Combination of gemcitabine and anti-PD-1 antibody enhances the anti-cancer effect of M1 macrophages and the Th1 response in a murine model of pancreatic cancer liver metastasis

SUPPLEMENTARY METHODS

Histological and immunohistochemical analyses

Immunohistochemistry and histopathology were performed on paraffin-embedded tissue sections and frozen tissue sections. Frozen sections were fixed with 4% formaldehyde; followed by incubation with the following primary Abs: rat anti-mouse CD8a (clone: 53-6.7), rat anti-mouse CD4 (clone: H129.19), rat anti-mouse CD11b (clone: M1/70) (BD Pharmingen), anti-CD279 (PD-1; clone: 29F.1A12; BioLegend, San Diego, CA), rat anti-mouse CD274/PD-L1 (clone: MIH6; LifeSpan BioSciences, Seattle, WA), and rat anti-mouse CD86 (clone: GL-1; Abcam plc., Cambridge, UK). Paraffin sections were incubated overnight at 4°C with rat anti-mouse F4/80 (clone: BM8; Thermo Fisher Scientific, Waltham, MA), rat anti-mouse Ly6C (clone: ER-MP20; Abcam plc.), rat anti-mouse Ly6G (clone: RB6-8C5; Abcam plc.), or rabbit anti-mouse CD206 (polyclonal; Abcam plc.). Following incubation with the primary Ab, the samples were incubated with a secondary Ab conjugated to a horseradish peroxidase-labeled dextran polymer (Histofine® Simple Stain™ Mouse MAX PO, Rat or Rabbit; Nichirei Corporation, Tokyo, Japan). After adding the diaminobenzidine substrate solution (DAB®; Dako

ChemMate EnVision Kit/HRP; Dako, Kyoto, Japan), the sections were counterstained with hematoxylin. The DAB+ area was quantified by using the color deconvolution tool within ImageJ software.[1]

DNA microarray analysis

PDAC liver metastasis model mice received treatments twice a week from day 7 to day 33. Peripheral blood was collected on day 34 and RNA was isolated by using a Mouse RiboPure™-Blood RNA Isolation Kit (Invitrogen). The DNA microarray process was performed as described previously.[2] BRB-ArrayTools v.4.6.0 (http://linus.nci.nih.gov/BRB-ArrayTools.html) was used for DNA microarray analysis; quantile normalization was applied and only genes with a p-value of log-ratio variation > 0.01 were included for the unsupervised dendrogram sample clustering. Additionally, class comparison analysis was performed and the differentially expressed genes among classes were obtained (parametric p-value < 0.05); the upregulated genes were used for enrichment analysis by pathway maps (p-value < 0.05; MetaCore; Clarivate Analytics, Philadelphia, PA).

RNA isolation and quantitative real-time PCR

RNA was isolated from macrophages or tumor-infiltrating inflammatory cells (TICs) using an Isospin Cell & Tissue RNA Kit (Nippon Gene, Tokyo, Japan). Gene

expression was analyzed by quantitative real-time PCR (qRT-PCR) with the QuantStudio 12K Flex RealTime PCR system (Applied Biosystems, Foster City, CA). For cDNA synthesis, 100 ng RNA was reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. PCR was conducted with the cDNA mixed with qPCR MasterMix Plus® (Eurogentec, Seraing, Belgium) and the following hydrolyzed Taqman® Gene Expression Assay probes: *Il6*, *Il12a*, *Il12b*, *Il1b*, *Tnf*, *Arg1*, *Il10*, *Tgfb1*, *Mmp9*, *Cxcl10*, *Ccl2*, *Pdcd1*, and *Prf1*. Relative expression levels were calculated with *Gapdh* as a reference gene using the $2^{-\Delta\Delta}$Ct method.

Flow cytometry

Isolated TICs were suspended in flow cytometry (FCM) buffer (PBS supplemented with 2% bovine serum albumin; Sigma-Aldrich) and stained with Abs for surface markers. After surface staining, intracellular staining was performed by using an Inside Stain Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) as per the protocol. All samples were processed with FACS ARIA II® or BD Accuri™ C6 cytometers (BD Biosciences). The data were analyzed using FlowJo™ software (v.10.4.1; Tree Star, Inc., Ashland, OR).

Abs used for FCM

The following Abs were used for surface marker evaluation: PerCP-conjugated anti-mouse CD8a (clone: 53-6.7), APC-Cy7-conjugated anti-mouse CD11b (clone: M1/70), PerCP-Cy5.5-conjugated anti-mouse Ly-6C (clone: AL-21), PE-conjugated anti-mouse Ly-6G (clone: 1A8), PE-conjugated anti-mouse CD25 (clone: 3C7; BD Pharmingen); VioGreen-conjugated anti-mouse CD4 (clone: GK1.5; Miltenyi Biotec); and FITC-conjugated anti-mouse F4/80 (clone: BM8), Alexa Fluor® 647-conjugated anti-mouse F4/80 (clone: BM8), and PE-conjugated anti-CD206 (clone: C068C2; BioLegend). After surface staining, the following Abs were used for intracellular staining: FITC-conjugated anti-mouse FoxP3 (clone: SB168a; SouthernBiotech, Birmingham, AL); and Alexa Fluor® 647-conjugated IL-10 (clone: JES5-16E3), Alexa Fluor® 647-conjugated anti-mouse Tbet (clone: 4B10), and Alexa Fluor® 700 anti-mouse IFN-γ (clone: XMG1.2; BioLegend). Macrophages used for the *in vitro* polarization test were labeled with the following Abs: FITC-conjugated anti-CD11b (clone: M1/70) and APC-conjugated anti-CD86 (clone: GL1; BD Pharmingen); and PE-conjugated anti-CD206 (clone: C068C2; BioLegend).

IFN-γ secretion assay

TICs were isolated from the murine PDAC liver metastasis model on day 30 after receiving a single dose of the treatments on day 28. TICs were stimulated for the first 2 days with recombinant human IL-2 (Wako Pure Chemical Industries, Osaka,

Japan) at a concentration of 100 U/mL in RPMI 1640 medium/10% FBS together with beads with anti-CD3 Ab and anti-CD28 Ab covalently bound to the surface (Dynabeads™ Mouse T-Activator CD3/CD28; Thermo Fisher Scientific), followed by 16 h of further co-culture with PAN02 cells with the same culture conditions. Next, a Mouse IFN-γ Secretion Assay and Detection Kit (Miltenyi Biotec) was used as per the protocol to stain IFN-γ-secreting CD8+ T cells; the positive cells were quantified by FCM.

Arginase assay

TICs were isolated from the murine PDAC liver metastasis model on day 30 after receiving a single dose of the treatments on day 28. They were stained with FITC-conjugated anti-CD11b (clone: M1/70) and PerCP-Vio700™-conjugated anti-Ly6G (clone: 1A8) Abs (Miltenyi Biotec) and then the CD11b+Ly6G+ cells were sorted with a FACS ARIA II® cytometer (BD Biosciences). The sorted cells were used directly for the test using a QuantiChrom™ Arginase Assay Kit (BioAssay Systems, Hayward, CA) as per the manufacturer's protocol: the cells were lysed, and after centrifugation, the supernatants were used for the urea reaction. A Tecan Sunrise™ microplate reader (Tecan Group Ltd., Männedorf, Switzerland) was used for optical density reading at 430 nm; arginase activity was calculated as described in the protocol.

In vitro macrophage polarization test

Splenocytes were isolated from liver metastasis PDAC mice that received no treatment, anti-PD-1 Ab, GEM, or GEM plus anti-PD-1 Ab. The cells were activated by co-culture with X-ray-irradiated (35 Gy) PAN02 cells for 7 days; the culture medium was supplemented with murine interleukin 2 (IL-2; 100 U/mL) and anti-biotin MACSiBead™ particles pre-loaded with biotinylated anti-CD3e and anti-CD28 Abs (T Cell Activation/Expansion Kit; Miltenyi Biotec). Meanwhile, bone marrow-derived macrophages (BMDMs) were generated from bone marrow cells of C57BL/6J mice. Briefly, bone marrow cells were cultured in DMEM supplemented with 10% heat inactivated FBS and P/S, and then stimulated with 25 ng/mL recombinant murine macrophage colony-stimulating factor (R&D Systems, Inc., Minneapolis, MN) until complete macrophage differentiation.[3] The BMDMs were incubated with activated splenocytes at a macrophage/splenocyte ratio of 1:20 for 48 h, and then FCM and qRT-PCR analysis of macrophages were performed.

Statistical analysis

Prism 8 software (GraphPad Software, San Diego, CA) was used for statistical analyses; precisely, one-way analysis of variance (ANOVA) followed by Tukey's HSD *post hoc* test. Kaplan–Meier curves were generated to estimate overall survival, while for group comparisons, the log-rank test was performed. All

of the other tests were verified by Student's t test. Statistical significance was determined to be $p < 0.05$.

SUPPLEMENTARY REFERENCES

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3. Assouvie A, Daley-Bauer LP, Rousselet G. Growing Murine Bone Marrow-Derived Macrophages. *Methods Mol Biol* 2018;1784:29-33.

4. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 1958;53:457-481.

SUPPLEMENTARY FIGURES

Supplementary Figure S1. Reproducibility validation and disease progression of PDAC liver metastasis established using the PAN02 cell line. (A) Macroscopic images of the liver, spleen, pancreas, and lung isolated from PDAC liver metastasis mice at 7 (n = 3) and 34 days (n = 20) after injection of PAN02 cells. (B)

Macroscopic images of mice at necropsy showing disease progression at day 34 (n = 30). (C) SPECT/CT images of F-18 fluorodeoxyglucose in the PDAC liver metastasis model at 24 days after injection of PAN02 cells showing tumor progression only in the liver (image from a representative scanned mouse out of 3 PDAC mice). The bar indicates the intensities as percent of injected dose per mL (%ID/mL); the color scale ranges from 4% to 12% ID/mL.

Supplementary Figure S2. Immunohistochemical analysis of tumors for PD-1+ cells. Magnification: ×200; bars: 100 μm. Quantification of cell infiltration by using ImageJ (each area analyzed = 1.576 mm^2, 3 different areas were analyzed for each sample). White bar: no treatment; light gray bar: anti-PD-1 Ab; dark gray bar: GEM; black bar: GEM plus anti-PD-1 Ab; bars represent mean ± SEM; one-way ANOVA followed by Tukey's HSD *post hoc* test was performed as statistical analysis; ****$p < 0.0001$.

Supplementary Figure S3. (A–E) FCM analysis of lymphoid-lineage cells within TICs isolated from tumors of PDAC liver metastasis mice treated with: no treatment, anti-PD-1 Ab, GEM, or GEM plus anti-PD-1 Ab on day 28. A representative scatterplot is depicted for each condition and lymphoid-lineage cell type: (A) CD4+ within TICs, (B) CD8a+ within TICs, (C) Tbet+IFN-γ+ (Th1) within CD4+ TICs, (D) IFN-γ+ within CD8+ TICs, and (E) CD25+FoxP3+ (regulatory T

cells) within CD4+ TICs followed by scatterplots of IL-10+ cells within regulatory T cells.

Supplementary Figure S4. Treatment effect on IFN-γ secretion capabilities of CD8+ TICs. PDAC liver metastasis mice received a single dose of the indicated treatment on day 28, and TICs were isolated on day 30, followed by 2 days of stimulation with IL-2 and anti-CD3/CD28 Abs conjugated to beads; after a further 16 h co-culture with PAN02 cells, FCM was performed for the quantification of IFN-γ secretion within CD8+ cells. (A) Representative scatterplots of IFN-γ+ cells within the CD8+ fraction. (B) IFN-γ+ fraction within CD8+ cells, (n = 6); bars represent mean ± SEM; Kruskal–Wallis rank sum test followed by the Conover *post hoc* test was performed as statistical analysis.

Supplementary Figure S5. Treatment effect on G-MDSCs and their suppressive activity. PDAC liver metastasis mice received a single dose of the indicated treatment on day 28, and TICs were isolated on day 30, followed by FCM analysis and cell sorting of CD11b+Ly6G+ G-MDSCs for the arginase activity assay; n = 5. (A) Representative scatterplots of the CD11b+Ly6G+ fraction within TICs. (B) CD11b+Ly6G+ fraction within TICs. (C) Arginase activity of sorted G-MDSCs (n = 5; 350,000 CD11b+Ly6G+ TICs). (B, C) Bars represent mean ± SEM; one-way

ANOVA followed by Tukey's HSD *post hoc* test was performed as statistical analysis; **p < 0.01, ***p < 0.001.

Supplementary Figure S6. (A, B) FCM analysis of myeloid-lineage cells within TICs isolated from tumors of PDAC liver metastasis mice treated with: no treatment, anti-PD-1 Ab, GEM, or GEM plus anti-PD-1 Ab on day 28. A representative scatterplot is depicted for each condition and myeloid-lineage cell type. (A) Ly6C+Ly6G- inflammatory monocytes (IMs) within CD11b+F4/80+ TICs. (B) M1 (CD206-) and M2 (CD206+) macrophages within CD11b+F4/80^{high} TICs.

Supplementary Figure S7. Unsupervised cluster analysis of gene expression in peripheral blood cells by DNA microarray. The PDAC liver metastasis mice received treatment 8 times, twice a week from day 7 to day 33: GEM plus anti-PD-1 Ab (n = 3), GEM (n = 3), anti-PD-1 Ab (n = 4), or no treatment (n = 3). On day 34, whole peripheral blood was collected and total RNA was isolated for gene expression analysis by DNA microarray. (A) All treatment conditions (5,710 filtered genes). (B) GEM treatment vs. no treatment (5,073 filtered genes). (C) GEM plus anti-PD-1 Ab vs. no treatment (4,194 filtered genes). (D) anti-PD-1 Ab vs. no treatment (4,587 filtered genes). (E) GEM treatment vs. GEM plus anti-PD-1 Ab (4,891 filtered genes).

SUPPLEMENTARY TABLES

Supplementary Table S1. Kaplan-Meier survival probability estimates for each time period.

No Treatment				
Interval (days)	At risk	Died	Survived	Kaplan-Meier survival probability estimate[a]
0-14	7	0	7	1.00
15-28	7	0	7	1.00
29-42	7	7	0	0.00
43-56	0	0	0	0.00
57-70	0	0	0	0.00
71-84	0	0	0	0.00
Anti-PD-1 Ab				
Interval (days)	At risk	Died	Survived	Kaplan-Meier survival probability estimate[a]
0-14	12	0	12	1.00
15-28	12	0	12	1.00
29-42	12	12	0	0.00
43-56	0	0	0	0.00
57-70	0	0	0	0.00
71-84	0	0	0	0.00
GEM				
Interval (days)	At risk	Died	Survived	Kaplan-Meier survival probability estimate[a]
0-14	7	0	7	1.00
15-28	7	0	7	1.00
29-42	7	0	7	1.00
43-56	7	4	3	0.43
57-70	3	3	0	0.00
71-84	0	0	0	0.00
GEM plus aPD-1 Ab				
Interval (days)	At risk	Died	Survived	Kaplan-Meier survival probability estimate[a]
0-14	6	0	6	1.00
15-28	6	0	6	1.00
29-42	6	0	6	1.00
43-56	6	1	5	0.83
57-70	5	4	1	0.17
71-84	1	1	0	0.00
GEM plus aPD-1 Ab plus aCD8 Ab				
Interval (days)	At risk	Died	Survived	Kaplan-Meier survival probability estimate[a]
0-14	7	0	7	1.00
15-28	7	0	7	1.00
29-42	7	0	7	1.00
43-56	7	3	4	0.57
57-70	4	4	0	0.00
71-84	0	0	0	0.00

[a] The Kaplan-Meier product-limit estimator was used for the calculation of the survival probability estimate.[4]