Additional files

Preclinical Proof-of-Concept Analyses

Preclinical studies were conducted to determine the change in gene expression in human peripheral blood cells after EP4 antagonism. To this end, the EP4-agonist PGE1-OH at a concentration of 100 nM was incubated for 3 or 24 hours with peripheral blood mononuclear cells (PBMCs) isolated from 5–6 healthy donors. Cells were harvested and analyzed by RNAseq. Genes with a false discovery rate of > 0.05 were excluded from analysis. Additionally, human PBMCs from healthy donors (n = 3) were incubated for 4 hours with PEG1-OH. mRNA was isolated from the cells and tumor necrosis factor (TNF)-α and C-X-C motif chemokine ligand (CXCL)10 transcripts were quantified by reverse transcription-polymerase chain reaction (RT-PCR).

Additional file 1:

Table S1. The TaqMan® Low Density Array (TLDA) gene-expression panels used for determination of the gene-expression profile in tumor biopsies (**A**) and whole blood (**B**).

A

TLDA gene-expression panel for tumor biopsies					
IL-1R1	CD40	CXCL2	IFNb	IRF5	REN
14-3-3	CD40LG	CXCL3	IFNG	IRF9	S100A4
ACTB[a]	CD45	CXCL5	IL-10	ITGAM	S100A9
AREG	CD45RO	CXCL9	IL-12A	ITGAX	TARC
ARG1	CD68	CXCR3	IL-13	LAG3	TBX21
CCL2	CD69	EOMES	IL-15	MCP1	TFRC[a]
CCL24	CD80	eotaxin	IL-17A	mrc1	TGFB1
CCL3	CD86	EREG	IL-1B	NR4A2	TIM3
CCL5	CD8b	fibronectin	CD70	OAS1	TNFa
CCR2	CD95	FOXP3	IL-2	PD-1	TNFR
CCR5	CREB	GAPDH[a]	IL-24	PD-L1	TNFSF10
CD163	CSF1	granzyme-B	IL-2RA	PD-L2	VCAM1
CD1C	CSF2	GUSB[a]	IL-4	perforin	VEGFA

CD244	CTLA4	ICAM1	IL-6	PTGER2	LGALS
CD28	CX3CL1	IDO1	IL-7	PTGER4	RORC
CD3E	CXCL1	IFIT1	IL-8	PTGES	
CD4	CXCL10	IFNa	iNOS	PTGS2	

B

TLDA gene-expression panel for whole blood					
AREG	CD4	CXCL1	IFNa	IL-12A	PTGES
ARG1	CD40	CXCL10	IFNb	iNOS	PTGS2
B7-H3	CD40LG	CXCL2	IFNG	IRF5	REN
B7-H4	CD45	CXCL5	IL-10	ITGAM	RORC
CCL2	CD45RO	CXCL9	IL-13	ITGAX	S100A4
CCL22	CD68	CXCR3	IL-17A	KLRK1	S100A9
CCL5	CD69	EOMES	IL-17F	LAG3	TGFB1
CCR2	CD70	EPCAM	IL-1B	LGALS1	TBX21
CCR5	CD80	EREG	IL-2	mrc1	TFRC
CCR7	CD86	FOXP3	IL-24	MS4A1	TIM3
CD1C	CD8A	GAPDH[a]	IL-2RA	PD-1	TNF
CD163	CLEC4C	granzyme-A	IL-4	PD-L1	TNFSF10
CD244	CSF1	granzyme-B	IL-6	PD-L2	VCAM-1
CD27	CSF2	GUSB[a]	IL-7	perforin	VEGFA
CD28	CTLA4	ICAM1	IL-7R	PTGER2	
CD3E	CX3CL1	IDO1	IL-8	PTGER4	

[a]Denotes control gene.

Additional file 2:

Table S2. Sample collection schedule for biomarker and pharmacokinetic analysis.

Biomarker sample	Time points	Analyses
Serum / plasma protein	Predose C1D1, 24 hours after first dose, C1D8, C1D15	TAM / MDSC-related cytokines/chemokines
Blood mRNA	Predose C1D1, 24 hours after first dose, C1D8, C1D15	Immune gene expression
Paired tumor biopsy	Predose and during C2	T cell and macrophage infiltration
Whole blood	Baseline, predose C1D1, C1D8, C1D15, C2D1, C3D1, C5D1	Circulating TBNK and MDSC

C#, cycle #; D#, day#; MDSC, myeloid-derived suppressor cell; mRNA, messenger ribonucleic acid; TAM, tumor-associated macrophages; TBNK, T cells, B cells, and natural killer cells.

Hong DS, *et al. J Immunother Cancer* 2020; 8:e000222. doi: 10.1136/jitc-2019-000222

Additional file 3:

Table S3. The immune-cell–related biomarker panel used for determination of serum protein levels.

Immune-cell–related biomarker panel			
IFN-γ	TNF-α	TNF-β	MIP-1β
IL-10	GM-CSF	VEGF-A	TARC
IL-12p70	IL-12p40	Eotaxin (CCL11)	Renin
IL-13	IL-15	Eotaxin-3	sCD163
IL-1β	IL-16	IL-8 CP[a]	CCL5
IL-2	IL-17	IP-10 (CXCL10)	CXCL1
IL-4	IL-1α	MCP-1 (CCL2)	CXCL2
IL-6	IL-5	MCP-4 (CCL13)	CXCL5
IL-8 (CXCL8)	IL-7	MIP-1α	Arginase 1

[a]IL-8 and IL-8 CP are the same cytokine measured in different assay panels

CCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; IL-8 CP, interleukin-8 chemokine panel; IP, interferon γ-induced protein; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; sCD163, soluble cluster of differentiation 163; TARC, thymus and activation-regulated chemokine; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

Additional file 4:

Table S4. Summary of clinical pharmacokinetic parameters for E7046 and the M1 metabolite (cycle 1 day 8).

Parameter Mean (SD)[a]	E7046 Dose, mg			
	125 (n = 5)	**250 (n = 8)**	**500 (n = 7)**	**750 (n = 7)**
E7046				
C_{max} (ng/mL)	177.9 (105.87)	604.0 (549.58)	1516.3 (1193.88)	1003.3 (527.67)
$AUC_{(0-t)}$, ng•h/mL	962.8 (740.83)	2270.0 (909.87)	6060.0 (2364.85)	5934.3 (2431.38)
$t_{1/2}$ (h)	11.1 (0.00)	8.9 (1.01)	6.1 (3.45)	8.7 (1.86)
T_{max} (h)[a]	4.7 (0.5–24.0)	0.5 (0.5–2.0)	2.0 (0.5–4.1)	2.0 (0.5–10.0)
M1				
C_{max} (ng/mL)	4572.8 (2668.29)	14936.3 (8547.71)	21228.6 (8268.15)	25385.7 (12655.09)
$AUC_{(0-t)}$, ng•h/mL	31316.0 (14868.70)	100662.5 (44561.19)	176057.1 (92837.62)	238571.4 (85379.27)
$t_{1/2}$ (h)	10.2 (0.00)	10.0 (1.87)	6.4 (6.13)	8.6 (0.92)
T_{max} (h)[a]	4.7 (1.0–24.0)	1.1 (0.5–4.0)	2.0 (1.0–4.1)	2.7 (1.0–10.0)

AUC, area under the plasma concentration-time curve; C_{max}, maximum concentration; t_{max}, time at maximum concentration occurs; $t_{1/2}$, elimination half-life; SD, standard deviation.

[a]Except as noted, t_{max} is reported as median (range)

Additional file 5:

Table S5. Efficacy results by E7046 dose cohort by irRECIST.

Patients, n (%)	E7046 Dose, mg				
	125 (n = 8)	**250 (n = 8)**	**500 (n = 7)**	**750 (n = 7)**	**Combined (N = 30)**
Best overall response					
Complete response	0	0	0	0	0
Partial response	0	0	0	0	0
Stable disease	2 (25)	2 (25)	0	3 (42.9)	7 (23.3)
Progressive disease	3 (37.5)	3 (37.5)	4 (57.1)	3 (42.9)	13 (43.3)
Unknown / not evaluable	3 (37.5)	3 (37.5)	3 (42.9)	1 (14.3)	10 (33.3)
Objective response rate	0	0	0	0	0
Disease control rate[a] (95% CI)	2 (25)	2 (25)	0	3 (42.9)	7 (23.3)
Median overall survival, months (95% CI)	4.3 (1.5, 14.0)	4 (1.1, 9.5)	3.7 (2.8, 3.8)	5.1 (1.3, 8.8)	---
Median progression-free survival, months (95% CI)	1.5 (1.1, 4.0)	1.4 (1.1, 4.1)	1.3 (1.2, 1.4)	1.3 (0.9, NE)	---
Death	0	1 (12.5)	0	0	1 (3.3)

[a]Disease control rate, irCR + irPR + irSD ≥ 5 weeks.

CI, confidence interval; irCR, complete response by irRECIST criteria; irPR, partial response by irRECIST criteria; irSD, stable disease by irRECIST criteria; irRECIST, immune-related Response Evaluation Criteria In Solid Tumors.

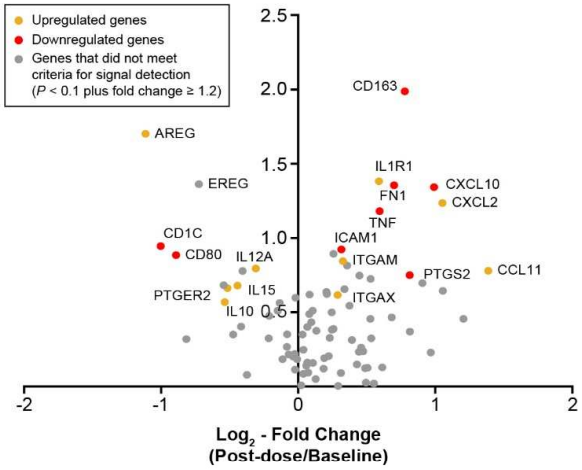
Additional file 6:

Fig. S1 Gene-expression analysis of 92 EP4-regulated and immune-related genes (A) Patients treated with E7046 had paired tumor biopsies evaluable for analysis (B)A volcano plot shows the change in gene expression for 90 examined genes from baseline to post-dose (C) The changes in gene expression levels from baseline to post-dose (C2D1) for *TNF-α* and *CXCL10* in paired tumor biopsies as measured by quantitative RT-PCR (TLDA assay, Thermo Fisher Scientific) are shown

A

Tumor Type	Dose (mg)	Clinical Response
Pancreatic Cancer	125	PD
Colorectal Cancer	250	PD
Hepatocellular Carcinoma	250	Long SD
Pancreatic Cancer	250	PD
Colorectal Cancer	500	PD
Lung Cancer	500	PD
Colorectal Cancer	750	Long SD
Colorectal Cancer	750	PD
Pancreatic Cancer	750	Short SD
Pancreatic Cancer	750	PD

B



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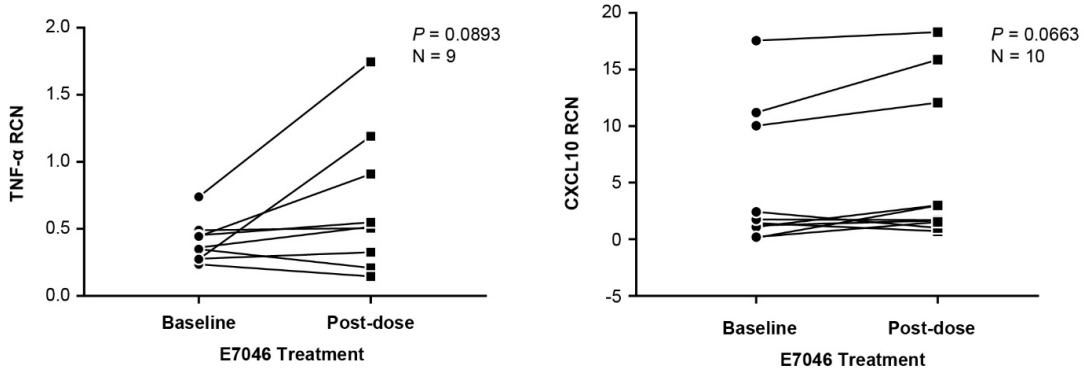
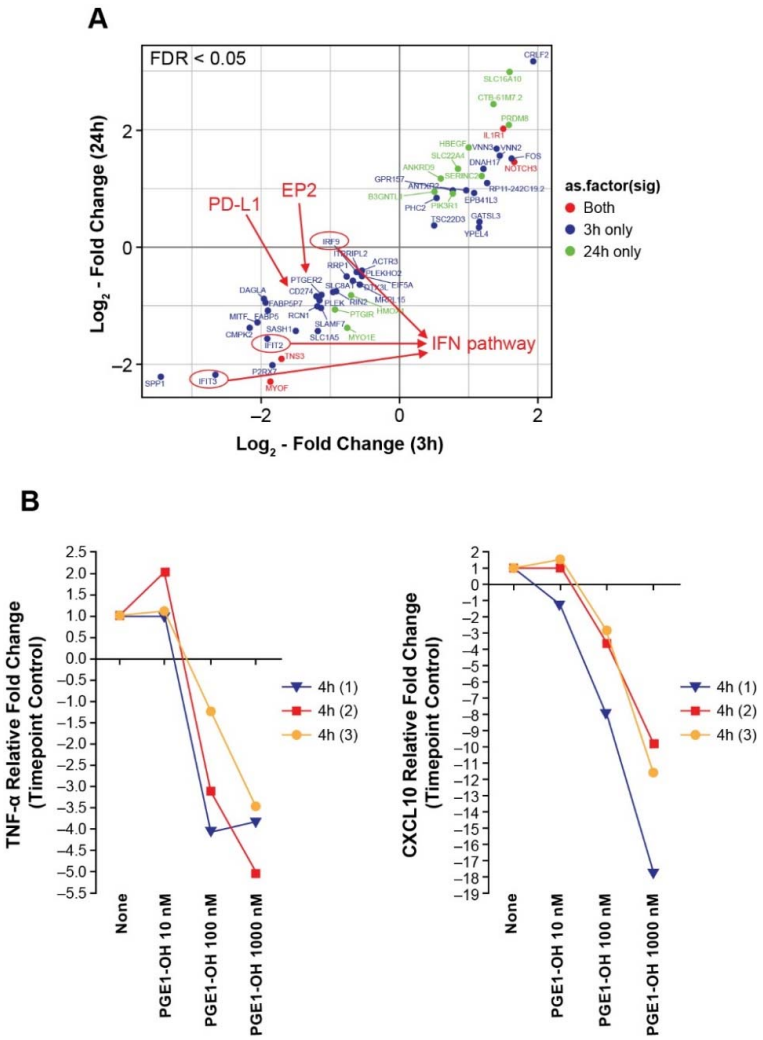


PD, progressive disease; RCN, relative copy number; RT-PCR, reverse transcription polymerase chain reaction; SD, stable disease; TLDA, TaqMan® Low Density Array; TNF, tumor necrosis factor.

Additional file 7:

Fig. S2. (**A**) Antagonism of EP4 in human PBMCs from healthy donors (N=5-6) for 3 hours or 24 hours with the PGE1-OH (100 nM) induced a change in gene expression levels of several genes; (**B**) Human PBMCs from 3 healthy donors were incubated for 4 hours with PEG1-OH and the gene expression levels of *TNF-α* and *CXCL10* as assessed by quantitative RT-PCR are shown.

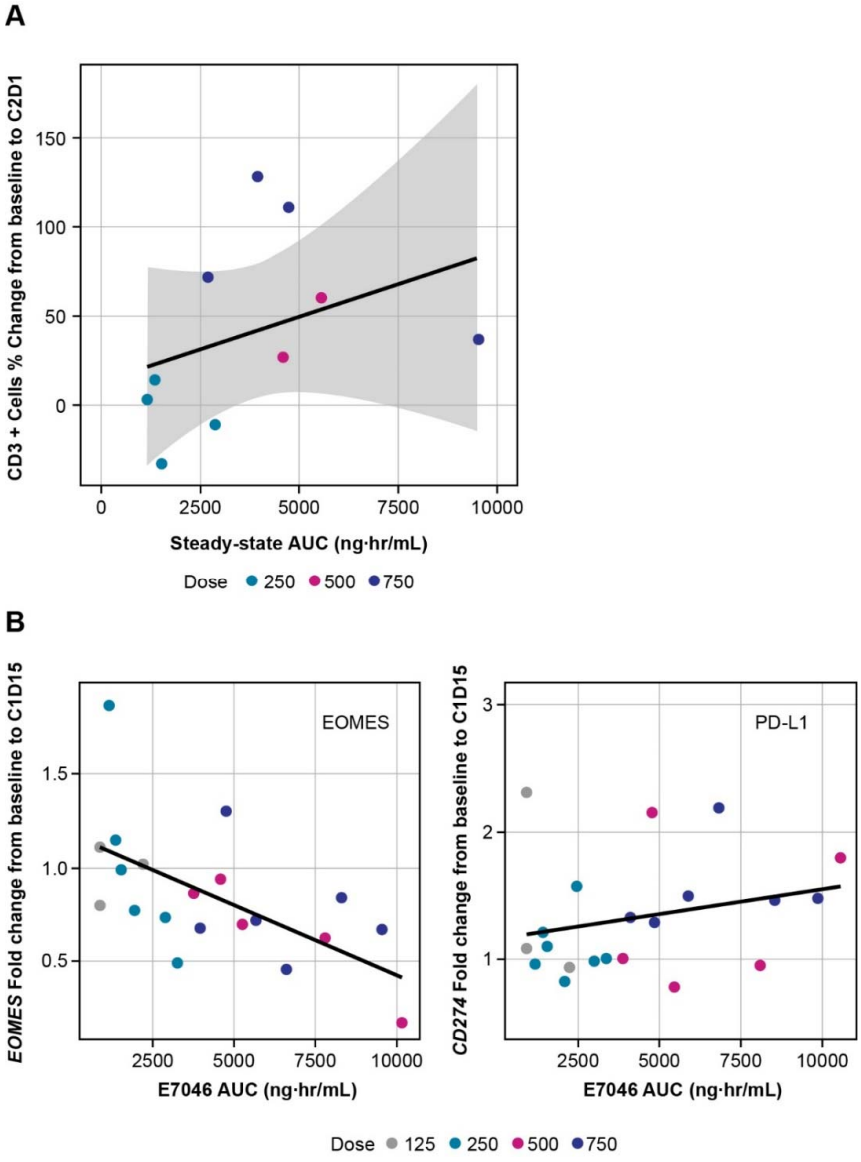


In the key of part A, "Both" indicates that the changes were observed at 3h and 24 h.

PBMC, peripheral blood mononuclear cells; RT-PCR, reverse transcription polymerase chain reaction; TNF, tumor necrosis factor.

Additional file 8:

Fig. S3. (A) Increased T cell infiltration in paired tumor biopsies from baseline to C2D1 (**B**) and the changes in the blood biomarker expression levels of *EOMES* (left) and *CD274* (right; gene encoding PD-L1), versus the extent of E7046 exposure from baseline to C1D15 are shown



AUC, area under the plasma concentration-time curve; C, cycle; CD, cluster of differentiation; D, day; EOMES, eomesodermin; PD-L1, programmed death ligand 1.