Chemotherapy-Derived Inflammatory Responses Accelerate the Formation of Immunosuppressive Myeloid Cells in the Tissue Microenvironment of Human Pancreatic Cancer

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic malignancies. PDAC builds a tumor microenvironment that plays critical roles in tumor progression and metastasis. However, the relationship between chemotherapy and modulation of PDAC-induced tumor microenvironment remains poorly understood. In this study, we report a role of chemotherapy-derived inflammatory response in the enrichment of PDAC microenvironment with immunosuppressive myeloid cells. Granulocyte macrophage colony-stimulating factor (GM-CSF) is a major cytokine associated with oncogenic KRAS in PDAC cells. GM-CSF production was significantly enhanced in various PDAC cell lines or PDAC tumor tissues from patients after treatment with chemotherapy, which induced the differentiation of monocytes into myeloid-derived suppressor cells (MDSC). Furthermore, blockade of GM-CSF with monoclonal antibodies helped to restore T-cell proliferation when cocultured with monocytes stimulated with tumor supernatants. GM-CSF expression was also observed in primary tumors and correlated with poor prognosis in PDAC patients. Together, these results describe a role of GM-CSF in the modification of chemotherapy-treated PDAC microenvironment and suggest that the targeting of GM-CSF may benefit PDAC patients' refractory to current anticancer regimens by defeating MDSC-mediated immune escape.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive cancer characterized by high mortality and poor prognosis, where in advanced cases the average of life expectancy is less than 1 year (1, 2). A recent study of cancer incidence and mortality has projected PDAC to become the second leading cause of cancer-related death by 2030 in the United States (3). In spite of recent progress in treatment strategies, the current protocols of chemotherapy remain insufficient to cure the patients (4, 5). Recently, we and other groups have reported a new concept of "adjuvant surgery" in which PDAC patients are treated with preoperative chemotherapy, followed by surgical resection, which contributes to long-term survival for locally advanced cases (6, 7).

Unfortunately, this procedure can be applied in only a small population of selected patients that were characterized with high outcome of preoperative chemotherapy (6, 7). Thus, new therapeutic strategies for improving chemotherapeutic response are critically needed to improve the clinical outcomes in advanced PDAC, which in turn depend on the deep understanding of changes induced in tumor microenvironment under chemotherapeutic conditions. In this context, it has recently become clear that anticancer chemotherapeutic agents can modify the tumor microenvironment, and the therapeutic effects mediated by these agents are considerably dependent on the host immunologic reaction (8, 9). In addition, the complex interaction between tumor cells and other cellular components of tumor microenvironment such as cancer-associated fibroblasts and myeloid cells has great impact on invasion, metastasis, and acquiring of chemo-resistant phenotypes (10, 11). PDAC microenvironment constitutes molecular and cellular components with inflammatory features, such as pancreatic stellate cells and immune cells, which affect PDAC progress (12, 13). Accumulating evidence has unveiled the role of KRAS oncogene in the formation of desmoplastic and inflammatory microenvironment via the secretion of multiple cytokines and chemokines (14). Thus, the understanding of the interaction between tumor microenvironment and immune cell and cytotoxic therapies is essential for the improvement of PDAC treatment.

Myeloid-derived suppressor cells (MDSC) are heterogeneous populations of immune cells derived from progenitor cells in
bone marrow, which accumulate in tumor microenvironment via various pathologic mechanisms, and contribute to tumor progression by damping T-cell immunity and promoting angiogenesis (15, 16). Cytokines such as colony-stimulating factors (e.g., GM-CSF and granulocyte colony-stimulating factor) are key molecules involved in the generation of MDSCs (17, 18). Oncogenic KRAS is the most frequently mutated gene in PDAC and has been shown to be involved in PDAC development and growth (19, 20). Importantly, oncocgenic KRAS is associated with overexpression of GM-CSF, which induces MDSC formation in PDAC microenvironments, which in turn prompt the development and progression of PDAC in genetically engineered mouse models (21, 22). Moreover, targeted depletion of MDSCs was effective to increase the intratumoral accumulation of activated T cells and thus improved the therapeutic efficacies of immunotherapy in murine models of PDAC and other cancers (23). However, little is known about the role of MDSCs in human PDAC, especially in clinical therapeutic settings, for example, chemotherapy-treated conditions.

In the current study, we show phenotypic and functional changes of monocytes under chemotherapy-treated human PDAC conditions. Human monocytes differentiated into HLA-DRlow/negative MDSC phenotype when cultured in conditioned medium (CM) of human PDAC cells. Moreover, HLA-DRlow/negative cell formation was enhanced when human monocytes were cultured in CM of chemotherapy-treated human PDAC cells. Gene and protein expression of GM-CSF or other inflammatory factors in human PDAC cell lines were upregulated after treatment with anticancer cytotoxic agents such as gemcitabine and fluorouracil (5-FU). Blockade of GM-CSF in the supernatants of PDAC cell culture with specific monoclonal antibodies resulted in recovery of T-cell proliferation when cocultured with monocytes stimulated with PDAC CM. Consistent with these results, we found that PDAC tumor tissues in chemotherapy-treated cancer patients recruited more cells that express MDSC markers compared with nontreated group.

In conclusion, targeting of PDAC with chemotherapy may activate inflammatory signals that induce the production of multiple sets of cytokines and chemokines in tumor cells. Among these, GM-CSF has emerged as a critical factor that link inflammatory signals with the creation of immunosuppressive microenvironment via the acceleration of monocytes differentiation into MDSCs. Together, our results give a new insight into how chemotherapy may results in counterproductive effects, and highlight the candidate molecules to be targeted in future improvement of PDAC treatment.

Materials and Methods

Ethics

Human PDAC samples were obtained from surgical specimens after obtaining informed consent from all patients. Blood samples were obtained from healthy volunteers and PBMCs were separated using cell separating tube (BD Biosciences). Both procedures were ethically approved by the committees in the Institutional Review Board of Hokkaido University Hospital (Sapporo, Japan; No. 013-0389, 013-0390).

Human PDAC tissue samples

For tissue microarray (TMA), PDAC tissue samples were obtained from 99 resected PDACs in our institute between 1994 and 2005. TMAs were constructed as described in our previous report (24). Patients without information about survival or broken and poor samples were omitted from analysis. A total of 68 patients were subjected to analysis. The characteristics of patients for TMA study were summarized in Supplementary Table S1. Evaluation procedure was performed as previously reported with a little modification. The intensity of GM-CSF staining was classified according to a three-level scale: 0, weak or equivalent staining compared with normal pancreas; 1+, strong and partial staining to cytoplasm of cancer cell; 2+, strong and diffuse staining to cytoplasm. Scoring was evaluated by two independent investigators.

The 15 patients that underwent surgery in our institute and were evaluated (Fig. 5) are overlap cohorts described in our previous report resected in our institute between 2006 and 2010 (25). The characteristics of these patients are summarized in Supplementary Table S2–S3. Immunohistochemical testing and evaluation of myeloid cells were performed according to previous reports (25). Briefly, five areas of most abundant myeloid cells distribution were selected in high-power field (>400). Average counted numbers of areas were compared. All specimens were evaluated by two independent investigators.

Cell lines

Human PDAC cell lines (Capan-1, Capan-2, PANC-1, MIA-PaCa-2, and BxPC-3), human cervical cancer cell line (HeLa), and human leukemia cell line (Jurkat) were purchased from ATCC. PK-45-P and PK-1 were purchased from RIKEN. PCI-43 and PCI-43-P5 were previously established from surgically resected primary carcinoma tissues in our institute (26). All cell lines were cultured in an appropriate medium as indicated by manufactures or references. For CM used in monocyte culture, Capan-1 and PANC-1 cells were cultured in RPMI1640 (WAKO) supplemented with 10% FBS (Cell Culture Bioscience), 1% penicillin/streptomycin, 10 mmol/L HEPES, 1% l-glutamine, 1 mmol/L sodium pyruvate, 1% nonessential amino acids (all from Life Technologies), and 50 μmol/L L-mercaptoethanol (WAKO) in accordance with optimizing conditions for monocytes.

In vitro human monocyte culture

To examine the effects of PDAC-derived factors on monocyte differentiation, we established the following in vitro models. For normal condition, the supernatants of PDAC cell culture were harvested when cells became 80% confluent and passed through 0.2 μm filter (Sartorius Stedim Biotech). To mimic clinical pharmacologic settings in PDAC patients, gemcitabine (1–30 μmol/L) or 5-FU (10 μg/mL) were applied at concentrations similar to that used in clinic (1–30 μmol/L). PDAC cell cultures were pulsed with GEM or 5-FU for 60 minutes followed by washing 5 times with sterilized PBS and changing to fresh media. After 72 hours, supernatants were collected and passed through 0.2 μm filter as described above. Human peripheral monocytes were purified from PBMC of healthy donors using CD14+ selection by magnetic cell sorting systems according to manufacturer’s protocols (Miltenyi Biotec) and cultured in the presence of supernatants prepared from normal PDAC or chemotherapy-treated PDAC cells for 6 days. On day 6, gene expression and protein analysis were evaluated by quantitative RT-PCR or flow cytometry, respectively. In some experiments, cytokines in the supernatants of PDAC cell culture were neutralized using anti-human GM-CSF (clone BVD2-23B6; Biolegend, 10 μg/mL), anti-human IL6 (clone
human cell surface markers were purchased from BD Biosciences (anti- HLA-DR and anti-CD15), Beckman Coulter (anti-CD11b and anti-CD33), Miltenyi Biotec (anti-CD14), or Biolegend (anti-CCR2 and anti-CX3CR1). Fluorescent antibodies used for the staining of human cell surface markers were purchased from Biolegend (anti-CD11b and anti-CD33), Miltenyi Biotec (anti-CD14), or Biolegend (anti-CD14 or anti-CD16/32). Samples were run on FACs Canto II (BD Biosciences) and analyzed using FlowJo software V7.6.5.

Flow cytometry

Single-cell suspensions were used for flow cytometry analysis after treatment with human FcR blocker (Miltenyi Biotec) or anti-mouse CD16/32 (BD Biosciences) and stained with appropriate fluorescent antibodies according to manufacturer’s instructions. Fluorescent antibodies used for the staining of human cell surface markers were purchased from BD Biosciences (anti-CCR2 and anti-CX3CR1). Fluorescent antibodies used for the staining of mouse cell surface markers were purchased from Biolegend (anti-CD11b and anti-Gr1). Pre-warmed with protease inhibitors aprotinin and phenylmethylsulfonyl-fluoride. Protein samples were resolved using 10% SDS-PAGE and were then transferred to polyethylene di fluoride membrane (GE Healthcare). Membranes were probed with primary antibodies against target molecules followed by reaction with secondary antibodies conjugated to horseradish peroxidase for appropriate incubation time. Antibodies against ERK, p-ERK, AKT, and p-AKT were purchased from Cell Signaling Technology; antibodies against β-actin were purchased from Millipore; secondary antibodies were purchased from Jackson ImmunoResearch. Immunoreactivity was detected by an Enhanced Chemiluminescence Detection System (GE Healthcare). Equal loading of proteins was confirmed with β-actin.

Quantitative RT-PCR

RNA was extracted from cells using RNeasy Plus Mini Kit (Qiagen) according to the manufacturer’s protocol, and used for cDNA synthesis (Prime Script RT Master Mix, Takara Bio). cDNA products were used to amplify target genes using Power SYBR Green (Life Technologies) and specific primer (Supplementary Table S3). PCR reactions and data analysis were performed in a StepOne Real-Time PCR System (Applied Biosystems), using the comparative C0 method and the housekeeping gene GAPDH. Primers used in this study are as follows:

**GAPDH** (forward: 5’-AACAGCGACACCCACTCCTC-3’; reverse: 5’-ATACACGGAAATGACCTGACA-3’), M-CSF (forward: 5’-GCCCTGGCCGAACCTCTCA-3’; reverse: 5’-ACCTGCTAGGATGGCCTTGG-3’), GM-CSF (forward: 5’-ATGATGCGCAGGCCTACAA-3’; reverse: 5’-CTGGCTCCAGACCTGCAAAG-3’), IL6 (forward: 5’-GGCACTGGGAAAAAACACC-3’; reverse: 5’-GGCAAGCTCCTCAGTGAACC-3’), IL8 (forward: 5’-CTGGCCCAACAAGATAATT-3’; reverse: 5’-ATGCTGACGCAACCATT-3’), IL1B (forward: 5’-ATACTGACGGACAGTCC-3’; reverse: 5’-GCCAACAGCCACAGATT-3’), TNF (forward: 5’-CAGATGAGTTTGCCGAA-3’; reverse: 5’-AGGAAGGCCCTAAGGCT-3’), VEGF-A (forward: 5’-CTACCTCCACATCATGCAAG-3’; reverse: 5’-GCGATGCTGGCCTGATAGA-3’), CXCL-12 (forward: 5’-CTACAGGTTCCATGGC-3’; reverse: 5’-CAGGCAGGTCTTCATC-3’), SDF (forward: 5’-AGGAGCTGTCCTTAAATG-3’; reverse: 5’-TGGCCCTTGTAACGTG-3’), TGFB1 (forward: 5’-GGGACTTACCACCGGC-3’; reverse: 5’-GGCGAACATGATG-3’), CCL-2 (forward: 5’-CAGCAAGTGTCCCAAATC-3’; reverse: 5’-TGGAATCCTGAACCCCTGC-3’), NOS2 (forward: 5’-TCAAGAAGCTTGGCACC-3’; reverse: 5’-AAATGGTGGGCTTGGTGG-3’), ARG1 (forward: 5’-ATGCTGCCAGGGACTGACC-3’; reverse: 5’-TGCAACGTGCTTCTGC-3’), IL-10 (forward: 5’-GAGATGCTGGCCTCAG-3’; reverse: 5’-ACATGGCGCCTTGCTG-3’). Primers specificity was confirmed by peak melt curve before using. All experiments were performed in duplicate for each sample.

Cytokine measurement

Cytokines were measured using commercial ELISA kits according to the manufacturer’s instructions. The kits for GM-CSF and IL8 were purchased from Biolegend. The kit for IL6 was purchased from R&D Systems. All measurements were performed using supernatants from three independent cell cultures.

Western blotting

Total cell lysates were prepared using RIPA buffer supplemented with protease inhibitors aprotinin and phenylmethysulfonyl-fluoride. Protein samples were resolved using 10% SDS-PAGE and were then transferred to polyethylene di fluoride membrane (GE Healthcare). Membranes were probed with primary antibodies against target molecules followed by reaction with secondary antibodies conjugated to horseradish peroxidase for appropriate incubation time. Antibodies against ERK, p-ERK, AKT, and p-AKT were purchased from Cell Signaling Technology; antibodies against β-actin were purchased from Millipore; secondary antibodies were purchased from Jackson ImmunoResearch. Immunoreactivity was detected by an Enhanced Chemiluminescence Detection System (GE Healthcare). Equal loading of proteins was confirmed with β-actin.

NF-kB luciferase reporter assay

Promoter activities of NF-kB in cultured cells were monitored using Ready-To-Glow secreted luciferase reporter system (Clontech). Briefly, Capan-1 cells were transfected with secreting luciferase reporter plasmid encoding NF-kB using Lipofectamine 2000 (Invitrogen), and stable clones were selected by G418. Stable clones were stimulated with GEM or 5-FU and luciferase activities in the supernatants were detected at the indicated time points. Luciferase activities were compensated by cell number.

T-cell proliferation assay

Autologous reactions of monocytes and CD4+ or CD8+ T cells were estimated by 3H-thymidine incorporation assay. Briefly, human CD4+ or CD8+ T cells were isolated from PBMC of healthy donors using CD4+ T Cell Isolation Kit and CD8+ T Cell Isolation Kit (Miltenyi Biotec). CD4+ or CD8+ T cells were cultured in the presence of 3 μg/ml of anti-CD3 antibody (OKT3; eBioscience) and 1 μg/ml of anti-CD28 antibody (CD28.2; Biolegend). Stimulated CD4+ or CD8+ T cells were then cocultured with monocytes differentiated in the presence of tumor supernatants at the indicated conditions T-cell/monocyte ratios. 3H-thymidine incorporations were counted after 72 hours culture.

Immunohistochemical staining of formalin-fixed paraffin-embedded tissues

Paraffin-embedded specimens were cut into thin slices and mounted on glass slide. Sections were deparaffinized in xylene and rehydrated in ethanol. Antigen retrieval was performed by boiling for 20 minutes in citrate buffer (pH 6.0) or Tris-EDTA buffer (pH 9.0). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol. Non-specific reactions were blocked with original blocking cocktails; the equal quantity of 10% normal goat serum (Nichirei), protein-block serum-free ready-to-use (Dako), and antibody diluted with background reducing components (Dako). Immunohistochemical reactions were carried out using the enzyme polymer methods with Histofine series (Nichirei). Primary antibodies were mounted into slides for 60 minutes at room temperature or overnight at 4°C followed by 20 minutes incubation with secondary antibodies at room temperature. Antibodies used for FFPE were purchased from LSbio (GM-CSF: LS-C104671 clone), Abcam (CD14: ab49755 clone, HLA-DR: EPR3692 clone), and Biolegend (CD66b: 6708; R&D Systems, 2 μg/mL), or anti-human IL8 (clone 6217; R&D Systems, 2 μg/mL).

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and used according to the manufacturer’s instructions. The list of primary antibodies with their reactive conditions is listed in Supplementary Table S4. Immunohistochemical reactions were visualized with DAB or Fast Red II (Nichirei) followed by counterstaining with hematoxylin and mounted on coverslips.

Statistical analysis

Parametric statistics were applied for in vitro data and Student t test was used for comparison between groups. For mouse or human data, nonparametric statistics were applied in which Mann–Whitney U test, Fisher exact test, or $\chi^2$ test was used as appropriate. Overall survival was calculated from the date of operation to the date of last follow-up or date of patient death. The Kaplan–Meier method was used to estimate overall survival, and survival differences were estimated by the log-rank test. Except where indicated, the values were presented as mean ± SEM. $P$ was considered statistically significant when $<0.05$. All data were analyzed using StatFlex software v6.0.

Results

Human monocytes differentiate into MDSCs when cultured in the supernatants of PDAC cell culture

PDAC cells secrete multiple inflammatory cytokines and growth factors. To assess how PDAC cell–derived soluble factors influence human myeloid cells differentiation, we generated in vitro culture models using CM from two PDAC cell lines: Capan-1 and PANC-1 (Fig. 1A). We found that human monocytes formed different morphologies in response to PDAC tumor supernatants. Monocytes differentiated into spindle adherent cells when cultured in normal medium, whereas the supernatants of PDAC cells induce monocytes differentiation into circular immature cells. Scale bars, 100 μm. C, flow cytometry analysis of CD14, CD33, and HLA-DR expression in monocytes cultured in normal medium (control), Capan-1 CM, or PANC-1 CM. PDAC CM–treated monocytes were CD14$^+$CD33$^+$HLA-DR$^{\text{low}}$ cells resembling mo-MDSC. D, HLA-DR expression levels in cultured monocytes at day 6. HLA-DR expressions were significantly decreased when monocytes were cultured in PDAC CM ($n=3$ donors). E, flow cytometry analysis of NOS2 and ARG1 in monocytes cultured in normal medium (control), Capan-1 or PANC-1 CM. Gray histogram, isotype; black line, control medium; gray line, Capan-1 or PANC-1 CM. PDAC CM–treated monocytes showed high levels of NOS2 and ARG1 compared with control. F, flow cytometry analysis of CD11b, CD15, CCR2 and CX3CR1 expression in monocytes cultured in Capan-1 or PANC-1 CM. Gray histogram, isotype; black line, Capan-1 or PANC-1 CM. PDAC CM–treated monocytes showed expression of CD11b and CCR2 but lack the expression of CD15 or CX3CR1. Flow cytometry results are shown as representative multiple independent experiments. $^*$, $P < 0.05$; $^{**}$, $P < 0.01$. 

Except where indicated, the values were presented as mean ± SEM. $P$ was considered statistically significant when $<0.05$. All data were analyzed using StatFlex software v6.0.
cultured in normal medium, while monocytes that were differentiated in the presence of Capan-1 or PANC-1 supernatants formed floating immature cells (Fig. 1B). Previous reports suggested that PDAC induces the accumulation of MDSCs in tumor regions in genetically engineered mouse models (21, 22). Monocyte-derived MDSCs (mo-MDSCs) from cancer patients express the monocyte macrophage marker CD14 and the common myeloid marker CD33, but lack or show lower expression of mature myeloid markers HLA-DR (27). We found that human monocytes expressed CD14 and CD33, while HLA-DR expression was relatively lower in monocytes cultured in PBS-treated PDAC CM compared with normal medium (Fig. 1C and D). Mo-MDSCs suppress T-cell immunity via nitric oxide synthase 2 (NOS2) or Arginase 1 (ARG1; refs. 28, 29). Thus, we next evaluated the expression levels of these two enzymes in monocytes induced by PDAC CM. PDAC CM–treated monocytes showed high expression of both NOS2 and ARG1 (Fig. 1E). In addition, we examined the expression of other myeloid lineage markers, and found that PDAC CM–treated monocytes express the common myeloid marker CD11b, chemokine receptor 2 (CCR2), but lack the expression of granulocyte or tissue resident macrophage marker CD15 or CX3C chemokine receptor 1 (CX3CR1; Fig. 1F; ref. 30). Together, these data demonstrated that human peripheral monocytes differentiated into mo-MDSCs when stimulated with PDAC CM.

The supernatants of chemotherapy-treated PDAC cells enhance the differentiation of human monocytes into MDSCs

Next, we examined whether the differentiation patterns of monocytes are altered in chemotherapy-treated PDAC microenvironment. To do so, we established an in vitro culture model using Capan-1 cell line treated with gemcitabine or 5-FU (Fig. 2A). Interestingly, after 6 days of culture, monocytes showed morphologic changes when cultured in the supernatants of chemotherapy-treated PDAC cells, represented by increased diameters (Fig. 2B and Supplementary Fig. S1) and formation of cytoplasmic vacuoles that were not observed in monocytes cultured in PBS-treated PDAC CM or normal medium. Scale bars, 20 μm. D and E, flow cytometry analysis shows high forward and side scatter voltage signals (top) and increased frequencies of HLA-DRlow/negative fraction (bottom) in monocytes cultured in gemcitabine-treated PDAC CM compared with PBS-treated PDAC CM. (n = 3 donors). F, enhanced expression of NOS2 in monocytes cultured in the supernatants of gemcitabine (GEM)-treated Capan-1 cells. Data are shown as representative of two independent experiments. *, P < 0.05; **, P < 0.01.
of HLA-DR\textsuperscript{low/negative} immature monocytes that have been previously reported (27). To evaluate the immunosuppressive features of monocytes differentiated in gemcitabine-treated PDAC CM, we analyzed expression levels of ARG1, IL-10, TGF-β1, and NOS2. Although no significant changes were observed in the expression of ARG1, IL-10, or TGF-β1 (data not shown), NOS2 expression was significantly increased in monocytes differentiated in gemcitabine-treated PDAC CM (Fig. 2F). MDSCs are usually characterized by lack or low expression of HLA-DR and high expression of NOS2 (28, 31). Accordingly, these data suggest that the supernatants of chemotherapy-treated PDAC cells accelerate the differentiation of monocytes into MDSCs with enhanced molecular patterns.

Treatment with chemotherapy amplifies the expression of GM-CSF and other inflammatory cytokines in PDAC cells via the activation of MAPK signaling pathway and NF-κB transcription

MDSCs are immunosuppressive myeloid cells that contribute to tumor progression and immune evasion. Accumulating evidence has unveiled that GM-CSF and other tumor-derived molecules are necessary for the induction of preferential expansion of MDSCs in tumor microenvironment (32–34). To identify factors in the supernatants of chemotherapy-treated PDAC cells responsible for monocytes differentiation into MDSCs, we investigated expression profiles of various cytokines and chemokines in Capan-1 or Panc-1 cell lines. Following stimulation with gemcitabine or 5-FU, several cytokines and chemokines were upregulated in both cell lines (Fig. 3A and B and Supplementary Fig. S2). In particular, the expression of GM-CSF, IL6, and IL8 was increased in the supernatants of chemotherapy-treated Capan-1 cells (Fig. 3C and Supplementary Fig. S3). In the next experiment, we focused on GM-CSF since both cell lines showed a significant enhancement in GM-CSF production after treatment with gemcitabine or 5-FU. In addition, GM-CSF is well known for its role as an essential factor of MDSC proliferation and differentiation in PDAC (22). In oncogenic KRAS-mediated PDAC murine model, GM-CSF is regulated by MAPK or PI3K signaling pathway, two major downstream pathways of KRAS oncogene (21). Thus, we next compared the activation status of these two pathways through the evaluation of ERK phosphorylation as an indicator for MAPK pathway, or AKT for PI3K pathway in normal or chemotherapy-treated conditions. We found that gemcitabine treatment enhances the phosphorylation of ERK (Fig. 3D) but not AKT (data not shown) in a time-dependent manner. NF-κB is a major transcription factor that induces the expression of inflammatory cytokines, including GM-CSF (35, 36). Thus, we next examined whether gemcitabine treatment may induce promoter activities of NF-κB in PDAC cells. In a luciferase assay, we found that NF-κB–luciferase activities were enhanced after chemotherapy treatment (Fig. 3E). These data indicate that chemotherapy enhances the production of multiple inflammatory cytokines including GM-CSF by amplifying the activation status of MAPK signaling pathway and NF-κB promoter activities in PDAC cells.

Neutralization of GM-CSF in the supernatants of chemotherapy-treated PDAC cells blocks monocyte differentiation into MDSCs and helps recovery of T-cell proliferation

The supernatants of chemotherapy-treated PDAC cells were enriched with GM-CSF, and induced morphologic and phenotypic changes in monocytes. To further examine the contribution of GM-CSF in these changes, we utilized a specific monoclonal antibody to neutralize GM-CSF in chemotherapy-treated Capan-1 CM. Interestingly, we found that the neutralization of GM-CSF has resulted in decreased forward and side scatter voltage signals as well as HLA-DR\textsuperscript{low/negative} fractions (Fig. 4A), and abolished the formation of cytoplasmic vacuoles that were observed in the case of gemcitabine-treated Capan-1 CM (Fig. 4B). These data indicate that GM-CSF is one of the major factors of monocyte differentiation in the supernatants of chemotherapy-treated PDAC cells.

MDSCs are heterogeneous populations of cells that are defined by their ability to potently suppress T-cell response by NOS2-dependent mechanism (31). As described above, the supernatants of chemotherapy-treated PDAC cells were enriched with GM-CSF, and induced high expression of NOS2 in MDSCs differentiated from monocytes. To confirm the immunosuppressive potential of MDSCs generated from monocytes in the presence of PDAC supernatants, we cocultured these MDSCs with CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells and examined T-cell aggregation and proliferation after stimulation. Interestingly, MDSCs generated from monocytes by normal Capan-1 CM suppressed aggregation and proliferation of stimulated CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells, which was further suppressed by MDSCs generated by gemcitabine-treated Capan-1 CM (Fig. 4C and D and Supplementary Fig. S4). Importantly, the neutralization of GM-CSF in gemcitabine-treated Capan-1 CM was effective to abolish these immunosuppressive functions and contribute to the recovery of T-cell function as observed by enhanced aggregation and proliferation (Fig. 4C and D and Supplementary Fig. S4). Together, these data highlight the role of GM-CSF in the enhancement of MDSCs formation in chemotherapy-treated PDAC microenvironment, and suggest that the neutralization of GM-CSF may contribute to block the formation of MDSCs and thus the recovery T-cell response.

GM-CSF is expressed in various human PDAC cell lines and tumor tissues and serves as a poor prognostic indicator for PDAC patients

To investigate whether GM-CSF expression is a common feature of PDAC cells, we examined the expression of GM-CSF in human samples. Quantitative PCR analysis showed high expression of GM-CSF in all PDAC cell lines with some variations (Fig. 5A). Next, immunohistochemistry staining was used to examine protein levels of GM-CSF in PDAC tissues of 68 resected primary tumors by IHC. PDAC tissues also showed variety in GM-CSF expression (Fig. 5B). The intensity of GM-CSF staining was classified as high or low as described in Materials and Methods (Fig. 5B and C), and scores were used to generate Kaplan–Meier survival curve. We found that survival rates were significantly lower in patients with high expression of GM-CSF (Fig. 5D). These data suggest that GM-CSF, a MDSC-inducing cytokine, is generally expressed in human PDAC, and correlates with poor prognosis.

Finally, to examine the impact of tumor microenvironment on MDSCs differentiation in human PDAC tissues under therapeutic conditions, we assessed MDSC marker expression in tumor-infiltrating myeloid cells in PDAC patients treated with preoperative chemotherapy including gemcitabine in our institute (Supplementary Table S3). We found that tumor-infiltrating CD14\textsuperscript{+} cells in PDAC patients treated with preoperative chemotherapy show no or weak expression of HLA-DR compared with
patients without preoperative chemotherapy treatment (Fig. 5E and F). These data indicate that CD14⁺ HLA-DR⁻ cells constitute a dominant fraction in PDAC tissues following chemotherapy, an observation that might be a contrast to a previous mouse study (37). Furthermore, we investigated the expression of CD66b, a marker of granulocytic MDSC (G-MDSC; ref. 38), and found that the frequencies of tumor-infiltrating CD66b⁺ cells were significantly higher in PDAC patients after chemotherapy treatment (Fig. 5G and H). On the other hand, no significant difference was observed in the frequencies of CD68⁺ macrophages between the two groups (Supplementary Fig. S5). Taken together, these results suggest that chemotherapy treatment accelerates the formation of both mo-MDSCs and G-MDSCs in human PDAC tissues, consistent with previous experiments.

Discussion

Most of PDAC cancer cases are diagnosed at late stages, which make surgical resection of the tumor or the organ difficult if not impossible (39). Chemotherapy has been suggested as a possible strategy for the treatment of PDAC patients; however, clinical response mediated by anticancer cytotoxic agents against PDAC is
so limited, and it is unlikely that chemotherapy alone will provide durable clinical benefit for the majority of PDAC patients. Thus, new combination protocols are suggested to gain cumulative or synergistic benefit in large population of patients. One good example is the treatment with radical surgery, which was accompanied by favorable clinical outcomes in some clinical cases (6, 7). Moreover, recent progress has been achieved in the protocols of "neoadjuvant chemotherapy" against PDAC (40, 41). These new protocols enable the analysis of molecular and pathologic patterns of chemotherapy-treated PDAC. For example, recent preoperative chemotherapy protocols helped to identify the molecular patterns of T cells, showing increased accumulation in tumor tissues in PDAC or oesophageal cancer patients (25, 42, 43). In addition, in this study we have reported for the first time the distribution of MDSC markers in PDAC patients after chemotherapy treatment, in which MDSCs were the dominant cells in cancer regions. However, the real therapeutic effects of chemotherapy in PDAC treatment still poorly understood, since a large proportion of PDAC patients develop chemoresistance and thus cannot receive surgical therapy. Therefore, further studies are critically needed to identify the molecular mechanism of chemoresistance in PDAC.

It is now well established that the antitumor activities of chemotherapy considerably rely on the complex interaction between tumor and immune system of the host (9, 44). Moreover, accumulating evidence has unveiled the importance of the interaction between tumor cells and myeloid cells in inducing chemoresistance and metastasis (11, 45). This is also applicable in the case of PDAC, and the deep understanding of this complex interaction in tumor microenvironment is a key concept for the improvement of chemotherapeutic response against PDAC. To
understand how PDAC cells influence tumor microenvironment in chemotherapy-treated condition, we first analyzed monocyte differentiation patterns using in vitro culture models. When stimulated with the supernatants of chemotherapy-treated PDAC cells, human monocytes differentiated into immunosuppressive cells that resemble MDSCs, showing similar morphology and shared the same molecular markers. Interestingly, the supernatants of chemotherapy-treated PDAC cells were found to be enriched with GM-CSF and other inflammatory factors that induce the differentiation of monocytes into MDSCs. Consistent with this, immunostaining of tumor tissues of PDAC patients treated with chemotherapy has shown enhancement in MDSC markers compared with normal tissues. Thus, chemotherapy itself may result in counterproductive effects in which the formation of immunosuppressive and tumorigenic myeloid cells is enhanced at the microenvironment of PDAC.

MDSCs are a heterogeneous population of immature myeloid cells that negatively regulate the antitumor immune responses (15). MDSCs also support tumor immune evasion by suppressing T-cell immunity and promote angiogenesis and
tumor progression (21, 22, 46). Accumulation of MDSCs has been correlated with tumor progression in patients (39). In addition, a recent report has suggested that MDSCs contribute to senescence evasion and chemoresistance in tumor (11). In PDAC, MDSCs were found to be induced by MAPK or PI3K pathway–dependent GM-CSF, and significantly correlated with tumor development and prognosis (21, 22). Importantly, we have found that GM-CSF production was dramatically enhanced in several PDAC cell lines as well as tumor tissues in PDAC patients after treatment with chemotherapy, which was accompanied by increased frequencies of MDSCs. One possible mechanism is the activation of MAPK and NF-kB signaling pathway as a consequent of chemotherapy-induced DNA-damage response (47). However, detailed mechanism should be elucidated in future studies.

GM-CSF may play two different roles at the tumor microenvironment of PDAC. First, GM-CSF may help to induce or activate anticancer immune responses through the priming of immunostimulatory dendritic cells. On the basis of this concept, GVAX, a GM-CSF gene-transferred tumor cell vaccine, has been developed for the treatment of advanced PDAC patients, but the clinical outcome was lower than what was expected (48). Alternatively, GM-CSF may induce the formation of MDSCs. One possible mechanism of these conflicting roles of GM-CSF is the enrichment of PDAC microenvironment with danger-associated molecular patterns (DAMP) after chemotherapy treatment. DAMPs are released from tumor cells killed by anticancer cytotoxic agents, and signaling mediated by these DAMPs may be involved in the alteration of cellular differentiation pattern (49, 50), which should be clarified in future studies.

Our data indicate that MDSCs were increased after treatment of PDAC with chemotherapy, which was related to enhancement in GM-CSF production. The neutralization of GM-CSF with antibodies was effective to reduce MDSC frequencies, and help the recovery of T-cell function (Fig. 6). Depletion of MDSCs has been recently suggested for PDAC treatment (23). In this context, the targeting of GM-CSF may constitute an additional option to further improve current protocols of PDAC treatment.

In conclusion, our data identify a role of chemotherapy-derived inflammatory response, in particular GM-CSF, in the enrichment of PDAC microenvironment with MDSCs. Here, we suggest that the targeting of MDSCs by direct depletion and/or the neutralization of tumor-derived GM-CSF in combination with current therapeutic regimens constitute a promising strategy for the treatment of PDAC patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions

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