Targeting S1PR1/STAT3 loop abrogates desmoplasia and chemosensitizes gemcitabine to pancreatic cancer

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Table S1: FTY720 and in combination with gemcitabine inhibited metastasis and ascites in an orthotopic model of pancreatic cancer

Treatment group	Mice	Number of mice with dissemination (metastasis)			Presence of ascites	
		Abdominal cavity	Intestine and colon	Liver	Mild	Severe
Vehicle	n=6	6	2	3	2	3
FTY720	n=6	5	1	1	0	1
Gemcitabine	n=6	4	2	1	1	4
FTY + Gem	n=5	2	0	0	2	0

Table S2:

Immune & Inflammatory Responses:

Immunostimulatory Factors: IFNG, IL2, IL12A, IL12B, IL15, TNF.

Immunosuppressive Factors: CD274 (PD-L1), CSF2 (GM-CSF), CTLA4, CXCL12 (SDF1), CXCL5, IDO1 (IDO), IL10, IL13, IL4, IL8, MIF, NOS2 (iNOS), PDCD1 (PD1), PTGS2 (COX2), TGFB1, VEGFA.

Pro-Inflammatory Genes: CCL2 (MCP-1), CCL20 (MIP-3A), IFNG, IL1A, IL1B, IL2, IL6, IL12A, IL12B, IL17A, IL23A, PTGS2 (COX2), TLR4, TNF, VEGFA.

Anti-Inflammatory Genes: IL4, IL10, IL13, TGFB1.

Enzymatic Modulators of Inflammation & Immunity: AICDA (AID), GZMA, GZMB, IDO1 (IDO), NOS2 (iNOS), PTGS2 (COX2).

Antigen Presentation: HLA-A, HLA-B, HLA-C, MICA, MICB.

Chemokines: CCL2 (MCP-1), CCL4 (MIP-1B), CCL5 (RANTES), CCL18 (PARC), CCL20 (MIP-3A), CCL21, CCL22 (MDC), CCL28, CXCL1, CXCL2, CXCL5, CXCL9 (MIG), CXCL10 (IP-10), CXCL11 (I-TAC, IP-9), CXCL12 (SDF1).

Chemokine Receptors: ACKR3 (CXCR7), CCR1, CCR2, CCR4, CCR7, CCR9, CCR10, CXCR1 (IL8RA), CXCR2 (IL8RB), CXCR3, CXCR4, CXCR5.

Interleukins: IL1A, IL1B, IL2, IL4, IL6, IL8, IL10, IL12A, IL12B, IL13, IL15, IL17A, IL23A.

Other Cytokines: KITLG (SCF), MIF, SPP1, TNF, TNFSF10 (TRAIL).

Growth Factors & Receptors: CSF1 (MCSF), CSF2 (GM-CSF), CSF3 (GCSF), EGF, EGFR, IGF1, TGFB1, VEGFA.

Signal Transduction:

Interferon Signaling: GBP1, IFNG, IL6, IRF1.

Interferon-Responsive Genes: CCL2 (MCP-1), CCL5 (RANTES), CXCL9 (MIG), CXCL10 (IP-10), GBP1, IRF1, MYD88, STAT1, TLR3, TNFSF10 (TRAIL).

NFκB Targets: BCL2L1 (BCL-XL), CCL2 (MCP-1), CCL5 (RANTES), CSF1 (MCSF), CSF2 (GM-CSF), CSF3 (GCSF), IFNG, IL8, TNF.

STAT Targets: CCL2 (MCP-1), CCL4 (MIP-1B), CCL5 (RANTES), CSF1 (MCSF), CSF2 (GM-CSF), CSF3 (GCSF), CXCL9 (MIG), CXCL10 (IP-10), CXCL11 (I-TAC, IP-9), CXCL12 (SDF1), IL1B, IL6, IL8, IL10, IL17A, IL23A, MYC.

Toll-Like Receptor Signaling: TLR2, TLR3, TLR4, MYD88.

Transcription Factors: FOXP3, HIF1A, IRF1, MYC, NFKB1, STAT1, STAT3, TP53 (p53).

Apoptosis:

Pro-Apoptotic: FASLG (TNFSF6), TNF, TNFSF10 (TRAIL), TP53 (p53).

Anti-Apoptotic: BCL2, BCL2L1 (BCL-XL), MYC, STAT3.

Table S3: List of primers used to check the expression of various gemcitabine metabolizing enzymes and EMT markers

S. No	Primer name	Primer sequence
1	hDCK-F	CAGCTTGCCTCTCTGAATGG
2	hDCK-R	TCCAGTCATGCCAGTCTTGA
3	hCDA-F	CTGCAGGCAAGTCATGAGAG
4	hCDA-R	GCATTCTCTGGCTGTCACTG
5	hDCTD-F	TGCTACATGTGCTGGAAGGA
6	hDCTD-R	TGCCACCCTATCTCGGATTC
7	hRRM1-F	GGAGGAATTGGTGTTGCTGT
8	hRRM1-R	GCTGCTCTTCCTTTCCTGTG
9	hRRM2-F	CCCGCTGTTTCTATGGCTTC
10	hRRM2-R	CCCAGTCTGCCTTCTTCTTG
11	hABCC5-F	AGAACTCGACCGTTGGAATGC
12	hABCC5-R	TCATCCAGGATTCTGAGCTGAG
13	hTWIST1-F	GGCTCAGCTACGCCTTCTC
14	hTWIST1-R	TCCTTCTCTGGAAACAATGACA
15	hTWIST2-F	TCTGACAAGCTGAGCAAGATCC
16	hTWIST2-R	CTGCAGCTGGTCATCTTATTGTC
17	hSlug-F	AGATGCATATTCGGACCCAC
18	hSlug-R	CCTCATGTTTGTGCAGGAGA
19	hSnail-F	AATCGGAAGCCTAACTACAGCGAG
20	hSnail-R	CCTTGGCCTCAGAGAGCTGG
21	hE-cadherin-F	GAAGGTGACAGAGCCTCTGGAT
22	hE-cadherin-R	GATCGGTTACCGTGATCAAAATC
23	hGli1-F	AGATGAATCACCAAAAAGGG
24	hGli1-R	ATATCACCTTCCAAGGGTTC
25	hGli3-F	CTCCATTGCATATGACTTCC
26	hGli3-R	GCGGATATAGTCCATGTAGG
27	hShh-F	GAGCGATTTAAGGAACTCAC
28	hShh-R	CCTTACACCTCTGAGTCATC
29	hIl6-F	AAATTCGGTACATCCTCGACGGCA
30	hIl6-R	GTGCCTCTTTGCTGCTTTCACACA
31	hβ-Actin-F	CCAGCTCACCATGGATGATG
32	hβ-Actin-R	ATGCCGGAGCCGTTGTC

Supplementary Figure legends

Figure S1: FTY720 inhibited the growth and induces apoptosis in pancreatic cancer cell lines

(A) The cytotoxic effect of FTY720 was assessed using MTT assay on mouse pancreatic acinar cells and **(B)** Human pancreatic ductal epithelia cells (HPDE) after 24 and 48 h **(C)**PAN 02 cells were treated with FTYT720 (10 μM and 15 μM) and the rate of apoptosis was quantified after 24 h using annexin V-FITC staining. Scatter plot from FACS (left panel) and quantification of live and apoptotic population (right panel)**(D)** The effect of FTY720 on cell cycle was quantified using propidium iodide staining after treating PAN 02 cells with 10 μM of FTY720. Data is presented as mean ± S.D and representative data from at least 3 independents experiments is shown

Figure S2: FTY720 exhibited anti-migratory property in the pancreatic cancer cell lines

(A) The anti-migratory capability of FTY720 was checked using wound healing assay. Briefly, a scratch was made when the PAN 02cells reach 90% confluence and treated with an increased dose of FTY720 and the closure of wound was monitored at 12 h and 24 h of time intervals. Representative images (top panel) and quantification (bottom panel).

Figure S3: FTY720 enhanced the effect of gemcitabine in vitro

(A)PAN 02 cells were treated with FTY720 in combination with gemcitabine and the loss in mitochondrial membrane potential was quantified using DiCO6(3) staining after 24 h

Figure S4: FTY720 in combination with gemcitabine reduces the tumor burden invivo

(A) Bioluminescent quantification of MIA PaCa-2 luc using IVIS measurement. Quantification was done and the data for day15 and 30 were plotted. **(B)** Necropsy images of mice with orthotopically implanted pancreatic cancer.

Figure S5: FTY720 in combination with gemcitabine altered NF-kB dependent gene expression and other inflammatory genes

(A) Heat map showing the expression of NF-kB driven genes after performing RT^2 profiler array. Briefly RNA was isolated from each sample was used for RT2 profiler array. Values are expressed in fold. Representative data from each experiment is shown here.

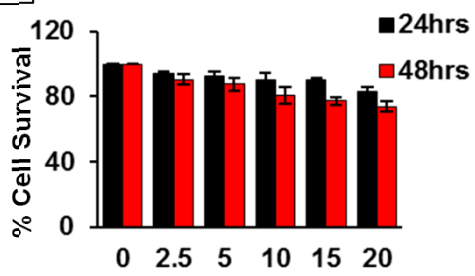
(B)and**(C)**Heat map showing the expression of genes associated with inflammation and immunity after performing RT^2 profiler array. Briefly RNA was isolated from each sample was used for RT2 profiler array. Values are expressed in fold. Representative data from each experiment is shown here.

Figure S6: FTY720 in combination with gemcitabine reduced tumor burden in syngeneic mice model

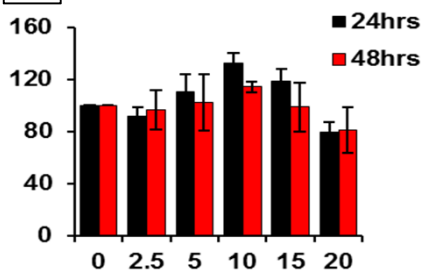
(A) Bioluminescent quantification of PAN 02- luc using IVIS measurement. Quantification was done and the data for day15 and 30 were plotted. **(B)** Necropsy images of mice with

orthotopically implanted pancreatic cancer. **(C)** Quantification data of myofibroblast density **(D)** Quantification data of collagen deposition in tumor. $p \leq 0.001$

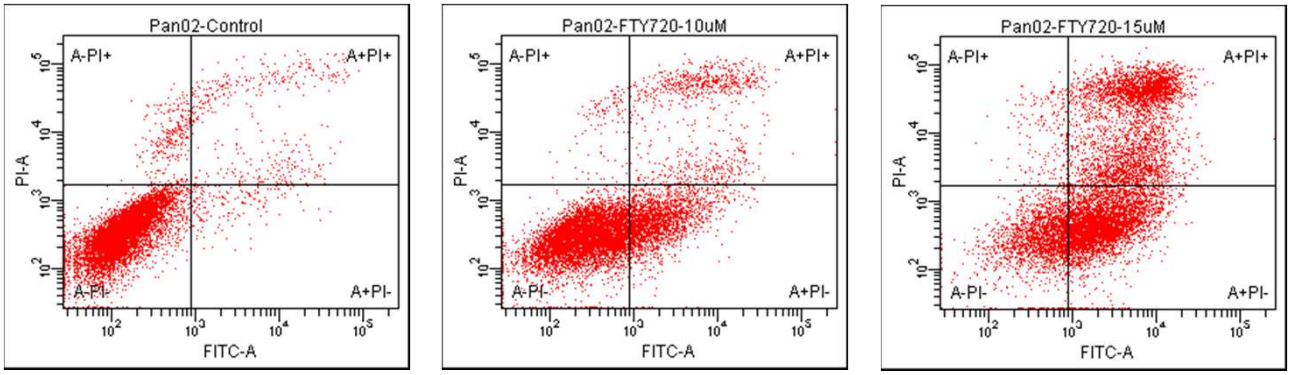
A

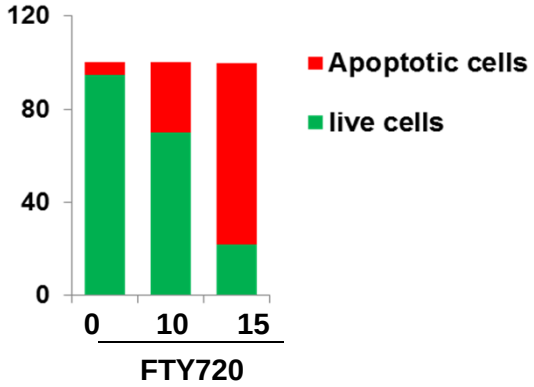

% Cell Survival
120
80
40
0
24hrs
48hrs
0 2.5 5 10 15 20

B

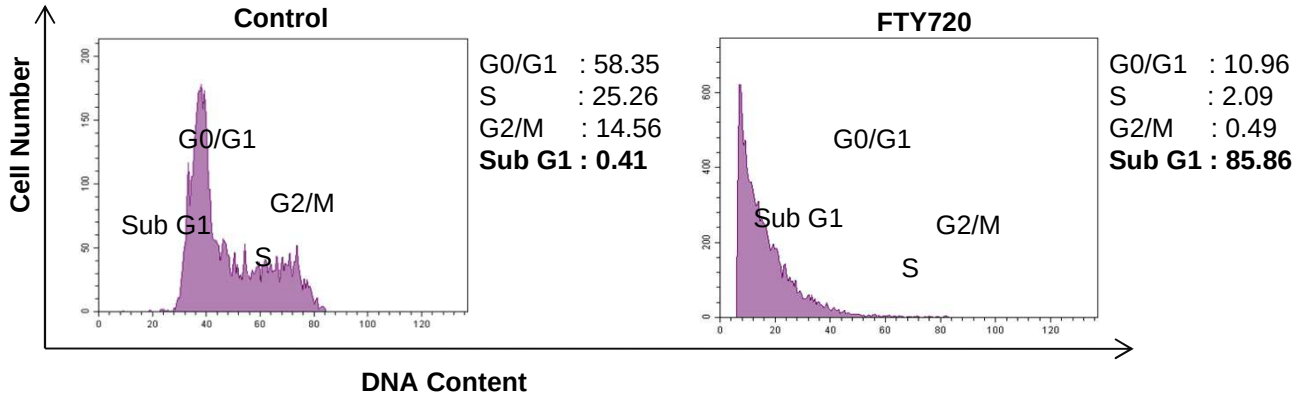

160
120
80
40
0
24hrs
48hrs
0 2.5 5 10 15 20

C

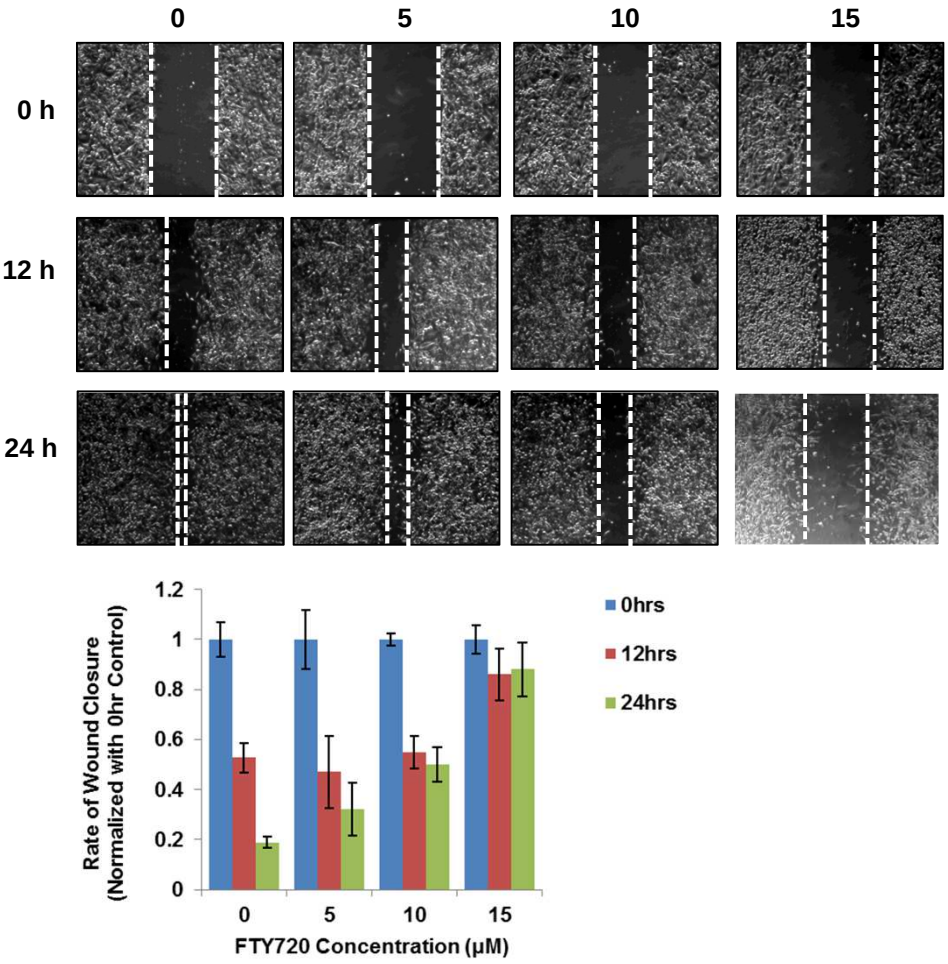

Pan02-Control
Pan02-FTY720-10uM
Pan02-FTY720-15uM
A-PI+
A+PI+
A-PI-
A+PI-
PI-A
FITC-A


120
80
40
0
Apoptotic cells
live cells
0 10 15
FTY720

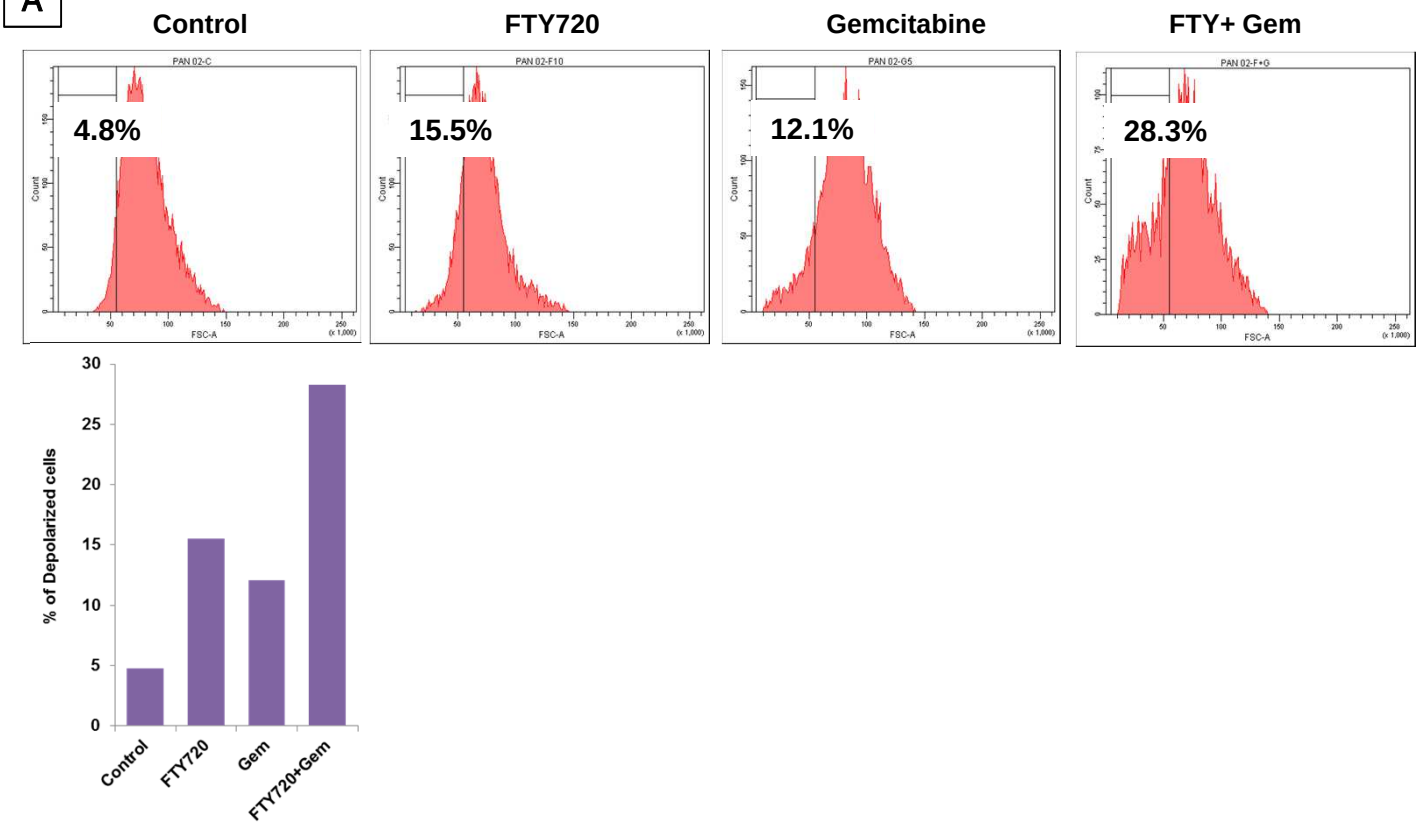
D


Control
G0/G1 : 58.35
S : 25.26
G2/M : 14.56
Sub G1 : 0.41
FTY720
G0/G1 : 10.96
S : 2.09
G2/M : 0.49
Sub G1 : 85.86
Cell Number
DNA Content
G0/G1
Sub G1
G2/M
S

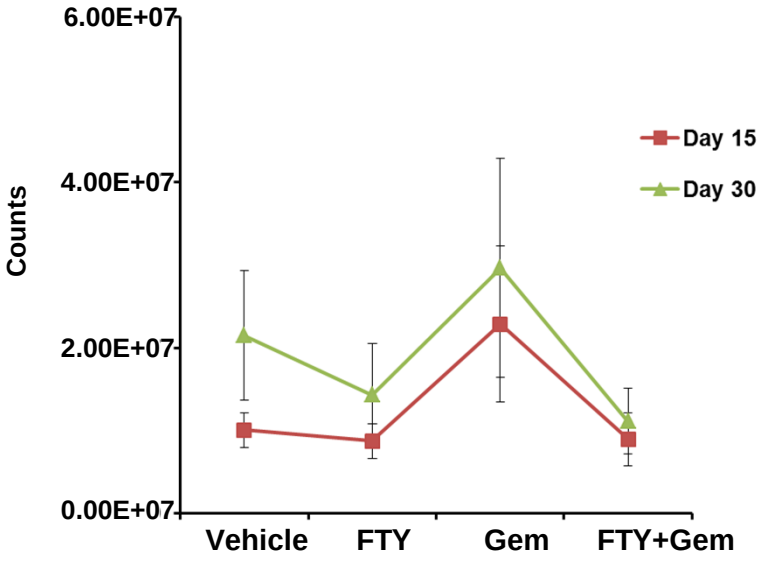
A


0
5
10
15
0 h
12 h
24 h
Rate of Wound Closure
(Normalized with 0hr Control)
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
FTY720 Concentration (μM)
0hrs
12hrs
24hrs

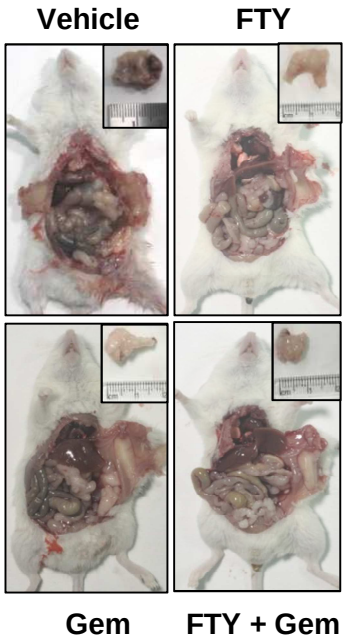
A


Control
FTY720
Gemcitabine
FTY+ Gem
4.8%
15.5%
12.1%
28.3%
% of Depolarized cells
30
25
20
15
10
5
0
Control
FTY720
Gem
FTY720+Gem

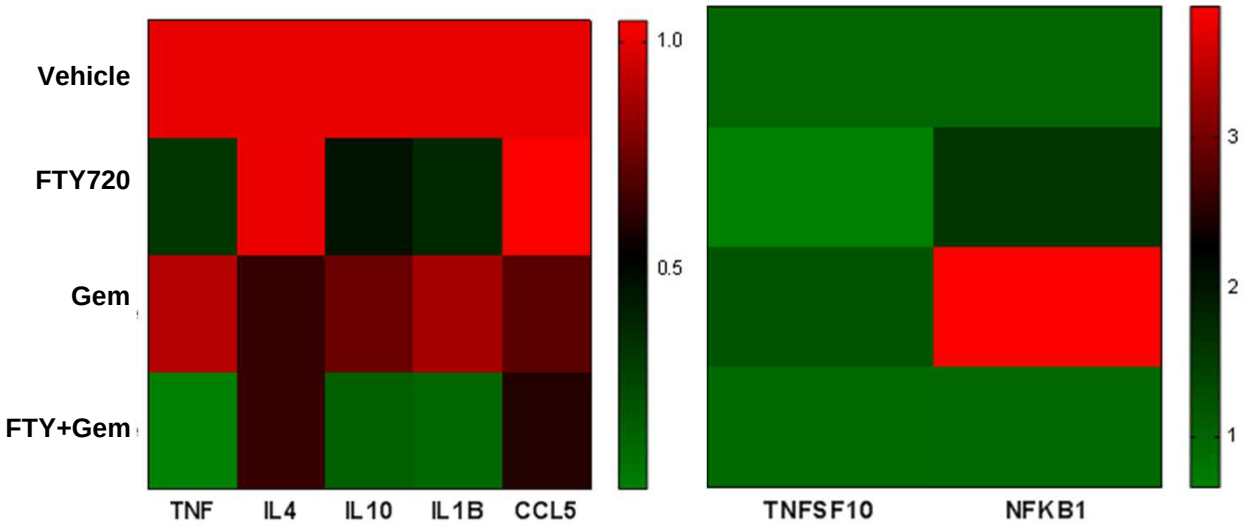
A


6.00E+07
4.00E+07
2.00E+07
0.00E+07
Counts
Day 15
Day 30
Vehicle
FTY
Gem
FTY+Gem

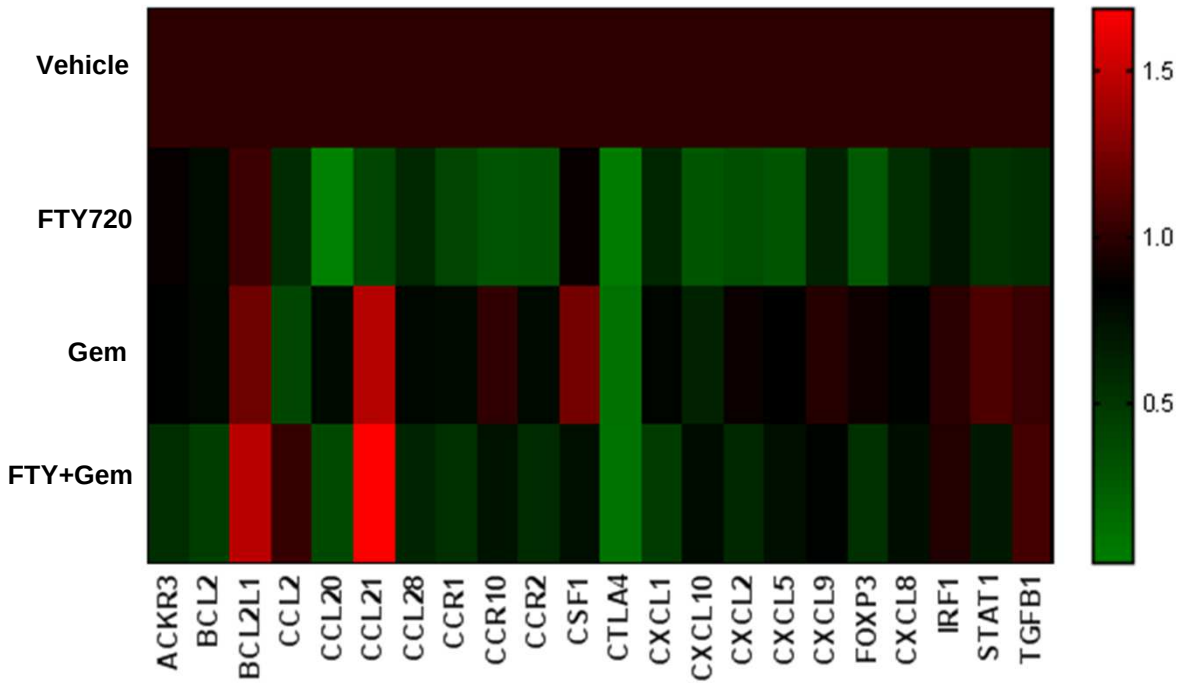
B


Vehicle
FTY
Gem
FTY + Gem

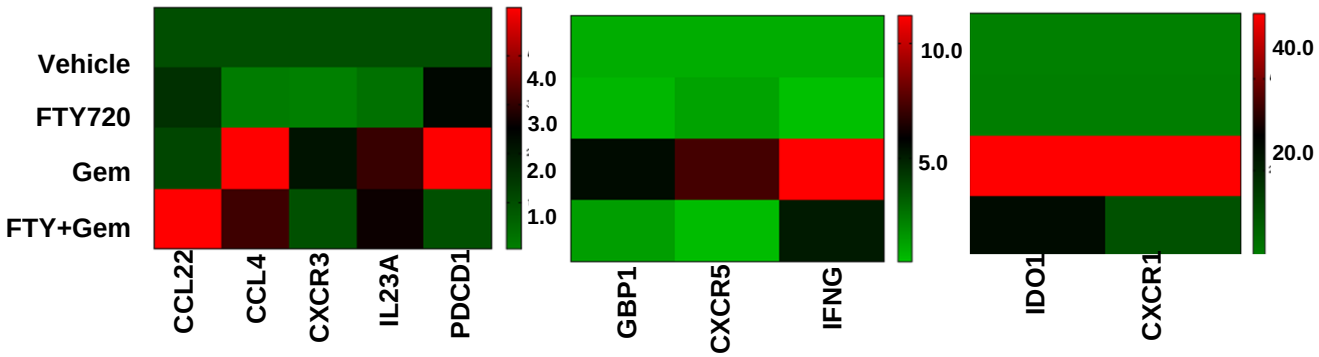
A


Vehicle
FTY720
Gem
FTY+Gem
1.0
0.5
3
2
1
TNF
IL4
IL10
IL1B
CCL5
TNFSF10
NFKB1

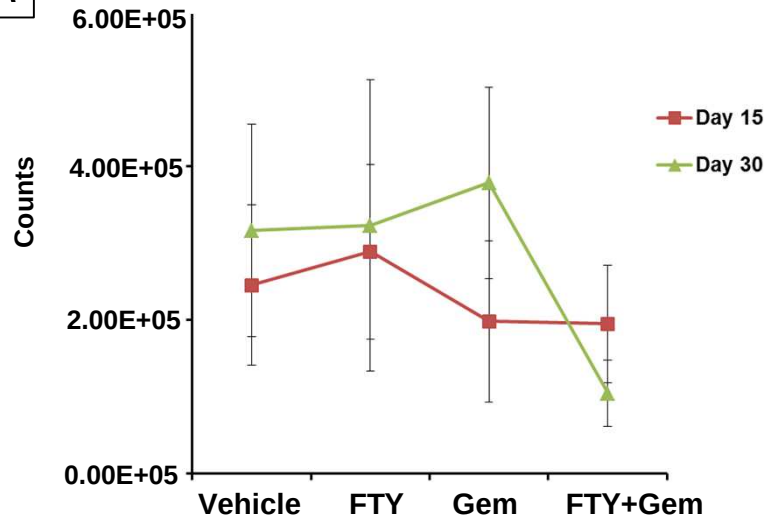
B


Vehicle
FTY720
Gem
FTY+Gem
1.5
1.0
0.5
ACKR3
BCL2
BCL2L1
CCL2
CCL20
CCL21
CCL28
CCR1
CCR10
CCR2
CSF1
CTLA4
CXCL1
CXCL10
CXCL2
CXCL5
CXCL9
FOXP3
CXCL8
IRF1
STAT1
TGFB1

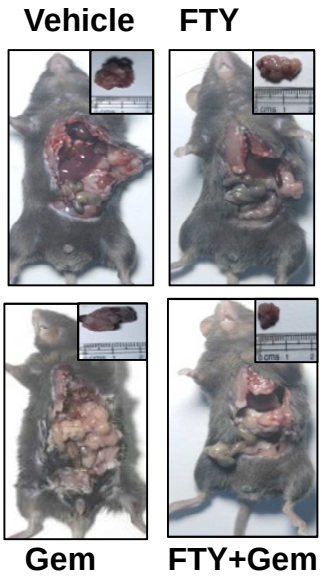
C


Vehicle
FTY720
Gem
FTY+Gem
4.0
3.0
2.0
1.0
10.0
5.0
40.0
20.0
CCL22
CCL4
CXCR3
IL23A
PDCD1
GBP1
CXCR5
IFNG
IDO1
CXCR1

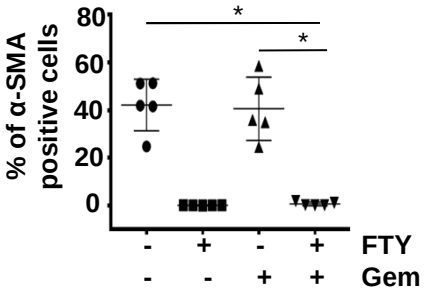
A


6.00E+05
4.00E+05
2.00E+05
0.00E+05
Counts
Day 15
Day 30
Vehicle
FTY
Gem
FTY+Gem

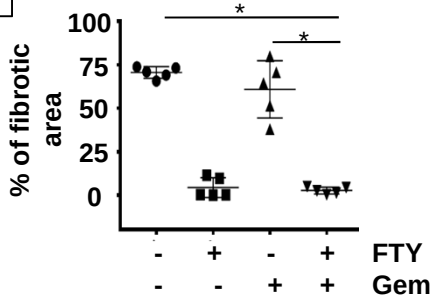
B


Vehicle
FTY
Gem
FTY+Gem

C


% of α-SMA positive cells
80
60
40
20
0
*
*
FTY
Gem

D


% of fibrotic area
100
75
50
25
0
*
*
FTY
Gem