Supplemental Information

Blockade of Fas signaling in breast cancer cells suppresses tumor growth and metastasis via disruption of Fas signaling-initiated cancer-related inflammation

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Supplemental Table 1. Clinical characteristics of the patients

Variable	Cohort 1 (N=119)	Cohort 2 (N=30)	*P* Value
Age - year			0.724
Median	50	52	
Range	29-83	31-66	
Tumor size - no. (%)			0.347
< 5 cm	108 (83.1)	27 (90.0)	
≥ 5 cm	22 (16.9)	3 (10.0)	
Histological grade - no. (%)			0.097
< II	40 (30.8)	14 (46.7)	
≥ II	90 (69.2)	16 (53.3))	
LN invasion - no. (%)			0.546
Negative	122 (93.8)	29 (96.7)	
Positive	8 (6.2)	1 (3.3)	
TNM stage – no. (%)			0.475
I~I I	82 (63.1)	21 (70.0)	
III	48 (36.9)	9 (30.0)	

P value of less than 0.05 was considered to indicate statistical significance. P values were calculated in SPSS 17.0 using chi-square test, except for age, which was calculated using t-tests. Abbreviations: LN, lymph node; TNM, tumor-node-metastasis.

Supplemental Table 2. Univariate and multivariate analysis of factors associated with overall survival

Clinical variables	Cohort 1 N=119	
	Hazard ratio (95% CI)	P value
Univariate analysis		
Fas (higher versus lower) [1]	2.92 (1.42-5.96)	0.003
Age (≥50 versus<50 years)	1.11 (0.57-2.17)	0.753
Histological grade (>II versus I/II)	1.32 (0.63-2.75)	0.460
Tumor size (≥5 cm versus <5 cm)	2.09 (0.98-4.47)	0.056
LN invasion (yes versus no)	0.82 (0.42-1.59)	0.559
TNM stage (III versus I-II)	2.02 (1.03-3.96)	0.038
Multivariate analysis		
Fas (higher versus lower) [1]	2.45 (1.16-5.18)	0.018
Age (≥50 versus<50 years)	1.18 (0.58-2.38)	0.649
Histological grade (>II versus I/II)	1.37 (0.65-2.90)	0.399
Tumor size (≥5 cm versus <5 cm)	1.73 (0.73-4.08)	0.206
LN invasion (yes versus no)	0.38 (0.14-1.04)	0.058
TNM stage (III versus I-II)	2.68 (0.92-7.79)	0.069

Analysis was conducted on breast cancer patients of Cohort 1. Hazard ratios (95% confidence interval) and P values were calculated using univariate or multivariate Cox proportional hazards regression in SPSS 17.0.

Fas expression was measured by defining regions of interest (ROI) using automated cell acquisition and quantification software for immunohistochemistry (Histoquest). Staining intensity more than 5% was considered positive and more than 50% was considered higher Fas expression.

Abbreviations: CI, confidence interval; TNM, LN, lymph node; tumor-node-metastasis.

Supplemental Table 3. Fas expression and clinical characteristics of the patients

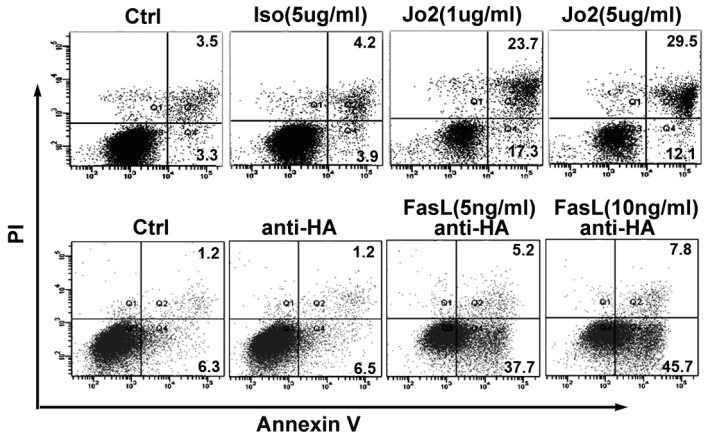
	Cohort1			Cohort2		
Variable	N/L	H	p	N/L	H	p
Age - year			0.745			0.789
< 50	30	25		4	7	
≥ 50	33	31		6	13	
Tumor size - no.			0.047			0.719
< 5 cm	56	42		9	18	
≥ 5 cm	7	14		1	2	
Histological grade - no.			0.647			0.650
< II	20	20		5	10	
≥ II	43	36		5	10	
LN invasion - no.			0.442			0.700
Negative	28	21		9	20	
Positive	35	35		0	1	
TNM stage – no.			0.000			0.012
I~I I	31	12		10	11	
III	25	51		0	9	

P values were calculated in SPSS 17.0 using Pearson chi-square test. And P value of less than 0.05 was considered to indicate statistical significance.

Abbreviations: N/L, negative/lower; H, higher; LN, lymph node; TNM, tumor-node-metastasis.

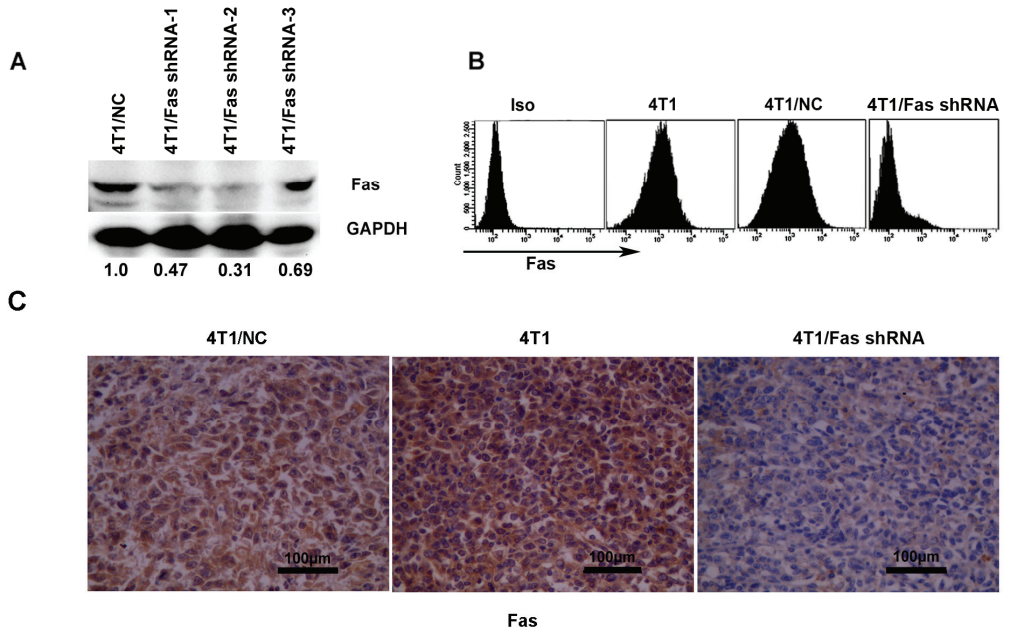
Fas expression was measured by defining regions of interest (ROI) using automated cell acquisition and quantification software for immunohistochemistry (Histoquest) in a FACS-like manner of scattergram analysis. Staining intensity more than 5% was considered positive and more than 50% was considered higher Fas expression.

Liu et al, Suppl. Figure 1



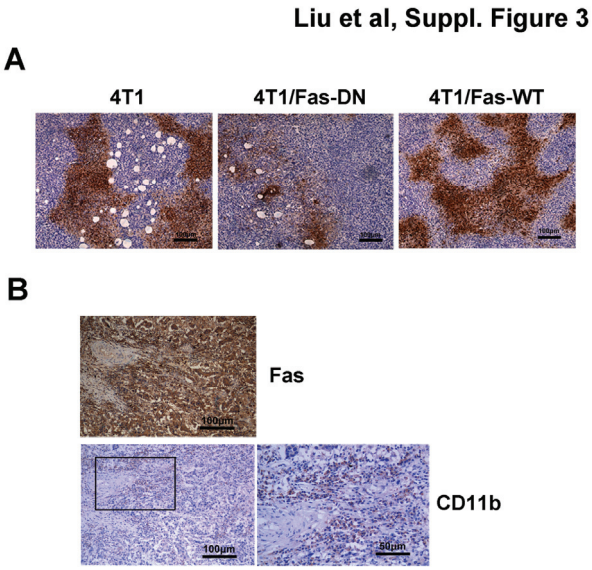
Supplemental Figure 1. Susceptibility of Fas-induced apoptosis in murine embryonic liver cells (BNL CL.2)

A. BNL CL.2 cells were treated with or without agonistic anti-Fas antibody, Jo2, or cross-linking Fas ligand at the indicated concentrations for 12 hours. Apoptosis cells were measured by staining with Annexin V-FITC and PI and detected by FACS.



Supplemental Figure 2. The expression of Fas in stably Fas-silenced 4T1 cells and tumor tissue derived from tumor-bearing mice

A, B. Expression of Fas in staby Fas-silenced 4T1 cell clones was identified by Western blot assay (A) and flow cytometry (B). C. Expression of Fas in tumor tissue derived from tumor-bearing mice was detected by immunohistochemistry (IHC). The results represent 3 independent experiments with similar results, bar represents 100μm.



Supplemental Figure 3. The infiltration and location of MDSCs in tumor tissue derived from tumor-bearing mice and human breast cancer tissues

A. $Gr1^+$ MDSCs infiltration and location in tumor tissue derived from tumor-bearing mice was detected by IHC. One representative image is shown, bar represents 100μm. B. One representative image of infiltration and location of $CD11b^+$ MDSCs in human breast cancer tissues with Fas higher expression.

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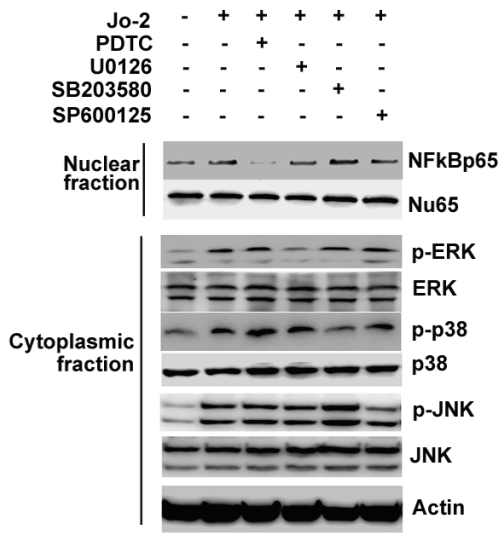
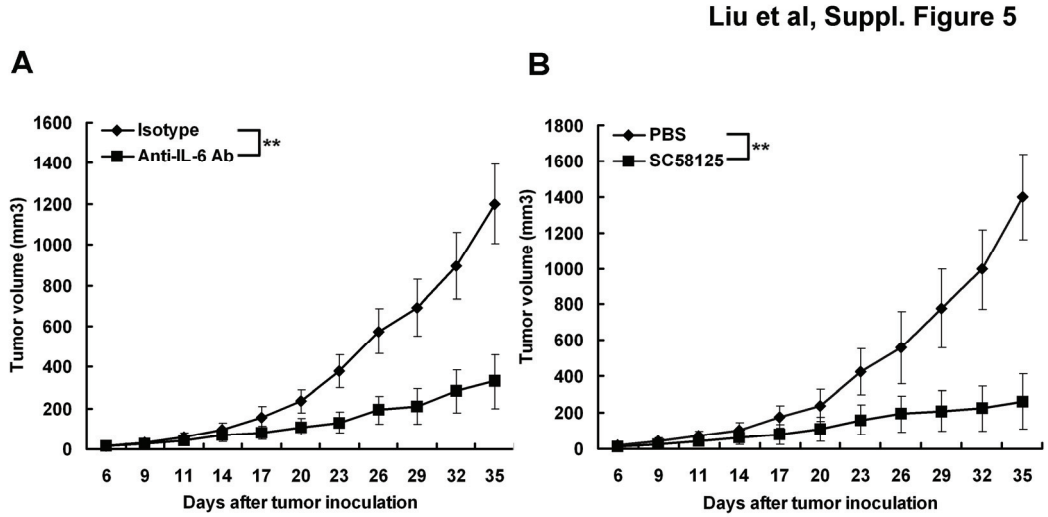


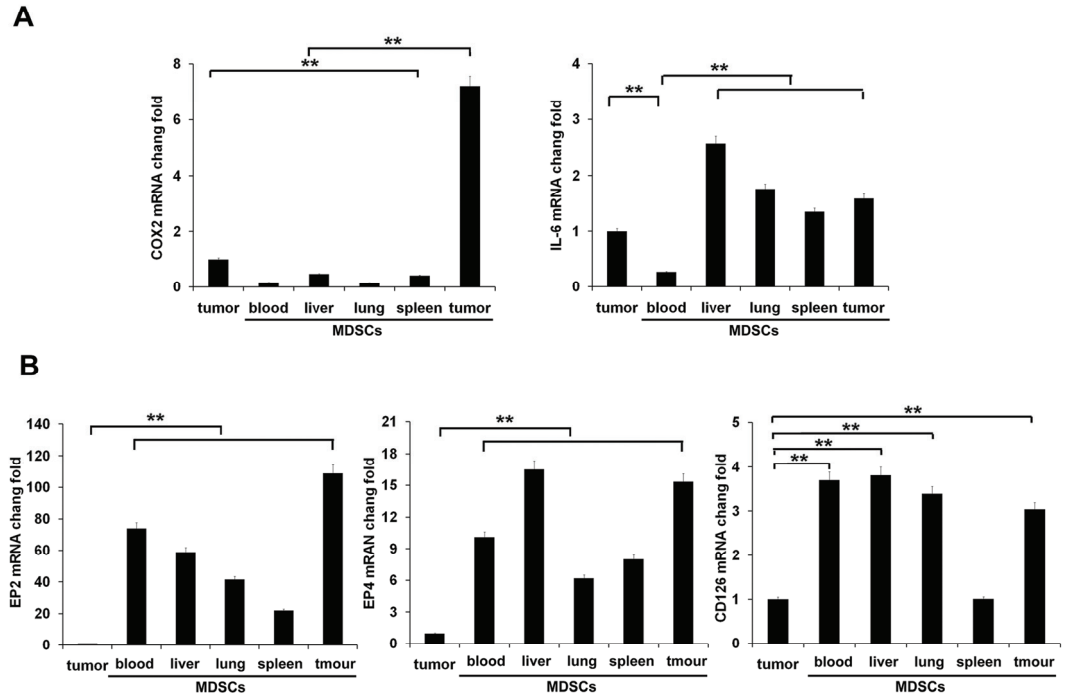
Supplemental Figure 4. Blocking effect of signaling inhibitors on MAPK and NF-κB signaling pathway in breast cancer cells

1×10^6 4T1 cells were pre-treated with MER1/2 inhibitor, U0126 (30μM); JNK/SAPK inhibitor, SP600125 (40μM); p38 MAPK inhibitor, SB203580 (30μM); and NF-κB inhibitor, PDTC (15μM), for 30 min prior to Jo2 simulation. The blocking effect of signaling inhibitors in MAPK and NF-κB signal pathways was detected by Western blot analysis.



Supplemental Figure 5. Suppression of tumor growth *in vivo* by anti-IL6 Ab or COX2 inhibitor

A, B. 5×10^5 4T1 cells were inoculated subcutaneously into the flank of BALB/c mice, 125μg anti-IL-6 Ab and control Isotype (mouse IgG) (A), or COX2 inhibitor SC58125 (5mg/kg) and PBS (B) were injected *i.p.* once a day for 14 days. Tumor growth was monitored and analyzed as described in the Materials and Methods. Tumor size was compared at various time points and *P* values are denoted. **, $p<0.01$.



Supplemental Figure 6. Expression of IL-6, COX2, and their receptors in primary tumor tissue and MDSCs derived from 4T1-bearing mice

A, B. 5×10^5 4T1/siFas and 4T1/NC cells were inoculated subcutaneously into the flank of BALB/c mice. 40 days later, peripheral blood, spleen, liver, lung, and draining lymph node (DLN) and tumor tissues from tumor-bearing mice were collected. The expression of IL-6, COX2 (A) and their receptors (CD126, EP2/EP4) (B) in primary tumor tissue and MDSCs sorting by $Gr1^+CD11b^+$ cells from liver, lung, spleen, peripheral blood, and primary tumor tissue derived from 4T1-bearing mice were analyzed by real-time PCR. **, $p<0.01$.