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Impact of combination therapy with anti-PD-1 blockade and a STAT3 inhibitor on the tumor-infiltrating lymphocyte status

Tadashi Ashizawa[a], Akira Iizuka[a], Chie Maeda[a], Emiko Tanaka[a], Ryota Kondou[a], Haruo Miyata[a], Takashi Sugino[b], Takuya Kawata[b], Shoichi Deguchi[c], Koichi Mitsuya[c], Nakamasa Hayashi[c], Akira Asai[e], Mamoru Ito[f], Ken Yamaguchi[d], Yasuto Akiyama[a,*]

[a] Immunotherapy Division, Shizuoka Cancer Center Research Institute, 1007 Shimonagakubo, Nagaizumi-cho, Sunto-gun, Shizuoka 411-8777, Japan
[b] Division of Pathology, 1007 Shimonagakubo, Nagaizumi-cho, Sunto-gun, Shizuoka 411-8777, Japan
[c] Division of Neurosurgery, 1007 Shimonagakubo, Nagaizumi-cho, Sunto-gun, Shizuoka 411-8777, Japan
[d] Office of the president, Shizuoka Cancer Center Hospital, 1007 Shimonagakubo, Nagaizumi-cho, Sunto-gun, Shizuoka 411-8777, Japan
[e] Graduate School of Pharmaceutical Sciences, University of Shizuoka, Yada 52-1, Suruga-ku, Shizuoka, 422-8526, Japan
[f] Research Division, Central Institute for Experimental Animals, Kawasaki-ku, Kawasaki, Kanagawa 210-0821, Japan

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ABSTRACT

Recently, clinical studies using anti-immune checkpoint molecule antibodies have been successful in solid tumors, such as melanoma and non-small cell lung cancers. However, pancreatic cancers are still intractable and difficult to treat once recurrence or metastasis occurs; thus, novel combined use of immune checkpoint blockade (ICB) with molecular targeted drugs is considered a therapeutic option.

Previously, we developed a novel humanized MHC-double knockout (dKO) NOG mouse model and demonstrated that an anti-PD-1 antibody or a STAT3 inhibitor showed anti-tumor effects through an immunological mechanism.

In the current study, using a humanized mouse model, we aimed to develop a combination therapy with an anti-PD-1 antibody and a STAT3 inhibitor (STX-0119) for use *in vivo* against pancreatic cancer. In an *in vitro* investigation, STX-0119 showed weak to moderate cytotoxic activity against several pancreatic cancer cell lines, which exhibited activated pSTAT3 and weak PD-L1 expression.

However, unexpectedly, an *in vivo* study indicated that the combination of the anti-PD-1 antibody with STX-0119 remarkably reduced the anti-tumor effect and TIL numbers despite the effective anti-tumor activity against pancreatic cancer was produced individually by STX-0119 and the anti-PD-1 antibody.

These results suggested that the combination of an anti-PD-1 antibody with specific signal inhibiting drugs should be carefully evaluated to avoid unexpected side effects, and such studies might contribute to the development of an effective combination regimen of ICB with cancer-targeting drugs such as tyrosine kinase inhibitors (TKIs).

1. Introduction

Pancreatic cancer is an intractable cancer that has a very poor prognosis with a 5-year survival rate of 7% [1]. Resectable cases comprise only 20% and even after macroscopically curative resection, most patients tend to have recurrence and distant metastasis, resulting in a low survival rate (5-year survival rate up to 25%) [2,3].

For pancreatic cancer patients that undergo potential curative resection, it is recommended that gemcitabine or S-1, an oral fluoropyrimidine derivative, is given as an adjuvant chemotherapy after surgery. Importantly, adjuvant chemotherapy with S-1 showed significantly better 5-year overall survival than adjuvant chemotherapy with gemcitabine in the phase 3 randomized JASPAC 01 clinical trial [4].

In addition, for locally advanced pancreatic cancers combination chemotherapies, such as gemcitabine plus nab-paclitaxel and

Abbreviations: ICB, immune checkpoint blockade; TME, tumor microenvironment; TIL, tumor-infiltrating lymphocyte; MHC, major histocompatibility complex; NOG, NOD/Shi-*scid*-IL2rγ^{null}; PBMC, peripheral blood mononuclear cell; MDSC, myeloid-derived suppressor cell; TAM, tumor-associated macrophages; TKI, tyrosine kinase inhibitor

* Corresponding author.
E-mail address: y.akiyama@scchr.jp (Y. Akiyama).

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Table 1
Effect of STX-0119 on the proliferation of pancreatic cancer cell lines.

Cell lines	STX-0119 (IC50, μM)	WP1066 (IC50, μM)
AsPC-1	43.1	4.3
BxPC-3	49.8	3.6
Capan-1	11.3	0.8
Capan-2	31.2	4.9
FA-6	8.4	1.3
KP-1N	39.5	3.5
KP-2	11.7	3.2
KP-3	16.4	3.6
MIAPaCa-2	9.6	2.0
PANC-1	37.7	5.2
PSN-1	11.1	3.3
SUIT-4	23.8	4.2
YPK-1	24.2	1.6
YPK-2	19.4	2.5

FOLFIRINOX (fluorouracil, leucovorin, irinotecan and oxaliplatin), are applicable therapeutic regimens [5,6]. However, with the aim of developing a curative regimen, a novel therapeutic regimen to obtain long-term and durable cancer control needs to be investigated.

With regard to cancer immunotherapy, since the development of immune checkpoint blockade (ICB) therapy, several types of solid cancer have been treated with ICB, which contributed to the epoch-making success of the cancer immunotherapy field [7,8].

However, in pancreatic cancer patients, obvious therapeutic efficacy for anti-programmed death (PD)-1/anti-programmed death ligand 1 (PD-L1) antibodies has not yet been reported [9,10].

With advances in the study of the tumor microenvironment (TME), several mechanisms responsible for immunosuppression in the tumor have been elucidated, such as tumorigenic signaling activation through driver mutations, immunosuppressive cytokines and immune cell accumulation, cancer-associated fibroblasts (CAF) generation and cancer-favoring metabolic skewing [11–14]. In particular the signals that activate transforming growth factor (TGF)-β and signal transducer and activator of transcription (STAT)3 pathways are representative candidates of possible factors involved in tumor immunosuppression and resistance to chemo-radiation [15–19]. Previously, we reported the promotive effect of STX-0119 on tumor-infiltrating lymphocyte (TIL) accumulation in the glioma tumors using a humanized major histocompatibility complex (MHC)-double knockout (dKO) NOD/Shi-*scid*-IL2rγ^{null} (NOG) mouse model [20].

In the present study, with the aim of developing efficient combination therapy with an anti-PD-1 antibody and a STAT3 inhibitor, we investigated the efficacy of a combination therapy with an anti-PD-1 antibody and STX-0119 at a preclinical level using a humanized MHC-dKO NOG mouse model.

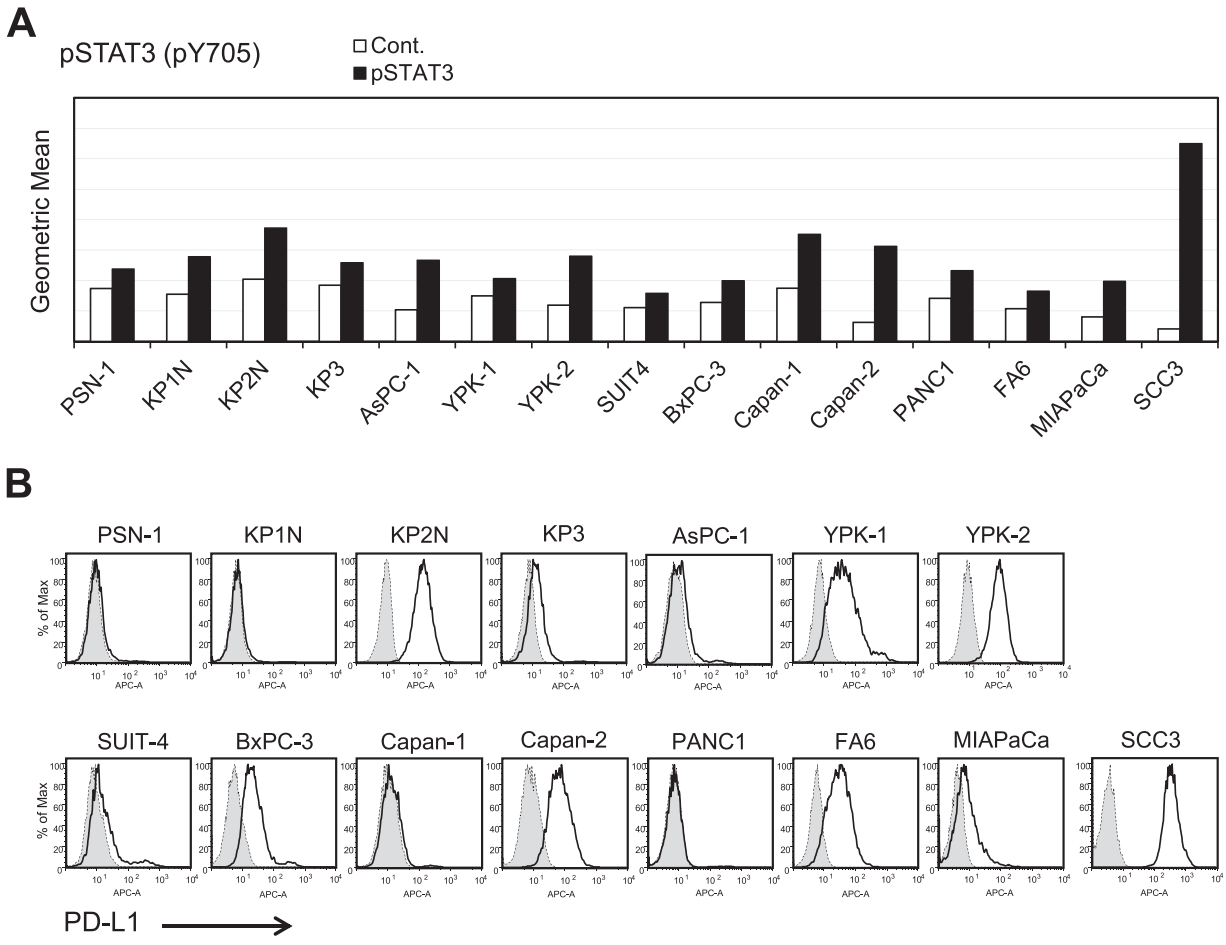
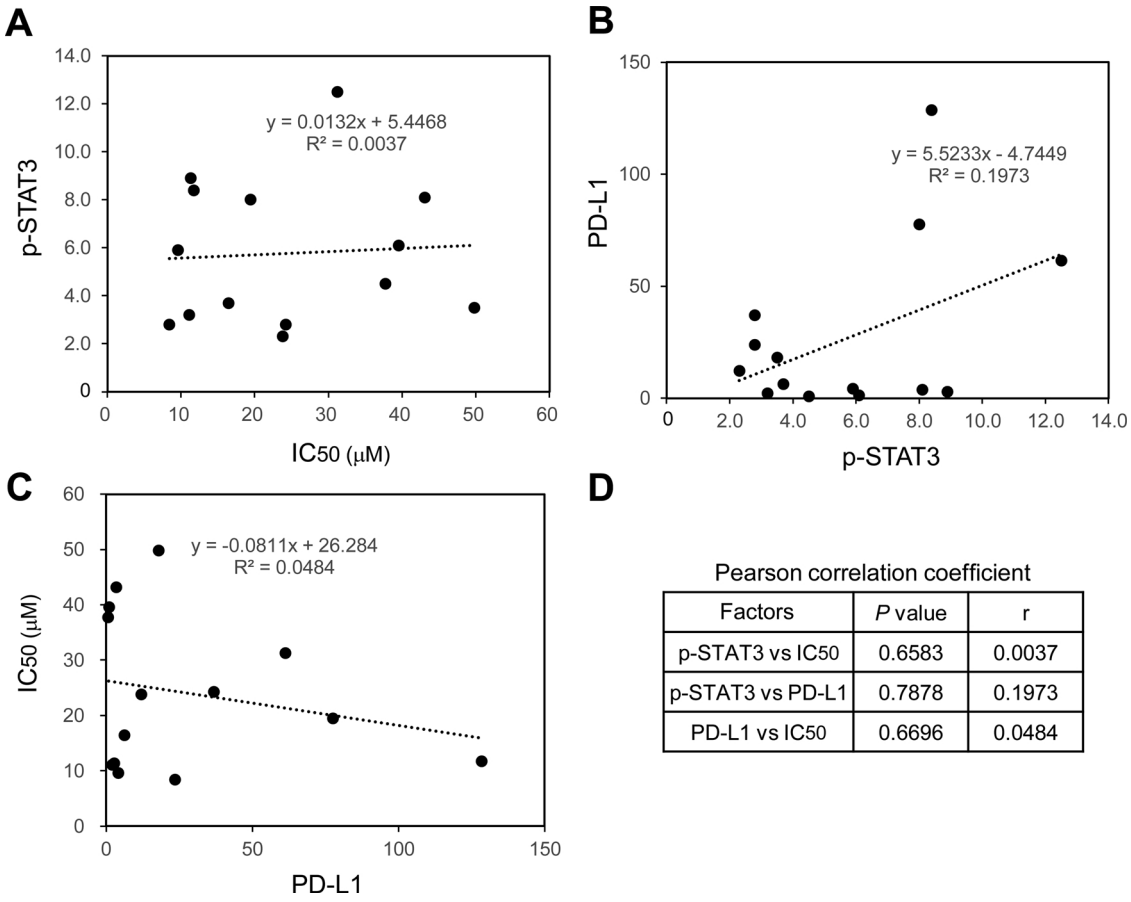


Fig. 1. Phosphorylated STAT3 and PD-L1 expression levels on 14 human pancreatic cancer cell lines. (A) Phosphorylated STAT3 (py705) expression measured by intracellular staining using a PE-conjugated anti-pSTAT3 (Tyr 705) monoclonal antibody for flow cytometry. Cell lines that showed a geometric mean value higher than 5 after subtracting background value (control antibody) from the anti-pSTAT3 antibody value were rated as positive. Open column: isotype control antibody, closed column: anti-pSTAT3 antibody. (B) PD-L1 expression in pancreatic cancer cell lines assessed using an APC-conjugated anti-PD-L1 antibody. Cell lines that showed a stain intensity value higher than 10 after subtracting background value (control antibody) from the anti-PD-L1-APC antibody value were rated as positive. Thin dashed line: isotype control antibody, thick solid line: anti-PD-L1 antibody.



Factors	*P* value	r
p-STAT3 vs IC50	0.6583	0.0037
p-STAT3 vs PD-L1	0.7878	0.1973
PD-L1 vs IC50	0.6696	0.0484

Fig. 2. Correlation analysis of the STX-0119 IC50 value and pSTAT3 and PD-L1 expression levels using the Spearman correlation test. (A) STX-0119 IC50 versus pSTAT3, (B) pSTAT3 versus PD-L1, and (C) PD-L1 versus STX-0119 IC50. (D) A coefficient and *P* value were calculated for every comparison. Values of $P < 0.05$ were considered statistically significant. Specifically, pSTAT3 and PD-L1 values were the staining intensities determined by flow cytometric analysis, from which the isotype antibody-based intensity was subtracted. Each dot represents the value of a pancreatic cell line.

2. Materials and methods

2.1. Cell lines and antibodies

The 14 pancreatic cancer cell lines used in this study (AsPC-1, BxPC-3, Capan-1, Capan-2, FA-6, KP-1 N, KP-2, KP-3, MIAPaCa-2, PANC-1, PSN-1, SUIT-4, YPK-1, and YPK-2) were described previously [21]. The following antibodies were used:

anti-CD4, anti-CD8, anti-CD11b, anti-CD14, anti-CD19, anti-CD25, anti-CD33, anti-CD45, anti-CD45RA, anti-CD45RO, anti-CD56, anti-FoxP3, anti-IL-7 receptor (CD127), anti-C-C chemokine receptor type7 (CCR7), anti-lymphocyte-activating gene 3 (LAG3), anti-programmed death-ligand 1 (PD-L1), anti-PD-1 (CD279), anti-T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), and anti-Ki-67 (MIB-1) antibodies, which were described previously [20]. These antibodies were used at the dilution ranging from 1:10 ~ 1:25.

STX-0119 was supplied by the Center for Drug Discovery, University of Shizuoka (Shizuoka, Japan). WP1066, a STAT3 inhibitor, was purchased from Selleck Chemicals (Houston, TX, USA) [22]. A biosimilar of the anti-PD-1 monoclonal antibody nivolumab was manufactured in our laboratory and used for *in vivo* experiments [23].

2.2. Cell proliferation assay

The protocol of the cell proliferation assay used was described previously [24]. Briefly, 1×10^4 pancreatic cancer cells were seeded into each well of a 96-well microculture plate (Corning, NY) with inhibitors ranging in concentration from 0.1 to 100 μM. After 4 day-incubation of cells with inhibitors, the WST-1 substrate was added to the culture, and the optical density (OD) was measured at 450 and 620 nm using an immunoreader (Nivo, PerkinElmer Inc., Waltham, MA, USA). WP-1066 was used as a reference compound for a STAT3 inhibitor only in the cell proliferation assay.

2.3. Flow cytometric analysis

Pancreatic cancer cells were stained with an anti-phosphorylated STAT3-PE-conjugated (BD Biosciences, Franklin Lakes, NJ, USA) or anti-PD-L1-APC-conjugated (BioLegend Inc., San Diego, CA, USA) antibody and analyzed on an FACS Canto II flow cytometer (BD Biosciences, San Diego, CA, USA). For TIL analysis, the staining protocol was described previously [20]. Briefly, isolated human $CD45^+$ cells were stained with various primary antibodies, fixed and analyzed on an FACS flow cytometer. The human cells were identified by gating the mouse $CD45^-PI^-$ and human $CD45^+$ fractions.

2.4. In vivo experiment using humanized NOG-dKO mice

Six to eight-week-old NOG-dKO mice were kindly supplied by Dr. Mamoru Ito at the Central Institute for Experimental Animals (Kawasaki, Japan). All animals were cared for and treated humanely according to the guidelines for the welfare and use of animals in cancer research, and experimental procedures were approved by the Animal Care and Use Committee of Shizuoka Cancer Center Research Institute.

Briefly, on day 1, human PBMCs with the HLA-A*0201 genotype were intravenously (i.v.) administered to each mouse that was X-ray

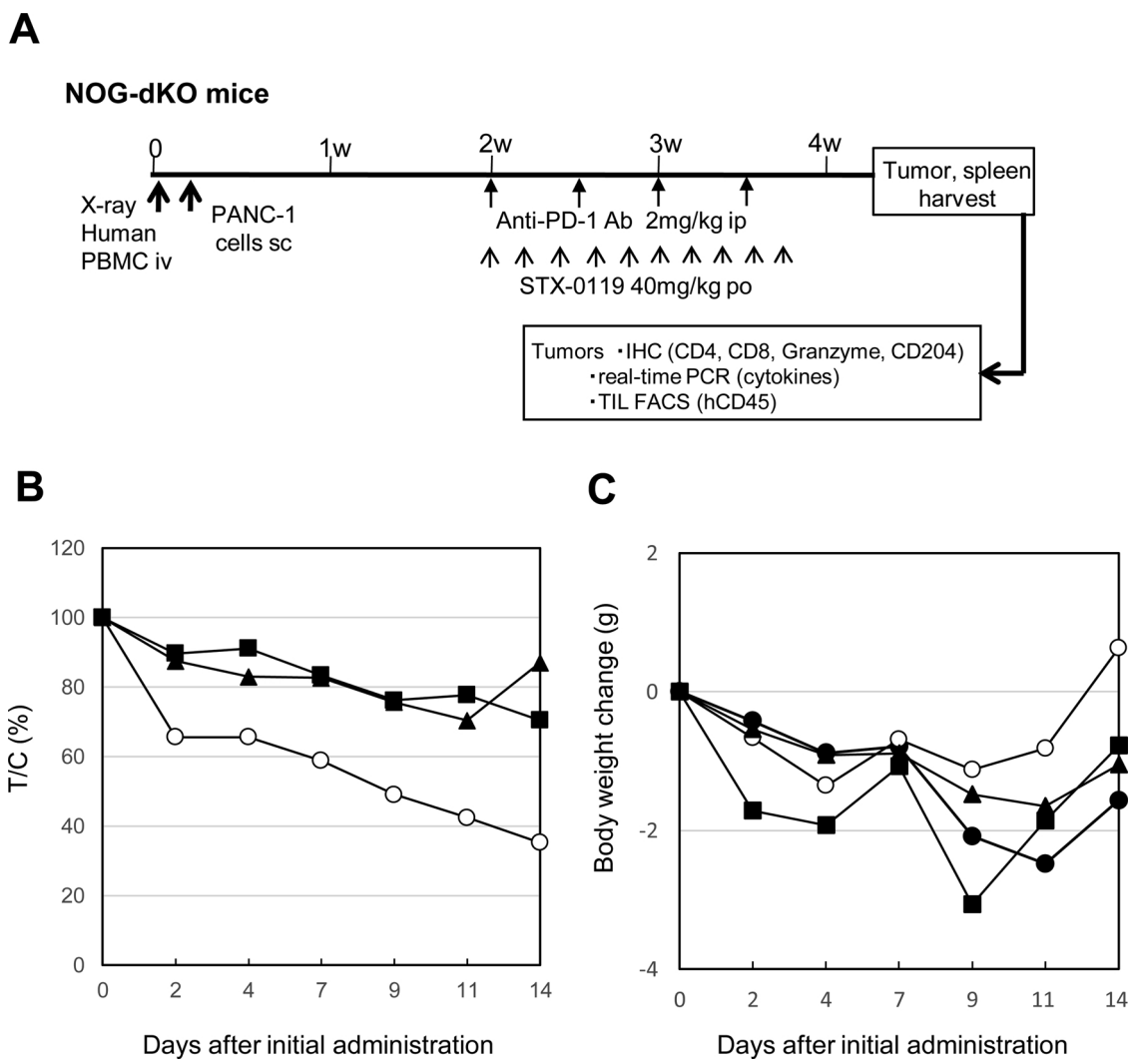


Fig. 3. Impact of STX-0119 and/or anti-PD-1 antibody treatment on PANC-1 tumor growth. (A) The schedule of the *in vivo* experiment using humanized dKO-NOG mice. (B) PANC-1 tumor growth inhibition by STX-0119 and/or anti-PD-1 antibody treatment. The tumor/control (T/C) value was calculated as the mean V/V_0 value of the treated group versus that of the control group [23]. (C) Body weight reduction in the STX-0119 and/or anti-PD-1 antibody-treated groups. Each point represents the mean value of 5 mice in a group. Closed circle: control (untreated), open circle: STX-0119-treated, closed triangle: anti-PD-1 Ab-treated, and closed square: STX-0119 plus anti-PD-1 Ab-treated.

irradiated on day 0, and 1×10^6 PANC-1 pancreatic cancer cells with the HLA-A*0201 genotype were subcutaneously transplanted. On day 14, daily oral administration of STX-0119 at a dose of 40 mg/kg was started and continued for 10 days. For the combination treatment, an anti-PD-1 monoclonal antibody was injected intraperitoneally at a dose of 2 mg/kg on days 14, 17, 21 and 24. Tumor volume was calculated on the basis of the National Cancer Institute formula as follows: tumor volume (mm^3) = length (mm) x [width (mm)]^2 x 1/2. On day 28, PANC-1 tumors, spleens and PBMCs were harvested from control and treated group mice and used for FACS, real-time PCR and IHC analyses.

2.5. Real time PCR analysis of cytokine expression

Real-time PCR analysis of cytokine genes using a QuantStudio 12 K Flex instrument was performed as described previously [20]. Briefly, PCR primers for following cytokines were used: *CCL3, CCL4*, colony-stimulating factor (*CSF*)*-1, GM-CSF*, *CXCL10*, interferon *(IFN)*-γ, *IL-23A, IL-7, MKI67*, Natural cytotoxicity triggering receptor (*NCR)1*, *TGF-β1*, tumor necrosis factor *(TNF)-α* and glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)*.

2.6. Immunohistochemistry (IHC)

Anti-CD4 and anti-CD8 antibodies (Thermo Fisher Scientific, Waltham, MA, USA), anti-FoxP3 antibody (Abcam, Cambridge, UK), an anti-granzyme B antibody (DAKO, Glostrup, Denmark), and an anti-CD204 antibody (TransGenic Inc., Kobe, Japan) were purchased and used for immunohistochemical analysis. More than 10 areas of tumor tissue in each immunostained section were analyzed and positive cell numbers were measured at high magnification (200x) using Winroof image analysis software.

2.7. Statistical analysis

Data were analyzed using a two-tailed unpaired Student t test for comparisons of two groups. For TIL number analysis in IHC experiments, a nonparametric method, Shirley-Williams' multiple comparison test was used. Correlations among the STX-0119 IC50 value and pSTAT3 and PD-L1 expression levels were analyzed using the Spearman correlation test. Values of $P < 0.05$ were considered statistically significant.

Table 2
Subpopulations of tumor-infiltrating lymphocytes from PANC1 Tumors treated with anti-PD1 antibody and/or STX-0119.

Cell populations	Control	STX-0119	p value	PD-1	p value	PD-1 + STX-0119	p value
Separation data							
Total cell # ($x10^5$)	2.77 ± 0.56	2.6 ± 1.5	0.623	3.3 ± 1.4	0.369	1.3 ± 0.4	0.736
Human $CD45^+$ cell (%)	15.3 ± 7.8	13.6 ± 2.1	0.584	13.8 ± 1.7	0.519	11.3 ± 3.8	0.960
Human $CD45^+$ cell# ($x10^5$)	0.46 ± 0.29	0.4 ± 0.1	0.703	0.5 ± 0.2	0.672	0.2 ± 0.1	0.105
Frequency of subpopulations							
$hCD3^+/hCD45^+mCD45^-$ cells (%)	35.6 ± 20.8	57.7 ± 7.5	**0.044**	47.6 ± 7.5	0.116	58.7 ± 6.4	**0.033**
$hCD4^+hCD3^+/hCD45^+$ cells (%)	10.3 ± 5.7	16.6 ± 3.5	0.100	13.4 ± 2.1	0.121	16.5 ± 5.3	0.198
$hCD8^+hCD3^+/hCD45^+$ cells (%)	7.5 ± 3.9	10.3 ± 3.6	0.285	16.2 ± 7.0	0.205	6.8 ± 6.4	0.871
$hCD56^+/hCD45^+$ cells (%)	16.0 ± 2.2	15.0 ± 2.5	0.373	14.3 ± 2.9	0.438	13.8 ± 3.6	0.497
$hCD19^+/hCD45^+$ cells (%)	38.0 ± 15.5	28.7 ± 6.3	0.863	36.4 ± 2.3	0.347	22.3 ± 4.8	0.673
$hCD25^+FoxP3^+/hCD4^+$ cells (%)	0.4 ± 0.6	0.7 ± 0.5	0.675	0.5 ± 0.8	0.946	0.4 ± 0.5	0.823
$hCD11b^+hCD33^+/hCD45^+$ cells (%)	9.4 ± 5.1	16.3 ± 6.3	0.230	31.2 ± 5.8	**0.023**	29.4 ± 7.4	0.050
$PD\text{-}L1^+/hCD45^+$ cells (%)	33.0 ± 6.6	44.2 ± 8.2	0.143	42.8 ± 3.0	0.110	31.0 ± 2.1	0.348
$hCD45RO^+hCD127^+/hCD4^+/hCD45^+$ cells (%)	10.0 ± 6.5	10.7 ± 4.6	0.620	25.9 ± 1.5	**0.003**	36.2 ± 5.9	0.015
$PD\text{-}1^+/CD3^+$ cells (%)	88.1 ± 7.3	88.6 ± 3.8	0.270	89.3 ± 3.9	0.264	90.1 ± 2.7	0.255
$TIM3^+LAG3^+/hCD3^+$ cells (%)	20.1 ± 3.3	21.3 ± 5.2	0.306	19.4 ± 5.3	0.403	14.9 ± 3.1	0.676
Cell# of subpopulations							
$hCD3^+/hCD45^+mCD45^-$ cell#(x105)	0.9 ± 0.4	1.5 ± 1.0	0.430	1.5 ± 0.7	0.286	0.8 ± 0.3	0.708
$hCD4^+hCD3^+/hCD45^+$ cell#(x105)	0.3 ± 0.1	0.4 ± 0.3	0.458	0.4 ± 0.2	0.229	0.2 ± 0.1	0.794
$hCD8^+hCD3^+/hCD45^+$ cell#(x105)	0.2 ± 0.1	0.2 ± 0.0	0.322	0.6 ± 0.4	0.322	0.1 ± 0.1	0.639
$hCD3^+/hCD45^+mCD45^-$ cell#(x105/g)	12.7 ± 9.7	36.7 ± 7.1	**0.048**	49.3 ± 34.0	0.217	22.4 ± 7.5	0.324
$hCD4^+hCD3^+/hCD45^+$ cell#(x105/g)	3.6 ± 2.6	10.5 ± 2.9	0.067	13.0 ± 7.5	0.169	5.7 ± 0.8	0.333
$hCD8^+hCD3^+/hCD45^+$ cell#(x105/g)	2.3 ± 0.8	6.3 ± 1.7	**0.040**	13.7 ± 5.0	**0.034**	1.8 ± 1.6	0.689

3. Results

3.1. STX-0119 inhibits the proliferation of pancreatic cancer cell lines

STX-0119 showed a moderately weak inhibitory effect on the proliferation of pancreatic cancer cells, but relatively greater inhibition of FA-6 and MIAPaCA-2 cells (Table 1). In contrast, WP1066 exhibited potent growth-inhibition on every cell line.

3.2. Expression levels of phosphorylated STAT3 and PD-L1

Flow cytometric analysis demonstrated the upregulation of phosphorylated STAT3 expression in 7 cell lines, and increased expression of PD-L1 was observed in 7 cell lines (Fig. 1A and B). Additionally, there

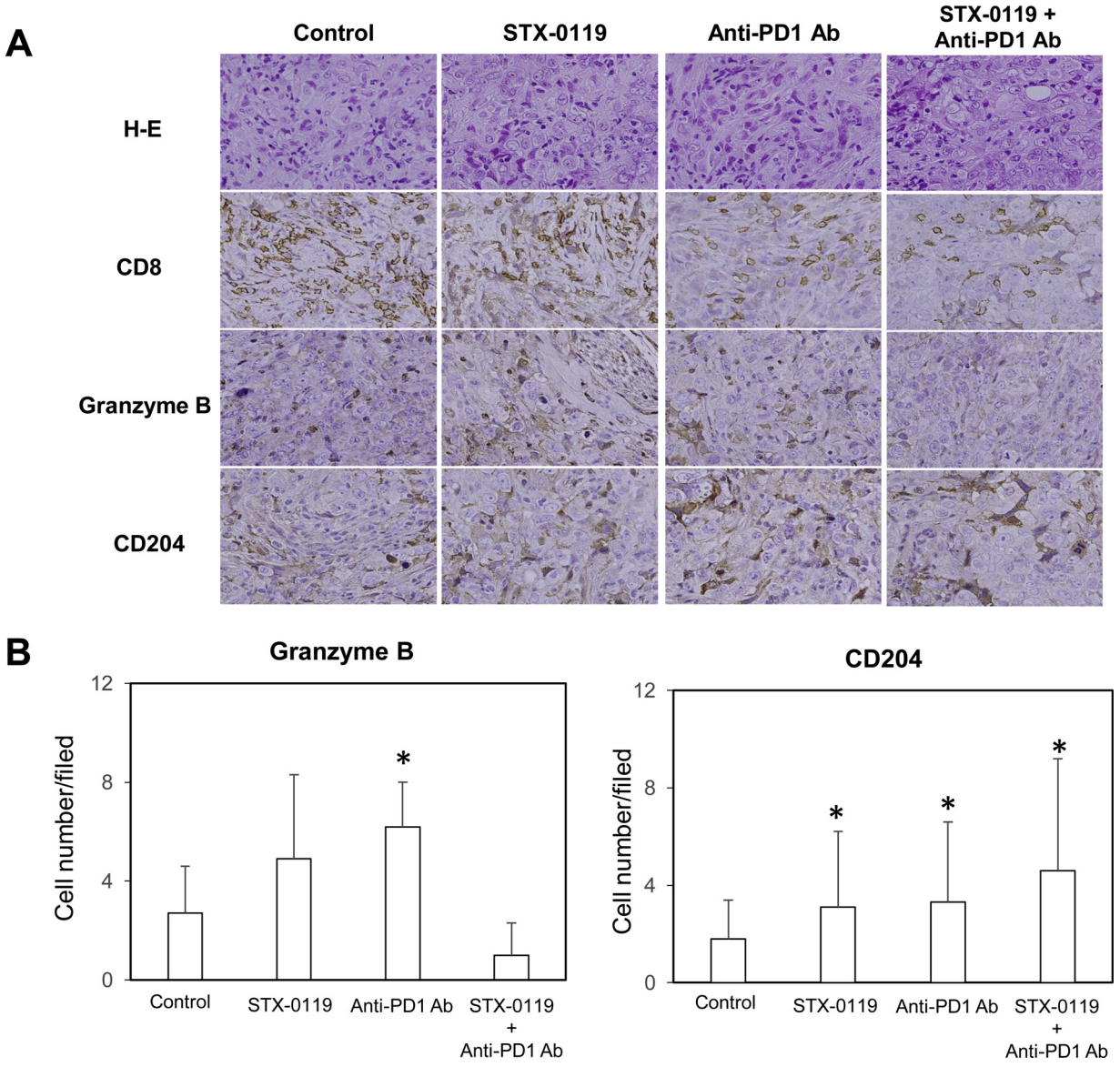


Fig. 4. IHC analysis of PANC-1 tumors in humanized dKO NOG mice treated with STX-0119 and/or an anti-PD-1 antibody. (A) H-E staining and immunostaining with anti-CD8, anti-granzyme B and anti-CD204 antibodies. Magnification 400 × . (B) Granzyme B-positive T cell or CD204-positive cell numbers in PANC-1 tumors treated with STX-0119 and/or an anti-PD-1 antibody. Positive cell counts per field were compared between the control and other treated groups. *; $P < 0.05$ and **; $P < 0.01$ indicate statistical significance.

were no obvious associations between the IC50 values of STX-0119 and phosphorylated STAT3 levels or between IC50 values and PD-L1 levels (Fig. 2).

3.3. Impact of STX-0119 and/or anti-PD-1 antibody treatment on PANC-1 tumor growth

The schedule for the *in vivo* experiment is shown in Fig. 3A. STX-0119 significantly suppressed PANC-1 tumor growth by more than 50% compared with control (untreated tumor), and an anti-PD-1 antibody showed moderate growth-inhibition by 20% (Fig. 3B). However, the combination of STX-0119 with the anti-PD-1 antibody did not show any additional growth inhibition, and the combination-treated group exhibited more weight loss as a side effect of therapy than other groups.

3.4. Characterization of TILs derived from tumors treated with STX-0119 and/or the anti-PD-1 antibody

Human $CD45^+$ TIL numbers recovered from tumor tissues samples were not different between the control and STX-0119 or anti-PD-1 antibody-treated group. However, the proportion of human $CD3^+$ T-cells significantly increased in the STX-0119 and anti-PD-1 antibody-treated groups, and in particular, the number of $CD8^+$ T-cells normalized to tumor weight increased more than 3-fold in the STX-0119 and anti-PD-1 antibody-treated group compared with the control group (Table 2). In contrast, in the combination regimen-treated group, human $CD45^+$ TIL numbers reduced to less than half of the number seen in the control group, and remarkably, $CD3^+$ T-cell numbers were strongly down-regulated compared with $CD11b^+$ macrophage-lineage cell numbers. Overtime, the promotive effect of STX-0119 and the anti-PD-1 antibody on human T-cell accumulation in the tumor site was diminished in the combination regimen-treated group.

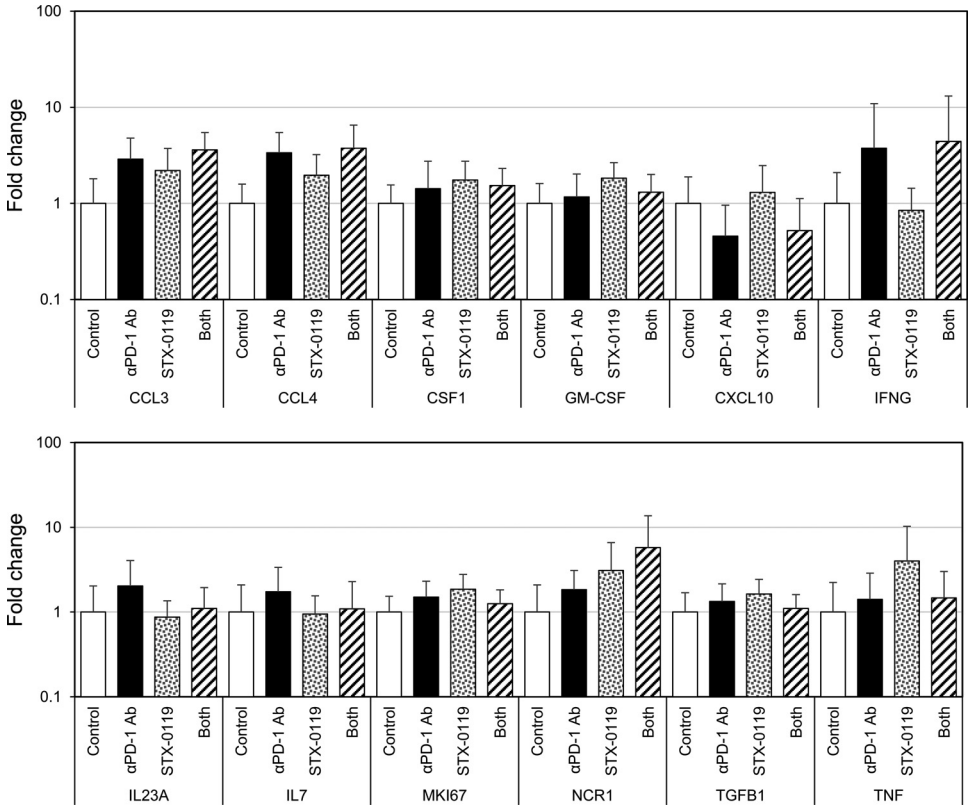


Fig. 5. Cytokine gene expression in PBMCs treated with STX-0119 and/or an anti-PD-1 antibody. The expression level of each gene in the control group was set to 1. The horizontal axis shows the fold change in mRNA expression in treated PBMCs compared with control PBMCs. Each column shows the mean value of triplicate samples.
Open column: control, closed column: anti-PD-1 Ab-treated, dotted column: STX-0119-treated, and hatched column: STX-0119 plus anti-PD-1 Ab-treated.

3.5. IHC analysis of STX-0119 and/or anti-PD-L1 antibody-treated tumors

The numbers of granzyme B-positive activated T cells were measured using Winroof image analysis software and compared between the control tumor group and other groups. Granzyme B^+ T cell numbers increased 2-fold and approximately 3-fold in the STX-0119-treated and anti-PD-1 antibody-treated tumors, respectively compared with the control tumor (Fig. 4A and B). However, the combination therapy-treated tumors showed a remarkable reduction in Granzyme B^+ T cell numbers.

3.6. Cytokine profiling of STX-0119 and/or anti-PD-1 antibody-treated PBMCs

Real time PCR analysis revealed the upregulation of T cell-attracting chemokine genes, such as *CCL3* and *CCL4*, and the Th1 cytokine gene *IFN*-γ in anti-PD-1 antibody monotherapy-treated or anti-PD-1 antibody combined with STX-0119-treated PBMCs (Fig. 5). Interestingly, the *NCR1* gene specific for activated human natural killer cells was most upregulated in the combination therapy-treated PBMCs.

4. Discussion

The STAT3 pathway is one of numerous oncogenic signaling pathways, and is constitutively activated in various human cancer cells. Activated STAT3 signaling is involved in not only the upregulation of genes crucial for cancer survival, proliferation, angiogenesis and cancer metastasis but also the promotion of gene expression underlying the immunosuppressive mechanisms in the TME [15,16]. Specifically, STAT3 signaling activation in the TME can trigger and activate diverse immunosuppressive factors, such as the production of immune-suppressive cytokines (IL-6, IL-10, TGF-beta, VEGF etc.), the accumulation of immunoregulatory cells [regulatory T cells, myeloid-derived suppressor cells (MDSCs), and M2-type tumor-associated macrophages (TAMs)], the metabolomic shift typical of the Warburg effect, and the attraction of CAFs, which may contribute to the impairment of effector T cell-mediated killing of cancer cells [25–28].

The association of pancreatic cancer development with STAT3 signaling has been demonstrated by several studies, in which the promotive effects of STAT3 activation on pancreatic cancer development, progression, liver metastasis and chemoradiation resistance have been shown [17–19,29,30]. Interestingly, Lesina et al. reported that the activation of STAT3/SOCS3 was required to promote Kras (G12D)-harboring pancreatic intraepithelial neoplasia (PanIN) to progress to pancreatic ductal adenocarcinoma [29]. Additionally, Lu *et al.* demonstrated that STAT3 activation-induced chronic inflammation impaired the anti-tumor effect of anti-PD-1 blockade therapy on an *in vivo* pancreatic cancer model [17].

Based on the evidence that mono-immunotherapy anti-PD-1/PD-L1 blockade had a poor anti-tumor effect on pancreatic cancers in a previous clinical trial, the development of a novel efficient combination therapy is crucially needed [31]. A preliminary phase I clinical trial of STAT3 inhibitors has been performed, but clinical studies have been discontinued because of poor clinical activity [32,33]. Recently, clinical trials of the novel JAK-STAT inhibitor ruxolitinib or anti-PD-1 antibody pembrolizumab in addition to chemotherapy have been performed in advanced pancreatic cancer patients [34,35].

More interestingly, Wang et al. reported that RAF (KRAS) inhibitor (AZ628) and STAT3 inhibitor (BP-1-102) combination markedly inhibited *in vitro* and *in vivo* the proliferation of KRAS-mutant lung cancer cells in a synergistic manner by abrogation of MEK/ERK signaling pathway activation. These results might suggest that the combination of RAF and STAT3 inhibitors is also an effective therapy for treating KRAS-mutant pancreatic cancers [36]. However, there should be careful monitoring established so that combination treatment with ICB and signal-targeting molecules such as tyrosine kinase inhibitors (TKIs) can be performed safely, because the combination of prior ICB with the new EGFR TKI osimertinib exhibited an increased frequency of interstitial pneumonitis compared with osimertinib treatment without prior ICB therapy [37,38].

Regarding the failure of the combination regimen of STX-0119 and the anti-PD-1 antibody in our study, the precise reasons or mechanism remain to be elucidated. Based on our previous report that STAT3 inhibitor promoted TIL accumulation *in vivo* consisting of IFN-γ-producing effector T cells and specific observations demonstrated by Wherry et al. [39], the combination of the STAT3 inhibitor and anti-PD-1 antibody might promotes the apoptosis of activated effector T cells through increased expression of inhibitory receptors and exhaustion mechanism under the condition of continuous antigen stimulation such as chronic inflammation. However, the specific reasons involved in a remarkable reduction in TIL number is still not obvious.

In addition, the expression of an activated NK cell marker was upregulated in most PBMCs treated with the combination of the anti-PD-1 antibody and STAT3 inhibitor, which suggested that the addition of the STAT3 inhibitor to anti-PD-1 blockade had an additional activating effect on NK cells in the blood despite the reduction in T cell numbers in the tumor.

In the current study, our group investigated the anti-tumor effect and safety of a combination regimen using a humanized MHC-dKO NOG mouse model. This method might be a useful tool to evaluate efficacy and safety at the preclinical stage in the context of a novel combination regimen of ICB and a TKI, as it that is difficult to predict serious adverse effects in conventional nude-mouse model.

Declaration of Competing Interest

The authors have no competing interests to declare.

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References

[1] T. Kamisawa, L.D. Wood, T. Itoi, K. Takaori, Pancreatic cancer, Lancet 388 (2016) 73–85.
[2] T. Seufferlein, J.B. Bachet, E. Van Cutsem, P. Rougier, ESMO Guidelines Working Group, Pancreatic adenocarcinoma: ESMO-ESDO clinical practice guidelines for diagnosis, treatment and follow-up, Ann. Oncol. Suppl 7 (2012) vii33–40.
[3] N.B. Jamieson, S.M. Denley, J. Logue, D.J. MacKenzie, A.K. Foulis, E.J. Dickson, et al., A prospective comparison of the prognostic value of tumor- and patient-related factors in patients undergoing potentially curative surgery for pancreatic ductal adenocarcinoma, Ann. Surg. Oncol. 18 (2011) 2318–2328.
[4] K. Uesaka, N. Boku, A. Fukutomi, Y. Okamura, M. Konishi, I. Matsumoto, et al., Adjuvant chemotherapy of S-1 versus gemcitabine for resected pancreatic cancer: a phase 3, open-label, randomized, non-inferiority trial (JASPAC 01), Lancer 388 (2016) 248–257.
[5] D.D. Von Hoff, T. Ervin, F.P. Arena, E.G. Chiorean, J. Infante, M. Moore, et al., Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine, N. Engl. J. Med. 369 (2013) 1691–1703.
[6] J. Mizusawa, A. Fukutomi, H. Katayama, H. Ishii, T. Ioka, T. Okusaka, et al., Protocol digest of randomized phase II study of modified FOLFIRINOX versus gemcitabine plus nab-paclitaxel combination therapy for locally advanced pancreatic cancer: japan clinical oncology group study (JCOG1407), Pancreatology 18 (2018) 841–845.
[7] F.S. Hodi, S.J. O`Day, D.F. McDermott, R.W. Weber, J.A. Sosman, J.B. Haanen, et al., Improved survival with ipilimumab in patients with metastatic melanoma, N. Engl. J. Med. 363 (2010) 711–723.
[8] S.L. Topalian, F.S. Hodi, J.R. Brahmer, S.N. Gettinger, D.C. Smith, D.F. McDermott, Safety, activity, and immune correlates of anti-PD-1 antibody in cancer, N. Engl. J. Med. 366 (2012) 2443–2454.
[9] M. Feng, G. Xiong, Z. Cao, G. Yang, S. Zheng, X. Song, et al., PD-1/PD-L1 and immunotherapy for pancreatic cancer, Cancer Lett. 407 (2017) 57–65.
[10] J.R. Brahmer, S.S. Tykodi, L.Q. Chow, W.J. Hwu, S.L. Topalian, P. Hwu, et al., Safety and activity of anti-PD-L1 antibody in patients with advanced cancer, N. Engl. J. Med. 366 (2012) 2455-2365.
[11] D.S. Chen, I. Mellman, Elements of cancer immunity and the cancer-immune set point, Nature 541 (2017) 321–330.
[12] P. Sharma, S. Hu-Lieskovan, J.A. Wargo, A. Ribas, Primary, adaptive, and acquired resistance to cancer immunotherapy, Cell 168 (2017) 707–723.
[13] G.P. Dunn, A.T. Bruce, H. Ikeda, L.J. Old, R.D. Schreiber, Cancer immunoediting: from immunosurveillance to tumor escape, Nat. Immunol. 3 (2002) 991–998.
[14] M. Yarchoan, B.A. Johnson 3rd, E.R. Lutz, D.A. Laheru, E.M. Jaffee, Targeting neoantigens to augment antitumor immunity, Nat. Rev. Cancer 17 (2017) 209–222.
[15] H. Yu, M. Kortylewski, D. Pardoll, Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment, Nat. Rev. Immunol. 7 (2007) 41–51.
[16] M. Kortylewski, H. Yu, Role of Stat3 in suppressing anti-tumor immunity, Curr. Opin. Immunol. 20 (2008) 228–233.
[17] C. Lu, A. Talukder, N.M. Savage, N. Singh, K. Liu, JAK-STAT-mediated chronic inflammation impairs cytotoxic T lymphocyte activation to decrease anti-PD-1 immunotherapy efficacy in pancreatic cancer, Oncoimmunolgy 6 (2017) e1291106, , https://doi.org/10.1080/2162402X.2017.1291106.
[18] N. Sahu, E. Chan, F. Chu, T. Pham, H. Coeppen, W. Forrest, et al., Cotargeting of MEK and PDGFR/STAT3 pathways to treat pancreatic ductal adenocarcinoma, Mol. Cancer Ther. 16 (2017) 1729–1738.
[19] X. Wu, W. Tang, R.T. Marquez, K. Li, C.A. Highfill, F. He, et al., Overcoming chemo/radio-resistance of pancreatic cancer by inhibiting STAT3 signaling, Oncotarget 7 (2016) 11708–11723.
[20] Y. Akiyama, C. Nonomura, T. Ashizawa, A. Iizuka, R. Kondou, H. Miyata, et al., The anti-tumor activity of the STAT3 inhibitor STX-0119 occurs via promotion of tumor-infiltrating lymphocyte accumulation in temozolomide-resistant glioblastoma cell line, Immunol. Lett. 190 (2017) 20–25.
[21] K. Sasaki, K. Sato, Y. Akiyama, K. Yanagihara, M. Oka, K. Yamaguchi, Peptidomics-based approach reveals the secretion of the 29-residue COOH-terminal fragment of the putative tumor suppressor protein DMBT1 from pancreatic adenocarcinoma cell line, Cancer Res. 62 (2002) 4894–4898.
[22] A. Iwamaru, S. Szymanski, E. Iwado, H. Aoki, T. Yokokawa, I. Fokt, et al., A novel inhibitor of the STAT3 pathway induces apoptosis in malignant glioma cells both *in vitro* and *in vivo*, Oncogene 26 (2007) 2435–2444.
[23] T. Ashizawa, A. Iizuka, C. Nonomura, R. Kondou, C. Maeda, H. Miyata, et al., Antitumor effect pf programmed death-1 (PD-1) blockade in humanized the NOG-MHC double knockout mouse, Clin. Cancer Res. 23 (2017) 149–158.
[24] T. Ashizawa, Y. Akiyama, H. Miyata, A. Iizuka, M. Komiyama, A. Kume, et al., Effect of the STAT3 inhibitor STX-0119 on the proliferation of a temozolomide-resistant glioblastoma cell line, Int. J. Oncol. 45 (2014) 411–418.
[25] V. Thorsson, D.L. Gibbs, S.D. Brown, D. Wolf, D.S. Bortone, T.H. Ou Yang, et al., The immune landscape of cancer, Immunity 48 (2018) 812–830.
[26] C.Y. Ock, B. Keam, S. Kim, J.S. Lee, M. Kim, T.M. Kim, et al., Pan cancer immunogenomic perspective on the tumor microenvironment based on PD-L1 and CD8 T-cell infiltration, Clin. Cancer Res. 22 (2016) 2261–2270.
[27] k. Sideras, H. Braat, J. Kwe k keboom, C.H. van Eijc k, M.P. Peppelenbosch, S. Sleijfer, et al., Role of the immune system in pancreatic cancer progression and immune modulating treatment strategies, Cancer Treat. Rev. 40 (2014) 513–522.
[28] J. Hutcheson, U. Balaji, M.R. Polembka, M.B. Wachsmann, P.A. McCue, E.S. Knudsen, Immunologic and metabolic features of pancreatic ductal adenocarcinoma define prognostic subtypes of disease, Clin. Cancer Res. 22 (2016) 3606–3617.
[29] M. Lesina, M.U. Kurkowski, K. Ludes, S. Rose-John, M. Treiber, G. Klöppel, et al., Stat3/Socs3 activation by IL-6 transsignaling promotes progression of pancreatic intraepithelial neoplasia and development of pancreatic cancer, Cancer Cell 19 (2011) 456–469.
[30] R.B. Corcoran, G. Contino, V. Deschpande, A. Tzatsos, C. Conrad, C.H. Benes, et al., STAT3 plays a critical role in KRAS-induced pancreatic tumorigenesis, Cancer Res. 71 (2011) 5020–5029.
[31] M.J. Smyth, S.F. Ngiow, A. Ribas, M.W. Teng, Combination cancer immunotherapies tailored to the tumour microenvironment, Nat. Rev. Clin. Oncol. 13 (2016) 143–158.
[32] E.R. Plimack, P.M. Lorusso, P. McCoon, W. Tang, A.D. Krebs, G. Curt, et al., AZD1480: a phase I study of a novel JAK2 inhibitor in solid tumors, Oncologist 18 (2013) 819–820.
[33] D.Y. Oh, S.H. Lee, S.W. Han, M.J. Kim, T.M. Kim, T.Y. Kim, et al., Phase I study of OPB-31121, an oral STAT3 inhibitor, in patients with advanced solid tumors, Cancer Res. Treat. 47 (2015) 607–615.
[34] H.I. Hurwitz, N. Uppal, S.A. Wagner, J.C. Bendell, J.T. Beck, S.M. Wade 3rdet al., Randomized, double-blind, phase II study of Ruxolitinib or placebo in combination with capecitabine in patients with metastatic pancreatic cancer for whom therapy with gemcitabine has failed, J. Clin. Oncol. 33 (2015) 4039–4047.
[35] G.J. Weiss, L. Blaydorn, J. Beck, K. Bornemann-Kolatzki, H. Urnovitz, E. Schütz, et al., Phase Ib/II study of gemcitabine, nab-paclitaxel, and pembrolizumab in metastatic pancreatic adenocarcinoma, Invest. New Drugs 36 (2018) 96–102.
[36] Z. Wang, Yin M, P. Chu, M. Lou, STAT3 inhibitor sensitized KRAS-mutant lung cancers to RAF inhibitor by activating MEK-ERK signaling pathway, Aging 11 (2019), https://doi.org/10.18632/aging.102244.
[37] M.J. Ahn, J.M. Sun, S.H. Lee, J.S. Ahn, K. Park, EGFR TKI combination with immunotherapy in non-small cell lung cancer, Expert Opin. Drug Saf. 16 (2017) 465–469.
[38] M. Kotake, H. Murakami, H. Kenmotsu, T. Naito, T. Takahashi, High incidence of interstitial lung disease following practical use of osimertinib in patients who had undergone immediate prior nivolumab therapy, Ann. Oncol. 28 (2017) 669–670.
[39] E.J. Wherry, T cell exhaustion, Nat. Immunol. 12 (2011) 492–499.