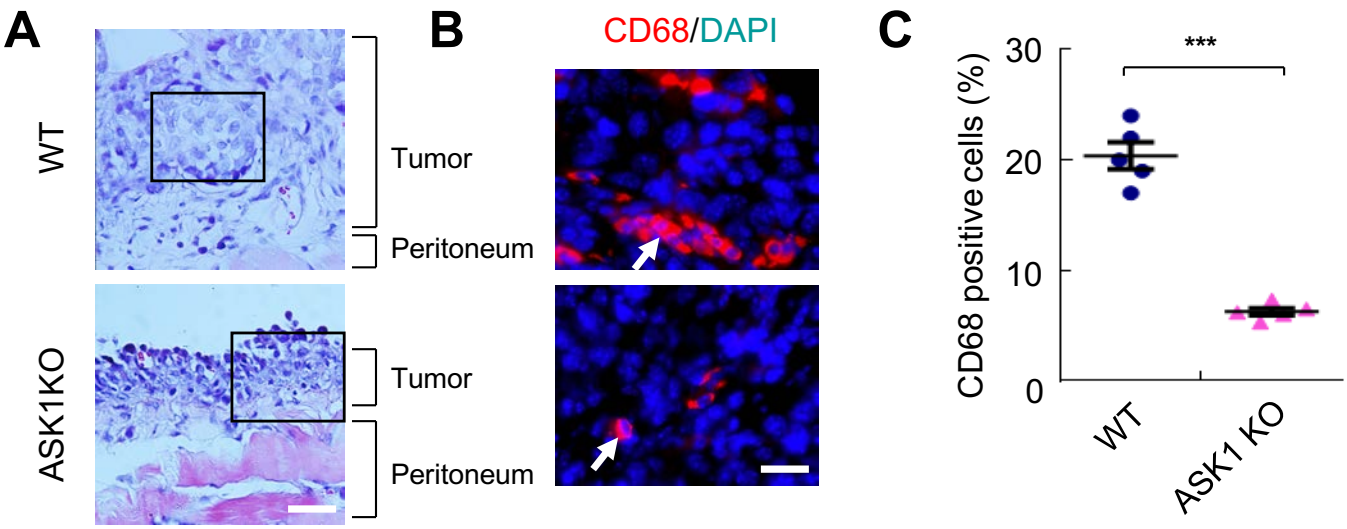
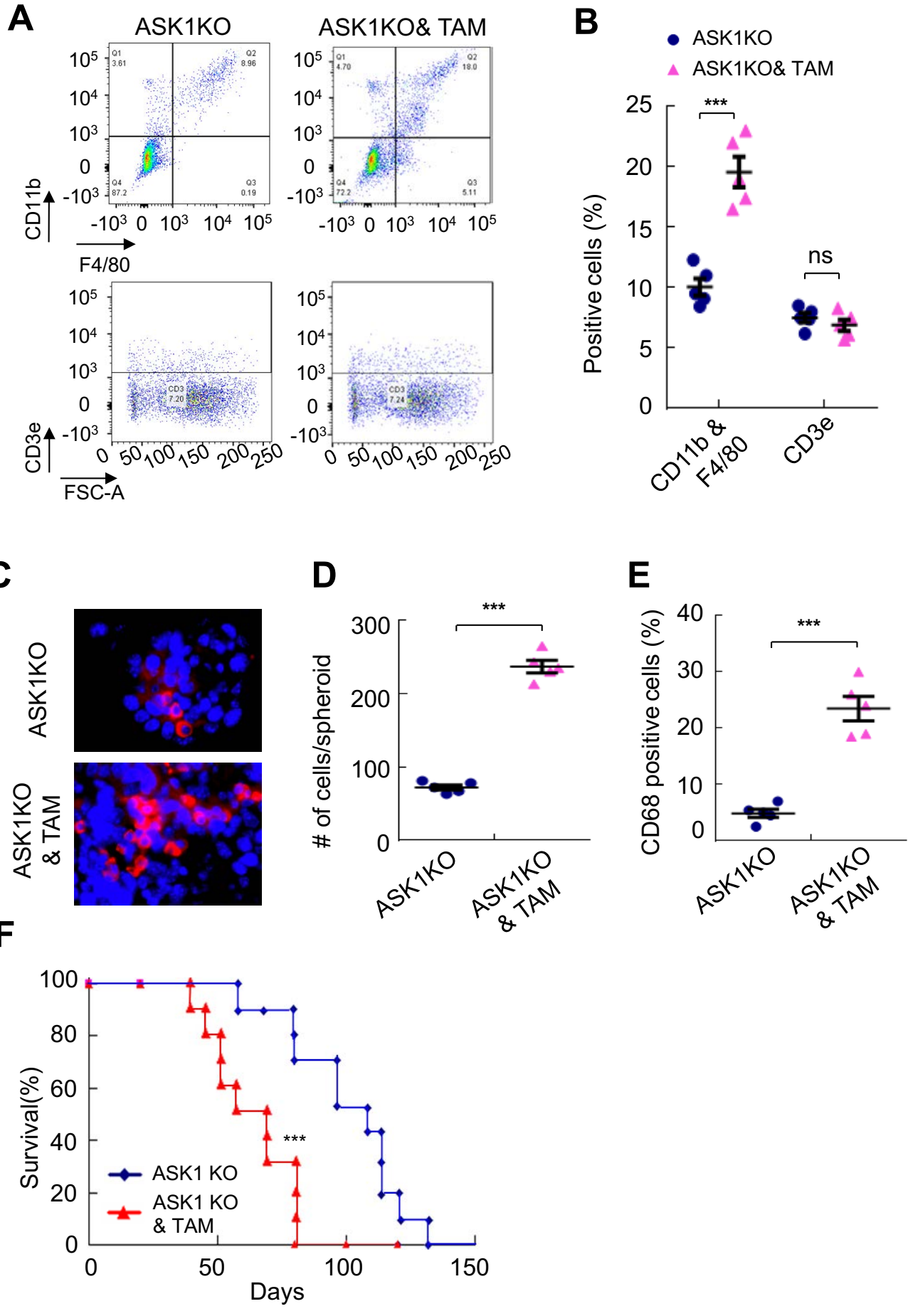
SUPPLEMENTAL FIGURES

Yin et al

ASK1-dependent endothelial cell activation play a critical role in ovarian cancer growth and metastasis



Supplementary Figure 1. Attenuated peritoneal macrophages in ASK1KO mice. In the orthotopic mouse OC model, peritoneal tumor implantations harvested at 8 weeks were subjected to H&E staining (**A**) and immunostaining with CD68 (**B**). Peritoneum and tumor implantation are indicated. Scale bar: 25 mm. % of $CD68^+$ cells were quantified in (**C**). Data presented are means ± SEM, n=10, ***, P<0.001 (two-sided student's *t*-test).

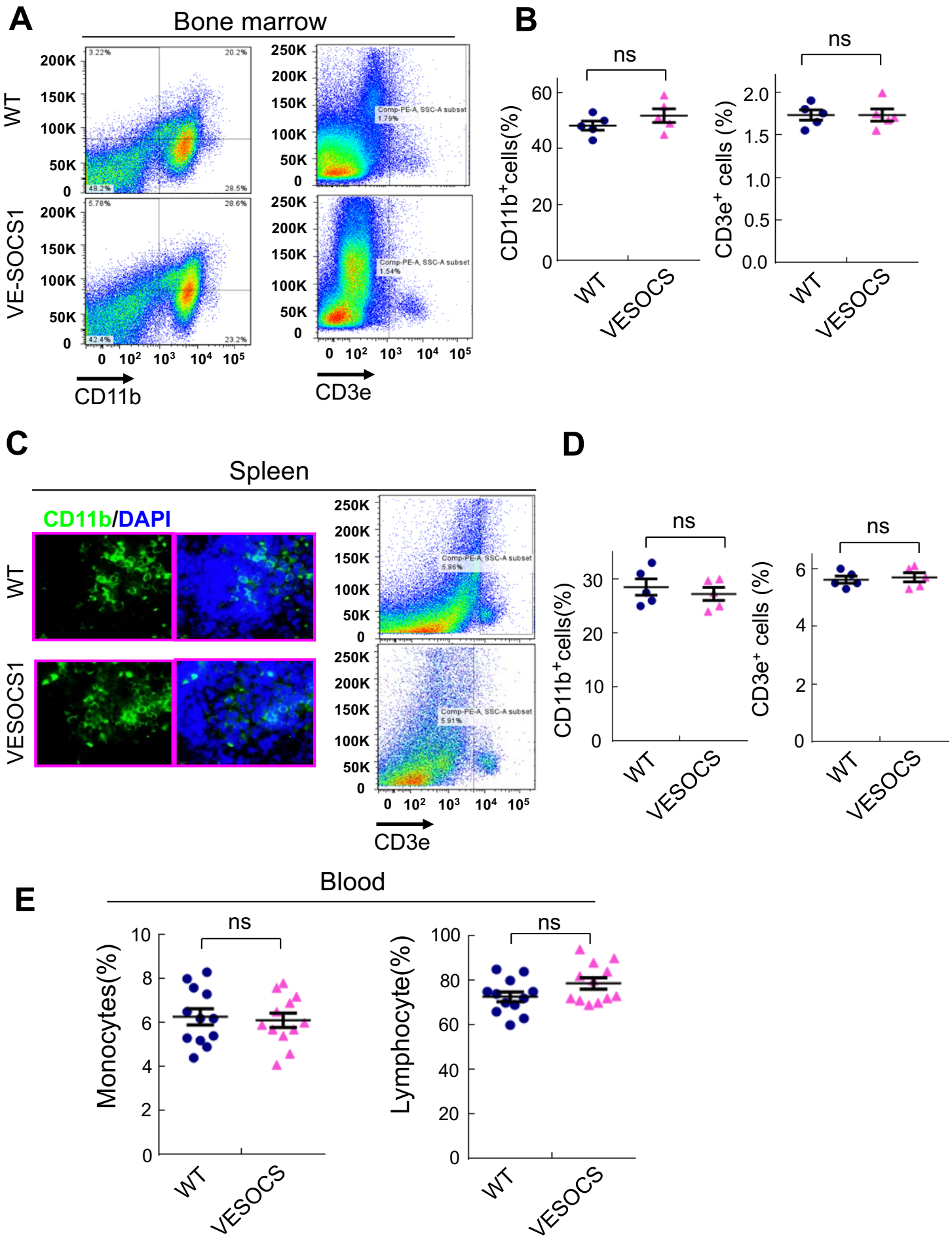

A
ASK1KO
ASK1KO& TAM
CD11b
F4/80
CD3e
FSC-A
B
ASK1KO
ASK1KO& TAM
Positive cells (%)
***
ns
CD11b & F4/80
CD3e
C
ASK1KO
ASK1KO & TAM
D
# of cells/spheroid
***
ASK1KO
ASK1KO & TAM
E
CD68 positive cells (%)
***
ASK1KO
ASK1KO & TAM
F
Survival(%)
***
ASK1 KO
ASK1 KO & TAM
Days

Supplementary Figure 2. TAMs promote tumor progression in ASK1KO mice. 20 days post-ID8 tumor injection in the orthotopic mouse OC model, half of the recipient mice were received $1x10^6$ $F4/80^+CD206^+$ tumor-associated macrophages (TAMs) isolated from ovarian cancer-bearing WT or ASK1KO donor mice.

(A-B) FACS and statistical analyses of CD11b/F4/80 and CD3e positive cells in peritoneal implantation 9 weeks post-ID8 injection. n=5, ***P<0.01.

(**C-E**) Effects of TAM on spheroid formation of ASK1KO mice. Spheroids from ascites were collected at week 8 and were examined by CD68 staining (C). Total cell number (D) and CD68 positive cell number (E) of spheroids were quantified.

(F) Survival curves of ASK1KO and ASK1 KO with TAM-injection mice after ID8 peritoneal injection. n=12, ***P<0.001.


A
Bone marrow
WT
VE-SOCS1
CD11b
CD3e
B
ns
CD11b+cells(%)
CD3e+ cells (%)
WT
VESOCS
C
Spleen
CD11b/DAPI
WT
VESOCS1
CD3e
D
ns
CD11b+cells(%)
CD3e+ cells (%)
WT
VESOCS
E
Blood
ns
Monocytes(%)
Lymphocyte(%)
WT
VESOCS

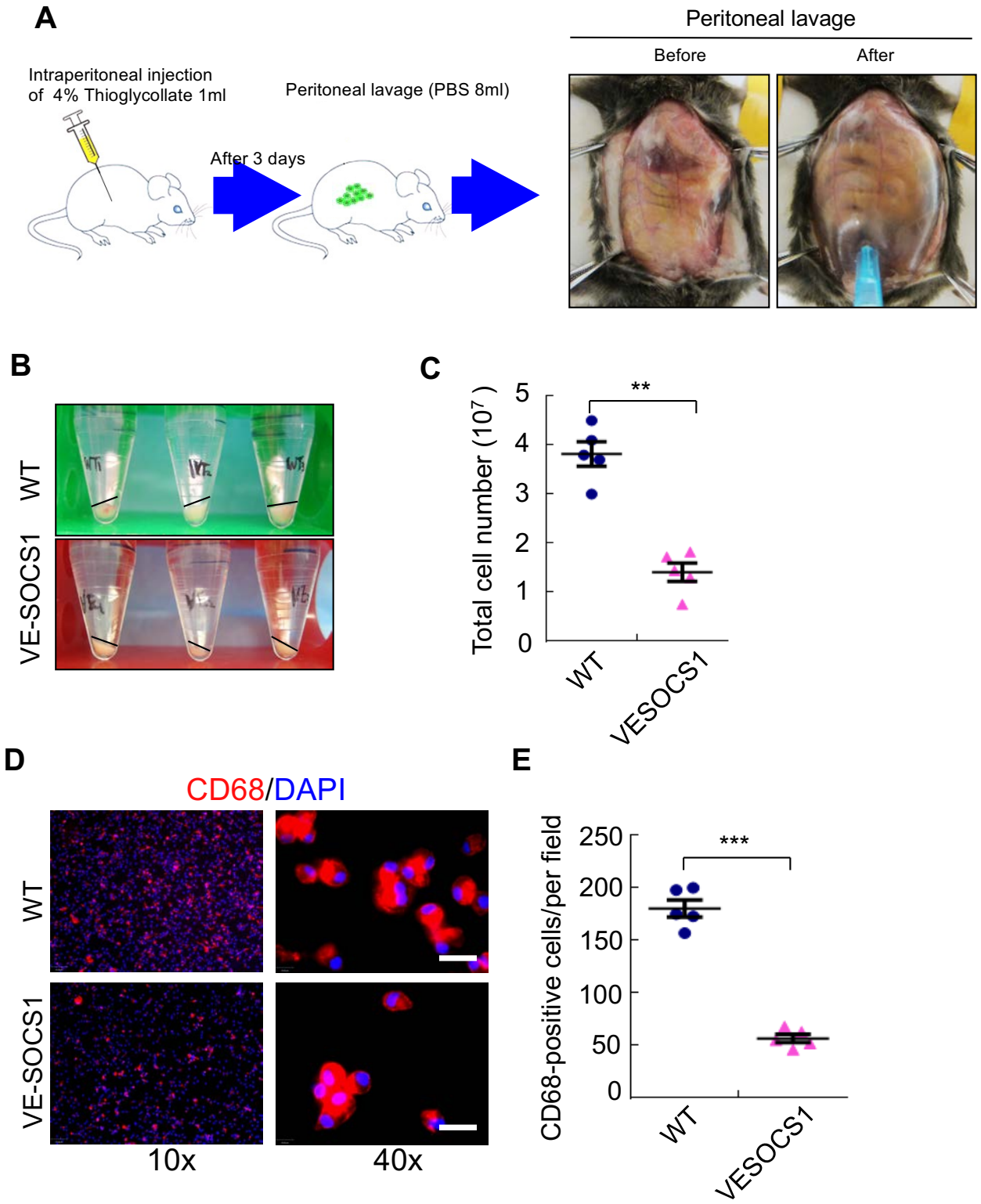
Supplementary Figure 3. Vascular expression of SOCS1 does not alter basal levels of macrophages in mouse tissues.

(**A,B**) Identification and statistical analysis of CD11b and CD3e positive cells in bone marrows of WT and VESOCS1 mice by FACS. (n=5 mice per group).

(**C,D**) Identification and statistical analysis of CD11b and CD3e positive cells in spleens of WT and VESOCS1 mice by immunostaining and FACS. (n=5 mice per group).

(E) Statistical analysis of monocytes and lymphocytes in blood of WT and VESOCS1 mice by CBC. (n=12 mice per group).

All data are presented as means ± SEM, n.s, $P>0.05$ (two-tailed student's *t*-test)



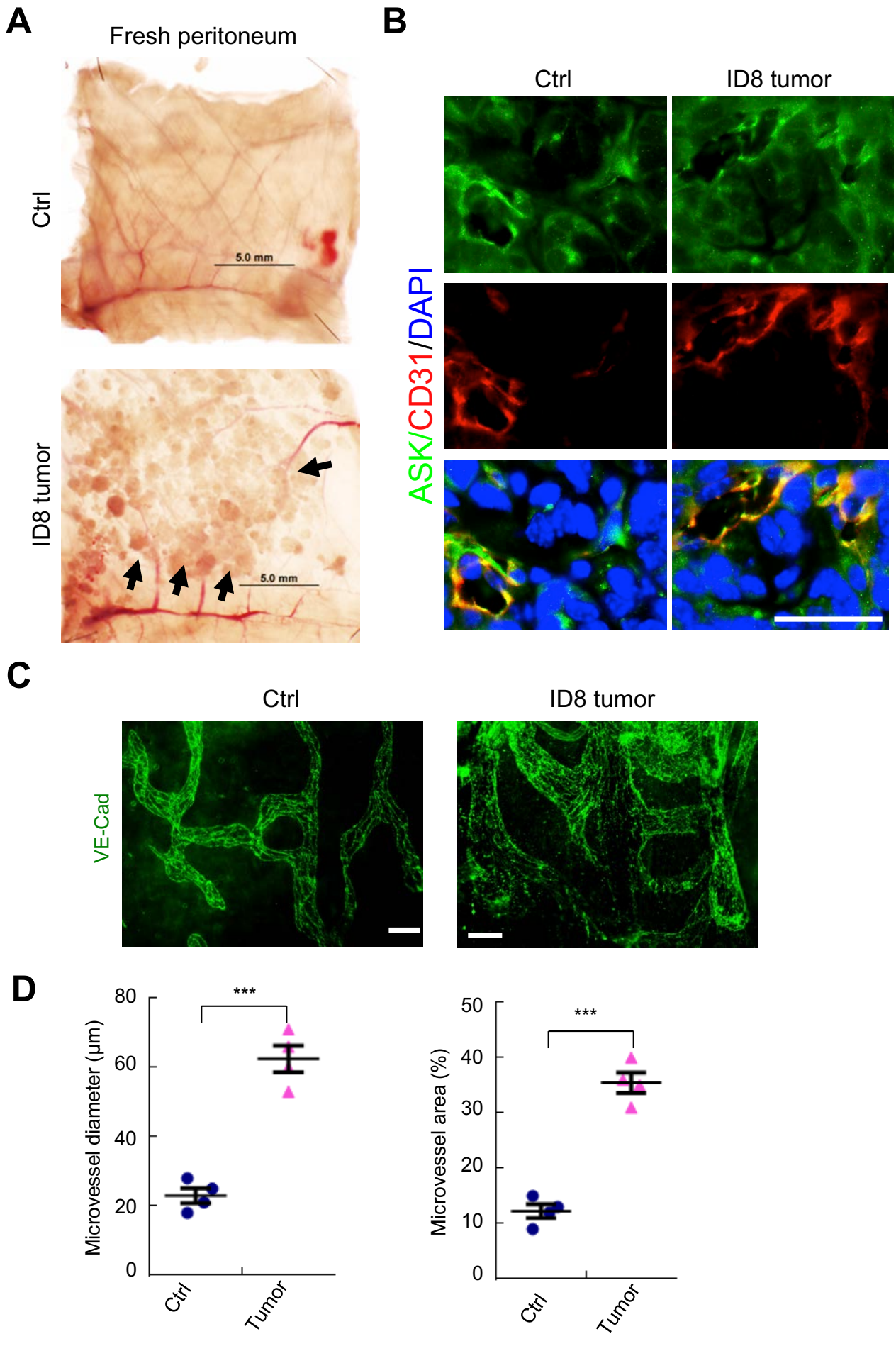
Supplementary Figure 4. Macrophage infiltration into peritoneal cavity is inhibited after thioglycollate induction in VESOCS1 mice.

(A) Mice were subjected to intraperitoneal injection of 4% thioglycollate (TG). Total peritoneal cells were harvested on day 3 post-TG injection.

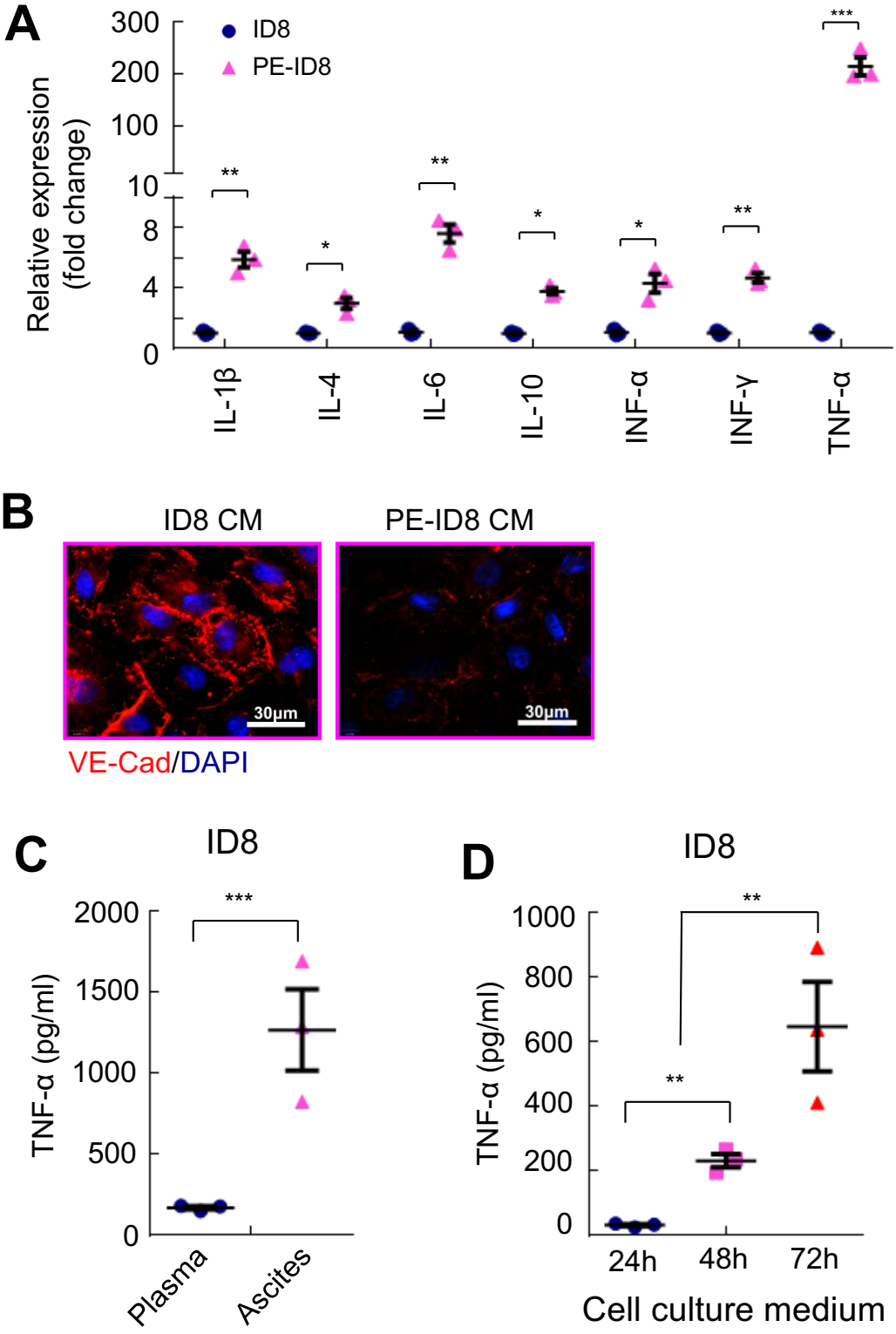
(B-C) Quantificatiosn of total ascitic cells by counting.

(D-E) Immunofluorescent staining and quantifications of CD68-positive cells. Scale bar, 30 μm.

Data are presented as means ± SEM, n=5, **, *P*<0.01; ***, *P*<0.001 (two-sided student's t-test).

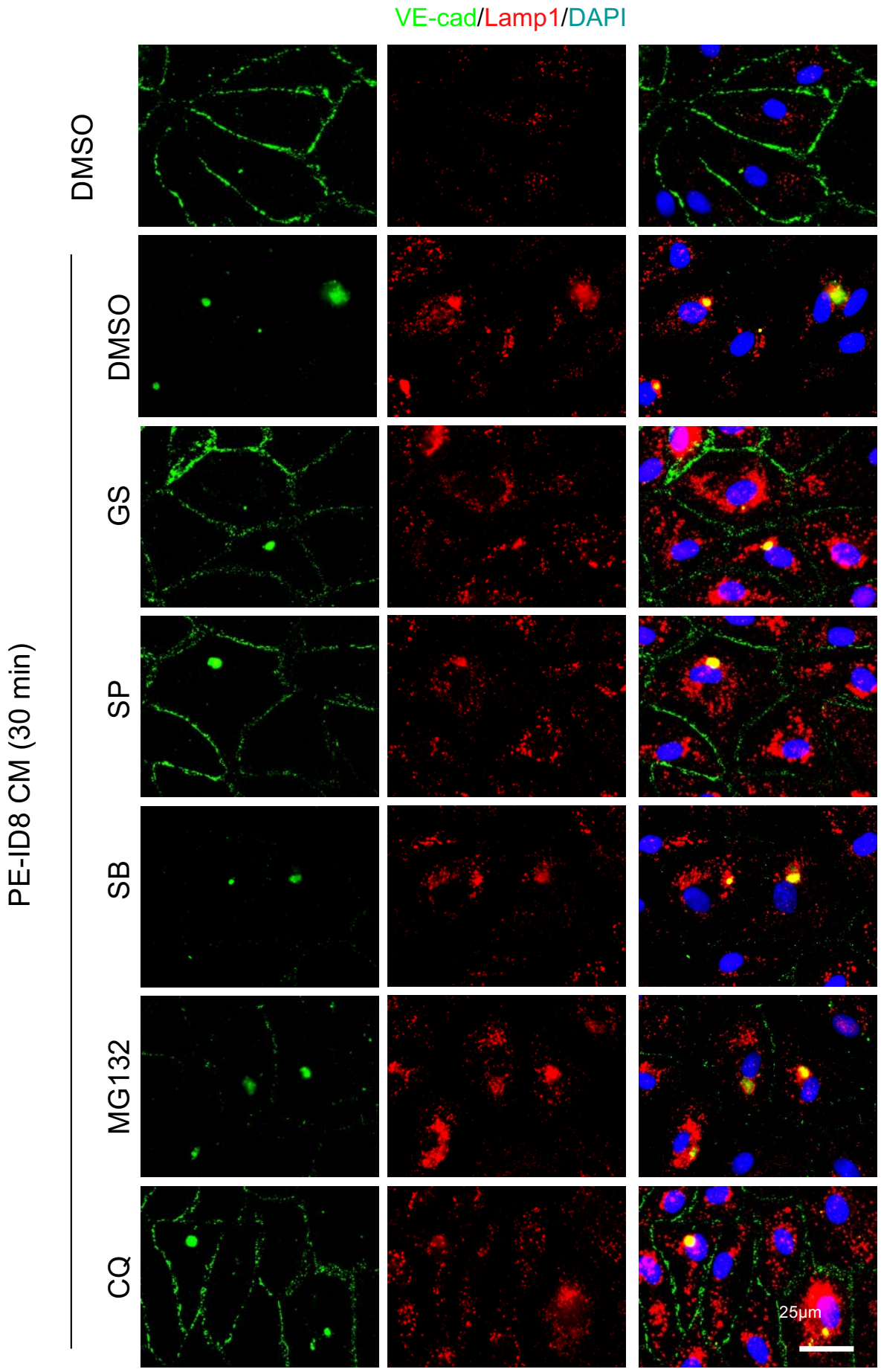

A
Fresh peritoneum
Ctrl
ID8 tumor
5.0 mm
5.0 mm
B
Ctrl
ID8 tumor
ASK/CD31/DAPI
C
Ctrl
ID8 tumor
VE-Cad
D
Microvessel diameter (μm)
80
60
40
20
0
***
Ctrl
Tumor
Microvessel area (%)
50
40
30
20
10
0
***
Ctrl
Tumor

Supplementary Figure 5. Tumor cells induce reduced VE-cadherin expression in peritoneal microvessels with disrupted junctions
Diaphrams were harvested from OC-bearing mice at 8 weeks post-ID8 cell implantation.
(A) Distribution of tumor implantations along peritoneal vessel growth. Arrows indicate accumulation of implantations along with the microvessels. Scale bar, 5 mm. Arrows indicate tumor cells attach to peritoneal vessels.
(B) Sections of peritoneum from Ctrl and ID8 –bearing mice were subjected to co-immunostaining with anti-ASK1 and EC marker CD31. Merged images show ASK1 expression in peritoneal vessels. Scale bar, 100 μm.
(C) Whole mount staining of VE-cadherin in peritoneum. Scale bar, 100 μm.
(D) Statistical analyses of vessel diameters and microvascular areas.
All data are presented as means ± SEM, n=4, ***, $P<0.001$ (two-tailed student's *t*-test).


A
ID8
PE-ID8
Relative expression (fold change)
300
200
100
10
8
4
0
IL-1β
IL-4
IL-6
IL-10
INF-α
INF-γ
TNF-α
B
ID8 CM
PE-ID8 CM
30μm
30μm
VE-Cad/DAPI
C
ID8
TNF-α (pg/ml)
2000
1500
1000
500
0
Plasma
Ascites
D
ID8
TNF-α (pg/ml)
1000
800
600
400
200
0
24h
48h
72h
Cell culture medium

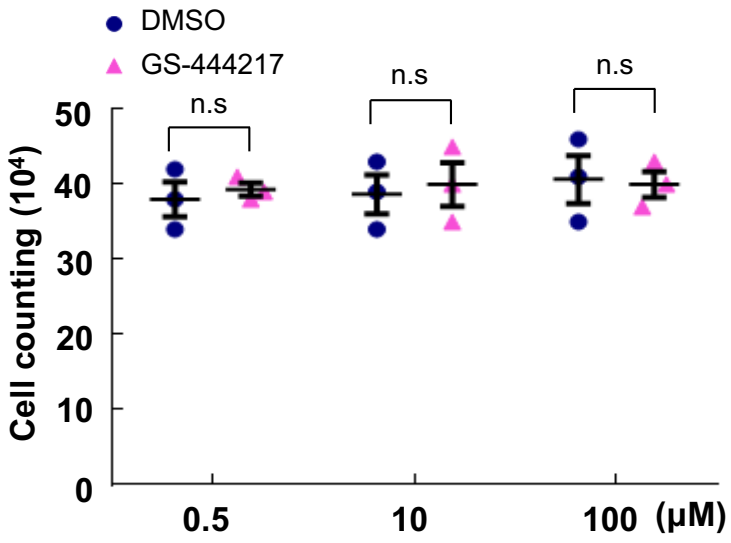
Supplementary Figure 6. Ovarian cancer cells express high levels of proinflammatory cytokines in peritoneal cavity that induces disruptions of EC junctions.

(A) Gene expression of cytokines in ID8 and peritoneal derived ID8 (PE-ID8) tumor cells isolated from 4- week OC tumor-bearing mice. Gene expressions of a panel of cytokines were determined by qRT-PCR. Relative gene expressions are presented as fold changes by taking ID8 cells as 1.0. Data are presented as means ± SEM, n=3; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ (two-sided student's t-test).
(**B)** Immunostaining of VE-Cadherin in mouse lung microvascular ECs (MLMVECs) exposed to conditional media (CM) from ID8 or PE-ID8 cells for 1 h. Scale bar, 30 μm.
(**C**) ELISA analysis of TNF-α protein expression in ascites at 8 weeks post-tumor injection. Plasma was used as a negative control. n=3, ***, $P<0.001$ (two-sided student's *t*-test).
(**D**) ELISA analysis of TNF-α protein expression in supernatants of culture tumor cells at 24, 48 and 72 hours. Data are presented as means ± SEM, n=3.


VE-cad/Lamp1/DAPI
DMSO
PE-ID8 CM (30 min)
DMSO
GS
SP
SB
MG132
CQ
25μm

Supplementary Figure 7. ASK1 mediates tumor condition media-induced VE-cadherin degradation.

Mouse lung microvessel ECs (MLMVECs) were cultured in condition media of PE-ID8 (ID8 cells isolated from OC tumor-bearing mice) for 1 h in the absence or presence of various inhibitors as indicated (10 μM each): SP600125 (JNK inhibitor), GS444217 (ASK1 inhibitor), SB203580 (p38 MAP kinase inhibitor), MG132 (proteasome inhibitor) and chloroquine (lysosome inhibitor). Cells were subjected to immunostaining with anti-VE-cadherin and anti-Lamp1 followed by counterstaining with DAPI.



Supplementary Figure 8. ASK1 inhibitor GS-444217 had no affect on tumor cell proliferation and survival in vitro.

(A) ID8 cells were cultured in the presence of DMSO or ASK1 inhibitor at 0.5, 10 and 100μM for 24 h, and cell numbers were counted. Data are presented as means ± SEM, n=3. No significant differences between DMSO and GS444217 groups (two-sided student's t-test).

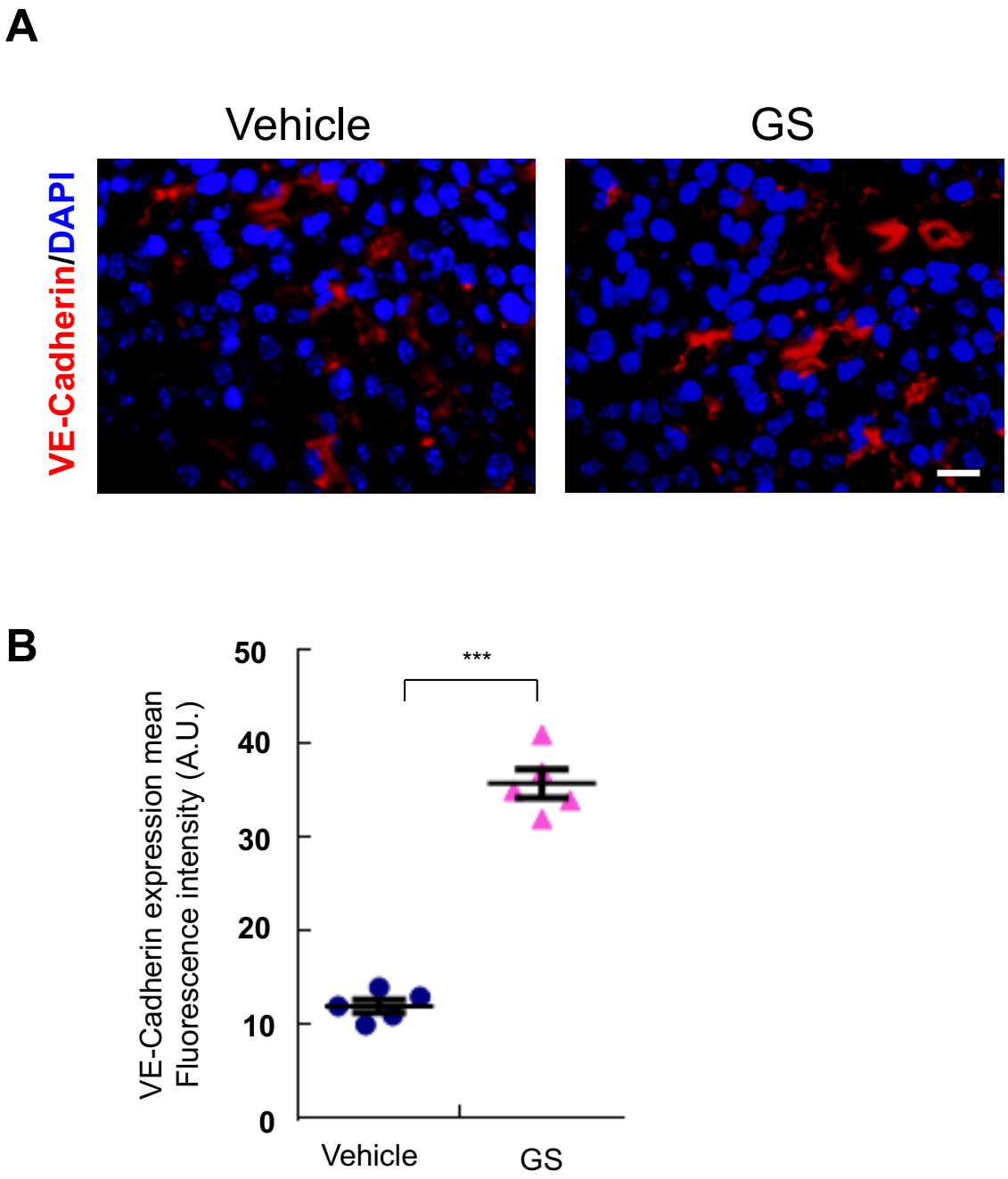
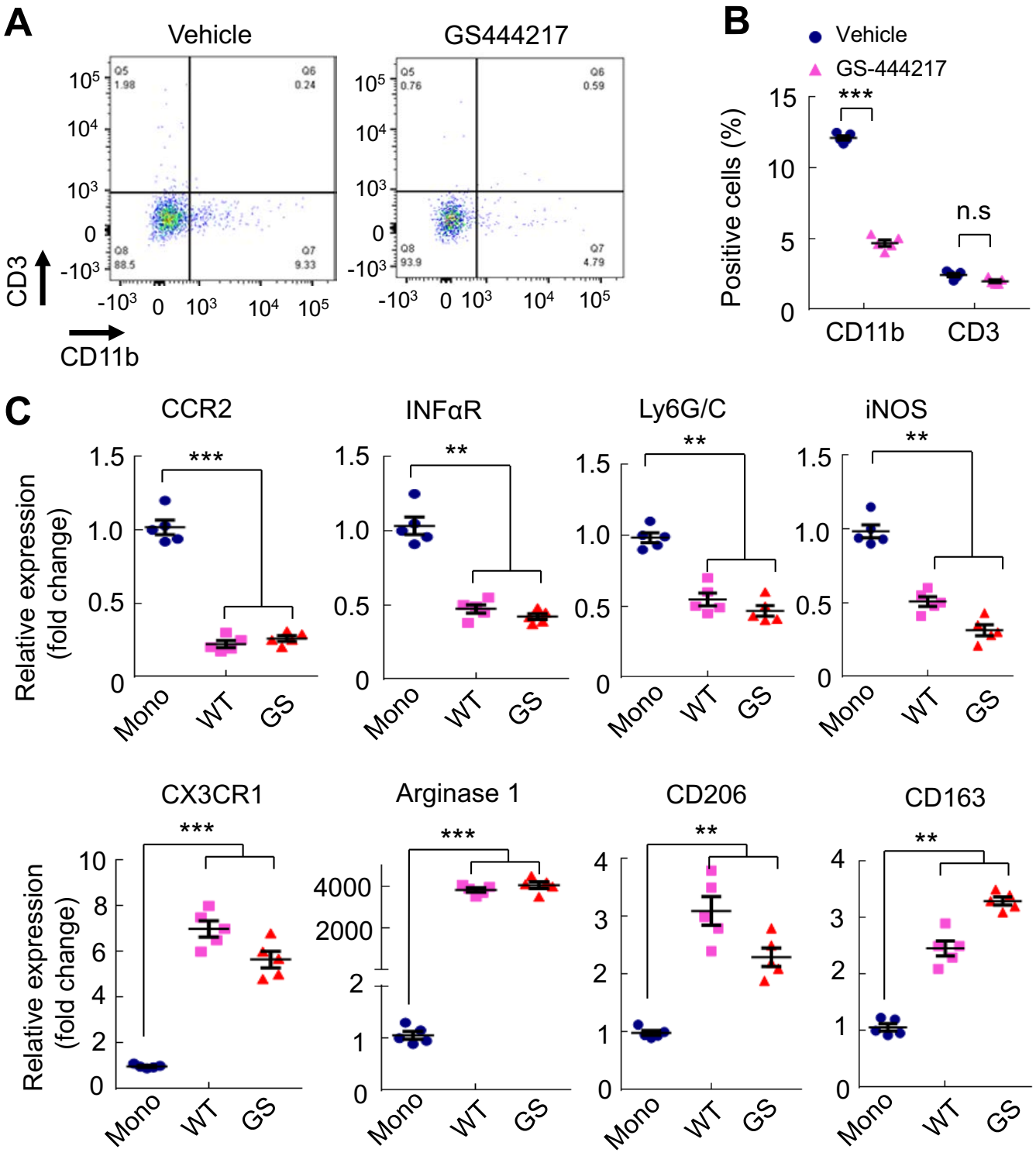


Figure 9. ASK1 inhibitor enhanced VE-cadherin expression in peritoneal microvessels.
ID8-implanted mice were randomly divided into two groups on day 7 post-implantation, and were fed with ASK1 inhibitor (GS444217) and vehicle control, respectively. Peritoneum were harvested at 8 weeks post-ID8 injection. Immunostaining of VE-Cadherin (**A**) with quantification of immunofluorescence intensity (**B**) are shown. Scale bar, 30 μm. Data are presented as means ± SEM, n=5; **, *P*<0.01 (two-sided student's t-test).



Supplementary Figure 10. ASK1 inhibitor enhanced macrophage infiltration into peritoneal cavity but did not prevent polarization.

ID8-implanted mice were randomly divided into two groups on day 7 post-implantation, and were fed with ASK1 inhibitor (GS444217) and vehicle control, respectively. Total peritoneal cells were harvested at 8 weeks post-ID8 injection.

(**A, B**) FACS and statistical analysis of CD11b and CD3e cells. All data are presented as means ± SEM, n=5, ***, P<0.001 (two-sided student's t-test).

(**C**) M1 subtype-specific and M2 subtype-specific markers were determined by qRT-PCR. Peripheral blood monocytes ($CD11b^+$) were used as a control. All data are presented as means ± SEM, n=5, **, P<0.01; ***, P<0.001 (two-sided student's t-test).

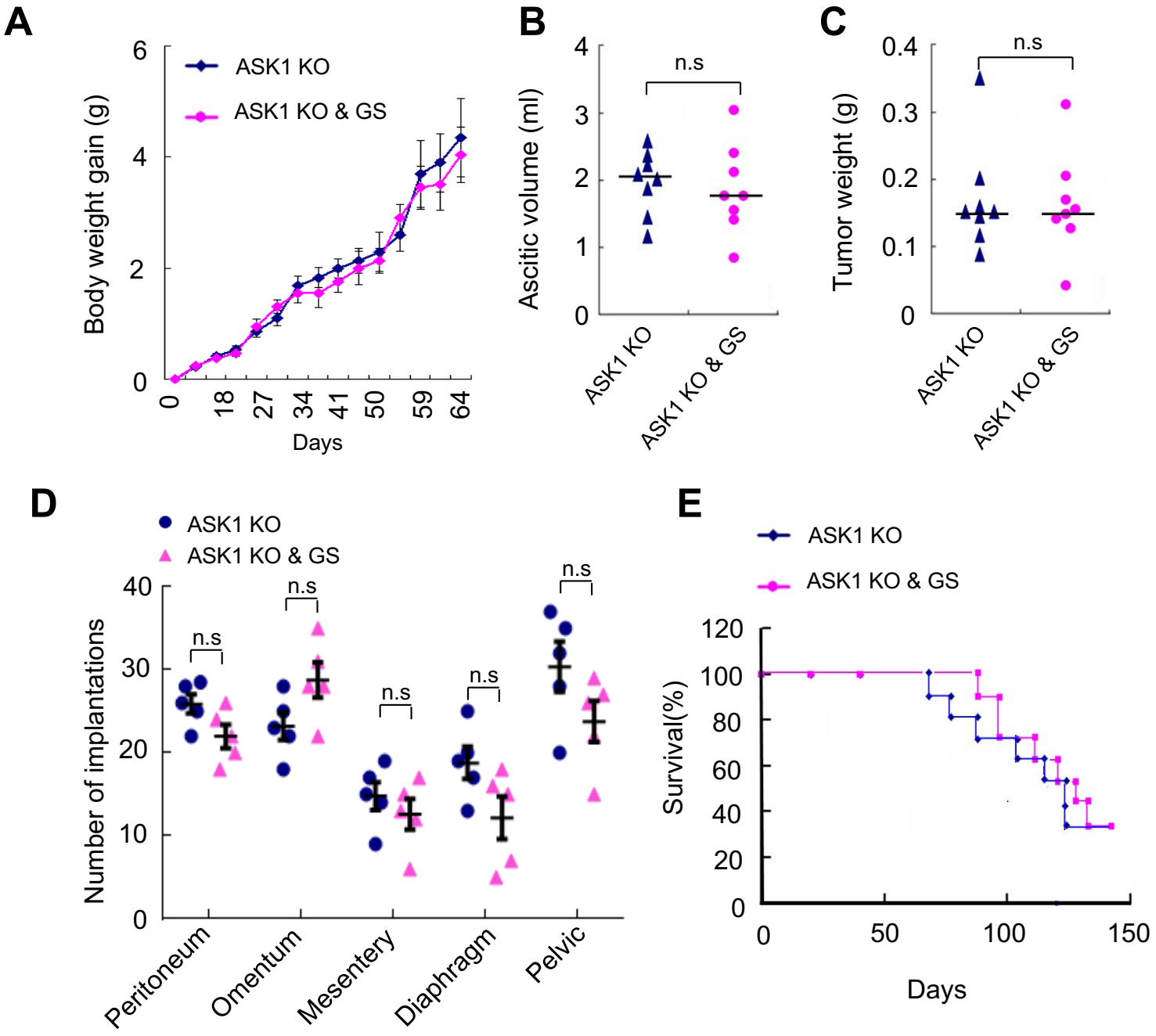


Figure 11. ASK1 inhibitor had no effects on tumor growth, ascites formation, abdominal cavity metastasis, survival rate in ASK1KO mice.

ID8-implanted ASK1KO mice were randomly divided into two groups on day 7 post-implantation, and were fed with ASK1 inhibitor (GS444217) and vehicle control, respectively. Total peritoneal cells were harvested at 8 weeks post-ID8 injection.

(**A-C**) Measurement and statistical analysis of increased body weight, tumor weight and ascites volume in ASK1 KO female recipient mice with or without GS treatment.

(**D**) Statistical analysis of tumor metastasis in peritoneum, omentum, mesentery, diaphragm and pelvic implantation ASK1 KO female recipient mice with or without GS treatment at nine weeks post- injection of ID8 ovarian cancer cells.

(**E**) Survival curves of ASK1 KO female recipient mice with or without GS treatment after ID8 peritoneal injection. (n=12).

Supplemental Table 1: Antibodies

Antibody name	Company	Cat No.	Usage	Dilution
β-Actin, rabbit	Gene Tex Inc	GTX109639	WB	1:2000
ASK1	Santa Cruz	sc-5294	WB	1:500
β-Catenin	BD Pharmingen	51-9001922	IF	1:200
CD11b, rat	BD Pharmingen	557395	IF	1:100
CD31, rabbit	abcam	ab32457	IF, WB	1:100,1:1000
CD68, mouse	abcam	ab31630	IF	1:100
CD68(KP1)	Santa Cruz	sc-20060	IHC	1:50
Claudin 5	Invitrogen	1248404A	WB	1:1000
GAPDH, rabbit	Cell signaling	#2118	WB	1:2000
JNK	Santa Cruz	sc-7345	WB	1:500
p-JNK	Cell signaling	#4671	WB	1:1000
Ki67, rabbit	Cell signaling	#9129	IF	1:100
P38, rabbit	Santa Cruz	sc-535	WB	1:1000
p-P38, rabbit	Cell signaling	#4511	WB	1:1000
SOCS1	Santa Cruz	sc-9021	WB	1:300
V-Cadherin	BD Pharmingen	51-9001928	IF, WB	1:200; 1:1000
ZO-1	Invitrogen	1244877A	WB	1:1000
FACS				
FITC-CD11b,	Biolegend	101205	FACS	1:200
PE-F4/80	Biolegend	123109	FACS	1:200
APC-F4/80	Biolegend	100311	FACS	1:200
PE-CD206	Biolegend	141705	FACS	1:200

Supplemental Table 2: Primes for qPCR

Primer	**Forward Sequence**	**Reverse Sequence**
Mouse		
ARG1	CTCCAAGCCAAAGTCCTTAGAG	AGGAGCTGTCATTAGGGACATC
ASK1	ACTCCAGTCCCTGAAGGAAAT	CAGTAGACCTTGTTGTGTGGTG
CD3e	GAGAGAGAATTCTGAGAGGATGCGG	GTCAGACTGCTCTCTGATTCAGGCC
CD11b	GGCTCCGGTAGCATCAACAA	ATCTTGGGCTAGGGTTTCTCT
CD163	GGTGGACACAGAATGGTTCTTC	CCAGGAGCGTTAGTGACAGC
CD206	CTCTGTTCAGCTATTGGACGC	TGGCACTCCCAAACATAATTTGA
CCR2	ATCCACGGCATACTATCAACATC	CAAGGCTCACCATCATCGTAG
CX3CR1	ACGAAATGCGAAATCATGTGC	CTGTGTCGTCTCCAGGACAA
IL-1 β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
IL-4	ACTTGAGAGAGATCATCGGCA	AGCTCCATGAGAACACTAGAGTT
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC

IL-10	AGAAGCATGGCCCAGAAATCA	GGCCTTGTAGACACCTTGGT
INF-α	CTTCCACAGGATCACTGTGTACCT	TTCTGCTCTGACCACCTCCC
INF-γ	GAACTGGCAAAAGGATGGTGA	TGTGGGTTGTTGACCTCAAAC
iNOS	ACATCGACCCGTCCACAGTAT	CAGAGGGGTAGGCTTGTCTC
INFαR	CTTCCACAGGATCACTGTGTACCT	TTCTGCTCTGACCACCTCCC
Ly6G/C	GACTTCCTGCAACACAACTACC	ACAGCATTACCAGTGATCTCAGT
SOCS1	GAGACCTTCGACTGCCTTTTC	GTCAGATCTGGAAGGGGAAGG
TNFα	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG