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Original Article

Co-inhibition of TIGIT, PD1, and Tim3 reverses dysfunction of Wilms tumor protein-1 (WT1)-specific CD8+ T lymphocytes after dendritic cell vaccination in gastric cancer

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Abstract: Dendritic cell (DC) vaccines have been shown to stimulate tumor antigen-specific CD8+ T cells; however, this strategy has demonstrated variable clinical efficacy likely due to immune escape mechanisms that can induce tumor-specific CD8+ T cell dysfunction. Herein, we evaluated the functional characteristics of DC vaccine-induced CD8+ T cells with regard to immune checkpoint inhibitors in gastric cancer patients who were administered Wilms tumor protein-1 (WT1)-targeted DC vaccine. We observed the upregulation of the inhibitory molecule, TIGIT and the inhibitory T cell co-receptors PD1 and Tim3 in limiting WT1-specific CD8+ T cell growth and function in GC patients. TIGIT-expressing PD1+Tim3- CD8+ T cells were the largest subset, while TIGIT+PD1+Tim3+ was the most dysfunctional subset of WT1-specific CD8+ T cells in gastric cancer patients. Importantly, the co-inhibition of TIGIT, PD1, and Tim3 pathways enhanced the growth, proliferation, and cytokine production of WT1-specific CD8+ T cells. In conclusion, our data suggests that targeting TIGIT, PD1, and Tim3 pathways may be important in reversing immune escape in patients with advanced gastric cancer.

Keywords: TIGIT, PD1, Tim3, gastric cancer, Wilms tumor antigen, DC vaccination

Introduction

Gastric cancer (GC) is the fourth most common cancer and the second leading cause of cancer-related deaths worldwide [1]. Due to confounding gastrointestinal symptoms, most patients often have advanced disease at the time of diagnosis, and median survival time for patients with GC is less than 1 year [2]. Surgical resection remains the first-line therapy option for GC, with few alternatives available for targeted immunotherapies. Therefore, there is a need to understand the mechanisms that promote progression of GC in order to develop treatment strategies that may be more efficacious.

Tumor antigens are often recognized as self-antigens by T cells, thus inducing weak immune responses and resulting in tolerance. However, while tumor-specific cytotoxic CD8+ T lymphocytes (CTLs) can recognize tumor antigens, CTLs may become unresponsive to the tumor, and thus fail to impede tumor growth [3]. Recent reports suggest that CTLs may become dysfunctional and exhibit characteristics of anergy, including lack of proliferation and reduced secretion of effector cytokines [4, 5]. These factors contribute to the immunosuppressive tumor microenvironment (TME), and may be enhanced by activation of inhibitory checkpoint signaling pathways. Indeed, recent therapies have focused on targeting inhibitory

pathways, such as PD1 and PD-L1, to promote anti-tumor CTL responses both *in vitro* and *in vivo* [6-11]. Inhibition of immune checkpoint using anti-PD1 antibodies has been efficacious, with improved outcomes seen in patients with advanced PD-L1-positive GC. Other promising strategies have targeted the co-inhibitory receptors, CTLA-4 and PD1 [11]. While the exact mechanisms underlying blockade of inhibitory checkpoint signaling pathways are still unclear, recent evidence has shown that anti-PD1 therapy response is improved by the presence of CTLs within the TME. As various factors collectively contribute to the immunosuppressive TME, it is reasonable to postulate that targeting multiple inhibitory pathways within the TME may be useful for patients with advanced malignancies, including GC.

TIGIT (T cell immunoreceptor with Ig- and ITIM domains) is an inhibitory receptor expressed by activated T cells, regulatory T cells (Tregs), and natural killer (NK) cells. TIGIT is ligated to the adhesion molecules CD155 (Necl-5) and CD112 (nectin-2), which are important for T cell and NK cell-mediated cytotoxicity against tumors [12-17]. CD155 and CD112 also bind to other ligands including CD226 (DNAM-1), the costimulatory partner to TIGIT, and interacts with LFA-1 to positively regulate T cell responses [18, 19]. Moreover, CD155 and CD112 are important in anti-tumor T cell and NK cell-mediated cytotoxicity [15, 16]. CD155 is expressed in epithelial cells, endothelial cells, platelets, dendritic cells (DCs), activated T cells [12, 16, 20, 21], and in various tumors, including GC [22]. Furthermore, a previous report indicated that TIGIT exerted its immunosuppressive effects by promoting IL-10 production in DCs through CD155, thus inhibiting CD4+ T cell proliferation and function [12]. Nevertheless, other studies have demonstrated that TIGIT constrains T cell functions directly by competing with CD226 [17, 23]. Recently, it has been reported high expression of TIGIT by tumor-infiltrating lymphocytes (TILs) in non-small cell lung cancer (NSCLC) and colon cancer, which was correlated with PD1 expression, and dysfunctional tumor antigen-specific CTLs [24]. Another T cell inhibitory receptor that has received much attention is Tim3 (T cell immunoglobulin and mucin-domain containing-3). The expression of Tim3 has been observed in dysfunctional CTLs in both solid tumors and hematological cancers [25, 26]. Interestingly, these studies found that Tim3+CD8+ T cells also expressed PD1, and these Tim3+PD1+ CTLs exhibited greater dysfunction in their effector function as compared to PD1+ CTLs, suggesting a synergistic relationship between these co-inhibitory receptors in cancer.

A recent promising immunotherapy modality is DC-based vaccines pulsed with tumor antigens [27]. Wilms' tumor protein-1 (WT1) is an oncogenic tumor antigen, which has been used in DC-based vaccines for hematological and solid tumors [28]. Studies have demonstrated that this DC-based vaccination can induce WT1-specific CTLs and contribute to tumor regression in the clinical setting [29]. The goal of DC-based vaccines is to produce tumor-specific CTL response [30]; therefore, the generation of sustained memory CTL responses is of utmost importance.

In the present study, we aimed to evaluate the functional impact of WT1-targeted DC vaccination on the expression of TIGIT, PD1 and Tim3. Patients with advanced GC were immunized with DCs pulsed with WT1 peptides and the utility of TIGIT+PD1+Tim3 blockade on anti-tumor response was evaluated.

Materials and methods

Patients and ethics statement

Ten HLA-A2*2402-restricted patients with stage III/IV gastric cancer were enrolled in this Phase I study. All patients provided written informed consent prior to study initiation. This study was conducted in accordance with guidelines established by the Declaration of Helsinki. The Institute Ethical Committee of the Affiliated Hospital of Capital Medical University approved the study protocol.

Study protocol

Peripheral blood mononuclear cells (PBMCs) were freshly isolated from each patient using Ficoll density gradient centrifugation. Monocytes were incubated for five days in AIM-V medium (Gibco) containing GM-SCF (granulocyte macrophage colony-stimulating factor; 100 ng/mL) and IL-4 (50 ng/mL) to generate immature DCs. Next, DCs were collected from

Table 1. Summary of patient characteristics, clinical outcomes and immune responses of DCs-based immunotherapy

Patient No.	Age (yrs) Sex	Clinical stage	Clinical outcomes		Immunological responses	Overall survival (mos)
			Recist	Clinical effect		
1	68, M	III	SD	Yes	+	10.5
2	65, M	IV	SD	Yes	+	13.2
3	47, M	IV	SD	Yes	+	20.6
4	57, F	III	SD	Yes	+	19.2
5	75, M	IV	PD	Yes	+	12.1
6	59, M	III	PD	No	-	7.3
7	49, M	IV	PD	Yes	+	10.1
8	52, M	IV	SD	Yes	+	23.1
9	67, M	III	SD	Yes	+	21.3
10	56, F	IV	PD	No	-	9.6

monocytes isolated using CD14+ cell magnetic selection kit (Miltenyi Biotech) and maturation of monocyte-derived DCs was achieved by incubation with 10 ng/mL TNF-α for 24 hours.

For each vaccination course, DCs were pulsed with MHC-I-restricted WT1 epitopes, depending on their HLA status. Briefly, mature DCs were incubated with WT1 peptides restricted to HLA-A*0204 (a modified-type, 9-mer WT1 peptide residues 235-243: CYTWNQMNL; NeoMPS Inc.) for 30 minutes and washed with saline. Approximately 1×10^7 DCs were injected into the axillary region of each patient (intradermal) with OK-432, a streptococcal preparation, at 2-week intervals for at least 6 sessions (one course) before clinical and immunological evaluation. Additional vaccinations were administered if a positive treatment response or no adverse effect was observed after one vaccination course.

Phenotype analysis of WT1-specific CTLs

PBMCs were isolated from each patient and CD8+ T lymphocytes were purified using MACS Column Technology (Miltenyi Biotec). CTLs were subsequently incubated with APC-labeled HLA-A*2402/WT1 235-243 tetramers. HIVenv/HLA-A24 peptides and matched isotype IgG were used as negative controls. CTLs were stained with the following antibodies: CD8-FITC, TIGIT-biotin or IgG2a-biotin or PD1-PE or Tim3-PE or IgG2a-PE (BD Pharmingen) and streptavidin-ECD (Invitrogen) conjugated antibodies. Cell viability was evaluated using a LIVE/DEAD violet amine reactive dye.

Functional analysis of WT1-specific CTLs

Functional analysis of WT1-specific CTLs was performed via cytokine production assays as previously reported [8]. Briefly, T cells were purified from isolated PBMCs, and CD4+ and CD8+ T cells were separately incubated with an equal number of non-CD3 autologous cells pulsed with 10 μg/mL WT1 peptide prior to cell surface staining.

For *in vitro* stimulation assays, PBMCs were incubated for 6 days in culture medium containing IL-2 (50 IU/mL) and WT1 peptide (10 μg/mL). Cells were exposed to the following three blocking monoclonal antibodies (either individually, combination of any two, or combination of all three): 10 μg/mL anti-TIGIT (BPS Bioscience, CA, USA), anti-PD1 (clone EH12.2H7; BioLegend), and anti-Tim3 (clone 2E2; Merck-Millipore). Isotype antibodies were used as control. After the 6-day incubation, cells were restimulated for 6 hours with WT1 peptide (10 μg/mL) followed by incubation with Brefeldin A (10 μg/mL, Sigma-Aldrich) to enhance intracellular cytokine staining. Cells were subsequently incubated with CD8-PE, CD4-PE-Cy7, CD14-ECD, CD19-ECD, CD56-biotin, streptavidin-ECD and intracellularly stained with IFN-γ-FITC, IL-2-APC and TNFα-Alexa 700 (BD Pharmingen) antibodies. Cell viability was evaluated using a LIVE/DEAD violet amine reactive dye. Cells were analyzed via flow cytometry using an LSR II (BD Biosciences) and data were analyzed using FlowJo v8.8.7 (Tree Star, Inc.).

For *in vitro* proliferation assays, CFSE-labeled PBMCs were treated with IL-2 and WT1 peptides, and incubated for 6 days with the blocking antibodies as described above. After the 6-day incubation, cells were incubated with APC-labeled HLA-A2/WT1 235-243 tetramers, CD8-PE, CD4-PE-Cy7, CD14-ECD, CD19-ECD, CD56-biotin, streptavidin-ECD conjugated antibodies and reagents. Cells were analyzed via flow cytometry using an LSRII (BD Biosciences) and data were analyzed using FlowJo v8.8.7 (Tree Star, Inc.).

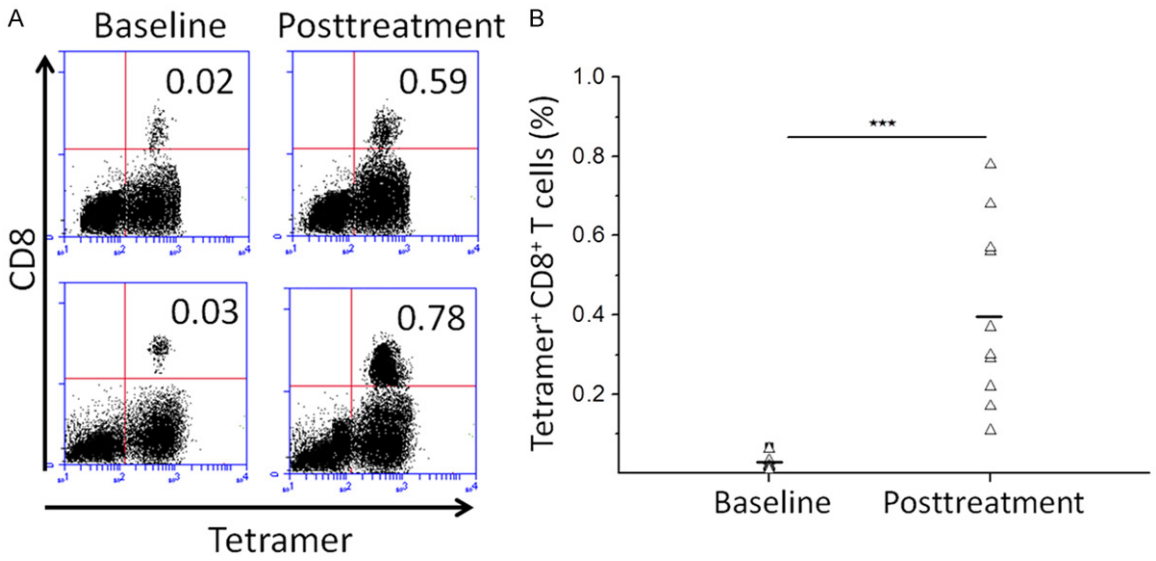

Figure 1. Expression of WT1-specific CD8+ T lymphocytes following DC vaccination. A. The expression of WT1-specific CD8+ T lymphocytes of selected GC patients before and after DC vaccination using a tetramer assay. Data shown are representative of two independent experiments performed in duplicate. B. Data from the 10 patients with advanced GC showing the proportion of WT1-specific CD8+ T lymphocytes expressed as a percentage of CD8+ cells. The black horizontal bar shows the median. Each data-point represents the proportion of WT1-specific CTLs for each patient. Differences between values before and after vaccination achieved statistical significance (***$P < 0.001$).

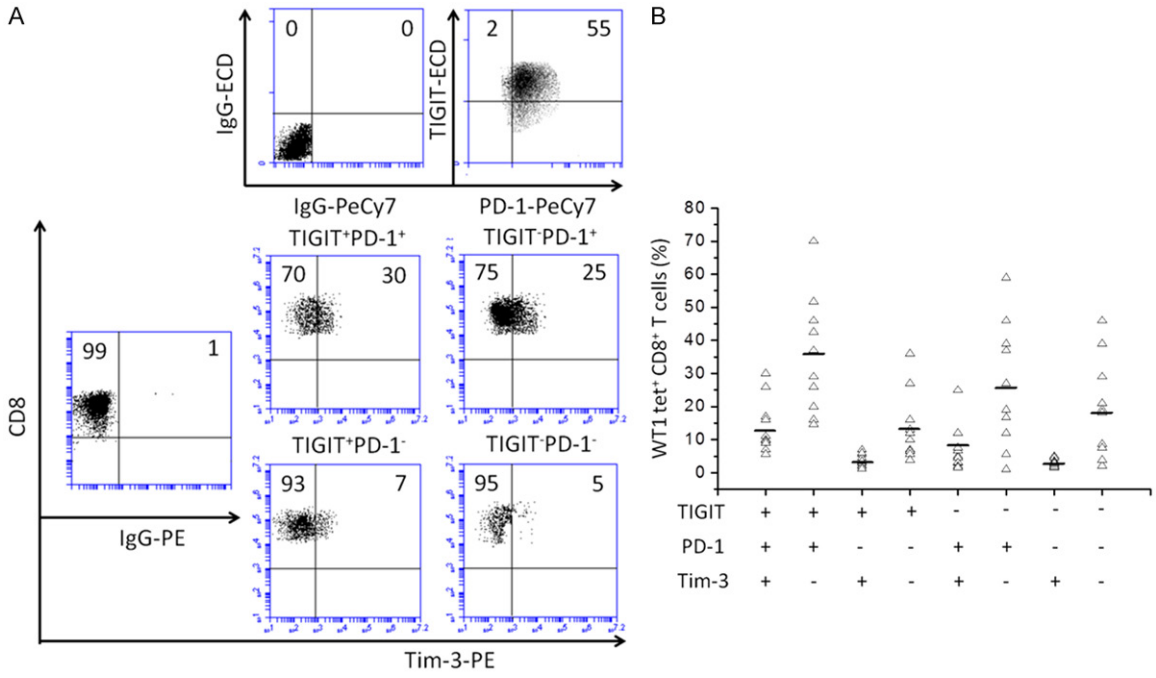

Figure 2. TIGIT is upregulated and co-expressed with PD1 and Tim3 on WT1-specific CD8+ T cells following DC vaccination. A. Dot plots from one representative gastric cancer patient showing TIGIT, PD1, and Tim3 expression on WT1-specific CD8+ T cells. Values indicate the proportion of CD8+ T cells expressing PD1 and/or TIGIT among tet+ CD8+ T cells and expressing Tim3 within different subsets of tet+ CD8+ T cells defined by TIGIT and PD1 expression. B. Pooled data from 10 gastric cancer patients showing the distribution of WT1-specific and total CD8+ T cells based on TIGIT, PD1, and Tim3 expression. Horizontal bars depict the mean percentage of TIGIT and/or PD1 and/or Tim3 expression on tet+ CD8+ T cells. Each data-point represents the proportion of WT1-specific CTLs for each patient. Data shown are representative of two independent experiments performed in duplicate.

Statistical analysis

T cell responses to the DC vaccines were evaluated with tetramer analysis (Medical & Biological Laboratories Co., Ltd.) or WT1-specific cytokine-producing T cells. Wilcoxon signed rank test was used to evaluate the significance of T cell responses and paired baseline vs. post-treatment results from the same patient. Tests were two-sided and $P < 0.05$ was considered statistically significant. Data were analyzed using SigmaStat 3.5 software (Systat Software, Inc.).

Results

Baseline clinical characteristics

The baseline clinical characteristics of the 10 patients with advanced (stage III/IV) GC are presented in **Table 1**. Of the 10 patients, 2 were females and the mean age was 59.5 years old. Majority (6 patients) had stage IV at the time of diagnosis, and the average time since diagnosis was 1.6 months.

WT1-specific CD8+ T lymphocytes are detectable after DC vaccination in gastric cancer patients

We examined the *ex vivo* frequency of WT1-specific CD8+ T lymphocytes with WT1 peptide/HLA-A*2402 tetramers isolated from PBMCs collected from the 10 patients with advanced GC. The assays were performed prior to the first DC vaccination course and after completion of vaccination course. The expression of WT1-specific CTLs significantly increased following DC vaccination (**Figure 1**). Previous studies demonstrated that upregulated co-inhibitory molecules expression in tumor antigen-specific CTLs was positively correlated with the expression of activation markers, and were further enhanced upon TCR activation with tumor antigen *ex vivo* [7, 8]. Moreover, the co-expression of PD1 and Tim3 has been reported to play a critical role in CTL dysfunction and regulating the proliferation and expansion of tumor antigen-specific CD8+ T cells [25, 26]. To investigate whether vaccination with DCs pulsed with WT1 peptides influenced the expression of inhibitory receptors, we assessed the expression of TIGIT in combination with PD1 and Tim3 by vaccination-induced WT1-specific CTLs before and after vaccine therapy in all patients (**Figure 2**). We observed that the mean proportion of upregulation of TIGIT in WT1-specific CTLs was significantly higher than total CD8+ T cell subsets and majority of TIGIT+WT1-specific CTLs co-upregulated PD1 expression. We further found that TIGIT+PD1+Tim3- (35.3% ± 10.2%) represented the largest WT1-specific CD8+ T cell subset as compared to TIGIT+PD1+Tim3+ (14.1 ± 5.6%), TIGIT+PD1-Tim3- (13.7% ± 3.1%), TIGIT-PD1+Tim3- (26.3% ± 13.2%) and TIGIT-PD1-Tim3- (19.4% ± 9.6%) CTL subsets (**Figure 2**).

Impaired cytokine production by TIGIT+PD1+Tim3+WT1-specific CTLs following DC vaccination

TIGIT+PD1+Tim3- and TIGIT+PD1+Tim3+WT1-specific CTLs produced significantly less IFN-γ than TIGIT-PD1+Tim3- ($P = 0.02$) (**Figure 3A**). In addition, TIGIT+PD1+Tim3+WT1-specific CTLs produced significantly less IFN-γ than TIGIT+PD1+Tim3- CTLs ($P = 0.04$). We also observed that TIGIT+PD1+Tim3+WT1-specific CTLs produced significantly less TNF and IL-2 than TIGIT+PD1+Tim3- (both $P = 0.02$). Interestingly, TIGIT+PD1+Tim3+ CTLs, but not TIGIT+PD1+Tim3-WT1-specific CTLs, produced significantly less IL-2 than TIGIT-PD1-Tim3- ($P = 0.004$) (**Figure 3**). However, we did not observe any significant differences in cytokine secretion between TIGIT-PD1-Tim3- and TIGIT-PD1+Tim3-WT1-specific CTLs.

TIGIT inhibition increases the proportions of cytokine-producing WT1-specific CD8+ T cells and synergizes with PD1 and Tim3 inhibition

We next investigated the effect of TIGIT pathway inhibition alone or combined with PD1 and/or Tim3 pathway inhibition in the proportions of WT1-specific CTLs that produced effector cytokines in response to WT1 peptide and anti-TIGIT mAbs, as compared to IgG control antibodies. The frequencies of IFN-γ, TNF-α and IL-2 secretion by WT1-specific CTLs was enhanced in the presence of anti-TIGIT and anti-PD1 mAbs when compared to either anti-TIGIT mAbs alone, anti-PD1 mAbs alone, and IgG control antibodies, resulting in a 2.0-fold, 2.2-fold and 2.7-fold change in the proportions of IFN-γ, TNF-α-, and IL-2-producing WT1-specific CTLs, respectively (**Figure 4**). No synergistic effect was observed in the presence of

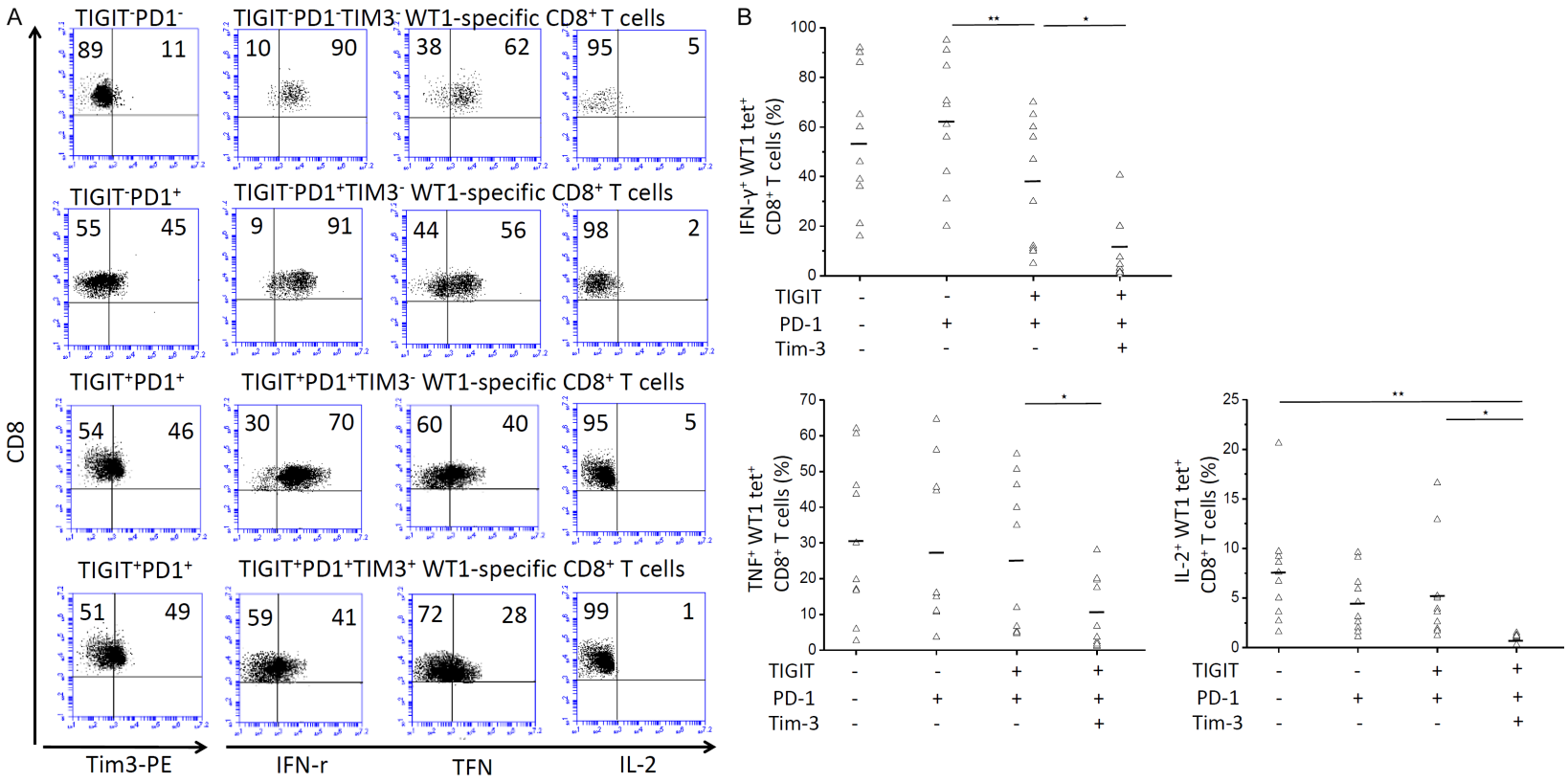

Figure 3. Impaired effector function of WT1-specific CTLs following DC vaccination. A. Dot plots from one representative gastric cancer patients showing the proportions of cytokine-producing WT1-specific CD8+ T cells based on TIGIT, PD1 and Tim3 expression. B. Pooled data from ten gastric cancer patients showing the proportion of cytokine-producing WT1-specific CTLs based on TIGIT, PD1 and Tim3 expression. Values indicate the percentages of cytokine-producing CTLs among Tim3+ and/or Tim3- fractions of TIGIT-PD1-, TIGIT-PD1+ and TIGIT+PD1+ WT1-specific CTLs. The *P* values were obtained by the Wilcoxon signed rank test. **P* < 0.05; ***P* < 0.01. Each data-point represents the proportion of WT1-specific CTLs for each patient. Data shown are representative of two independent experiments performed in duplicate.

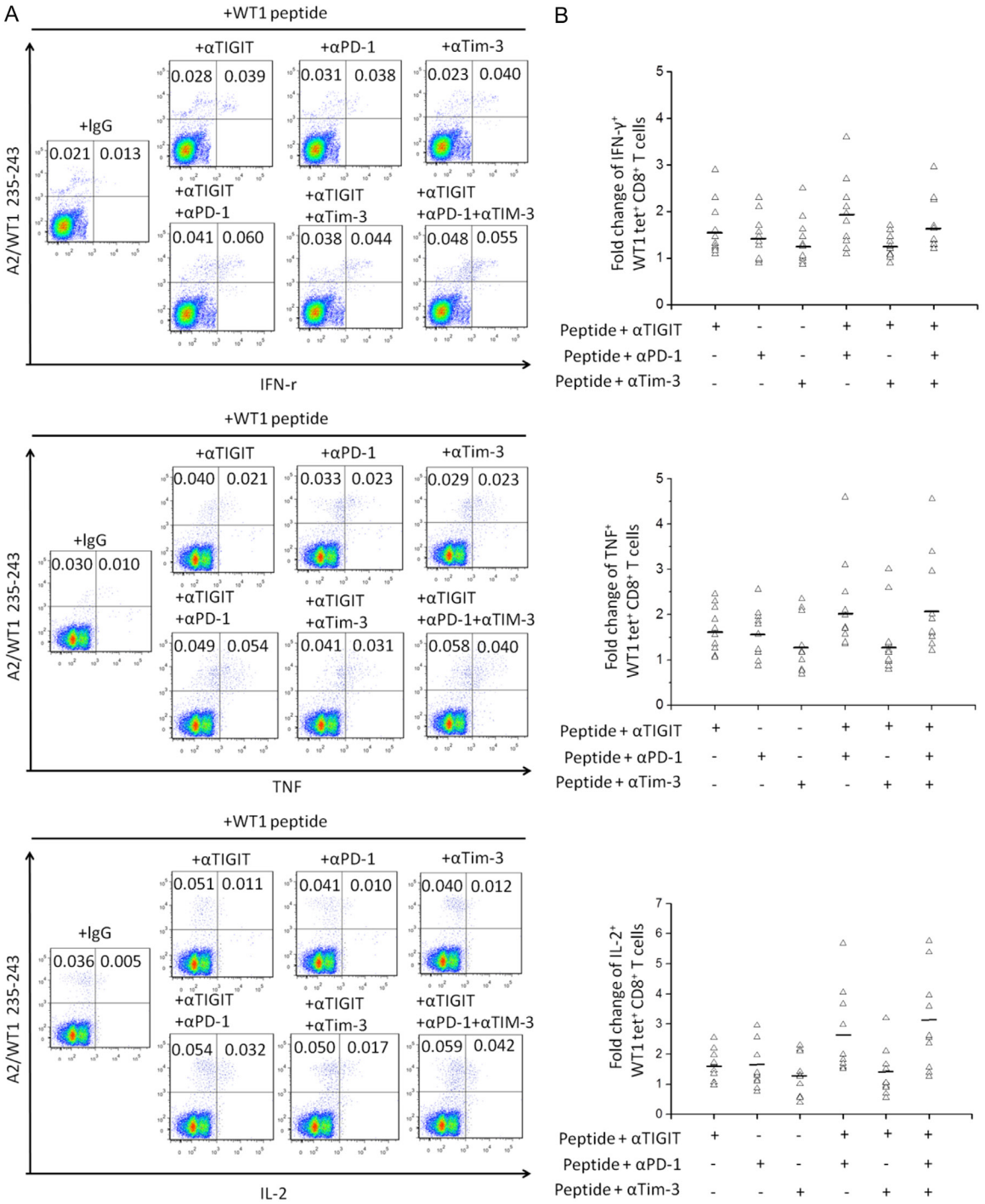

Figure 4. TIGIT inhibition synergizes with PD1 and Tim3 inhibition to promote the frequencies of cytokine-producing WT1-specific CD8+ T cells. A. Dot plots from one representative gastric cancer patient showing the proportions of IFN-γ-, TNF- and IL-2-producing A2/WT1 235-243 tet+ CD8+ T cells among total CD8+ T cells. PBMCs from gastric cancer patients were incubated for 6 days with WT1 235-243 peptide and blocking mAbs against TIGIT (αTIGIT) and/or PD1 (αPD1) and/or Tim3 (αTim3) or isotype control mAbs (IgG), prior to evaluating intracellular cytokine production of A2/WT1 235-243 tet+ CD8+ T cells in response to the cognate peptide. B. Fold changes of the frequencies of IFN-γ-, TNF- and IL-2-producing A2/WT1 235-243 tet+ CD8+ T cells after a 6-day *in vitro* stimulation with cognate peptide and the indicated mAb (n = 10). Each data-point represents the proportion of WT1-specific CTLs for each patient. Data shown are representative of two independent experiments performed in duplicate.

both anti-TIGIT and anti-Tim3 mAbs. The triple blockade further amplified the proportions of WT1-specific CTLs that secreted IL-2, but not IFN-γ and TNF-α, resulting in a 3.0-fold, 1.7-fold, and 2.2-fold change in the proportions of IL-2-, IFN-γ-, and TNF-α-producing WT1-specific CD8+ T cells, respectively, as compared to all other experimental conditions including TIGIT/PD1 blockade. Notably, the effects of TIGIT, PD1, and Tim3 inhibitions were observed only upon exposure to WT1 peptide, but not in response to an irrelevant peptide (data not shown).

TIGIT inhibition promoted the proportions of proliferating and total WT1-specific CD8+ T cells and synergizes with PD1 and Tim3 inhibitions

Finally, we assessed the effects of TIGIT, PD1, and Tim3 pathway inhibition on the growth of vaccine-induced WT1-specific CTLs *in vitro*. CFSE-labeled PBMCs isolated from eight GC patients after DC immunization were incubated for 6 days with WT1235-243 peptide in the presence of blocking mAbs against TIGIT and/or PD1 and/or Tim3 or IgG control antibodies. After 6 days, cells were restimulated (6 hours) with WT1 or HIV peptide, before evaluating the proportions of proliferating ($CSFE^{lo}$) and total A2/WT1235-243 tet+ CTLs. We observed that TIGIT inhibition promoted the proportions of $CFSE^{lo}$ and total A2/WT1235-243 tet+ CTLs as compared to stimulation with IgG control antibodies, resulting in a 1.7-fold and 1.6-fold change in the proportions of $CFSE^{lo}$ and total A2/WT1235-243 tet+ CTLs, respectively (**Figure 5**). In contrast to TIGIT blockade alone, TIGIT and PD1 inhibition further augmented the proportions of $CFSE^{lo}$ and total A2/WT1235-243 tet+ CTLs as compared to TIGIT blockade alone or PD1 blockade alone, resulting in a 2.7-fold and 1.7-fold change in the proportions of $CFSE^{lo}$ and total A2/WT1235-243 tet+ CD8+ T cells, respectively. In addition, the triple TIGIT/PD1/Tim3 inhibitions further promoted the proportions of $CFSE^{lo}$ and total A2/WT1235-243 tet+ CD8+ T cells as compared to TIGIT/PD1 or TIGIT/Tim3 blockade. This data suggested that there was a synergistic effect of TIGIT, PD1 and Tim3 blockades on DC vaccination-induced WT1-specific CTLs expansion, resulting in the greatest rise in the proportions of $CFSE^{lo}$ and total A2/WT1235-243 tet+ CTLs (3.0-fold and 2.0-fold respectively) as compared to other groups (**Figure 5**). No noteworthy proliferation of WT1-specific CTLs was observed upon exposure to an irrelevant peptide with or without inhibition (data not shown).

Side effects and clinical outcome

Of the 10 patients, 3 received one round of DC vaccination, while 7 received more than one course. All treatment-emergent adverse events (TEAEs) are shown in **Table 2**. We observed no Grade 3 or higher toxicity. Neither complete response nor partial response was observed. Moreover, patients with stable disease had better survival outcomes as compared to patients with advanced disease ($P < 0.05$, **Table 1**).

Discussion

In the present study, we report a novel subset of dysfunctional WT1-specific CTLs in stage III/IV gastric cancer patients. We found that the majority of WT1-specific CTLs in PBMCs from patients who received DC vaccination-based immunotherapy targeting WT1 co-expressed TIGIT, PD1, and Tim3; moreover, TIGIT+PD1+Tim3+WT1-specific CTLs were the most dysfunctional subset among the circulating WT1-specific CTLs. Our findings are consistent with the recent report that found higher TIGIT expression by CD8+ TILs in human solid tumors, including gastric, lung, breast, colon, uterine, melanoma and renal cancers, and TIGIT was frequently co-expressed with PD1 in CD8+ TILs in human NSCLC and colon cancer samples [24].

Furthermore, we demonstrated that TIGIT+PD1+Tim3+ and TIGIT+PD1+Tim3-WT1-specific CTLs represented two distinct dysfunctional subsets of tumor antigen-specific CTLs primed by DC vaccination, suggesting that there is a graded loss in T cell function in response to DC vaccination in patients with advanced gastric cancer. These findings increase our understanding of molecular mechanisms that drive T cell exhaustion in gastric cancer patients and provide potential targets to revert T cell anergy in cancer [31, 32].

Substantial evidence suggests that factors mediating TIGIT upregulation in response to tumor antigen-specific dysfunctional T cells may differ from factors that regulate PD1 and

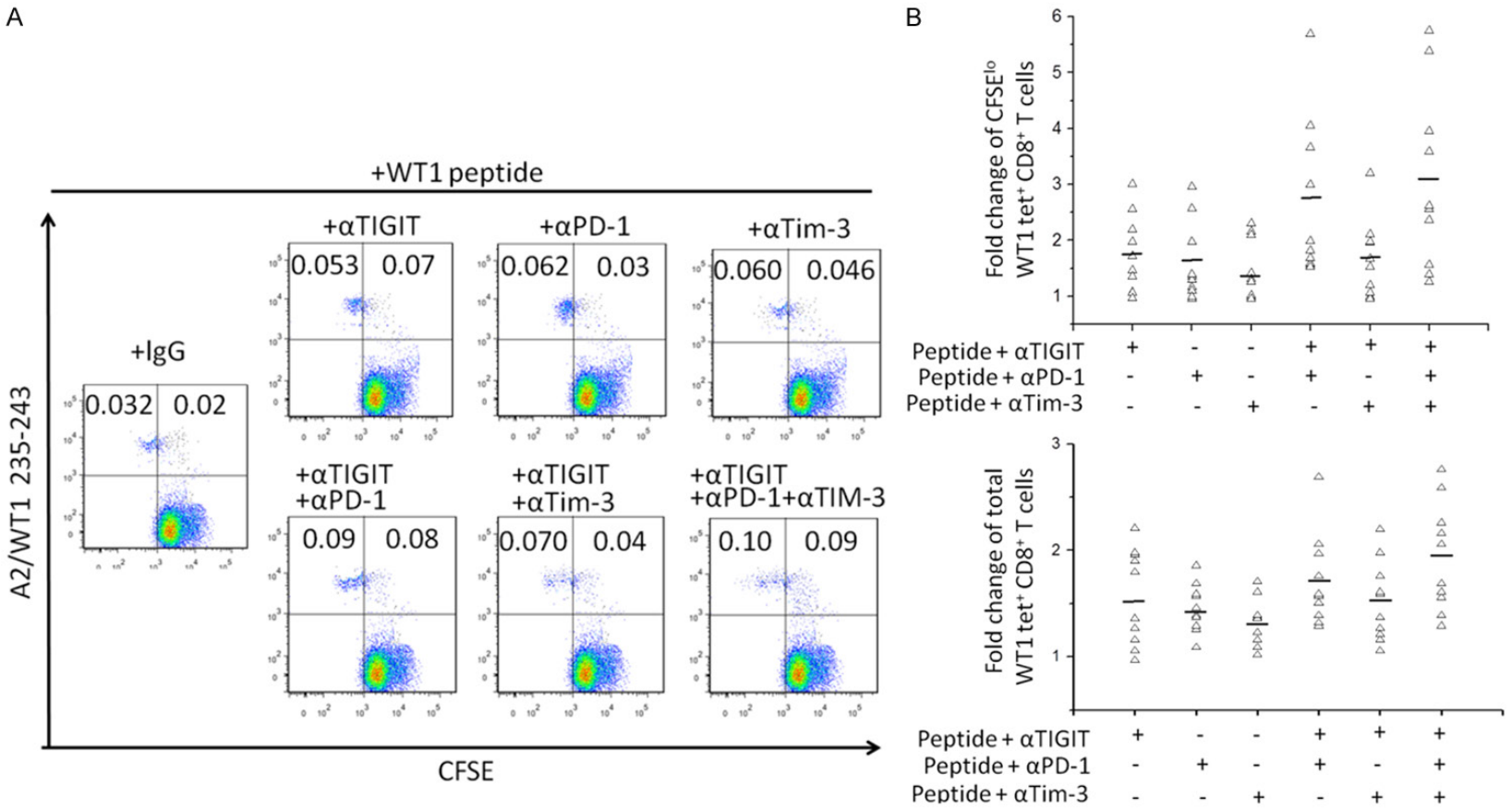

Figure 5. TIGIT inhibition synergizes with PD1 and Tim3 blockade to increase the proportions of proliferating and total WT1-specific CD8+ T cells. A. Dot plots from one representative gastric cancer patients showing the percentages of $CFSE^{lo}$ A2/WT1 235-243 tet+ CTLs among total CD8+ T cells. CFSE-labeled PBMCs from gastric cancer patients were incubated for 6 days with WT1 235-243 peptide and blocking mAbs against TIGIT (αTIGIT) and/or PD1 (αPD1) and/or Tim3 (αTim3) or isotype control mAbs (IgG). B. Fold changes of the proportions of $CFSE^{lo}$ and total A2/WT1 235-243 tet+ CD8+ T cells after a 6-day IVS with cognate peptide and the indicated mAbs (n = 10). Each data-point represents the proportion of WT1-specific CTLs for each patient. Data shown are representative of two independent experiments performed in duplicate.

Table 2. Safety and toxicity

Grade/ Toxicity	Fatigue	Flu symptoms	Fever	Chill, Rigor	Mild erythema at the injection site
1/2	3/0	3/0	6/0	3/0	10/0
3/4	0/0	0/0	0/0	0/0	0/0

Tim3 expression [12, 17, 23]. For example, a previous study found that tumor antigen-specific CD8+ T cells promoted TIGIT expression after 24 hours of *in vitro* stimulation with antigen, while PD1 upregulation occurred after 96 hours of stimulation [6]. In the present study, we did not observe significant upregulation of TIGIT expression TIGIT+PD1+Tim3- and TIGIT+PD1+Tim3+WT1-specific CTLs, suggesting that TIGIT expression may not be correlated with the severity of T cell dysfunction in patients with advanced GC. Moreover, WT1-specific CTLs upregulated PD1 and Tim3 expression, but not TIGIT, upon prolonged stimulation. This data suggests that upregulation of TIGIT by dysfunctional WT1-specific CTLs occurs earlier in the T cell activation process, with no further impact seen during chronic stimulation.

Adaptive T cell immune responses to tumors are influenced by high antigen load and the immunosuppressive TME. Therefore, with constant antigen exposure in the TME, it is reasonable to postulate that subsets of tumor antigen-specific T cells lose their effector function through exhaustion from high antigen load and anergy due to suboptimal priming. Previously, a report found that NY-ESO-1-specific CD8+ T cells did not respond to PD1 blockade, thus PD1 inhibition failed to restore tumor-specific CD8+ T cell dysfunction [8]. Similarly, we observed that PD1 inhibition alone did not reverse WT1-specific T cell dysfunction. There are several suppositions that might explain the failure of PD1 inhibition to revert the T dysfunction experienced by PD1+ exhausted T cells. First, tumor-specific exhausted T cells express varying levels of PD1, and $PD1^{high}$ T cell subsets appear to be less responsive to PD1 blockade than $PD1^{int/low}$ CD8+ T cells [8, 33]. Consistent with this observation, we found higher PD1 expression in the partially dysfunctional TIGIT+PD1+Tim3-WT1-specific CTL subset than on TIGIT-PD1+Tim3- subset, but were still lower than the most dysfunctional TIGIT+PD1+Tim3+ subset. Second, exhausted T cells upregulate multiple inhibitory receptors, including PD1, CTLA-4, Tim3 and LAG-3 [5, 34]. The co-expression of these inhibitory receptors is associated with higher T cell dysfunction; thus, an ideal strategy in reversing CTL dysfunction may be to target multiple inhibitory receptors. Altogether, our findings support the combined use of PD1 and Tim3 blockades, in conjunction with tumor vaccines to enhance vaccine-induced tumor antigen-specific CTL responses. Such a strategy might improve the clinical outcomes for patients with advanced cancer [35]. Consistent with these observations, we observed that most DC vaccine-induced WT1-specific CTLs detected *ex vivo* upregulated PD1 and TIGIT expression, and a marginal proportion also upregulated Tim3. The levels of TIGIT, PD1, and Tim3 expression by vaccination-induced WT1-specific CTLs after DC vaccination inversely correlated with their function and expansion. Moreover, TIGIT blockade, in combination with PD1 and Tim3 inhibition further augmented the proportions of IL-2-producing, proliferating and total WT1-specific CD8+ T cells among total CD8+ T cells.

In summary, our data demonstrated that DC vaccination stimulated WT1-specific CTLs with enhanced IFN-γ production, cytotoxicity, and lytic capacities that promote TIGIT and PD1. We also show that TIGIT and PD1 regulated the growth of DC vaccination-induced CTLs after WT1-specific DC vaccination. We further demonstrated that TIGIT and PD1 blockades enhanced the growth and function of vaccine-induced CTLs. Importantly, TIGIT blockade synergizes with PD1 and Tim3 blockade to enhance WT1-specific CD8+ T cell growth and function. Our findings support the hypothesis of targeting of TIGIT, PD1, and Tim3 pathways to revert tumor-induced T cell dysfunction and promote antitumor CD8+ T cell responses in patients with advanced GC. Potentially, these patients might respond favorably to the combination of these inhibitory receptors with DC vaccination-based immunotherapy.

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Disclosure of conflict of interest

None.

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