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. Cancer Res; 75(13); 2629–40. ©2015 AACR.

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-derived inflammatory responses 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 (6, 9). In addition, the complex interaction between tumor cells and other cellular components of the 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, oncogenic 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 result 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 95 patients were subjected to analysis. The characteristics of patients for TMA study are 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 manufacturers 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 2-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 (Sartouris 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 cells 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...
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-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 mouse cell surface markers were purchased from Biolegend (anti-CD11b and anti-Gr1). Samples were run on FACScanto II (BD Biosciences) and analyzed using FlowJo software V7.6.5.

Quantitative RT-PCR

RNA was extracted from cells using RNasy 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 Ct method and the housekeeping gene GAPDH. Primers used in this study are as follows:

- GAPDH: forward: 5′-AACACGGACACCCCATCTCCT-3′; reverse: 5′-ATACCGAAATGAGCCTGACAA-3′; M-CSF (forward: 5′-GGCTGCGTCCGAACTCTA-3′; reverse: 5′-ACTGCCAGTAGATGCTTTCG-3′), GM-CSF (forward: 5′-ATGATGGCAGCGCCACTACAA-3′; reverse: 5′-CTGCTGCCCCAGCGTAAGAG-3′), IL6 (forward: 5′-GGCAGTGGACGAAGAACAAG-3′; reverse: 5′-GGAAGTTGCTCATTGAAGTCC-3′), IL8 (forward: 5′-ATACCTGAGTCCGAGCT-3′; reverse: 5′-GCCAACAGCAAGATAA-3′; reverse: 5′-ATTGCGATCTGGCAAACCT-3′; reverse: 5′-GCCAACAGCAAGATAA-3′; reverse: 5′-ATACCGAGTGTTGCTGAGAA-3′; reverse: 5′-AGGGCTTCAG-3′; reverse: 5′-GCCAACAGCAAGATAA-3′; reverse: 5′-ATACCGAGTGTTGCTGAGAA-3′; reverse: 5′-AGGGCTTCAG-3′; reverse: 5′-GCCAACAGCAAGATAA-3′; reverse: 5′-ATACCGAGTGTTGCTGAGAA-3′; reverse: 5′-AGGGCTTCAG-3′; reverse: 5′-GCCAACAGCAAGATAA-3′; reverse: 5′-ATACCGAGTGTTGCTGAGAA-3′; reverse: 5′-AGGGCTTCAG-3′;
- IL8 were purchased from Biolegend. The kit for IL6 was purchased according to the manufacturer’s protocol, and used for detecting cytokines in the supernatants at the indicated conditions at different T-cell/monocyte ratios. 1H-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. Nonspecific 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 diluent 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: ab9755 clone, HLA-DR: EPR3692 clone), and Biolegend (CD66b:...
G10F5), 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 χ² 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.

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 macrophage-like cells when cultured in normal medium, whereas the supernatants of PDAC cells induced 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-DRlow 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. Capan-1 or PANC-1 CM–treated monocytes showed high levels of NOS2 and ARG1 compared with control. F, flow cytometry analysis of CD11b, CD15, CCR2 and CXCR1 expression in monocytes cultured in Capan-1 or PANC-1 CM. Capan-1 or PANC-1 CM–treated monocytes showed expression of CD11b and CCR2 but lack the expression of CD15 or CXCR1. Flow cytometry results are shown as representative multiple independent experiments. *, P < 0.05; **, P < 0.01.
cultured in normal medium, while monocytes that were differentiat-
ed in the presence of Capan-1 or PANC-1 supernatants formed floating immature cells (Fig. 1B). Previous reports sug-
gested that PDAC induces the accumulation of MDSCs in tumor regions in genetically engineered mouse models (21, 22). Mono-
cyte-derived MDSCs (mo-MDSCs) from cancer patients express
the monocyte macrophage marker CD14 and the common mye-
loid 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 rela-
tively lower in monocytes cultured in the presence of PDAC
supernatant 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 eval-
uated the expression levels of these two enzymes in monocytes
induced by PDAC CM.

Next, we examined whether the differentiation patterns of
monocytes are altered in chemotherapy-treated PDAC microen-
vironment. To do so, we established an in vitro culture model using
Capan-1 cell line (27). We found that human monocytes
expressed CD14 and CD33, while HLA-DR expression was rela-
tively lower in monocytes cultured in the presence of PDAC
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Mo-MDSCs suppress T-cell immunity via nitric oxide synthase 2
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uated 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 com-
mon 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

GM-CSF and Modulation of PDAC Microenvironment

Figure 2. Supernatants of chemotherapy-treated PDAC cells induce
morphologic changes in monocytes with enhanced MDSC markers. A,
a scheme of culture protocol used to study the effects of chemotherapy-
treated PDAC microenvironment on monocytes differentiation. Capan-1
cells were pulsed with gemcitabine (GEM; 1 μmol/L or 30 μmol/L) or 5-FU
(10 μg/mL) for 1 hour, followed by careful wash with sterilized PBS and
changed into fresh medium. CM was collected after 72 hours and applied to
human peripheral CD14+ monocytes as described above. B, morphologic
changes in monocytes cultured in gencitabine-treated PDAC CM at day
6. These cells were larger in size than monocytes cultured in PBS-treated
PDAC CM. Scale bars, 100 μm. C, May Giemsa staining showed unique
cytoplasmic vacuoles in monocytes cultured in gencitabine (GEM)-
treated PDAC CM (red arrows) but not 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 gencitabine-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.

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of HLA-DR<sup>low/negative</sup> 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, TGF-β1, and 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 up-regulated 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 oncopgene (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 chemotherapytreated 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<sup>low/negative</sup> 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 potentially 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<sup>+</sup> or CD8<sup>+</sup> 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<sup>+</sup> or CD8<sup>+</sup> 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 of 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 variation in GM-CSF expression (Fig. 5B). The intensity of GM-CSF was regulated in both cell lines (Fig. 3A and B and Supplementary Fig. S3). We found that tumor-infiltrating MDSCs are heterogeneous populations of cells that are defined by their ability to potentially 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<sup>+</sup> or CD8<sup>+</sup> 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<sup>+</sup> or CD8<sup>+</sup> 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 of T-cell response.

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Finally, to examine the impact of tumor microenvironment on MDSCs differentiation in human PDAC tissues under chemo-therapeutic conditions, we assessed MDSC marker expression in tumor-infiltrating myeloid cells in PDAC patients treated with neoadjuvant chemotherapy including gemcitabine in our institute (Supplementary Table S3). We found that tumor-infiltrating CD4<sup>+</sup> cells in PDAC patients treated with neoadjuvant 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 populations 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 patients after chemotherapy treatment, in which MDSCs were the dominant cells in cancer regions. However, the real therapeutic effects of chemotherapy in PDAC treatment are 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

Figure 4. Blockade of GM-CSF contributes to the reversal of morphologic and phenotypic changes induced in monocytes by chemotherapy-treated PDAC CM. A, flow cytometry analysis shows decreased forward and side scatter voltage signals (top) and decreased frequencies of HLA-DRlow/negative fraction (bottom) in monocytes cultured in gemcitabine-treated PDAC CM after depletion of GM-CSF (anti-GM-CSF, 10 μg/mL). B, microscopic examination and May Giemsa staining showed decrease in cell size (top) and disappearance of cytoplasmic vacuoles (bottom) that were observed in gemcitabine-treated PDAC CM after treatment with anti-GM-CSF. Scale bars, 100 μm for photomicrographs and 20 μm for May Giemsa staining. C, photomicrographs of T-cell aggregate. MDSCs were cocultured with autologous CD4 T cells stimulated with anti-CD3/28 for 72 hours at the indicated ratio. Data are shown as representative of two independent experiments. Scale bar, 10 μm. D, T-cell proliferation assay. MDSCs were cocultured with autologous CD4 T cells stimulated with anti-CD3/28 for 72 hours at the indicated ratio and T-cell proliferation was measured by H3-thymidine uptake. Neutralization of GM-CSF in gemcitabine-treated Capan-1 CM was effective to abolish the immunosuppressive functions and contribute to the recovery of CD4 T-cell function as observed by enhanced aggregation and proliferation. Data are shown as representative of two independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
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

Figure 5.
GM-CSF expression is observed in various PDAC cell lines and tumor tissues of PDAC patients, and related to the enhancement of MDSC markers after treatment with preoperative chemotherapy. A, quantitative RT-PCR analysis of GM-CSF in various PDAC cell and non-PDAC cell lines. GM-CSF expression was normalized to GAPDH. Data are shown as representative of three independent experiments. B, immunohistochemical staining of GM-CSF in PDAC region or normal region of pancreatic tissues from PDAC patients, Scale bar, 100 μm, C, the intensity of GM-CSF staining was classified according to a three-level scale: 0, 1+, 2+, and 71% of patients were GM-CSF high criteria. D, Kaplan–Meier survival analysis of overall survival in 68 resected PDAC samples. GM-CSF–high population showed significantly lower survival rates. E, immunohistochemical staining of CD14 and HLA-DR in pancreatic tissues of PDAC patients before or after treatment with preoperative chemotherapy. Scale bar, 100 μm, F, frequencies of CD14\(^+\) HLA-DR\(^+\) (left) and percentage of HLA-DR\(^+\) cells to total CD14\(^+\) cells (middle) and total CD14\(^+\) (right) in pancreatic tissues of PDAC patients before or after preoperative chemotherapy. G, immunohistochemical staining of CD66b in pancreatic tissues of PDAC patients before or after treatment with preoperative chemotherapy. Scale bar, 100 μm, H, frequencies of CD66b\(^+\) in pancreatic tissues of PDAC patients before or after preoperative chemotherapy. For F and H, bars indicate the median value and the box encompasses the 25th and 75th percentiles. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
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 regimes constitute a promising strategy for the treatment of PDAC patients.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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