ORIGINAL ARTICLE

Lauren classification identifies distinct prognostic value and functional status of intratumoral CD8+ T cells in gastric cancer

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Abstract

With dichotomous etiology and pathogenesis, intestinal type and diffuse type gastric cancers vary in their clinical and molecular features to the point of representing distinct entities. However, the differences of tumor-infiltrating immune cells within the two types of gastric cancer have not been well researched. This study was aimed to evaluate the functional impact of Lauren classification on immune contexture in gastric cancer patients. Tumor tissues of gastric cancer patients from Zhongshan Hospital and gastric cancer data from The Cancer Genome Atlas (TCGA) cohort were analyzed. By immunohistochemistry and flow cytometry, we found that intratumoral CD8+ T cells were more abundant but less functional in diffuse type as compared with those in intestinal type tumor tissues. Survival analysis indicated that CD8+ T cells yielded favorable prognosis only in intestinal type patients other than diffuse type cancer patients. Moreover, such diffuse type-associated CD8+ T cell dysfunction was featured by elevated expression of immunosuppressive factors including interleukin-10 (IL-10), transforming growth factor-β1 (TGF-β1) and indoleamine 2,3-dioxygenase 1 (IDO1). In summary, we found that the density, prognostic significance and functional status of intratumoral CD8+ T cells varied with Lauren subtypes in gastric cancer. These results further indicated Lauren classification might be a potential therapeutic marker, and should be considered in therapeutic decisions, especially immunotherapeutic eligibility.

Keywords CD8+ T cells · Gastric cancer · Lauren classification · Prognosis

Introduction

Gastric cancer is a complex, heterogenous entity that encompasses tumors with varying histological types, molecular profiles and biological behaviors. Lauren [1] described two distinct histological types of gastric cancer: intestinal type and diffuse type, based on the tissue structure. Besides the morphological differences, the two types of gastric cancer also differ in their molecular features, which consequently

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contribute to distinct therapeutic response to chemotherapy regimens within the two subtypes of gastric cancer [2–4]. Furthermore, the Lauren classification has already been used to indicate trastuzumab application according to NCCN guidelines [5].

In the past 10 years, tumor immune microenvironment has drawn increasing attention because of the promising clinical trial data of immunotherapy [6]. To our best knowledge, previous studies which referred to heterogeneity between intestinal type and diffuse type gastric cancers were predominantly concentrated on tumor cells. Few studies investigated the difference in tumor immune microenvironment of the two types of gastric cancer [7]. Therefore, with the coming of immunotherapy era, uncovering the heterogeneity in immune microenvironment between the two Lauren subtypes of gastric cancer might shed light on the identification of patients who may respond to immunotherapy.

A central role in immune protection against cancer is orchestrated by T-lymphocytes, particularly CD8$^+$ T cells [8]. Their infiltration in tumor cell nests is usually associated with a favorable prognosis and may predict therapeutic response to immune checkpoint inhibitors [9–12]. A very recent study by Pernot S has paid attention to the differential infiltration of CD8$^+$ T cells within the two Lauren subtypes of gastric cancer [13]. However, the functional differences between intestinal type-associated CD8$^+$ T cells and diffuse type-associated CD8$^+$ T cells were neglected in Pernot S' study. Thus, we analyzed both the number and the functional status of intratumoral CD8$^+$ T cells in larger cohorts in our study. It was shown that CD8$^+$ T cells were more abundant but less functional in diffuse type gastric cancer. Furthermore, we found elevated expression of immunosuppressive factors including interleukin-10 (IL-10), transforming growth factor-β1 (TGF-β1) and indoleamine 2,3-dioxygenase 1 (IDO1) could depict the diffuse type-associated CD8$^+$ T cell dysfunction in gastric cancer.

Materials and methods

Patients

Initially, the study recruited 496 patients, but only 468 patients had comprehensive information about clinicopathological data and survival outcomes for complete analysis. After immunohistochemistry, specimens of 12 patients were lost. Consequently, 456 patients were included as the Zhongshan Tissue Microarray (TMA) cohort. A total of 235 gastric cancer patients with Lauren subtype identification, survival data and complete RNA-seq information from The Cancer Genome Atlas (TCGA) database were enrolled as TCGA cohort. Fresh tumor tissue samples were obtained from 63 gastric cancer patients during surgery at Zhongshan Hospital

from April to November 2018 (Zhongshan FCM cohort). The clinical characteristics of all gastric cancer patients are summarized in Table S1. Due to the small number and the similarity with diffuse type gastric cancer in clinical and prognostic aspects [14], the Lauren classification mixed type gastric cancer was combined into the diffuse type in our analysis as previous studies [15, 16]. Overall survival was calculated from the date of gastrectomy to the date of death or the last follow-up. The median follow-up time was 33.3 and 43.5 months in TCGA cohort and Zhongshan TMA cohort, respectively. Written informed consent was obtained from each patient included, and the protocol of all study cohorts was approved by the institutional review board and ethics committee of Zhongshan Hospital, Fudan University.

Assay methods

The protocol of immunohistochemistry and used antibodies are shown in Table S2. After immunostaining, sections were scanned at high-power magnification (×200) and three randomly selected fields were captured by NIS-Elements F3.2 software. The semiquantitative H-score system described previously was used for IL-10, TGF-β1 and IDO1 level evaluation [17]. Density of CD8$^+$ and granzyme B (GZMB)$^+$ T cells was counted as the number of positive staining cells per high magnification view (×200). CD8$^+$ and GZMB$^+$ T cell counting, IL-10, TGF-β1 and IDO1 expression for each field were evaluated by two pathologists who were blinded to the clinical data. In case of disagreement, the pictures were reviewed and a consensus was reached by the two observers. Finally, three fields were averaged as the ultimate score of each sample. 45/HPF and − 0.54 were determined as the cutoff values to discriminate high and low CD8$^+$ T cell infiltration subgroups in Zhongshan TMA and TCGA cohort by minimum P method with the use of X-tile software (Version 3.6.1, Yale University).

For flow cytometry, CD8$^+$ T cell number was detected in all 63 patients by flow cytometry, but functional phenotype of CD8$^+$ T cells was detected only in 43 patients, of whom the formalin-fixed paraffin-embedded tissues were retrospectively collected for immunohistochemistry. Paired normal gastric mucosa tissues were obtained from 16 patients for flow cytometry analysis. The protocol for flow cytometry and antibodies used are shown in Table S3. Flow cytometry results were analyzed by FlowJo software (TreeStar).

The RNA-seq data for TCGA gastric cancer cohort were downloaded from the UCSC Xena at Jan. 2018 (https://xenabrowser.net/datapages/). To estimate CD8$^+$ T cell infiltration level in the TCGA cohort, the average expression level of predefined CD8$^+$ T cell gene set was determined utilizing normalization value to the mean and standard deviation among samples [18].

Statistical analysis

Survival analyses were performed by Kaplan–Meier method, log-rank test and Cox hazards regression model. Univariate and multivariate linear regression models of log-transformed $CD8^+$ T cell numbers (per high magnification view and per 10^5 cells in Zhongshan TMA cohort and Zhongshan FCM cohort, respectively) versus clinicopathological variables were fitted. Student's t test and Spearman's correlation test were used for differential and correlation analysis. For all tests in this study, a P value of <0.05 was considered statistically significant. All analyses were conducted using Graph-Pad Prism (Version 6.00) and IBM SPSS Statistics (version 21) software.

Results

Intratumoral $CD8^+$ T cells exhibit more abundant manner in diffuse type gastric cancer patients

By means of immunohistochemistry, we detected the expression of CD8 and GZMB, which is regarded as a functional tissue marker of $CD8^+$ T cells, in sequential slides of Zhongshan TMA cohort. We found that the density of intratumoral $CD8^+$ T cells was higher in diffuse type than in intestinal type gastric cancer ($P < 0.001$, Fig. 1a, b). Interestingly, there was no significant difference of intratumoral $GZMB^+$ cell density between the two subtypes of gastric cancer (Fig. 1a, b). We further conducted flow cytometry analysis, and the results also showed that diffuse type gastric cancer tissues contained higher proportion of $CD8^+$ T cells among $CD45^+$ leukocytes and higher total $CD8^+$ T cell number, compared with intestinal type gastric cancer tissues ($P = 0.011$ and $P = 0.001$, Fig. 1c, d). Multivariate linear

Fig. 1 Distribution of tumor-infiltrating $CD8^+$ T cells by Lauren classification. **a** Representative immunohistochemistry images of CD8 and GZMB staining in gastric cancer tissues with different Lauren subtypes (200× magnification). **b** Density of $CD8^+$ and $GZMB^+$ T cell infiltration in intestinal type ($n = 286$) and diffuse type ($n = 170$) gastric cancer patients from Zhongshan TMA cohort. **c** Representative images from flow cytometry showing $CD8^+$ T cells proportions in intestinal type and diffuse type gastric cancer. **d** Proportions among all $CD45^+$ leukocytes and absolute numbers of tumor-infiltrating $CD8^+$ T cells in intestinal type ($n = 36$) and diffuse type ($n = 27$) gastric cancer patients from Zhongshan FCM cohort. **e** Univariate and multivariate associations between tumor-infiltrating $CD8^+$ T cells and clinicopathological variables in Zhongshan TMA cohort and FCM cohort. Data are represented as mean ± SD, and P values are calculated using unpaired two-tailed t test. *GZMB* granzyme B, *TMA* tissue microarray, *FCM* flow cytometry
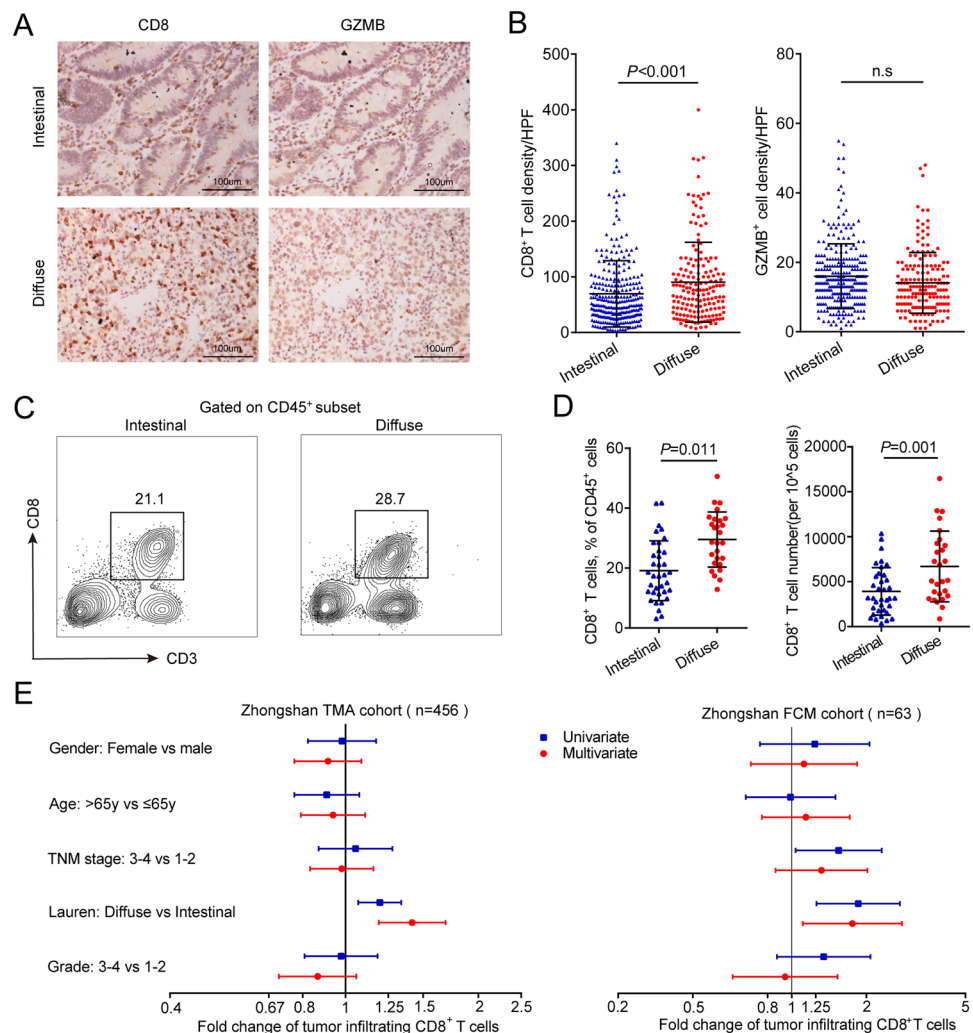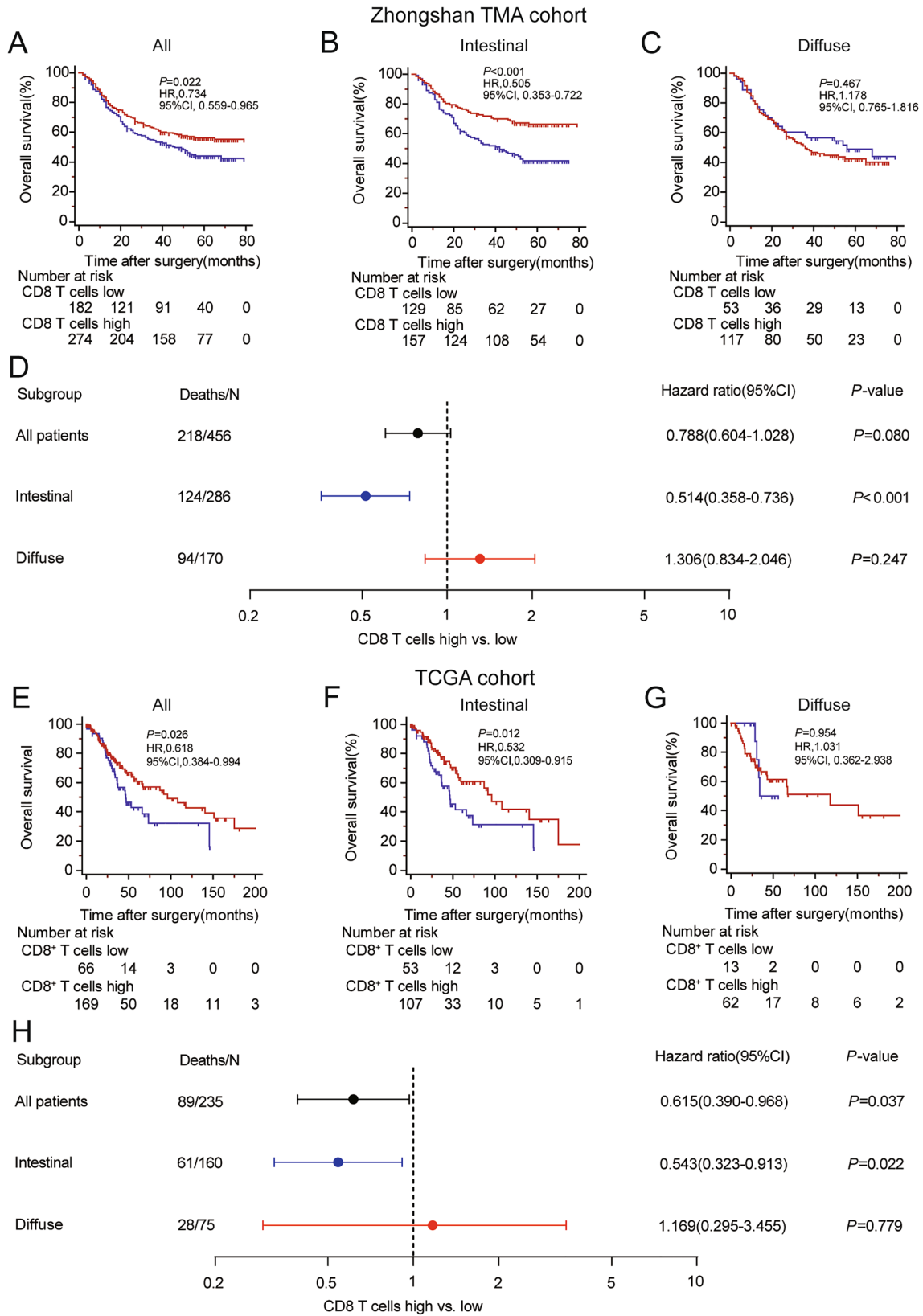
◄**Fig. 2** Prognostic value of CD8$^+$ T cell infiltration for gastric cancer patients with different Lauren subtypes. **a–c** Kaplan–Meier curves of overall survivals stratified by CD8$^+$ T cell infiltration for all patients (**a**), intestinal type patients (**b**) and diffuse type patients (**c**) in the Zhongshan TMA cohort. Blue curves, CD8$^+$T cells low infiltration; red curves, CD8$^+$ T cells high infiltration. **d** Forest plot of CD8$^+$ T cell infiltration on survival analysis adjusted for TNM stage, age, gender and tumor grade by multivariate Cox regression model in Zhongshan TMA cohort. **e–g** Kaplan–Meier curves of overall survivals stratified by CD8$^+$ T cell infiltration for all patients (**e**), intestinal type patients (**f**) and diffuse type patients (**g**) in the TCGA cohort. Blue curves, CD8$^+$T cells low infiltration; red curves, CD8$^+$ T cells high infiltration. **h** Forest plot for CD8$^+$ T cell infiltration on survival analysis adjusted for TNM stage, age, gender and tumor grade by multivariate Cox regression model in the TCGA cohort. *TNM* tumor nodes metastasis, *TMA* tissue microarray, *TCGA* the cancer genome atlas

regression model was constructed to evaluate whether Lauren classification could still discriminate CD8$^+$ T cell infiltration when adjusted by other clinicopathological variables. As we hypothesized, there was still significant difference in intratumoral CD8$^+$ T cell infiltration between intestinal and diffuse types of gastric cancer in multivariate linear regression model (Fig. 1e). We then evaluated the expression of predefined CD8$^+$ T cell signature genes in TCGA cohort and found higher expression of CD8$^+$ T cell signature genes in diffuse type than in intestinal type tumors ($P < 0.001$, Figure S1A and B). Considering the distinct pathogenesis of gastric cancer with different Lauren subtypes, we investigated the CD8$^+$ T cell infiltration pattern in peritumoral gastric mucosa. However, no significant difference was found between CD8$^+$ T cells from peritumoral tissues of patients with intestinal type and diffuse type tumors (Figure S2A and B). Altogether, these data demonstrated that intratumoral CD8$^+$ T cells are more abundant in diffuse type than that in intestinal type gastric cancer.

Intratumoral CD8$^+$ T cells yield favorable prognostic value in intestinal type gastric cancer patients

CD8$^+$ T cells are recognized as the main effector cells in anti-tumor immunity, and increased CD8$^+$ T cell infiltration predicts better prognosis in many solid cancers such as hepatocellular carcinoma [19], ovarian cancer [20], non-small cell lung cancer [21] and breast cancer [22]. However, previous studies on gastric cancer indicated that CD8$^+$ T cell infiltration could not stratify overall survival and even indicated a poor prognosis [23, 24]. Since the Lauren classification might have a potential impact on CD8$^+$ T cells, we consequently investigated whether the prognostic value of CD8$^+$ T cells would vary with Lauren classification in gastric cancer. We evaluated the prognostic value of CD8$^+$T cells in Zhongshan TMA cohort and TCGA cohort. Patients with higher density of intratumoral CD8$^+$ T cells have a slightly improved prognosis than those with lower density of

intratumoral CD8$^+$ T cells in both Zhongshan TMA cohort ($P = 0.022$, HR = 0.734, 95%CI 0.559–0.965, Fig. 2a) and TCGA cohort ($P = 0.026$, HR = 0.618, 95%CI 0.384–0.994, Fig. 2e). However, when subgroup analysis was performed, CD8$^+$ T cell infiltration could only indicate better prognosis in intestinal type patients ($P < 0.001$, HR = 0.505, 95%CI 0.353–0.722 and $P = 0.012$, HR = 0.532, 95%CI 0.309–0.915, respectively, Fig. 2b, f), rather than diffuse type patients ($P = 0.467$, HR = 1.178, 95%CI 0.765–1.816 and $P = 0.954$, HR = 1.031, 95%CI 0.362–2.938, respectively, Fig. 2c, g). To further validate different prognostic values of CD8$^+$ T cells between these two subtypes of gastric cancer, multivariate Cox regression model including gender, age, TNM stage, tumor grade and CD8$^+$ T cell infiltration was constructed in patients with different Lauren subtypes. Consistently, CD8$^+$ T cell infiltration could still predict better prognosis in intestinal type patients ($P < 0.001$, HR = 0.514, 95%CI 0.358–0.736 and $P = 0.022$, HR = 0.543, 95%CI 0.323–0.913, Fig. 2d, h), rather than diffuse type patients ($P = 0.247$ HR = 1.306, 95%CI 0.834–2.046 and $P = 0.779$ HR = 1.169, 95%CI 0.295–3.455, Fig. 2d, h) in the two independent cohort. The full Cox multivariate analysis is shown in Table S4. These data suggested that intratumoral CD8$^+$ T cells yielded favorable prognostic value merely in intestinal type gastric cancer patients.

Intratumoral CD8$^+$ T cells perform dysfunctional phenotype in diffuse type gastric cancer patients

Considering the discrepant prognostic value of CD8$^+$ T cells in patients with different Lauren subtypes, we therefore assessed whether phenotypic and functional properties of CD8$^+$ T cells also varied with Lauren classification by flow cytometry. As we hypothesized, flow cytometry showed visibly reduced expression of effector molecules including CD107a, granzyme B (GZMB), perforin (PRF1) and interferon gamma (IFN-γ) on intratumoral CD8$^+$ T cells from diffuse type gastric cancer ($P = 0.007$, $P < 0.001$, $P < 0.001$ and $P = 0.001$, respectively; Fig. 3a). Meanwhile, we detected the expression of immune checkpoint molecules including programmed cell death-1 (PD-1), cytotoxic T-lymphocyte associated protein-4 (CTLA-4), T cell immunoglobulin domain and mucin domain-3 (TIM-3) and lymphocyte activation gene-3 (LAG-3), which were generally considered as T cell dysfunctional markers in tumor tissues. Interestingly, we only observed significantly higher expression of PD-1 on CD8$^+$ T cells ($P = 0.026$, Fig. 3b) from diffuse type gastric cancer. As to CTLA-4, TIM-3 and LAG-3 expression, no significant difference was found between CD8$^+$ T cells from the two subtypes of gastric cancer (Fig. 3b). Analyses for normal gastric mucosa showed the phenotypical difference was only observed in intratumoral CD8$^+$ T cells but not in CD8$^+$ T cells from normal gastric mucosa (Figure S2D and
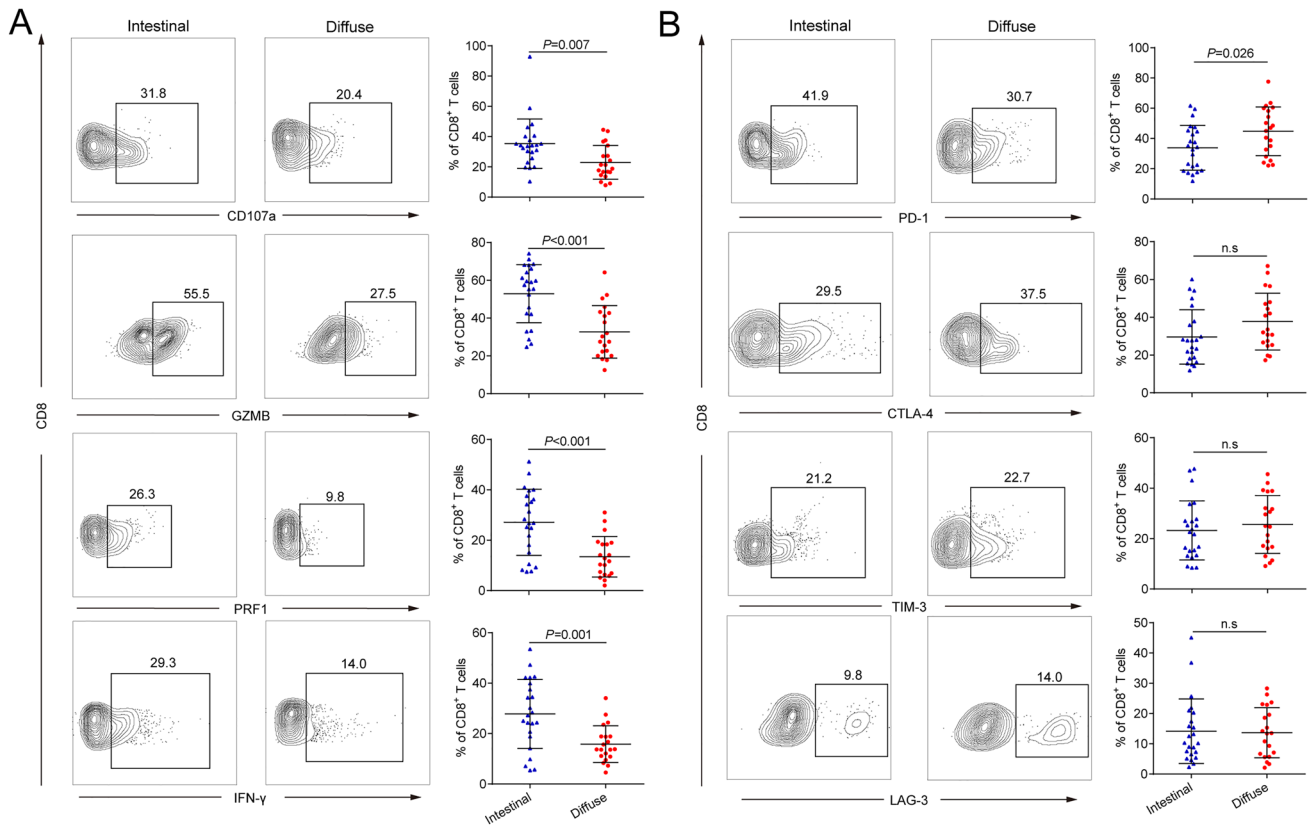
Fig. 3 Phenotypic and functional properties of CD8+ T cells by Lauren subtypes. **a** Flow cytometrical analysis of effector molecules CD107a, GZMB, PRF1 and IFN-γ expression on CD8+ T cells from intestinal ($n=23$) and diffuse type ($n=20$) gastric cancer tissues. **b** Flow cytometrical analysis of effector molecules PD-1, CTLA-4, TIM-3 and LAG-3 expression on CD8+ T cells from intestinal ($n=23$) and diffuse type ($n=20$) gastric cancer tissues. Data are rep-resented as mean $\pm$ SD and *P* values are calculated using unpaired two-tailed t test. *GZMB* granzyme B, *IFN-γ* interferon gamma, *PRF1* perforin, *PD-1* programmed cell death 1, *CTLA-4* cytotoxic T-lymphocyte associated protein 4, *TIM-3* T cell immunoglobulin domain and mucin domain 3, *LAG-3* lymphocyte activation gene-3, *n.s* no significance

E). Conclusively, these data revealed that intratumoral CD8+ T cells performed dysfunctional phenotype in diffuse type gastric cancer patients.

Intratumoral CD8+ T cell dysfunction correlates with lavish immunosuppressive factors in diffuse type gastric cancer patients

Besides immune checkpoint molecules, some immunoregulatory cytokines produced in the tumor microenvironment also contribute to T cell dysfunction. Thus, we tended to investigate whether these two subtypes of gastric cancer express different immunosuppressive factors. We analyzed the expression of factors well known for their immunosuppressive function [25] in TCGA tumor mRNA expression data. Notably, we found elevated expression of IL-10, TGF-β1 and IDO1 (Fig. 4a) in diffuse type gastric cancer. This finding was verified by immunohistochemistry in Zhongshan TMA cohort (Fig. 4b and Figure S3). To further validate the

association between CD8+ T cell dysfunction and expression of immunosuppressive factors, we retrospectively collected formalin-fixed paraffin-embedded tissues of the 43 patients with CD8+ T cell function data from Zhongshan FCM cohort and detected the expression of immunosuppressive factors including IL-10, TGF-β1 and IDO1. Consistent with the results in the Zhongshan TMA cohort, diffuse type gastric cancer showed higher expression level of IL-10, TGF-β1 and IDO1 in the FCM cohort (Fig. 4c). Then, we analyzed whether suppressive factor expression was correlated with CD8+ T cell dysfunction in Zhongshan FCM cohort. A 2D heatmap showed generally higher expression of immunosuppressive factors and lower expression of effector molecules on CD8+ T cells in diffuse type versus intestinal type tumors (Fig. 4d). Correlation analysis showed that the expression of effector molecules on CD8+ T cells was negatively correlated with the expression level of immunosuppressive factors including IL-10, TGF-β1 and IDO1 in gastric cancer (Fig. 4e). In conclusion, these results indicated that intratumoral
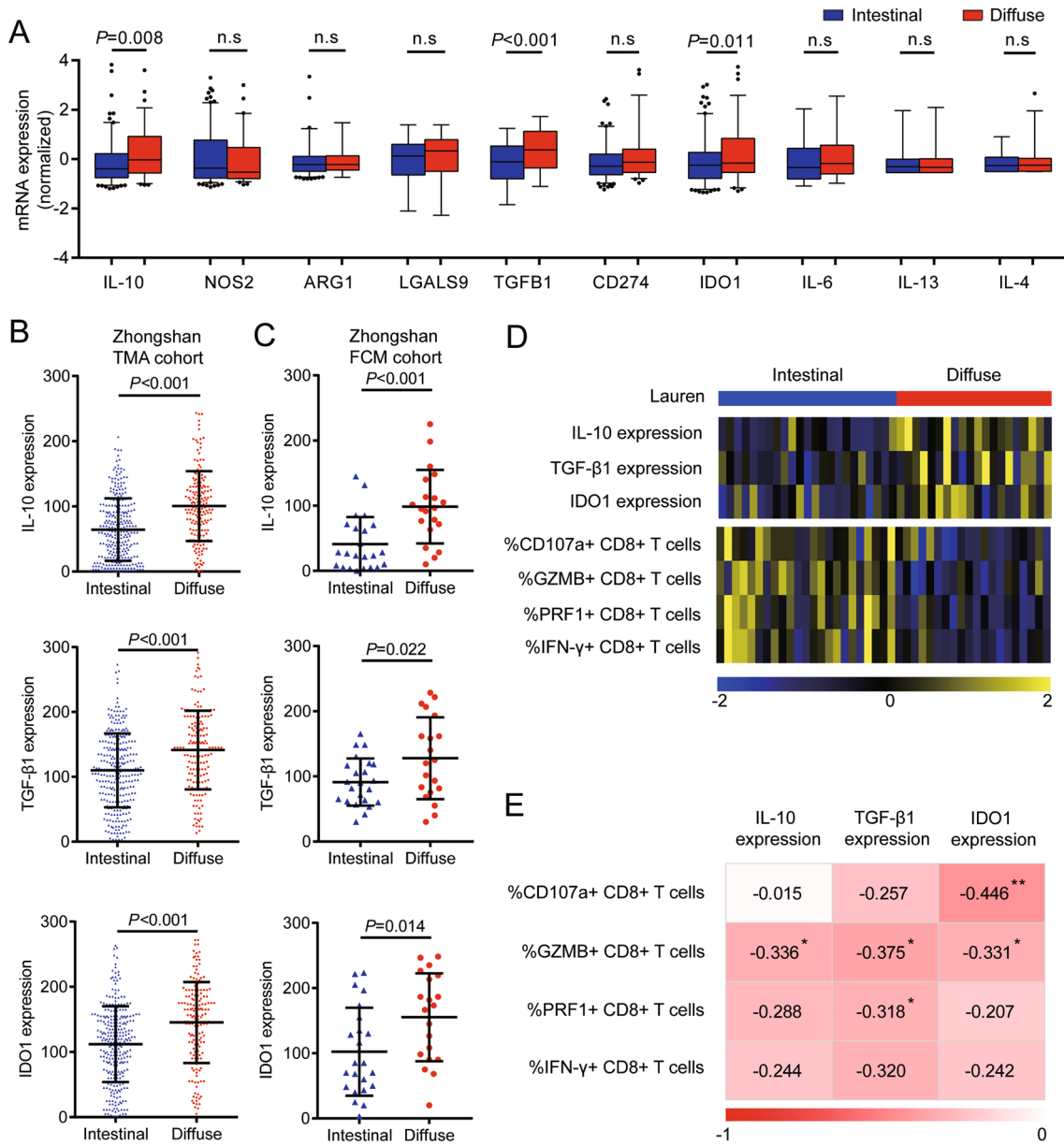
Fig. 4 CD8^{+} T cell dysfunction in diffuse type gastric cancer correlates with the lavish expression of immunosuppressive factors. **a** mRNA expression of immunosuppressive factors by different Lauren subtypes of gastric cancer in the TCGA cohort ($n=235$). **b** Expression of IL-10, TGF-β1 and IDO1 in gastric cancer tissues with different Lauren subtypes detected by immunohistochemistry in Zhongshan TMA cohort ($n=456$). **c** Expression of IL-10, TGF-β1 and IDO1 in gastric cancer tissues with different Lauren subtypes detected by immunohistochemistry in Zhongshan FCM cohort ($n=43$). **d** A 2D heatmap showing different expressions of immuno-suppressive factors and CD8^{+} T cell functional status in Zhongshan FCM cohort ($n=43$). Colors show row-normalized z-score of immuno-suppressive factor expression scores or cell populations percentages. **e** Association between expression level of immunosuppressive factors and functional status of tumor-infiltrating CD8^{+} T cells. Data are represented as mean $\pm$ SD and P values are calculated using unpaired two-tailed t test. *IL-10* interleukin-10, *TGF-β1* transforming growth factor-β1, *TMA* tissue microarray, *FCM* flow cytometry, *n.s* no significance

CD8^{+} T cell dysfunction might be correlated with lavish immunosuppressive factors in diffuse type gastric cancer patients.

Discussion

It has been reported that immune infiltration and its prognostic significance might be distinguished by histological types in many tumors like breast cancer, non-small cell lung

cancer and ovarian cancer [26–28]. Although the Lauren classification system dates back to 1965, it is still widely accepted and has been proved to be one of the most meaningful classifications by histology for gastric cancer. Thus, we conducted this study to test the hypothesis that Lauren classification might signify distinct immune environment signature in gastric cancer.

In this study, a higher number of CD8+ T cells was found in diffuse type tumors, compared with intestinal type tumors. Interestingly, our results seemed opposite to Pernot S', which found more CD8+ T cell infiltration in intestinal type of gastric cancer [13]. We speculated that this might be caused by the heterogeneity of patients, because the patients in Pernot S' study were all metastasis cases, which only played a small part of our study. Remarkably, a different picture emerged when we focused on functional status instead of only counting their numbers. In this regard, the correlations were inverse, providing evidence for better CD8+ T cell function in intestinal type gastric cancer, with significantly higher fractions of CD8+ T cells expressing CD107a, GZMB, IFN-γ and PRF1. Indeed, our findings are essentially consistent with the conclusion of studies on epithelial–mesenchymal transition (EMT) and anti-tumor immune response. EMT process, a biological program featured by loss of cell adhesion and increased invasive behavior, is seen as a hallmark of diffuse type gastric cancer [29, 30]. Tumors with a higher level of EMT usually exhibit more T cell infiltration in other solid tumors [31, 32]. However, despite the positive correlation of EMT score and T cell infiltration, tumors with high EMT score often exhibit immunosuppressive microenvironment featured by T cell dysfunction [33, 34]. Consequently, it's logical that intratumoral CD8+ T cells are more abundant but less functional in diffuse type gastric cancer, which usually exhibits higher degree of epithelial mesenchymal transformation.

Immunosuppressive factors like IL-10, TGF-β1 and IDO1 have been shown to induce EMT [35–37], which gives possible explanations to CD8+ T cell dysfunction in diffuse type tumors with higher mesenchymal transition level. Furthermore, in mesenchymal type tumors, these immunosuppressive factors may attenuate response to immune checkpoint inhibitors (ICPIs), regardless of T cell infiltration level [33, 38], while in two recent clinical trials, diffuse type gastric cancer really tended to show lower response rate to immune checkpoint inhibitors despite increased CD8+ T cell infiltration [39, 40]. Therefore, Lauren classification may add significant information to identification of gastric cancer patients who may benefit from ICPIs, with easier clinical application. Additionally, combination with targeting IL-10, TGF-β1 or IDO1 may be potentially effective to improve the effects of ICPIs in diffuse type gastric cancer in view of the aberrant expression of these immunosuppressive factors in this type of tumors.

Several limitations should be addressed in this study. We concentrated on CD8+ T cells in the present study. However, considering the intricate interactions between immune contextures, future work will be required to provide comprehensive examination of the immune landscape among Lauren subtypes using the high-dimensional analytical tools like cytometry by time of flight and next-generation sequencing. Also, the underlying mechanism how Lauren classification shapes the immune microenvironment should be uncovered by studies in the future.

In summary, our findings indicated that intratumoral CD8+ T cells were more abundant but less functional in diffuse type gastric cancer as compared with that in intestinal type. We showed for the first time that CD8+ T cell infiltration in diffuse type gastric cancer might not be associated with better survival. Increased CD8+ T cell infiltration was found accompanied by elevated expression of IL-10, TGF-β1 and IDO1 in diffuse type gastric cancer, which may contribute to impaired CD8+ T cell function in this type of tumors. These results indicate that ICPIs may be more effective in intestinal type gastric cancer rather than diffuse type disease. Importantly, we identified possible targets that were overexpressed in diffuse type gastric cancer and could provide the foundation for rational immune therapy combination strategies.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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