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Identification of Co-inhibitory Receptors PD-1 and TIM-3 on T Cells from Gastric Cancer Patients

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Abstract

The critical role of tumor antigen-specific immune responses to restrain tumor growth in patients with gastric cancer furthers the need to dissect the co-inhibitory pathways involved in tumor infiltrating lymphocyte (TIL) exhaustion/dysfunction. It was previously reported that PD-1 expression was significantly increased on $CD4^+$ and $CD8^+$ T cells from patients with gastric cancer and in gastric cancer tissues, compared to normal donors. In this study, we observed up-regulation of TIM-3 and PD-1 expression on peripheral T cells and up-regulation of CTLA-4, TIGIT, TIM-3 and PD-1 expression on TILs from gastric cancer patients. Furthermore, the percentages of $PD\text{-}1^+/TIM\text{-}3^+$ and $PD\text{-}1^+/TIM\text{-}3^-$ peripheral T cells was significantly higher in gastric cancer patients than normal donors, and the percentage of $PD\text{-}1^+/TIM\text{-}3^+$ TILs was significantly higher than the percentage of $PD\text{-}1^+/TIM\text{-}3^+$ peripheral T cells in gastric cancer patients. $PD\text{-}1^+/TIM\text{-}3^+$ cells represent the predominant fraction of TILs. PD-1 blockade enhanced cytokine production and the cytotoxicity of TILs and exhibited a synergistic effect with TIM-3 blockade. Moreover, the expression of PD-1 was associated with the age, tumor size and lymph node metastasis of patients. Collectively, our results suggest that the use of anti-PD-1 blockade in combination with anti-TIM-3 blockade could restore the function of TILs in patients with gastric cancer.

Keywords: Gastric cancer; Co-inhibitory receptor; PD-1; TIM-3; Cancer immunotherapy

Introduction

Gastric cancer (GC) is the second leading cause of cancer-related deaths worldwide, causing approximately 700,000 deaths annually [1]. The five-year overall survival rates of advanced gastric cancer patients are still very poor when multimodal treatment strategies involving surgery, perioperative chemotherapy, and radiation are used [2,3]. It has been demonstrated that a high density of cytotoxic T cells and memory T cells infiltrating the tumor is associated with a better disease outcome for gastric cancer patients [4]. Adjuvant adoptive cell transfer (ACT) immunotherapy using autologous cytokine-induced killer (CIK) cells for gastric cancer patients is better than traditional chemotherapy alone [5]. However, immunosuppression remains serious after surgery, chemotherapy and immunotherapy for gastric cancer [6]. Tumor cells can induce increased expression of co-inhibitory receptors on TILs, leading to TIL exhaustion and decreasing the tumor-specific response. Thus, not all gastric cancer patients receiving ACT immunotherapy have an overall response [7]. The co-inhibitory molecules up-regulated on TILs in gastric cancer can be targeted to augment the host immune system, leading to improved survival [3]. Regrettably, until now, no systematically analysed data have shown a leading role for any particular co-inhibitory molecule in gastric cancer. Therefore, a comprehensive study of co-inhibitory molecule expression in gastric cancer is important both for fully understanding this disease and as a basis for developing new treatment options.

Over two decades of study, a class of immune checkpoint proteins, including cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed death-1 (PD-1), T-cell immunoglobulin and mucin protein-3 (TIM-3), T cell Ig and ITIM domain (TIGIT), has been found to be crucial for regulating the responses of T cells [8]. In the normal physiological state, immune checkpoints are critical for the maintenance of self-tolerance and for protecting normal tissues from damage by the immune system [9]. Long term contact with tumor cells in the tumor micro-environment can induce tumor- specific lymphocytes to dysregulate the expression of certain immune checkpoint proteins, resulting in lymphocyte dysfunction [10]. Preclinical findings have shown that antibodies that block the inhibitory signals of checkpoint proteins enhance antigen-specific T cell responses [8,11]. Thus, immune checkpoint proteins are potential targets for human anti-tumor immune therapy.

Recent studies have shown that co-expression of immune checkpoint molecules occurs frequently on tumor-specific T cells, as well as on pathogen-specific T cells in chronic infections [11]. For example, a population of exhausted T cells co-expresses PD-1 and TIM-3 in a lymphocytic choriomeningitis virus (LCMV) model [12]. In HIV and HCV patients, T cells with coexpression of TIM-3 and PD-1 comprise the smallest group of $CD8^+$ T cells and exhibit exhaustion [13,14]. Several mouse tumor models show that PD-1 and TIM-3 coexpression occurs on approximately half of tumor infiltrating lymphocytes (TILs), and PD-1+/$TIM\text{-}3^+$ TILs display the most severe

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dysfunctional phenotype as defined by failure to proliferate and produce IL-2, TNF-α, and IFN-γ. Combined blocking of the PD-1 and TIM-3 signaling pathways is more effective in suppressing tumor growth than blocking either signaling pathway alone [15,16]. In human cancer, TIM-3 is highly expressed on PD-1$^+$ NY-ESO-1-specific CD8$^+$ T cells; PD-1$^+$/TIM-3$^+$ NY-ESO-1-specific CD8$^+$ T cells are more dysfunctional than PD-1$^+$/TIM-3$^-$ and PD-1$^-$/TIM-3$^-$ NY-ESO-1-specific CD8$^+$ T cells, producing less IL-2, TNF-α, and IFN-γ. TIM-3 signaling pathway blockade enhanced the proliferation and cytokine production of NY-ESO-1-specific CD8$^+$ T cells and exerted synergistic effects with PD-1 signaling pathway blockade [17].

In this study, we show that the expression of two co-inhibitory receptors, PD-1 and TIM-3, was significantly increased in peripheral T cells from gastric cancer patients compared to normal donors. The expression of PD-1 and TIM-3 was further increased in TILs compared to peripheral T cells from gastric cancer patients. Either PD-1 pathway blockade or TIM-3 pathway blockade *in vitro* promoted cytokine production by TILs, and combined PD-1 and TIM-3 blockade had cooperation on cytokine production in TILs. The cytotoxicity of *in vitro* activated TILs in the presence of PD-1 and TIM-3 blockade was significantly higher than that in the presence of either PD-1 or TIM-3 blockade alone. Our results suggest that targeting the PD-1 and TIM-3 pathways is an effective therapeutic strategy for gastric cancer.

Materials and Methods

Study subjects

15 patients with a pathological diagnosis of gastric cancer who were treated at The First Affiliated Hospital of Soochow University were enrolled in this study. None of the patients received immunotherapy before surgery. Nine healthy normal donors were obtained from The Second Affiliated Hospital of Soochow University. The study protocol was approved by the Ethics Committee at The First Affiliated Hospital of Soochow University, and all participants provided written informed consent. The gastric adenocarcinoma line AGS was kindly provided by Dr. Quansheng Zhou's laboratory at Soochow University. AGS-GFP was generated by transducing lentiviral vectors encoding green fluorescent protein (GFP) genes into AGS cells.

Isolation of TILs

TILs were isolated by dissociating tumor tissue with the plunger portion of syringes on mesh sieves before centrifugation on a Ficoll-Paque Plus (GE) gradient. TILs were activated by incubating with the Human Lymphocytes Activation and Amplification Kit (PersonGen) and IL-2 for 2 weeks. The activated TILs were then used in the next experiments.

Surface and intracellular cytokine staining

Single cell suspensions were incubated with CD3-FITC (clone HIT3a) or CD4-FITC (clone RPA-T4) or CD8-FITC (clone SK1), TIM-3-PE (clone 2E2) and PD-1-APC (clone MIH4). 7-AAD (BD) was used to assess the viability of the cells. Alternatively, cells were stained with the following antibodies: TIGIT-PE-Cy7 (clone MBSA43) and CTLA-4-PE-Cy5 (clone BNI3). For *in vitro* cytokine production assays, activated TILs were mixed with AGS cells at a ratio of 10:1 and were incubated for 5h with GolgiStop (BD) in the presence of 10μg/ml anti-TIM-3 (clone 2E2) or anti-PD-1 (PersonGen) or isotype control antibodies (Invitrogen) as described previously [17]. Cells were then stained with CD4-FITC (clone RPA-T4) and CD8-PE-Cy7 (clone SK1). Intracellular staining was performed with IL-2-PE (clone MQ1-17H12), IFN-γ-PerCP-Cy5.5 (clone B27) and TNF-α-APC (clone Mab11). LIVE/DEAD near-IR fluorescent reactive dye (Invitrogen) was used to assess the viability of the cells. All data were collected on a FACSAria III (BD) and analyzed with FlowJo software (Tree Star, Inc.).

Cytotoxicity analysis

AGS-GFP cells were mixed with TILs at a ratio of 1:6 and incubated for 4 h in the presence of 10 μg/ml anti-TIM-3 (clone 2E2) or anti-PD-1 (PersonGen) or isotype control antibody (Invitrogen), followed by 7-AAD (BD) staining. The percentage of live AGS cells (GFP positive and 7-AAD negative) was determined. The percentage of dead AGS cells was calculated using the formula: percent total AGS minus percent live AGS divided by percent total AGS × 100 as described previously [18].

Statistics

Statistical hypotheses were tested with paired t-tests, unpaired t-tests or Wilcoxon signed rank tests (for paired results of MFI analysis from the same patient) using GraphPad Prism software. Tests were two-sided, and $P<0.05$ was considered significant. Because rank tests are not sensitive to the actual values in a comparison, only to their ranks, differing sets of values can produce identical p-values.

Results

PD-1 and TIM-3 expression are up-regulated on T cells in gastric cancer patients

To determine which co-inhibitory molecules may be important for TIL exhaustion in gastric cancer, we assessed the expression of CTLA-4, TIGIT, PD-1 and TIM-3 on CD3$^+$, CD4$^+$ and CD8$^+$ T cells isolated from gastric cancer tissue, adjacent gastric mucosa (AGM), and peripheral blood mononuclear cells (PBMCs) from gastric cancer patients, as well as PBMCs from normal donors.

CTLA-4 was weakly expressed on peripheral blood CD3$^+$ and CD4$^+$ T cells from gastric cancer patients, but was highly expressed on peripheral blood CD8$^+$ T cells from gastric cancer patients. A higher percentage of CD3$^+$, CD4$^+$ and CD8$^+$ TILs expressed CTLA-4 compared to T cells from the peripheral blood of gastric cancer patients (Figure 1a and Figure S1). TIGIT expression on CD3$^+$ and CD4$^+$ TILs was higher than expression on CD3$^+$ and CD4$^+$ peripheral blood T cells from GC patients. However, the percentage of TIGIT$^+$ CD8$^+$ TILs was lower than that of peripheral blood CD8$^+$ T cells. The percentage of TIGIT positive cells was highest in CD3$^+$ and CD4$^+$ T cells from AGM (Figure 1b and Figure S1). The percentage of CD3$^+$ TILs expressing PD-1 (60.18 ± 23.57%) was significantly higher than that of CD3$^+$ PBMCs from GC patients (37.10 ± 19.91%, p=0.0288; Figure 2a and Figure S2). The frequency of CD3$^+$ T cells expressing PD-1 was 3-fold higher in PBMCs from GC patients than in PBMCs from normal donors (11.70 ± 6.436%, p=0.0020; Figure 2a and Figure S2). The percentage of CD3$^+$ TILs expressing TIM-3 (44.89 ± 29.42%) was significantly higher than that of CD3$^+$ PBMCs from GC patients (26.77 ± 17.25%, p=0.0119; Figure 2b and Figure S2), which in turn was significantly higher than that of CD3$^+$ PBMCs from normal donors (10.78 ± 11.54%, p=0.0314; Figure 2b and Figure S2). The pattern of PD-1 and TIM-3 expression on CD4$^+$ and CD8$^+$ T cells was the same as that on CD3$^+$ T cells (Figures 3a, 3b, 4a and 4b). Similar observations were made when analysing TIGIT, PD-1 and TIM- 3 expression by MFI on TILs and PBMCs from GC patients (Figures 1b and 5). Furthermore, we observed that the expression of PD-1 was positively correlated with lymph node status (p=0.0477), age (p=0.0305) and tumor size (p=0.0305) of patients (Table 1).

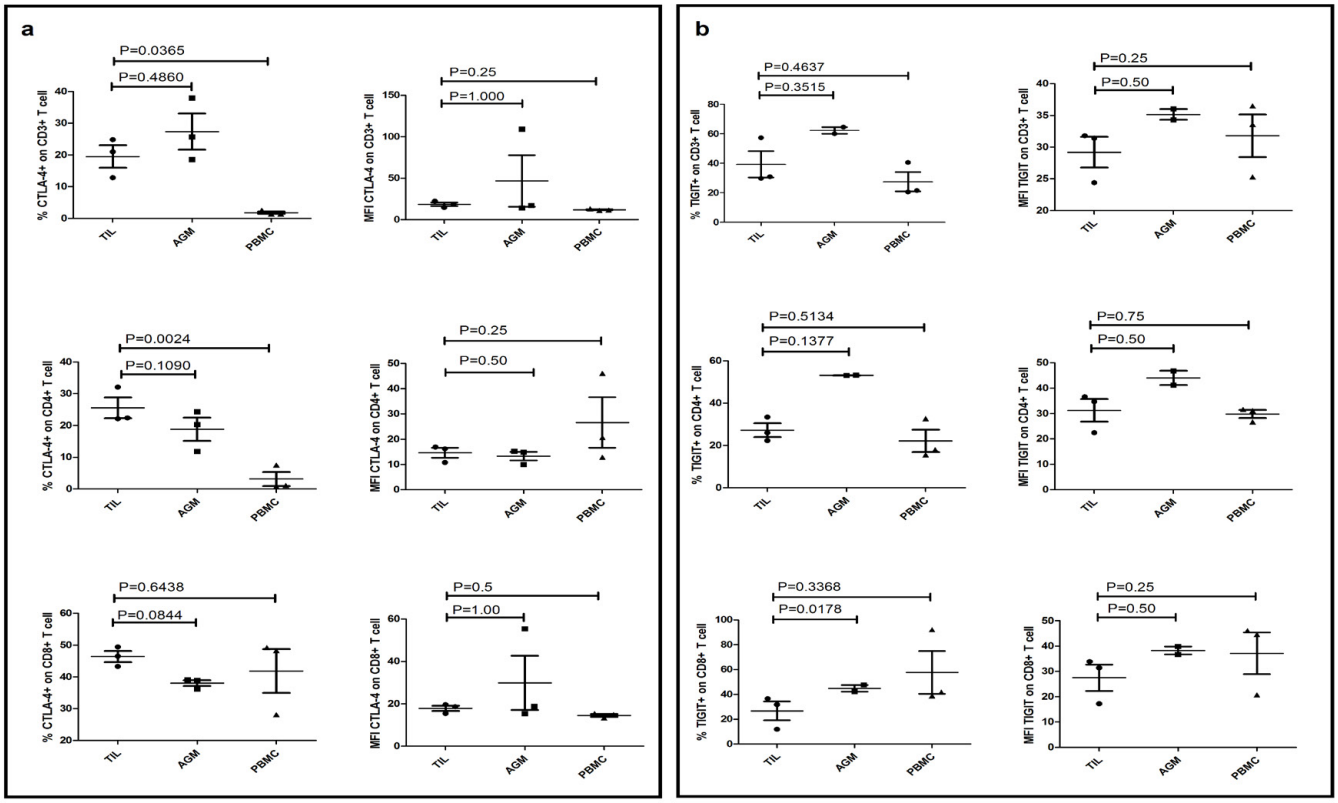

Figure 1: **Pooled data showing the CTLA-4 and TIGIT expression on T cells.** Pooled data from gastric cancer patient peripheral blood mononuclear cells (PBMC), adjacent gastric mucosa (AGM) and tumor infiltrating lymphocytes (TIL) showing CTLA-4 (**a,** n=3) and TIGIT (**b,** n=3) expression on $CD3^+$, $CD4^+$ and $CD8^+$ T cells. The horizontal bars indicate means. The error bars indicate SEM. The p-values were calculated using the paired *t*-tests.

Clinical pathological parameters	Cases(n)	Frequecy of PD- 1$^+$CD3 $^+$ TILs (%, Mean ± SEM)	p-value	Frequecy of TIM- 3$^+$ CD3$^+$ TILs (%, Mean ± SEM)	p-value
Sex					
Male	11	61.09 ± 5.946	0.8137	42.61 ± 9.042	0.6362
Female	4	57.67 ± 17.92		51.17 ± 15.57	
Age(years)					
>70	8	48.28 ± 8.625	0.0305	31.75 ± 9.007	0.0611
≥ 70	7	73.77 ± 5.312		59.91 ± 10.48	
Tumor size(cm)					
≤ 4	8	48.28 ± 8.625	0.0305	31.75 ± 9.007	0.0611
>4	7	73.77 ± 5.312		59.91 ± 10.48	
Histological grade					
Moderate-well differentiated	6	60.18 ± 11.67	0.9999	47.77 ± 16.54	0.7695
Poor differentiated	9	60.18 ± 7.162		42.97 ± 7.286	
TNM stage					
Stage I-II	6	51.05 ± 7.529	0.2338	31.40 ± 9.677	0.1536
Stage III-IV	9	66.26 ± 8.533		53.88 ± 10.20	
Lymph node metastasis					
Negative	4	40.60 ± 5.493	0.0477	25.90 ± 14.28	0.1365
Positive	11	67.30 ± 6.957		51.79 ± 8.368	
Abbreviations: TIL: Tumor Infiltrating Lymphocyte; SEM: Standard Error of Mean					

Table 1: Correlation of clinicopathological features of the patients with gastric cancer and PD-1/TIM-3 expression levels in $CD3^+$ TILs.

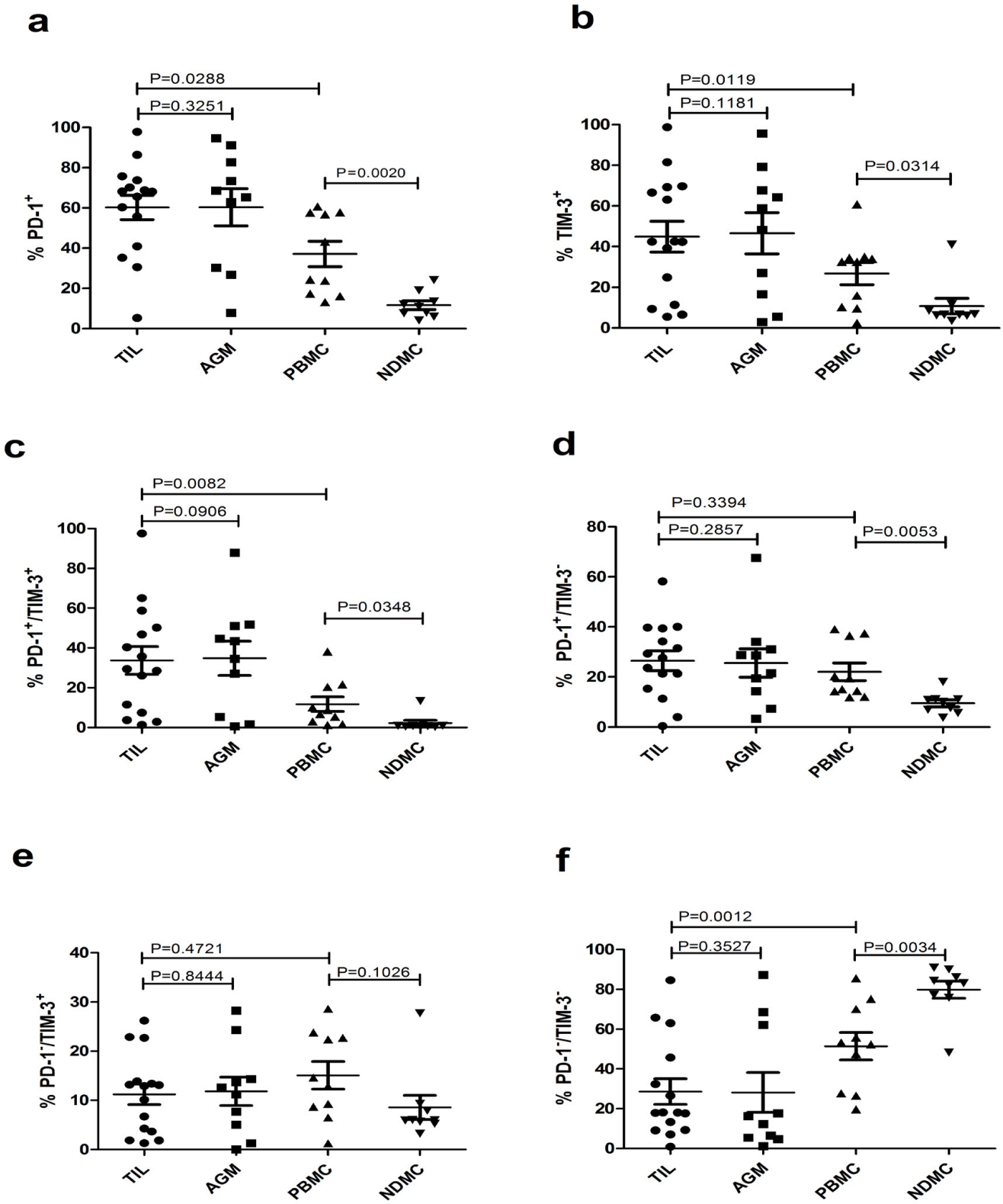

Figure 2: Pooled date showing the PD-1 and TIM-3 expression on CD3⁺ T cells. Pooled data showing the percentage (%) of PD-1⁺ **(a)**; TIM-3⁺ **(b)**; PD-1⁺/TIM-3⁺ **(c)**; PD-1⁺/TIM-3⁻ **(d)**; PD-1⁻/TIM- 3⁺ **(e)** and PD-1⁻/TIM-3⁻ **(f)** on CD3⁺ T cells from tumor infiltrating lymphocytes (TIL, n=15), adjacent gastric mucosa (AGM, n=10) and peripheral blood mononuclear cells (PBMC, n=10) of gastric cancer patients, and on CD3⁺ T cells from PBMCs of normal donors (NDMC, n=9). The horizontal bars indicate means. The error bars indicate SEM. The p-values between TIL and AGM/PBMC were calculated using the paired t-tests. The p-values between PBMC and NDMC were calculated using the unpaired t-tests.

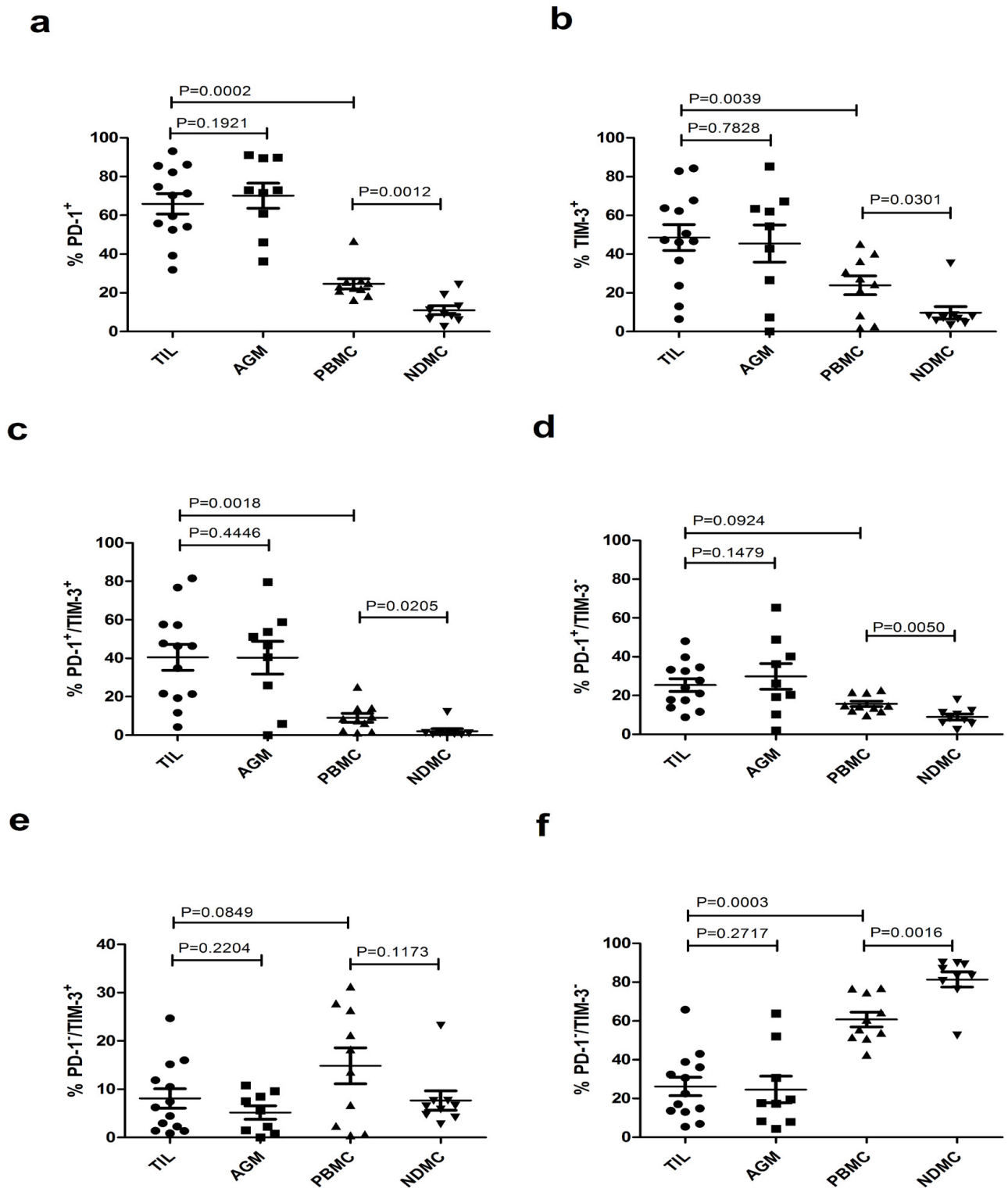

Figure 3: Pooled data showing the PD-1 and TIM-3 expression on CD4⁺ T cells. Pooled data showing the percentage (%) of PD-1⁺ **(a)**; TIM-3⁺ **(b)**; PD-1⁺/TIM-3⁺ **(c)**; PD-1⁺/TIM-3⁻ **(d)**; PD-1⁻/TIM- 3⁺ **(e)** and PD-1⁻/TIM-3⁻ **(f)** on CD4⁺ T cells from TILs (n=13), AGM (n=9) and PBMCs (n=10) of gastric cancer patients and PBMCs of normal donors (NDMC, n=9). The horizontal bars indicate means. The error bars indicate SEM. The p-values between TIL and AGM/PBMC were calculated using the paired t-tests. The p-values between PBMC and NDMC were calculated using the unpaired t-tests.

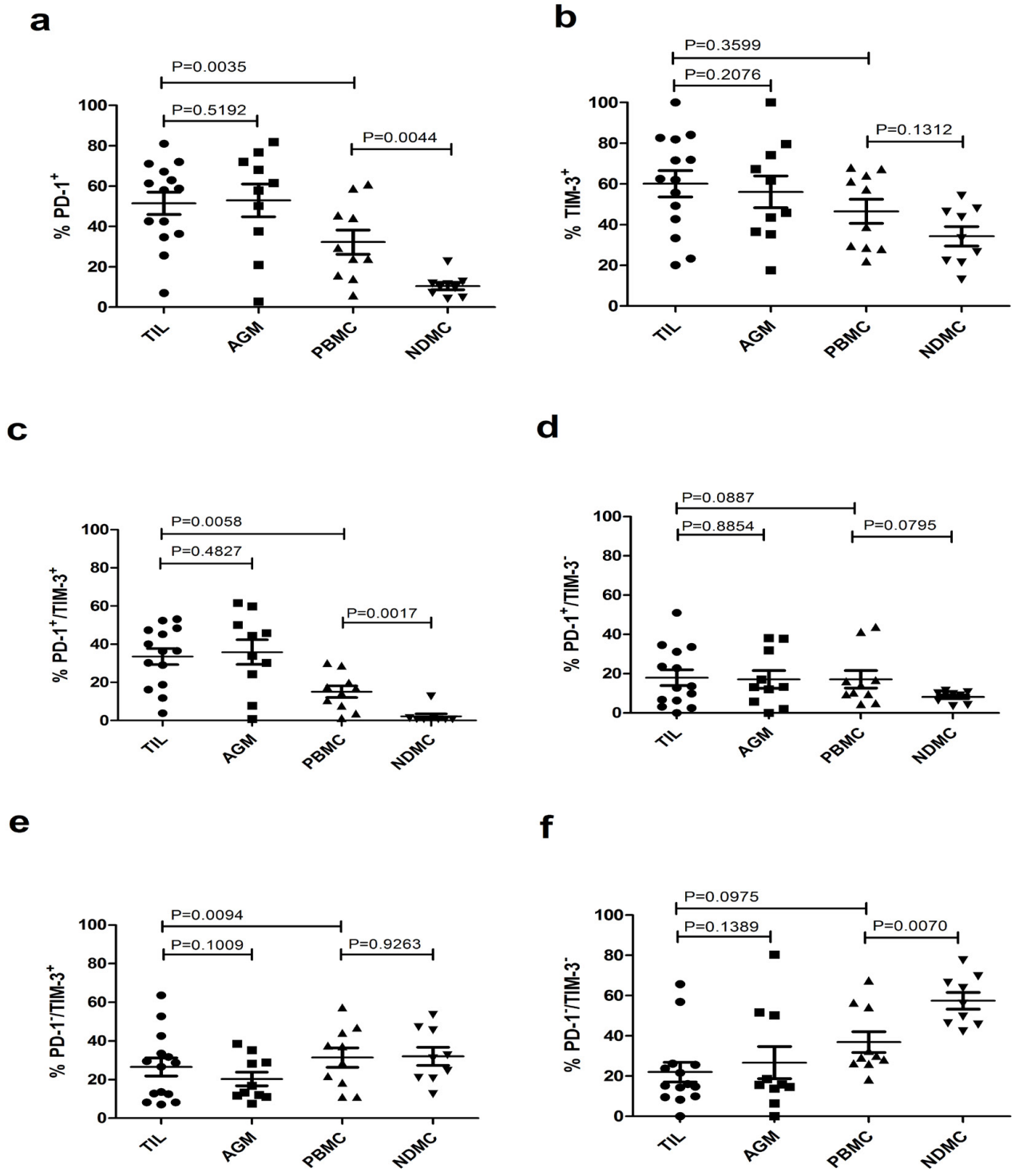

Figure 4: Pooled data showing the PD-1 and TIM-3 expression on CD8⁺ T cells. Pooled data showing the percentage (%) of PD-1⁺ **(a)**; TIM-3⁺ **(b)**; PD-1⁺/TIM-3⁺ **(c)**, PD-1⁺/TIM-3⁻ **(d)**; PD-1⁻/TIM-3⁺ **(e)** and PD-1⁻/TIM-3⁻ **(f)** on CD8⁺ T cells from TILs (n=14), AGM (n=10) and PBMCs (n=10) of gastric cancer patients, and PBMCs of normal donors (NDMC, n=9). The horizontal bars indicate means. The error bars indicate SEM. The p-values between TIL and AGM/PBMC were calculated using the paired t-tests. The p-values between PBMC and NDMC were calculated using the unpaired t-tests.

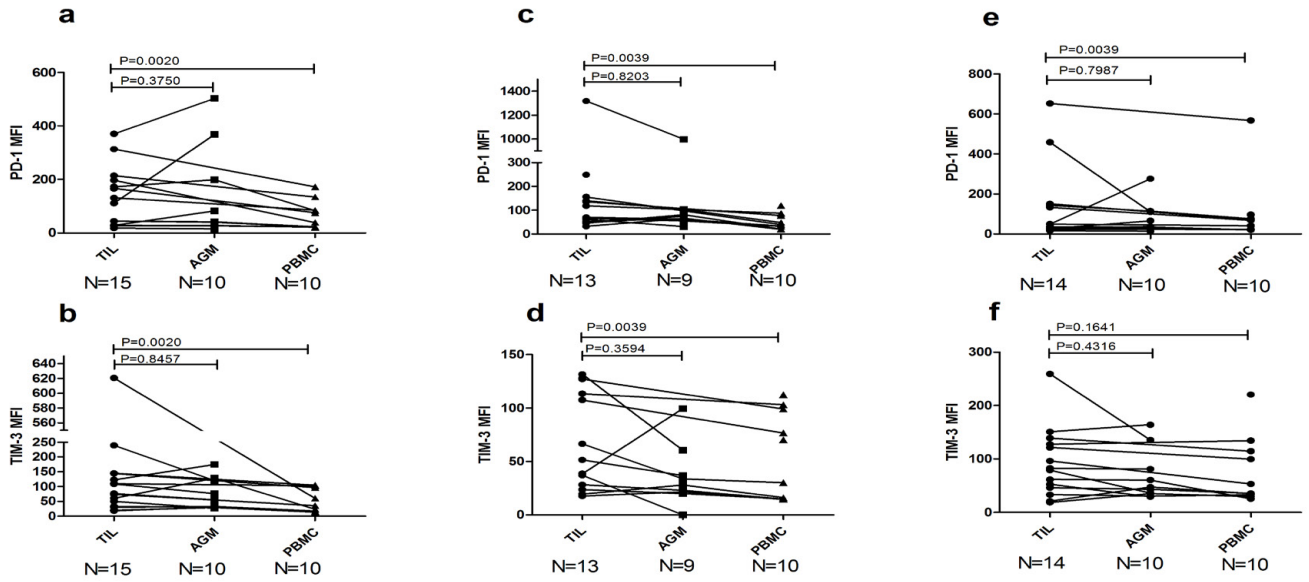

Figure 5: Data showing the mean fluorescence intensity (MFI) of PD-1 and TIM-3 expression. Pooled data showing the mean fluorescence intensity (MFI) of PD-1 and TIM-3 expression on $CD3^+$ **(a** and **b)**; $CD4^+$ **(c** and **d)** and $CD8^+$ **(e** and **f)** T cells from the TILs, AGM and PBMCs of gastric cancer patients. The p-values were calculated using the Wilcoxon signed rank test. The p-values were calculated using the Wilcoxon signed rank tests.

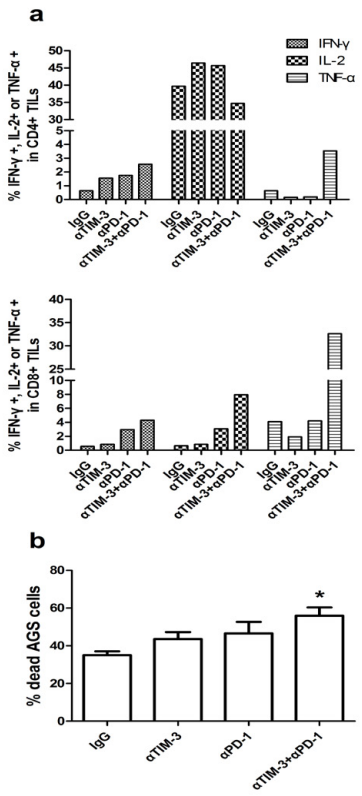

Figure 6: Blockade of TIM-3 and/or PD-1 pathways enhances cytokine production and the cytotoxicity of TILs. (a) Expression of cytokines in $CD4^+$ and $CD8^+$ TILs was evaluated in the presence of blocking anti-TIM-3 (αTIM-3) and/or anti-PD-1 (α-PD-1) mAbs or an isotype control antibody (IgG) (n=1); **(b)** In the presence of blocking anti-TIM-3 (αTIM-3) and/or anti-PD-1 (αPD-1) mAbs or an isotype control antibody (IgG), AGS-GFP cells were co-cultured with TIL at a 1:6 ratio for 4h, and the percentage of dead AGS cells was measured by flow cytometry with 7-AAD (n=3); *p<0.05, vs IgG.

Up-regulation of PD-1^+/TIM-3^+ T cells in PBMCs and TILs from GC patients

Because both PD-1 and TIM-3 were up-regulated on a fraction of peripheral blood T cells in GC patients and further up-regulated on a majority of TILs, we next determined whether PD-1 and TIM-3 are expressed on identical or distinct T cell subsets. $CD3^+$ T cells from GC patient PBMCs had a significantly higher percentage of PD-1^+/TIM-3^+ cells than $CD3^+$ T cells from normal donor PBMCs (11.70 ± 11.70% vs. 2.228 ± 4.196%, p=0.0348; Figure 2c and Figure S2). The percentage of PD-1^+/TIM-3^- cells also increased in the $CD3^+$ T cell population in GC patient PBMCs compared to normal donor PBMCs (22.06 ± 11.13% vs. 9.467 ± 4.177 %, p=0.0053; Figure 2d and Figure S2). Furthermore, $CD3^+$ TILs had a significantly higher percentage of PD- 1^+/TIM-3^+ cells than $CD3^+$ peripheral T cells from GC patients (33.68 ± 27.20% vs. 11.70 ± 11.70%, p=0.0082; Figure 2c and Figure S2). The frequencies of PD-1^+/TIM-3^+ cells among $CD4^+$ and $CD8^+$ T cells were similar to $CD3^+$ T cells (Figures 3c, 4c and Figure S2). Our results demonstrate that PD-1^+/TIM-3^+ cells make up the largest fraction of TILs (the frequencies of PD-1^+/TIM-3^+ cells in $CD3^+$, $CD4^+$ and $CD8^+$ TILs were 33.68 ± 27.20%, 40.47 ± 24.25% and 33.52 ± 15.80%, respectively; Figures 2c, 3c and 4c).

PD-1 and TIM-3 blockade enhances cytokine production in TILs

To determine whether PD-1 and TIM-3 are effective targets for gastric cancer immunotherapy, we measured the effect of blocking the PD-1 and/or TIM-3 pathways The TILs used in our blocking assays were proliferated for two weeks on cytokine production in TILs. Blockade of the TIM-3 pathway led to a small increase in the percentage of IFN-γ^+ and IL-2^+ cells in $CD4^+$ TILs, while no change was seen in $CD8^+$ TILs. Blockade of the PD-1 pathway led to a small increase in the percentage of $CD4^+$ and $CD8^+$ TILs producing IFN-γ and IL-2, but did not increase TNF-α production (Figure 6a and Figure S3). When compared with blocking the PD-1 or TIM-3 pathways alone, blocking both pathways

simultaneously led to an obvious increase in the percentages of $CD4^+$ and $CD8^+$ TILs secreting IFN-γ and TNF-α, and the percentage of $CD8^+$ TILs secreting IL-2 (Figure 6a and Figure S3). Combined PD-1/TIM-3 pathway inhibition had the most striking effect on TNF-α production in $CD8^+$ TILs, where 32% of the cells secreted TNF-α in response to blockade of both pathways, compared to less than 5% of cells secreting TNF-α after blockade of either pathway alone (Figure 6a and Figure S3).

PD-1 and TIM-3 blockade enhances the cytotoxicity of TILs

Using flow cytometry with 7-AAD to detect dead AGS cells, we observed that both PD-1 blockade alone and TIM-3 blockade alone enhanced the cytotoxicity of TILs to AGS cells (Figure 6b). Moreover, the cytotoxicity of TILs, as seen by increased AGS cell death, was enhanced when blockade of PD-1 and TIM-3 pathways was combined compared to the inhibition of either pathway alone (Figure 6b).

Discussion

PD-1 has been shown to be up-regulated on TILs in patients with melanoma, renal cell carcinoma and non-small cell lung cancer [19]. In a recent clinical study, PD-1 expression was found to be up-regulated on $CD4^+$ and $CD8^+$ T cells from gastric cancer patients, and positively correlated with PD-L1 expression [20]. A study of polymorphisms in the TIM-3 gene illustrated that specific genetic variants located in the TIM-3 gene promoter region were associated with susceptibility to gastric cancer [21]. However, no direct results were shown to address whether TIM-3 was up- regulated on T cells from GC patients, or whether expression of TIM-3 was related to PD-1 expression. Our results determined that both PD-1 and TIM-3 were up-regulated on peripheral T cells and TILs from GC patients, and that more than a third of TILs from GC patients were $PD\text{-}1^+/TIM\text{-}3^+$, a 10 to 14-fold increase compared to peripheral T cells from normal donors (Figures 2a, 2b, 3a, 3b, 4a and 4b). The levels of $PD\text{-}1^+/TIM\text{-}3^+$ peripheral T cells from GC patients was increased 5 to 7-fold compared to those from normal donors (Figures 2c, 3c and 4c). The expression of PD-1 and/or TIM-3 is associated with T cell exhaustion or dysfunction [18, 22-24]. Our results suggest that TILs display a more severe exhausted phenotype than peripheral T cells from GC patients, and that peripheral T cells from GC patients exhibit a more severe exhausted phenotype than peripheral T cells from normal donors.

Previous studies have suggested that the co-inhibitory molecule TIGIT may be up-regulated on TILs via long term interaction with tumor antigen [8]. However, we observed the percentage of $TIGIT^+$ cells on $CD3^+$ and $CD4^+$ T cells from AGM was higher than that of $CD3^+$ and $CD4^+$ TILs (Figure 1b). These paradoxical results, with lower TIGIT expression on $CD8^+$ TILs, need to be studied further. Additionally, we also observed that the differences in PD-1 and TIM-3 expression between TILs and adjacent gastric mucosa were not statistically significant (Figures 2a, 2b; 3a, 3b and 4a, 4b). These results suggest that the adjacent gastric mucosa may also be in a suppressed immune state, similar to gastric cancer tissue, resulting in T cell exhaustion.

We observed an increase in $PD\text{-}1^+/TIM\text{-}3^+$ cells and $PD\text{-}1^+/TIM\text{-}3^-$ cells in TILs and peripheral T cells (exception of $PD\text{-}1^+/TIM\text{-}3^-$ cells in $CD8^+$ TILs and peripheral $CD8^+$ T cells) from GC patients compared to peripheral cells from normal donors, but no increase in $PD\text{-}1^-/TIM\text{-}3^+$ cells, suggesting that increased expression of TIM-3 occurs exclusively on T cells that co-express PD-1; however, increased expression of PD-1 can occur in the absence of TIM-3 expression. Furthermore, we observed that the fold increase in PD-1 expression (3-/5-fold increase) from normal donor PBMCs to gastric cancer patient PBMCs/TILs was slightly more than that of TIM-3 expression (2.5-/4.5-fold increase), suggesting that PD-1 up-regulation occurs not only on peripheral T cells, but also on TILs from patients with gastric cancer, and may occur a little earlier than TIM-3 up-regulation. These results demonstrate that, in GC patients, TIM-3 up-regulation was positively associated with PD-1 up-regulation.

It has been shown that programmed death-ligand-1 (PD-L1) is up-regulated in gastric cancer, and that increased PD-L1 expression correlates with decreased overall survival [25,26]. Other studies have shown that PD-1 is also up-regulated in gastric cancer, and $PD\text{-}1^+$ TILs produce less IFN-γ than PD-1- TILs [18]. Here, we demonstrate that blockade of signaling through PD-1 using an anti-PD-1 antibody enhanced IFN-γ and IL-2 production in $CD4^+$ and $CD8^+$ T cells and increased the cytotoxicity of TILs towards GC cells (Figure 6). Our results imply that using an anti-PD-1 blocking antibody has a broad prospect in gastric cancer immunotherapy.

To assess the effects of anti-TIM-3 antibody on tumors, previous studies characterized the comparatively defective cytokine effector function of $PD\text{-}1^+/TIM\text{-}3^+$ $CD8^+$ T cell populations in experimental tumors in mice, or NY-ESO-1 specific $PD\text{-}1^+/TIM\text{-}3^+$ $CD8^+$ T cells in advanced melanoma in humans, or both $CD8^+$ and $CD4^+$ T cells in WTMCA2 sarcomas, CT26 and MC38 colon adenocarcinomas in mice [15-17]. We have demonstrated that an anti-TIM-3 blocking antibody increases IFN-γ and IL-2 production in $CD4^+$ T cells isolated from human gastric cancer tissue. However, we did not observe an increase in cytokine production in $CD8^+$ T cells after treatment with an anti-TIM-3 blocking antibody, which was contrary to previously published results [15-17]. The TILs used in our blocking assays were proliferated for two weeks *in vitro*, and were stimulated to some extent while in culture, leading to TILs that were unable to respond to single agent anti- TIM-3 blockade. Interestingly, we observed that $CD8^+$ TILs produced more IFN-γ, IL-2 and TNF-α after blocking both the TIM-3 and PD-1 signaling pathways than after blocking either pathway alone (Figure 6a). A similar observation was made in $CD4^+$ TILs, where combined treatment with an anti-TIM-3 blocking antibody and an anti-PD-1 blocking antibody enhanced IFN-γ and TNF-α production compared to blockade of either pathway alone. Despite of the $CD4^+$ T cells producing less IL-2 after combined TIM-3 and PD-1 blockade compared to blocking either pathway alone (Figure 6a). The cytotoxicity of TILs in the presence of PD-1 and TIM-3 blockade was significantly higher than that in the presence of either PD-1 or TIM-3 blockade alone (Figure 6b).

Additionally, as gastric cancer cells from patients were not acquired, AGS was used as targets to activate TILs. However, the response of TILs to AGS-GFP cells in cytotoxicity or cytokine production assay may be dependent on allogeneic recognition, not tumor specific recognition. Although the cytotoxicity and cytokine production of TILs to AGS-GFP cells were enhanced in the presence of antibody blockade, the efficiency of TILs to autologous gastric cancer cells was required to further explore.

In summary, our data demonstrate that the expression of PD-1 and TIM-3 was significantly increased in peripheral T cells from gastric cancer patients compared to normal donors. The expression of PD-1 and TIM-3 was further increased in TILs compared to peripheral T cells from gastric cancer patients. Moreover, the expression of PD-1 was strongly correlated with the age, tumor size and lymph node status of patients. The cytotoxicity and cytokine production of *in vitro* activated TILs in the presence of PD-1 and TIM-3 blockade was

significantly higher than that in the presence of either PD-1 or TIM-3 blockade alone. Therefore, blockade of both the TIM-3 and PD-1 signaling pathways shows the potential to be an effective treatment for gastric cancer.

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Disclosure Statement

All authors of this paper have no conflict of interest to declare.

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