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Intratumor *Fusobacterium nucleatum* promotes the progression of pancreatic cancer via the CXCL1-CXCR2 axis

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Abstract

Intratumor bacteria modify the tumor immune microenvironment and influence outcomes of various tumors. Periodontal pathogen *Fusobacterium nucleatum* has been detected in pancreatic cancer tissues and is associated with poor prognosis. However, it remains unclear how *F. nucleatum* affects pancreatic cancer. Here, we compared clinical features with *F. nucleatum* colonization in pancreatic cancer tissues. *F. nucleatum* was detected in 15.5% (13/84) of pancreatic cancer patients. The tumor size was significantly larger in the *F. nucleatum*-positive group than in the negative group. To clarify the biological effect of intratumor *F. nucleatum* on pancreatic cancer progression, we performed migration/invasion assays and cytokine array analysis of cancer cells cocultured with *F. nucleatum*. *F. nucleatum* promoted CXCL1 secretion from pancreatic cancer cells, leading to cancer progression through autocrine signaling. Intratumor *F. nucleatum* suppressed tumor-infiltrating CD8⁺ T cells by recruiting myeloid-derived suppressor cells (MDSCs) to the tumor in an *F. nucleatum*-injected subcutaneous pancreatic cancer mouse model, resulting in tumor progression. Furthermore, tumor growth accelerated by *F. nucleatum* was suppressed by MDSC depletion or cytokine inhibitors. Intratumor *F. nucleatum* promoted pancreatic cancer progression through autocrine and paracrine mechanisms of the CXCL1-CXCR2 axis. Blockade of the CXCL1-CXCR2 axis may be a novel therapeutic approach for patients with intratumor *F. nucleatum*-positive pancreatic cancer.

KEYWORDS

CXCL1, CXCR2, *Fusobacterium nucleatum*, pancreatic cancer, tumor immune microenvironment

Abbreviations: BHI, brain heart infusion; CXCL1, C-X-C motif chemokine ligand 1; CXCR2, C-X-C motif chemokine receptor 2; IL-8, interleukin-8; KPC, *LSL-Kras*^{G12D/+}; *LSL-Trp53*^{R172H/+}; *Pdx-1-Cre*; MDSCs, myeloid-derived suppressor cells; TCGA, The Cancer Genome Atlas.

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1 | INTRODUCTION

Pancreatic cancer is among the deadliest cancers worldwide, and the 5-year survival rate of patients with pancreatic cancer is only 12%.¹ Conventional therapies for pancreatic cancer do not provide an adequate prognosis, and novel treatment approaches are required.² The gut microbiota acts as the primary mediator of tumor responses to chemotherapy and immunotherapy in cancer patients.^{3,4} Dysbiosis, the loss of gut microbial diversity, suppresses antitumor immunity, promotes cancer-associated inflammation, and increases resistance to cancer therapy.^{5–7}

In addition to the gut microbiota, intratumor bacteria have been highlighted as a key regulator of tumor behavior.⁸ Colonization of intratumor bacteria has been reported not only in gastrointestinal cancers, such as colorectal cancers⁹ and esophageal cancers,^{10,11} but also in non-gastrointestinal cancers, including breast cancers^{12,13} and pancreatic cancers.^{14–17} Pushalkar et al.¹⁵ showed that depletion of intratumor bacteria by antibiotics enhanced the efficacy of immune checkpoint inhibitors by upregulating the expression of programmed cell death protein 1 in a mouse model of pancreatic cancer, suggesting that intratumor bacteria modify the tumor immune microenvironment of pancreatic cancer.

Fusobacterium nucleatum is a gram-negative anaerobic periodontal pathogen that has been detected in esophageal cancer^{10,11} and colorectal cancer.^{18,19} *Fusobacterium* adhesin A, an outer membrane protein derived from *F. nucleatum*, attaches to E-cadherin on cancer cells and promotes colorectal cancer cell proliferation by activating β -catenin signaling.¹⁶ *Fusobacterium* species have also been detected in pancreatic cancer tissues by 16S rRNA gene sequencing, and intratumor *Fusobacterium* species are associated with a poor prognosis of pancreatic cancer.¹⁷ These findings suggest that *Fusobacterium* species are potential therapeutic targets. However, it remains unclear how *F. nucleatum* affects pancreatic cancer.

We hypothesized that *F. nucleatum* is a key molecule promoting pancreatic cancer progression by modifying tumor biology and the tumor immune microenvironment. To clarify the effect of intratumor *F. nucleatum* on pancreatic cancer progression, we investigated the biological alterations of cancer cells by migration and invasion assays in direct cocultures with *F. nucleatum* in vitro experiments. Cytokine array analysis of the culture supernatant from cancer cells cocultured with *F. nucleatum* identified the key molecules that modified pancreatic cancer behavior. Additionally, we revealed that inhibition of *F. nucleatum*-specific molecules restored the antitumor microenvironment and suppressed tumor growth in a subcutaneous tumor mouse model injected with *F. nucleatum* intratumorally.

2 | MATERIALS AND METHODS

2.1 | Human pancreatic ductal adenocarcinoma tissue samples

We reviewed patients who underwent pancreatic resection for pancreatic ductal adenocarcinoma with curative intent at the Department of Surgery and Oncology, Kyushu University Hospital

(Fukuoka, Japan) between 2012 and 2020 with available fresh-frozen tissue. Patients with positive intraoperative ascites cytology, noncancer death, and acinar cell carcinoma at pathological diagnosis were excluded. Normal pancreatic tissues were obtained from nontumor areas in patients with resected pancreatic neuroendocrine tumors. Informed consent was received from all patients in accordance with the Declaration of Helsinki. Clinical data including histopathological findings were obtained from electronic medical records.

2.2 | Bacterial strains and growth conditions

Fusobacterium nucleatum strain ATCC 23726⁹ and *Porphyromonas gingivalis* strain ATCC 33277²⁰ were purchased from the American Type Culture Collection (ATCC). Detailed procedures are described in Appendix S1.

2.3 | Human cell culture

SUIT-2 cells were purchased from the Japan Health Science Research Resources Bank and maintained as described previously.²¹ Passages three to eight were used in assays.

2.4 | Mice

LSL-Kras^{G12D/+}; *LSL-Trp53*^{R172H/+}; *Pdx-1-Cre*; (KPC) mice have been described previously.^{22–24} KPC mice were bred and maintained at Charles River Laboratories Japan, Inc. and the Research Animal Facility at Kyushu University in accordance with Kyushu University guidelines. Four-week-old female C57BL/6 mice were purchased from CLEA Inc.

2.5 | Murine cells and culture conditions

Murine pancreatic cancer cells were established in our laboratory from primary tumors of KPC mice using the outgrowth method as described previously.²⁴ Murine pancreatic cancer cells were maintained in Dulbecco's modified Eagle's medium (Sigma Chemical Co.) containing 10% FBS, 100 μ g/mL streptomycin, and 100 U/mL penicillin.

2.6 | Matrigel invasion and migration assays

Detailed procedures are described in Appendix S1.

2.7 | Cell proliferation assay

Detailed procedures are described in Appendix S1.

2.8 | DNA extraction

Detailed procedures are described in Appendix S1.

2.9 | Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

We examined the expression levels of the following genes using qRT-PCR. Primer sequences are listed in Table 1. We defined a Cycle threshold value of >40 or nonspecific amplification in the melting curve analysis as not detected. All RT-PCRs were performed in triplicate. Cases in which *F. nucleatum* was detected in at least two replicates were defined as positive for *F. nucleatum*. Detailed procedures are described in Appendix S1.

2.10 | Immunohistochemistry

Detailed procedures are described in Appendix S1.

2.11 | Cytokine array

Detailed procedures are described in Appendix S1.

2.12 | The Cancer Genome Atlas (TCGA)

Detailed procedures are described in Appendix S1.

2.13 | Flow cytometry

Detailed procedures are described in Appendix S1.

2.14 | In vivo experiments

KPC cells (KPCF453-PC; 5×10^5) were suspended in 50 μ L Dulbecco's modified Eagle's medium and subcutaneously injected into the abdomen skin of 5-week-old female C57/B6 mice. Seven days after implantation, three to six mice were used in each of the bacterium-injected group and non-bacterium-injected group. Bacterium-injected group, *F. nucleatum* or *P. gingivalis* was suspended at a dose of 5×10^7 colony forming unit in 50 μ L BHI medium and injected into each mouse tumor every 3 days per week for 2 weeks; control group, mice were injected with 50 μ L BHI medium only. The tumor volume was calculated using the following formula: $\pi/6 \times (L \times W \times W)$, where *L* is the largest tumor diameter, and *W* is the smallest tumor diameter.

TABLE 1 Primers used for quantitative reverse-transcription polymerase chain reactions.

Gene	Primer	Sequence 5'-3'
<i>Fusobacterium nucleatum</i>	Forward	CAACCATTACTTTAACTCTA CCATGTTCA
	Reverse	GTTGACTTTACAGAAGGAGA TTATGTAAAAATC
	FAM probe	GTTGACTTTACAGAAGG AGATTA
SLCO2A1	Forward	ATCCCCAAAGCACCTGGTTT
	Reverse	AGAGGCCAAGATAGTCC TGGTAA
	VIC probe	CCATCCATGTCCTCATCTC
Human CXCL1	Forward	GAAAGCTTGCTCAATCCTG
	Reverse	CTCCCTTCTGGTCAGTTGGA
Human IL-8	Forward	CACTGCGCCAACAC AGAAAT
	Reverse	GCCCTCTTCAAAAACTT CTCCAC
Human GAPDH	Forward	GCACCGTCAAGGCT GAGAAC
	Reverse	TGGTGAAGACGCCAGTGGGA
Mouse Cxcl1	Forward	CTGGGATTACCTCAAG AACATC
	Reverse	CAGGGTCAAGGCAAGCCTC
Mouse Gapdh	Forward	TGTGTCCGTCGTGGATCTGA
	Reverse	TTGCTGTTGAAGTCGCA GGAG

2.15 | In vivo drug treatments

In some experiments, mice were injected with six doses of a C-X-C motif chemokine receptor 2 (CXCR2) antagonist (5 mg/kg, i.p.; SB225002, Selleck) or neutralizing anti-C-X-C motif chemokine ligand 1 (CXCL1) mAb (4 mg/kg, intratumor injection; R&D Systems) at 72-h intervals unless specified otherwise. Control-group mice were injected with dimethyl sulfoxide as described previously.²⁵ To deplete myeloid-derived suppressor cells (MDSCs), six doses of 100 μ g/dose of InVivoMAb anti-mouse Ly6G²⁶ (clone 1A8; BioXCell, Cat # BP0075-1) or isotype control antibody (clone 2A3, BioXcell, Cat # BP0089) diluted in InVivoPure pH 7.0 Dilution Buffer (BioXcell, Cat # IP0070) was intraperitoneally injected at 72-h intervals unless specified otherwise.

2.16 | Statistical analysis

The χ^2 test was used to analyze the correlation between intratumor *F. nucleatum* and clinicopathological characteristics in qRT-PCR analyses. Survival analyses were performed by the Kaplan–Meier method and compared using the log-rank test. Results are presented

as the mean \pm standard deviation (SD). For each result, group comparisons were conducted using Student's *t*-test. Statistical significance was defined as $p < 0.05$. Statistical analyses were performed using GraphPad Prism 8 (v8.4.3; GraphPad Software) or JMP software (v17.0.0; SAS Institute).

3 | RESULTS

3.1 | Intratumor *F. nucleatum* affects pancreatic tumor behavior

To confirm bacterial colonization in human pancreatic cancers, we performed immunohistochemistry using antibodies against bacterial lipopolysaccharide, a molecule in the outer membrane of bacteria.²⁷

Lipopolysaccharide was detected in pancreatic tumor tissue, but not in normal pancreatic tissue (Figure 1A). To assess colonization of *F. nucleatum* in the human pancreas, we performed qRT-PCR analysis of *F. nucleatum* DNA in fresh-frozen tissues of 84 resected pancreatic tumors and 41 normal pancreatic tissues.¹¹ *F. nucleatum* DNA was detected in 13 cases (15.5%) of pancreatic tumors, while only three cases of normal tissues (7.3%) were positive ($p < 0.05$; Figure 1B). Tumors with *F. nucleatum* colonization were significantly larger and more frequently showed retroperitoneal invasion and multiple lymph node metastases (more than four) compared with tumors without *F. nucleatum* colonization ($p < 0.01$; Figure 1C–E). Patients with *F. nucleatum* in the tumor had shorter disease-free survival and overall survival times (Figure 1F). These findings suggest that intratumor *F. nucleatum* promotes pancreatic tumor growth and its metastasis, leading to a poor prognosis.

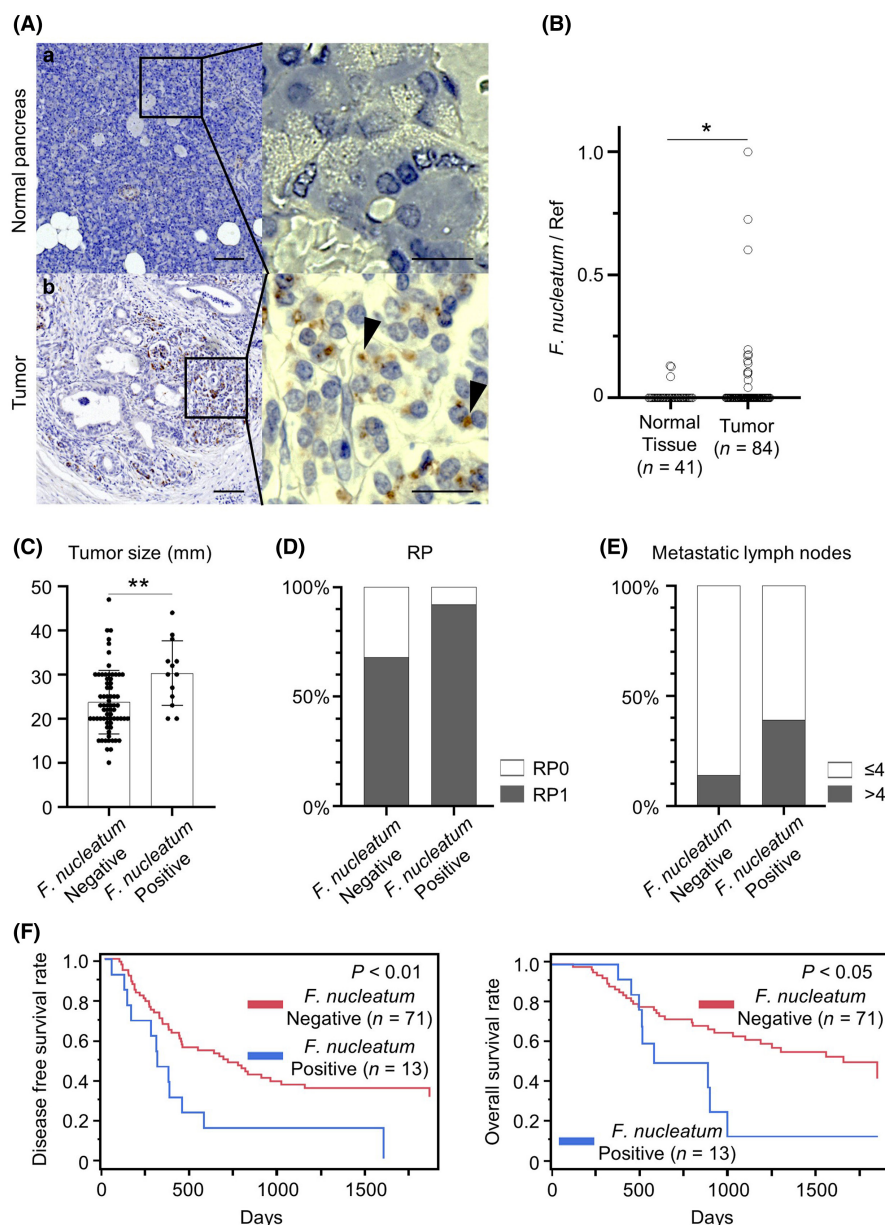


FIGURE 1 Characterization of intratumor *Fusobacterium nucleatum* in pancreatic cancer. (A) Immunohistochemistry of bacterial lipopolysaccharide in normal pancreatic tissue (A-a: upper case) and pancreatic tumor tissue (A-b: lower case). Arrowheads in the insets indicate positive staining of bacterial lipopolysaccharide in the pancreatic tumor (original magnification: $\times 40$; scale bar $100\mu\text{m}$, insets: $\times 400$; scale bar, $20\mu\text{m}$). (B) Relative abundance of *F. nucleatum* in normal pancreatic tissues ($N=41$) versus pancreatic tumor tissues ($N=84$). (C–E) The relationship of the tumor size (C), retroperitoneal invasion (D), and multiple lymph node metastases (more than four) (E) in pancreatic cancer patients with or without intratumor *F. nucleatum*. (F) Kaplan–Meier curves demonstrating disease-free survival (left) and overall survival (right) of pancreatic cancer patients with or without intratumor *F. nucleatum*. * $p < 0.05$, ** $p < 0.01$.

3.2 | *Fusobacterium nucleatum* stimulates CXCL1 and IL-8 secretion from pancreatic cancer cells, promoting cell migration in an autocrine manner

To evaluate the effect of *F. nucleatum* on the biological activities of human pancreatic cancer cells, we performed proliferation, migration, and invasion assays using a coculture system of a pancreatic cancer cell line SUIT-2 cocultured with *F. nucleatum*. As a result, we found significant promotion of cell migration and invasion compared with monocultured SUIT-2 cells ($p < 0.05$; Figure 2A,B). Another periodontal pathogen, *P. gingivalis*, did not promote migration or invasion of SUIT-2 cells. Pancreatic cancer cell proliferation did not change after coculture with *F. nucleatum* or *P. gingivalis* (Figure 2C). To assess the molecules that changed the biological activities of cancer cells, we performed cytokine array analysis of the culture supernatant from SUIT-2 cells cocultured with periodontal pathogens. CXCL family cytokines, CXCL1 and interleukin-8 (IL-8) were upregulated in the culture supernatants of SUIT-2 cells cocultured with *F. nucleatum*, but not with *P. gingivalis* (Figure 2D). Increased mRNA expression of CXCL1 and IL-8 in *F. nucleatum*-cocultured SUIT-2 cells was also confirmed by qRT-PCR ($p < 0.01$; Figure 2E). CXCL1 and IL-8 secreted by cancer cells bind to CXCR2 expressed on cancer cells and induce autocrine signaling to promote cancer cell migration.^{28,29} Therefore, we assessed the effect of CXCL1 and IL-8 on cancer cell activity by inhibiting CXCR2. CXCR2-specific inhibitor SB225002 significantly suppressed the migration of *F. nucleatum*-cocultured SUIT-2 cells ($p < 0.01$; Figure 2F), suggesting that enhanced migration of pancreatic cancer cells by *F. nucleatum* is mediated through autocrine signaling.

3.3 | CXCL1 and IL-8 expression in pancreatic cancer patients correlates with a poor prognosis

Because in vitro experiments showed that *F. nucleatum* upregulated CXCL1 expression in cancer cells, we confirmed the association between *F. nucleatum* colonization and CXCL1 expression in human pancreatic cancer tissues. Pancreatic cancers colonized with *F. nucleatum* showed significantly higher CXCL1 expression than those without *F. nucleatum* colonization ($p < 0.05$; Figure 3A). Next, we investigated CXCL1 and IL-8 expressions in human pancreatic tumor samples using publicly available data provided by TCGA and the Genotype-Tissue Expression project. Patient samples were compared with noncancerous control tissues using GEPIA.³⁰ CXCL1 and IL-8 expression levels were higher in tumor samples compared with normal samples ($p < 0.01$; Figure 3B). High expression of CXCL1 and IL-8 was significantly associated with poor overall survival in all pancreatic cancer patient data from TCGA (Figure 3C). These findings indicate that high expression of CXCL1 and IL-8 in pancreatic tumors contributes to the aggressiveness of pancreatic cancer.

3.4 | Intratumor *F. nucleatum* modifies the tumor immune microenvironment in pancreatic cancer

To investigate the effects of intratumor *F. nucleatum* on the tumor immune microenvironment in vivo experiments, we established subcutaneous pancreatic cancer models with KPC cells in C57BL/6 mice and injected bacteria into the tumors as shown in Figure 4A. Intratumor *F. nucleatum* increased the tumor volume compared with the control or *P. gingivalis* ($p < 0.05$; Figure 4B). Cxcl1 expression was significantly increased in *F. nucleatum*-injected tumors compared with the control and *P. gingivalis*-injected tumors ($p < 0.05$; Figure 4C). To investigate the effect of intratumor *F. nucleatum* on the tumor immune microenvironment, flow cytometric analysis was used to evaluate tumor-infiltrating immune cells. The composition of tumor-infiltrating immune cells showed significantly increased infiltration of CD11b⁺ Gr-1⁺ MDSCs into *F. nucleatum*-injected tumors ($p < 0.05$; Figure 4D). Conversely, CD8⁺ T cells were decreased in *F. nucleatum*-injected tumors compared with the control and *P. gingivalis*-injected tumors (Figure 4D). Infiltration of dendritic cells and macrophages was not affected by intratumor bacteria (Figure 4D). Immunohistochemistry confirmed that colonization of *F. nucleatum* in tumors increased the number of CD11b⁺ cells and reduced the number of CD8a⁺ cells compared with the control ($*p < 0.05$, $**p < 0.01$; Figure 4E). These data suggest that intratumor *F. nucleatum* promotes in vivo tumor growth and contributes to the establishment of an immunosuppressive tumor microenvironment in pancreatic cancer.

3.5 | Intratumor *F. nucleatum* promotes pancreatic cancer progression through modification of the tumor immune microenvironment via the CXCL1-CXCR2 axis

To investigate whether intratumor *F. nucleatum* promotes tumor progression via CXCR2 signaling, we administrated the CXCR2 antagonist in *F. nucleatum*-injected subcutaneous pancreatic cancer models (Figure 5A). Tumor growth promoted by *F. nucleatum* was significantly suppressed by CXCR2 inhibition ($*p < 0.05$, $**p < 0.01$; Figure 5B,C). Increased infiltration of MDSCs in *F. nucleatum*-injected tumors was also suppressed by CXCR2 inhibition, although a change in CD8⁺ T cell infiltration was not observed ($*p < 0.05$, $**p < 0.01$; Figure 5D). Infiltration of dendritic cells and macrophages did not change after CXCR2 inhibition. In increased two cytokines (CXCL1 and IL-8) detected in the supernatant of human pancreatic cancer cells treated with *F. nucleatum*, IL-8 is not expressed in mice³¹; therefore, we investigated the role of CXCL1 in modification of the tumor immune microenvironment in *F. nucleatum*-injected pancreatic tumors. Intratumor administration of the anti-CXCL1 Ab into *F. nucleatum*-injected tumors suppressed tumor growth in mice ($p < 0.05$; Figure 5E–G) with suppressed MDSC infiltration and reversal of CD8⁺ T cell infiltration ($p < 0.01$; Figure 5H). These data indicate that intratumor *F. nucleatum* promotes pancreatic cancer

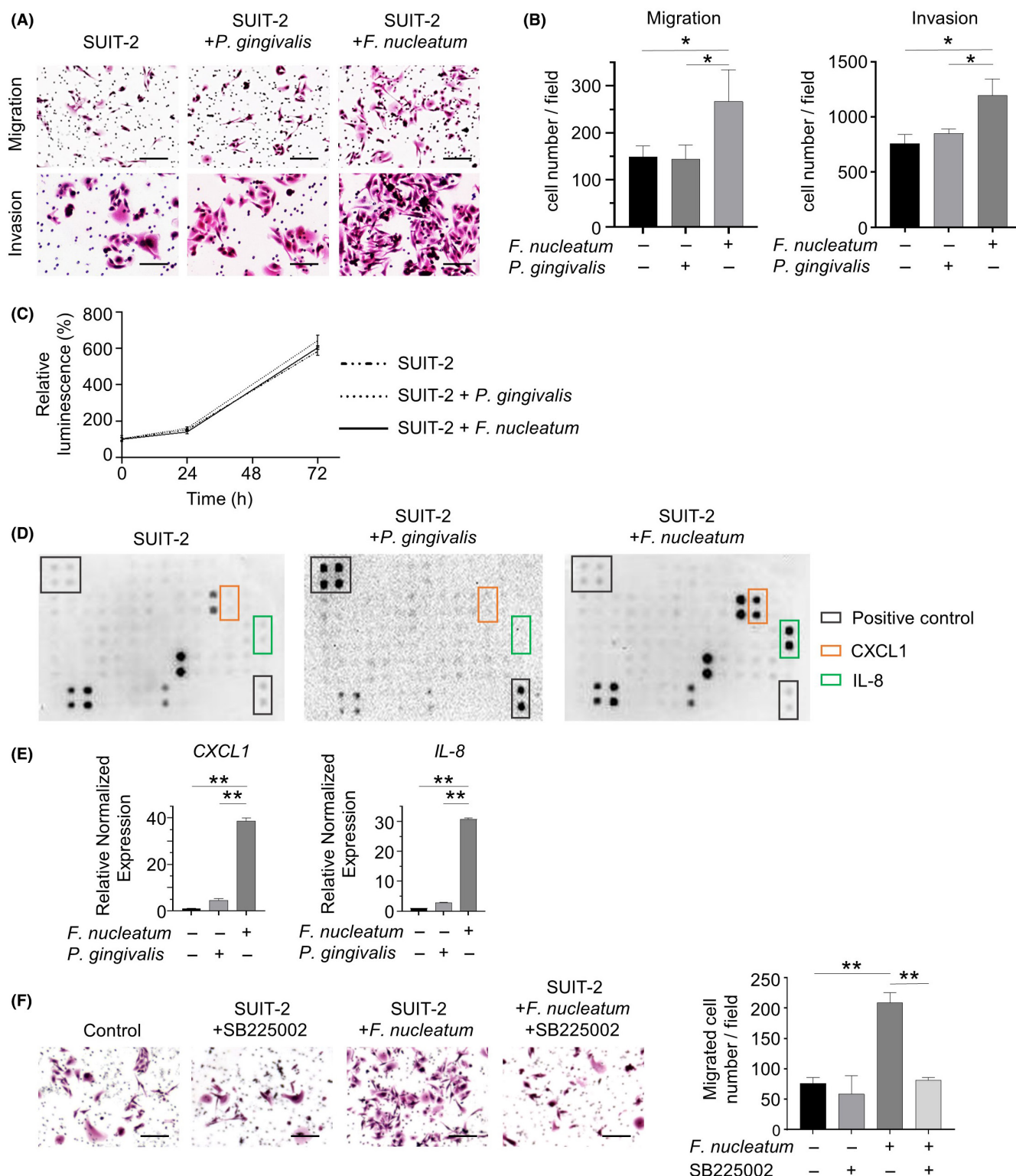


FIGURE 2 Effects of *Fusobacterium nucleatum* on migration and invasion of pancreatic cancer cells. (A) Representative photomicrographs of migrating and invading SUIT-2 cells cocultured with *F. nucleatum* and *P. gingivalis* or monocultured. (B) *F. nucleatum* promoted the migration and invasion of SUIT-2 cells compared with *P. gingivalis*-cocultured and monocultured cells. (C) Proliferation rate of SUIT-2 cells cocultured with *F. nucleatum* was similar to that of *P. gingivalis*-cocultured and monocultured SUIT-2 cells. (D) Representative human cytokine array images of conditioned medium derived from SUIT-2 cells cocultured with *F. nucleatum* and *P. gingivalis* or monocultured. Black boxes: positive controls; orange boxes: CXCL1; green boxes: IL-8. CXCL1 and IL-8 were upregulated in the conditioned medium of SUIT-2 cells cocultured with *F. nucleatum*, but not with *P. gingivalis* or monocultured. (E) CXCL1 (left) and IL-8 (right) expression was increased in SUIT-2 cells cocultured with *F. nucleatum*, but not with *P. gingivalis*. (F) Representative photomicrographs of migrating *F. nucleatum*-cocultured SUIT-2 cells with or without the CXCR2 inhibitor (SB225002). The CXCR2 inhibitor significantly suppressed migration of *F. nucleatum*-cocultured SUIT-2 cells. Original magnification: $\times 40$; scale bar 100 μm . * $p < 0.05$, ** $p < 0.01$.

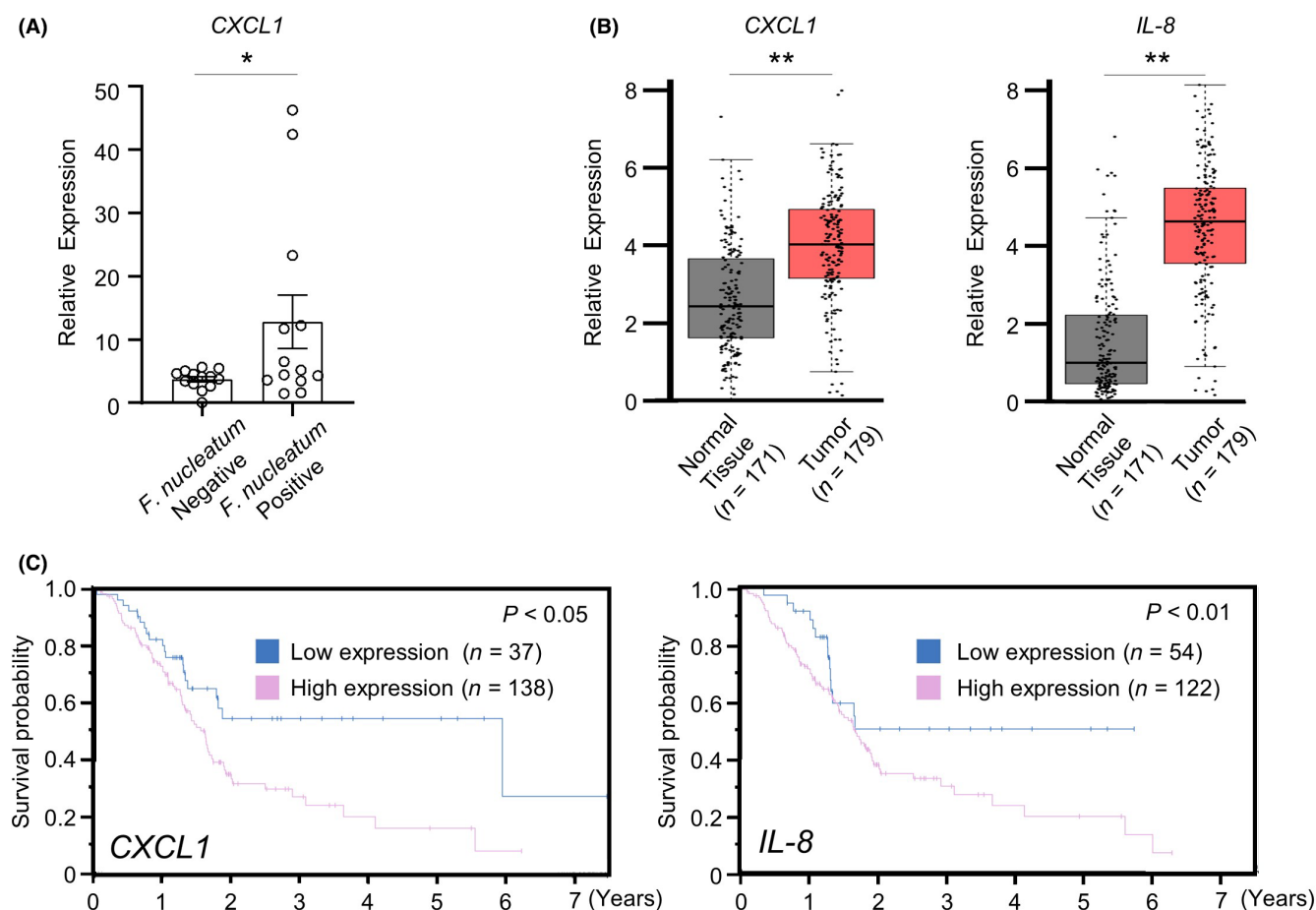


FIGURE 3 Comparison of CXCL1 and IL-8 expression in tumors and the prognosis of pancreatic cancer patients. (A) CXCL1 expression in human pancreatic cancer tissues in the *Fusobacterium nucleatum*-negative group ($N = 13$) versus the *F. nucleatum*-positive group ($N = 13$). (B) Gene expression profiling interactive analysis of CXCL1 (left) and IL-8 (right) in normal (gray bars) and tumor (pink bars) samples from pancreatic cancer data of The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression databases. (C) Kaplan-Meier analysis of overall survival of TCGA pancreatic cancer patients with high and low expression of CXCL1 (left) and IL-8 (right). * $p < 0.05$, ** $p < 0.01$.

progression via the CXCL1-CXCR2 axis by affecting cancer cells themselves in an autocrine manner and modifying the tumor immune microenvironment in a paracrine manner.

3.6 | Intratumor *F. nucleatum* promotes pancreatic cancer progression through recruitment of MDSCs to the tumor immune microenvironment

The results so far indicate that intratumor *F. nucleatum* induces CXCR2-mediated recruitment of MDSCs to the tumor immune microenvironment, resulting in tumor progression. Finally, we investigated whether MDSC recruitment induced by intratumor *F. nucleatum* was critical to suppress antitumor immunity and tumor progression. To abrogate MDSC infiltration in pancreatic tumors, we injected anti-Ly6G Ab intraperitoneally (Figure 6A). Tumor growth promoted by *F. nucleatum* was significantly suppressed by the anti-Ly6G Ab treatment (* $p < 0.05$, ** $p < 0.01$; Figure 6B,C). Flow cytometry confirmed

that anti-Ly6G Ab administration significantly suppressed the infiltration of MDSCs in *F. nucleatum*-injected tumors, but not that of dendritic cells or macrophages ($p < 0.05$; Figure 6D). *F. nucleatum*-induced effects on the infiltration of MDSCs or CD8⁺ T cells did not observe with anti-Ly6G Ab administration (Figure 6D), which was confirmed by immunohistochemistry ($p < 0.01$; Figure 6E). Taken together, our findings suggest that intratumor *F. nucleatum* facilitates progression by modifying the tumor immune environment, inducing MDSC infiltration and inhibiting CD8⁺ T cell proliferation.

4 | DISCUSSION

In this study, we elucidated the mechanisms by which intratumor *F. nucleatum* promoted pancreatic cancer progression. *Fusobacterium* species in tumors have been reported to impair the prognosis of pancreatic cancer patients, but the underlying mechanisms have not been elucidated so far.¹⁷ We found that tumors with *F. nucleatum*

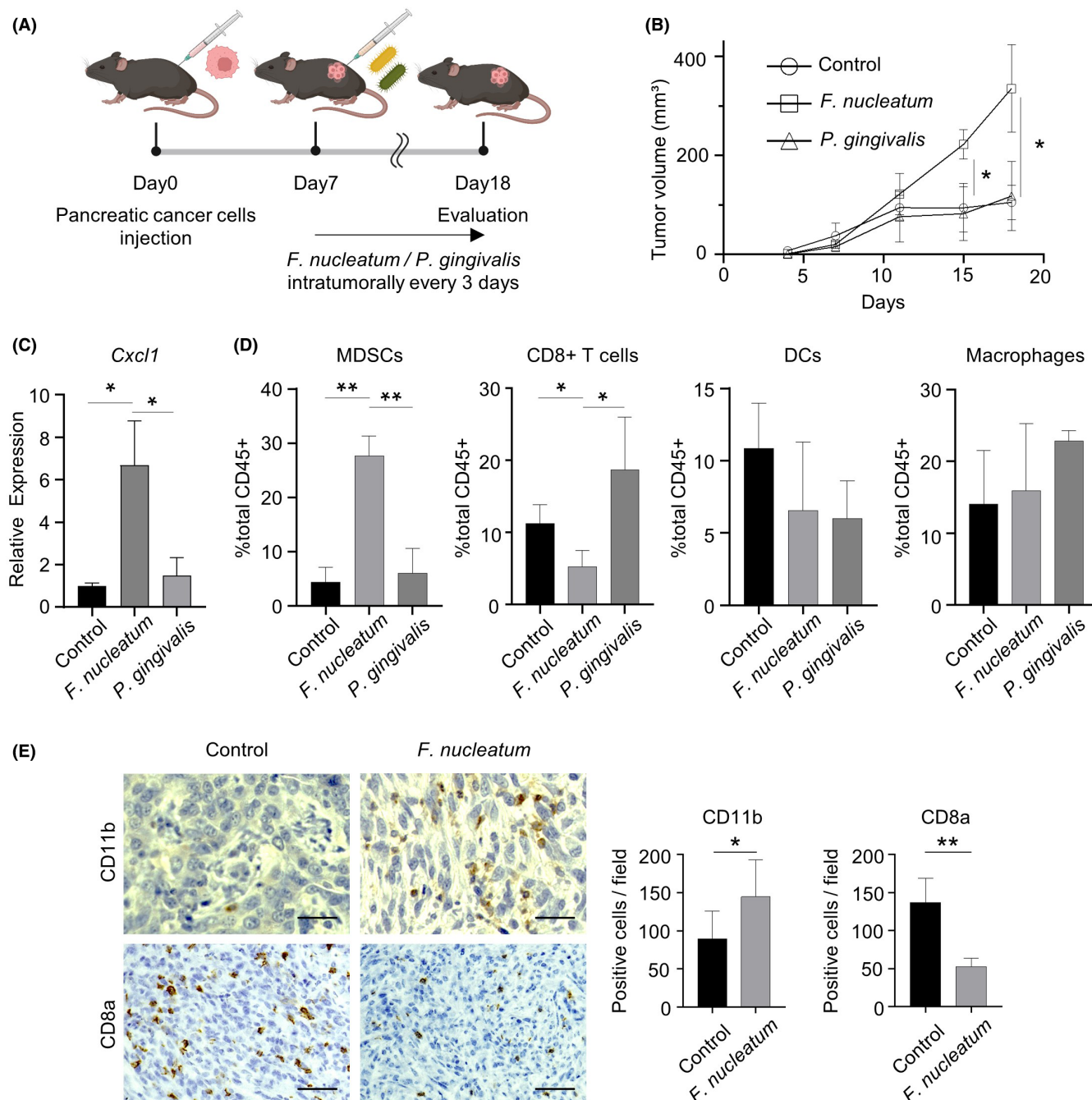


FIGURE 4 Effects of intratumor *Fusobacterium nucleatum* on in vivo tumor growth of pancreatic cancer. (A) Experimental schema. KPC cells were subcutaneously implanted into C57/B6 mice. From 1 week after implantation, the mice were intratumorally injected with *F. nucleatum* or *P. gingivalis* every 3 days for 11 days. Control mice were intratumorally injected with BHI medium. (B) Representative quantified graph of tumor growth curve. Comparisons of the tumor volume in control, *F. nucleatum*, and *P. gingivalis* are shown. (C) *Cxcl1* expression was increased in *F. nucleatum*-injected tumors compared with the control and *P. gingivalis*-injected tumors. (D) Representative flow cytometric analysis of CD11b⁺Gr-1⁺ MDSCs, CD8⁺ T cells, CD11c⁺MHCII⁺F4/80⁻ dendritic cells, and F4/80⁺ macrophages gated on CD45⁺ cells. (E) Representative immunohistochemical staining of CD11b⁺ myeloid cells and CD8a⁺ T cells. Original magnification, $\times 100$. Scale bar, 50 μ m. * $p < 0.05$, ** $p < 0.01$.

colonization were significantly larger and more frequently showed retroperitoneal invasion compared with tumors without *F. nucleatum* colonization, suggesting that intratumor *F. nucleatum* contributes to the aggressiveness of pancreatic cancer. *F. nucleatum* stimulated

pancreatic cancer cells to secrete CXCL1, leading to cancer progression through two pathways. First, in an autocrine manner, *F. nucleatum* promoted pancreatic cancer cell migration and invasion, which was confirmed by in vitro coculture experiments with *F. nucleatum*;

second, in a paracrine manner, *F. nucleatum* suppressed tumor-infiltrating CD8⁺ T cells by recruiting MDSCs to the tumor, which was confirmed by in vivo experiments (Figure 7). Finally, we revealed that inhibition of the CXCL1-CXCR2 axis restored antitumor immunity and suppressed tumor growth in *F. nucleatum*-injected mice. To the best of our knowledge, we are the first to show that intratumor *F. nucleatum* promotes the progression of pancreatic cancer by modifying the tumor immune microenvironment.

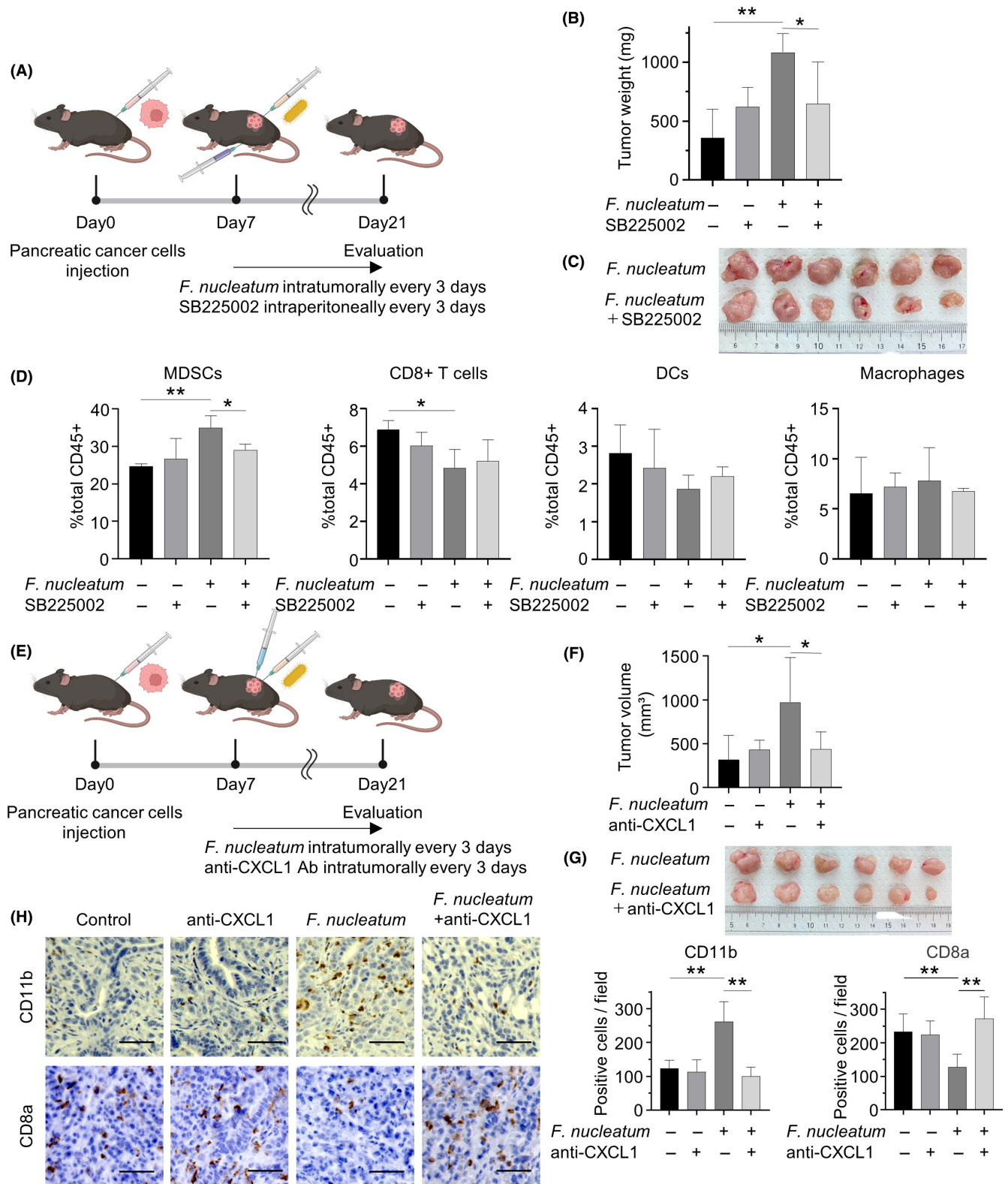
Bacterial colonization in human pancreatic tumors has been reported in several studies.^{14–17,32} Neiman et al.³² detected bacterial DNA in more than 60% of pancreatic tumors by 16S rRNA gene sequencing. Depletion of intratumor bacteria by antibiotics reshapes the tumor immune microenvironment, by decreasing MDSCs and increasing CD8⁺ T cell activation in tumors.¹⁵ Riquelme et al.¹⁴ showed that the composition and diversity of intratumor bacteria were associated with improved CD8⁺ T cell responses and prolonged survival of pancreatic cancer patients. Specifically, enrichment of *Sachharopolyspora*, *Pseudoxanthomas*, and *Streptomyces* in a tumor was positively correlated with tumor-infiltrating CD8⁺ T cells. This suggests that pancreatic tumors contain a mixture of functionally different bacterial species that modify the tumor immune microenvironment positively or negatively. Intratumor bacteria that modify antitumor immunity are a therapeutic target, but functional analysis of a specific bacterium has not been reported. This study showed that intratumor *F. nucleatum* promoted tumor growth by mediating antitumor immunity represented by suppression of tumor-infiltrating CD8⁺ T cells. The negative effect of intratumor *F. nucleatum* on tumors was confirmed by clinicopathological analysis of resected pancreatic cancers with *F. nucleatum* colonization. Selective antibiotic depletion of specific bacteria promoting pancreatic cancer progression such as *F. nucleatum* might be a novel approach to immunostimulatory therapy of pancreatic cancer.

CXCL1 is a chemoattractant for immune cells, mediating inflammatory responses at an infectious site.³³ CXCL1 is mainly secreted from cancer cells, specifically binds to its receptor, CXCR2, expressed on MDSCs and cancer cells, and promotes sarcoma tumor growth in a paracrine manner.³⁴ Binding of CXCL1 to CXCR2 on prostate cancer cells promotes migration and invasion via NF- κ B activation through the Akt pathway in an autocrine manner.³⁵ It is also known that CXCL1 recruits CXCR2⁺ MDSCs to tumors, which suppresses CD8⁺ T cell proliferation at the site and establishes an immunosuppressive microenvironment.³⁴ Our TCGA analysis showed that CXCL1 was highly expressed in pancreatic tumors and correlated with a poor prognosis of pancreatic cancer patients. Considering the aforementioned findings about CXCL1 in other tumors, the CXCL1-CXCR2 axis may be a candidate therapeutic target in pancreatic cancer. However, a previous report has demonstrated that depletion of CXCR2 alone does not suppress pancreatic tumors in in vivo experiments.³⁶ Our study revealed that CXCL1 secretion from cancer cells was enhanced by *F. nucleatum*. Inhibition of the

CXCL1-CXCR2 axis by the anti-CXCL1 Ab or CXCR2 inhibitor restored antitumor immunity and suppressed the growth of *F. nucleatum*-injected tumors. This effect was not observed in tumors without *F. nucleatum* injection, suggesting that the contribution of the CXCL1-CXCR2 axis to tumor growth is limited to *F. nucleatum*-colonized tumors. We found that a certain proportion of human pancreatic cancers harbored *F. nucleatum*, indicating that inhibition of the CXCL1-CXCR2 axis might be effective only in these cases. The composition of intratumor bacteria would influence the tumor immune microenvironment, and a therapeutic strategy in accordance with the intratumor bacteria might be proposed in the future.

While a large cohort study has demonstrated that periodontitis is a risk factor of pancreatic cancer development,³⁷ the causal relationship between periodontal pathogens and pancreatic cancer progression has not been revealed. Periodontal pathogens are mainly composed of *F. nucleatum*, *P. gingivalis*, *Tannerella denticola*, and *Tannerella forsythia*.³⁸ Among them, *F. nucleatum* and *P. gingivalis* are reported to promote the proliferation of pancreatic cancer cells by enhancing Akt signaling and cyclin D1 expression in in vitro experiments.^{33,39} In this study, we found that intratumor *F. nucleatum* promoted tumor growth by modifying the tumor immune microenvironment in multiple in vivo experiments. This is the first evidence that periodontal pathogens in pancreatic tumors alter antitumor immunity and contribute to tumor progression. Although the relationship between oral and pancreatic microbiomes has not been revealed yet, it is known that some bacteria in pancreatic tumors originate from the duodenum.¹⁶ It is also shown that bacteria administered to mice by oral gavage migrate to the pancreas and activate Ki67⁺ cells in tumors.⁴⁰ It is reasonable that the oral microbiome partially represents the intratumor microbiome in the pancreas. Considering that the intratumor microbiome affects malignancy, analysis of the oral microbiome might provide useful information about therapeutic options for pancreatic cancer.

We acknowledge there are some limitations in this study. First, we did not observe interactions between intratumor bacteria and immune cells. Intratumor injection of bacteria might directly act on immune cells and modify antitumor immunity. However, *F. nucleatum* promoted CXCL1 secretion from cancer cells, and depletion of CXCL1 changed the composition of intratumor immune cells. Interactions between cancer cells and intratumor bacteria should affect the progression of pancreatic cancer. The direct effect of intratumor bacteria on immune cells in the tumor will be elucidated in the next study. Second, we did not find that *P. gingivalis*, which has been reported to be epidemiologically associated with pancreatic cancer,⁴¹ promoted pancreatic cancer progression. *P. gingivalis* varies in virulence among strains,⁴² and the virulent strain W83 has been reported to promote pancreatic cancer progression.⁴⁰ We used the less virulent strain ATCC 33277 in this study, which may have led to the different results from the previous report.



In conclusion, *F. nucleatum* can colonize pancreatic cancer and affect the prognosis of pancreatic cancer patients. Intratumor *F. nucleatum* promotes CXCL1 secretion from cancer cells, contributing to cancer progression in an autocrine and paracrine manners. The

CXCL1-CXCR2 axis is activated by *F. nucleatum* to create an immunosuppressive tumor microenvironment, and blockade of this axis suppresses tumor growth. Our results suggest a novel therapeutic approach for intratumor *F. nucleatum*-positive pancreatic cancer patients.

FIGURE 5 Effects of intratumor *Fusobacterium nucleatum* on the tumor immune microenvironment via CXCR2. (A) Experimental schema. KPC cells were subcutaneously implanted into C57/B6 mice. From 1 week after implantation, the mice were intratumorally injected *F. nucleatum* or BHI and treated with or without CXCR2 inhibitor every 3 days for 2 weeks. (B) Representative quantified graph of tumor weight. (C) Representative in situ images of tumors. Tumor growth promoted by *F. nucleatum* was significantly suppressed by CXCR2 inhibition. (D) Representative flow cytometric analysis of CD11b⁺Gr-1⁺ MDSCs, CD8⁺ T cells, CD11c⁺MHCII⁺F4/80⁺ dendritic cells, and F4/80⁺ macrophages gated on CD45⁺ cells. (E) Experimental schema. KPC cells were subcutaneously implanted into C57/B6 mice. From 1 week after implantation, the mice were intratumorally injected with *F. nucleatum* or BHI and treated with or without an anti-CXCL1 Ab every 3 days for 2 weeks. (F) Representative quantified graph of tumor volumes. (G) Representative in situ images of tumors. Tumor growth promoted by *F. nucleatum* was significantly suppressed by the anti-CXCL1 Ab. (H) Representative immunohistochemical staining of CD11b⁺ myeloid cells and CD8a⁺ T cells. Original magnification, $\times 100$. Scale bar, 50 μ m. * $p < 0.05$, ** $p < 0.01$.

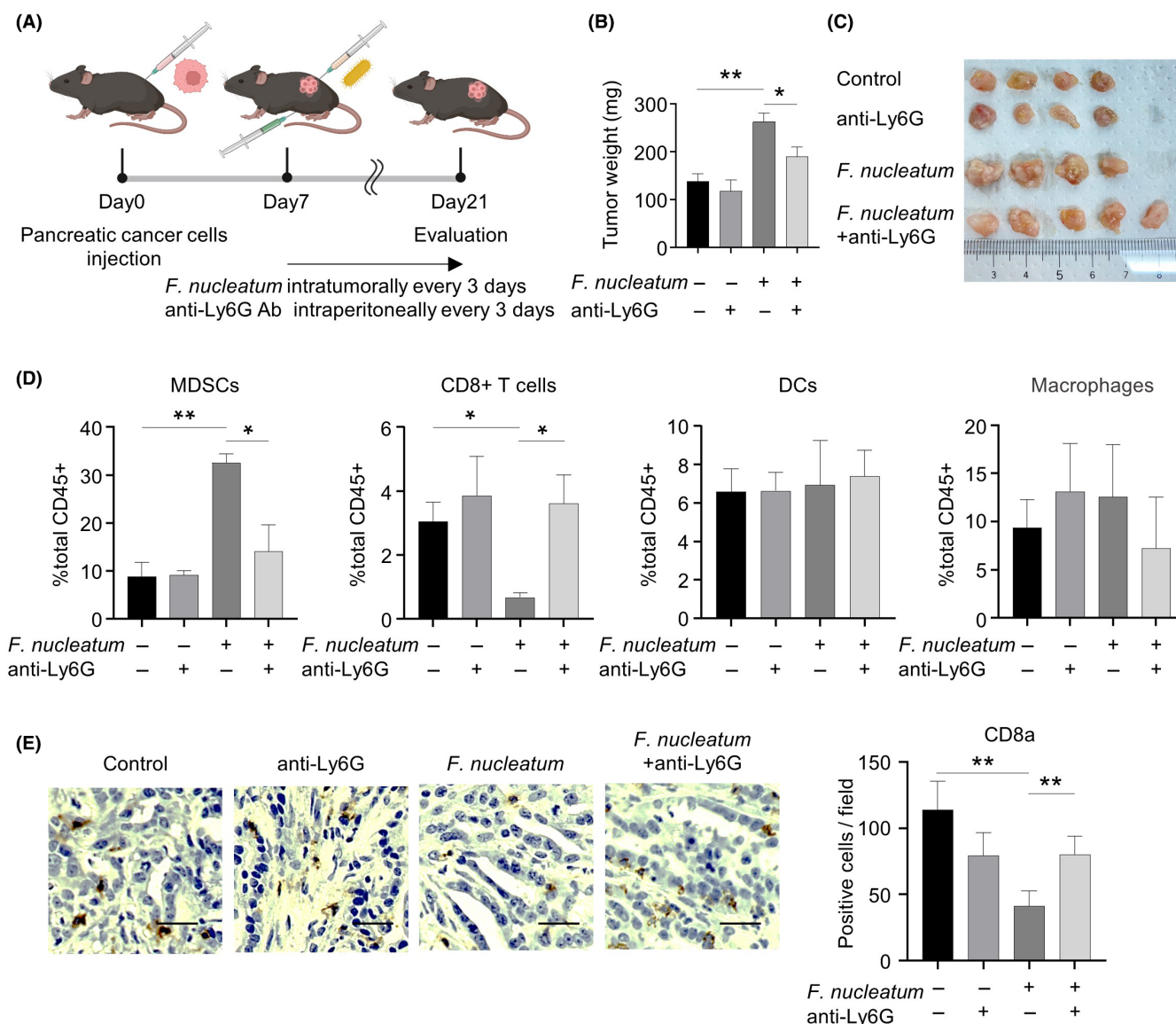


FIGURE 6 Effects of intratumor *Fusobacterium nucleatum* on myeloid-derived suppressor cell (MDSC) recruitment to the tumor immune microenvironment. (A) Experimental schema. KPC cells were subcutaneously implanted into C57/B6 mice. From 1 week after implantation, the mice were intratumorally injected with *F. nucleatum* or BHI and treated with an anti-Ly6G Ab or isotype control Ab every 3 days for 2 weeks. (B) Representative quantified graph of tumor weights. (C) Representative in situ images of tumors. Tumor growth promoted by *F. nucleatum* was significantly suppressed by the anti-Ly6G Ab. (D) Representative flow cytometric analysis of CD11b⁺Gr-1⁺ MDSCs, CD8⁺ T cells, CD11c⁺MHCII⁺F4/80⁺ dendritic cells, and F4/80⁺ macrophages gated on CD45⁺ cells. (E) Representative immunohistochemical staining of CD8a⁺ T cells. Original magnification, $\times 100$. Scale bar, 50 μ m. * $p < 0.05$, ** $p < 0.01$.

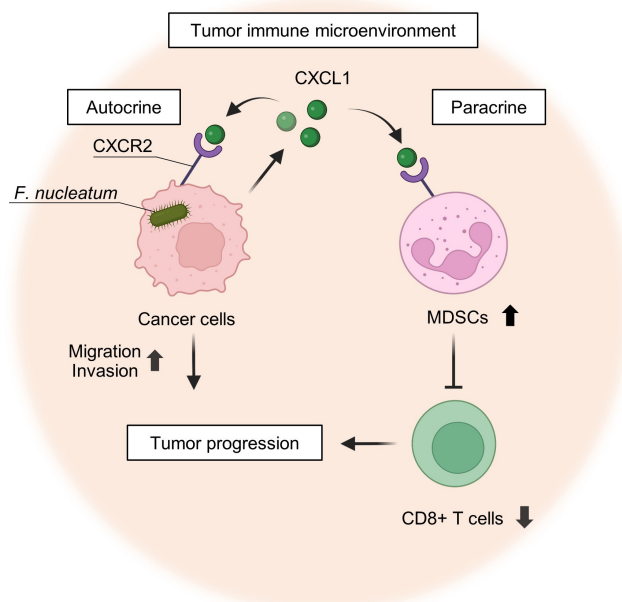


FIGURE 7 Model of the mechanism of *Fusobacterium nucleatum* stimulating CXCL1 secretion from pancreatic cancer cells, leading to cancer progression through two pathways: first, in an autocrine manner, *F. nucleatum* promoted pancreatic cancer cell migration and invasion, which was confirmed by in vitro coculture experiments with *F. nucleatum*; second, in a paracrine manner, *F. nucleatum* suppressed tumor-infiltrating CD8⁺ T cells by recruiting MDSCs in vivo experiments.

AUTHOR CONTRIBUTIONS

M.H. performed most experiments, analyzed data, and wrote the manuscript. N.I. coordinated the project, supervised manuscript writing, and contributed to data interpretation and discussion. K.N. supervised the study and contributed to data interpretation, designed the experimental approach, and critically revised the manuscript. H.L., P.Z., S.D., K.O., N.H., A.K., C.I., and T.A. performed mouse breeding and assisted with animal experiments and assays. N.T., Y.Y., and Y.O. performed immunohistochemistry and contributed resources. K.O. contributed to discussion and supervised manuscript writing. M.N. supervised the study and manuscript writing. All authors approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest. Yoshinao Oda, the coauthor for this study, is the Associate Editor of *Cancer Science*.

ETHICS STATEMENT

Approval of the research protocol by an Institutional Reviewer Board: The study was approved by the Ethics Committee of Kyushu University (approval numbers: 30-230 and 858-00; Fukuoka, Japan).

Informed Consent: N/A.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: All mouse experiments were conducted in accordance with the guidelines of the institutional animal committee of Kyushu University (approval number: A21-383).

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SUPPORTING INFORMATION

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

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