Chemotherapy-derived inflammatory responses accelerate the formation of immunosuppressive myeloid cells in the tissue microenvironment of human pancreatic cancer.

Shintaro Takeuchi¹,², Muhammad Baghdadi², Takahiro Tsuchikawa¹, Haruka Wada², Toru Nakamura¹, Hirotake Abe¹,², Sayaka Nakanishi², Yuu Usui², Kohtaro Higuchi², Mizuna Takahashi¹, Kazuho Inoko¹, Syoki Sato¹, Hironobu Takano¹, Toshiaki Shichinohe¹, Ken-ichiro Seino²* and Satoshi Hirano¹*

¹ Department of Gastroenterological Surgery II, Hokkaido University Graduate School of Medicine, Kita-15, Nishi-7, Sapporo 060-8638, Japan
² Division of Immunobiology, Institute for Genetic Medicine, Hokkaido University, Kita-15 Nishi-7, Sapporo 060-0815, Japan

*Corresponding authors:
Ken-ichiro Seino, MD, PhD
Division of Immunobiology, Institute for Genetic Medicine, Hokkaido University
Kita-15, Nishi-7, Sapporo 060-0815, Japan
Tel: 81-11-706-5532
Satoshi Hirano, MD, PhD
Department of Gastroenterological Surgery II, Graduate School of Medicine, Hokkaido University
Kita-15, Nishi-7, Sapporo 060-8638, Japan
Tel: 81-11-706-7714
Fax: 81-11-706-7158
E-mail: satto@med.hokudai.ac.jp
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. 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 (MDSCs). Furthermore, blockade of GM-CSF with monoclonal antibodies helped to restore T cell proliferation when co-cultured 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 MDSCs-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 regimens 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 pre-operative 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 pre-operative 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 anti-cancer chemotherapeutic agents can modify the tumor microenvironment, and the therapeutic effects mediated by these agents are considerably dependent on the host immunological reaction (8, 9). Additionally, the complex interaction between tumor cells and other cellular
components of tumor microenvironment such as cancer associated fibroblasts (CAF) and myeloid cells has great impact on invasion, metastasis and acquiring of chemo-resistant phenotypes (10, 11). PDAC microenvironment constitutes of molecular and cellular components with inflammatory features, such as pancreatic stellate cells (PaSC) 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 (MDSCs) are heterogeneous populations of immune cells derived from progenitor cells in bone marrow, which accumulate in tumor microenvironment via various pathological 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 G-CSF) 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 MDSCs 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 intra-tumoral 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 present study, we show phenotypic and functional changes of monocytes under chemotherapy-treated human PDAC conditions. Human monocytes differentiated into HLA-DR^{low/negative} MDSC phenotype when cultured in conditioned medium of human PDAC cells. Moreover, HLA-DR^{low/negative} cells formation was enhanced when human monocytes were cultured in conditioned medium 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. Blockade of GM-CSF in the supernatants of PDAC cell culture with specific monoclonal antibodies resulted in recovery of T cell proliferation when co-cultured with monocytes stimulated with PDAC conditioned medium. Consistent with these results, we found that PDAC tumor tissues in chemotherapy-treated cancer patients
recruited more cells which express MDSC markers compared to non-treated 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 Bioscience). Both procedures were ethically approved by the committees in the Institutional review Board of Hokkaido University Hospital (No. 013-0389, 013-0390).

Human PDAC tissue samples

For tissue microarray (TMA), PDAC tissue samples were obtained from 99 resected PDAC in our institute between 1994 and 2005. TMA was constructed as described in our previous report (24). Patients without information about survival or broken and poor samples were omitted from analysis. Total 68 patients were subjected to analysis. The characteristics of patients for TMA study are summarized in supplementary table 1. 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 were evaluated in the comparison study (figure 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 2-3. Immunohistochemistry 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, MIAPaCa-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 conditioned medium used in monocyte culture, Capan-1 and PANC-1 cells were cultured in RPMI 1640 (WAKO) supplemented with 10% fetal bovine serum (Cell Culture Bioscience), 1% penicillin/streptomycin, 10mM HEPES, 1% L-glutamine, 1mM sodium pyruvate, 1%
non-essential amino acids (All from Life technologies), and 50μM 2-mercaptoethanol (WAKO) in accordance with optimizing conditions for monocytes.

In vitro human monocyte culture

To examine the effects of PDAC-derived factors on monocytes 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 0.2μm filter (Sartourius Stedim Biotech). To mimic clinical pharmacological settings in PDAC patients, gemcitabine (GEM, 1-30μM) or fluorouracil (5-FU, 10μg/ml) were applied at concentrations similar to that used in clinic (1-30μM). PDAC cells were pulsed with GEM or 5-FU for 60 minutes followed by wash for 5 times with sterilized PBS and change to fresh media. After 72 hours, supernatants were collected and passed through 0.2μm filter as described above. Human peripheral monocytes were purified from PBMC of healthy donors using CD14 positive selection by magnetic cell sorting systems according to manufacture’s protocols (Miltenyi Biotech) 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, 10μg/ml),
anti-human IL-6 (Clone 6708; R&D systems, 2μg/ml), or anti-human IL-8 (Clone 6217;
R&D systems, 2μg/ml).

Flow cytometry

Single cell suspensions were used for flow cytometry analysis after treatment with
Human FcR blocker (Miltenyi Biotech) or anti-mouse CD16/32 (BD Biosciences) and
staining with appropriate fluorescent antibodies according to manufacturer’s
instruction. 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 FACS canto II (BD Biosciences) and analysed
using FlowJo software V7.6.5

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,
cDNA products were used to amplify target genes using Power SYBR Green (Life Technologies) and specific primer (Supplementary Table 3). PCR reactions and data analysis were performed in a StepOne Real-time PCR system (Applied Biosystems), using the comparative C<sub>T</sub> method and the housekeeping gene GAPDH. Primers used in this study are as follows:

- **GAPDH** (Forward: 5'-AACAGCGACACCCACTCCTC-3' Reverse: 5'-ATACCAGGAAATGAGCTTGACAA-3'),
- **M-CSF** (Forward: 5'-GCCTGCGTCCGAACCTTCTA-3' Reverse: 5'-ACTGCTAGGGATGGCTTTGG-3'),
- **GM-CSF** (Forward: 5'-ATGGCTCCAGCAGTGCAAG-3' Reverse: 5'-CTGGCTCCCAGCAGTCAAAG-3'),
- **IL-6** (Forward: 5'-CTGCGCCAACACAGAAAATTA-3' Reverse: 5'-ATTGCATCTGCAACCTAC-3'),
- **IL-8** (Forward: 5'-ATGATGGCCAGCCACTACAA-3' Reverse: 5'-ACTGCTAGGGATGGCTTTGG-3'),
- **IL-1B** (Forward: 5'-ATCAGCTGAAGTGCTGGCAAC-3' Reverse: 5'-AGGAAGGCCTAAGGTCCACT-3'),
- **PTCS2** (Forward: 5'-AGGGCTTCAGCATAAGCGT-3' Reverse: 5'-GTTCCACCCGCAGTGCAAG-3'),
- **TNF** (Forward: 5'-CACAGTGCTGGCAAC-3' Reverse: 5'-AGGAAGGCCTAAGGTCCACT-3'),
- **VEGF-A** (Forward: 5'-CTACCTCCACCAGTGCAAG-3' Reverse: 5'-GCAGTAGCTGCGCTGATAGA-3'),
- **CXCL-12** (Forward: 5'-GCAGTAGCTGCGCTGATAGA-3')
5’-CTACAGATGCCCATGCCGAT-3’ Reverse: 5’-CAGCCGGGCTACAATCTGAA-3’),

SCF (Forward: 5’-AGCCAGCTCCCTTAGGAATGA-3’ Reverse: 5’-GAACCGTGGATGTCCACTT-3’),

TGF-B1 (Forward: 5’-TGCCCTTGTAAGACTTGGCTG-3’),

CCL-2 (Forward: 5’-CAGCAAGTGTCACCAAGAAGCTG-3’ Reverse: 5’-TGGAATCCTGAACCCACTTCTGC-3’),

NOS2 (Forward: 5’-TGGAATCCTGAACCCACTTCTGC-3’),

Cytokine measurement

Cytokines were measured using commercial ELISA kits according to the manufacturer’s instructions. The kits for GM-CSF and IL-8 were purchased from Biolegend. The kit for IL-6 was purchased from R&D systems. All measurements were performed using supernatants from three independent cell cultures.

Primers specificity was confirmed by peak melt curve before using. All experiments were performed in duplicate for each sample.
**Western blotting**

Total cell lysates were prepared using RIPA buffer supplemented with protease inhibitors aprotinin and PMSF. Protein samples were resolved using 10% SDS-PAGE and were then transferred to PVDF membrane (GE Healthcare). Membranes were probed with primary antibodies against target molecules followed by reaction with secondary antibodies conjugated to horseradish peroxidase (HRP) for appropriate incubation time. Antibodies against ERK, p-ERK, AKT and p-AKT were purchased from Cell Signaling; 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-κB luciferase reporter assay**

Promoter activities of NF-κB 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-κB 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 3μg/ml of anti-CD3 antibody (OKT3; eBioscience) and 1μg/ml of anti-CD28 antibody (CD28.2; Biolegend). Stimulated CD4⁺ or CD8⁺ T cells were then co-cultured with monocytes differentiated in the presence of tumor supernatants at the indicated conditions at different T cell / monocyte ratios. ³H-thymidine incorporations were counted after 72 hours culture.

**Immunohistochemical staining of formalin fixed paraffin embedded tissues (FFPE)**

Paraffin-embedded specimens were cut into thin slices and mounted on slide glass. 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: ab49755 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 4. 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’s *t*-test was used for comparison between groups. For mouse or human data, non-parametric statistics were applied in which Man-Whitney *U* test, Fisher’s exact test, or $\chi^2$ test were 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 cells-derived soluble factors influence human myeloid cells differentiation, we generated in vitro culture models using conditioned medium (CM) from 2 PDAC cell lines: Capan-1 and PANC-1 (Figure 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, while monocytes that were differentiated in the presence of Capan-1 or PANC-1 supernatants formed floating immature cells (Figure 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 the presence of PDAC supernatants compared to normal medium (Figure 1C and 1D). Mo-MDSCs suppress T cell immunity via nitric oxide synthase 2 (NOS2) or Arginase 1 (ARG1) (28,
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 (Figure 1E). Additionally, 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) (30) (Figure 1F). 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 if the differentiation patterns of monocytes are altered in chemotherapy-treated PDAC microenvironment. To do so, we established in vitro culture model using Capan-1 cell line treated with gemcitabine (GEM) or Fluorouracil (5-FU) (Figure 2A). Interestingly, after 6 days of culture, monocytes showed morphological changes when cultured in the supernatants of chemotherapy-treated PDAC cells, represented by increased diameters (Figure 2B and Supplementary Fig. S1) and formation of cytoplasmic vacuoles that were not observed in monocytes.
cultured in normal medium or normal PDAC supernatant (Figure 2C). These monocytes showed high forward and side scatter voltage signals in flowcytometry analysis, which was consistent with gross examination (Figure 2D). Additionally, the HLA-DR\textsubscript{low/negative} fraction was increased in monocytes differentiated in the supernatants of chemotherapy-treated PDAC cells (Figure 2D, E and Supplementary Fig. S1). These changes are consistent with the phenotype of HLA-DR\textsubscript{low/negative} immature monocytes that have been previously reported (27). To evaluate the immunosuppressive features of monocytes differentiated in GEM-treated PDAC CM, we analysed 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 GEM-treated PDAC CM (Figure 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 signalling 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 (33, 34). To identify factors in the supernatants of chemotherapy-treated PDAC cells responsible for monocytes differentiation into MDSCs, we investigated expression profiles of various cytokine and chemokine in Capan-1 or PANC-1 cell lines. Following stimulation with GEM or 5-FU, several cytokines and chemokines were upregulated in both cell lines (Figure 3A, B and Supplementary Fig. S2). In particular, the expression of GM-CSF, IL-6 and IL-8 was increased in the supernatants of chemotherapy-treated Capan-1 cells (Figure 3C, 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 GEM 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 signalling 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 GEM treatment enhances
the phosphorylation of ERK (Figure 3D) but not AKT (data not shown) in a
time-dependent manner. NF-κB is a major transcription factor which induces the
expression of inflammatory cytokines including GM-CSF (35, 36). Thus, we next
examined if GEM 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 (Figure 3E). These data indicate that chemotherapy
enhances the production of multiple inflammatory cytokines including GM-CSF by
amplifying the activation status of MAPK signalling pathway and NF-κB promoter
activities in PDAC cells.

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

The supernatants of chemotherapy-treated PDAC cells were enriched with GM-CSF,
and induced morphological 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^{low/negative} fractions (Figure
4A), and abolished the formation of cytoplasmic vacuoles that were observed in the case of GEM-treated Capan-1 CM (Figure 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 co-cultured these MDSCs with CD4+ or CD8+ 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+ or CD8+ T cells, which was further suppressed by MDSCs generated by GEM-treated Capan-1 CM (Figure 4C, D and Supplementary Fig. S4). Importantly, the neutralization of GM-CSF in GEM-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 (Figure 4C, 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
(Figure 5A). Next, immunohistochemistry staining was used to examine protein levels
of GM-CSF in PDAC tissues of 68 resected primary tumors by tissue microarray.
PDAC tissues also showed variety in GM-CSF expression (Figure 5B). The intensity
of GM-CSF staining was classified as high or low as described in material and
methods (Figure 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 (Figure 5D). These data suggest that GM-CSF, a MDSC
inducing cytokine, is generally expressed in human PDAC, and correlate with poor
prognosis.

Finally, to examine the impact of tumor microenvironment on MDSCs differentiation
in human PDAC tissues under chemotherapeutic conditions, we assessed MDSC marker expression in tumor-infiltrating myeloid cells in PDAC patients treated with pre-operative chemotherapy including GEM in our institute (Supplementary table 3). We found that tumor-infiltrating CD14$^+$ cells in PDAC patients treated with pre-operative chemotherapy show no or weak expression of HLA-DR compared to patients without pre-operative chemotherapy treatment (Figure 5E and F). These data indicate that CD14$^+$HLA-DR$^-$ cells constitute a dominant fraction in PDAC tissues following chemotherapy. Furthermore, we investigated the expression of CD66b, a marker of granulocytic MDSC (G-MDSC) (38), and found that the frequencies of tumor-infiltrating CD66b$^+$ cells were significantly higher in PDAC patients after chemotherapy treatment (Figure 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, in 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 favourable 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 pathological patterns of chemotherapy-treated PDAC. For example, recent pre-operative 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).

Additionally, 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 analysed 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 which 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 to
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 anti-tumor 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). Additionally, 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-κB signalling pathway as a consequent of chemotherapy-induced DNA-damage response (DDR) (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 (DC). Based on 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 MDSC. One
possible mechanism of these conflict roles of GM-CSF is the enrichment of PDAC
microenvironment with DAMPs (Danger-associated molecular patterns) after
chemotherapy treatment. DAMPs are released from tumor cells killed by anticancer
cytotoxic agents, and signalling 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 (Figure 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 statement

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Hiraku Shida for technical assistance in making slides for immunochemical staining, and Tomohiro Shimizu and Katsuji Marukawa for kindly supporting in cytospin techniques. Finally, the authors would like to express heartful thanks to the later Dr. Masaki Miyamoto for the guidance and mentorship of this research.
References


myeloid cells antagonize senescence in cancer. Nature advance online publication.


immunosuppressive strength among myeloid-derived suppressor cell subsets is determined by GM-CSF. *European Journal of Immunology* 40: 22-35.


Figure Legend

Figure 1 Supernatants of human PDAC cell culture induce the differentiation of monocytes into MDSCs

(A) A scheme of culture protocol used to study the effects of PDAC-derived factors on monocytes differentiation. Human peripheral CD14+ monocytes were purified from healthy donor and cultured in PDAC CM for 6 days. (B) Representative photomicrographs of monocytes cultured for 6 days in normal medium, Capan-1 CM or PANC-1 CM. Monocytes differentiate into spindle macrophage-like cells when cultured in normal medium, whereas the supernatants of PDAC cells induce monocytes differentiation into circular immature cells. Scale bars: 100μm. (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 was 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. Capan-1 or PANC-1 CM-treated monocytes show high levels of NOS2 and ARG1 compared to control. (F) Flow
cytometry analysis of CD11b, CD15, CCR2 and CXCR1 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 CXCR1. Flowcytometry results are shown as representative multiple independent experiments. \(^* P < 0.05; \ ^{**} P < 0.01.

Figure 2: Supernatants of chemotherapy-treated PDAC cells induce morphological 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 GEM (1μM or 30μM) or 5-FU (10μg/ml) for 1 hour, followed by careful wash with sterilized PBS, and change into fresh medium. Conditioned medium was collected after 72 hour and applied to human peripheral CD14\(^+\) monocytes as described above.

(B) Morphological changes in monocytes cultured in GEM-treated PDAC CM at day 6. These cells were larger in size than monocytes cultured in PBS-treated PDAC CM. Scale pars: 100μm. (C) May Giemsa staining showed unique cytoplasmic vacuoles in monocytes cultured in GEM-treated PDAC CM (red arrows) but not PBS-treated PDAC CM or normal medium. Scale bars: 20μm (D and E) Flowcytometry analysis shows high forward and side scatter voltage signals (upper panel) and increased
frequencies of HLA-DR\textsuperscript{low/negative} fraction (lower panel) in monocytes cultured in GEM-treated PDAC CM compared to PBS-treated PDAC CM. \( (n=3\) donors). \( (F) \)

Enhanced expression of NOS2 in monocytes cultured in the supernatants of GEM-treated Capan-1 cells. Data are shown as representative of 2 independent experiments. \( \ast P < 0.05; \ast \ast P < 0.01. \)

\textbf{Figure 3} Chemotherapy treatment amplifies the expression of multiple MDSCs-inducing cytokines in PDAC cells via MAPK pathway-mediated signal

(A) and (B) Quantitative RT-PCR analysis for various cytokines and chemokines in PBS or GEM-treated (A) or 5-FU-treated (B) Capan-1 cells after 72 hours of stimulation. Data from PBS-treated cells were set as 1. Data is shown as representative of 3 independent experiments. (C) ELISA measurement of GM-CSF in the supernatants of PBS or chemotherapy-treaded Capan-1 cells after 72 hours of stimulation. GM-CSF production is enhanced after chemotherapy treatment in a dose-dependent manner. Data is shown as representative of 2 independent experiments. (D) Western blotting of p-ERK or total ERK, p-AKT or total AKT, and \( \beta \)-Actin of PBS or GEM-treated Capan-1 cells stimulated for the indicated time. GEM enhances the phosphorylation of ERK in a time-dependent manner. Similar results were obtained from multiple independent experiments. \( (E) \) A time course of luciferase
activity of Nfκb promoter-luciferase reporter plasmid in Capan-1 cells stimulated with GEM (upper panel) or f-FU (lower panel). Data is shown as representative of 2 independent experiments. * P < 0.05; ** P < 0.01; *** P < 0.001.

**Figure 4** Blockade of GM-CSF contributes to the reversal of morphological and phenotypic changes induced in monocytes by chemotherapy-treated PDAC CM

(A) Flowcytometry analysis shows decreased forward and side scatter voltage signals (upper panel) and decreased frequencies of HLA-DR^{low/negative} fraction (lower panel) in monocytes cultured in GEM-treated PDAC CM after depletion of GM-CSF (anti-GM-CSF: 10μg/ml). (B) Microscopic examination and May Giemsa staining showed decrease in cell size (upper panel) and disappearance of cytoplasmic vacuoles (lower panel) that were observed in GEM-treated PDAC CM after treatment with anti-GM-CSF. Scale bars: 100μm for photomicrographs and 20μm for May Giemsa staining. (C) Photomicrographs of T cell aggregate. MDSCs were co-cultured 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: 10μm. (D) T cell proliferation assay. MDSCs were co-cultured with autologous CD4^+ T cells stimulated with anti-CD3/28 for 72 hours at the indicated ratio, and T cell proliferation was measured by H^3 thymidine uptake. Neutralization of GM-CSF in
GEM-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.

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 pre-operative 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 is shown as representative of 3 independent experiments. (B) Immunohistochemistry staining of GM-CSF in PDAC region or normal region of pancreatic tissues from PDAC patients. Scale bar: 100μm. (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) Immunohistochemistry staining of CD14 and HLA-DR in pancreatic tissues of PDAC patients before or after treatment with pre-operative chemotherapy. Scale bar: 100μm. (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 pre-operative chemotherapy. (G) Immunohistochemistry staining of CD66b in pancreatic tissues of PDAC patients before or after treatment with pre-operative chemotherapy. Scale bar: 100μm. (H) Frequencies of CD66b$^+$ in pancreatic tissues of PDAC patients before or after pre-operative chemotherapy. For F and H, bars indicate the median value and the box encompasses the 25$^{th}$ and 75$^{th}$ percentiles. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

**Figure 6 Mechanism of chemotherapy-mediated induction of MDSCs**

A scheme of mechanism by which chemotherapy induces MDSC formation in PDAC microenvironment is shown. Chemotherapy induces activation of MAPK signal pathway and NF-κB promoter activities leading to enhancement in GM-CSF production which in turn enhance the differentiation of monocytes into MDSCs. Anti-GM-CSF Ab may offer a promising tool to block monocytes differentiation into MDSCs, and thus help the recovery of effective antitumor T cell response.
Supplementary Figure Legend

Supplementary figure.1 5-FU-treated Capan-1 supernatants induce the differentiation of monocytes into MDSCs

Flow cytometry analysis of FSC, SSC and HLA-DR expression in monocytes cultured in normal medium (control), 5-FU- treated Capan-1 CM.

Supplementary figure.2 Gemcitabine amplifies the expression of multiple MDSCs-inducing cytokines including GM-CSF in PANC-1 cell line

Quantitative RT-PCR analysis of various cytokines and chemokines in PBS- or GEM-treated PANC-1 cells after 72 hours of stimulation. Data from PBS-treated cells were set as 1. Data is shown as representative of 3 independent experiments. "P < 0.05; ""P < 0.01; """"P < 0.001.

Supplementary figure.3 Gemcitabine amplifies the expression of IL-6 or IL8 in Capan-1 cells

ELISA measurement of IL-6 and IL-8 in the supernatants of PBS- or GEM-treaded Capan-1 cells after 72 hours of stimulation. Both cytokine productions were enhanced after GEM treatment in a dose-dependent manner. Data is shown as representative of 2 independent experiments.
Supplementary figure.4 Blockade of GM-CSF contributes to the recovery of CD8$^+$ T cells proliferation when cultured with monocytes stimulated with chemotherapy-treated PDAC CM

(A) Photomicrographs of T cell aggregate. CD8$^+$ T cells stimulated with anti-CD3/28 were co-cultured with autologous MDSCs for 72 hours at the indicated ratio. Representative of two independent experiments. Scale bar: 10μm. (B) CD8$^+$ T cell proliferation was measured by $^3$H thymidine uptake. Neutralization of GM-CSF in GEM-treated Capan-1 CM contribute to the recovery of CD8$^+$ T cell proliferation. Data are shown as representative of two independent experiments. * $P < 0.05; ** P < 0.01; *** P < 0.001.

Supplementary figure.5 No significant difference in the frequencies of CD68$^+$ macrophages in cancer patients after treatment with pre-operative chemotherapy

Frequencies of CD68$^+$ cells in pancreatic tissues of PDAC patients before or after pre-operative chemotherapy.
Figure 1

A) Diagram illustrating the process of generating conditioned medium. PDAC cell line is cultured, conditioned medium is generated, and CD14+ peripheral monocytes are cultured in the conditioned medium for 6 days, followed by gene expression and protein analysis.

B) Images of cells in different media conditions: Media, Capan-1 CM, and PANC-1 CM.

C) Flow cytometry analysis showing CD33, CD14, CD11b, CD15, CCR2, and CX3CR1 expression in Control, Capan-1 CM, and PANC-1 CM conditions. The percentage of CD14+ cells is shown as 1%, 39%, and 27% for respective conditions.

D) Graph showing HLA-DR relative MFI for Control, Capan-1 CM, and PANC-1 CM. The bar graph shows a significant increase in HLA-DR expression in both conditioned media compared to control.

E) Gene expression analysis showing NOS2 and ARG1 expression in Control, Capan-1 CM, and PANC-1 CM conditions.

F) Protein expression analysis showing CD11b, CD15, CCR2, and CX3CR1 expression in Capan-1 CM and PANC-1 CM conditions, with isotype and conditioned medium conditions compared.
Figure 2
Figure 3
Figure 4

A) Capan-1 CM

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>GEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-GM-CSF</td>
<td>(-)</td>
<td>(-)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>FSC</th>
<th>SSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>18%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>73%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>62%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B) GEM-treated Capan-1 CM + α-GM-CSF

C) Media vs. GEM-treated Capan-1 CM

<table>
<thead>
<tr>
<th></th>
<th>Media Control</th>
<th>α-GM-CSF (-)</th>
<th>α-GM-CSF (+)</th>
</tr>
</thead>
</table>

D) CPM (×10³)

<table>
<thead>
<tr>
<th>T-cell/MDSC</th>
<th>4:1</th>
<th>2:1</th>
<th>1:1</th>
<th>1:0</th>
</tr>
</thead>
<tbody>
<tr>
<td>αCD3/28</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4
GM-CSF (normalized to GAPDH)

B

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>2+</td>
<td>0</td>
</tr>
<tr>
<td>1+</td>
<td>low</td>
</tr>
<tr>
<td>0</td>
<td>high</td>
</tr>
</tbody>
</table>


C

- 0: GM-CSF low (29%)
- 1+: GM-CSF moderate (43%)
- 2+: GM-CSF high (71%)

D

*P=0.02998, log-rank test

GM-CSF low (n=20)
Median survival: 25.7 months

GM-CSF high (n=48)
Median survival: 13.9 months

E

Pre-operative chemotherapy
(+)
Case1  Case2  Case3

CD14/HLA-DR

G

Pre-operative chemotherapy
(+)
Case1  Case2  Case3

CD66b

F

CD14 HLA-DR 
+ -

H

CD66b 
+ -

Figure 5
Chemotherapy

PDAC cancer cell

MAPK
NF-κB

Multiple inflammatory factors (GM-CSF, etc.)

Monocyte

MDSC

PDAC progression ↑
Antitumor immunity ↓

Anti-GM-CSF

T cell immunity ↑

Figure 6