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RESEARCH

Prognostic nutrition index reveals LAG3 in cytotoxic CD8+ T cells and MHC class II in gastric cancer cells

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Abstract

Background The prognostic nutrition index (PNI) has recently been highlighted as a predictor of immune checkpoint (IC) inhibitor efficacy in gastric cancer (GC). Although LAG3, an IC molecule, has gained considerable attention, its association with PNI remains unexplored.

Materials and methods We retrospectively analyzed clinical data from 796 GC patients who underwent radical gastrectomy to identify which previously reported nutritional index had the greatest impact on prognosis. Single-cell RNA sequencing was performed on 38 GC tissues, and multiplex immunofluorescence staining was conducted on 59 GC tissues to evaluate the relationship between nutritional indices and IC molecule expression in cytotoxic CD8-positive T cells.

Results A low preoperative PNI was identified as the strongest predictor of poor prognosis among the nutritional indices in GC patients. The expression of not only PDCD1 (encoding PD1) but also LAG3 in cytotoxic CD8-positive T cells was significantly higher in GC with low PNI compared to those with high PNI. Among cytotoxic CD8-positive T cells, the proportion of LAG3-positive cells was greater than that of PDCD1-positive cells, particularly in GC with low PNI, and most LAG3-positive cells did not co-express PDCD1. Additionally, the expression of MHC class II, a ligand for LAG3, was higher in GC cells with high levels of epithelial–mesenchymal transition-related molecules in GC with low PNI compared to those with high PNI.

Conclusions PNI can reflect LAG3 expression in cytotoxic CD8-positive T cells and MHC class II expression in GC cells.

Keywords Gastric cancer · Prognostic nutrition index · LAG3 · MHC class II

Introduction

Gastric cancer (GC) ranks as the third leading cause of cancer-related deaths [1]. Currently, therapies targeting immune checkpoint (IC) molecules, such as programmed cell death protein 1 (PD1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4), are widely adopted for various solid tumors. CD8-positive T cells expressing these IC molecules are inactivated through interactions with their respective ligands. Treatment with nivolumab, anti-PD1 antibodies, has improved overall survival (OS) in patients with advanced GC; however, approximately 60% of GC patients do not respond to these antibodies [2]. Therefore, the development of effective therapeutic targets and predictive biomarkers for GC treatment is urgently needed.

Various IC molecules have been extensively targeted in cancer therapies to improve treatment outcomes. Among these, combination therapy using nivolumab and ipilimumab, an anti-CTLA4 antibody, has become the standard treatment for advanced esophageal squamous cell carcinoma [3]. Recently, relatlimab, an anti-lymphocyte

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activation gene 3 (LAG3) antibody, has shown remarkable benefit in melanoma patients resistant to anti-PD1 therapy [4, 5]. LAG3 is a critical inhibitory receptor that regulates the activation and apoptosis of CD8-positive T cells by interacting with ligands such as major histocompatibility complex (MHC) class II, galectin 3 (GAL3), and fibrinogen-like protein 1 (FGL1) [6, 7]. These interactions make LAG3 a compelling therapeutic target within the realm of IC molecules. However, the widespread use of IC inhibitors has been associated with a significant increase in immune-related adverse events (irAEs). Notably, nearly 60% of melanoma patients treated with nivolumab plus ipilimumab experience serious irAEs, often leading to treatment discontinuation [4]. To improve patient selection for IC therapies, there is an urgent need for predictive biomarkers. The combined positive score (CPS) has been successfully employed to predict responses to nivolumab therapy in GC patients with CPS > 5 [8]. However, despite the growing interest in LAG3 as a therapeutic target, no serum biomarkers have been reported to predict its efficacy, underscoring a significant gap in clinical practice.

Previous studies have demonstrated that nutritional indices, including the prognostic nutritional index (PNI), the Glasgow prognostic score (GPS), and the C-reactive protein–albumin–lymphocyte (CALLY) index, can be predictive prognostic factors for a variety of solid tumors [9–12]. In GC patients, PNI was a predictive indicator of OS and relapse-free survival (RFS) [13], while the preoperative lymphocyte-to-monocyte ratio (LMR) was a prognostic factor in resectable GC patients [14]. Recently, the preoperative CALLY index was independently associated with a poor prognosis for GC patients after gastrectomy [15]. In advanced GC, neutrophil–lymphocyte ratio (NLR) was a predictive biomarker for nivolumab efficacy [12]. GC patients with low PNI had a poorer prognosis after nivolumab treatment [16], suggesting that the PNI helps select nivolumab-resistant GC patients. However, the identification of IC molecules effective for patients with nivolumab-resistant GC remains a critical challenge. Additionally, the association between nutritional indices and IC molecules beyond PD1 has been poorly elucidated.

This study aimed to investigate the correlation between nutritional indices and IC molecules in cytotoxic CD8-positive T cells of GC patients. We found that the expression of not only PD1 but also LAG3 in cytotoxic CD8-positive T cells was significantly higher in GC patients with low PNI compared to those with high PNI. Notably, among IC molecules, LAG3 was the most highly expressed in cytotoxic CD8-positive T cells, particularly in GC patients with low PNI. Furthermore, MHC class II, a ligand for LAG3, was significantly more highly expressed in GC cells of patients with low PNI than in those with high PNI. These findings suggest that the PNI **can** reflect LAG3 expression in cytotoxic CD8-positive T cells and MHC class II expression in GC cells.

Material and methods

Study population

We retrospectively collected the clinical data of 796 patients who underwent radical surgery for GC at Kyushu University Hospital (Fukuoka, Japan) between 2006 and 2018 (Supplemental Table 1). The nutritional indices used in this study were calculated based on previous literature using blood test parameters [9–12, 14, 17–21]. For example, PNI was calculated using the formula: PNI = Serum Albumin (g/dL) × 10 + Total Lymphocyte Count (/mm^3) × 0.005. Additionally, LMR was determined as: LMR = Total Lymphocyte Count/Total Monocyte Count. CALLY index was defined as: CALLY index = (Albumin × Lymphocyte)/(CRP × 10^4). Each nutritional index was classified into two groups based on reference values when defined and median values when not defined. A total of 38 patients with histologically confirmed GC were included in the single-cell RNA sequencing (scRNA-seq) analysis. Specimens for scRNA-seq were obtained from patients who underwent gastrectomy for GC at Kyushu University Hospital between 2019 and 2023. For analysis using clinical data, we excluded patients with microscopic (R1) and macroscopic (R2) resection. Patients received postoperative follow-up for 5 years or until recurrence or death. Recurrence of the disease was identified based on radiological or pathological findings. A 12-month regimen of S-1, an oral fluoropyrimidine derivative, or a 6-month course of capecitabine combined with oxaliplatin was recommended as postoperative adjuvant therapy for all patients, unless contraindicated by their condition or declined by the patient. Preoperative blood tests were conducted shortly before surgery, typically within 1–3 days of the procedure.

Sample preparation for scRNA-seq and scRNA-seq analysis

scRNA-seq analysis was performed as described previously [22, 23]. Freshly obtained tumor and adjacent normal tissue specimens were minced into 1–2 mm^3 pieces and then enzymatically digested in Liberase™ TH (Merck, Darmstadt, Hessen, Germany), containing type I/II collagenase and thermolysin, and Trypsin (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 30 min at 37 °C. The reaction was quenched by adding 2% fetal bovine serum (FBS; Thermo Fisher Scientific). The cell suspensions were filtered through a 70-µm filter (Corning Incorporated, Corning, NY, USA) and centrifuged at 400×*g* for 5 min

at 4 °C. Next, red blood cells in the cell suspension were lysed using Red Blood Cell Lysis Solution (Miltenyi Biotec, Bergisch Gladbach, North Rhine-Westphalia, Germany). The cells were then resuspended in phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA; Merck) and dead cells were removed using Dead Cell Removal MicroBeads (Miltenyi Biotec). The cell suspensions were centrifuged at 500×*g* for 10 min at 4 °C and resuspended in PBS containing 0.2% BSA. The suspensions were then filtered through a 40-µm filter (Corning Incorporated) and used for scRNA-seq. cDNA libraries were generated using a Chromium Next GEM Single Cell 3′ Reagent Kit v3.1 (10× Genomics, Pleasanton, California, USA) and a Chromium Next GEM Single Cell 5′ Kit v2 for Dual Index (10× Genomics) and then sequenced on the NovaSeq6000 (Illumina, San Diego, California, USA) or DNBSEQ-G400 (MGI Tech, Shenzhen, Guangdong Province, China) platforms.

The raw sequencing data were aligned to the GRCh38 human reference genome and processed using the Cell Ranger software package (version 5.0.0) to generate a matrix of normalized gene counts per cell for each sample. The matrix was imported and processed using the Seurat R package (version 4.3.0). All functions were run with default parameters, unless otherwise specified. Low-quality cells [< 200 UMI (unique. molecular identifiers)/cell, > 6000 genes per cell, < 3 cells per gene, and > 25% mitochondrial genes] were filtered out. The identified doublets were subsequently filtered out with the "DoubletFinder" R package (version 2.0.3). The "CellCycleScoring" and "SCTransform" functions were used to remove the potential influence of the cell cycle and batch effects on the scRNA-seq data. In addition, integration was conducted using "Harmony (version 0.1.1)" to accurately remove batch effects. To normalize the data and merge high-quality samples, the "SCTransform" function was performed. After principal component analysis with the "RunPCA" function, clusters were estimated using the "FindNeighbor" function and then visualized with the "FindClusters" function using the Uniform Manifold Approximation and Projection (UMAP) method. Supplemental Table 2 shows the gene signature scoring used in the present study [24, 25].

Multiplex immunofluorescence staining

Paraffin-embedded tissues were prepared from GC patients, and multiplex immunofluorescence staining was performed on 4-µm-thick sections according to standard procedures. Sections were deparaffinized in xylene and rehydrated using an ethanol series. Endogenous peroxidase activity was blocked using methanol containing 0.3% hydrogen peroxidase for 30 min. To retrieve antigenic epitopes, the sections were boiled for 20 min in Tris–EDTA buffer (pH 9.0) using a pressure cooker. The sections were blocked with 3% BSA in PBS for 30 min and incubated overnight with primary antibodies at 4 °C (Supplementary Table S3). Next, sections were incubated with secondary antibodies for 1 h at room temperature. Autofluorescence and endogenous immunoglobulins were blocked using the Vector True VIEW Autofluorescence Quenching Kit (SP-8400, Vector Laboratories, Newark, California, USA). Three hot spot images were captured, and cells were counted using an optical microscope (BZ-X800, Keyence, Osaka, Japan). Images were analyzed using an image analyzer (BZ-X800 Analyzer, Keyence). These stained samples were analyzed by pathologists who were blind to sample clinicopathology.

Statistical analysis

All statistical analyses, except for those related to scRNA-seq data, were conducted using GraphPad Prism (version 9.3.1, RRID:SCR_002798). Survival analysis was performed using the Kaplan–Meier method, while univariate analysis was conducted with the log-rank test. The Cox proportional hazards model was applied for multivariate analysis. Group comparisons were carried out using the Mann–Whitney U test or the Kruskal–Wallis test, with Dunnett's test employed for multiple comparisons. For scRNA-seq data analysis, the Wilcoxon rank-sum test and Kruskal–Wallis test were utilized. Correlation analysis between groups was assessed using Spearman's correlation coefficient. A P value of less than 0.05 was considered statistically significant ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$).

Results

Correlation between preoperative nutrition indices and prognosis in GC patients after radical gastrectomy

To elucidate which previously reported nutritional indices strongly correlate with prognosis in GC patients after radical gastrectomy, we retrospectively analyzed clinicopathological data from 796 GC patients, including preoperative blood test results and adjuvant chemotherapy records (Fig. 1A). Kaplan–Meier analysis and log-rank tests revealed that low PNI, low LMR, and low CALLY were significantly associated with higher hazard ratios compared with their respective high-value groups (Fig. 1B). Among these, low PNI was the strongest prognostic factor, demonstrating the highest hazard ratio (HR). Cox proportional hazards analysis further confirmed that low PNI is an independent predictor of poor prognosis in GC patients undergoing radical gastrectomy (supplemental Fig. 1A and supplemental Table 2).

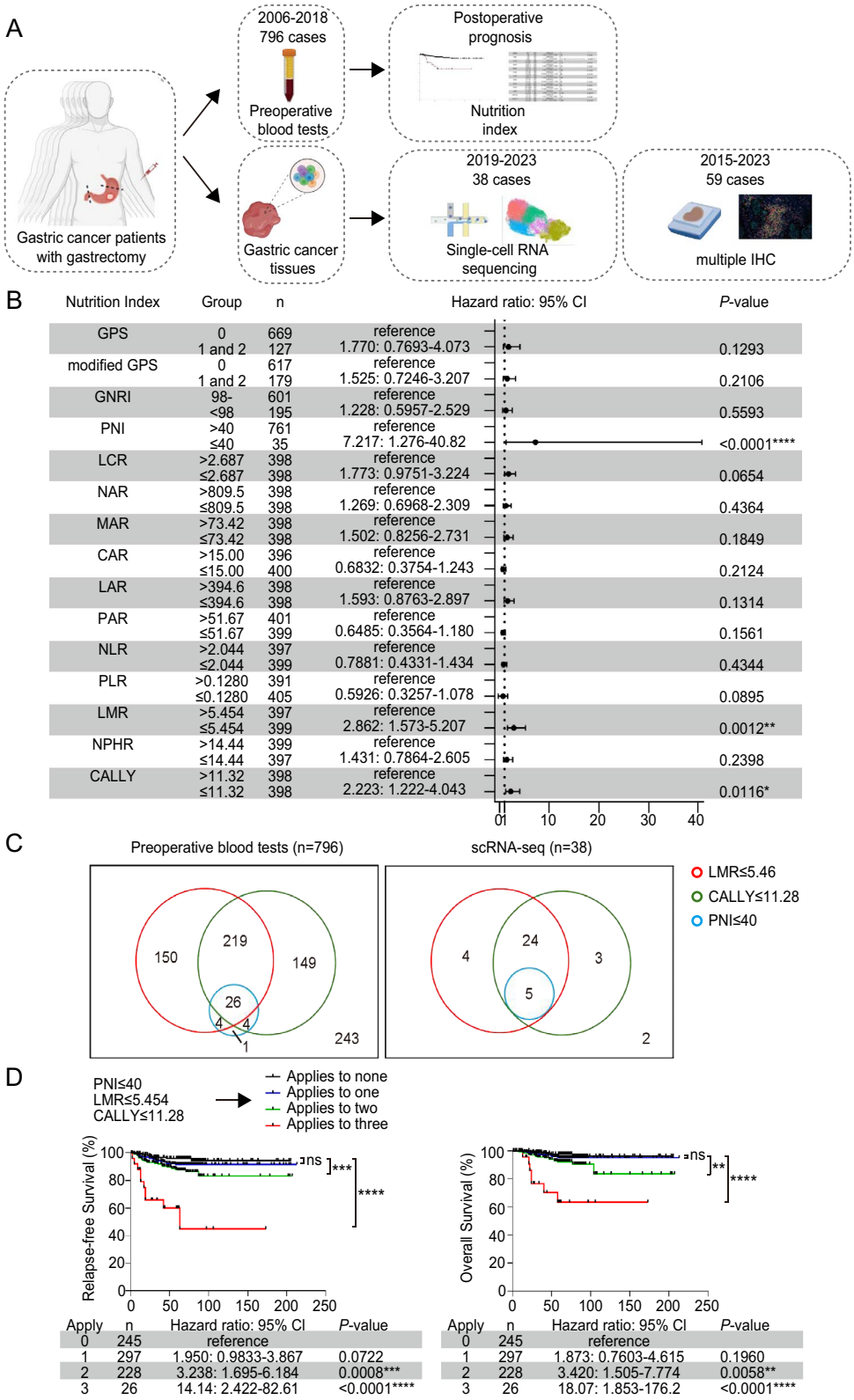


Fig. 1 Relationship between preoperative nutritional indices and prognosis in GC patients undergoing radical gastrectomy. **A** Schematic diagram of the experimental design. **B** Hazard ratios (performed using the log-rank test) for nutritional indices in GC patients categorized into two groups based on nutritional indices. **C** Venn diagram showing the distribution of patients with low LMR, low CALLY, and low PNI among preoperative blood test (left) and scRNA-seq samples (right). **D** Overall survival analysis (performed using the Kaplan–Meier plotter) among groups based on nutritional indices (low PNI, low LMR, and low CALLY). GC, gastric cancer; LMR, lymphocyte-to-monocyte ratio; CALLY, C-reactive protein–albumin–lymphocyte index; PNI, prognostic nutritional index; scRNA-seq, single-cell RNA sequencing

Next, we analyzed the distribution of patients with low PNI, low LMR, and low CALLY indices. Among patients identified with low PNI through preoperative blood tests and scRNA-seq data, 77.5% (31/40) had concurrent low LMR and low CALLY values (Fig. 1C). Kaplan–Meier curves and log-rank tests demonstrated that GC patients with low PNI, LMR, and CALLY values had significantly shorter relapse-free survival and overall survival following radical surgery (Fig. 1D). These findings underscore the critical role of preoperative PNI as a robust prognostic indicator among nutritional indices in GC patients undergoing radical gastrectomy.

Association between PNI and IC molecule expression in cytotoxic CD8-positive T cells

Cytotoxic CD8-positive T cells, which are a subset of CD8-positive T cells, play a central role in tumor antigen-specific anti-tumor immunity and are distinguished by their high levels of IC molecule expression [26]. To investigate the relationship between nutritional indices, including PNI, and the expression of IC molecules in these cells, we examined gene expression in 286,241 single cells derived from 38 GC tissues and 16 adjacent normal tissues (Adj. tissues) (Fig. 1A and Supplemental Table 4). Among these, five GC patients had low PNI (Fig. 1C, right). Unbiased clustering revealed 14 major clusters, which were assigned to known cell populations based on canonical marker genes (Supplemental Fig. 2A, B). To identify CD8-positive T cells, we focused on the T cell and natural killer (NK) cell clusters (C2 and C3 clusters). These clusters were further subdivided into six subclusters through re-clustering (Supplemental Fig. 2C). Based on CD8A expression, we designated the T1 and T3 clusters as CD8-positive T cells (Supplemental Fig. 1D). The CD8-positive T cell clusters were then re-clustered, resulting in five distinct subclusters with varying gene expression profiles (Fig. 2A). Among them, the CD8-1 (LAG3+HAVCR2+) and CD8-4 (PDCD1+TIGIT+) subclusters exhibited high levels of IC molecules, GZMB, and PRF1. Consequently, these subclusters were classified as cytotoxic CD8-positive T cells, in accordance with a previous study [26] (Supplemental Fig. 2E, F). Furthermore, we examined the correlation between nutritional indices and IC molecule expression in cytotoxic CD8-positive T cells for each patient with scRNA-seq data. While IC molecule expression in these cells tended to be higher in patients with low PNI than in those with high PNI, no clear association was observed between IC molecules and LMR or CALLY (Fig. 2B). Moreover, LMR showed a significant inverse correlation with CTLA4 and HAVCR2 (which encodes TIM3), while CALLY exhibited a significant inverse correlation only with HAVCR2. In contrast, PNI demonstrated a significant inverse correlation with all IC molecules in cytotoxic CD8-positive T cells (Fig. 2C). These results indicate that PNI reflects the expression of IC molecules in cytotoxic CD8-positive T cells.

Identification of IC molecules strongly correlated with PNI in cytotoxic CD8-positive T cells

To identify therapeutic targets among IC molecules in GC with low PNI, we assessed their expression in cytotoxic CD8-positive T cells. LAG3-positive cells constituted the highest percentage among IC molecule-positive cells (Fig. 3A). The expression levels of all IC molecules were significantly higher in GC with low PNI than in those with high PNI (Fig. 3B). Notably, cytotoxic CD8-positive T cells exhibited the lowest levels and proportions of HAVCR2 expression (Fig. 3A, B). Based on this observation, we focused our evaluation on IC molecules other than HAVCR2. GC with low PNI showed significantly higher average expression levels of PDCD1 (which encodes PD1) and LAG3 compared to GC with high PNI (Fig. 3C). Consequently, we next evaluated PD1 and LAG3 in CD8-positive cells using multiplex immunofluorescence staining. The percentage of PD1-positive cells among CD8-positive cells and the number of double-positive PD1 and CD8 T cells were significantly higher in GC with low PNI than in GC with high PNI (Fig. 3D, E). Similarly, the percentage of LAG3-positive cells among CD8-positive cells and the number of double-positive LAG3 and CD8 T cells were significantly elevated in GC with low PNI compared to GC with high PNI (Fig. 3F, G). Among cytotoxic CD8-positive T cells, LAG3-positive cells were more abundant than PDCD1-positive cells, particularly in GC with low PNI compared to GC with high PNI (Fig. 3H). Notably, most LAG3-positive cells did not co-express PDCD1, suggesting that LAG3 represents a distinct therapeutic target for GC patients who do not respond to PD1-targeted therapy. Furthermore, PNI was significantly inversely correlated with the percentage of LAG3-positive cells among CD8-positive cells and the number of double-positive LAG3 and CD8 T cells (Fig. 3I). Receiver operating characteristic (ROC) curve analysis demonstrated that PNI had significant diagnostic performance for both the percentage of LAG3-positive cells among CD8-positive T cells and the number of double-positive LAG3 and CD8 T cells, with area under curve (AUC) values of 0.8187 and 0.7632, respectively (Fig. 3J). Additionally, ROC curve analysis showed that PNI was predictive of average LAG3 expression levels in cytotoxic CD8-positive T cells, with an AUC value of 0.7697 (Fig. 3K). These findings underscore the potential of LAG3 as a pivotal therapeutic target for cytotoxic CD8-positive T cells in GC patients with low PNI.

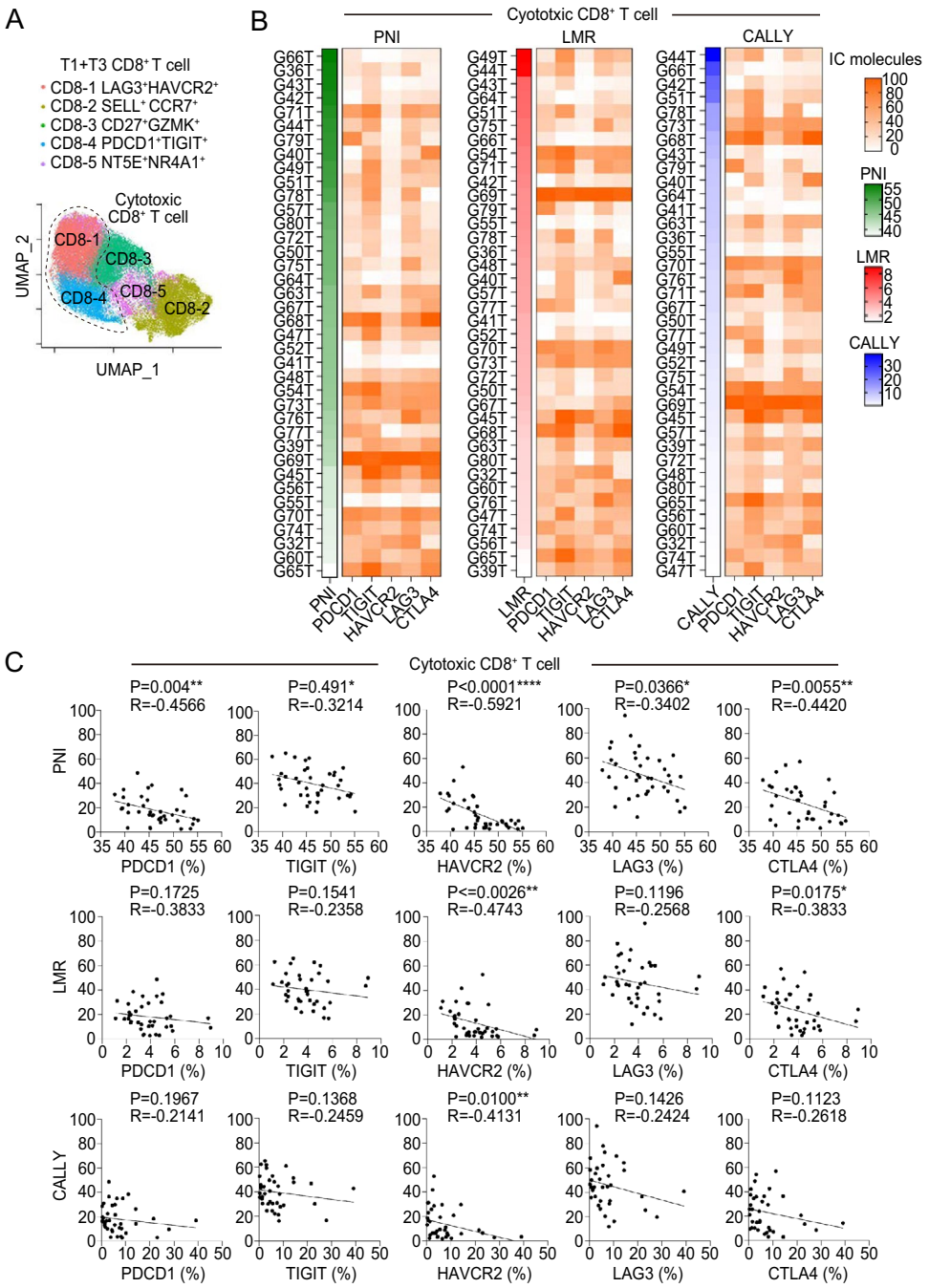


Fig. 2 Link between PNI and IC molecule expression in cytotoxic CD8-positive T cells. **A** UMAP plot of 47,972 CD8-positive T cells with scRNA-seq data from 38 GC patients, representing the formation of five main clusters. **B** Heatmaps depicting the correlation between nutritional indices and the expression of IC molecules in cytotoxic CD8-positive T cells from the scRNA-seq data of each GC patient. **C** Correlation analysis of the scRNA-seq data of GC patients based on nutritional indices and the expression of IC molecules in cytotoxic CD8-positive T cells. PNI, prognostic nutritional index; IC, immune checkpoint; UMAP, uniform manifold approximation and projection; scRNA-seq, single-cell RNA sequencing; GC, gastric cancer

LAG3 ligand expression in GC with low PNI

LAG3 ligands include MHC class II, GAL3, and FGL1 [6], with MHC class II directly suppressing CD8-positive T cells through LAG3 [27]. Given that GAL3 and FGL1 were scarcely expressed in our scRNA-seq data from GC patients, we focused on MHC class II. Professional antigen-presenting cells (APCs), such as macrophages, dendritic cells (DCs), and B cells, are known to express MHC class II molecules [28]. To investigate their contribution, we analyzed MHC class II expression in professional APCs by re-clustering the myeloid cell cluster (C7 cluster) and identifying macrophages (M1 cluster) and DCs (M4/M5/M6 clusters) based on their expression profiles (Fig. S3A, B). Similarly, we re-clustered the B cell and plasma cell clusters (C4/C5/C6/C9/C13 clusters) and identified the B1 cluster as B cells based on CD19 and MS4A1 expression (Fig. S3C, D). In these professional APCs, the expression of MHC class II-related genes was not higher in GC with low PNI compared to GC with high PNI (Fig. 4A). These results suggest that the interaction between LAG3 on

CD8-positive T cells and MHC class II on professional APCs is unlikely to be a crucial pathway for LAG3-positive CD8-positive T cells in GC with low PNI.

Recently, elevated MHC class II expression in cancer cells has been reported in various solid tumors [29]. Additionally, LAG3 expression is upregulated in MHC class II-positive melanoma and non-small cell lung cancers that have acquired resistance to anti-PD1 antibodies [30]. Based on this, we evaluated MHC class II-positive GC cells and LAG3-positive CD8-positive T cells using multiplex immunofluorescence staining. In GC with low PNI, MHC class II-positive cancer cells and LAG3-positive CD8-positive T cells were observed in closer proximity compared to GC with high PNI (Fig. 4B). To elucidate the characteristics of GC cells with high MHC class II expression, we isolated the epithelium clusters (C1/C14 clusters) and identified six subclusters (Fig. 4D). Among these, the E4 subcluster, characterized by high VIM and TWIST expression, was more prevalent in GC with low PNI than in GC with high PNI (Fig. 4D, E). Furthermore, the E4 subcluster exhibited significantly higher MHC class II-related gene expression compared to other epithelial subclusters (Fig. 4F). Notably, the expression of MHC class II-related genes was markedly higher in GC with low PNI than in GC with high PNI within this subcluster (Fig. 4F). Multiplex immunofluorescence staining further confirmed that the percentage of HLA-DR/DP/DQ-positive GC cells was significantly higher in GC with low PNI compared to GC with high PNI (Fig. 4G, H). Additionally, the percentage of double-positive HLA-DR/DP/DQ and Vimentin GC cells was significantly higher in GC with low PNI (Fig. 4G, H). These findings suggest a potential relationship between MHC class II in GC cells and LAG3-positive cytotoxic CD8-positive T cells in GC with low PNI.

Investigation of the mechanisms linking PNI to LAG3 expression

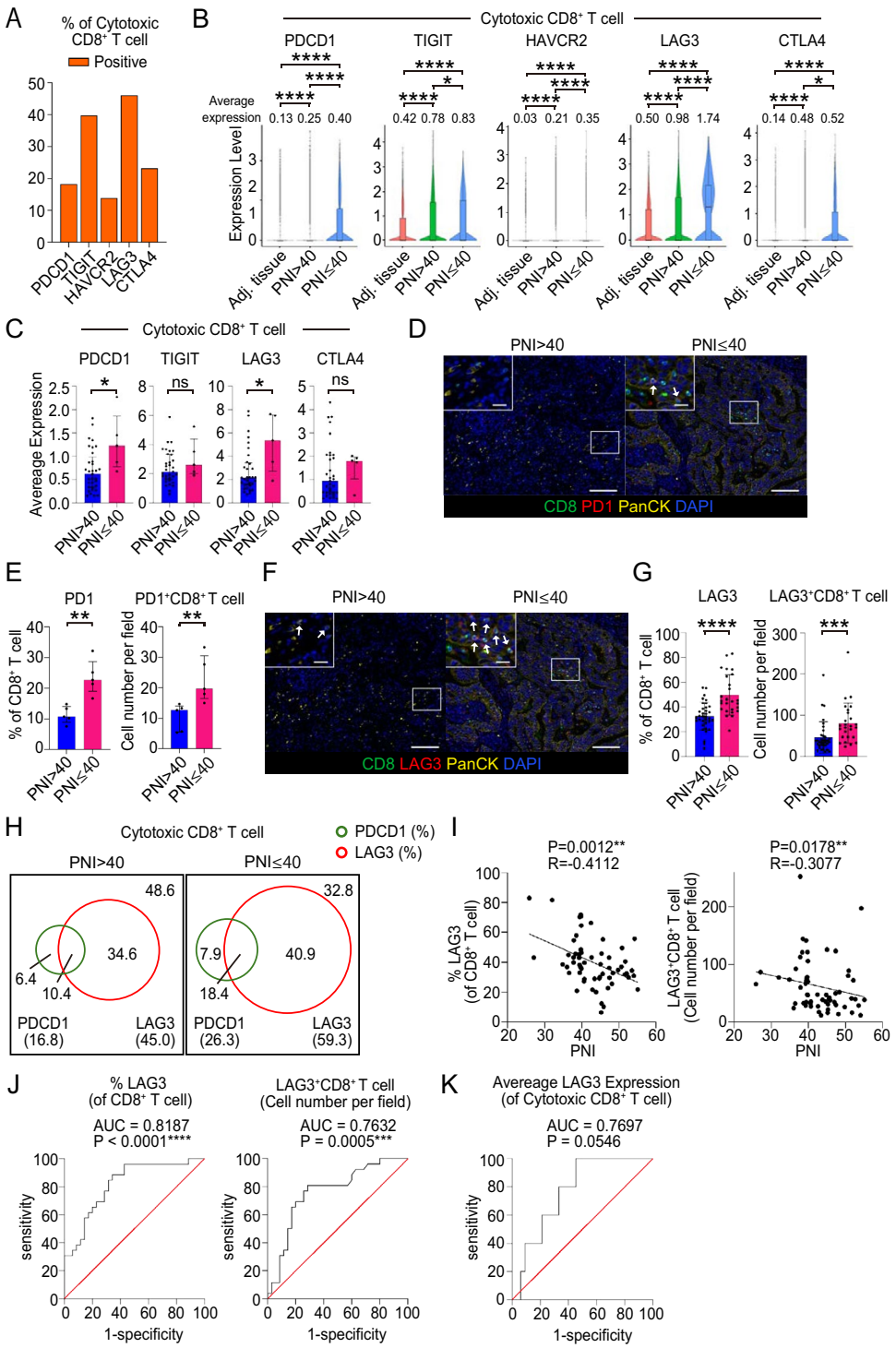
To investigate the mechanisms underlying the correlation between PNI and LAG3 expression, we examined the specific components of PNI—albumin levels and lymphocyte count—and their relationship with LAG3 expression in cytotoxic CD8-positive T cells. Our findings revealed that, although neither albumin levels nor lymphocyte count emerged as significant prognostic factors (Fig. 5A), distinct patterns were observed. Specifically, lymphocyte count showed a significant inverse correlation with the average LAG3 expression in cytotoxic CD8-positive T cells, while albumin levels demonstrated a significant inverse correlation with the average expression of MHC class II-related genes in the E4 cluster (Fig. 5B, C). These findings suggest that the specific components of PNI are reflected in distinct immunological pathways, with lymphocyte count influencing LAG3 expression in cytotoxic CD8-positive T cells and albumin levels reflecting MHC class II-related gene expression in GC cells.

LAG3 binds to MHC class II, suppressing the activation of LAG3-positive T cells [7]. Moreover, the interaction between LAG3 and MHC class II is critical in regulating apoptosis, particularly by enhancing cell death in LAG3-expressing cells [31, 32]. Based on these previous findings, we further examined the expression of apoptosis-related genes [25] and the activation marker CD69 in cytotoxic CD8-positive T cells to elucidate the significance of the LAG3-MHC class II pathway. Our results showed that GC with low PNI exhibited significantly higher pro-apoptotic gene expression and substantially lower anti-apoptotic gene expression compared to those with high PNI (Fig. 5D). Additionally, the activation marker CD69 was expressed at significantly lower levels in the low PNI group than in the high PNI group (Fig. 5D). Furthermore, GC patients with low PNI exhibited a closer association between MHC class II-positive GC cells and LAG3-positive CD8-positive T cells than those with high PNI (Fig. 4B). These findings highlight the potential role of the interaction between LAG3 in cytotoxic CD8-positive T cells and MHC class II in t.

Furthermore, gene set enrichment analysis (GSEA) revealed that immune response pathways were significantly enriched in cytotoxic CD8-positive T cells from GC with low PNI compared to those with high PNI (Fig. 5E). IFNγ is a cytokine that plays a key role in activating the immune response [33]. The scRNA-seq data demonstrated high IFNG expression in T and NK cell clusters (C2/C3 clusters) and revealed that IFNG expression in these clusters was significantly higher in GC with low PNI than in GC with high PNI (Fig. 5F). Expression of MHC class II in epithelial cells and LAG3 in immune cells is induced by IFNγ [34, 35]. Therefore, in GC with low PNI, high IFNG expression may contribute to the induction of LAG3 in CD8-positive T cells and MHC class II in GC cells.

Discussion

Nutrition is closely related to anti-tumor immunity. PNI, calculated from serum albumin levels and total lymphocyte count, reflects the nutritional and immune status of patients with cancer [13]. The present study revealed that preoperative low PNI was the poorest prognostic factor among previously studied nutritional indices in GC patients who underwent radical gastrectomy. A recent study demonstrated that low PNI is associated with poor prognosis in GC patients treated with anti-PD1 antibodies [36]. However, the relationship between PNI and IC molecules other than PD1 remains poorly understood. In a previous study, CD8-positive T cells were shown to



recognize tumor-specific antigens and subsequently express not only PD1 but also other IC molecules, such as LAG3 [37]. Although LAG3 has recently gained considerable attention, no studies have investigated the association between PNI and LAG3. To address this gap, we aimed to characterize the heterogeneous expression of IC molecules in tumor-infiltrating CD8-positive T cells and to examine their association with PNI. We found that GC with low PNI exhibited significantly higher expression levels of not only PDCD1 but also LAG3 in cytotoxic CD8-positive T cells compared to GC with high PNI. Among cytotoxic CD8-positive T cells, the proportion of LAG3-positive cells was greater than that of PDCD1-positive cells, particularly in GC with low PNI. Importantly, the percentage of LAG3-positive

◂**Fig. 3** Identification of IC molecules closely associated with PNI in cytotoxic CD8-positive T cells. **A** The percentage of cells positive for IC molecules in cytotoxic CD8-positive T cells from scRNA-seq data. **B** Violin plots representing the expression levels of IC molecules in cytotoxic CD8-positive T cells from Adj. tissue, high PNI, and low PNI groups. **C** The average expression level of IC molecules in cytotoxic CD8-positive T cells from high PNI and low PNI groups in each sample. **D** Representative images of CD8, PD1, and PanCK immunostaining and DAPI staining in GC tissues. Scale bars, 100 μm and 20 μm for the higher magnification boxed area. Arrows indicate CD8/PD1-positive cells. **E** Quantification of tumor-infiltrating PD1/CD8-positive T cells per field in GC tissues with high PNI and low PNI. **F** Representative images of CD8, LAG3, and PanCK immunostaining and DAPI staining in GC tissues. Scale bars, 100 μm and 20 μm for the higher magnification boxed area. Arrows indicate CD8/LAG3-positive cells. **G** Quantification of the percentage of LAG3-positive cells among CD8-positive cells (left) and the number of double-positive LAG3 and CD8 T cells (right) per field in GC tissues with high PNI and low PNI. **H** Venn diagram showing distribution of PDCD1- and LAG3-positive cytotoxic CD8-positive T cells in high PNI (left) and low PNI (right) groups. **I** Correlation analysis based on PNI and the percentage of LAG3-positive cells among CD8-positive cells (left)/the number of double-positive LAG3 and CD8 T cells (right). **J** Receiver operating characteristic curves showing the diagnostic performance of PNI for the percentage of LAG3-positive cells among CD8-positive T cells (left) and the number of double-positive LAG3 and CD8 T cells per field (right). **K** Receiver operating characteristic curves showing the diagnostic performance of PNI for the percentage of average LAG3 expression in cytotoxic CD8-positive T cells from the scRNA-seq data. IC, immune checkpoint; PNI, prognostic nutritional index; scRNA-seq, single-cell RNA sequencing; GC, gastric cancer

cells among cytotoxic CD8-positive T cells was higher than that of cells expressing other IC molecules. These findings suggest that LAG3 could serve as a promising therapeutic target for GC patients with low PNI.

A previous study indicated that the expression of MHC class II in epithelial cells and LAG3 in immune cells is induced by IFNγ [34, 35]. Additionally, bulk RNA sequencing data showed that the expression levels of MHC class II-related genes and IFNG were elevated in Epstein-Barr virus (EBV)-positive GC [38]. Our study revealed that IFNG expression in T and NK cells was significantly higher in GC with low PNI than in GC with high PNI. Moreover, among the five GC patients whose samples were used for our scRNA-seq analysis, one was EBV-positive, and two were human epidermal growth factor receptor 2 (HER2)-positive. EBV expresses multiple virus-related antigens, such as EBNA-2 and EBNA-3A, while HER2 serves as a tumor-associated antigen that enhances immune responses through antigen processing by dendritic cells [39–41]. Our study demonstrated that immune response pathways showed significant enrichment in cytotoxic CD8-positive T cells from GC patients with low PNI compared to those with high PNI. Additionally, lymphocyte count was significantly inversely correlated with the average LAG3 expression in cytotoxic CD8-positive T cells, while albumin levels showed a significant inverse correlation with the average expression of MHC class II-related genes in GC cells. Furthermore, cytotoxic CD8-positive T cells exhibited higher expression levels of pro-apoptotic genes and lower expression levels of anti-apoptotic genes in GC with low PNI compared to GC with high PNI. Tumor-related inflammation can suppress albumin synthesis and impair anti-tumor immunity [42, 43]. Moreover, immune checkpoint molecules, including LAG3, inhibit T cell proliferation and contribute to peripheral lymphopenia in solid tumors [7, 44]. These findings suggest that in a tumor-associated antigen-enriched TME, tumor-related inflammation is triggered, leading to increased IFNγ production, suppressed albumin synthesis, and upregulated MHC class II expression in GC cells. Simultaneously, CD8-positive T cells express LAG3, which interacts with MHC class II, promoting apoptosis and leading to peripheral lymphocyte reduction. Consequently, GC patients with low PNI exhibit higher LAG3 expression in CD8-positive T cells and higher MHC class II expression in GC cells compared to those with high PNI.

In recent years, IC inhibitors have revolutionized cancer treatment. However, the emergence of resistance to IC inhibitors and rapid tumor progression following IC blockade have become significant concerns [2, 45]. Therefore, there is an urgent need for simple and accessible biomarkers to identify patients who are most likely to benefit from IC therapy. The PNI, which can be easily calculated from routine blood tests, serves as a convenient clinical marker. This study demonstrated that in GC patients with low PNI, LAG3 expression in cytotoxic CD8-positive T cells and the expression of its ligand, MHC class II, in GC cells were high. Additionally, ROC curve analysis demonstrated that PNI could predict the average LAG3 expression levels in cytotoxic CD8-positive T cells. These findings suggest that PNI could serve as a useful biomarker for identifying GC patients who may benefit from LAG3 blockade, thereby enabling more precise patient selection for IC inhibitor therapy. Furthermore, our study showed that among cytotoxic CD8-positive T cells, the proportion of LAG3-positive cells exceeded that of PDCD1-positive cells, particularly in GC with low PNI, and most LAG3-positive cells did not co-express PDCD1. This finding suggests that LAG3-targeted therapy could be a promising treatment option for GC patients who are resistant to PD1 therapy. Taken together, our findings highlight the potential of PNI as a biomarker for guiding personalized immunotherapy strategies in GC. However, our study only establishes a correlation between PNI and the LAG3–MHC class II axis in GC patients and does not directly assess cell–cell interactions, which remains a limitation. Moreover, the number of samples analyzed by scRNA-seq and immunofluorescence staining was limited, and we cannot rule out the possibility that our findings reflect patient-specific results. To confirm a broader correlation between

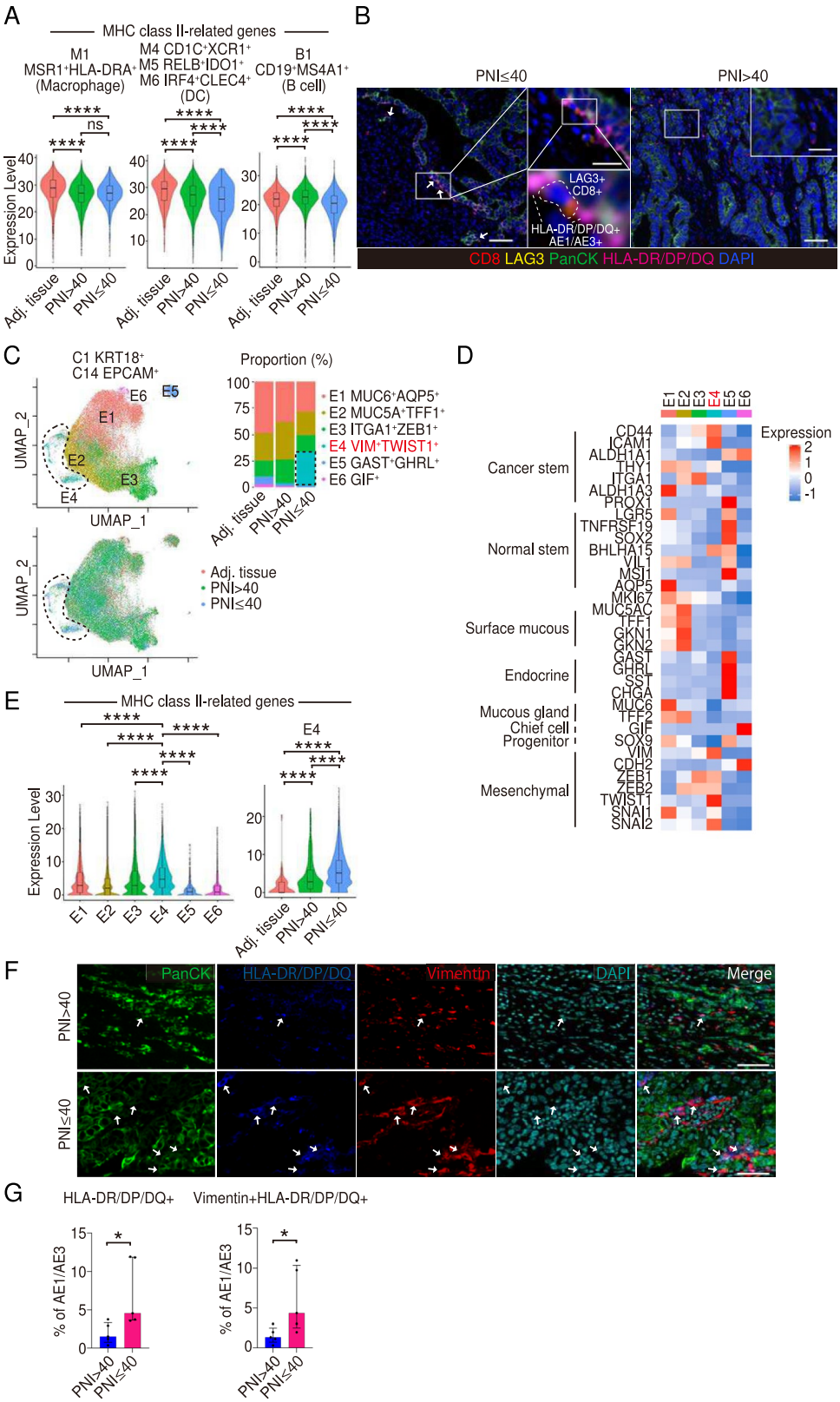


Fig. 4 Expression patterns of LAG3 ligands in GC with low PNI. **A** Violin plots representing the expression levels of MHC class II-related genes in professional APCs from Adj. tissue, high PNI, and low PNI GC groups. **B** Representative images of CD8, LAG3, PanCK, and HLA-DR/DP/DQ immunostaining and DAPI staining in GC tissues. Scale bars, 50 μm and 20 μm for the higher magnification boxed area. Arrows indicate the proximity of CD8/LAG3-positive cells to PanCK/HLA-DR/DP/DQ-positive cells. **C** UMAP plots of 55,772 epithelial cells, representing the formation of six main clusters (upper row, left), the location of epithelial cells in the UMAP for each PNI group (bottom row), and the proportions of each subcluster in epithelial cells (upper row, right). **D** Heatmaps depicting the mean cluster expression of a panel of genes. **E** Violin plots showing the expression levels of MHC class II-related genes in each epithelial subcluster (left) and in each group among E4 subcluster (right). **F** Representative images of PanCK, HLA-DR/DP/DQ, and Vimentin immunostaining and DAPI staining in GC tissues. Scale bars, 50 μm. Arrows indicate triple-positive PanCK, HLA-DR/DP/DQ, and Vimentin cells. **G** Quantification of double-positive PanCK and HLA-DR/DP/DQ cells (left) and triple-positive PanCK, HLA-DR/DP/DQ, and Vimentin cells (right) per field in GC tissues. GC, gastric cancer; PNI, prognostic nutritional index; APCs, antigen-presenting cells; scRNA-seq, single-cell RNA sequencing

PNI and the LAG3–MHC class II axis and further elucidate these interactions, future studies with larger sample sizes and functional experiments are required.

In conclusion, LAG3 expression in cytotoxic CD8-positive T cells was higher in GC with low PNI than in GC with high PNI. Among cytotoxic CD8-positive

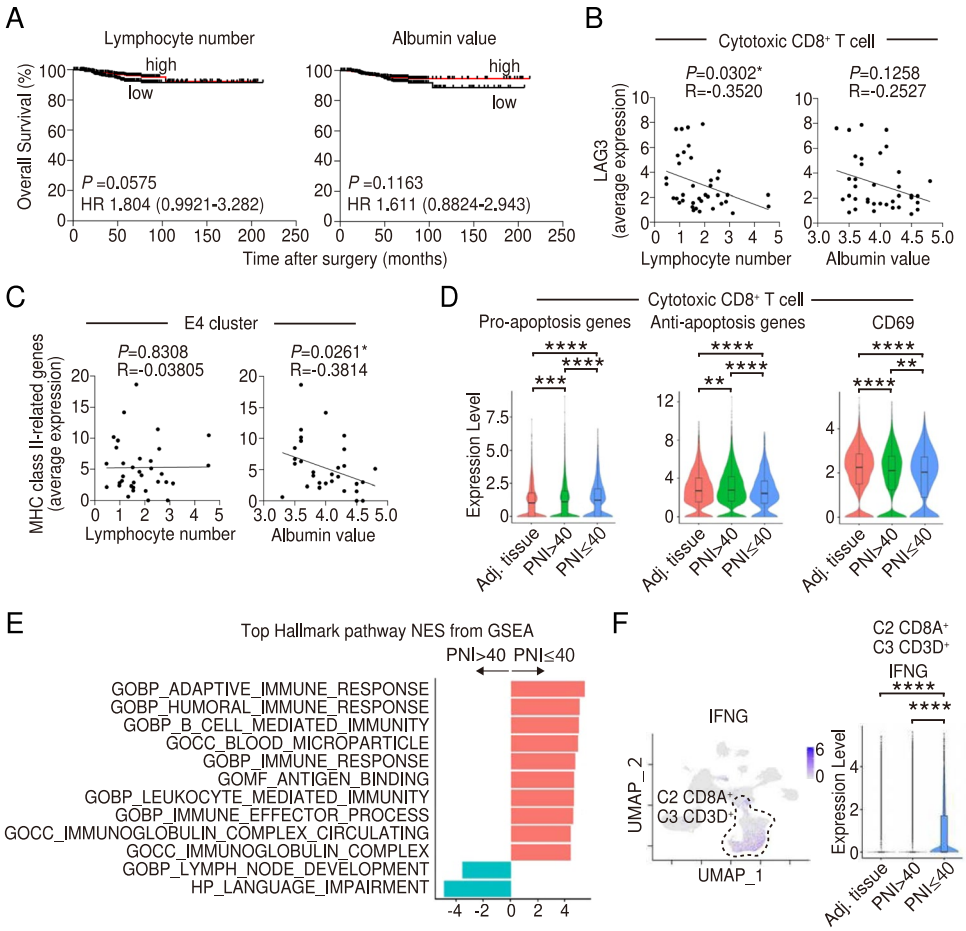


Fig. 5 Mechanisms connecting PNI to LAG3 expression. **A** Overall survival analysis (performed using the Kaplan–Meier plotter) among groups based on lymphocyte number and albumin value. **B** Correlation analysis based on average LAG3 expression in cytotoxic CD8-positive T cells and lymphocyte number (left)/albumin value (right) in GC patients. **C** Correlation analysis based on average expression of MHC class II-related genes in E4 subcluster and lymphocyte number (left)/albumin value (right) in GC patients. **D** Violin plots representing the expression levels of pro-apoptosis genes, anti- apoptosis genes, and CD69 in cytotoxic CD8-positive T cells from Adj. tissue, high PNI, and low PNI groups. **E** GSEA analysis in cytotoxic CD8-positive T cells based on PNI. **F** UMAP plots of all cells with IFNG expression (left) and violin plots representing IFNG expression levels of the T and NK cell clusters in each group (right). PNI, prognostic nutritional index; GC, gastric cancer; GSEA, gene set enrichment analysis; UMAP, uniform manifold approximation and projection

T cells, LAG3-positive cells were more abundant than PDCD1-positive cells, with this difference being more pronounced in GC with low PNI. Additionally, MHC class II expression was higher in GC cells with high levels of epithelial–mesenchymal transition-related molecules in GC with low PNI compared to GC with high PNI. Therefore, these findings suggest that PNI can reflect LAG3 expression in cytotoxic CD8-positive T cells and MHC class II expression in GC cells.

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Author Contributions CT designed the experimental approach, performed the experimental work, analyzed data, coordinated projects, and wrote the manuscript. KO designed the experimental approach, analyzed data, wrote the manuscript, coordinated projects, and contributed to data interpretation and discussion. CT, MI, BT, KS, TK, NK, YM, SH, and NT performed the experimental work. CI, KH, KS, YM, NI, and KN contributed to data interpretation and discussion. KO, KH, KS and YO provided and prepared human samples. Immunohistochemistry was analyzed by CT, YS, and YO. MN coordinated projects, wrote the manuscript, and contributed to data interpretation and discussion.

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Data Availability The scRNA-seq data from this study are available in the GEO database under the accession code GSE268238. Further details can be found in the article, Supplementary Information, or provided by the authors upon request.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval Clinical data, including histopathological findings, were obtained from the patients' electronic medical records. Written informed consent was obtained from all participating patients and the study was approved by the Ethics Committee of Kyushu University (approval number: 2023–79 and 22002-00) and conducted in accordance with the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government and the Declaration of Helsinki.

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