High-level of intratumoral GITR+ CD4 T cells associate with poor prognosis in gastric cancer

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Highlights
The expression of GITR varies greatly across T cell subsets and tissue types
GITR+ CD4 T cells contribute to the immunosuppressive microenvironment in human gastric cancer
GITR+ CD4 T cells correlate with worse prognosis in gastric cancer patients
GITR protein is mainly distributed intracellularly in PBMC-derived Treg cells

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High-level of intratumoral GITR+ CD4 T cells associate with poor prognosis in gastric cancer

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SUMMARY
Immunotherapy targeting glucocorticoid-induced TNFR-related protein (GITR) exhibited strong anti-tumor capacity in mouse model but poor efficacy in clinical trials. This may be attributed to the different GITR expression mode between human and mouse. In this study, we analyzed single-cell RNA sequencing (scRNA-seq) data of human gastric cancer (GC) and used flow to explore the GITR expression across T cell subsets and tissue types in GC patients. We revealed that GITR+ CD4 T cells, including regulatory CD4 T (Treg) cells and conventional CD4 T (Tconv) cells, might contribute to the immunosuppressive microenvironment in GC. The enrichment of these cells was associated with a worse prognosis. Moreover, we found the cellular distribution of GITR protein in Treg cells was microenvironment dependent. In conclusion, GITR is still an important immune checkpoint need to be studied.

INTRODUCTION
Immune checkpoint blockade (ICB), such as anti-programmed cell death protein 1 (PD-1), is now established as a new strategy of cancer treatment.3,4 However, the efficacy of anti-PD-1 therapy used in GC treatment is still limited.5,6 Only small proportion of GC patients respond to anti-PD-1 therapy.7,8 The clinical trial KEYNOTE-059 has shown anti-PD-1 treatment cannot improve the overall survival (OS) of GC patients.8 Therefore, identifying new immunotherapeutic targets for GC is urgently needed.9

GITR, also known as TNFRSF18/CD357/AITR, belongs to the tumor necrosis factor receptor superfamily (TNFRSF).10 It is constitutively expressed at high levels in Treg cells and activated T cells but at low levels in naive and memory T cells.10,11 The GITRL, ligand of GITR, is expressed on activated antigen-presenting cells including B cells and macrophages.10,12,13

GITR signaling, mediated by GITRL or agonistic mAb, can reduce the number of Treg cells and inhibit the suppressive functions of Treg cells.14–17 On the contrary, GITR signaling can increase effector T cells’ activation and proliferation and decrease suppression to Treg cells.15,18,19 In vivo studies have shown that DTA-1 (agonistic mAb of GITR) induces effective antitumor responses in different mice tumor models.20–22 When combined with other treatments (e.g. anti-PD-1, chemotherapy), it can achieve better efficacy.23 Preclinical studies also have confirmed treatments of GITR mAbs have manageable safety profiles, making GITR a potential immunotherapeutic target candidate for GC treatment.24,25 Most GITR-related researches are conducted in mouse tumor models; however, the function of GITR in human tumor microenvironment (TME) seems to be much more complicated. For instance, the GITR mAbs treatments have not shown satisfactory efficacy in clinical trials, suggesting the complex microenvironment of cancer patients need to be considered for the development of GITR treatment.10,24,26

In our study, high level of CD4 T cell infiltration and differential gene expression of GITR+ CD4 T cells in GC were identified via scRNA-seq analysis. We examined the expression pattern of GITR on T cells, including Treg cells, Tconv cells, and CD8 T cells, and analyzed the feature of GITR+ T cells in tumor tissues, peritumor tissues, tumor-draining lymph node (TDLN), and peripheral blood mononuclear cells (PBMCs) samples from GC patients. Our results showed that the abundance of intratumoral GITR+ Treg cells and GITR+ Tconv cells is associated with immunosuppressive tumor microenvironment and worse OS in patients.

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with GC. In the end, we found the distribution of GITR protein varied largely in Treg cells from different tissues.

RESULTS

GITR+ CD4 T cells were important in human GC

To overview the TME of human GC, we first analyzed scRNA-seq data of GC patients, which contained 26 tumor samples and 11 peritumor samples. Based on canonical markers defined in the literature, we categorized single cells into 13 major cell clusters (Figures 1A and 1B). The CD4 T cells were divided into Tconv cells and Treg cells. We identified 4 cell subsets (Tconv cells, Treg cells, B cells, and mast cells) that were significantly elevated in GC compared with peritumor tissues (Figure 1C). A number of studies have shown that T cells are critical in the antitumor immune response.27,28 Tconv cells could secret effector cytokines and help cytotoxic CD8 T cells to kill tumors in the TME.28 In contrast to Tconv cells’ effector function, Treg cells, a subset of T cells, possess strong immunosuppressive function, promote immune escape of tumor cells, and are predicted to be associated with poor prognosis in various tumors.29–32 To validate the role of GITR in Treg and Tconv cells, we performed differentially expressed gene analysis (Figures 1D and 1E and Tables S1 and S2). The results indicated that the top upregulated genes in GITR+ Treg cells included LAYN, TNFRSF9, IL2RA, and CCR8, suggesting the high inhibitory capacity of GITR+ Treg cells.33,34 Unexpectedly, GITR+ Tconv cells exhibited similar gene expression signature as GITR+ Treg cells. The correlation analysis of immune cell subclusters revealed complex associations between GITR+ CD4 T cells and other immune cells (Figure 1F).

The expression of GITR+ T cells in GC patients

Flow cytometry was used to analyze the expression of GITR in different types of T cells in tumor, peritumor, PBMCs, and TDLN samples from GC patients. The gating strategy was shown in Figure S1A. As shown in Figures 2A–2E, different tissues showed similar trend: GITR was highly expressed on Treg cells followed by Tconv cells and CD8 T cells. Treg cells from tumor tissues had the highest expression level of GITR (71.58%), followed by peritumor tissue (60.64%), TDLN (53.46%), and PBMCs (12.62%) (Figures 2F and 2G). Similar trend of GITR expression was also observed in Tconv cells from different tissue types (tumor 23.92%, peritumor tissue 11.97%, TDLN 9.77%, and PBMCs 2.84%) but at much lower levels (Figure 2H). As for CD8 T cells, they seldom expressed GITR (tumor 3.09%, peritumor tissue 1.77%, TDLN 2.20%, and PBMCs 0.85%) (Figure 2I). In summary, Treg cells were the major source of GITR in immune microenvironments, especially in tumor microenvironment. The percentage of GITR+ Tconv cells varied greatly among tissues. CD8 T cells, from various tissues, rarely had GITR expression, which might partially explain the poor efficacy of GITR agonistic mAbs treatments.

We further analyzed the correlation among the percentage of GITR+ cells in Treg cells, Tconv cells, and CD8 T cells. Although the expression of GITR differed among patients, we found the expression of GITR on Treg cells and Tconv cells were strongly correlated in different tissues (Figures S1B, S1E, S1H, and S1K). Meanwhile, the correlation of GITR expression between Tconv cells and CD8 T cells was only detected in tumor tissues (Figure S1C).

Furthermore, we categorized GC patients into different groups according to their clinical characteristics and compared their GITR expression in tumor tissues (Table S3). It showed that male patients had higher GITR expression on Tconv cells than female patients. Unexpectedly, significantly higher GITR expression on Tconv cells was detected in early T stage tumors as well as tumors with stronger proliferation capacity (higher Ki67%).

Figure 1. Single-cell Atlas of human GC

(A) Bubble plot depicting the expression of canonical marker genes in tumor-infiltrating immune cell clusters. The size of the circle represents the percentage of cells expressing the gene in that specific immune cell cluster, whereas the color represents the average expression of the gene.

(B) UMAP plot representation of 13 unique cell clusters color coded by their corresponding immune cell subtype. Each dot in the UMAP represents a single cell.

(C) Comparison of the infiltration levels of major cell types in 26 tumor tissues and 11 peritumor tissues from GC patients. Box plot center, box, and whiskers correspond to the median, IQR, and 1.5 × IQR, respectively.

(D and E) Volcano plot shows differentially expressed genes between GITR+ (red dots) and GITR− (green dots) CD4 T cells, including (D) Treg cells and (E) Tconv cells. The names of the partial significant genes are indicated in the plots. See also Tables S1 and S2.

(F) Pairwise Spearman’s correlations of immune cell subclusters are performed, using the average expression of marker genes. The color gradient shows the range of correlation coefficients from −1 (blue) to 1 (red). Data are represented as mean +/− SEM and were analyzed by unpaired Student’s t test in (C). *p < 0.05, **p < 0.01, ns: not significant.
Figure 2. Expression of GITR protein among T cell subsets from different tissues of GC patients
(A) Schematic gating of GITR on Treg cells (left), Tconv cells (middle), and CD8 T cell (right) from tumor tissues of GC patients.
(B–E) Expression of GITR among T cell subsets from (B) tumor tissues, (C) peritumor tissues, (D) TDLN, and (E) PBMC.
(F) Schematic gating of GITR on Treg cells from different tissues of GC patients.
(G–I) Expression of GITR on (G) Treg cells, (H) Tconv cells, and (I) CD8 T cells from different tissues. The schematic gating strategy for analysis of human T cells from GC or other tissues used in Figure 2 is shown in Figure S1A. Data are represented as mean ±/SEM and were analyzed by unpaired Student’s t test in (B–E and G–I). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Intratumoral GITR+ Treg cells possessed stronger immune-suppressive functions

When Treg cells exhibit high GITR expression, they are thought to play important roles in promoting tumor growth within the tumor immune microenvironment. To explore the features of intratumoral GITR+ T cells, positive percentage and MFI were used, as indicators, to evaluate parameters that can be divided into two subsets in flow and cannot, respectively. Schematic gating and representative histogram plots of each marker were shown in Figure S2. Notably, we found that GITR+ Treg cells from GC patients expressed higher levels of FOXP3 and CD25 (Figures 3A and 3B), which had been reported to represent a more stable state of Treg cells. In addition to high FOXP3 and CD25 expression, we found GITR+ Treg cells had higher levels of immune checkpoint molecules, including CTLA-4, PD-1, OX40, LAG3, and CD39 (Figures 3C–3G), indicating GITR+ Treg cells might exhibit powerful suppressive functions. The in vitro suppression assay confirmed that the GITR+ Treg cells had stronger immune suppressive capacity than the GITR− Treg cells (Figure 3H).

GITR+ Tconv cells exhibited less effector function but more immunosuppressive phenotype

As mentioned earlier, GITR+ Tconv cells also showed strong correlation with the clinical characteristics of GC patients. And several studies have reported GITR+ CD25low CD4 T cells exhibit immune regulatory
functions and help to maintain immune homeostasis. Thus, we also explored the functions of GITR+ Tconv cells. We found GITR+ Tconv cells in GC were weaker in effector function, with less interferon gamma (IFN-\(\gamma\)) and tumor necrosis factor alpha (TNF-\(\alpha\)) secretion, but stronger in regulatory function with higher CTLA-4 expression (Figures 4A–4D). GITR+ Tconv cells and GITR+ CD8 T cells, from GC samples, displayed similar expression pattern for immune checkpoint molecules as shown in Figures 4E–4H and 5D–5G, respectively. However, different from GITR+ Treg cells and GITR+ Tconv cells, GITR+ CD8 T cells were more activated and displayed increased tumor killing capacity (Figures 5A–5C). Furthermore, our results also suggested that the prospect for anti-GITR treatment in combination with other ICB therapies might be promising.

High-level of intratumoral GITR+ CD4 T cells was associated with higher Treg cells and less CD8 T cell infiltration

To further investigate the functions of GITR+ T cells in immune microenvironment, we looked into the correlation of GITR+ T cells by assessing the Treg/CD4 ratio and CD8/CD3 ratio. First, we analyzed the percentage of Treg cells and CD8 T cells in different tissue types. We found that tumor contained significantly more Treg cells than other tissues (tumor 18.02%, peritumor tissue 8.31%, TDLN 7.31%, PBMCs 5.09%) (Figure 6A). The percentage of CD8 T cells in tumor (29.56%) was lower than that in peritumor tissues (34.37%), although the difference was not significant. PBMCs (27.52%) ranked the third and TDLN (20.37%) was the lowest (Figure 6B).

Statistical analysis had revealed the significant positive correlations between the percentage of GITR+ Treg cells and Treg/CD4 ratio as well as the percentage of GITR+ Tconv cells and Treg/CD4 ratio in GC tumor samples (Figures 6C and 6E), whereas the density of CD8 T cells was negatively correlated with them (Figures 6D and 6F). Our results indicated the existence of a stronger immunosuppressive microenvironment, in GC patients, formed by higher density of GITR+ Treg cells and GITR+ Tconv cells. GITR+ CD8 T cells showed no significant impact on immune microenvironment, probably because the percentage of GITR+ CD8 T cells was low (Figures 6G and 6H).

In addition, we also examined the effect of GITR+ T cells on the immune microenvironment of other tissues (Figure S4). We could only observe positive correlation between GITR+ Tconv and Treg ratio in TDLN and PBMCs but not peritumor tissues (Figures S4D and S4F). GITR+ Treg and GITR+ Tconv ratio in tumor and peritumor tissues was positively correlated (Figures S4A and S4D). It indicated GITR+ T cells, in different environments, exhibited different functions.
High-level of intratumoral GITR+ CD4 T cells was associated with poor prognosis

As GITR+ CD4 T cells tended to cultivate the immune-suppressive TME, we investigated the prognostic value of tumor-infiltrating GITR+ Treg cells and GITR+ Tconv cells. We constructed tumor microarray (TMA) and used multiple immunofluorescence (IF) to examine the expression of GITR in FOXP3+ CD4+ T cells and FOXP3−/CD4+ T cells in GC tumor samples (Figure 7A). Depending on the GITR expression level, we divided GC patients into four groups, including GITR+ Treg high, GITR+ Treg low, GITR+ Tconv high, and GITR+ Tconv low (TMA cohort, n = 84) (Figure 7B). The clinical characteristics of GC patients were presented in Table S4. The survival curves revealed that GC patients with high level of tumor infiltrating GITR+ Treg cells had shorter OS than patients with low tumor infiltrating GITR+ Treg cells (Figure 7C). Similarly, higher density of GITR+ Tconv cells indicated worse prognosis (Figure 7F). To validate our finding, we further constructed another two cohorts (TCGA cohort, n = 366 and GSE15459 cohort, n = 191) by bioinformatic analysis (STAR methods).2 Both cohorts showed GITR+ CD4 T cells predicted shorter OS of GC patients (Figures 7D–7E, 7G, and 7H).

GITR protein distribution varied greatly in Treg cells from different tissues

In general, immune checkpoints are expressed on cell surface and function by binding to their ligands.41 However, there are studies reported that a proportion of ICs is expressed intracellularly.42 As GITR expression levels on T cells differed across tissues (Figures 2G–2I), we speculated whether T cells contained a portion of intracellular GITR. Flow cytometric analysis had revealed that nearly all Treg cells expressed GITR, which were designated as total GITR (GITR[TOT]) (Figure 8A). Among GC patients, Treg cells from tumors expressed the highest level of GITR (93.23%), followed by peritumor tissues (86.70%), TDLN (74.30%), and PBMCs (73.63%). We also measured the expression of surface GITR (GITR[SUR]) on Treg cells (Figure 2G). By comparison, we found the GITR[TOT] expression level was significantly higher than GITR[SUR] on Treg cells, suggesting a large proportion of GITR was stored intracellularly (GITR[ICS]) (Figure 8B). In addition, unlike Treg cells from other tissues, Treg cells from PBMCs of GC patients expressed most GITR intracellularly (Figure 8C). We further investigated the location of GITR in Treg cells using confocal microscope. Under confocal microscopy, we observed two GITR expression modes, on cell surface (Figure 8D) and intracellular (Figure 8E), in Treg cells from the tumor tissues and PBMCs of GC patients, respectively. We also observed clear images of intracellular GITR protein in induced Treg (iTreg) cells (Figure 8F), which also could be validated by another two GITR antibodies (Figure S6C).

We also explored GITR expression mode on Tconv cells (Figures 8G–8I, S7A, and S7B) and CD8 T cells (Figures 8J–8L, S7C, and S7D). Unlike Treg cells, we found Tconv cells and CD8 T cells mainly stored...
DISCUSSION

In our study, we analyzed scRNA-seq data of GC patients and identified the accumulation of CD4 T cells in TME. The GITR+ CD4 T cell subsets seemed to play important roles. Next, we examined GITR expression on different T cells in GC patients. We confirmed GITR was mainly expressed by Treg cells, followed by Tconv cells and CD8 T cells in different tissue types. We also compared GITR expression among tissues and found the expression of GITR in Treg cells and Tconv cells varied largely across tissues, which was different from consistent low GITR expression on CD8 T cells. The different distribution of GITR+ Treg cells and GITR+ Tconv cells among tissues may be attributed to following reasons. First, T cells residing in non-lymphoid tissues adapt distinct phenotypic and functional properties relative to their circulating counterparts in the peripheral blood. Apart from that, GITR expression can be upregulated by T cell receptor (TCR) stimulus. This might be because the stimulation of TCR, by
Figure 7. High-level of tumor-infiltrating GITR+ CD4 is associated with poor prognosis

(A) Human GC specimen stained with DAPI (blue), FOXP3 (red), CD4 (green), and GITR (yellow). The lower panel is merged view of the four upper images. The scale bar (200 µm) is placed at the left corner of each image.
ever, different from mice tumor model, CD8 T cells expressed extremely low level of GITR in human GC. In some immune treatment might be very promising. However, a study reported that the PD-1 expression balance between levels of other immune checkpoints such as CTLA-4, PD-1, and OX40, suggesting that the combination studies were needed for GITR+ Tconv cells. In addition, we observed that GITR+ T cells expressed high effector functions but more immune-suppressive phenotype. More detailed analysis such as subsets exhibited stronger immune-suppressive functions. Intratumoral GITR+ Tconv cells possessed weaker Then we analyzed the features of GITR+ T cells and found that GITR+ Treg cells were more activated and restrained GITR expression intracellularly, which could be a cross-generational change in the history of the factors or the mechanisms underlining the phenomenon, we might be able to block this process and tors that could facilitate the externalization of intracellular GITR in tissues (except PBMCs). If we could find GITR synthesis, but the externalization of intracellular GITR. In other words, there might be some key fac-

Various molecules, activates T cells, which consistently express GITR at high level. For example, in stomach, Helicobacter pylori infection leads to the release of specific molecules, from damaged cells, which may stimulate TCR and activate T cells.5,23 In TME, tumor-derived neoantigens may also activate T cells via TCR stimulation, which may account for the highest expression of GITR in GC tumor tissues.3,53 In the end, we observed that there was a large part of GITR expressed intracellularly in Treg cells (especially Treg cells derived from PBMCs). Previous studies have shown that GITR is highly expressed when T cells are activated.8. We hypothesized that most increase of cell surface GITR expressions were not because of GITR synthesis, but the externalization of intracellular GITR. In other words, there might be some key factors that could facilitate the externalization of intracellular GITR in tissues (except PBMCs). If we could find the factors or the mechanisms underlining the phenomenon, we might be able to block this process and restrain GITR expression intracellularly, which could be a cross-generational change in the history of immunotherapy.

Apart from Treg cells, we found Tconv cells and CD8 T cells also expressed a small fraction of GITR on cell surface. In other words, it is feasible to activate the effector CD8 T cells by promoting the expression of GITR Sur or to optimize the specificity of GITR-targeting drugs.

Above all, we speculate that GITR-targeting therapy cannot get satisfactory efficacy in clinical trials may be attributed to the following reasons. First, except intratumoral Treg cells, peritumor-tissue-derived Treg cells also expressed a relatively high level of GITR, especially on the surface of Treg cells. We speculate the large proportion of GITR expressed within peritumor tissues might reduce the efficacy of GITR agonistic mAbs used in clinical trials. More precise GITR-targeting drugs are needed for cancer treatments. Second, previous studies show that GITR-induced depletion of Treg cells is not sufficient to generate significant antitumor immunity, and effective engagement of GITR on effector T cells is also needed.56–58 However, different from mice tumor model, CD8 T cells expressed extremely low level of GITR in human GC.59 So the GITR-induced antitumor response may not be as efficient as in mice. Moreover, most GITR-targeting therapies only focus on inhibiting Treg cells and activating CD8 T cells but neglect their effects on CD4 T cells.13 Schoenhals et al. have reported the efficacy of GITR-related therapy depends more on CD4 T cells followed by CD8 T cells in preclinical model.60 However, based on our results, we report that GITR+ Tconv cells are less effective but show more immune-suppressive phenotype in human GC. In other words, agonistic anti-GITR therapy may not promote effector functions of Tconv cells in human cancers.
Moreover, different GITR distribution in T cells across tissues may affect the efficacy of GITR-targeting therapy. Some strategies may be used to improve the treatment targeting GITR. First, combine with ICB for GITR+ T cells expressing high level of immune checkpoints. Studies have reported the combination treatment of GITR and ICB is more effective.26,60 Apart from ICB, other treatment could also be used in GITR-targeting therapy, such as antibodies promoting ADCC59,61,62 and radiotherapy.60 The approaches are mutually exclusive and could improve the efficacy of anti-GITR therapy via other mechanisms. Recently, rational protein engineering seems to boost GITR-mediated antitumor immunity.63 What’s more, if possible, based on our results, it is valuable to figure out the mechanism of GITR externalization for the upregulation of GITR expression on CD8 T cells.

In conclusion, our study has revealed the complex expression mode of GITR in T cell subtypes from various tissues of GC patients. GITR+ Treg cells and GITR+ Tconv cells tend to be immune-suppressive in tumor immune microenvironment and associate with poor prognosis for GC patients. Moreover, there is a notable amount of GITR expressed intracellularly in T cells and it may be valuable to figure out the mechanisms for the externalization of GITR.

Limitations of the study
In this study, we mainly focus on GITR expression on T cells. There are other studies reported that GITR is expressed by other cells in TME, such as NK cells64,65 and tumor cells,64,67 which may also be functional and affect the efficacy of anti-GITR antibodies. And in this study, we have only reported the phenomenon such as distribution of GITR in T cells. More experiments need to be performed to reveal the mechanism underlying them.

STARMethods
Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105529.

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AUTHOR CONTRIBUTIONS
S.K., Y.G., and F.X. designed the study. S.K., Z.W., and H.G. collected and prepared the samples. S.K., Y.G., F.X., and Y.Y. were responsible for experiments. D.X. and X.L. were responsible for the pathologic diagnosis of patients. F.X. analyzed and interpreted the data. S.K. and J.C. wrote the manuscript. X.X., F.Y., C.Z., Z.Z., G.Z., B.L., and W.Z. revised the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| PE anti-human GITR (108–17) | Biolegend | Cat# 371204, RRID:AB_2616841 |
| BV 711 anti-human GITR (108–17) | Biolegend | Cat# 371212, RRID:AB_2687161 |
| PE anti-human GITR (DT5D3) | Miltenyi Biotec | Cat# 130-121-331, RRID: AB_2784152 |
| BV650 anti-human CD3 | Biolegend | Cat# 300468, RRID:AB_2629574 |
| BV510 anti-human CD8 | Biolegend | Cat# 344732, RRID:AB_2564624 |
| PE/Cyanine7 anti-human CD4 | Biolegend | Cat# 300512, RRID:AB_314080 |
| PerCP/Cyanine5.5 anti-human CD4 | Biolegend | Cat# 300530, RRID:AB_893322 |
| FITC anti-human CD4 | Biolegend | Cat# 300506, RRID:AB_314074 |
| BV421 anti-human CD4 | Biolegend | Cat# 300531, RRID:AB_314074 |
| APC anti-human FOXP3 | Thermo Fisher Scientific | Cat# 17-4777-42, RRID:AB_10900084 |
| FITC anti-human CD25 | Biolegend | Cat# 356104, RRID:AB_2561863 |
| PE/Cyanine7 anti-human CD127 | Thermo Fisher Scientific | Cat# 25-1278-42, RRID:AB_1659672 |
| PE/Cyanine7 anti-human CD152 (CTLA-4) | Biolegend | Cat# 349914, RRID:AB_2563098 |
| BV421 anti-human CD279 (PD-1) | Biolegend | Cat# 329920, RRID:AB_10960742 |
| PerCP/Cyanine5.5 anti-human CD134 (OX40) | Biolegend | Cat# 350010, RRID:AB_10719224 |
| Alexa Fluor 647 anti-human CD223 (LAG-3) | Biolegend | Cat# 369204, RRID:AB_2566478 |
| APC anti-human CD39 | Biolegend | Cat# 328209, RRID:AB_1953233 |
| FITC anti-human IFN-gamma | Biolegend | Cat# 502507, RRID:AB_315232 |
| BV421 anti-human TNF-alpha | Biolegend | Cat# 502932, RRID:AB_10960738 |
| PE anti-human/mouse Granzyme B Recombinant | Biolegend | Cat# 98377, RRID:AB_2747370 |
| FoxP3 (D2WBE) Rabbit mAb | Cell Signaling Technology | Cat# 98377, RRID:AB_2747370 |
| CD4 (EP204) Rabbit mAb | Cell Signaling Technology | Cat# 48274, |
| GITR (D919D) Rabbit mAb | Cell Signaling Technology | Cat# 68014, RRID:AB_2997940 |
| CD4 (EPR6855) Rabbit mAb | abcam | Cat# ab133416, RRID:AB_2750883 |
| GITR (EPR20566) Rabbit mAb | abcam | Cat# ab223841, RRID:AB_2889261 |
| GITR (DT5D3) Mouse mAb | Miltenyi Biotec | Cat# 130-092-885, RRID:AB_871549 |
| CD4 (4E10F3) Mouse pAb | Servicebio | Cat# GB13064-2, RRID:AB_2892096 |
| **Biological samples**      |        |            |
| GC patient tumor tissues | Renji Hospital, School of Medicine, Shanghai Jiao Tong University | N/A |
| GC patient peritumor tissues | Renji Hospital, School of Medicine, Shanghai Jiao Tong University | N/A |
| GC patient TDLN | Renji Hospital, School of Medicine, Shanghai Jiao Tong University | N/A |
| GC patient peripheral blood | Renji Hospital, School of Medicine, Shanghai Jiao Tong University | N/A |
| formalin-fixed paraffin-embedded GC blocks | Renji Hospital, School of Medicine, Shanghai Jiao Tong University | N/A |
| **Deposited data**        |        |            |
| Processed scRNA-seq data of human GC and peritumor tissue | Kumar et al., | GEO: GSE183904 |
| Processed RNA-seq data of human GC | The Cancer Genome Atlas | https://xenabrowser.net |
| Processed RNA-seq data of human GC | Lei et al., | GEO: GSE15459 |
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Wenyi Zhao (zhaowy2win@yeah.net).

Materials availability
This study did not generate new unique reagents.

Data and code availability
- This paper analyzes existing, publicly available data. Single-cell RNA-seq data used in Figure 1 were downloaded from the Gene Expression Omnibus repository: GSE183904. Original data of survival analysis for Figure 7 in this paper were obtained from The Cancer Genome Atlas (TCGA) database and GSE15459. These accession numbers for the datasets are also listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human subject
A total of 33 patients, pathologically diagnosed with GC at Ren Ji Hospital Shanghai Jiao Tong University, were enrolled in this study. Paired, fresh tumor and peritumor tissue samples (at least 5 cm from matched tumor tissues), TDLN and peripheral blood samples were collected. Detailed clinical and pathological information including age, gender, and tumor size were listed in Table S3. The tissue and blood samples were used to isolate peripheral and tissue-infiltrating immune cells for flow cytometry analysis. A retrospective analysis of 84 primary GC patients (TMA cohort) who underwent gastrectomy at Ren Ji Hospital, from January 2016–December 2017, was also carried out. The final follow-up date, for all cases examined, was January 20, 2022. OS time was defined as the interval between the gastrectomy and patient death or survival by the end of the final follow-up date. All patients received the standard treatments such as D2 radical resection and first-line adjuvant chemotherapy according to the NCCN guide. Formalin-fixed paraffin embedded (FFPE) tissue blocks of tumor were collected for TMA construction and IF. The available clinical features of TMA cohort were summarized in Table S4.

We excluded the following types of patients: (1) patients with recurrent GC after the radical operation, (2) patients receiving neoadjuvant chemotherapy or previous radiotherapy, (3) patients suffering from other malignant tumors, and (4) patients with autoimmune or immunodeficiency diseases. Tumor TNM stage was assigned based on pathological tumor, node, and metastasis staging per the American Joint Committee on Cancer (AJCC 8th edition) staging system.

All patients were given informed consent for collection of clinical information, tissue collection, research testing under Institutional Review Board (IRB)-approved protocols (2017-114-CR-02 and KY2022-174-B) at Renji Hospital Shanghai Jiao Tong University.

METHOD DETAILS

Unsupervised clustering and markers identification
The digital expression matrices, in which the low-quality cells had been filtered out by the original authors, were imported into the Scanpy module for standard downstream pipeline analysis. Firstly, the data were normalized and scaled. Principle component analysis (PCA) of 2,192 genes variably expressed across all 158,415 cells was performed. Subsequently, BBKNN was used to remove the batch effect from different samples and create the neighbor graph with parameter n_pcs equal to 30. Then, we used Uniform Manifold Approximation and Projection (UMAP) to embed the graph in two dimensions and the Leiden graph-clustering to cluster the neighborhood graph of cells with resolutions parameter equal to 1. This identified a number of cell populations that could be easily assigned to known cell lines by tagging genes. Besides, in order to perform more explicit sub-groups of T cells, we extracted T cells according to high expression of CD3E and re-clustered them with steps similar to those described above. After then, the obtained objects were input into the R package Seurat for visualization and differential expressed gene analysis.
Differentially expressed gene analysis for GITR+ and GITR- CD4 T Cells

To define feature genes between GITR+ and GITR- CD4 T cells (including Treg and Tconv), differential expression analysis was carried out using the “FindMarkers” function implemented in the Seurat package, with log-scaled fold change ≥ 0.25 and adjusted p value < 0.05 (Wilcoxon Rank Sum test). Differentially expressed genes passing the criteria were shown in Tables S1 and S2.

Cell isolation

Fresh tissue samples were surgically removed from patients and immersed in a complete medium containing 90% Dulbecco’s modified eagle medium (DMEM; Cat# 11054001, GIBCO) and 10% fetal bovine serum (FBS; Cat# 16140071, GIBCO), and transported to the lab in a refrigerated container within 4 h. Tissues were cut into pieces (diameter 1–3 mm) and incubated in 3 mL complete medium containing 2 mg/mL type IV collagenase (Cat# C5138, Sigma) and 50 U/mL DNase I (Cat# DN25, Sigma) for 40 min at 37°C on a shaker (200 rpm). Then the dissociated cell suspensions were filtered through 50 μm nylon meshes to obtain single-cell suspensions. PBMC were isolated by density gradient centrifugation (speed at 2000 rpm; Acceleration ramp 3 and Braking ramp 0) for 20 min using Ficoll-Paque Plus (Cat# 17-1440-03–1, GE Healthcare).

Flow cytometry and sorting

Single cell suspensions were then stained with various fluorochrome-conjugated antibodies (as shown in key resources table) in FACS buffer (PBS containing 2% FBS). After being washed with FACS buffer, cells were incubated for 30 min at 4°C in dark for surface markers staining and dead cells were labeled with Fixable Viability Dye (Cat# 65-0865-14, eBioscience). Cells were then fixed and permeabilized with Fixation/Permeabilization concentrate (Cat# 00-5521-00, eBioscience) for 40 min at room temperature (22–24°C) in dark. Intracellular targets were stained for 40 min at 4°C in dark followed by two wash steps with permeabilization buffer (Cat# 00-8333-56, eBioscience). To determine cytokine expression, cells were stimulated with 0.25 μg/mL PMA (Cat#P1585, Sigma), 0.25 μg/mL ionomycin (Cat#I3909, Sigma) and 0.1% golgistop (Cat#554724, BD Bioscience) for 4 h before staining. For sample acquisition, a BD LSRFortessa X-20 cell analyzer embedded with FACS DIVA software (BD Bioscience) was used. Results were analyzed with FlowJo v10 (FlowJo LLC).

As for flow sorting, tumor-infiltrating lymphocytes (TIL) were isolated from GC single cell suspensions by density gradient centrifugation (speed at 2000 rpm; Acceleration ramp 6 and Braking ramp 2) for 25 min using Percoll (Cat# 17-0891-01, GE Healthcare). After being stained and labeled with Fixable Viability Dye, CD4+CD25hiCD127lo T cells were sorted upon GITR expression using a BD FACSAria III (BD Biosciences).

In vitro Treg suppression assay

CD8 T cells were isolated from PBMCs of healthy donor using CD8 MicroBeads (Cat# 130-045-201, Miltenyi Biotec) and utilized in the Treg suppression assay. Purified CD8 T cells (1 × 10^3) were labeled with Cell Trace Violet and then cultured in 96 well round bottom plates in 200 μL complete media. Anti-CD3/CD28 Dyna-Beads (Cat# 11132D, GIBCO) were added along with cultured flow sorted Treg populations (GITR+ Treg and GITR- Treg) at the indicated ratios. Cells were incubated at 37°C for 72 h. The proliferation and suppression of CD8 T cells were assessed by flow cytometry. Proliferation of responder T cells was evaluated by CellTrace Violet dilution. Percent suppression of CD8 T cell was calculated with values representing the ratio of total divided peaks to both divided and non-divided peaks, and then normalized to the anti-CD3/CD28 alone experimental group.

PBMC paraffin embedding and TMA construction

PBMCs were fixed with 5% paraformaldehyde (Cat# G1101, Servicebio) for 10 min. The cellular pellet, after centrifugation, were mixed with 45°C agar solution (Cat# 110GR100, BioFroxx). PBMCs containing agar were solidified into agar block at 4°C for 30 min. Then the PBMCs containing agar block was paraffin embedded after being gradient dehydrated ethanol and transparent by ethanol and Xylene, respectively.

We collected FFPE GC tissue blocks from the pathology department of Renji Hospital. The diagnosis of each case was confirmed by two senior pathologists through a review of H&E-stained slides. Representative FFPE blocks were chosen to be punched onto glass slides for the construction of TMA. Tumor tissues from corresponding patients were consecutively aligned on the TMA, and the TMA was constructed using a tissue arrayer with 5 μm thickness.
Multiplexed immunofluorescence staining (tyramide signal amplification, TSA)

Multiplex staining was performed using PANO 7-plex IHC kit (Cat# 0004100100, Panovue) according to manufacturer’s instruction. CD4 (EP204), FoxP3 (D2W8E), GITR (D9I9D) antibodies were sequentially applied, followed by horseradish peroxidase-conjugated secondary antibody incubation and tyramide signal amplification. The slides were microwave heat-treated after each tyramide signal amplification. Nuclei were stained with DAPI after all the antigens above had been labeled. The stained slides were scanned to obtain multispectral images using the Mantra System (PerkinElmer), which captured the fluorescent spectra at 20-nm wavelength intervals from 420 to 720 nm with identical exposure time. The densities of GITR + Treg and GITR + Tconv were recorded as the mean number of cells/HPF from 6 randomized fields counted by 2 independent pathologists (each with 3 fields) who were blinded from the clinical data.

In vitro cell culture

Human CD4⁺ CD25loCD45RAhi naive T cells were flow sorted from the PBMCs of healthy donor using BD FACSAria II (BD Bioscience). Cells were differentiated into induced Treg (iTreg) using anti-CD3/CD28 DynaBeads at a 1:4 bead-to-cell ratio in cell culture media [X-VIVO (Cat# 04-418Q, Lonza), supplemented with 10% FBS (Cat# 10100147C, Gibco), 1% L-Glutamine-100X (Cat# 335050061, Gibco), 1% MEM Non-Essential Amino Acids-100X (Cat# 11140050, Gibco), 1 mM sodium pyruvate (Cat# 11360070, Gibco), 1% Antibiotic-Antimycotic 100x (Cat# 15240112, Gibco)], 100 U/mL rhIL-2 (Cat# 200–02, Peprotech) and 5 ng/mL hTGF-β1 (Cat# 7754-BH-100/CF, R&D). After 5 days of culture, cells were characterized by intracellular flow cytometry for Foxp3 expression.

Indirect immunofluorescence staining and confocal microscopy

iTregs were seeded into a 15 mm cell culture plate coated with lysine (Cat# P4707, Sigma), fixed with 4% paraformaldehyde for 10 minutes, blocked in PBS containing 5% BSA and 0.2% Triton X-100 for 1 h, and then incubated with primary antibodies overnight at 4°C. The cells were then washed three times with PBS and incubated with Alexa Fluor 488/555-conjugated secondary antibodies (Cat# A32731, Cat# A32727, eBioscience) and DAPI (Cat# C1002, Beyotime) for 1 h at room temperature. Immunofluorescence (both indirect immunofluorescence and TSA immunofluorescence) was detected using a Leica TCS Sp8 STED confocal microscope.

QUANTIFICATION AND STATISTICAL ANALYSIS

Survival analysis

We analyzed the association of GITR + CD4 T cell density and OS in TMA cohort. We further validated the correlation between GITR + CD4 T cell and prognosis of GC patients in TCGA and GSE15459 databases. For survival analysis, the samples in the database were divided into high and low expression groups based on the mean expression of feature genes. We analyzed the gene signature of GITR + Treg and GITR + Tconv from the single-cell RNA sequencing data of gastric cancer (obtained from GSE183904). The optimal cutoff value was calculated using the R package survminer. Kaplan–Meier survival curves were then plotted to show the differences in survival time. The log-rank p values, reported by the Cox regression models, implemented in the R package were used to determine the statistical significance. The GITR + Treg feature genes included CD4, FOXP3, TNFRSF18, IL2RA, IKZF2, LAYN, CTLA4, IL21R, BATF, TNFRSF9, and CCR8, while the GITR + Tconv feature genes included CD4, TNFRSF18, CCL5, GZMA, KIR2DL4, HOPX, TYROBP, FCER1G, AREG, TMIGD2, TRDC, KRT86, GNLY, CLIC3, and KLRC1.

Statistical analyses

Comparisons of the percentage of different cell types between tumor and paraneoplastic tissues in the scRNA-seq data were calculated by t-test. The Spearman’s correlations of different cell infiltration ratios were calculated using the rcorr function in the Hmisc package. Other statistical analyses were performed using SPSS 23.0 and GraphPad Prism 8.0. T-tests and ANOVA were used to examine associations between continuous variables. Chi-square tests were performed to analyze relationships between categorical variables. Pearson’s correlation analysis and linear regression analysis were used to assess correlations between variables. All p values were two-tailed, and p < 0.05 was considered statistically significant.