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ORIGINAL ARTICLE

CD39 expression defines exhausted CD4$^+$ T cells associated with poor survival and immune evasion in human gastric cancer

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Abstract

Objectives. CD4$^+$ T cell helper and regulatory function in human cancers has been well characterised. However, the definition of tumor-infiltrating CD4$^+$ T cell exhaustion and how it contributes to the immune response and disease progression in human gastric cancer (GC) remain largely unknown. **Methods.** A total of 128 GC patients were enrolled in the study. The expression of CD39 and PD-1 on CD4$^+$ T cells in the different samples was analysed by flow cytometry. GC-infiltrating CD4$^+$ T cell subpopulations based on CD39 expression were phenotypically and functionally assessed. The role of CD39 in the immune response of GC-infiltrating T cells was investigated by inhibiting CD39 enzymatic activity. **Results.** In comparison with CD4$^+$ T cells from the non-tumor tissues, significantly more GC-infiltrating CD4$^+$ T cells expressed CD39. Most GC-infiltrating CD39$^+$CD4$^+$ T cells exhibited CD45RA$^-$CCR7$^-$ effector–memory phenotype expressing more exhaustion-associated inhibitory molecules and transcription factors and produced less TNF-α, IFN-γ and cytolytic molecules than their CD39$^-$CD4$^+$ counterparts. Moreover, *ex vivo* inhibition of CD39 enzymatic activity enhanced their functional potential reflected by TNF-α and IFN-γ production. Finally, increased percentages of GC-infiltrating CD39$^+$CD4$^+$ T cells were positively associated with disease progression and patients' poorer overall survival. **Conclusion.** Our study demonstrates that CD39 expression defines GC-infiltrating CD4$^+$ T cell exhaustion and their immunosuppressive function. Targeting CD39 may be a promising therapeutic strategy for treating GC patients.

Keywords: CD39, CD4$^+$ T cells, exhaustion, gastric cancer, immunotherapy

INTRODUCTION

Tumor progression is influenced by the cross-talk between cancer cells and hosts immune elements including T cell infiltration at the tumor site, especially CD8$^+$ T cells that are often associated with a better clinical outcome in human cancers.[1–3] However, T cell infiltration does not always ensure antitumor immunity, especially during tumor progression,[4,5] and this insufficient T cell antitumor immunity has been acknowledged as a result of T cell exhaustion characterised by the downregulation of effector function. Most studies on T cell exhaustion have primarily focused on CD8$^+$ T cells because of their direct tumor-killing roles.[6–8] As a result, whether other tumor-infiltrating immune cells, including CD4$^+$ T cells, are also exhausted remains underappreciated.

There is increasing evidence that CD4$^+$ T cells play a crucial role in antitumor adaptive immunity.[9] Studies have indicated that CD4$^+$ T cells not only licence dendritic cells to allow optimal priming of CD8$^+$ T cell antitumor immune response but also lyse tumor cells directly by releasing cytokines and cytolytic molecules.[10–13] Nevertheless, paralleling CD8$^+$ T cell exhaustion, tumor-infiltrating CD4$^+$ T cells can also progressively lose their effector activity after upregulating multiple immune inhibitory molecules such as programmed cell death protein 1 (PD-1).[14–16] In addition to reinvigorating CD8$^+$ T cells, targeting PD-1 has also been reported to restore CD4$^+$ T cell antitumor activity, suggesting that CD4$^+$ T cell exhaustion is associated with their expression of PD-1.[17] Additionally, one recent study points out that CD39 expression, rather than PD-1, is more reflective of CD4$^+$ T cell exhaustion in human cancers, for CD4$^+$ T cells expressing CD39 have higher levels of PD-1 expression and produce fewer effector cytokines.[18] Nevertheless, the relationship between CD39 or PD-1 expression by tumor-infiltrating CD4$^+$ T cells and their effector function in human gastric cancer (GC) remains unclear.

In the present study, we investigate the exhausted feature of tumor-infiltrating CD4$^+$ T cells and their potential roles in GC. We show that CD39 marks GC-infiltrating CD4$^+$ T cell exhaustion, association with tumor progression and immune evasion. We further demonstrate that POM-1, an inhibitor of CD39 enzymatic activity, has effectively improved the effector function of GC-infiltrating CD4$^+$ T cells. Thus, strategies that inhibit CD39 activity could potentially be immunotherapeutic agents against GC.

RESULTS

GC patients show increased percentages of CD39$^+$CD4$^+$ T cells in their tumor tissues

To explore whether tumor-infiltrating CD4$^+$ T cells in GC patients were exhausted, their PD-1 expression was first examined (Figure 1a, Supplementary figure 1). Although the percentages of PD-1$^+$CD4$^+$ T cells in the non-tumor and tumor tissues were significantly increased when compared with their peripheral blood CD4$^+$ T cells, there was no significant difference in the percentages of PD-1$^+$CD4$^+$ T cells between the non-tumor and tumor tissues, and similar observations were made when analysing PD-1 expression on CD4$^+$ T cells as the mean fluorescence intensity (MFI) (Figure 1b and c), suggesting that the PD-1 expression levels on tumor-infiltrating CD4$^+$ T cells were not altered. We then assessed CD39 expression on CD4$^+$ T cells in the different samples from GC patients (Figure 1d). The percentages of CD39$^+$CD4$^+$ T cells in the non-tumor and tumor tissues were significantly higher than those in the peripheral blood, and the tumor tissues also contained higher percentages of CD39$^+$CD4$^+$ T cells than those in the non-tumor tissues (Figure 1e). In addition, the MFI of CD39 expression on CD39$^+$CD4$^+$ T cells in the tumor tissues was significantly higher than their counterparts in the peripheral blood and non-tumor tissues (Figure 1f). Moreover, data from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) also showed that compared with the non-tumor tissues, the tumor tissues from GC patients contained more CD4$^+$ T cells expressing CD39, but fewer CD4$^+$ T cells expressing PD-1 (Supplementary figure 2). These data indicate that more CD4$^+$ T cells express CD39 in the tumor tissues of GC patients.

GC-infiltrating CD39$^+$CD4$^+$ T cells are activated and display an exhausted phenotype

Inasmuch as an increased CD39$^+$CD4$^+$ T cell percentage in the tumor tissues, we delineated the phenotype of these cells. The differentiation status of CD4$^+$ T cells was divided into naive (Tn, CD45RA$^+$CCR7$^+$), central memory (Tcm, CD45RA$^-$CCR7$^+$), effector memory (Tem, CD45RA$^-$CCR7$^-$) and terminally differentiated effector memory (Temra, CD45RA$^+$CCR7$^-$). CD4$^+$ T cells in the tumor tissues were mainly composed

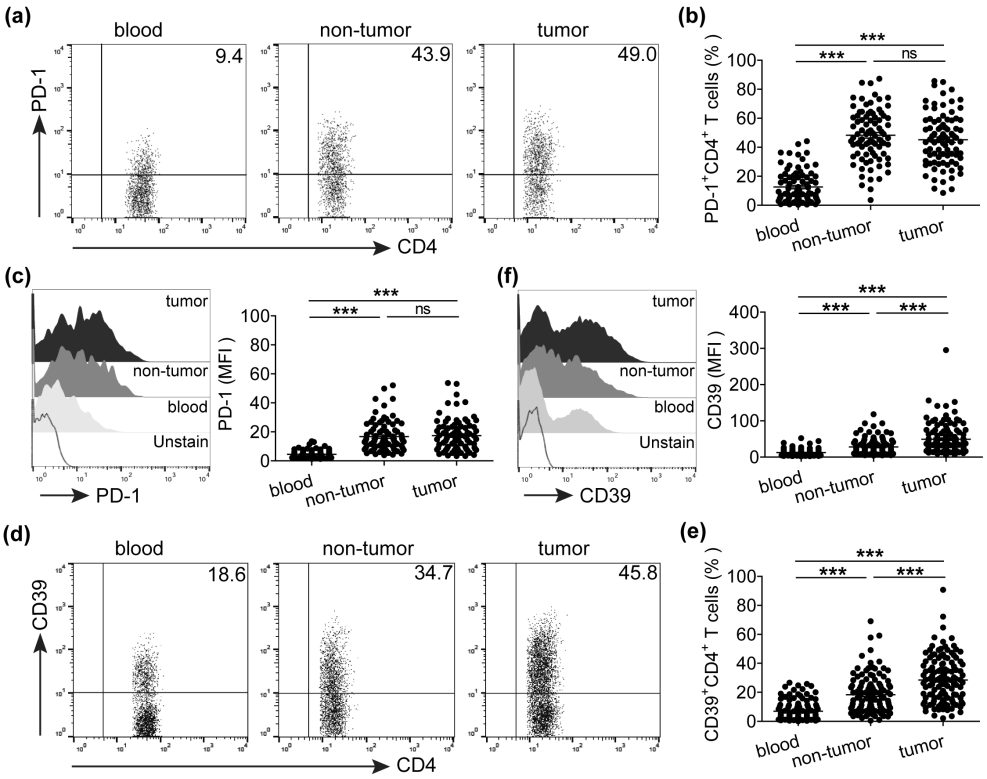

Figure 1. PD-1 and CD39 expression on CD4^{+} T cells in the peripheral blood, non-tumor and tumor tissues of GC patients. **(a)** A representative flow cytometry analysis of PD-1 expression on CD4^{+} T cells in the peripheral blood, non-tumor and tumor tissues of the same patient. **(b)** The percentages of PD-1^{+} cells in CD4^{+} T cells were statistically analysed in the peripheral blood, non-tumor and tumor tissues of GC patients (n = 85). **(c)** The MFI of PD-1 expression on CD4^{+} T cells was analysed in the peripheral blood, non-tumor and tumor tissues of GC patients (n = 85). **(d)** A representative flow cytometry analysis of CD39 expression on CD4^{+} T cells in the peripheral blood, non-tumor and tumor tissues of the same patient. **(e)** The percentages of CD39^{+} cells in CD4^{+} T cells were statistically analysed in the peripheral blood, non-tumor and tumor tissues of GC patients (peripheral blood: n = 124, non-tumor tissues: n = 115, tumor tissues: n = 128). **(f)** The MFI of CD39 expression on CD4^{+} T cells was analysed in the peripheral blood, non-tumor and tumor tissues of GC patients (peripheral blood: n = 124, non-tumor tissues: n = 115, and tumor tissues: n = 128). ***P < 0.001, ns, not significant: Kruskal–Wallis test and Dunn's multiple-comparisons test.

of the Tem subset, and their percentages were significantly higher than those in the peripheral blood, whereas the percentages of Tn, Tcm and Temra subsets were significantly decreased (Figure 2a). CD39^{+}CD4^{+} T cells in the tumor tissues were also primarily composed of the Tem subset, but their percentages of Tn, Tcm, Tem and Temra subsets were similar to those of their CD39^{-}CD4^{+} counterparts (Figure 2b), implying that most GC-infiltrating CD39^{+}CD4^{+} T cells were effector memory cells.

We next analysed the expression of other surface molecules on the CD39^{+}CD4^{+} T cells of the tumor tissues. Although there was no significant difference in the expression of tissue-resident memory marker CD103, costimulatory molecules CD28 and CD27, lymph node homing marker CD62L between CD39^{+}CD4^{+} and CD39^{-}CD4^{+} T cells, and CD39^{+}CD4^{+} T cells exhibited a reduced expression of effector molecules CD127 and KLRG1 (Figure 2c). Additionally, significantly more CD39^{+}CD4^{+} T cells expressed activation molecules CD69 and HLA-DR, and costimulatory molecules ICOS, 4-1BB and OX40 than their CD39^{-}CD4^{+} counterparts. Furthermore, more CD39^{+}CD4^{+} T cells, than CD39^{-}CD4^{+} T cells, expressed multiple co-inhibitory molecules PD-1, CTLA-4, TIGIT, GITR and NKG2A, although the proportions of Tim-3, LAG-3, 2B4, BTLA and VISTA expression of CD39^{+}CD4^{+} T cells were similar to those of their CD39^{-}CD4^{+} counterparts (Figure 2d). These findings indicate that CD39^{+}CD4^{+} T cells in the tumor tissues are activated and exhibit an exhausted phenotype than their CD39^{-}CD4^{+} counterparts.

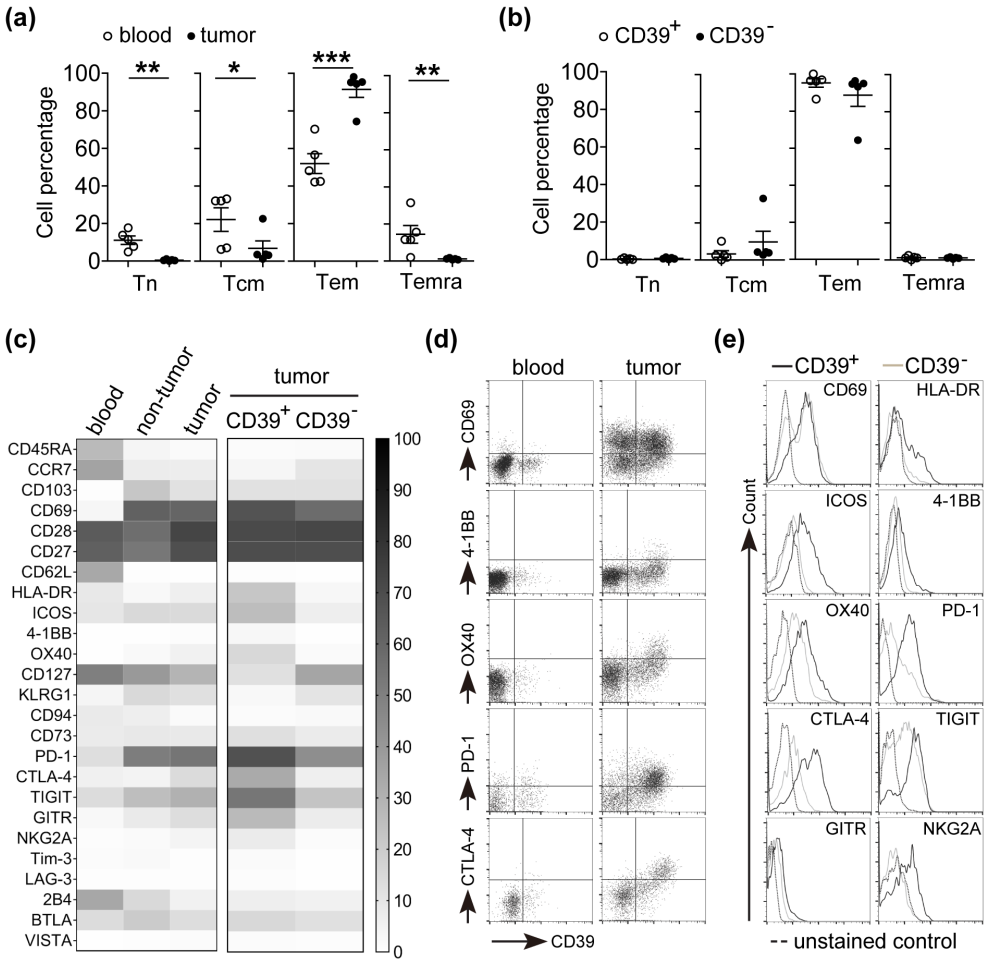

Figure 2. The phenotype of CD39$^+$CD4$^+$ T cells in the tumor tissues of GC patients. **(a)** The percentages of blood- and tumor-derived CD4$^+$ T cells indicated by CD45RA and CCR7 expression are shown: naive T cells (Tn, CD45RA$^+$CCR7$^+$), central memory T cells (Tcm, CD45RA$^-$CCR7$^+$), effector memory T cells (Tem, CD45RA$^-$CCR7$^-$) and terminally differentiated effector memory T cells (Temra, CD45RA$^+$CCR7$^-$; n = 5). **(b)** The percentages of GC-infiltrating CD39$^+$CD4$^+$ and CD39$^-$CD4$^+$ T cells indicated by CD45RA and CD27 expression are shown: Tn, Tcm, Tem and Temra (n = 5). **(c)** Flow cytometry analysis was used to determine the phenotypic characteristics of the following: CD4$^+$ T cells from paired peripheral blood, non-tumor and tumor tissues; and CD39$^+$CD4$^+$ and CD39$^-$CD4$^+$ T cells from the tumor tissues. Data represent mean of at least four GC patients (n = 4–10). **(d)** A representative dot plot for candidate markers versus CD39 expressed by CD4$^+$ T cells in the peripheral blood and tumor tissues. **(e)** Analysis of the representative markers expressed by GC-infiltrating CD39$^+$CD4$^+$ and CD39$^-$CD4$^+$ T cells. *P < 0.05, **P < 0.01 and ***P < 0.001: Student's t-test, Mann–Whitney U-test.

GC-infiltrating CD39$^+$CD4$^+$ T cells exhibit an exhausted functional potential

Based on the exhausted phenotype of CD39$^+$CD4$^+$ T cells in the tumor tissues, their functional profiles were further explored by measuring cytokines production capacity. We found that fewer CD39$^+$CD4$^+$ T cells were TNF-α^+, IFN-γ^+ and TNF-α^+IFN-γ^+ than their CD39$^-$CD4$^+$ counterparts (Figure 3a), suggesting that the functional potential of producing cytokines TNF-α and IFN-γ by GC-infiltrating CD39$^+$CD4$^+$ T cells was decreased. In addition, compared with CD39$^-$CD4$^+$ T cells, the percentages of CD39$^+$CD4$^+$ T cells expressing cytolytic molecules granzyme A, granzyme B, granzyme K, perforin and granulysin were all significantly decreased (Figure 3c, Supplementary figure 3), indicating an exhausted cytotoxic potential of GC-infiltrating CD39$^+$CD4$^+$ T cells.

To explore potential pathways involved in the formation of CD39$^+$CD4$^+$ T cell exhaustion in the tumor tissues, we analysed their expressions of several candidate function-associated transcription factors (Figure 4a). Flow cytometry analyses

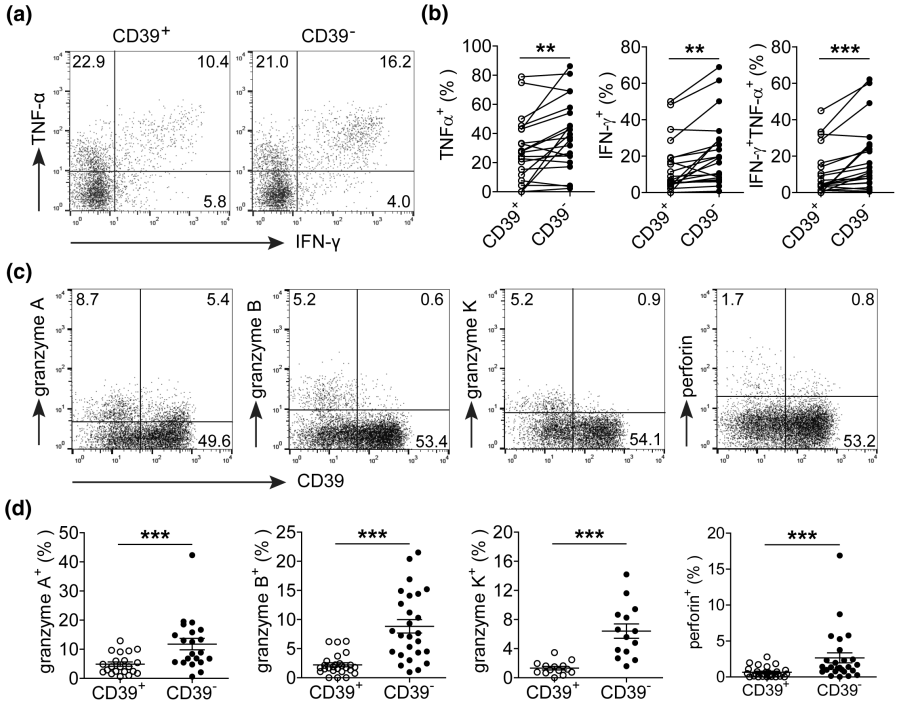

Figure 3. Functional characteristics of CD39$^+$CD4$^+$ T cells in the tumor tissues of GC patients. **(a)** A representative flow cytometry analysis of TNF-α versus IFN-γ expression in GC-infiltrating CD39$^+$CD4$^+$ and CD39$^-$CD4$^+$ T cells. **(b)** The percentages of TNF-α$^+$, IFN-γ$^+$ and TNF-α$^+$IFN-γ$^+$ cells were statistically analysed in GC-infiltrating CD39$^+$CD4$^+$ and CD39$^-$CD4$^+$ T cells (n = 21). **(c)** A representative flow cytometry analysis of granzyme A, granzyme B, granzyme K and perforin versus CD39 expression in GC-infiltrating CD4$^+$ T cells of the same patient. **(d)** The percentages of granzyme A$^+$, granzyme B$^+$, granzyme K$^+$ and perforin$^+$ cells were statistically analysed in GC-infiltrating CD39$^+$CD4$^+$ and CD39$^-$CD4$^+$ T cells (granzyme A: n = 21, granzyme B and perforin: n = 26, granzyme K: n = 14). **P < 0.01, ***P < 0.001: Student's t-test, Mann–Whitney U-test.

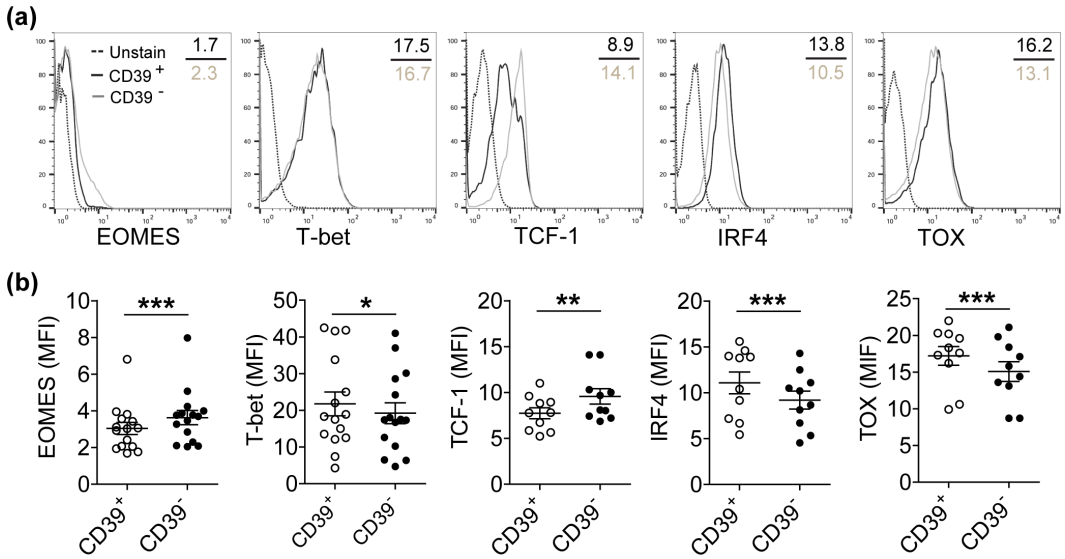

Figure 4. Transcription factor profiles of CD39$^+$CD4$^+$ T cells in the tumor tissues of GC patients. **(a)** A representative histogram plot for the expression of EOMES, T-bet, TCF-1, IRF4 and TOX in CD39$^+$CD4$^+$ and CD39$^-$CD4$^+$ T cells of the tumor tissues. **(b)** The mean fluorescence intensity (MFI) of EOMES$^+$, T-bet$^+$, TCF-1$^+$, IRF4$^+$ and TOX$^+$ cells in GC-infiltrating CD39$^+$CD4$^+$ and CD39$^-$CD4$^+$ T cells was statistically analysed (EOMES and T-bet: n = 15, IRF4, TOX and TCF-1: n = 10). *P < 0.05, **P < 0.01, ***P < 0.001: Student's t-test, Mann–Whitney U-test.

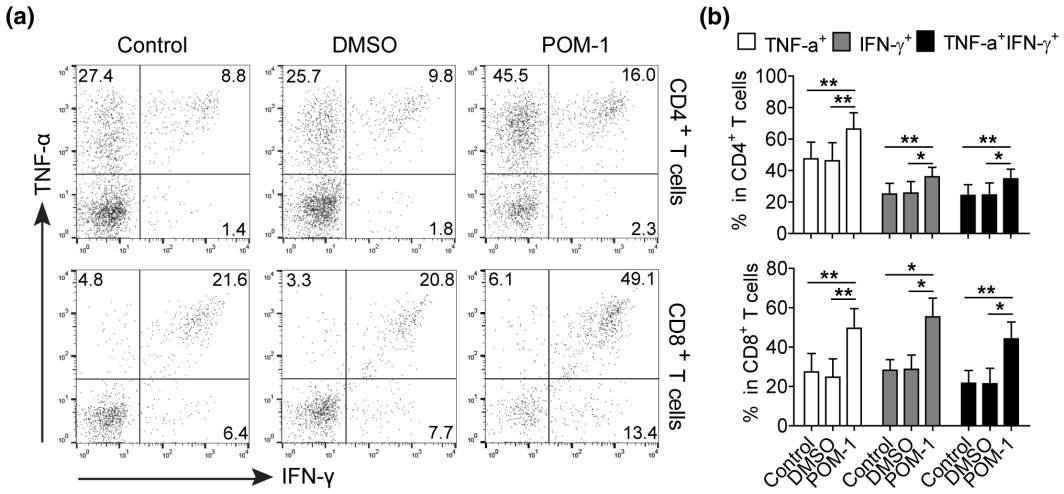

Figure 5. Effect of CD39 enzymatic inhibitor POM-1 on the production of TNF-α and IFN-γ in GC-infiltrating CD4$^+$ and CD8$^+$ T cells. **(a)** A representative flow cytometry analysis of TNF-α and IFN-γ expression in GC-infiltrating CD4$^+$ and CD8$^+$ T cells among the control, DMSO-treated and POM-1-treated groups. **(b)** Statistical analysis of TNF-α$^+$, IFN-γ$^+$ and TNF-α$^+$IFN-γ$^+$ cell percentages in GC-infiltrating CD4$^+$ and CD8$^+$ T cells among the control, DMSO-treated and POM-1-treated groups (n = 5). *P < 0.05, **P < 0.01: Student's t-test, Mann–Whitney U-test.

showed that compared with CD39$^-$CD4$^+$ T cells, CD39$^+$CD4$^+$ T cells displayed significantly lower MFI and percentages of effector-associated transcription factors EOMES and TCF-1 expression, but higher MFI and percentages of exhaustion-associated transcription factors IRF4 and TOX expression, and a slightly increased MFI and percentage of T-bet expression in CD39$^+$CD4$^+$ T cells was also observed (Figure 4b, Supplementary figure 4), suggesting that GC-infiltrating CD39$^+$CD4$^+$ T cells might intrinsically experience exhaustion.

Inhibition of CD39 enzymatic activity enhances the function of GC-infiltrating T cells

As GC-infiltrating CD39$^+$CD4$^+$ T cells showed an exhausted functional profile, we wondered whether CD39 inhibition could improve their effector function. Therefore, tumor-derived cell suspensions were cultured with a CD39 enzymatic inhibitor POM-1 or vehicle for 16 h, and then the production of cytokines TNF-α and IFN-γ in T cells was examined by flow cytometry. Compared with the control and DMSO-treated group, CD4$^+$ T cells in the POM-1-treated group exhibited significantly increased percentages of TNF-α and IFN-γ expression, and such increased percentages of TNF-α and IFN-γ expression in the POM-1-treated group was also observed in CD8$^+$ T cells (Figure 5a). Additionally, CD39$^+$CD4$^+$ T cells in the POM-1-treated group also showed higher percentages of TNF-α and IFN-γ expression than those in the control and DMSO-treated group, and similar observations were made when analysing the expression of these two cytokines in their CD39$^-$CD4$^+$ counterparts (Supplementary figure 5). These data suggest that inhibition of CD39 enzymatic activity could improve antitumor response of GC-infiltrating T cells.

Increased GC-infiltrating CD39$^+$CD4$^+$ T cell percentages correlate with tumor progression and poor overall survival

We next investigated the associations between the percentages of GC-infiltrating CD39$^+$CD4$^+$ T cells and patients' clinical parameters (Figure 6). In comparison with early disease stage (TNM stage I + II), the percentages of CD39$^+$CD4$^+$ T cells were significantly increased at advanced disease stage (TNM stage III + IV). More importantly, high CD39$^+$CD4$^+$ T cell percentages were shown to be positively correlated with patients' poorer overall survival according to the median value of all GC-infiltrating CD39$^+$CD4$^+$ T cell percentages. Multivariate analyses further verified that GC-infiltrating CD39$^+$CD4$^+$ T cell percentages could independently predict survival of patients (Supplementary table 3). Additionally, GC-infiltrating

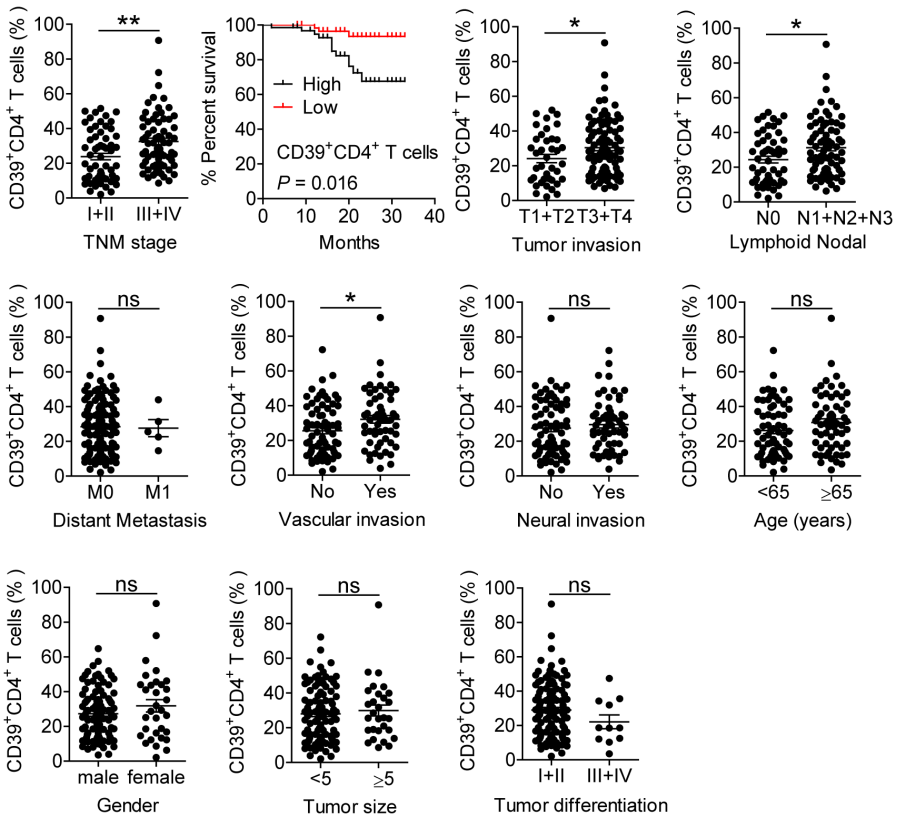

Figure 6. The correlations between GC-infiltrating CD39$^+$CD4$^+$ T cell percentages and clinical parameters. The percentages of GC-infiltrating CD39$^+$CD4$^+$ T cells were analysed for the correlations with TNM stage, tumor invasion, lymph node metastasis, distant metastasis, vascular invasion, neural invasion, age, gender, tumor size as well as the degree of tumor differentiation. Each dot represents one patient (n = 128). For the cumulative patients' overall survival curve, patients were divided into two groups based on the median value of GC-infiltrating CD39$^+$CD4$^+$ T cell percentages, and Kaplan–Meier plots were used to calculate the cumulative patients' overall survival time. *P < 0.05, **P < 0.01, ns, not significant: Student's t-test, Mann–Whitney U-test, log-rank test.

CD39$^+$CD4$^+$ T cell percentages were also positively correlated with tumor invasion, lymph node metastasis and vascular invasion status. However, no significant correlation was found between GC-infiltrating CD39$^+$CD4$^+$ T cell percentages and distant metastasis, neural invasion status, age, gender, tumor size and tumor differentiation. These results demonstrate that increased GC-infiltrating CD39$^+$CD4$^+$ T cell percentages are associated with tumor progression and patients' poorer clinical outcomes.

DISCUSSION

In this study, we showed an increased percentage of GC-infiltrating CD39$^+$CD4$^+$ T cells compared with that of the non-tumor tissues, and high percentages of these cells were associated with tumor progression and patients' poor overall survival. In addition, we demonstrated that CD39 expression defined CD4$^+$ T cell exhaustion, for GC-infiltrating CD39$^+$CD4$^+$ T cells expressed more co-inhibitory molecules and exhaustion-associated transcription factors and produced less effector cytokines and cytolytic molecules than their CD39$^-$CD4$^+$ counterparts. Finally, we demonstrated that CD39 inhibition enhanced TNF-α and IFN-γ production by cultured GC-infiltrating CD4$^+$ T cells, suggesting that targeting CD39 inhibition might be a viable immunotherapeutic approach against GC.

CD4$^+$ T cell helper and regulatory function in human cancer have been extensively examined, including GC.[19–21] However, the exhaustion feature of tumor-infiltrating CD4$^+$ T cells in GC has not yet been elucidated. Here, we examined the expression of PD-1 and CD39, two

typical markers of CD8$^+$ T cell exhaustion,[22,23] on GC-infiltrating CD4$^+$ T cells. We found that the PD-1$^+$CD4$^+$ T cell percentage and their PD-1 MFI were similar between the tumor and non-tumor tissues, whereas the CD39$^+$CD4$^+$ T cell percentage and their CD39 MFI in the tumor tissues were significantly increased compared with those in the non-tumor tissues, implying that CD39 was more closely linked to CD4$^+$ T cell exhaustion in GC patients.

Along with an increased percentage of GC-infiltrating CD39$^+$CD4$^+$ T cells, several exhaustion-associated features of these cells were uncovered. First, although GC-infiltrating CD39$^+$CD4$^+$ T cells were activated and exhibited an effector–memory phenotype, these cells showed high expressions of canonical and alternative inhibitory molecules including PD-1, CTLA-4, TIGIT, GITR and NKG2A.[24] Second, the antitumor potential of GC-infiltrating CD39$^+$CD4$^+$ T cells was impaired, as fewer such cells produced TNF-α and IFN-γ as well as granzyme A, granzyme B, granzyme K, granulysin and perforin. These effector cytokines and cytolytic molecules from CD4$^+$ T cells have been shown to be involved in killing tumor cells in an MHC class II-dependent or -independent fashion.[13,25] Third, in comparison with CD39$^-$CD4$^+$ T cells, GC-infiltrating CD39$^+$CD4$^+$ T cells upregulated transcription factors IRF4 and TOX, which have been reported to promote CD8$^+$ T cell exhaustion.[26,27] Finally, accompanied with disease progression, the percentages of GC-infiltrating CD39$^+$CD4$^+$ T cells were gradually increased and higher percentages of GC-infiltrating CD39$^+$CD4$^+$ T cells were positively associated with patients' poorer survival. In line with our findings, Li *et al*.[28] identified that, in lung and colorectal cancers, tumor-infiltrating CD39$^+$CD4$^+$ T cells evoked a similar exhausted state with significantly increased co-inhibitory molecules expression, which further supported that CD39 might serve as a valuable marker of exhausted CD4$^+$ T cells in GC.

As an ectonucleotidase, CD39 is also able to catalyse extracellular ATP into AMP, which is subsequently hydrolysed into adenosine by CD73 to impair CD8$^+$ T cell-mediated tumor control.[29] After inhibiting CD39 enzymatic function with POM-1, we found that a higher proportion of GC-infiltrating CD4$^+$ T cells produced TNF-α and IFN-γ, further demonstrating that CD39 expression on these CD4$^+$ T cells had imposed a suppressive role on their antitumor potential. Additionally, when CD4$^+$ T cells were divided into CD39$^+$ and CD39$^-$ subsets, CD39 inhibition enhanced TNF-α and IFN-γ production in both CD39$^+$ and CD39$^-$CD4$^+$ T cells, implying that CD39 might also mediate autoregulation of GC-infiltrating CD39$^+$CD4$^+$ T cells in addition to their suppressive activity to nearby T cells. Thus, our data support the hypothesis that CD39 expression not only defines exhausted CD4$^+$ T cells in GC patients but also contributes to limiting local antitumor immunity. Recently, some reports state that CD39 identifies the subset of tumor-specific CD4$^+$ T cells in human cancers and that the presence of such cells predicts improved efficacy of immune checkpoint blockade.[30,31] However, in our study, we did not investigate whether GC-infiltrating CD39$^+$CD4$^+$ T cells are able to recognise and kill tumor cells. Thus, further study to elucidate the association of CD39 expression on GC-infiltrating CD4$^+$ T cells with their tumor antigen specificity is warranted.

In conclusion, our results provide novel insights into the expression of CD39 on CD4$^+$ T cells with their potential roles and clinical implications in GC. Given ineffective clinical response to anti-PD-1 immunotherapy in most GC patients, CD39 may be an attractive therapeutic target to overcome resistance to PD-1 blockade by exhausted T cells, or perhaps it is necessary to target both molecules on the exhausted T cells simultaneously.

METHODS

Patients and specimens

The tumor tissues were obtained from 128 GC patients who underwent surgical resection between September 2020 and November 2022 at the Second Affiliated Hospital of the Third Military Medical University. Among these patients, the peripheral blood of 124 GC patients and the non-tumor tissues (at least 5 cm distant from the tumor site, Supplementary figure 6) of 115 GC patients were also collected. No treatment was conducted before sampling. The study was approved by the Ethics Committee of the Third Military Medical University, and written informed consent was obtained from all patients. The tumor node metastasis (TNM) stages of tumors were determined according to the guidelines of International Union against Cancer (Edn 8). The clinical characteristics of GC patients are presented in Supplementary table 1.

Cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation, and single-cell suspensions were obtained from the tumor and

non-tumor tissues by mechanical dissociation and enzymatic digestion as previously described.[32] In brief, tumor and non-tumor tissues were cut into small pieces and transferred to the tubes containing RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with DNase I (10 mg mL^{-1}, Roche, Basel, Switzerland) and collagenase IV (1 mg mL^{-1}, Invitrogen), the tubes were then placed in a gentle MACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) to obtain mechanically dissociated tissue suspensions. Under continuous rotation at 37°C, the dissociated tissue suspensions in the tubes were further digested for 1 h. Cell strainers of 70 μm were subsequently used to filter the digested tissue suspensions to collect single-cell suspensions. The single-cell suspensions were washed, resuspended in RPMI 1640 medium containing 10% foetal calf serum (Gibco, Bionova, Uruguay) and further cultured *ex vivo* or directly used for flow cytometric analysis.

Flow cytometric analysis

PBMCs isolated from the peripheral blood and the single-cell suspensions obtained from tissue digestions were labelled with Fixable Viability Stain 700 (FVS700, BD Pharmingen, San Diego, CA, USA) to exclude dead cells, and then washed and stained with appropriate surface antibodies for 30 min. Thereafter, the cells were further fixed and permeabilised for 30 min using Foxp3-Staining Buffer Set (Invitrogen), and finally, stained with intracellular antibodies specific to the studied cytokines, cytolytic molecules and transcription factors for 30 min. For intracellular staining of cytokines TNF-α and IFN-γ, the cell suspensions obtained from tumor digestions were incubated for 4 h with Leukocyte Activation Cocktail (BD Pharmingen) before staining. Isotype antibodies were used as controls. Cells were acquired on a BD FACSCanto cell analyser (BD Biosciences, San Diego, CA, USA). Data were analysed using the Flowjo software (10.8.1). The fluorochrome-labelled antibodies used in this study are listed in Supplementary table 2. The gating strategy for flow cytometry analysis is shown in the Supplementary figure 1. After excluding dead cells by FVS700, live cells were successively gated on $CD45^+$, $CD3^+$, $CD8^-$ and $CD4^+$ cells to isolate $CD4^+$ T cells ($FVS700^-CD45^+CD3^+CD8^-CD4^+$ cells). $CD4^+$ T cells were further divided into $CD39^+$ and $CD39^-$ cell subpopulations based on CD39 expression, and then the surface molecules, cytolytic molecules, transcription factors and effector cytokines expression were comparatively analysed between these two subpopulations. Unstained controls were included in the flow cytometry analysis.

CD39 enzymatic inhibition assay

The cell suspensions obtained from the tumor digestions were cultured in 96-well plates pre-coated with anti-CD3 (1 μg mL^{-1}) and anti-CD28 (2 μg mL^{-1}) antibodies (Biolegend, San Diego, CA, USA). An inhibitor of CD39 enzymatic activity (POM-1, 100 μM, MedChem Express, Monmouth Junction, NJ, USA) or vehicle (DMSO) was added in the culture system for 16 h. The cells were stimulated with Leukocyte Activation Cocktail (BD Pharmingen) during the last 4 h, and then the cells were collected for flow cytometric analysis.

Re-analysing public datasets

The publicly available single-cell RNA-sequencing (scRNA-seq) data of 30 GC patients were downloaded and selected for downstream analysis and building signature matrix files.[33] Specifically, for the pre-processing step, the cells were filtered by the original authors based on three metrics (genes/features shared by 3 or more cells, 500 or more features and fewer than 6000 features, mitochondrial RNA percentages ≤ 20). Selected sample datasets were separately performed SCtransform normalisation and were combined using Seurat version 5.0.[34] Data scale and principal component analysis (PCA) were then conducted on the integrated dataset. Additionally, a clustering analysis was performed and immune cells were identified using the ScType R package and further sub-clustered and annotated with SingleR.[35,36] Then, the $CD4^+$ T cell cluster was divided into $CD39^+$ and $CD39^-$ subpopulations or PD-1^+ and PD-1^- subpopulations based on gene count thresholds (> 0) to construct distinct signature matrices. These signature matrices were applied to analyse The Cancer Genome Atlas (TCGA) datasets from 410 GC patients and Genotype-Tissue Expression (GTEx) datasets of non-diseased gastric tissues from 174 individuals using CIBERSORTx.[37] Samples with a *P*-value ≥ 0.05 in CIBERSORTx outputs were excluded from further analysis. Finally, the relative proportions of $CD39^+CD4^+$ or PD-1^+CD4^+ T cells in $CD4^+$ T cells between tumor and non-tumor tissues were analysed.

Statistical analysis

All data are summarised as mean ± standard error of the mean (SEM), and the GraphPad Prism 8.0 Software was used to perform statistical analysis. Two-tailed Student's *t*-tests or Mann–Whitney *U*-tests were used to evaluate the differences between groups, unless otherwise stated. The Kaplan–Meier method was used to calculate the cumulative survival time in months, and the log-rank test was applied to compare between groups. *P*-values less than 0.05 were considered to be statistically significant.

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AUTHOR CONTRIBUTIONS

Zhen-quan Duan: Data curation; formal analysis; investigation; methodology; validation; visualization; writing – original draft. **Yu-xian Li:** Data curation; formal analysis; investigation; methodology; validation; visualization. **Yuan Qiu:** Formal analysis; investigation; methodology; resources; validation; visualization. **Yang Shen:** Data curation; formal analysis; investigation; methodology. **Ying Wang:** Data curation; formal analysis; investigation; methodology. **Yuan-yuan Zhang:** Data curation; formal analysis; investigation; methodology. **Bao-hang Zhu:** Formal analysis; investigation; methodology. **Xiao-hong Yu:** Formal analysis; investigation; methodology. **Xue-ling Tan:** Formal analysis; investigation; methodology. **Weisan Chen:** Supervision; writing – review and editing. **Yuan Zhuang:** Writing – review and editing. **Ping Cheng:** Investigation; methodology. **Wei-jun Zhang:** Investigation; methodology. **Quan-ming Zou:** Data curation; formal analysis; project administration; supervision; writing – review and editing. **Dai-yuan Ma:** Data curation; formal analysis; funding acquisition; project administration; supervision; writing – review and editing. **Liu-sheng Peng:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; supervision; validation; visualization; writing – original draft; writing – review and editing.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

Data are available upon reasonable request. All data relevant to the study are either included in the article or uploaded as supplementary information.

ETHICS APPROVAL

The study was approved by the Ethics Committee of the Third Military Medical University. Full written informed consent was obtained from all patients.

REFERENCES

1. Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* 2012; **12**: 298–306.
2. Lee HE, Chae SW, Lee YJ *et al*. Prognostic implications of type and density of tumour-infiltrating lymphocytes in gastric cancer. *Br J Cancer* 2008; **99**: 1704–1711.
3. Zhang N, Cao M, Duan Y, Bai H, Li X, Wang Y. Prognostic role of tumor-infiltrating lymphocytes in gastric cancer: a meta-analysis and experimental validation. *Arch Med Sci* 2020; **16**: 1092–1103.
4. Chen DS, Mellman I. Elements of cancer immunity and the cancer-immune set point. *Nature* 2017; **541**: 21–30.
5. Zheng L, Qin S, Si W *et al*. Pan-cancer single-cell landscape of tumor-infiltrating T cells. *Science* 2021; **374**: e6474.
6. Raskov H, Orhan A, Christensen JP, Gogenur I. Cytotoxic $CD8^+$ T cells in cancer and cancer immunotherapy. *Br J Cancer* 2021; **124**: 359–367.
7. Blank CU, Haining WN, Held W *et al*. Defining T cell exhaustion. *Nat Rev Immunol* 2019; **19**: 665–674.
8. Oliveira G, Stromhaug K, Klaeger S *et al*. Phenotype, specificity and avidity of antitumour $CD8^+$ T cells in melanoma. *Nature* 2021; **596**: 119–125.
9. Speiser DE, Chijioke O, Schaeuble K, Münz C. $CD4^+$ T cells in cancer. *Nat Can* 2023; **4**: 317–329.
10. Ferris ST, Durai V, Wu R *et al*. cDC1 prime and are licensed by $CD4^+$ T cells to induce anti-tumour immunity. *Nature* 2020; **584**: 624–629.
11. Zander R, Schauder D, Xin G *et al*. $CD4^+$ T cell help is required for the formation of a cytolytic $CD8^+$ T cell subset that protects against chronic infection and cancer. *Immunity* 2019; **51**: 1028–1042.
12. Quezada SA, Simpson TR, Peggs KS *et al*. Tumor-reactive $CD4^+$ T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. *J Exp Med* 2010; **207**: 637–650.
13. Kruse B, Buzzai AC, Shridhar N *et al*. $CD4^+$ T cell-induced inflammatory cell death controls immune-evasive tumours. *Nature* 2023; **618**: 1033–1040.
14. Rad PS, Morikawa H, Kiani NA *et al*. Exhaustion of $CD4^+$ T-cells mediated by the Kynurenine pathway in melanoma. *Sci Rep* 2019; **9**: 12150.
15. Goods BA, Hernandez AL, Lowther DE *et al*. Functional differences between PD-1^+ and PD-1^- $CD4^+$ effector T cells in healthy donors and patients with glioblastoma multiforme. *PLoS One* 2017; **12**: e181538.
16. Miggelbrink AM, Jackson JD, Lorrey SJ *et al*. CD4 T-cell exhaustion: does it exist and what are its roles in cancer? *Clin Cancer Res* 2021; **27**: 5742–5752.
17. Fu J, Yu A, Xiao X *et al*. $CD4^+$ T cell exhaustion leads to adoptive transfer therapy failure which can be prevented by immune checkpoint blockade. *Am J Cancer Res* 2020; **10**: 4234–4250.
18. Balanca CC, Salvioni A, Scarlata CM *et al*. PD-1 blockade restores helper activity of tumor-infiltrating, exhausted PD-$1^{hi}$$CD39^+$ CD4 T cells. *JCI Insight* 2021; **6**: e142513.
19. Oliveira G, Stromhaug K, Cieri N *et al*. Landscape of helper and regulatory antitumour $CD4^+$ T cells in melanoma. *Nature* 2022; **605**: 532–538.
20. De Simone M, Arrigoni A, Rossetti G *et al*. Transcriptional landscape of human tissue lymphocytes unveils uniqueness of tumor-infiltrating T regulatory cells. *Immunity* 2016; **45**: 1135–1147.
21. Chen J, Liu K, Luo Y *et al*. Single-cell profiling of tumor immune microenvironment reveals immune irresponsiveness in gastric signet-ring cell carcinoma. *Gastroenterology* 2023; **165**: 88–103.
22. Mclane LM, Abdel-Hakeem MS, Wherry EJ. CD8 T cell exhaustion during chronic viral infection and cancer. *Annu Rev Immunol* 2019; **37**: 457–495.

23. Canale FP, Ramello MC, Nunez N *et al*. CD39 expression defines cell exhaustion in tumor-infiltrating $CD8^+$ T cells. *Cancer Res* 2018; **78**: 115–128.
24. Burugu S, Dancsok AR, Nielsen TO. Emerging targets in cancer immunotherapy. *Semin Cancer Biol* 2018; **52**: 39–52.
25. Oh DY, Kwek SS, Raju SS *et al*. Intratumoral $CD4^+$ T cells mediate anti-tumor cytotoxicity in human bladder cancer. *Cell* 2020; **181**: 1612–1625.
26. Man K, Gabriel SS, Liao Y *et al*. Transcription factor IRF4 promotes $CD8^+$ T cell exhaustion and limits the development of memory-like T cells during chronic infection. *Immunity* 2017; **47**: 1129–1141.
27. Khan O, Giles JR, Mcdonald S *et al*. TOX transcriptionally and epigenetically programs $CD8^+$ T cell exhaustion. *Nature* 2019; **571**: 211–218.
28. Li S, Zhuang S, Heit A *et al*. Bystander $CD4^+$ T cells infiltrate human tumors and are phenotypically distinct. *Onco Targets Ther* 2022; **11**: 2012961.
29. Moesta AK, Li XY, Smyth MJ. Targeting CD39 in cancer. *Nat Rev Immunol* 2020; **20**: 739–755.
30. Kortekaas KE, Santegoets SJ, Sturm G *et al*. CD39 identifies the $CD4^+$ tumor-specific T-cell population in human cancer. *Cancer Immunol Res* 2020; **8**: 1311–1321.
31. Martinez-Gomez C, Michelas M, Scarlata CM *et al*. Circulating exhausted PD-1^+CD39^+ helper CD4 T cells are tumor-antigen-specific and predict response to PD-1/PD-L1 Axis blockade. *Cancers (Basel)* 2022; **14**: 3679.
32. Shen Y, Li XL, Li YX *et al*. Distribution, phenotype, functional and clinical relevance of $CD8^+CD103^+$ tissue-resident memory T cells in human gastric cancer. *Cancer Immunol Immunother* 2022; **71**: 1645–1654.
33. Kumar V, Ramnarayanan K, Sundar R *et al*. Single-cell atlas of lineage states, tumor microenvironment, and subtype-specific expression programs in gastric cancer. *Cancer Discov* 2022; **12**: 670–691.
34. Hao Y, Stuart T, Kowalski MH *et al*. Dictionary learning for integrative, multimodal and scalable single-cell analysis. *Nat Biotechnol* 2024; **42**: 293–304.
35. Ianevski A, Giri AK, Aittokallio T. Fully automated and ultra-fast cell-type identification using specific marker combinations from single-cell transcriptomic data. *Nat Commun* 2022; **13**: 1246.
36. Aran D, Looney AP, Liu L *et al*. Reference-based analysis of lung single-cell sequencing reveals a transitional profibrotic macrophage. *Nat Immunol* 2019; **20**: 163–172.
37. Newman AM, Steen CB, Liu CL *et al*. Determining cell type abundance and expression from bulk tissues with digital cytometry. *Nat Biotechnol* 2019; **37**: 773–782.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.