STUDY

Twenty severe AA patients aged 13–41 years old (average, 21 years old) who had not received immunosuppressive therapy were enrolled in this study. Seven healthy volunteers were employed as control. Written informed consent was obtained from all participants or their guardians, as appropriate. The study was approved by the ethics committee of the Fourth Military Medical University, China, and all patients remained under continuous medical supervision and assistance.

IMMUNE-MEDIATED SEVERE AA MOUSE MODELS

The severe AA mice were induced as described previously, with minor modification [Bloom et al., 2004]. Briefly, B6D2F1 mice were radiated once by 5 Gy ^{60}Co-γ ray. Approximately 16–20 h later, these B6D2F1 mice were injected with 1×10^7 splenocytes from their C57BL/6 (B6) parent via the tail vein.

$CD4^+$ T CELL ISOLATION

For $CD4^+$ T cells isolated from the peripheral blood of severe AA patients, human peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized blood samples from severe AA patients or healthy controls using the Ficoll separate solution (Biochrom, Berlin, Germany). PBMCs collected from the interphase were washed twice and resuspended in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS; Life Technologies, Great Neck, NY), 2 mM L-glutamine, 1 mM Na pyruvate, 0.1 mM non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol (Sigma, St. Louis, MO) at 37°C in a humidified atmosphere with 5% CO_2. $CD4^+$ T cell in patients PBMCs were isolated using a $CD4^+$ T Cell isolation kit (130-096-533, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol.

For $CD4^+$ T cell isolation from the spleen of severe AA mouse models, the splenocytes from the AA mouse models were obtained by homogenizing the spleen in supplemented RPMI 1640 containing 10 μg/ml DNAse and centrifuging in the Ficoll separate solution. The collected splenocytes were used for $CD4^+$ T cell isolation using a $CD4^+$ T Cell isolation kit (130-095-248, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol.

FLOW CYTOMETRY

The $CD4^+$ T cells isolated above were stained with PE conjugated anti-human TIGIT antibodies (MBSA43, eBiosciences, San Diego, CA) for 30 min at 4°C. After washing twice with PBS–0.5% BSA, samples were analyzed using the LSRII flow cytometer (BD Biosciences, San Diego, CA). A PE-conjugated mouse IgG1 (P3.6.2.8.1, eBiosciences, San Diego, CA) was used as isotype control.

TIGIT KNOCKDOWN WITH siRNA

TIGIT-specific siRNA (sc-78458) and scramble negative control siRNA (sc-44233) were both purchased from Santa Cruz Biotechnology (Santa Cruz, CA). For transfection, 8 pmol siRNA in 100 μl RPMI 1640 media was mixed with 2 μl Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA) and incubated at room temperature for 15 min. Then, the mixtures were added to the cells with a final volume of 1 ml and incubated at 37°C with 5% CO_2.

TIGIT OVEREXPRESSION

A recombined plasmid bearing the open reading frame of human TIGIT cDNA (SC124474, pCMV6-XL5-TIGIT) was purchased from OriGene Technologies (Rockville, MD). Transfection was performed using the Lipofectamine™ RNAiMAX (Invitrogen) as suggested by the manufacturer.

RNA EXTRACTION AND QUANTITATIVE REAL-TIME PCR

Total RNA from human $CD4^+$ T cells or mouse BM mononuclear cells (BMMNCs) was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The cDNA was synthesized using M-MLV reverse transcriptase (Clontech, Palo Alto, CA) with oligo-dT primers according to the manufacturer's protocol. The RT-PCR reactions were performed based on the thermal cycler dice® real-time system. The PCR primers specific for TIGIT were 5′-CTGATACAGGCTGCCTTCCT-3′ (sense) and 5′-TGGGTCACTTCAGCTGTGTC-3′ (anti-sense); those for T-bet were 5′-GCTGGAGAAAAGAAGACAAGAAAG-3′ (sense) and 5′-AAGAAAAAACACACACCCACACAC-3′ (anti-sense); those for CXCR3 were 5′-AGCTTTGACCGCTACCTGAA-3′ (sense) and 5′-CTCACAAGCCCGAGTAGGAG-3′ (anti-sense); those for CD34 were 5′-TAGCACAGAACTTCCCAGCAAAC-3′ and 5′-CTCAGATCACAGTTCTGTGTCAGC-3′; those for stem cell factor (SCF) were 5′-GCGCTGCCTTTCCTTATGAA-3′ and 5′-TATTACTGCTACTGCTGTCA-3′; those for granulocyte/macrophage-colony-stimulating factor (GM-CSF) were 5′-ACCACCTATGCGGATTTCAT-3′ and 5′-TCATTACGCAGGCACAAAAG-3′; and those for β-actin were 5′-CAACTTGATGTATGAAGGCTTTGGT-3′ (forward) and 5′-ACTTTTATTGGTCTCAAGTCAGTGTACAG-3′ (reverse). The PCR procedure was as follows: 94°C for 4 min; 94°C for 20 s, 55°C for 30 s, and 72°C for 20 s; 2 s for plate reading for 35 cycles; and melt curve from 65 to 95°C. All reactions were performed in triplicate, and results were represented as relative mRNA expression data calculated according to the $2^{-\Delta\Delta CT}$ method [Livak and Schmittgen, 2001].

WESTERN BLOTTING

The pre-stimulated cells were harvested and the total protein of cells was prepared using RIPA lysis buffer (Beyotime, Nantong, China) according to the operating instructions. The protein concentration in the lysates was evaluated using a BCA protein assay kit (Beyotime). Then, Western blotting was performed according to the routine method with rabbit anti-human TIGIT polyclonal antibody, mouse anti-human T-bet monoclonal antibody, or rabbit anti-human β-actin polyclonal antibody. HRP-conjugated secondary antibody and the ECL reagents (Boehringer Mannheim, Mannheim, Germany) were used for development. All of the primary and secondary antibodies were purchased from Abcam (Cambridge, UK). All experiments were performed in triplicate, and the results were normalized according to β-actin.

CELL PROLIFERATION ASSAY

For cell activation, 5×10^4 $CD4^+$ T cells were stimulated with plate-bound anti-CD3ε (5 μg/ml, Abcam) and anti-CD28 antibodies (2.5 μg/ml, Abcam) in the presence or absence of agonistic anti-TIGIT (50 μg/ml, kindly provided by N. Joller) or IgG1 isotype control antibodies (Abcam) in RPMI 1640 media supplemented with 25 mM HEPES, 2 mM L-glutamine, 50 μg/ml of gentamycin and 10% FCS (completed medium) in 24-well round bottom plates at 37°C for 48 h. Proliferation was analyzed using $[^3H]$-thymidine incorporation assay. Briefly, $CD4^+$ T cells were pulsed with 1 mCi $[^3H]$ thymidine (Sigma) and incubated for an additional 18 h before incorporation was analyzed using a β-counter (Beckman, LS-0100C, Fullerton, CA). Results were expressed as counts per minute (cpm). Cell viability assay was performed using a CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay Kit (Promega, Madison, Wisconsin) according to the manufacturer's instructions. Data represent means ± SEM of triplicate wells and are representative of 3–5 independent experiments.

ELISA

For cytokine production analysis, an enzyme-linked immunosorbent assay (ELISA) was performed using human or mouse ELISA Set specific for IL-2, IFN-γ, TNF-α, and IL-10 (BD Biosciences, San Jose, CA) according to the manufacturer's instructions.

CONSTRUCTION AND PRODUCTION OF LENTIVIRUS EXPRESS TIGIT AND CELL TRANSFER

The cDNA for mouse TIGIT was amplified by polymerase chain reaction (PCR) using the TIGIT-specific primers. Then, the obtained TIGIT cDNA was sub-cloned into the pLV-EX2d-EF1A expression lentivector (Invitrogen Life Technologies) to generate the lentivector-expressed TIGIT, named TIGIT-LV. The empty lentivector was used as a positive control and named CON-LV. The lentivirus was packaged into 293FT cells using ViraPower Lentiviral packing mix (Invitrogen Life Technologies) according to manufacturer's instructions. For infection, the $CD4^+$ T cells isolated from mice spleen were incubated with lentivirus and selected with puromycin (20 ng/ml) for at least 2 weeks.

For cell transfer, splenocytes depleted of $CD4^+$ T cells were remixed with $CD4^+$ T cells infected with TIGIT-LV and were then injected into the recipient mouse through the tail vein (1×10^7/mouse) to generate AA mouse models. The wild-type or CON-LV infected $CD4^+$ T cells were transferred as a control.

RED BLOOD CELL COUNT

The blood was sampled from the tail vein. Red blood cells (RBCs) were counted using a Sysmex F-820 semi-automatic blood analyzer (Japan's east AsiaCompany).

PREPARATION OF MOUSE BONE MARROW MONONUCLEAR CELLS

Bone marrow was collected from femurs and tibiae harvested from mice that were killed by cervical dislocation. BMMNCs were isolated from marrow cells by density gradient centrifugation using Ficoll-Hypaque (Accurate Chemical & Scientific, Westbury, NY) according to the manufacturer's instructions.

STATISTICAL ANALYSIS

All data were derived from at least three independent experiments. Results were expressed as mean values and the standard error of the mean (SEM). The significance of differences was evaluated by the Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

FREQUENCY OF TIGIT-POSITIVE CD4$^+$ T CELLS WAS REDUCED WHEN SEVERE APLASTIC ANEMIA OCCURRED

As the well-documented Th1-mediated pathology for AA [Chen et al., 2005; Young et al., 2008], the molecular mechanisms driving CD4$^+$ T cell alterations have attracted the attention of numerous researchers. TIGIT is an ITIM-bearing inhibitory receptor and can negatively regulate T cell activation [Lozano et al., 2012]. Therefore, we addressed whether TIGIT expression on CD4$^+$ T cells was altered in the PBMCs of severe AA patients. As expected, the frequency of TIGIT-positive CD4$^+$ T cells was significantly decreased in 85% of severe AA patients (17/20) compared to that of healthy donors (Fig. 1A–D). In the immune-induced AA mouse models, a similar decline in TIGIT-positive CD4$^+$ T cells in the spleen was also observed (data not shown). The expression level of TIGIT on CD4$^+$ T cells from AA patients was also significantly decreased compared to that of healthy donors as shown by Western blot (Fig. 1E,F). These data suggested the decrease of TIGIT expression in CD4$^+$ T cells might confer the pathogenesis of AA and led us to investigate the potential role of TIGIT in CD4$^+$ T cells.

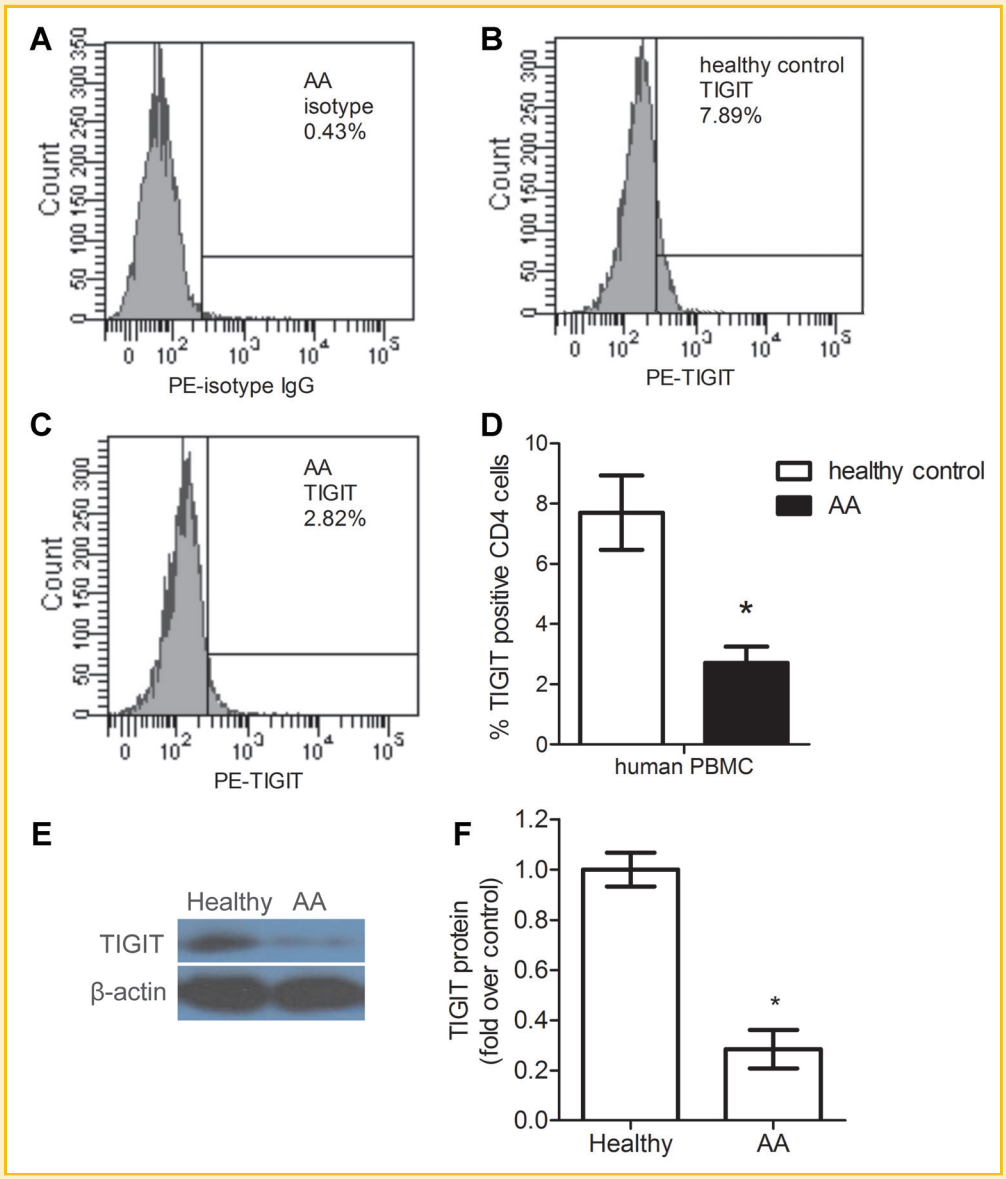

Fig. 1. The percentage of TIGIT-positive CD4$^+$ T cells in AA patients. A: CD4$^+$ T cells isolated from PBMCs of AA patients were stained with PE-conjugated mouse IgG1. CD4$^+$ T cells isolated from PBMCs of healthy control (B) or AA patients (C) were stained with PE-conjugated anti-TIGIT antibodies. TIGIT expression levels were analyzed using flow cytometry. D: Quantitative data of TIGIT-positive cells in total CD4$^+$ T cells isolated from PBMCs of AA patients ($n = 20$) and healthy control ($n = 7$). E: The expression level of TIGIT on CD4$^+$ T cells from PBMCs of healthy donors and AA patients were determined using Western blot. F: The quantitative data of E. Data were represented as mean ± SEM. *$P < 0.05$ was regarded as statistically significant.

TIGIT KNOCKDOWN PROMOTED THE ACTIVATION OF CD4$^+$ T CELLS

In light of the down-regulated TIGIT expression of CD4$^+$ T cells in severe AA patients, we sought to explore whether TIGIT down-regulation promoted CD4$^+$ T cell function. CD4$^+$ T cells isolated from PBMCs of healthy donors were transfected with siRNA-targeted TIGIT. RT-PCR and Western blot demonstrated that the mRNA and protein levels of TIGIT were both significantly decreased ($P < 0.05$) by TIGIT-siRNA transfection compared to the control group (Fig. 2A, B). Using the [^{3}H]-thymidine incorporation assay, we showed that agonistic anti-TIGIT antibody treatment delivered an inhibitory signal and significantly hindered the proliferation of CD4$^+$ T cells in the control group (Fig. 2C). This was in accordance with a previous report that agonism of the anti-TIGIT antibody inhibited the proliferation of mice splenocytes [Foks et al., 2013]. After TIGIT was silenced, proliferation of CD4$^+$ T cells was elevated compared to the control group, possibly as a result of elimination of the inhibitory signaling delivered by TIGIT (Fig. 2C). As expected, no significant decrease in the proliferation of TIGIT-silenced CD4$^+$ T cells was observed in response to agonistic anti-TIGIT antibody stimulation (Fig. 2C).

Next, several important cytokines secreted by Th1 cells were determined by ELISA. After anti-CD3ε/CD28 activation and agonistic anti-TIGIT antibody treatment, IL-2 and IFN-γ production in TIGIT-siRNA transfected CD4$^+$ T cells were significantly increased ($P < 0.05$, Fig. 2D). However, IL-10 production was significantly decreased ($P < 0.05$) and TNF-α production was only slightly inhibited ($P > 0.05$, Fig. 2D). These data indicate that CD4$^+$ T cell function was improved by TIGIT down-regulation.

FUNCTION OF CD4$^+$ T CELLS FROM PATIENTS WITH AA WAS IMPAIRED BY TIGIT OVEREXPRESSION

Activated Th1 and its mediators are essential for the progression of AA. As TIGIT was reported to trigger a negative regulation of T cell function [Lozano et al., 2012], we investigated whether the function of activated Th1 could be inhibited by TIGIT overexpression. CD4$^+$ T cells were isolated from the PBMCs of severe AA patients who were free from immunosuppressive therapy. As shown in Figures 3A and B, the transcription levels and protein levels of TIGIT were both significantly increased by pCMV6-XL5-TIGIT vector transfection ($P < 0.05$), indicating that TIGIT was successfully overexpressed in CD4$^+$ T cells. The proliferation assay revealed that agonistic anti-TIGIT antibody treatment inhibited the proliferation of CD4$^+$ T cells regardless of the TIGIT expression levels ($P < 0.05$, Fig. 3C). In the absence of the agonistic anti-TIGIT antibody, TIGIT overexpression significantly decreased the proliferation of CD4$^+$ T cells ($P < 0.05$, Fig. 3C). After treatment by agonistic anti-TIGIT antibody, a further decrease in the proliferation of TIGIT-overexpressed CD4$^+$ T cells was observed compared to the control group ($P < 0.05$, Fig. 3C).

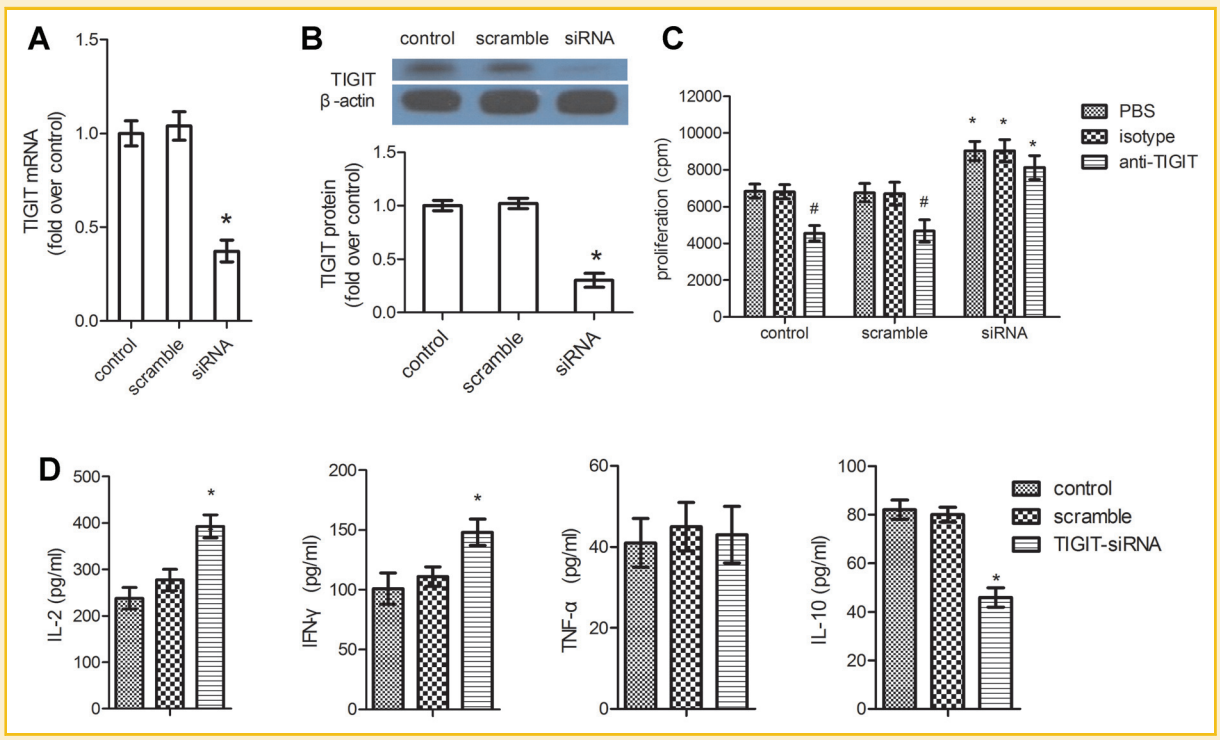

Fig. 2. Effect of TIGIT knockdown on CD4$^+$ T cell function. CD4$^+$ T cells isolated from healthy human volunteers were transfected with scramble siRNA or TIGIT-siRNA. The mRNA and protein levels of TIGIT in the control group, scramble group, and TIGIT-siRNA transfection group were determined by RT-PCR (A) and Western blot (B), respectively. The mRNA and protein levels were both normalized based on β-actin levels. C: Cells were activated by anti-CD3ε/CD28 antibodies in the absence or presence of agonistic anti-TIGIT antibodies. The IgG1 isotype was used as a control antibody. Proliferation of CD4$^+$ T cells was determined by the [^{3}H]-thymidine incorporation assay. D: Cells were activated by anti-CD3ε/CD28 antibodies in the presence of an agonistic anti-TIGIT antibody. The production of IL-2, IFN-γ, TNF-α, and IL-10 in the supernatants was determined using a corresponding ELISA Kit. Data are expressed as mean ± SEM. *$P < 0.05$ was regarded as statistically significant. *Compared to control group; #compared to PBS group in C.

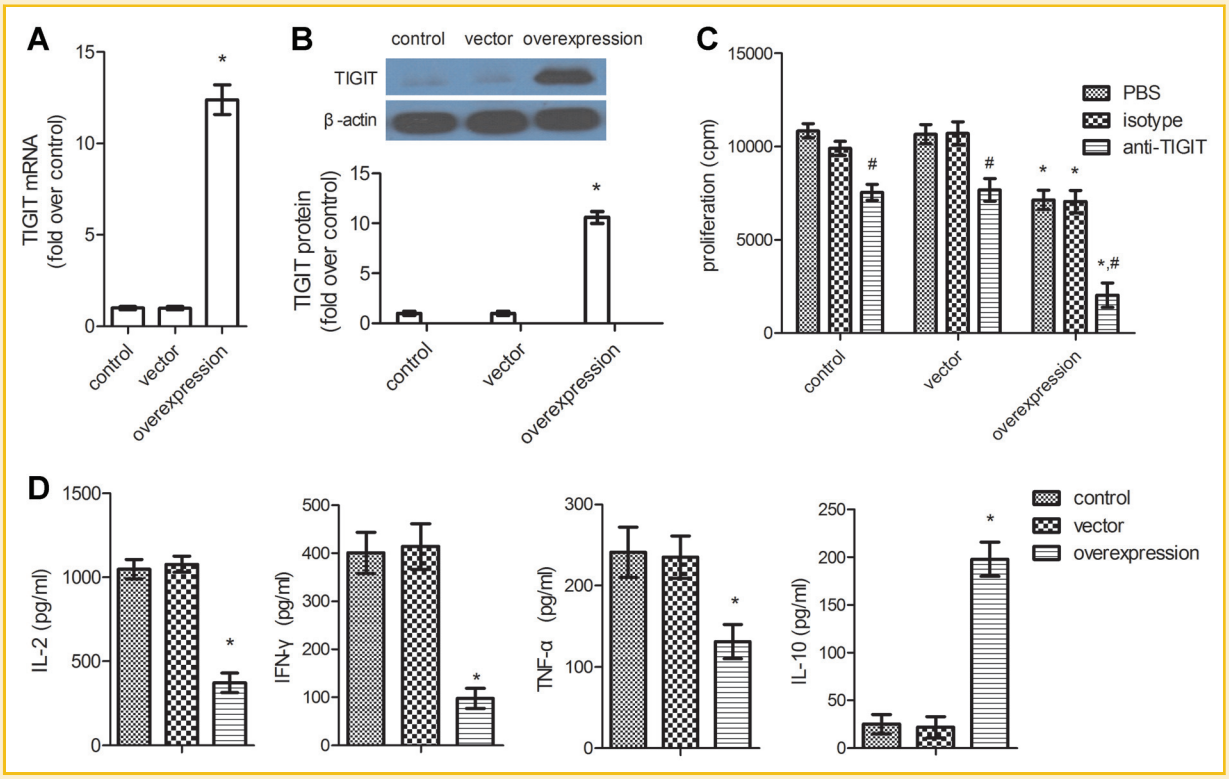

Fig. 3. Effect of TIGIT overexpression on $CD4^+$ T cell function. $CD4^+$ T cells isolated from patients with AA were transfected with the empty vector or pCMV6-XL5-TIGIT. The mRNA and protein levels of TIGIT in the control group, empty vector and overexpression group were determined by RT-PCR (A) and Western blot (B), respectively. The mRNA and protein levels were both normalized based on β-actin levels. C: Cells were activated by anti-CD3ε/CD28 antibodies in absence or presence of agonistic anti-TIGIT antibody. The IgG1 isotype was used as a control antibody. Proliferation of $CD4^+$ T cells was determined by $[^3H]$-thymidine incorporation assay. D: Cells were activated by anti-CD3ε/CD28 antibodies in the presence of agonistic anti-TIGIT antibody. The production of IL-2, IFN-γ, TNF-α, and IL-10 in the supernatants was determined using a corresponding ELISA Kit. Data are expressed as mean ± SEM. *$P < 0.05$ was regarded as statistically significant. *Compared to control group; #compared to PBS group in Figure 2C.

TIGIT overexpression was also accompanied by a significant decrease in IL-2, IFN-γ, and TNF-α production ($P < 0.05$, Fig. 3D). In contrast, IL-10 production was significantly increased as a result of TIGIT overexpression ($P < 0.05$, Fig. 3D). These data indicate that TIGIT overexpression impairs the function of $CD4^+$ T cells.

TIGIT OVEREXPRESSION DECREASED T-BET EXPRESSION ON $CD4^+$ T CELLS FROM PATIENTS WITH AA

T-bet, a T-box transcription factor encoded by the *TBX21* gene, is a master transcriptional regulator for Th1 cell differentiation and is critical for the production of Th1-associated cytokines such as IFN-γ [Szabo et al., 2000]. It has been previously shown that T-bet is up-regulated in patients with AA [Solomou et al., 2006; Shan et al., 2013], indicating a potent role of T-bet in the pathogenesis of AA. Therefore, we asked whether TIGIT regulation altered the expression of T-bet. $CD4^+$ T cells isolated from healthy donors were transfected with TIGIT-siRNA and activated by anti-CD3ε/CD28 antibodies in the presence of agonistic anti-TIGIT antibody. However, no significant increase was observed in the mRNA or protein levels of T-bet in TIGIT-silenced $CD4^+$ T cells (Fig. 4A,B). In $CD4^+$ T cells isolated from patients with AA, a significant decrease of mRNA and protein levels of T-bet was accompanied by TIGIT overexpression ($P < 0.05$, Fig. 4C,D). In addition, the expression of CXCR3, which was deemed a functional Th1 phenotype that allows Th1 $CD4^+$ T cells to become properly differentiated [Groom et al., 2012], was also significantly decreased in TIGIT-overexpressed $CD4^+$ T cells, as shown by the RT-PCR assay ($P < 0.05$, Fig. 4E).

TIGIT OVEREXPRESSION ON $CD4^+$ T CELLS AMELIORATED DISEASE IN AA MICE

The potent inhibitory function of TIGIT on $CD4^+$ T cells in vitro led us to investigate whether TIGIT overexpression could ameliorate disease in vivo. To address this question, $CD4^+$ T cells isolated from splenocytes of the donor mouse were infected by lentivirus expressing TIGIT and were transferred into the recipient mouse. As expected, mice receiving TIGIT-overexpressed $CD4^+$ T cells demonstrated a significant increase in the RBC counts (Fig. 5A) and a decrease in the plasma concentrations of INF-γ (Fig. 5B) and TNF-α compared to wild-type $CD4^+$ T cell-induced AA mice (Fig. 5C). However, the RBC counts and levels of plasma INF-γ and TNF-α in TIGIT-overexpressed $CD4^+$ T cell-induced AA mice were still significantly different from those of normal mice (Fig. 5A–C). To further identify the alterations in the degree of BM damage in mice, BMMNCs were isolated. The mRNA levels of CD34, SCF, and GM-CSF in BMMNCs were also up-regulated compared to the wild-type $CD4^+$ T cell-induced AA mice, but they were still lower than those of

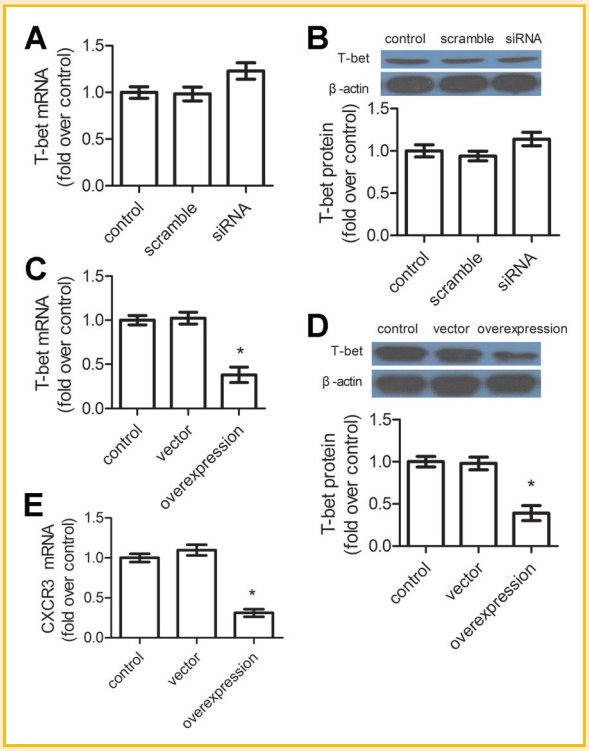

Fig. 4. The expression of T-bet was regulated by TIGIT. $CD4^+$ T cells isolated from healthy human volunteers was transfected with scramble siRNA or TIGIT-siRNA. Cells were activated by anti-CD3ε/CD28 antibodies in the presence of agonistic anti-TIGIT antibodies. T-bet expression levels were determined by RT-PCR (A) and Western blot (B), respectively. $CD4^+$ T cells isolated from patients with AA were transfected with empty vector or pCMV6-XL5-TIGIT and activated by anti-CD3ε/CD28 antibodies in the presence of agonistic anti-TIGIT antibody. T-bet expression levels were determined by RT-PCR (C) and Western blot (D), respectively. E: CXCR3 expression levels were determined by RT-PCR. All data were normalized based on β-actin levels and were expressed as mean ± SEM. $^*P < 0.05$ was regarded as statistically significant.

normal mice (Fig. 5D–F). At the 68 days follow-up, the median survival time of AA mice in the normal control group and CON-LV treatment group was 17.5 days (range from 11 to 26 days; Fig. 5G). However, mice that received TIGIT-LV infected $CD4^+$ T cells achieved a median survival of 55 days (range from 22 to 57 days; $P < 0.01$) with two out of ten mice being fully rescued from lethal AA (Fig. 5G). These observations indicate that disease in the immune-mediated AA mouse model could be alleviated by TIGIT over-expression on $CD4^+$ T cells.

DISCUSSION

AA is regarded an autoimmune disease, in which self-reactive T cells infiltrate into the BM and induce apoptosis of hematopoietic stem/progenitor cells [Zeng et al., 1999, 2004]. Recent studies have emphasized the important role of co-inhibitory molecules in autoimmune diseases. TIGIT is an inhibitory type 1 transmembrane protein expressed on $CD4^+$ T cells and was documented to deliver inhibitory effect on the [Boles et al., 2009; Yu et al., 2009; Lozano et al., 2012]. In the present study, a significant decrease in the percentage of TIGIT-positive $CD4^+$ T cells was observed in the peripheral blood from most AA patients (85%, 17/20). This finding led to the hypothesis that the pathogenesis of AA might be accelerated by TIGIT impairment on $CD4^+$ T cells. Tim-3, another co-inhibitory protein involved in the pathogenesis of several human autoimmune diseases, was found to be significantly decreased on $CD4^+$ T cells in AA patients compared to that of healthy controls [Shan et al., 2013]. However, expression of Tim-3 on PBMCs was increased in AA patients in another report [Zhang et al., 2008]. In the current study, although a significant decrease in the percentage of TIGIT-positive $CD4^+$ T cells was found in most AA patients, it was not found in 15% of AA patients (3/20). This might be due to the difference in the severity of AA between individuals.

As $CD4^+$ T cells play a critical role in the pathogenesis of AA, we explored the effect of TIGIT on the function of $CD4^+$ T cells. When TIGIT was knocked-down, the production of IL-2, IFN-γ, and TNF-α was increased, and cell proliferation was improved, whereas the production of IL-10 was hindered, as expected. This was consistent with findings of Lozano, who demonstrated that the knockdown of TIGIT expression by short hairpin RNA led to an increase in IFN-γ expression with a concomitant decrease in IL-10 expression [Lozano et al., 2012]. Using $CD4^+$ T cells isolated from AA patients, in which TIGIT was rarely expressed, overexpression of TIGIT exhibited opposite effects on the function of $CD4^+$ T cells compared to TIGIT knockdown. This was similar to the effect of agonistic anti-TIGIT monoclonal antibody treatment [Lozano et al., 2012].

In a previous study, T-bet expression in human $CD4^+$ T cells was increased by short hairpin RNA targeting TIGIT [Lozano et al., 2012]. Here, we showed that T-bet expression was significantly down-regulated by overexpression of TIGIT in $CD4^+$ T cells from AA patients but was only mildly up-regulated by siRNA transfection in healthy $CD4^+$ T cells. This might be reasoned by the normal low basal expression of TIGIT in healthy human $CD4^+$ T cells. T-bet was firstly identified as a Th1-specific transcription factor that directly binds to the IFN-γ promoter and activates the expression of the hallmark Th1 cytokine and represses that of the Th2 cytokines [Szabo et al., 2000; Mullen et al., 2001; Afkarian et al., 2002]. Thus, T-bet appears to act as a master switch for Th1 development. The expression of T-bet was regulated by the T cell receptor (TCR) signaling, and IFN-γ signaling through signal transducer and activator of transcription 1 (STAT1) and interleukin-12 (IL-12) signaling through STAT4 activation [Afkarian et al., 2002; Shin et al., 2009]. Transcription of T-bet could also be transactivated by the early growth response gene 1 (EGR1), which is a transcriptional regulator that contains a zinc-finger DNA-binding domain [Shin et al., 2009]. Recently, the NOTCH signaling pathway was reported to be involved in T-bet regulation [Roderick et al., 2013]. In both the current study and a previous study, T-bet was shown to be regulated by TIGIT in an unclear signaling pathway [Lozano et al., 2012]. However, the underlying mechanism still needs to be elucidated.

Although TIGIT overexpression did not abrogate BM failure in the present study, it alleviated the severity of BM failure with significantly higher RBC counts and mRNA levels of CD34, SCF, and GM-CSF. However, some limitations in this study should be

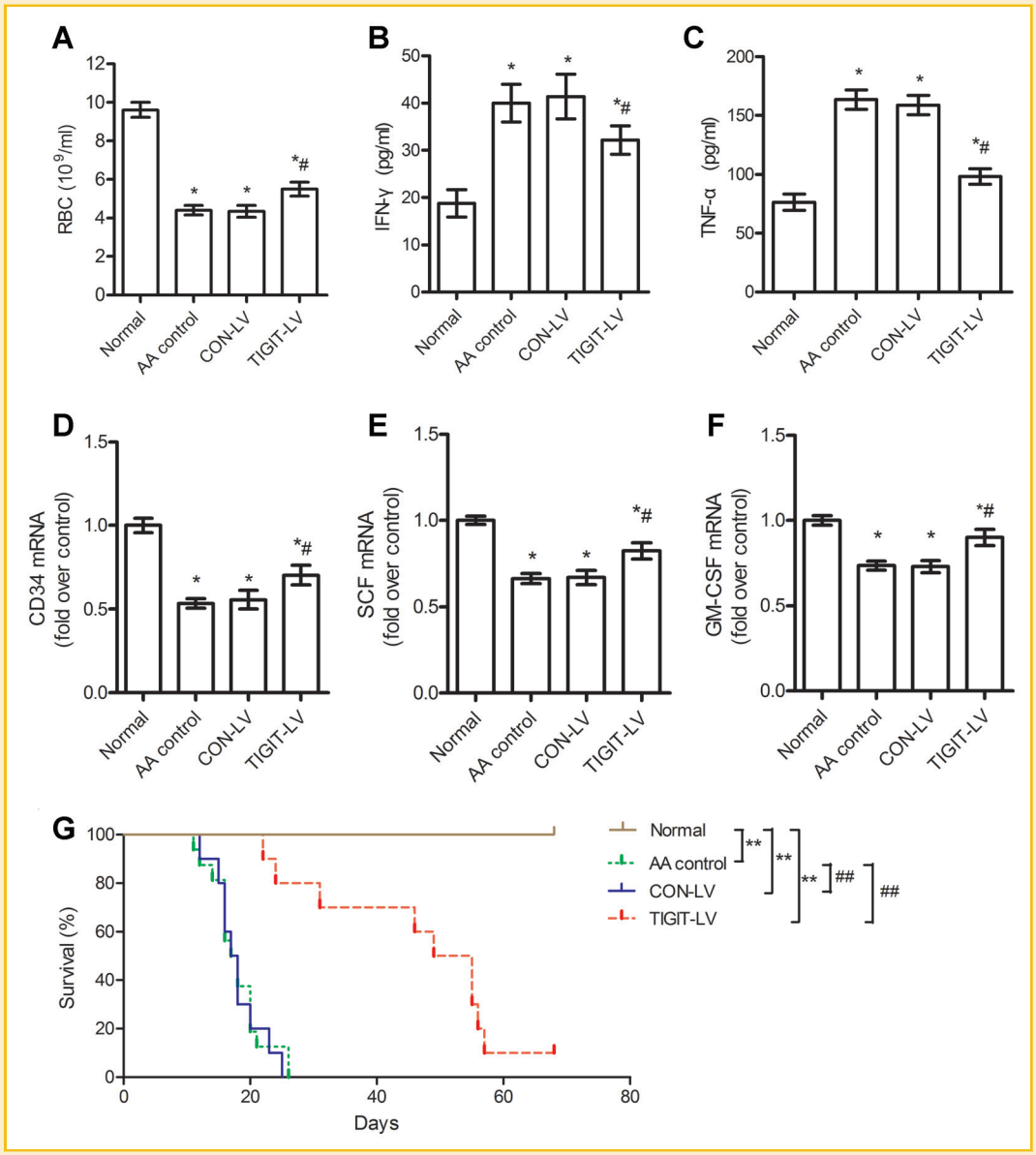

Fig. 5. TIGIT overexpression on $CD4^+$ T cells ameliorated disease in AA mice. $CD4^+$ T cells isolated from splenocytes of the donor mouse were infected by TIGIT-LV. Then, the TIGIT-overexpressed $CD4^+$ T cells were transferred to a radiated recipient mouse via tail vein. Wild-type $CD4^+$ T cells and CON-LV infected $CD4^+$ T cells transferred mice were used as a AA control and CON-LV control, respectively. Normal control group represented mice receiving equivalent normal saline without any $CD4^+$ T cells. A: Mice blood was sampled from tail veins. Red blood cells (RBCs) were counted using a Sysmex F-820 semi-automatic blood analyzer. B: IFN-γ and (C) TNF-α levels in serum were determined by ELISA. D–F: BMMNCs were isolated from mice. The mRNA levels of CD34, SCF, and GM-CSF were determined by RT-PCR. The above data were represented as mean ± SEM. The significance of differences was evaluated by the Student's *t*-test. $P < 0.05$ was regarded as statistically significant. *Compared to normal control group; #compared to AA control group. G: Survival estimates for AA mice induced with TIGIT-LV infected $CD4^+$ T cells (n = 16) compared with mice induced with normal $CD4^+$ T cells (n = 10) or CON-LV infected $CD4^+$ T cells (n = 10). Log-rank test was used to evaluate the significance of differences. * or #, $P < 0.05$; ** or ##, $P < 0.01$. *Compared to normal control group; #compared to AA control group.

noted. As it was difficult to specifically overexpress TIGIT in $CD4^+$ T cells in vivo in ready-made AA mouse models, the real therapeutic effect of TIGIT on AA was difficult to evaluate. To clarify this important point, a realistic method for the induction of AA in mouse models using splenocytes containing TIGIT-overexpressed $CD4^+$ T cells was performed. However, when TIGIT was overexpressed, the proliferation activity of $CD4^+$ T cells was suppressed. Thus, the shortage of $CD4^+$ T cells might have also contributed to the alleviated severity of BM failure. Previous studies had demonstrated that some inhibitory co-stimulator exhibited opposite function on T effector cells and T regulator cells (Tregs). Moorman [Moorman et al., 2012] showed that the inhibitory receptor Tim-3 expression on $Foxp3^+$ Tregs positively correlated with the proliferation of Tregs and blockade of Tim-3 on $CD4^+CD25^+$ T cells promoted expansion of T effector cells. Expression of PD-1 and LAG-3 also delivered active signals for Treg function [Goding et al., 2013]. In the present

study, $CD4^+CD25^+$ Tregs were not depleted in the $CD4^+$ T cells subset. Although the accurate effect of TIGIT overexpression on Tregs were not determined, we hypothesis TIGIT overexpressed Tregs might also contribute to the amelioration of AA.

In conclusion, our results demonstrated that TIGIT expression on $CD4^+$ T cells was decreased in AA patients. The function of $CD4^+$ T cells could be elevated by TIGIT knockdown and inhibited by TIGIT overexpression. The increased expression of TIGIT on $CD4^+$ T cells could ameliorate immune-mediated BM failure of AA in a mouse model. Our results provided a possible etiology of AA and a new potential strategy for the treatment of AA.

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