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Research paper

TIGIT expression is upregulated in T cells and causes T cell dysfunction independent of PD-1 and Tim-3 in adult B lineage acute lymphoblastic leukemia

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ABSTRACT

T cell immunoglobulin and ITIM domain (TIGIT) is a novel immune checkpoint receptor and plays critical roles in cancer immunity. Adult acute lymphoblastic leukemia (ALL) remains a treatment challenge despite years of research. In this study, we analyzed the status of TIGIT expression in circulating T cells from patients with adult ALL. Compared to the data in healthy controls, the expression of TIGIT in $CD4^+CD25^-$ T cells and $CD8^+$ T cells in adult ALL patients presented a small but significant upregulation. Stimulation via the CD3/CD28 route increased TIGIT mRNA expression at 24 h, which peaked at 48 h and was maintained at 72 h post-stimulation. The frequency of $TIGIT^+$ cells, on the other hand, consistently increased over time. ALL protein lysate or Wilms' Tumor 1 peptide could significantly increase the expression of TIGIT in ALL, but not healthy control T cells. Compared to $TIGIT^-$ cells, the $TIGIT^+$ cells presented significantly higher PD-1 and Tim-3 expression directly *ex vivo*, and significantly lower IL-2, IFN-γ, and TNF-α after CD3/CD28 stimulation. The high inhibitory molecule and low cytokine expression signature was especially pronounced in ALL $TIGIT^+$ $CD4^+CD25^-$ T cells and $TIGIT^+$ $CD8^+$ T cells. Blocking TIGIT alone could minimally increase cytokine expression independent of PD-1 and Tim-3 blocking, whereas blocking TIGIT, PD-1, and Tim-3 altogether was significantly more effective. Together, these data demonstrated that TIGIT regulated T cell function in adult ALL patients, and may serve as a treatment target for ALL.

1. Introduction

T cell immunoglobulin and ITIM domain (TIGIT) is a member of the CD28 family with inhibitory functions [1]. TIGIT expression can be found on activated CD4 and CD8 T cells, T regulatory (Treg) cells, type 1 regulatory T (Tr1) cells, follicular helper T (Tfh) cells, and natural killer (NK) cells [2–6]. TIGIT strongly binds CD155, which can be found on antigen-presenting cells (APCs), activated T cells, and tumor cells [7–9]. DNAM-1 is a co-stimulatory molecule that delivers a proinflammatory signal to CD155 [10,11]. TIGIT could negate DNAM-1 by disrupting the DNAM-1/CD155 interaction in a dose-dependent manner [12]. In addition, TIGIT/CD155 interaction could activate the ITIM in the cytoplasmic tail of CD155, resulting in the inhibition of IL-12 p40 subunit and the induction of IL-10 in dendritic cells [13], which then mediates subsequent tolerogenic effects. It is also shown that the cytoplasmic domain of TIGIT can directly deliver an inhibitory signal in TIGIT-expressing NK cells and T cells, resulting in reduced cytotoxicity and cytokine expression [12,14–16]. On the other hand, TIGIT upregulates the expression of anti-apoptosis molecule Bcl-xL, and may promote T cell survival [16]. Given that TIGIT is preferentially expressed by Treg cells, this pro-survival effect might promote the tolerogenic, rather than proinflammatory, immune responses [17]. Furthermore, co-expression of TIGIT with other immune checkpoint molecules, including PD-1, Tim-3, and Lag3, has a synergistic effect in suppressing T cell function [4,6,17,18].

Acute lymphoblastic leukemia (ALL) is developed due to abnormality in the proliferation and differentiation of lymphoid progenitor cells [19]. This malignancy is predominantly found in children, as the ratio of pediatric to adult cases is approximately 4 to 1 [19,20]. However, the adult cases are more difficult to manage, with a dismal 5-year survival rate of 30% to 40%, compared to 90% in children [21,22]. Treatment strategy for adult ALL is modeled after pediatric ALL, and although complete remission can be achieved in 80% to 90% of adult patients, approximately half will experience relapse. More research into the mechanistic and immunological features of the adult type of ALL is urgently needed.

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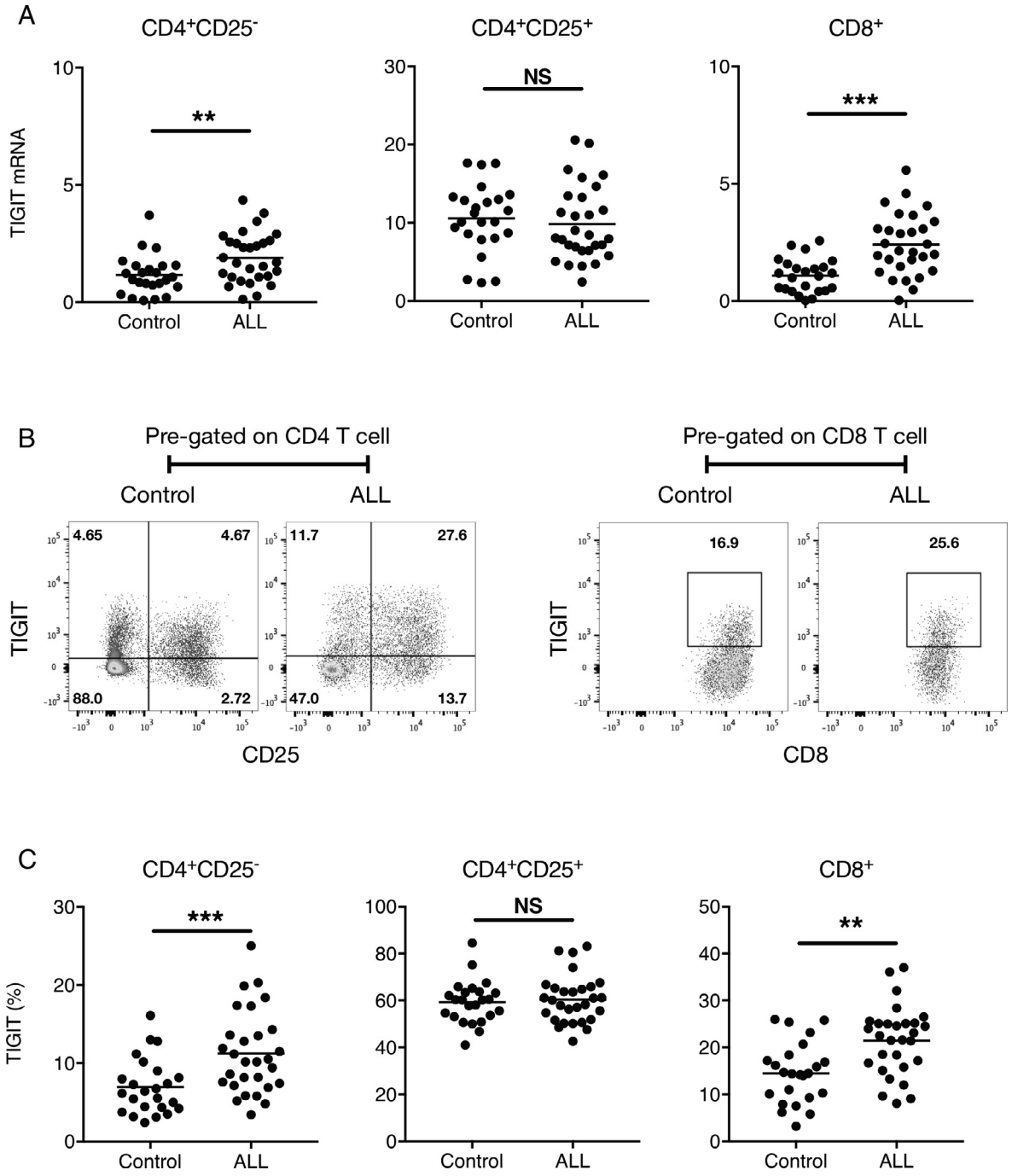

Fig. 1. TIGIT expression in circulating T cells. (A) TIGIT mRNA expression in circulating $CD4^+CD25^-$ T cells, $CD4^+CD25^+$ T cells, and $CD8^+$ T cells from healthy controls and ALL patients *ex vivo*. (B) Sample TIGIT gating in $CD4^+CD25^-$ T cells, $CD4^+CD25^+$ T cells, and $CD8^+$ T cells from healthy controls and ALL patients. (C) The frequencies of TIGIT-expressing cells in circulating $CD4^+CD25^-$ T cells, $CD4^+CD25^+$ T cells, and $CD8^+$ T cells from healthy controls and ALL patients *ex vivo*. Mann-Whitney test. NS, not significant. $*p < 0.05$. $**p < 0.01$. $***p < 0.001$.

The expression of TIGIT is significantly higher in CD8 T cells from acute myelogenous leukemia (AML) patients than in age- and sex-matched control patients. These $TIGIT^+$ CD8 T cells demonstrated features of exhaustion and dysfunction, while TIGIT knockdown could reverse functional defects [23]. Blockade of TIGIT and CD155 could also promote proinflammatory responses in malignancies of hematopoietic origins [24–26]. In adult ALL, the expression and function of TIGIT by proinflammatory $CD4^+$ and $CD8^+$ T cells and the effect of TIGIT blockade have not been analyzed. Hence, we performed the current study to investigate the above questions.

2. Methods

2.1. Subjects

A total of 29 adult B lineage ALL patients, including 18 males and 11 females between 22 and 41 years of age (median 34), and a total of 24 healthy controls, including 14 males and 10 females between 20 and 42 years of age (median 33), were included in this study. Diagnosis was performed at Liaocheng People's Hospital and confirmed via bone marrow biopsy. All patients were newly diagnosed without previous episodes of hematopoietic malignancies or any other malignancies. In

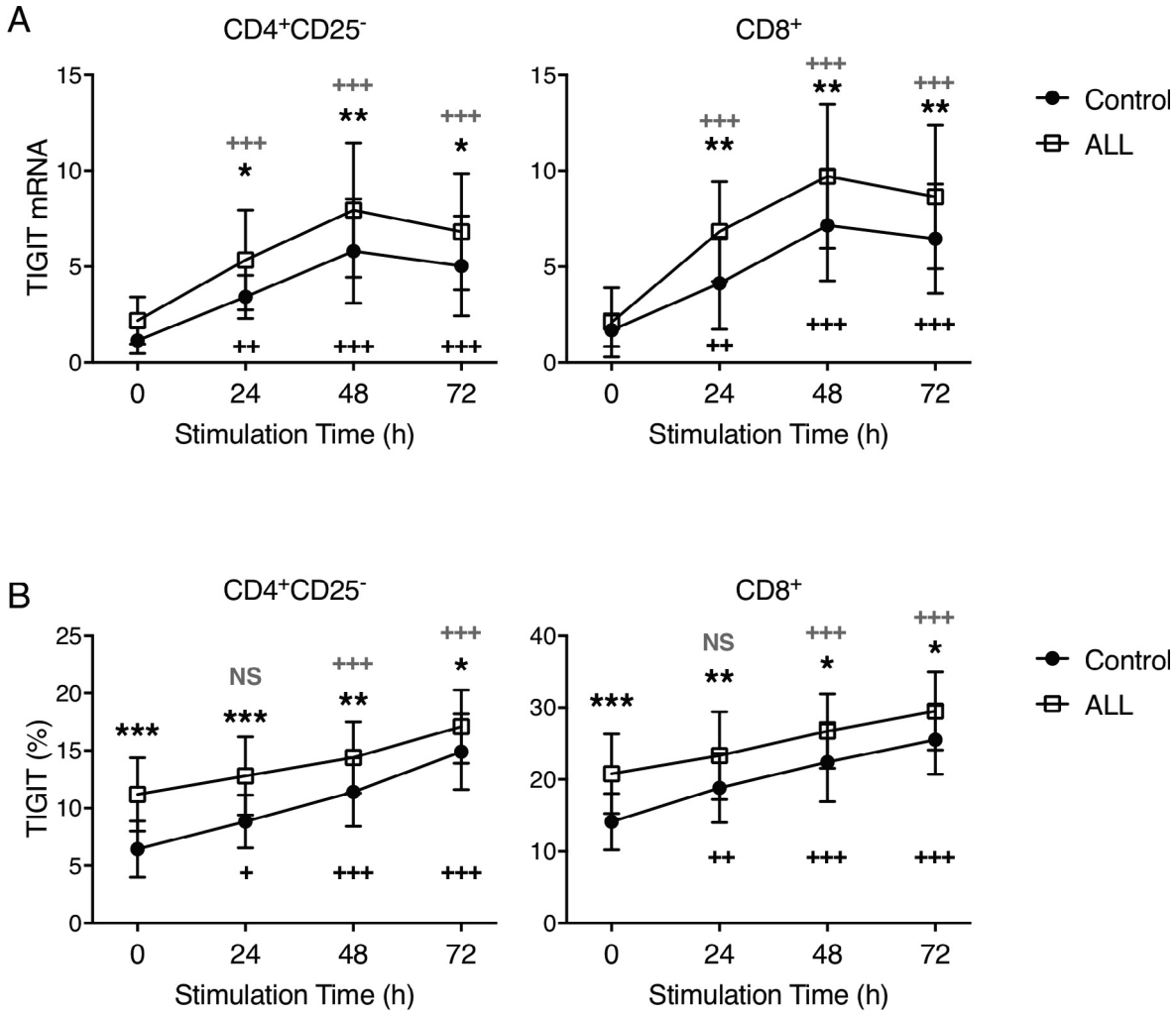

Fig. 2. TIGIT expression in TCR-stimulated T cells. Anti-CD3 and anti-CD28 stimulation beads were added to purified $CD4^+CD25^-$ T cells and purified $CD8^+$ T cells from healthy controls (N = 24) and ALL patients (N = 29) at time 0. (A) The TIGIT mRNA expression levels and (B) the frequency of TIGIT-expressing cells were examined in $CD4^+CD25^-$ T cells and $CD8^+$ T cells at time points 0, 24, 48 and 72 h post-stimulation. Two-way ANOVA followed by Sidak's multiple comparison test. Significant differences between the control group and the ALL group were labeled using asterisks, and significant differences between time 0 and each subsequent time points were labeled using plus signs (black for healthy control and gray for ALL). NS, not significant. $*/+p < 0.05$. $**/++p < 0.01$. $***/+++p < 0.001$.

addition, no treatment was given to the patients at the time of sample collection. The peripheral blood was collected from all the participants upon receiving written informed consent. The ethics review board of Liaocheng People's Hospital provided study approval.

2.2. Cell collection

Ficoll (Sigma) gradient centrifugation was performed to isolate peripheral blood mononuclear cells (PBMCs) from heparinized blood. $CD4^+$ T cells and $CD8^+$ T cells were isolated via magnetic negative selection using Human CD4 T Cell Enrichment Kit and Human CD8 T Cell Enrichment Kit (Stemcell) according to the manufacturer's instructions. Purity was consistently greater than 97%. The $CD4^+$ T cells were subsequently fractionated into $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ T cells by incubating $CD4^+$ T cells with PE-anti-human CD25 antibody (BioLegend), washing to remove excess antibody, and fractionating into PE^- and PE^+ cells, respectively, using Human PE Positive Selection Kit (Stemcell) according to the manufacturer's instruction. Purity was consistently greater than 95%.

2.3. Flow cytometry

Cells were first treated with Fixable Aqua Dead Cell Stain (Invitrogen). Anti-human CD3, CD4, CD8, CD25, PD-1, Tim-3, and/or TIGIT (BioLegend) monoclonal antibodies were added to cells under 4 °C in dark. After 30 min, the excess antibodies were removed by washing, and the cells were acquired in FACSCanto cytometer. The samples were subsequently analyzed in FlowJo version 10.0.7 (Tree Star).

2.4. Stimulation

To stimulate $CD4^+CD25^-$ T cells and $CD8^+$ T cells, PBMCs were plated in an anti-CD3/CD28-coated (BioLegend) 96-well round bottom plate at 1×10^5 cells per well per 200 μL complete media at 1 bead per T cell. In select experiments, the TIGIT, PD-1, and/or Tim-3 were antagonized using monoclonal anti-human TIGIT (71340, BPS Biosciences), anti-human PD-1 (AF1086, R&D Systems), and anti-human Tim-3 (2E2, BioLegend) at 10 μg/mL each. The cells were incubated inside a 37 °C humidified incubator with 5% CO_2 and were harvested every 24 h.

2.5. Gene expression

Total RNA was collected using the RNeasy Mini Kit (Qiagen). cDNA was then generated using iScript cDNA Synthesis Kit (Bio-Rad). The

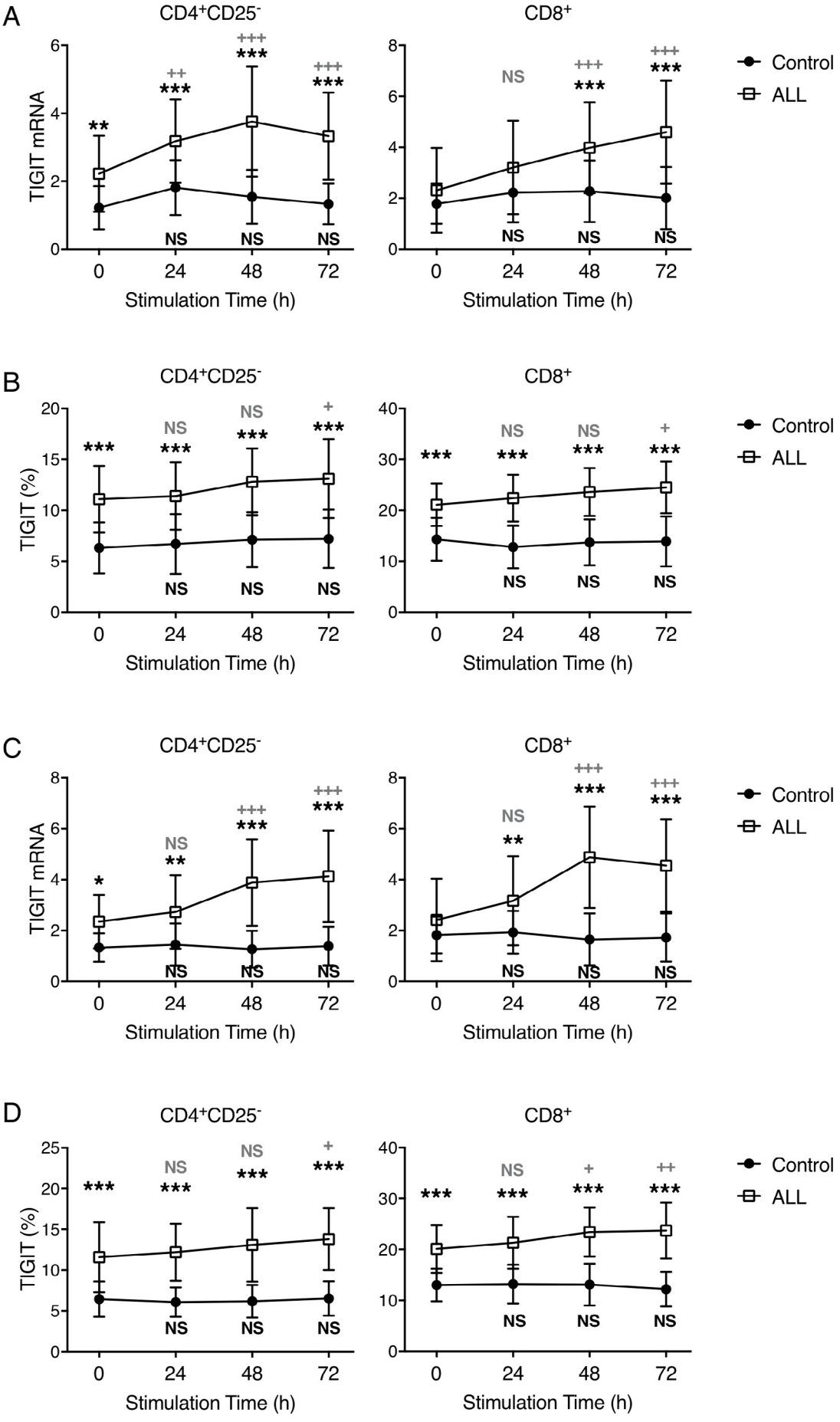

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Fig. 3. TIGIT expression in T cells after stimulation with ALL-associated antigens. (A) and (B) Purified $CD4^+CD25^-$ T cells and purified $CD8^+$ T cells from healthy controls (N = 24) and ALL patients (N = 29) were coincubated with autologous monocytes and 10 μg/mL CCRF-SB protein lysate for a total of 72 h. At time points 0, 24, 48 and 72 h post-stimulation, the $CD4^+CD25^-$ T cells and $CD8^+$ T cells were harvested and (A) the TIGIT mRNA expression levels and (B) the frequency of TIGIT-expressing cells were examined. (C) and (D) Purified $CD4^+CD25^-$ T cells and purified $CD8^+$ T cells from healthy controls (N = 24) and ALL patients (N = 29) were coincubated with autologous monocytes and 100 ng/mL WT1 peptides for a total of 72 h. At time points 0, 24, 48 and 72 h post-stimulation, the $CD4^+CD25^-$ T cells and $CD8^+$ T cells were harvested and (C) the TIGIT mRNA expression levels and (D) the frequency of TIGIT-expressing cells were examined. Two-way ANOVA followed by Sidak's multiple comparison test. Significant differences between the control group and the ALL group were labeled using asterisks, and significant differences between time 0 and each subsequent time points were labeled using plus signs (black for healthy control and gray for ALL). NS, not significant. $*/+p < 0.05$. $**/++p < 0.01$. $***/+++p < 0.001$.

following TaqMan assays (Thermo Fisher Scientific) for each gene were applied, including Hs00545087_m1 for TIGIT, Hs00174114_m1 for IL-2, Hs00989291_m1 for IFN-γ, and Hs00174128_m1 for TNF-α. The relative expression of the genes was normalized to B2M (Hs00187842_m1). All PCR reactions were performed in triplicates in the ABI PRISM 7000 System.

2.6. Statistical analysis

Statistical comparisons between two groups used Mann-Whitney test, and between multiple groups used two-way ANOVA, followed by Sidak's multiple comparisons. Two-tailed $p < 0.05$ was considered significant.

3. Results

3.1. TIGIT expression was upregulated in circulating non-Treg T cells from adult ALL patients

Adult B lineage ALL patients and age- and sex-matching healthy controls were recruited. The mRNA expression of the TIGIT gene was assessed in circulating $CD4^+CD25^-$ T cells, $CD4^+CD25^+$ T cells, and $CD8^+$ T cells directly ex vivo. Circulating $CD4^+CD25^-$ T cells and $CD8^+$ T cells from adult ALL patients presented significantly higher TIGIT expression than corresponding populations from healthy controls (Fig. 1A), while circulating $CD4^+CD25^+$ T cells from ALL patients presented comparable TIGIT expression with the $CD4^+CD25^+$ T cells from healthy controls (Fig. 1A). Subsequently, we analyzed the percentage of TIGIT-expressing circulating $CD4^+CD25^-$ T cells, $CD4^+CD25^+$ T cells, and $CD8^+$ T cells using flow cytometry analysis (Fig. 1B). In general, the frequencies of TIGIT-expressing cells in $CD4^+CD25^-$ T cells and $CD8^+$ T cells were significantly higher in ALL patients than in healthy controls (Fig. 1C). In $CD4^+CD25^+$ T cells, however, the frequencies of TIGIT-expressing cells were comparable between healthy controls and ALL patients. Additionally, the frequencies of TIGIT-expressing $CD4^+CD25^+$ T cells were significantly higher than the frequencies of TIGIT-expressing $CD4^+CD25^-$ T cells and $CD8^+$ T cells in both healthy controls and ALL patients ($p < 0.001$ for all comparisons, Kruskal-Wallis ANOVA followed by Dunn's test). Together, these data demonstrated that the circulating non-Treg T cells from ALL patients presented higher TIGIT expression than the corresponding cell types in healthy controls. No correlation could be found between the levels of TIGIT expression and the demographic characteristics, including the age and the sex, of the patients,

3.2. TIGIT expression was upregulated on non-Treg T cells upon prolonged TCR-specific stimulation

Prolonged antigen stimulation is thought to induce checkpoint molecules on T cells [27]. Hence, the induction of TIGIT upon anti-CD3/CD28 activation of T cells was examined. In both $CD4^+CD25^-$ T cells and $CD8^+$ T cells, the TIGIT mRNA was elevated at 24 h of anti-CD3/CD28 stimulation, peaked at 48 h, and was maintained at high levels at 72 h (Fig. 2A). The surface TIGIT protein expression in those cells correspondingly increased over time (Fig. 2B and C). Interestingly, the TIGIT expression, both in terms of mRNA and surface protein, was significantly higher in ALL patients than in healthy controls (Fig. 2A and C).

Instead of using pan-T cell anti-CD3/CD28 stimulation, we also investigated whether ALL-associated antigens, obtained by using ALL cell lysate, could alter TIGIT expression in $CD4^+CD25^-$ T cells and $CD8^+$ T cells. Briefly, protein lysate from CCRF-SB cells, a B lymphoblast ALL line, was added to primary monocytes collected from each of the healthy controls and ALL patients. Autologous $CD4^+CD25^-$ T cells or $CD8^+$ T cells were then added for a total of 72 h, and the level of TIGIT expression was examined by mRNA analysis and flow cytometry every 24 h. The $CD4^+CD25^-$ T cells and $CD8^+$ T cells from ALL patients presented higher TIGIT mRNA than the $CD4^+CD25^-$ T cells and $CD8^+$ T cells from healthy controls throughout the duration of the experiment (Fig. 3A). Interestingly, no upregulation of TIGIT mRNA was found in $CD4^+CD25^-$ T cells and $CD8^+$ T cells from healthy controls, while in contrast, the $CD4^+CD25^-$ T cells and $CD8^+$ T cells significantly increased TIGIT mRNA expression following stimulated with CCRF-SB lysate. The TIGIT expression on the cell surface was also higher in $CD4^+CD25^-$ T cells and $CD8^+$ T cells from ALL patients than $CD4^+CD25^-$ T cells and $CD8^+$ T cells from healthy controls (Fig. 3B). In ALL patients, the frequencies of $TIGIT^+$ cells were significantly higher at 72-hours post-stimulation than at time 0, while no significant changes were observed in healthy controls.

Wilms' Tumor 1 (WT1) is a zinc finger transcription factor that has been widely found in malignant cells of the hematopoietic origin [28]. To investigate whether there are specific responses toward WT1, WT1 peptides were loaded to primary monocytes collected from each of the healthy controls and ALL patients. The level of TIGIT upregulation was examined by mRNA analysis and flow cytometry. In the $CD4^+CD25^-$ T cells and $CD8^+$ T cells from healthy controls, no TIGIT upregulation was observed, while in the $CD4^+CD25^-$ T cells and $CD8^+$ T cells from ALL patients, both mRNA and surface TIGIT expression were upregulated (Fig. 3C and D). TIGIT expression in healthy controls was significantly higher than that in healthy controls throughout the duration of the experiment (Fig. 3C and D).

3.3. High TIGIT expression was associated with the expression of other inhibitory molecules

In chronic infections and malignancies, the immune system faces an inability to mediate antigen clearance, and it is thought that continued exposure to antigen, together with other factors, can result in an upregulation of PD-1 and Tim-3 on the surface of non-Treg $CD4^+CD25^-$ T cells and $CD8^+$ T cells and functional exhaustion of these cells [27]. Here, the $CD4^+CD25^-$ T cells and $CD8^+$ T cells were separated into $TIGIT^+$ and $TIGIT^-$ fractions, and the expression of PD-1 was examined (Fig. 4A). Interestingly, the frequency of PD-1-expressing cells was significantly higher in $TIGIT^+$ cells than in $TIGIT^-$ cells (Fig. 4B). Additionally, in $CD4^+CD25^-$ T cells and $CD8^+$ T cells, the $TIGIT^+$ cells from ALL patients presented higher PD-1 expression than the $TIGIT^+$ cells from controls (Fig. 4B). Similarly, the frequency of Tim-3-expressing cells was significantly higher in $TIGIT^+$ cells than in $TIGIT^-$ cells (Fig. 4C). The $TIGIT^+$ $CD4^+CD25^-$ T cells from ALL patients presented significantly higher Tim-3 expression than the corresponding cells from controls (Fig. 4D), and both $TIGIT^+$ and $TIGIT^-$ $CD8^+$ T cells from ALL patients presented significantly higher Tim-3 expression than

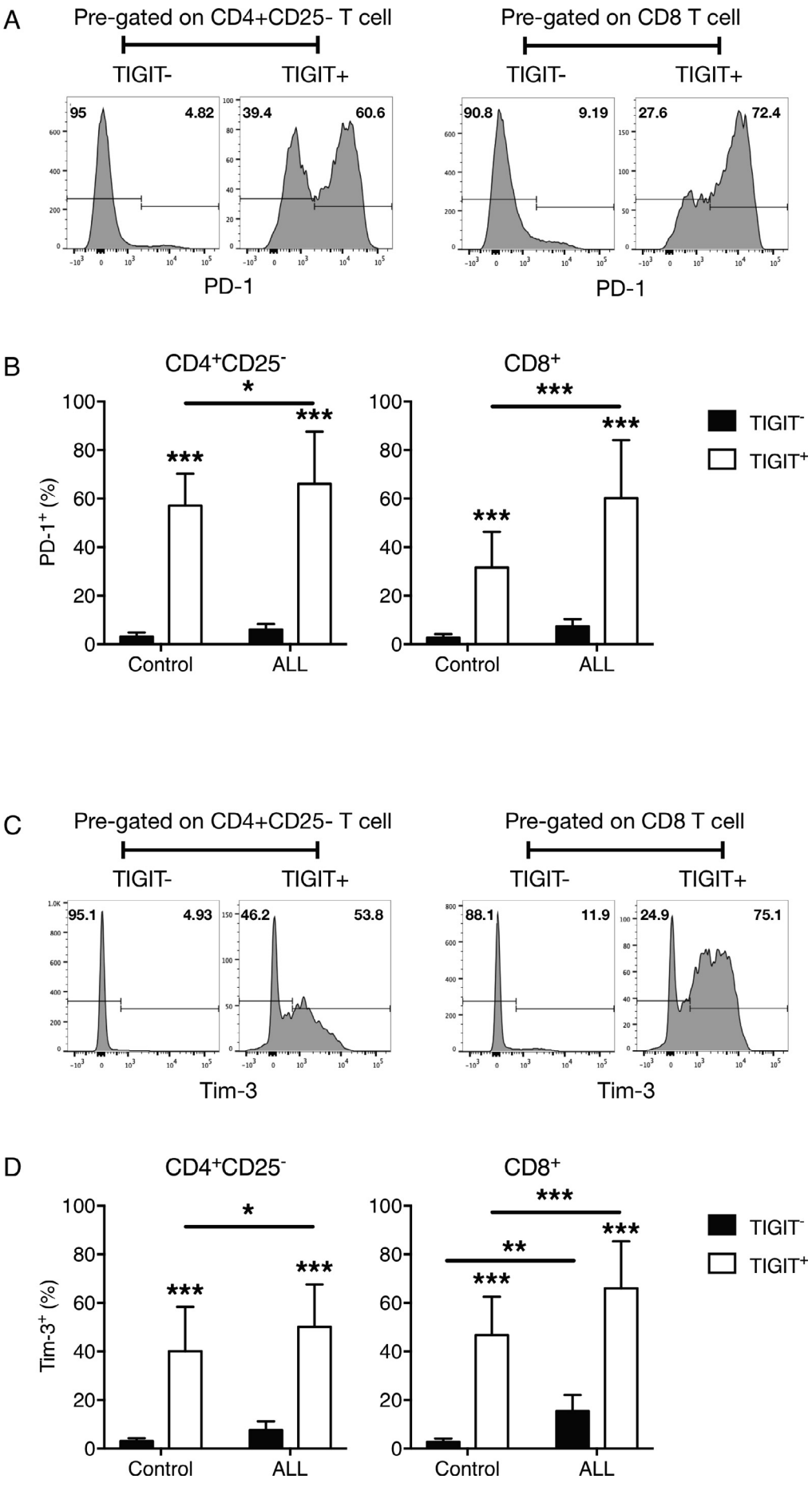

Fig. 4. TIGIT expression was associated with high PD-1 and Tim-3 expression. Unstimulated $CD4^+CD25^-$ T cells and $CD8^+$ T cells were separated into $TIGIT^+$ and $TIGIT^-$ cells. (A) Representative PD-1 expression in pre-gated $TIGIT^+$ and $TIGIT^-$ cells in one ALL individual. (B) The frequency of PD-1-expressing $CD4^+CD25^-$ T cells and $CD8^+$ T cells in 24 healthy controls and 29 ALL patients. (C) Representative Tim-3 expression in pre-gated $TIGIT^+$ and $TIGIT^-$ cells in one ALL individual. (B) The frequency of Tim-3-expressing $CD4^+CD25^-$ T cells and $CD8^+$ T cells in 24 healthy controls and 29 ALL patients. Two-way ANOVA followed by Sidak's multiple comparison test. $^*p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$.

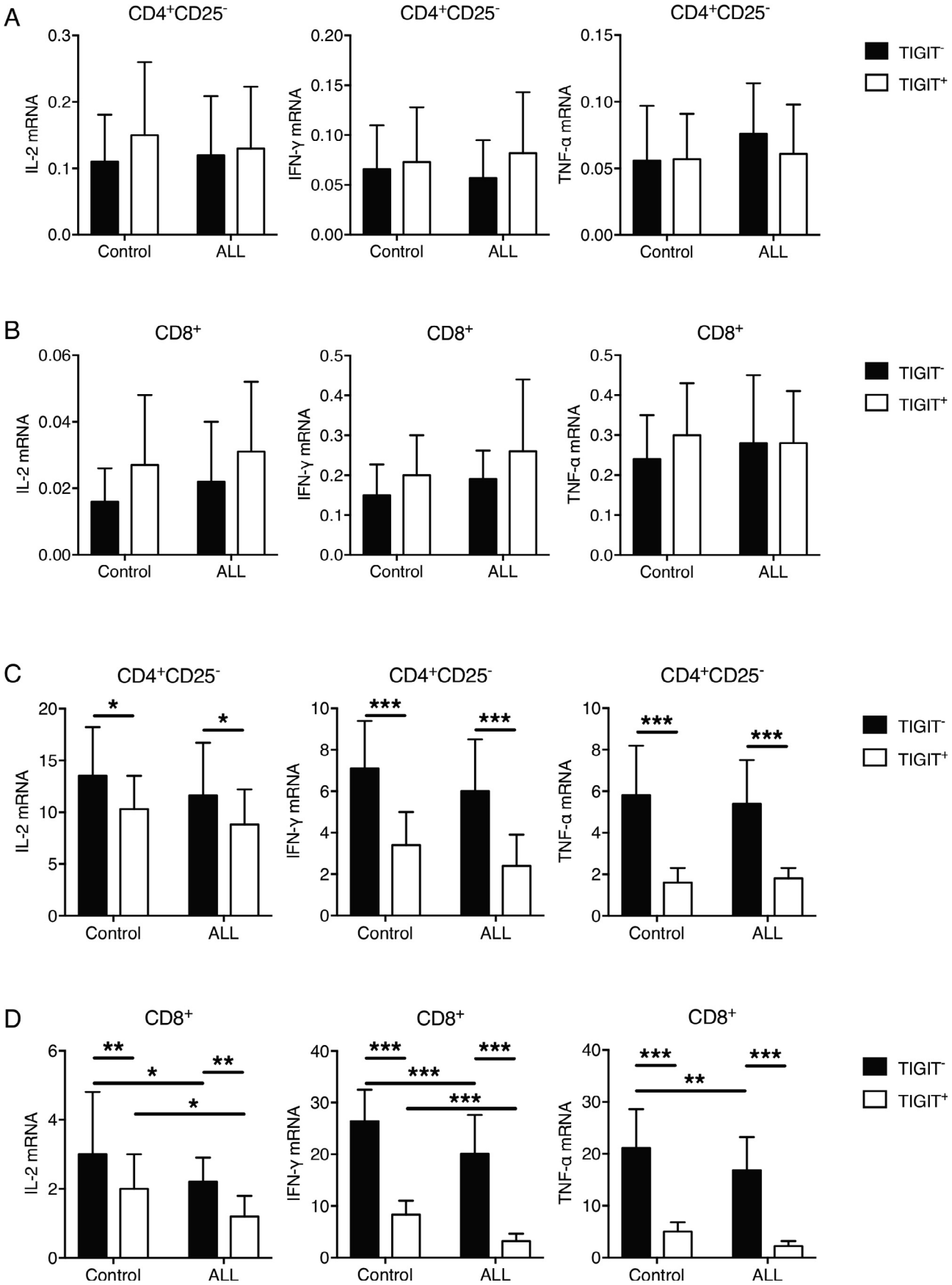

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Fig. 5. Cytokine expression by TIGIT$^{\pm}$ and TIGIT^{-} T cells. (A) and (B) CD4^{+}CD25^{-} T cells and CD8^{+} T cells were separated into TIGIT^{+} and TIGIT^{-} cells and were lysed immediately for mRNA analysis. (A) The mRNA levels of IL-2, IFN-γ, and TGF-α in unstimulated CD4^{+}CD25^{-} T cells. (B) The mRNA levels of IL-2, IFN-γ, and TGF-α in unstimulated CD8^{+} T cells. (C) and (D) CD4^{+}CD25^{-} T cells and CD8^{+} T cells were separated into TIGIT^{+} and TIGIT^{-} cells and were stimulated via anti-CD3/CD28 before lysis and mRNA investigation. (C) The mRNA levels of IL-2, IFN-γ, and TGF-α in anti-CD3/CD28-stimulated CD4^{+}CD25^{-} T cells. (C) The mRNA levels of IL-2, IFN-γ, and TGF-α in anti-CD3/CD28-stimulated CD8^{+} T cells. N = 24 for healthy controls and N = 29 for ALL patients. Two-way ANOVA followed by Sidak's multiple comparison test. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$.

the corresponding cells from controls (Fig. 4D).

3.4. High TIGIT expression predicted lower cytokine expression

The expression of IL-2, IFN-γ, and TNF-α were then examined in CD4^{+}CD25^{-} T cells and CD8^{+} T cells, pre-sorted into TIGIT^{+} and TIGIT^{-} subsets. In unstimulated CD4^{+}CD25^{-} T cells and CD8^{+} T cells, the expression of IL-2, IFN-γ, and TNF-α were low, with no significant differences observed between healthy controls and ALL patients or between TIGIT^{+} and TIGIT^{-} cells (Fig. 5A and B). Anti-CD3/CD28 stimulation could significantly elevate the expression of IL-2, IFN-γ, and TNF-α ($p < 0.001$ for all comparisons between unstimulated and stimulated cells). In anti-CD3/CD28-stimulated CD4^{+}CD25^{-} T cells, the TIGIT^{-} cells presented slightly higher levels of IL-2, IFN-γ, and TNF-αthan the TIGIT^{+} cells (Fig. 5C). These differences were observed in both healthy controls and ALL patients, with no additional difference between the two groups. In CD8^{+} T cells, the TIGIT^{-} cells also presented higher IL-2, IFN-γ, and TNF-α than the TIGIT^{+} cells (Fig. 5D). Additionally, the TIGIT^{-} CD8^{+} T cells from healthy controls presented higher IL-2, IFN-γ, and TNF-α expression than the TIGIT^{-} CD8^{+} T cell from ALL patients, and the TIGIT^{+} CD8^{+} T cells from healthy controls presented higher IL-2 and IFN-γ expression than the TIGIT^{+} CD8^{+} T cells from ALL patients.

3.5. TIGIT blocking could result in higher IFN-γ and TNF-α expression independent of PD-1 and Tim-3 blocking

Subsequently, we investigated whether the inhibition of TIGIT alone, or the inhibition of TIGIT, PD-1, and Tim-3 altogether, could upregulate cytokine expression in TIGIT^{+} CD4^{+}CD25^{-} T cells and TIGIT^{+} CD8^{+} T cells. Antagonistic anti-TIGIT, anti-PD-1, and anti-Tim-3 antibodies or their respective isotype control antibodies were added to TIGIT^{+} CD4^{+}CD25^{-} T cells and TIGIT^{+} CD8^{+} T cells. The cytokine expression was then evaluated. In healthy subjects, inhibition of TIGIT alone could significantly upregulate the expression of IFN-γ and TNF-α in TIGIT^{+} CD4^{+}CD25^{-} T cells (Fig. 6A), and significantly upregulate the expression of IL-2 and IFN-γ in TIGIT^{+} CD8^{+} T cells (Fig. 6B). Inhibition of TIGIT, PD-1, and Tim-3 altogether, on the other hand, could significantly upregulate all cytokines examined. The extent of the upregulation was significantly higher after TIGIT, PD-1, and Tim-3 triple inhibition than after TIGIT single inhibition. In ALL patients, we also observed that the inhibition of TIGIT, PD-1, and Tim-3 altogether significantly elevated the expression of IL-2, IFN-γ, and TNF-α in both TIGIT^{+} CD4^{+}CD25^{-} T cells and TIGIT^{+} CD8^{+} T cells (Fig. 6C and D). Blocking TIGIT alone, on the other hand, was only effective at upregulating TNF-α in TIGIT^{+} CD4^{+}CD25^{-} T cells (Fig. 6C), and at upregulating IFN-γ and TNF-α in TIGIT^{+} CD8^{+} T cells (Fig. 6D).

4. Discussion

TIGIT has crucial regulatory roles in the suppression of autoimmune responses, but may also inhibit antiviral and antitumor immunity [4,13,18,29,30]. Mechanistically, the TIGIT molecule contains an ITIM motif in the cytoplasmic region that propagates inhibitory signaling events. TIGIT can also promote the expression of IL-10 and suppress the expression of IL-12 by antigen-presenting macrophages and dendritic cells, thus helping establish a suppressive tumor microenvironment. Given the recent success of anti-PD-1 therapies in treating cancer, the possibility of targeting TIGIT in cancer patients should be investigated.

In this study, we analyzed the status of TIGIT expression in circulating T cells from patients with adult B-cell ALL. Though TIGIT was mostly found on CD4^{+}CD25^{+} T cells, a small fraction of CD4^{+}CD25^{-} non-Treg T cells and CD8^{+} T cells also expressed TIGIT, and compared to the corresponding cell types in healthy controls, the CD4^{+}CD25^{-} T cells and CD8^{+} T cells in adult ALL patients presented small but significantly higher levels of TIGIT expression, which could be further upregulated upon prolonged anti-CD3/CD28 stimulation. And over the course of stimulation, the CD4^{+}CD25^{-} T cells and CD8^{+} T cells from adult ALL patients consistently produce higher TIGIT mRNA and protein than those cells from healthy controls. We later found that ALL-associated antigens, such as protein lysate from a B lineage ALL cell line and WT1 peptide, could significantly the expression of TIGIT in ALL patients but not in healthy controls. Therefore, it is possible that TIGIT is upregulated due to antigen-specific stimulation in ALL patients. In both healthy controls and adult ALL patients, the levels of PD-1 and Tim-3 expression were much higher in TIGIT^{+} cells than in TIGIT^{-} cells. At the same time, the IL-2, IFN-γ, and TNF-α expression were much lower in TIGIT^{+} cells than in TIGIT^{-} cells. The high inhibitory molecule and low cytokine expression signature was especially pronounced in the TIGIT^{+} CD4^{+}CD25^{-} T cells and TIGIT^{+} CD8^{+} T cells from adult ALL patients. Blocking TIGIT could minimally increase the expression of some, but not all, cytokines in TIGIT^{+} CD4^{+}CD25^{-} T cells and CD8^{+} T cells. However, blocking TIGIT, PD-1, and Tim-3 altogether was significantly more effective. Together, these data demonstrated that adult ALL patients not only presented higher levels of TIGIT-expressing non-Treg T cells, but even within the TIGIT^{+} fraction, those from adult ALL patients appeared more dysfunctional than those from healthy controls. Blocking TIGIT could increase the cytokine expression by TIGIT^{+} cells independent of PD-1 and Tim-3 blocking, but the effect was minimal and was significantly less effective than the combined blocking of TIGIT, PD-1, and Tim-3.

Some of the above results were similar to previous investigations, for example, in adult AML patients, Kong et al. also demonstrated an upregulation of TIGIT in CD8^{+} tumor-infiltrating lymphocytes (TILs) [23]. However, the frequencies of TIGIT^{+} CD8^{+} T cells in their results were about 20% to 90%, with a mean value of about 50%, while in our results, the frequencies were around 10% to 40%. The differences in age, which was between 22 and 41 years of age (median 34) in our study and 60 ± 16 (mean ± SD) years with a range of 23–76 years in the study by Kong et al., might have contributed to a difference in TIGIT expression. In addition, Kong et al. found that TIGIT expression was positively association with PD-1 expression but was not associated with Tim-3 expression, while our study found that TIGIT expression was associated with both PD-1 and Tim-3. Further investigations are required to address these issues.

A major limitation of this study is the lack of association between TIGIT expression and the clinical features of the patients. In our investigation, we only collected one type of sample, the peripheral blood of patients who were newly diagnosed and untreated. In future investigations, samples from more patients at various stages of the disease should be collected to assess the association between TIGIT expression and various clinical features of the patients, including response to treatment, remission status and risk of relapse. Another issue is that TIGIT is a pleiotropic molecule with a variety of identified functions, many of which were not examined in this study or were unfeasible to investigate in an *in vitro* system. For example, it has been shown that

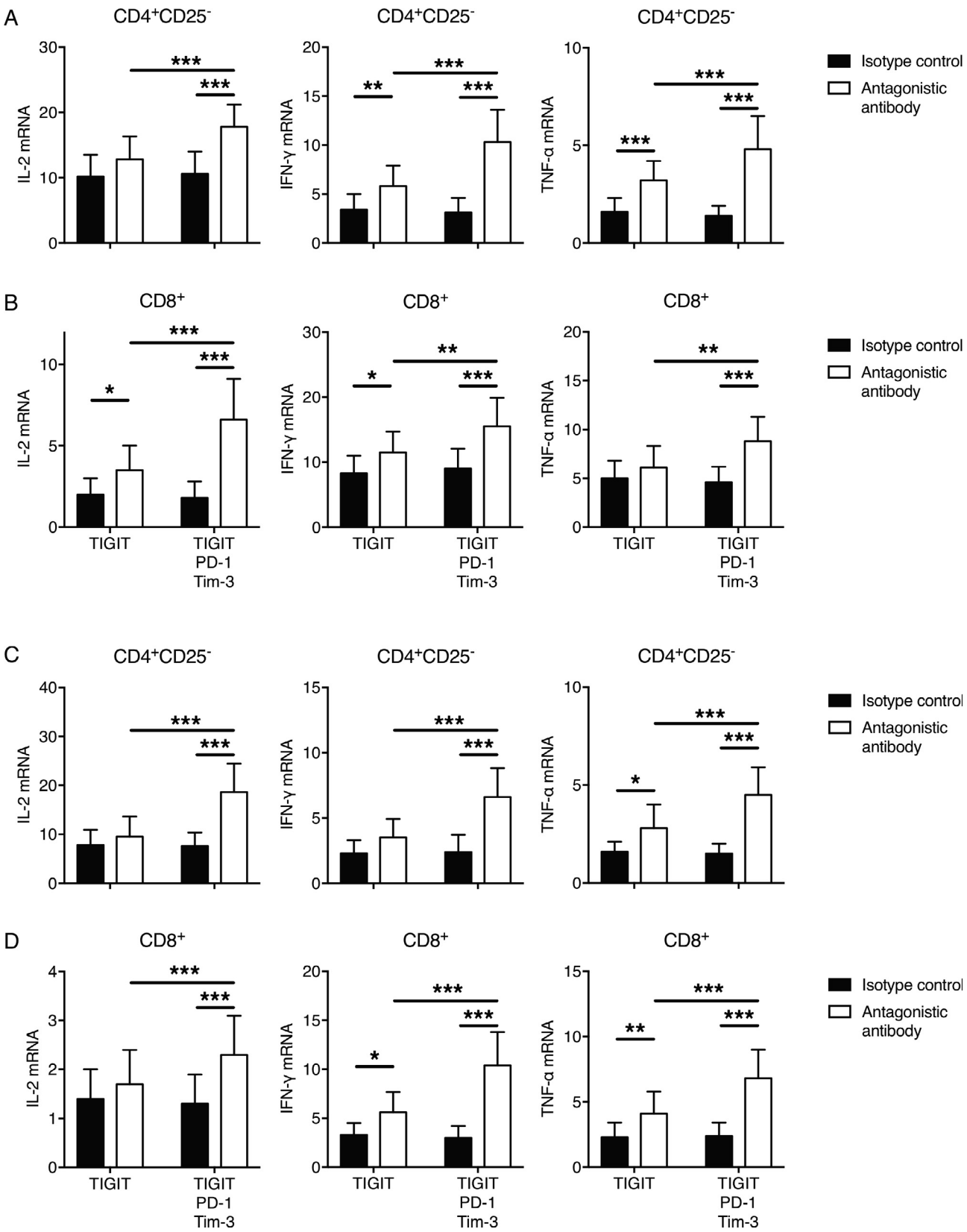

Fig. 6. Cytokine expression after TIGIT, PD-1, and Tim-3 blocking. Antagonistic anti-TIGIT antibody alone, or in combination with anagonistic anti-PD-1 and anti-Tim-3 antibodies, were added to $TIGIT^+$ $CD4^+CD25^-$ T cells and $TIGIT^+$ $CD8^+$ T cells. The cells were then stimulated via anti-CD3/CD28 and lysed for mRNA analysis. (A) The expression of IL-2, IFN-γ, and TGF-α in $CD4^+CD25^-$ T cells from 15 healthy subjects (B) The expression of IL-2, IFN-γ, and TGF-α in $CD8^+$ T cells from 15 healthy subjects. (C) The expression of IL-2, IFN-γ, and TGF-α in $CD4^+CD25^-$ T cells from 15 subjects (D) The expression of IL-2, IFN-γ, and TGF-α in $CD8^+$ T cells from 15 healthy subjects were examined. Two-way ANOVA followed by Sidak's multiple comparison test. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$.

blocking either PD-1 or TIGIT could increase the frequency of IFN-γ-expressing $CD8^+$ T cells in the lymph node but not in the tumor, while blocking both molecules could increase the frequency of IFN-γ-expressing $CD8^+$ T cells in the tumor [31]. Whether these molecules, PD-1 and TIGIT, collaborated on $CD8^+$ T cell trafficking requires further studies. How TIGIT might regulate the expression of chemokine receptors is also not examined. Interestingly, Chew et al. in HIV infected individuals demonstrated that although $TIGIT^+$ $CD8^+$ T cells presented lower IFN-γ, IL-2, and TNF-α expression than $TIGIT^-$ $CD8^+$ T cells, the expression of perforin and granzyme B were significantly higher in $TIGIT^+$ $CD8^+$ T cells than in $TIGIT^-$ $CD8^+$ T cells [18]. Whether this is also true for adult ALL patients and how would TIGIT blocking affect the cytotoxic function of $CD8^+$ T cells are yet unknown. In addition, TIGIT was shown to change macrophage polarization and skew the cytokine balance in the tumor microenvironment toward a more suppressive type [13,32]. How these properties of TIGIT might affect the progression of ALL is still unclear and requires further studies.

Declaration of Competing Interest

The authors declare no conflict of interest.

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