TLR2 signaling regulates T cell exclusion in pancreatic ductal adenocarcinoma

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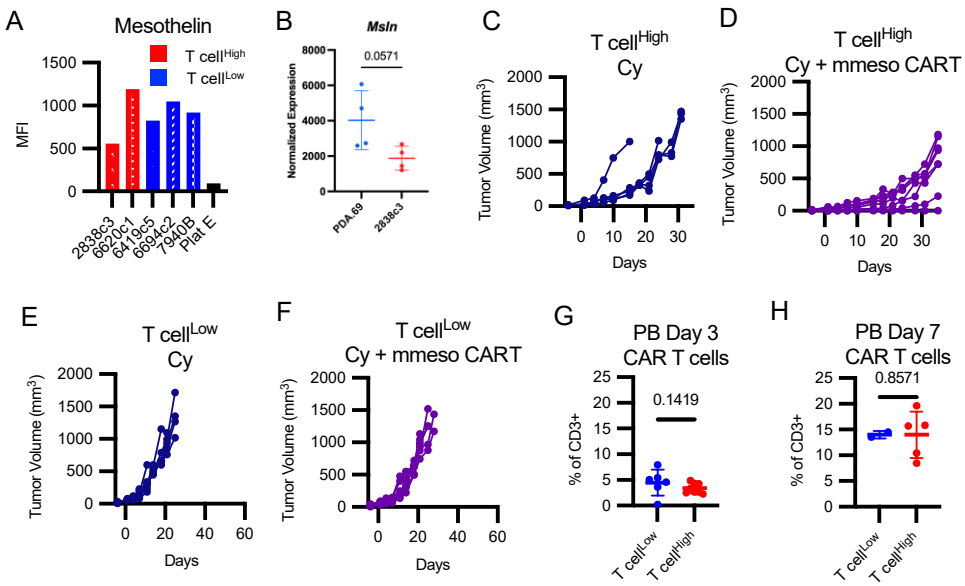
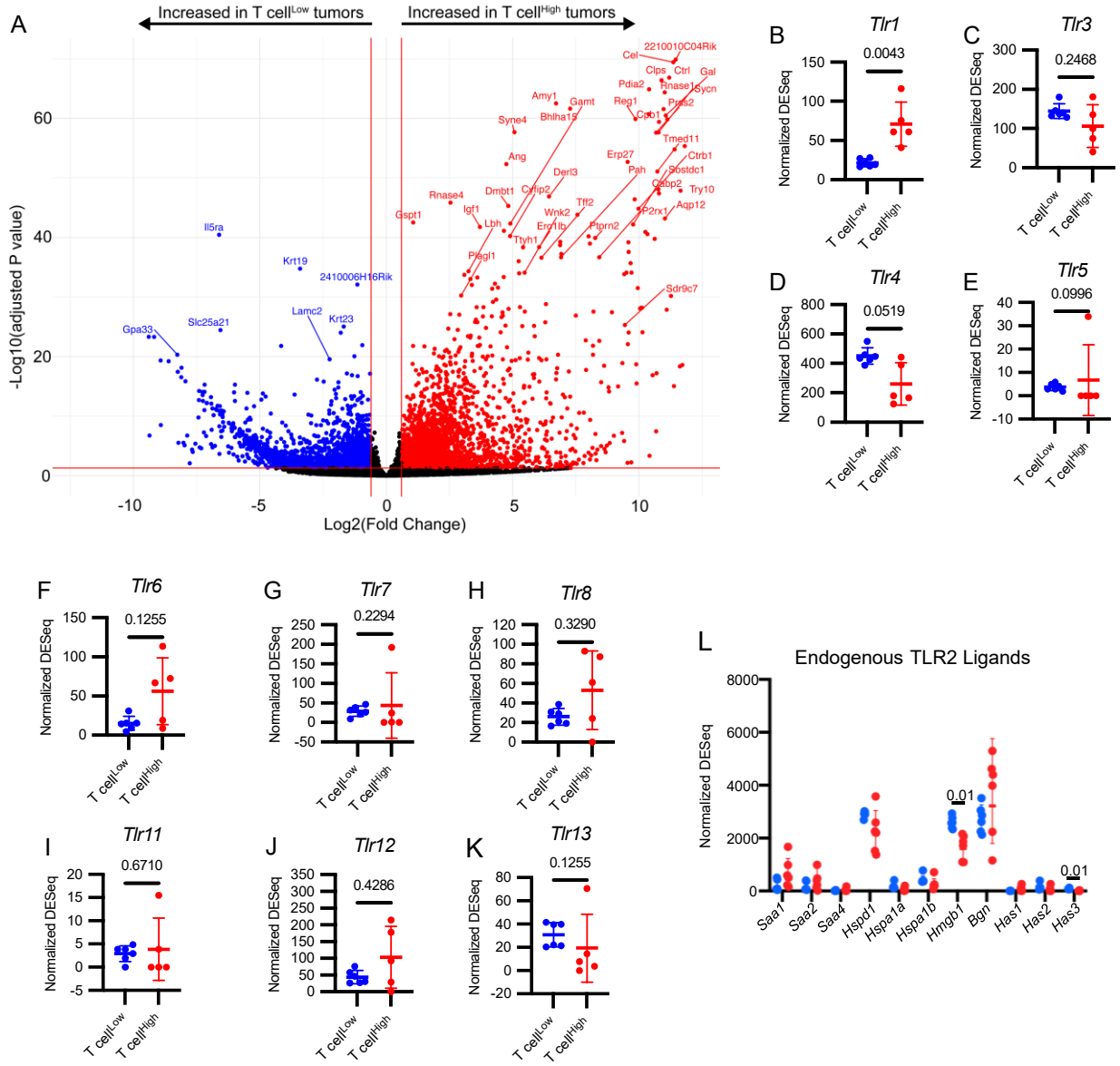
SUPPLEMENTAL MATERIALS

Supplemental Figure 1



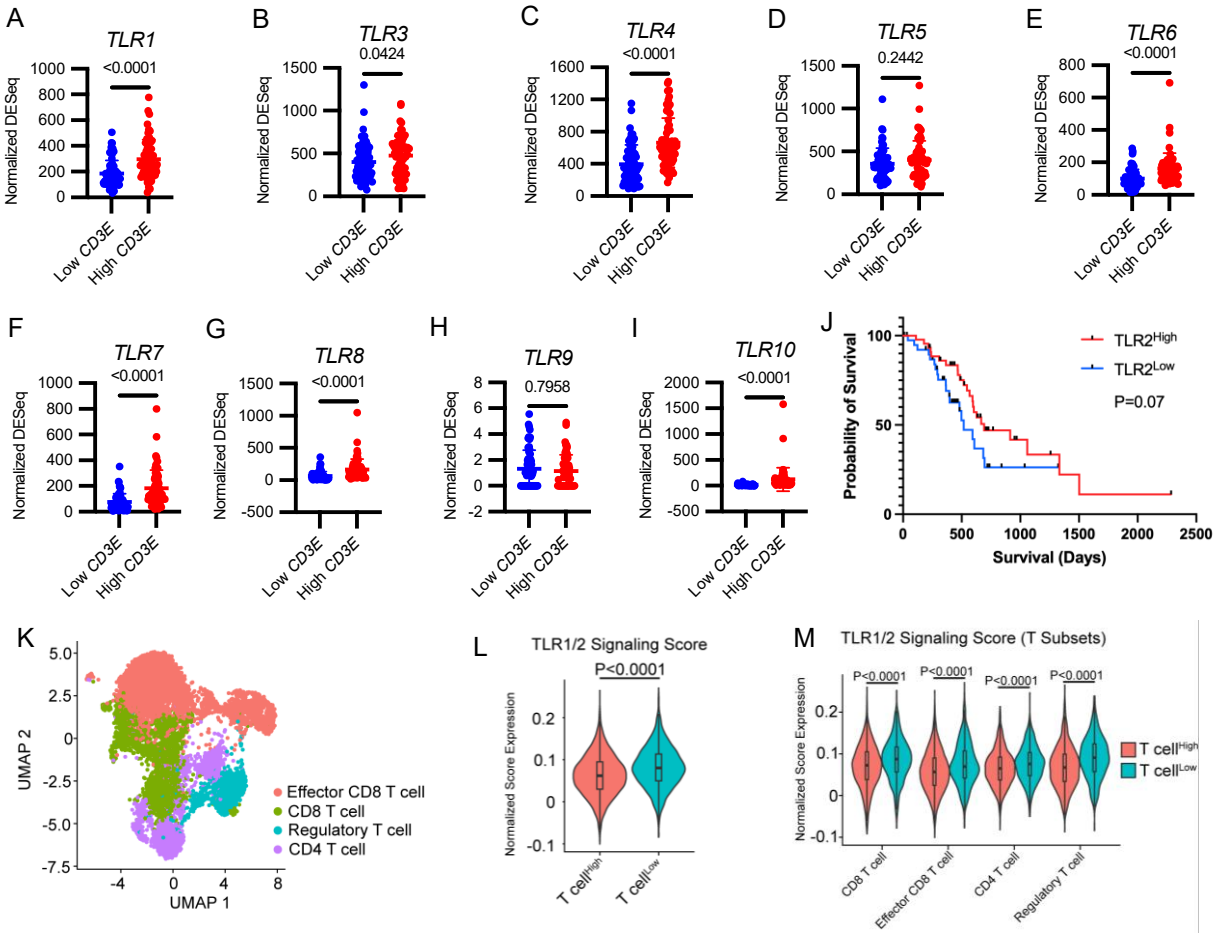
Supplemental Figure 1. Mesothelin expression and CART cell dynamics in T cell^{High} and T cell^{Low} PDAC models. A) Mesothelin MFI expression in T cell^{High} and T cell^{Low} cell lines. Plat E is a engineered 293T cell which does not express mesothelin. **B)** *Msln* expression measured by qRT-PCR in tumors derived *in vivo* from PDA.69 (T cell^{Low}) compared to 2838c3 (T cell^{High}). Mann Whitney test performed. **C- F**) Mice were treated as in Figure 1B. Individual tumor curves . **G)** Day 3 and **H**) Day 7 of CAR T cell expansion in peripheral blood from mice who received either T cell^{High} or T cell^{Low} tumors (Mann Whitney test performed).

Supplemental Figure 2



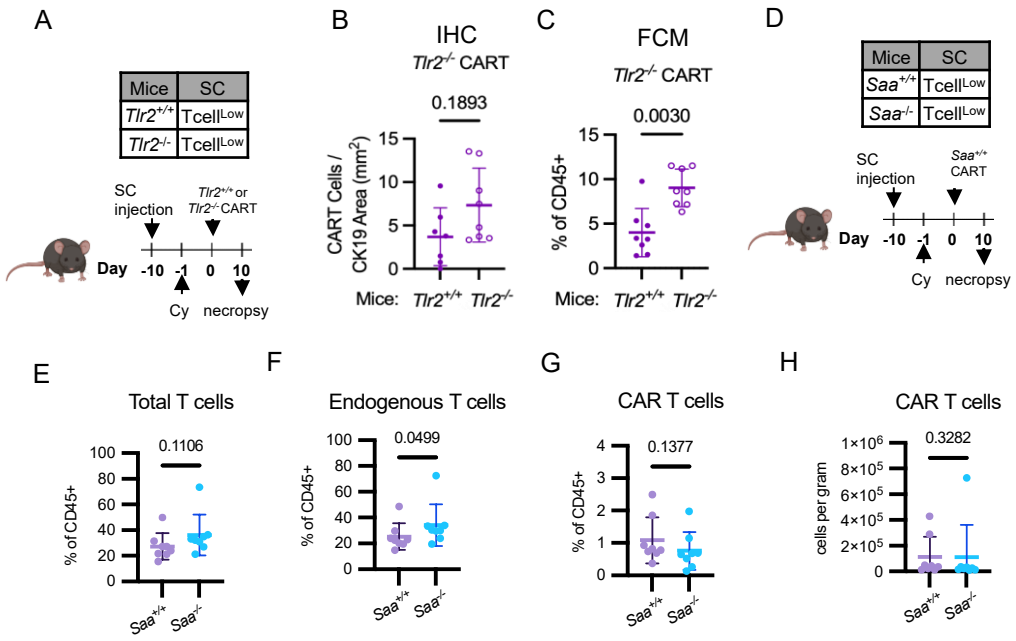
Supplemental Figure 2. Bulk RNA sequencing in T cell^{High} and T cell^{Low} mouse PDAC. A) Volcano plot showing upregulated genes in T cell^{High} and T cell^{Low} tumors. **B)** Normalized *Tlr1*, **C)** *Tlr3*, **D)** *Tlr4*, **E)** *Tlr5*, **F)** *Tlr6*, **G)** *Tlr7*, **H)** *Tlr8*, **I)** *Tlr11*, **J)** *Tlr12*, **K)** *Tlr13* DESeq expression from bulk RNA-seq data from Figure 2A-C (Mann Whitney tests performed). **L)** Normalized endogenous TLR2 ligands DESeq expression from bulk RNA-seq data from Figure 2A-C (Mann Whitney test with correction for multiple comparisons performed).

Supplemental Figure 3



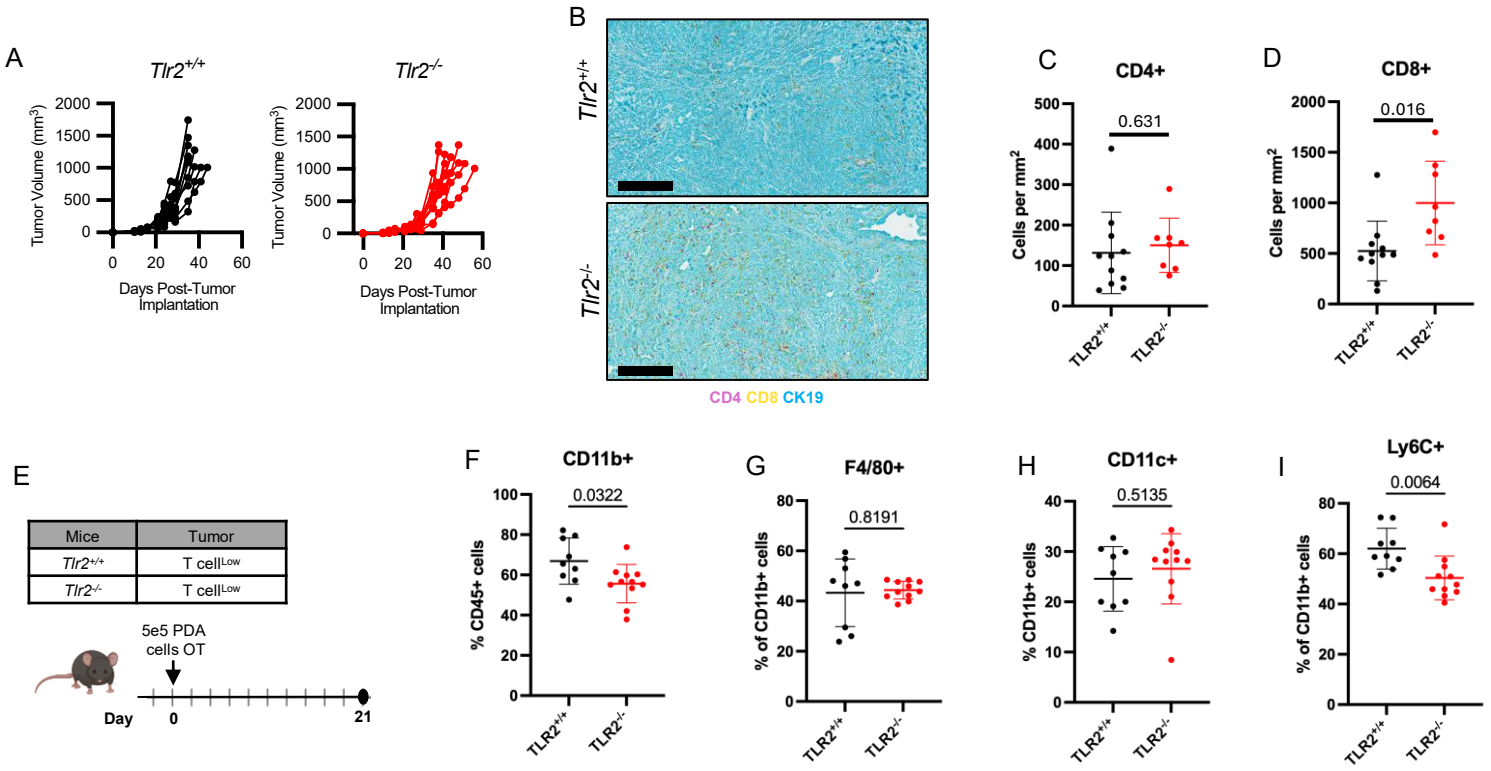
Supplemental Figure 3. TLR molecule expression and signaling in human PDAC. A) Normalized *TLR1*, **B)** *TLR3*, **C)** *TLR4*, **D)** *TLR5*, **E)** *TLR6*, **F)** *TLR7*, **G)** *TLR8*, **H)** *TLR9*, **I)** *TLR10* RNA expression of high (quartiles 3,4) versus low (quartiles 1,2) *CD3E* gene expression from TCGA data in Figure 2H-J. Mann Whitney tests performed. **J)** Kaplan-Meier curve showing overall survival of high CD3E tumor expressing patients subdivided by $TLR2^{High}$ and $TLR2^{Low}$ expression. Log-Rank test performed. **K)** UMAP clustering showing T cell population from Figure 2L. **L)** TLR1/2 signaling score in T cells between $T\ cell^{High}$ and $T\ cell^{Low}$ tumors. TLR1/2 signaling score derived from 115 genes in the "Reactome Toll Like Receptor TLR1 TR2 Cascade" gene set. Mann Whitney test performed. **M)** TLR1/2 signaling score between T cell subsets identified in J. Mann Whitney test performed.

Supplemental Figure 4



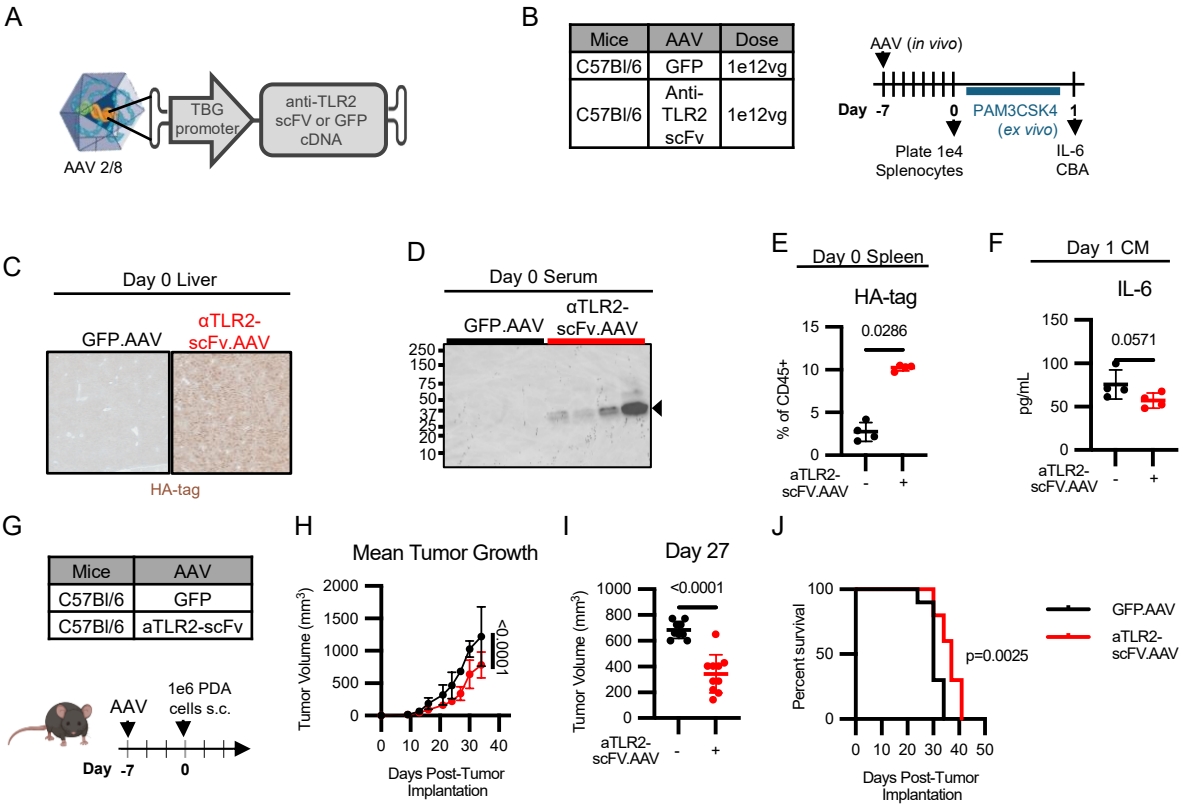
Supplemental Figure 4. Impact of SAA and TLR2 deficiency on endogenous T cell and meso-CAR T cell infiltration in T cell^{Low} PDAC model. A) Study design of G and H. T cell^{Low} cells (1e6) were implanted subcutaneously into *Saa*$^{+/+}$ and *Saa*$^{-/-}$ mice on day -10. Each group consisted of 8 mice. On day -1, mice received cyclophosphamide (120mg/kg dose, i.p.) and received meso *Tlr2*$^{+/+}$ or *Tlr2*$^{-/-}$ CAR-T cells (5-8e6 cells/mouse, i.v.) on days 0 with necropsy 10 days later. **B)** Immunohistochemistry showing Tlr2$^{-/-}$ CAR-T cell infiltration into PDAC tumors of *Tlr2*$^{+/+}$ and *Tlr2*$^{-/-}$ mice. **C)** Flow cytometry showing Tlr2$^{-/-}$ CAR-T cell infiltration into PDAC tumors of *Tlr2*$^{+/+}$ and *Tlr2*$^{-/-}$ mice. **D)** Study design of B-E. T cell^{Low} cells (1e6) were implanted subcutaneously into *Saa*$^{+/+}$ and *Saa*$^{-/-}$ mice on day -10. Each group consisted of 8 mice. On day -1, mice received cyclophosphamide (120mg/kg dose, i.p.) and received meso *Saa*$^{+/+}$ CAR-T cells (5-8e6 cells/mouse, i.v.) on days 0 with necropsy 10 days later. **E)** Analysis of Total T cell, **F)** Endogenous T cell, and **G)** CAR T cell percentages in the tumors via flow cytometry (Mann Whitney tests performed). **H)** Analysis of CAR T cells per gram in the tumors via flow cytometry (Mann Whitney tests performed).

Supplemental Figure 5



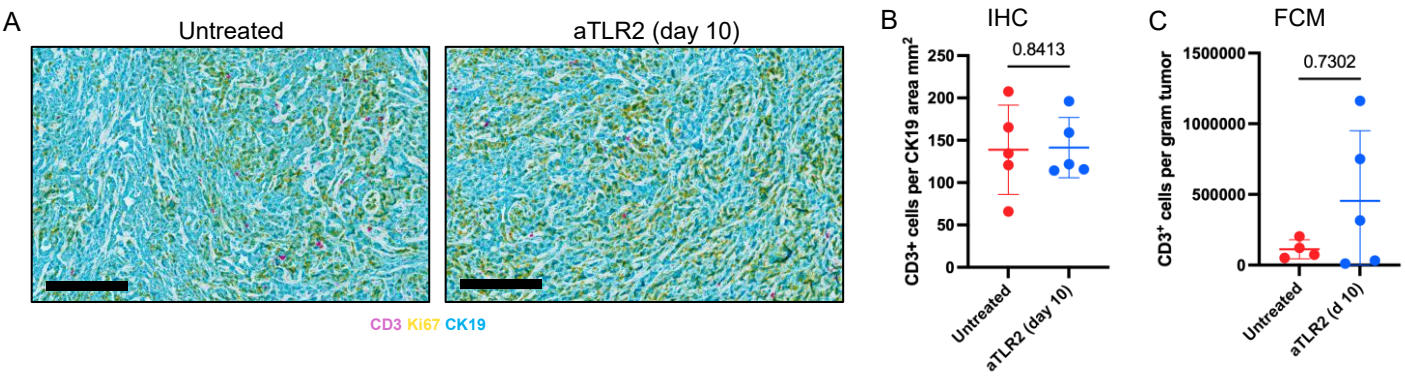
Supplemental Figure 5. T cell and myeloid cell profiling of T cell^{Low} tumors in Tlr2$^{+/+}$ vs Tlr2$^{-/-}$ mice. A) Mice were treated as in Figure 4A. Individual tumor curves are shown. **B)** Representative images of tumors stained for CD4 (pink), CD8 (yellow) and CK19 (blue). Scale bar, 300μm. **C)** Analysis of T cell (CD4 stained) per tumor area (CK19 stained) from A (Welch's test performed). **D)** Analysis of T cell (CD8 stained) per tumor area (CK19 stained) from A (Welch's test performed). **E)** Study schematic for F-G. T cell^{Low} (PDA.69) cells (5e5) were implanted orthotopically into *Tlr2*$^{+/+}$ or *Tlr2*$^{-/-}$ mice (n=9-11 mice/group) on Day 0. Data shown are representative of n=1 biological replicates. **F)** Analysis of CD11b+ myeloid cells as a percentage of CD45 cells in the tumors via flow cytometry (Mann Whitney test performed). **G)** Analysis of F4/80+ macrophages as a percentage of CD11b+ cells in the tumors via flow cytometry (Mann Whitney test performed). **H)** Analysis of CD11c+ dendritic cells as a percentage of CD11b+ cells in the tumors via flow cytometry (Mann Whitney test performed). **I)** Analysis of LY6c+ monocytes as a percentage of CD11b+ cells in the tumors via flow cytometry (Mann Whitney test performed).

Supplemental Figure 6



Supplemental Figure 6. Characterization and efficacy of TLR2-blocking scFv in PDAC model. A) Design of an AAV to express a TLR2 blocking scFv with an HA tag (αTLR2-scFv) in hepatocytes. **B)** Study schematic. Mice were treated with 1e12 viral genomes (vg) of the AAVs indicated. 7 days later, splenocytes were harvested and treated *ex vivo* with TLR2 agonist PAM3CSK4 (1000ng/mL) **C)** Immunohistochemistry showing expression of αTLR2-scFv in hepatocytes of mice treated as in (B). Livers were stained for HA. **D)** Western blot detecting αTLR2-scFv in serum of mice treated as in (B). Membranes were blotted for HA. Arrow indicates anti-TLR2-scFv at 27 kDa. **E)** Spleens were analyzed by flow cytometry for binding of αTLR2-scFv to splenocytes. scFv binding is accessed by detection of HA. **F)** IL-6 in media of splenocytes harvested from mice as in (B) and treated with PAM3CSK4 overnight. **G)** Study schematic. Mice were treated with AAVs (GFP or aTLR2-scFv). 7 days later, T cell^{Low} tumor cells were subcutaneously implanted. Mice were monitored for overall survival. **H**) Mean tumor growth curves (two-way ANOVA test performed) **I)** Tumor volume Day 27 (Mann Whitney test performed). **J)** Overall survival (Mantel Cox test performed).

Supplemental Figure 7



Supplemental Figure 7. Characterization of T cell tumor infiltration in therapeutic anti-TLR2 antibody treatment. A) Representative IHC images of untreated and anti-TLR2 treated mice. Anti-TLR2 treatment started on day 10 and was dosed at 0.2mg every 3-4 days. Tumors were collected on day 15. **B)** Quantification of A. Mann-Whitney test performed. **C)** Flow cytometry of tumors staining for $CD3^+$ cells from untreated and anti-TLR2 treated mice (day 10). Mann-Whitney test performed.

Supplemental Table 1

Application	Target Antigen/ Cell		Target Species	Host Species	Clone	Conjugation	Dilution	Vendor	Catalog #
Automated IHC	Primary Antibody	CD3	Mouse	Rabbit	Polyclonal	Unconj.	1:200	Abcam	ab5690
		Ki67	Mouse	Rabbit	D3B5	Unconj.	1:200	Cell Signaling	12202
		CK19	Mouse	Rabbit	EPNCIR127B	Unconj.	1:800	Abcam	ab133496
		GFP	Mouse	Goat	Polyclonal	Unconj.	1:300	Abcam	ab6673
		HA	Mouse	Chicken	Polyclonal	Unconj.	1:800	Abcam	ab1190
	Secondary Antibody	HQ	N/A	N/A	N/A	HRP	No dilution	Roche	760-4820
		IgG	Rabbit	Goat	Polyclonal	NP	No dilution	Roche	760-4817
		IgG	Rabbit	Goat	Polyclonal	HQ	No dilution	Roche	760-4815
		NP	N/A	Mouse	Monoclonal	AP	No dilution	Roche	760-4827
		IgG	Goat	N/A	N/A	HRP	No dilution	Roche	760-4647
		IgG	Chicken	Rabbit	Polyclonal	HRP	1:3000	Jackson ImmunoResearch	303-035- 003

Chromogen	Application(s) used	Vendor	Catalog #
Purple	Automated IHC	Roche	760-229
Teal	Automated IHC	Roche	760-247
Yellow	Automated IHC	Roche	760-239
DAB (Brown)	Automated IHC	Roche	760-159

Supplemental Table 1: Antibodies and reagents for immunohistochemistry.
IHC, immunohistochemistry

Supplemental Table 2

Application	Target Antigen/ Cell	Target Species	Host Species	Clone	Conjugation	Dilution	Vendor	Catalog #
FCM	CD11b	Mouse	Rat	M1/70	PerCp	1:100	BD Biosciences	561114
	CD11c	Mouse	Hamster	HL3	PE-Cy7	1:100	BD Biosciences	558079
	CD11c	Mouse	Hamster	N418	APC	1:100	BioLegend	117310
	CD11c	Mouse	Hamster	N418	FITC	1:100	BioLegend	117306
	CD19	Mouse	Rat	6D5	PB	1:100	BioLegend	115523
	CD3	Mouse	Rat	17A2	PE-Cy7	1:100	BioLegend	100220
	CD3	Mouse	Rat	17A2	PB	1:100	BioLegend	100214
	CD45	Mouse	Rat	30-F11	PerCp	1:100	BD Biosciences	550994
	CD45	Mouse	Rat	30-F11	PE-Cy7	1:100	BD Biosciences	552848
	CD45	Mouse	Rat	30-F11	APC-Cy7	1:100	BD Biosciences	561037
	CD45.1	Mouse	Mouse	A20	APC-Cy7	1:100	BioLegend	110716
	CD45.2	Mouse	Mouse	104	PE-Cy7	1:100	BioLegend	109830
	F4/80	Mouse	Rat	BM8	PB	1:100	BioLegend	123124
	F4/80	Mouse	Rat	BM8	APC	1:100	BioLegend	123116
	F4/80	Mouse	Rat	BM8	APC-Cy7	1:100	BioLegend	123118
	Goat anti-Rat	Rat	Goat	Poly4054	PE-Cy7	1:100	BioLegend	405413
	HA	N/A	Mouse	16B12	PE-Cy7	1:100	BioLegend	901528
	Ly6C	Mouse	Rat	HK1.4	PB	1:100	BioLegend	128014
	Ly6C	Mouse	Rat	HK1.5	FITC	1:100	BioLegend	128006
	Ly6G	Mouse	Rat	1A8	PB	1:100	BioLegend	127612
	Mesothelin	Mouse	Rat	B35	Unconjugated	1:200	Isbio	LS-C179484-100
	TLR2	Mouse	Rat	CB225	PE	1:100	BioLegend	148604

Supplementary Table 2: Antibodies for flow cytometry.
FCM, flow cytometry

Supplemental Table 3

Gene	Forward Sequence	Reverse Sequence
ltr	GGAACCCCTAGTGATGGAGTT	CGGCCTCAGTGAGCGA

Supplementary Table 3: Primer sequences.

Supplemental Table 4

Supplemental Table 4: Top 100 differentially expressed genes between T cell^{Low} vs T cell^{High} tumors.

Gene ID	Log2(Fold Change)	Log2(Fold Change) SE	P value	Adjusted P value
Hamp2	10.02095329	0.422064518	1.31E-124	2.13E-120
Cela2a	11.78088221	0.574808499	2.37E-93	1.93E-89
Cela1	10.98340098	0.543259789	6.86E-91	3.72E-87
Cckar	10.92199865	0.578754836	1.95E-79	7.95E-76
Tmed6	9.280159594	0.506629646	6.01E-75	1.96E-71
Pla2g1b	10.70418347	0.585181145	9.57E-75	2.60E-71
Chst2	6.209435988	0.340818529	3.64E-74	8.45E-71
2210010C04Rik	11.45159128	0.629720499	6.76E-74	1.38E-70
Cel	11.3556429	0.626607695	2.12E-73	3.83E-70
Ctrl	11.19432734	0.629322607	8.77E-71	1.43E-67
Clps	10.88934166	0.614334038	2.67E-70	3.95E-67
Pdia2	10.39605771	0.593266509	9.49E-69	1.29E-65
Rnase1	11.01596442	0.631258013	3.39E-68	4.25E-65
Amy1	6.718569909	0.390620217	2.67E-66	3.10E-63
Bhlha15	7.274057322	0.42596115	2.21E-65	2.40E-62
Cpb1	10.96876012	0.642755894	2.69E-65	2.74E-62
Cuzd1	10.382839	0.612639126	2.00E-64	1.91E-61
Gal	11.05718312	0.653728186	3.55E-64	3.21E-61
Reg1	9.861482965	0.585964648	1.48E-63	1.27E-60
Zg16	11.12281637	0.661386773	1.82E-63	1.48E-60
Cpa1	10.78570951	0.643584756	4.88E-63	3.78E-60
Syne4	5.071715152	0.30712988	2.95E-61	2.18E-58
Sycn	10.76897142	0.652356836	3.22E-61	2.28E-58
Prss2	10.67254483	0.646807686	3.65E-61	2.47E-58
Try4	11.80423965	0.729445636	6.70E-59	4.36E-56
Pnlip	11.40934665	0.708745891	2.64E-58	1.65E-55
1810018F18Rik	12.44226346	0.781806062	5.01E-57	3.02E-54
Erp27	9.548608753	0.604634962	3.51E-56	2.04E-53
Ang	4.75131454	0.30191297	8.39E-56	4.71E-53
Tmed11	10.72420241	0.689581909	1.55E-54	8.40E-52
Cabp2	10.76565493	0.712650495	1.47E-51	7.70E-49
Try10	11.64154875	0.772684462	2.70E-51	1.37E-48
Cpa2	10.78649867	0.71946553	8.24E-51	4.06E-48
Derl3	6.441117489	0.431961884	2.78E-50	1.33E-47
Slc38a5	9.826867207	0.662277343	8.32E-50	3.87E-47
Rnase4	2.540181349	0.172219139	3.09E-49	1.40E-46
Dmbt1	4.828967161	0.329394293	1.16E-48	5.10E-46
Ctrb1	9.982584948	0.684387484	3.44E-48	1.47E-45
1810010D01Rik	7.560580508	0.524144274	3.62E-47	1.51E-44
Aqp12	11.02465064	0.769884833	1.64E-46	6.69E-44
Gspt1	1.066570157	0.075037426	7.52E-46	2.98E-43
Gamt	4.909675923	0.346177861	1.18E-45	4.55E-43
Sostdc1	9.766803203	0.689851165	1.67E-45	6.32E-43
Igf1	3.707953555	0.263236943	4.63E-45	1.71E-42
ambiguous	4.654445208	0.332923422	2.05E-44	7.41E-42
Cela3b	10.25173583	0.735059491	3.29E-44	1.16E-41
Spink1	10.33290522	0.744301246	8.07E-44	2.79E-41
Il5ra	-6.610190489	0.47671447	1.02E-43	3.44E-41
Cyfip2	4.899417975	0.354304292	1.72E-43	5.72E-41
Dusp26	8.004555857	0.579278746	1.98E-43	6.45E-41
Ptprn2	8.265879555	0.600253568	3.83E-43	1.22E-40
Cela3a	10.61873677	0.77254428	5.45E-43	1.70E-40
Cldn10	6.886072606	0.50401335	1.70E-42	5.22E-40
Pnliprp1	9.582680966	0.702252179	2.14E-42	6.46E-40
Hsd17b13	8.054333343	0.591797934	3.50E-42	1.03E-39
Egf	6.877982612	0.507099016	6.60E-42	1.92E-39
Ero1lb	6.049735999	0.448000311	1.48E-41	4.24E-39
Ttyh1	5.413192941	0.401014581	1.59E-41	4.46E-39
Klk1b5	9.587301001	0.717423392	9.88E-41	2.72E-38
Fam174b	6.92876811	0.520688709	2.11E-40	5.72E-38
Pah	6.915388572	0.523565532	7.86E-40	2.10E-37
P2rx1	8.427221588	0.638100595	8.02E-40	2.11E-37
Tff2	6.138213786	0.465251646	9.59E-40	2.48E-37
Prss8	5.236457417	0.399995371	3.69E-39	9.39E-37
Klk1	9.664382013	0.741679554	8.22E-39	2.06E-36
Krt19	-3.408891696	0.26494253	6.95E-38	1.71E-35
Lbh	3.252310488	0.254307271	1.89E-37	4.59E-35
Wnk2	5.47196898	0.429379292	3.37E-37	7.95E-35
Amy2b	9.702994433	0.76135428	3.35E-37	7.95E-35
Klk1b11	9.502666461	0.74589067	3.54E-37	8.24E-35
Casp9	5.256417051	0.413157497	4.43E-37	1.02E-34
Gp2	9.41380807	0.741501688	6.25E-37	1.41E-34
Cachd1	3.091499835	0.243942017	8.34E-37	1.86E-34
Ggh	4.649057312	0.368870424	2.02E-36	4.44E-34
Hnmt	3.604358789	0.286256839	2.36E-36	5.11E-34
Mgll	3.32838687	0.265436522	4.55E-36	9.74E-34
Nupr1	4.299310321	0.346762806	2.67E-35	5.63E-33
2410006H16Rik	-1.143641083	0.092460621	3.85E-35	8.03E-33
Tbc1d30	3.38265381	0.273772897	4.54E-35	9.35E-33
Slc39a5	4.422754308	0.360478627	1.33E-34	2.70E-32
Klk1b3	9.949883329	0.81173814	1.53E-34	3.08E-32
Edem2	4.189907618	0.342375952	1.95E-34	3.88E-32
Plagl1	2.962957841	0.246551322	2.87E-33	5.63E-31
Ctrc	11.26329808	0.938366504	3.42E-33	6.63E-31
Cbs	4.349952272	0.365988781	1.41E-32	2.70E-30
Sel1l	4.219819241	0.357253593	3.39E-32	6.42E-30
Klf15	6.316787626	0.538223789	8.30E-32	1.55E-29
Slc7a8	5.291087842	0.454603097	2.61E-31	4.83E-29
Anpep	3.843262484	0.330306137	2.72E-31	4.98E-29
Mknk1	3.57508864	0.307315302	2.79E-31	5.05E-29
Try5	10.09596579	0.86904932	3.37E-31	6.02E-29
Prom2	10.11917382	0.871439067	3.58E-31	6.33E-29
Pnliprp2	10.0392399	0.866557234	4.90E-31	8.57E-29
Fam46c	4.836827863	0.41792858	5.63E-31	9.74E-29
Klk1b8	11.09353054	0.960958808	7.89E-31	1.35E-28
Pnmal2	5.483950506	0.47757171	1.61E-30	2.72E-28
Lrrc7	8.197481507	0.714059117	1.66E-30	2.79E-28
Gabra4	8.927151588	0.779138345	2.15E-30	3.57E-28
Ptger3	4.599637381	0.401659053	2.31E-30	3.79E-28
Angpt1	2.531806575	0.222223697	4.53E-30	7.37E-28

Supplemental Table 5

Characteristics of the cohort	
n=179	
Demographic information	
Age	65 [57-73]
Sex (Male)	98 (54.7)
Race	
African American	7 (3.9)
Asian	10 (5.6)
Hispanic	5 (2.8)
White	157 (84.9)
Not reported	5 (2.8)
Tumor characteristics	
T stage	
T1	6 (3.4)
T2	23(12.8)
T3	144 (80.4)
T4	4 (2.2)
Not reported	2 (1.2)
N stage	
N0	48 (26.8)
N1	127 (70.9)
Not reported	4 (2.3)
M stage	
M0	81 (45.3)
M1	5 (2.8)
Not reported	93 (52.0)
Location	3 (60)
Head of pancreas	132 (73.7
Body of pancreas	15 (8.4)
Tail of pancreas	13 (7.3)
Overlapping lesion	2 (1.1)
Not specified	17 (9.5)
Note: Continuous data is presented as mean ± standard deviation while count data is shown as number (%) or median [IQR].	

Supplemental Table 5: Patient characteristics of dataset used for analysis of patients with pancreas cancer.