

Tumor-infiltrating monocytic myeloid-derived suppressor cells contribute to the development of an immunosuppressive tumor microenvironment in gastric cancer

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10 **Short running head**

11 Monocytic myeloid-derived suppressor cells in gastric cancer

12

Abstract

Background

Gastric cancer (GC) is characterized by an immunosuppressive and treatment-resistant tumor immune microenvironment (TIME). Here, we investigated the roles of different immunosuppressive cell types in the development of the GC TIME.

Methods

Single-cell RNA sequencing (scRNA-seq) and multiplex immunostaining of samples from untreated or immune checkpoint inhibitor (ICI)-resistant GC patients were used to examine the correlation between certain immunosuppressive cells and the prognosis of GC patients.

Results

The results of the scRNA-seq analysis revealed that tumor-infiltrating monocytic myeloid-derived suppressor cells (TI-M-MDSCs) expressed higher levels of genes with immunosuppressive functions than other immunosuppressive cell types. Additionally, M-MDSCs in GC tissues expressed significantly higher levels of these markers than adjacent normal tissues. The M-MDSCs were most enriched in GC tissues relative to adjacent normal tissues. Among the immunosuppressive cell types assessed, the M-MDSCs were most enriched in GC tissues relative to adjacent normal tissues; moreover, their presence was most strongly associated with a poor prognosis. Immediate early response 3 (*IER3*), which we identified as a differentially expressed gene between

1 M-MDSCs of GC and adjacent normal tissues, was an independent
2 poor prognostic factor in GC patients ($P=0.0003$). IER3⁺ M-MDSCs
3 expressed higher levels of genes with immunosuppressive functions
4 than IER3⁻ M-MDSCs and were abundant in treatment-resistant GC
5 patients.

6 **Conclusions**

7 The present study suggests that TI-M-MDSCs, especially IER3⁺ ones,
8 may play a predominant role in the development of the
9 immunosuppressive and ICI-resistant GC TIME.

1 **Introduction**

2 Gastric cancer (GC) is the third leading cause of cancer deaths
3 worldwide [1]. Immune checkpoint inhibitors (ICIs) have
4 revolutionized cancer therapy and clinical studies using ICIs for
5 advanced GC have reported improved overall survival (OS) in these
6 patients². However, many GC patients do not respond to ICIs. For
7 instance, only 40% of GC patients experienced controlled disease
8 after nivolumab treatment [2]. Therefore, the question of how the
9 tumor immune microenvironment (TIME) in GC reduces the efficacy
10 of ICIs needs to be urgently addressed to develop effective anticancer
11 therapies.

12 Tumors develop an immunosuppressive TIME to enhance
13 cancer cell survival. The presence of various immunosuppressive
14 cells (ICs), such as regulatory T-cells (Tregs), myeloid-derived
15 suppressor cells (MDSCs), and regulatory macrophages (Mregs) in
16 the TIME are poor prognostic factors in GC [3–5]. These cell types
17 can suppress the functions of tumor-antigen-specific cytotoxic
18 T-cells [6]. Numerous studies in mice have revealed Gr-1 to be a
19 reliable pan-MDSC marker [7,8]. However, MDSC markers differ
20 between mice and humans and no reliable human pan-MDSC markers
21 have been identified to date [9]. The need to evaluate human MDSCs
22 with multiple markers is one of the factors delaying human MDSC
23 research. Recently, the introduction of multiplex
24 immunohistochemistry has helped overcome the limitations of
25 traditional histological studies of human MDSCs [10]. However,

tumor-infiltrating (TI)-MDSCs have different functions from peripheral blood and splenic MDSCs [11]. Moreover, monocytic MDSCs (M-MDSCs) in peripheral blood are a worse prognostic factor than Tregs in lung cancer patients [12]. Although human peripheral blood and splenic MDSCs have been relatively well studied [13,14], TI-MDSCs have been poorly characterized in humans.

To date, few M-MDSC-targeted therapies for cancer patients have reached clinical trial stage and thus many challenges remain in improving their efficacy. All-trans retinoic acid (tretinoin) induced the differentiation of MDSCs into mature macrophages without inhibitory functions [15]. Meanwhile, doxorubicin and gemcitabine enhanced antitumor effects in mice by eliminating or suppressing MDSCs [16]. However, in breast cancer patients, doxorubicin–cyclophosphamide chemotherapy actually increased the number of MDSCs in the peripheral blood [17]. The heterogeneity of MDSCs and cancer-specific differences make it challenging to characterize MDSCs and elucidate their functions for effective MDSC-targeted therapy. Therefore, the heterogeneity of MDSCs in patients with cancer needs to be understood.

In the present study, we focused on characterizing TI-ICs in the TIME of GC patients (GC TIME). The results of single-cell RNA sequencing (scRNA-seq) analysis revealed that M-MDSCs expressed higher levels of genes associated with immunosuppressive functions than other ICs. Additionally, multiplex immunohistochemistry revealed that, among all the ICs in the GC TIME, TI-M-MDSCs were

most strongly associated with a poor prognosis. Moreover, immediate early response 3 (IER3)⁺ M-MDSCs expressed higher levels of immunosuppressive-function-related genes and were more abundant in treatment-resistant GC patients. IER3 was an independent poor prognostic factor in GC. These findings indicate that targeting IER3⁺ TI-M-MDSCs may be a promising, cell-specific treatment strategy in patients with ICI-resistant GC; however, further studies are needed to validate this approach.

Materials and Methods

Sample collection

In the present study, 30 patients with pathologically diagnosed GC and esophageal squamous cell carcinoma (ESCC) were enrolled for scRNA-seq analysis. All specimens used in this study were obtained from patients who underwent surgery or biopsies for GC and ESCC at Kyushu University Hospital (Fukuoka, Japan). The clinical characteristics of the enrolled patients are summarized in Tables S1. All fresh specimens were obtained from tumors and adjacent normal tissues (Adj. tissue) immediately after surgical resection or biopsy.

10X Genomics single-cell 3' library preparation and scRNA-seq

Single-cell library preparation was performed according to the Single-cell 3' Reagent Kits v3.1 manual (10X Genomics, Pleasanton, California, USA), and other analyses described supplemental methods.

Single-cell gene expression normalization, quality control, dimensionality reduction, and annotation

Raw sequencing data were aligned to the GRCh38 human reference genome and processed to a matrix containing normalized gene counts versus cells per sample using the Cell Ranger software package (version 5.0.0). Low-quality cells (<200 UMI (Unique Molecular Identifiers) /cell, >6000 genes/cell, <3 cells/gene, and >25% mitochondrial genes) were excluded. To eliminate the potential influence of the cell cycle and batch effects on the data, “CellCycleScoring” and “SCTransform” functions were performed [18,19]. The “SCTransform” function was used for normalization, to enable merging of high-quality data from all samples [19]. The identified doublets were then filtered using the “DoubletFinder” R package [20]. Cell-cell interactions were assessed using “NicheNet” [21].

Identification of differentially expressed genes and pathway enrichment analysis

The “FindAllMarkers” function of the Seurat package was used to identify differentially expressed genes (DEGs) among different clusters. The DEGs were analyzed using a two-sided Wilcoxon rank-sum test with Bonferroni FDR correction. Pathway enrichment was performed using Gene Ontology (GO; Metascape) [22] and Gene Set Enrichment Analysis (GSEA; ver.4.3.2) [23].

Gene signature score evaluation

The gene signature scoring methods used in this study are shown in Table S2.

scRNA-seq analysis of a public dataset

See supplemental methods.

Survival analysis in TCGA dataset

Gene Expression Profiling Interactive Analysis 2 was performed to analyze the survival of patients with stomach adenocarcinoma (STAD) and esophageal carcinoma (ESCA) using TCGA datasets [24].

Immunohistochemistry/ Multiplex immunohistochemistry

See supplemental methods.

Statistical analysis

P-values <0.05 were considered as a measure of statistical significance ($*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$), and other analyses described supplemental methods.

Results

Proposal and validation of a new MDSC signature score

We analyzed a total of 125,898 single cells from 12 GC tissues and 10 Adj. tissue collected from 13 GC patients who underwent gastrectomy (Figure 1A, Table S1). The participants did not receive chemotherapy or radiotherapy prior to surgery. Unbiased clustering generated 16 main clusters and was annotated to 16 known clusters according to canonical marker genes and DEGs (Figure 1B, S1A–B, Table S3). To identify MDSC clusters, we focused on the expression of CD11b⁺, CD14⁺, CD15⁺, CD33⁺, and MHC class II⁺, as MDSC-specific cell surface markers [9]. However, MDSC clusters in this dataset were difficult to identify because these genes were not expressed in specific clusters. Therefore, we adopted the MDSC gene signature proposed by Alshetaiwi et al. and MDSC-specific, immunosuppression-related genes to detect MDSCs (Table S2) [25,26]. Additionally, we identified MDSCs based on monocyte marker, neutrophil marker, and *IL1B* expression, as described by Alshetaiwi et al. [25]. MDSC gene signature scoring for all clusters revealed that the myeloid cell (C8) and neutrophil (C9) clusters expressed high levels of the MDSC signature genes (Figure 1C). On the basis of these results, we speculated that the myeloid cluster (C8) included M-MDSCs, and the neutrophil cluster (C9) contained PMN-MDSCs. We next isolated the myeloid cell and neutrophil clusters and subdivided them into five subclusters (Figure 1D, 1F). Within the myeloid cluster, the M3 subcluster had the highest MDSC signature score [25] and MDSC-specific immunosuppression-related gene expression levels [26] (Figure 1E). Furthermore, the M3

1 cluster exhibited high expression levels of monocyte markers and
2 *IL1B* (Figure S1C), suggesting it contained M-MDSCs. However, we
3 found that the MDSC signature score in the neutrophil cluster was not
4 consistent with the expression levels of MDSC-specific
5 immunosuppression-related genes (Figure 1G). Furthermore, *IL1B*
6 expression levels and *IL1B*-expressing neutrophil frequencies were
7 highest in the P2 subcluster (Figure S1D), whereas the MDSC
8 signature score was the highest in the P3 subcluster. Meanwhile, P1,
9 P2, and P5 expressed the highest levels of MDSC-specific
10 immunosuppression-related genes among the neutrophil subclusters
11 (Figure 1G), suggesting that they contained PMN-MDSCs. In
12 accordance with a report that the MDSC signature score proposed by
13 Alshetaiwi et al. failed to identify M-MDSCs in human breast cancer
14 [25], it was also not overly effective at identifying PMN-MDSCs in
15 our GC dataset. Consequently, we proposed a new MDSC signature
16 score, which could more effectively identify MDSCs in scRNA-seq
17 datasets, irrespective of their MDSC-specific
18 immunosuppression-related gene expression. To identify overlapping
19 genes between M- and PMN-MDSCs, we searched for common genes
20 specific to these cell types. Our analysis revealed 71 DEGs between
21 M- and PMN-MDSCs (Figure 1H, Table S2). These DEGs included
22 genes involved in the immunosuppressive functions of MDSCs, such
23 as *IL1B* and *CYBB* [26]. To construct an MDSC gene set, we
24 identified five genes (*IL1B*, *CCR1*, *CXCL2*, *GRINA*, and *IER3*), which
25 were shared between the 71 DEGs identified in our study and the

genes proposed by Alshetaiwi et al. [25] (Figure 1H). These five genes were defined as the “new MDSC signature score” (Table S2). When this score was applied to the GC dataset, it showed that the M3, P1, P2, and P5 subclusters scored significantly higher than the others (Figure 1I–J, S1E).

To further validate the utility of the new MDSC signature score in identifying MDSCs, we tested its performance in the ESCC (original dataset, Table S1: ESCC samples) and HNC datasets (Figure S2A–F, S3A–F) [27]. The new MDSC signature score was used alongside canonical marker genes to identify clusters, including M-MDSCs and PMN-MDSCs, in the ESCC and HNC datasets. We found that the new MDSC signature score and MDSC-specific immunosuppressive function-related genes were consistent at identifying M-MDSCs (ESCC; M4, HNC; M4) and PMN-MDSCs (ESCC; P1, HNC; P1) (Figure S2F, S3F). In addition, these clusters expressed monocyte markers, neutrophil markers, and *IL1B*, as described by Alshetaiwi et al. (Figure S2D, S3D) [25]. These results indicated that a signature score based on five selected genes effectively identified MDSC clusters within scRNA-seq datasets. Thus, the new MDSC signature score could help differentiate PMN-MDSCs from neutrophils and M-MDSCs from other myeloid cells.

Comparison of the immunosuppressive functions of immunosuppressive cell types in GC

1 We next extracted ICs from the scRNA-seq dataset to investigate
2 which cell types had the most important immunosuppressive
3 functions within the GC TIME. We then identified and added the
4 subsets “epithelium”, “terminal-exhausted CD8 T-cells” (TE_CD8T),
5 and “progenitor-exhausted CD8 T-cells” (PE_CD8T) to evaluate their
6 cell-cell interactions within the GC TIME (Figure 2A, S4A–H). The
7 ratio of ICs to TE_CD8T and PE_CD8T subsets was significantly
8 higher in the GC and ESCC tissues than in Adj. tissues (Figure 2B,
9 S5A). Additionally, the increase in the number of M-MDSCs between
10 GC/ESCC tissues and Adj. tissues was most dramatic (Figure 2C,
11 S5B). Next, we evaluated the performance of the
12 “immunosuppression signature score”, which was constructed from
13 genes presented in Figure S2E (Table S2) in each immunosuppressed
14 cell. The genes for the immunosuppression signature score were
15 selected based on results from previous studies, which showed that
16 these genes were associated with immunosuppressive functions
17 [26,28]. We found that M-MDSCs scored highest in our GC dataset,
18 followed by Mregs, PMN-MDSC, and Tregs (Figure 2D). The
19 expression level of *IL1B* was remarkably higher in M-MDSCs with
20 gastric cancer than in other ICs (Figure 2E). Similar results were
21 obtained by a study using another GC dataset and the same ESCC
22 dataset as used in our study (Figure 2D, S5C) [29]. However,
23 PMN-MDSCs and M-MDSCs scored equally high in colon cancer
24 [30], indicating that PMN-MDSCs may have a more important role in
25 colon cancer than in other cancer types (Figure S5C). The expression

of each gene (i.e., *CYBB*, *TGFB1*, *IL6*, and *VEGFA*) was confirmed to be higher in PMN-MDSCs in colon cancer than in other cancers (Figure S5D). Furthermore, the scores were significantly higher in the PMN-MDSC, M-MDSC, Treg, and epithelium clusters of the GC than in those of Adj. tissue (Figure 2F). By contrast, the Mreg scores were not significantly different between the GC and Adj. tissues.

TCGA data were then used to evaluate the correlation between patient signature scores and their prognosis. The results suggested that GC and ESCC may have similar immunosuppressive TIMEs. Therefore, we used a combined STAD and ESCA TCGA dataset to show that the high-scoring group of patients had a significantly worse prognosis than the low-scoring group; a trend toward poor prognosis was also observed individually for high-scoring STAD and ESCA patients (Figure S5E–F). The M-MDSC signature score (DEGs; M3) correlated more strongly with prognosis than the PMN-MDSC (DEGs; P1, 2, 5) and Treg signature scores (DEGs; CD4-4) (Figure 2G, Table S2). The identification of DEGs between M-MDSCs in GC and Adj. tissues, revealed that *IER3* and *GRINA*, two of the new MDSC signature score genes, were also GC DEGs (Figure 2H, Table S2). In PMN-MDSCs, *IER3* and *IL1B* were observed among the GC DEGs (Figure 2H, Table S2). We found that the patients with high M-MDSCs DEG expression levels in tumor had a significantly worse prognosis than those with low M-MDSCs DEG expression levels in tumor; meanwhile, the expression of M-MDSCs DEGs in Adj. tissue was not significantly associated with

prognosis (Figure 2I). These results suggest that M-MDSCs can be important in the development of an immunosuppressive GC TIME.

Prognostic impact of TI-ICs in GC

To investigate TI-ICs and prognosis in GC tissues, we examined GC samples using immunohistochemistry; the clinical characteristics of these patients are presented in Table S4. First, we classified patients with FOXP3-expressing Tregs into low and high groups based on their median cell numbers (Figure 3A). We found no significant difference between the OS of patients allocated to the Treg-high and -low groups (Figure 3B). On the basis of previous studies showing that M-MDSCs are characterized as CD14⁺CD15⁻HLA-DR/DP/DQ⁻ cells and PMN-MDSC as CD14⁻CD15⁺HLA-DR/DP/DQ⁻ cells [31], we next evaluated MDSCs in GC and Adj. tissues using multiplex immunohistochemistry (Figure 3C). Our results showed that M-MDSC and PMN-MDSC numbers were significantly higher in GC tissues than in Adj. tissues (Figure 3D). Thus, GC patients were next divided into M-MDSC and PMN-MDSC -high and -low groups according to their median cell counts of M-MDSCs and PMN-MDSCs, respectively. The group with high M-MDSC counts and that with high PMN-MDSC counts had a significantly poorer prognosis than their corresponding low groups (Figure 3E). The HR for M-MDSCs was higher than that for PMN-MDSCs (HR: 6.499 vs. 2.923). Additionally, patients with high counts of M-MDSC and PMN-MDSC, or high counts of M-MDSCs and

low counts of PMN-MDSCs, had a significantly worse prognosis than those with low M-MDSC and PMN-MDSC counts (Figure 3F). These results suggest that the prognosis of GC patients is correlated with TI-M-MDSC numbers.

Identification of therapeutic target molecules in TI-M-MDSCs of patients with GC

IER3 was one of the DEGs identified in M-MDSCs/PMN-MDSCs between GC and Adj. tissues; it was also included in the new MDSC signature score (Figure 1H, 2H). Therefore, we next focused on *IER3* in M-MDSCs. Although *IER3* is associated with a poor prognosis in tongue cancer [32], no studies have demonstrated its significance in GC. Thus, to investigate the clinical impact of *IER3* in GC, we performed *IER3* staining of GC samples (Figure 4A). The data revealed that *IER3* expression was significantly higher in samples from patients who experienced GC recurrence than in samples from those who did not (Figure 4B). We next investigated the association between *IER3* expression and the recurrence rate and prognosis of GC patients after gastrectomy (Table 1). In the univariable analysis, stage, neoadjuvant chemotherapy, adjuvant chemotherapy, postoperative complications, and *IER3* expression were identified as significant risk factors for recurrence after gastrectomy ($P<0.05$). The multivariable analysis results demonstrated that stage and *IER3* expression were significant risk factors for recurrence ($P<0.05$). We

1 further validated the significance of *IER3* expression in GC using
2 propensity score matching analysis.

3 Before propensity score matching, 78 and 83 patients were
4 included in the *IER3*-low and *IER3*-high groups, respectively. There
5 was a significant difference in stage and adjuvant chemotherapy
6 parameters between these groups (Table 2). After propensity score
7 matching, the *IER3*-low and *IER3*-high groups each contained 44
8 patients. The *IER3*-high group had significantly worse relapse-free
9 survival (RFS) ($P=0.0251$, $HR=5.415$) and OS ($P=0.0290$, $HR=5.250$)
10 than the *IER3*-low group (Figure 4C).

11 We next examined *IER3*⁺ TI-MDSCs (Figure 4D) and found that the
12 percentages of *IER3*⁺ M-MDSC and *IER3*⁺ PMN-MDSC were
13 significantly higher in the GC than in Adj. tissues (Figure 4E). The
14 percentages of *IER3*-expressing cells were highest in the C8 and C9
15 clusters, and *IER3* expression was highest in M-MDSCs and
16 PMN-MDSCs (Figure S6A–D, S6F–G). In addition, the percentage of
17 *IER3*⁺ cells was higher in GC than in Adj. tissues (Figure S6E, S6H).
18 Subsequently, GC patients were divided into *IER3*[−] and *IER3*⁺ groups
19 according to the *IER3* expression levels of their M-MDSCs and
20 PMN-MDSCs (determined by scRNA-seq). The groups with either
21 *IER3*⁺ M-MDSCs or *IER3*⁺ PMN-MDSCs had a significantly higher
22 immunosuppressive signature scores than the corresponding *IER3*[−]
23 groups (Figure 4F).

24 To investigate intercellular communication between M-MDSCs and
25 other immune cells in the GC TIME, we performed systematic

analyses of potential cell-cell interactions using NicheNet [21]. We then focused on the interaction between *IER3* and *TNF* (the gene encoding tumor necrosis factor [TNF]) (Figure 4G, S6I) because *TNF* was more highly expressed in Mregs than in other cell types (Figure S6J). Previously, *IER3* was reported to regulate the biological effects of TNF [33]. Moreover, *TNF* is an M1-like macrophage marker gene and the interaction between M-MDSCs and macrophages is involved in immunosuppression [34]. Our findings revealed that *IER3* expression in TI-M-MDSCs was significantly higher in GC than in Adj. tissues (Figure 4H, left). By contrast, *TNF* expression in Mregs was lower in GC than in Adj. tissues (Figure 4H, right). Additionally, the numbers of *IER3*⁺ M-MDSCs and *TNF*⁺ Mregs were significantly inversely correlated (Figure 4I). Furthermore, patients with *IER3*⁺ M-MDSC high had macrophages with higher immunosuppressive signature scores than those with *IER3*⁺ M-MDSC low (Figure 4J). This observation supports the notion that *IER3* in M-MDSCs contributes to the polarization of macrophages toward the immunosuppressive M2 phenotype. Additionally, this result revealed that *IER3*⁺ M-MDSCs were more enriched for the "GOBP_LYMPHOCYTE_CHEMOTAXIS" signaling pathway than *IER3*⁻ M-MDSCs (Figure 4K, Table S5). *CCL3* is one of the core enrichment genes of the "GOBP_LYMPHOCYTE_CHEMOTAXIS" signaling pathway. We found that *CCL3* expression was significantly higher in *IER3*⁺ M-MDSCs than in *IER3*⁻ M-MDSCs (Figure 4L). *CCL3* induces MDSCs and CCR1⁺ tumor-associated macrophages

(TAMs) to enter the TIME [3,35]. We found that *CCR1* expression in M-MDSCs was significantly higher in Adj. tissues than in GC tissues (Figure 4M), suggesting that CCL3 in IER3⁺ M-MDSCs may recruit CCR1⁺ M-MDSCs from healthy tissues to the tumor. On the basis of these results, IER3 in TI-M-MDSC may be involved in the development of the immunosuppressive GC TIME.

Gene expression levels in Lauren's classification, tumor location, and stage

To explore differences in TIME in Lauren's classification, tumor location, and stage, we evaluated the immunosuppressive signature score and *IER3* expression in M-MDSCs, respectively. The immunosuppressive signature scores were higher in mixed, Body, and stage II/ III than in other groups (Figure S7A). Furthermore, *IER3* expression levels were higher in stage II/ III than in the other groups (Figure S7B). However, further studies are needed to uncover TIME in each group due to variations in patient backgrounds.

Role of IER3 in M-MDSCs in the GC TIME and resistance to anti-PD-1 antibody therapy

TI-MDSCs confer resistance to anti-PD-1 antibody (a popular ICI) therapy in mouse models of advanced GC [36]. Thus, we next evaluated the TIME in patients with HNC receiving anti-PD-1 antibody therapy [27]. Tumor-antigen-specific CD8⁺ T-cells often become exhausted in the TIME [37]. Therefore, CD8-1 and CD8-3

clusters were defined as the TE_CD8T and PE_CD8T subsets (Figure S8A, S8B). There was no significant difference between the cytotoxic signature scores [38] of the TE_CD8T subset in the pre- and post-treatment groups (Figure S8C, right). However, the PE_CD8T population of the post-treatment group had a significantly higher cytotoxic signature score than that of the pre-treatment group (Figure S8C, left). This result is consistent with that of a previous study, as it showed that PE_CD8T could respond to anti-PD-1 therapy while TE_CD8_T could not [39]. We found no significant difference between the immunosuppressive signature scores of PMN-MDSCs in the pre- and post-treatment groups; however, the immunosuppressive signature score of M-MDSCs was significantly higher after treatment (Figure S8D). To assess the ICI-resistant TIME in GC, we next performed scRNA-seq of samples from GC patients with progressive disease (PD) after receiving anti-PD-1 antibody treatment and chemotherapy (Chemo+anti-PD-1) (Figure 5A). We analyzed a total of 72,745 single cells from three Adj. tissue samples, three tumor samples from untreated GC patients (No treatment), two tumor samples from GC patients on chemotherapy (Chemo), and two tumor samples from patients on Chemo+anti-PD-1. The clinical characteristics of these patients were recorded (Table S1: GC-treatment samples). Unbiased clustering, using canonical marker genes and DEGs, generated 18 main clusters (Figure S9A–C). Subsequently, we isolated seven CD8 T-cell subclusters and identified the PE_CD8T and TE_CD8T subsets (Figure 5B, S9D). The

1 results showed that the cytotoxic signature score and the proportion
2 of the PE_CD8T subset were highest in the Chemo+anti-PD-1 group
3 (Figure 5C–D). Next, the M-MDSC subcluster was identified within
4 the C8 cluster (Figure 5E, S9E–F). The immunosuppressive signature
5 score and *IER3* expression of this M-MDSC subset were significantly
6 higher in the Chemo+anti-PD-1 group than in the other groups
7 (Figure 5F–G). In addition, the Chemo+anti-PD-1 group had the
8 highest frequency of M-MDSCs, most of which were *IER3*⁺ (Figure
9 5H, S9H). By contrast, the immunosuppressive signature score and
10 *IER3* expression of the PMN-MDSC subset (identified from the C10
11 cluster) were not significantly different between the
12 Chemo+anti-PD-1 and other groups (Figure 5I–K, S9G). However, the
13 number of PMN-MDSCs was highest in the Chemo+anti-PD-1 group
14 and half of these cells were *IER3*⁺ (Figure 5L, S9I). These findings
15 suggest that M-MDSCs, especially *IER3*⁺ M-MDSCs, contributed to
16 anti-PD-1 antibody therapy resistance in GC.

18 Discussion

19 The TIME is a highly complex and dynamic system,
20 in which immune cells play important roles. MDSC-induced cytotoxic
21 T-cell exhaustion and dysfunction are frequently observed in various
22 solid tumors [6,40]. Although all IC types are predictors of poor
23 prognosis in cancer, the presence of M-MDSCs in the peripheral
24 blood of patients with lung cancer was associated with a worse
25 prognosis than that of Tregs [3–5,12]. However, no studies have

1 evaluated which TI-ICs dominate in the development of the
2 immunosuppressive TIME. In the present study, TI-M-MDSCs in GC
3 TIME expressed higher levels of immunosuppression-related genes
4 than other ICs. Besides, the M-MDSCs were associated with the worst
5 prognostic among the ICs. These results suggest that M-MDSCs can
6 play a predominant role in the immunosuppressive GC TIME.

7 ICI is the standard treatment for recurrent or
8 unresectable GC [41]. However, because this mode of treatment is not
9 the standard neoadjuvant therapy in Japan, it is challenging to obtain
10 adequate numbers of specimens from GC patients receiving ICI
11 therapy. In the present study, we collected these rare, fresh samples
12 from anti-PD-1-antibody-treated GC patients during procedures such
13 as palliative surgery. These samples were then analyzed using
14 scRNA-seq. To the best of our knowledge, this is the first study to
15 analyze the scRNA-seq data generated from human GC samples
16 following anti-PD-1 antibody treatment. It revealed that, although
17 patients in the Chemo+anti-PD-1 group had progressive disease, their
18 exhausted CD8⁺ T-cells expressed significantly higher levels of
19 cytotoxicity-related genes than those of patients in the Chemo group,
20 consistent with the results of previous studies of other cancers [39].
21 However, the immunosuppression-related gene expression of
22 M-MDSCs was significantly higher in the Chemo+anti-PD-1 group
23 than in other groups. Moreover, M-MDSCs were most abundant in the
24 Chemo+anti-PD-1 group. These results suggest that TI-M-MDSCs
25 confer treatment resistance in GC.

In the present study, we identified IER3 as one of the new MDSC signature score genes and an important M-MDSC DEG in GC. Therefore, we focused on IER3 in TI-M-MDSCs in the GC TIME. Previously, IER3 was shown to be involved in protecting cells from apoptosis in a prognostic model of tongue cancer [32]. However, no studies had investigated the expression and function of IER3 in TI-MDSCs in the GC TIME. The present study demonstrated that IER3 was an independent poor prognostic factor in GC and that IER3⁺ TI-M-MDSCs were more abundant in GC tissues than in normal adjacent tissues. Based on these results, IER3⁺ MDSCs may be less susceptible to apoptosis and thus continue to exert their immunosuppressive function, which leads to a poor prognosis. Furthermore, M-MDSCs were more abundant in the treatment-resistant than -responsive GC patients, most of which were IER3⁺. IER3 participates in resistance to gemcitabine in pancreatic ductal adenocarcinoma [42]. These findings suggest that therapy for IER3⁺ M-MDSCs may potentially alter the therapy-resistant TIME. Our data indicated that IER3⁺ M-MDSC and TNF⁺ Mreg numbers were inversely correlated. Moreover, macrophages expressed higher levels of immunosuppression-related genes in GC patients with IER3-high M-MDSCs than in those with IER3-low M-MDSCs. Interacting M-MDSCs and macrophages contribute to immunosuppression; moreover, M-MDSCs can differentiated into TAMs [34,43]. Meanwhile, IER3 overexpression in T-cells prolongs their survival, suggesting IER3 protects T-cells from apoptosis [44]. These results

1 suggest that IER3⁺ M-MDSCs might be less susceptible to apoptosis
2 and subsequently differentiate into immunosuppressive macrophages.

3 M-MDSCs can be a promising therapeutic target;
4 however, it is difficult to develop M-MDSC-specific treatment
5 because there are no M-MDSC-specific molecules. The present study
6 demonstrated *IER3* was highly expressed in M-MDSCs but also in
7 other immune cells (Figure S6A–H). Therefore, M-MDSC-specific
8 therapy is still challenging. Recently, it has been reported that
9 myeloid cell-derived IL1 β was a promising therapeutic target in the
10 treatment of pancreatic cancer [45]. We also found that M-MDSCs
11 expressed higher levels of *IL1B* than other immunosuppressed or
12 cancer cells. (Figure 2E). These data suggest that IL1 β -targeting
13 treatment may be effective in gastric cancer patients with high
14 fraction of M-MDSCs.

15 In recent years, *Helicobacter pylori* (*H. pylori*) infection has
16 attracted attention for its relationship with monocytes. A previous
17 study revealed M2-like monocytes were significantly increased in *H.*
18 *pylori*-infected patients compared to the controls [46]. On the other
19 hand, monocyte adhesion played an essential role in the development
20 of *H.pylori* infection-associated gastric diseases [47]. The present
21 study did not include the sufficient number of *H.pylori* positive and
22 negative cases to reveal the relationships between *H. pylori* infection
23 and M-MDSCs. Therefore, further studies will be needed to elucidate
24 these relationships.

1 In conclusion, we have shown that TI-M-MDSCs, especially
2 IER3⁺ M-MDSCs, can be involved in the development of an
3 immunosuppressive and ICI-resistant GC TIME. Therefore, IER3⁺
4 M-MDSCs may be promising targets for novel GC therapies; although
5 this will need to be validated in further studies.

6

Declarations

Ethics approval and consent to participate

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: 22002-00 and 2020-7882020-503) and conducted in accordance with the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government and the Declaration of Helsinki.

Consent for publication

All authors have read and agreed with the manuscript.

Availability of data and materials

The scRNA-seq data generated in this study are available from the GEO database under accession code GSE231540. The remaining data are available within the manuscript, Supplementary Information, or from the corresponding author upon reasonable request.

Competing interests

The authors have no conflicts of interest.

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9 **Authors' Contributions**

10 C. Tsutsumi designed and performed the experiments, analyzed the
11 data, coordinated the research, and wrote the manuscript. K.
12 Ohuchida designed the experiments, analyzed the data, wrote the
13 manuscript, coordinated the research, and contributed to data
14 interpretation and discussion. N. Katayama, Y. Otsubo, S. Nakamura,
15 and K. Okuda performed the experiments. C. Iwamoto, Y. Mizuuchi,
16 N. Ikenaga, K. Nakata, E. Nagai, and T. Morisaki contributed to data
17 interpretation and discussion. K. Ohuchida, N. Torata, K. Horioka,
18 and K. Shindo obtained and prepared human samples.
19 Immunohistochemically-stained samples were analyzed by C.
20 Tsutsumi, Y. Yamada, and Y. Oda. M. Nakamura coordinated the
21 research, wrote the manuscript, and contributed to data interpretation
22 and discussion.

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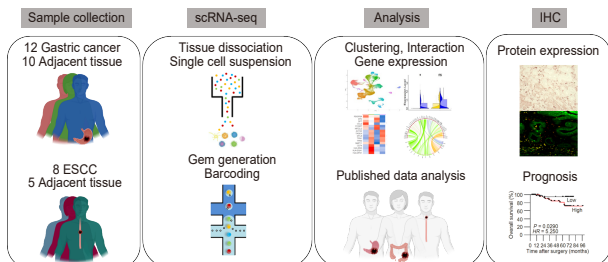
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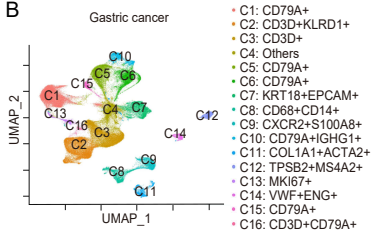
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Figure 1

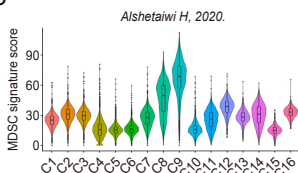
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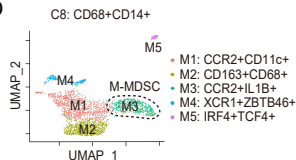
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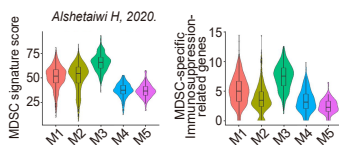
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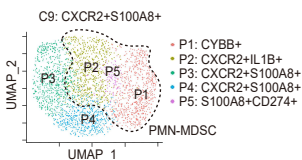
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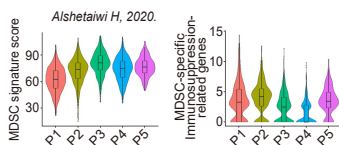
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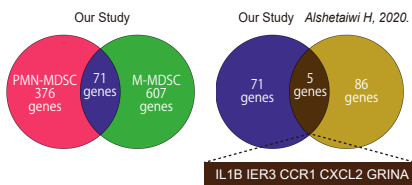
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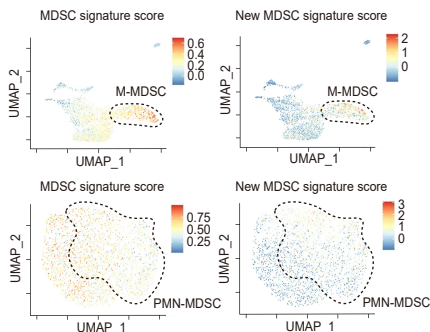
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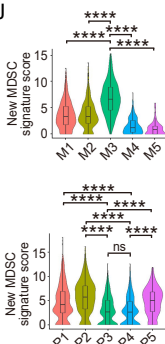


Figure 2

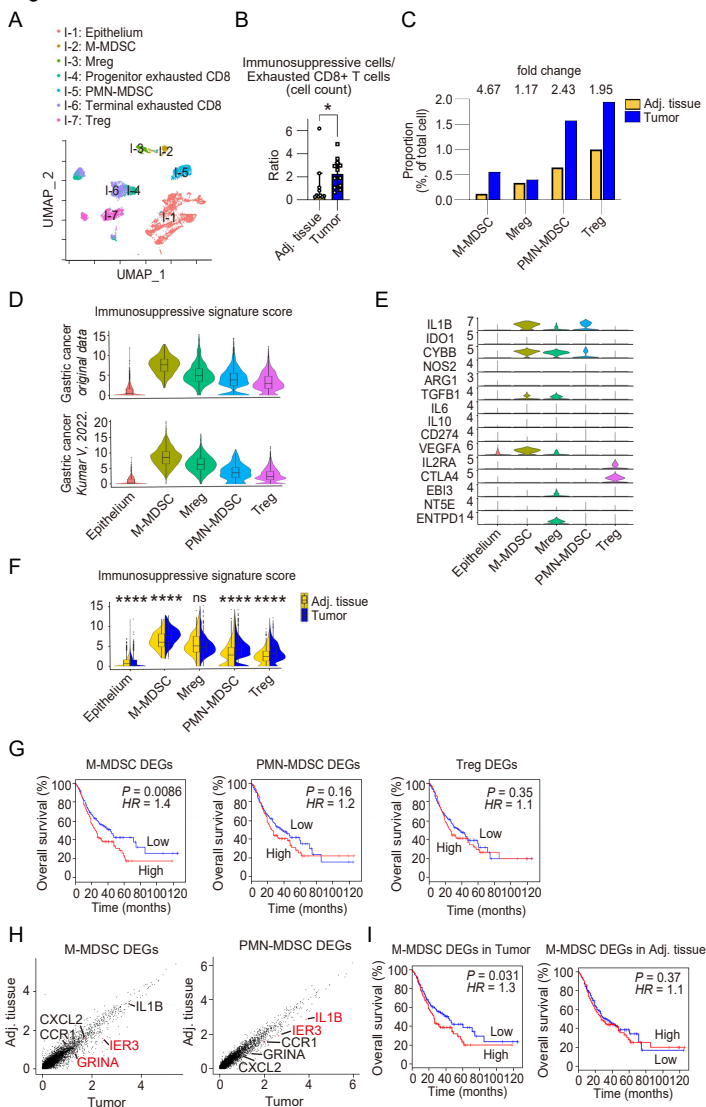


Figure 3

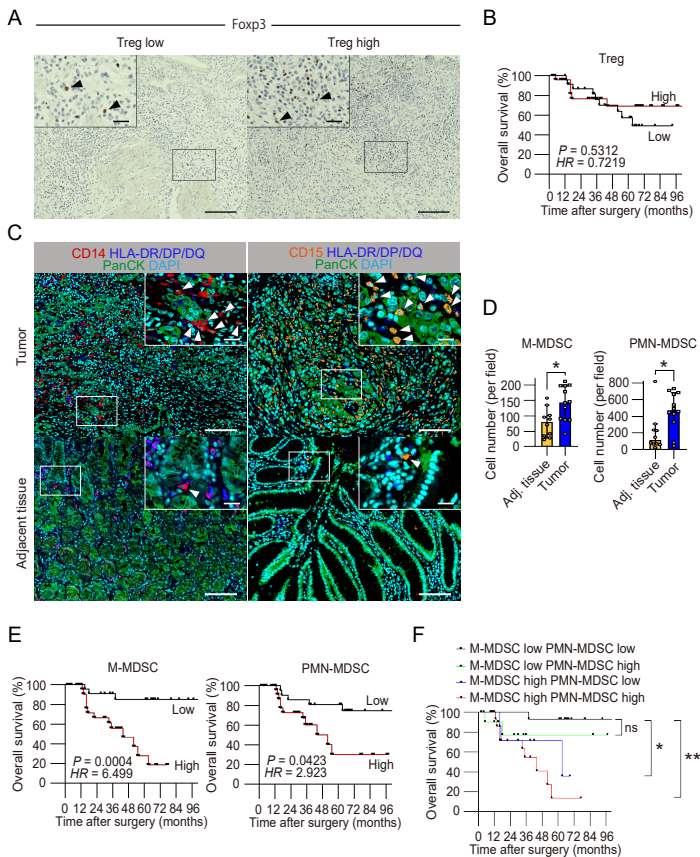


Figure 4

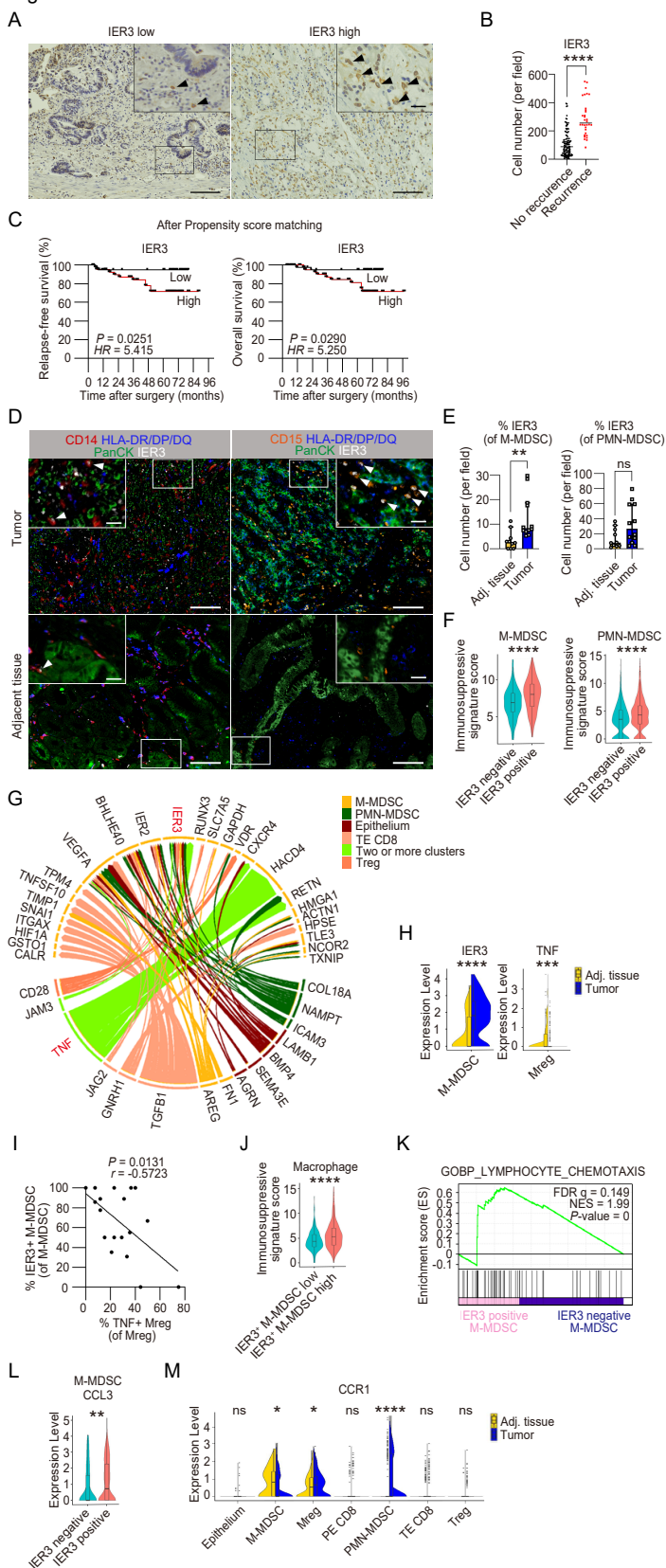


Figure 5

