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Supplemental Information

Extracellular vesicle-encapsulated CC16 as novel nanotherapeutics for treatment of acute lung injury

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Supplemental Figures

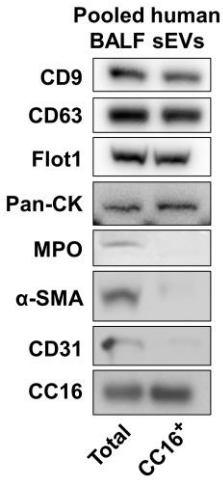

fig. S1. CC16 positive sEVs ($CC16^+$ sEVs) are mainly released from epithelial cells. BALF sEVs from pooled normal humans (n = 10) and pneumonia patients (n = 21) were examined by western blot. $CC16^+$ sEVs were purified by CC16 antibody-coated magnetic beads. sEVs positive markers (CD9, CD63, and Flot1) are detected in 50 μg of sEVs or $CC16^+$ sEVs. The Pan-CK, MPO, α-SMA, and CD31 were used as epithelial cell markers, neutrophil markers, smooth muscle cell markers, and endothelial cell markers, respectively.

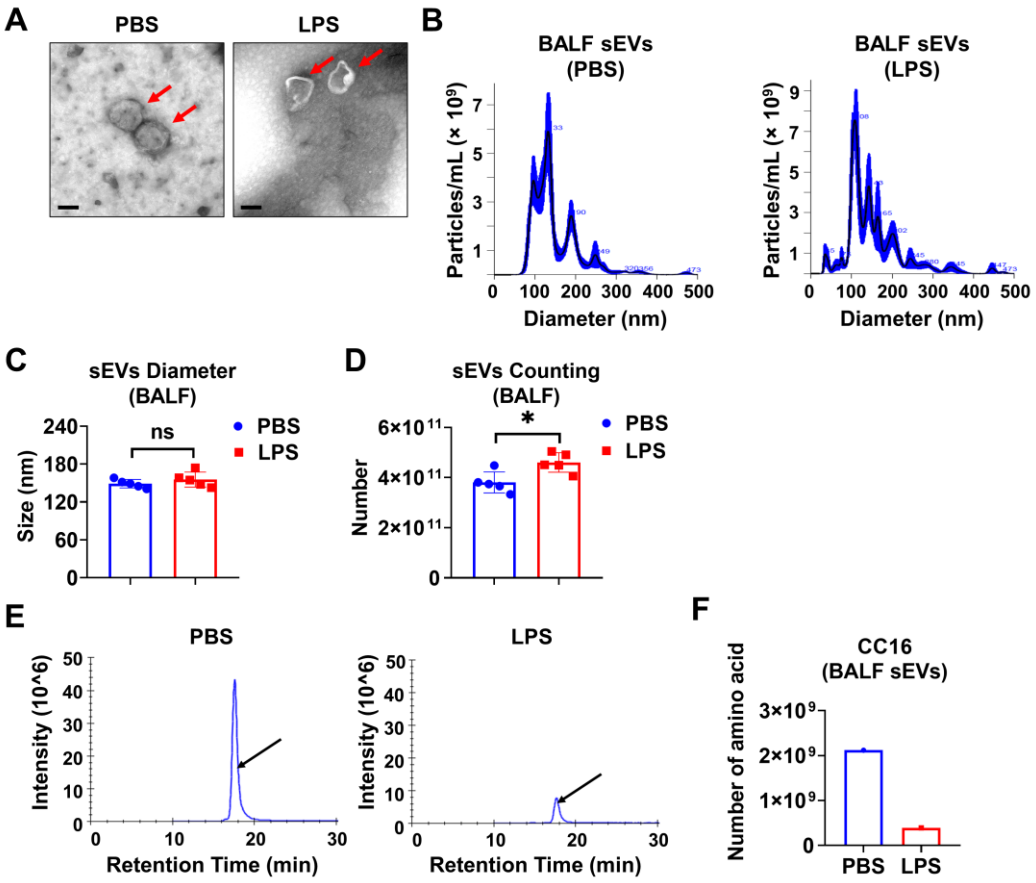

fig. S2. LPS treatment reduces sEV-carried CC16 derived from murine BALF. (**A-D**) Mice (n = 5 per group) received 50 µL PBS or 1 µg LPS (in 50 µL PBS) via i.t. Mice were sacrificed 24 h after treatment. BALF sEVs (200 µg) from PBS- or LPS-treated mice (n = 5) were isolated and then analyzed. Representative TEM images of sEVs were shown. Scale bar = 100 nm (**A**). Size distribution (**B**), average size (**C**), and particle numbers (**D**) were determined by NTA. Data are mean ± SD. The data were analyzed using two-tailed unpaired Student's t-test. ns; $p > 0.05$; *, $p < 0.05$. (**E and F**) MS was used to measure the number of CC16-derived amino acids in 20 µg pooled BALF sEVs from PBS- or LPS-treated mice (n = 5).

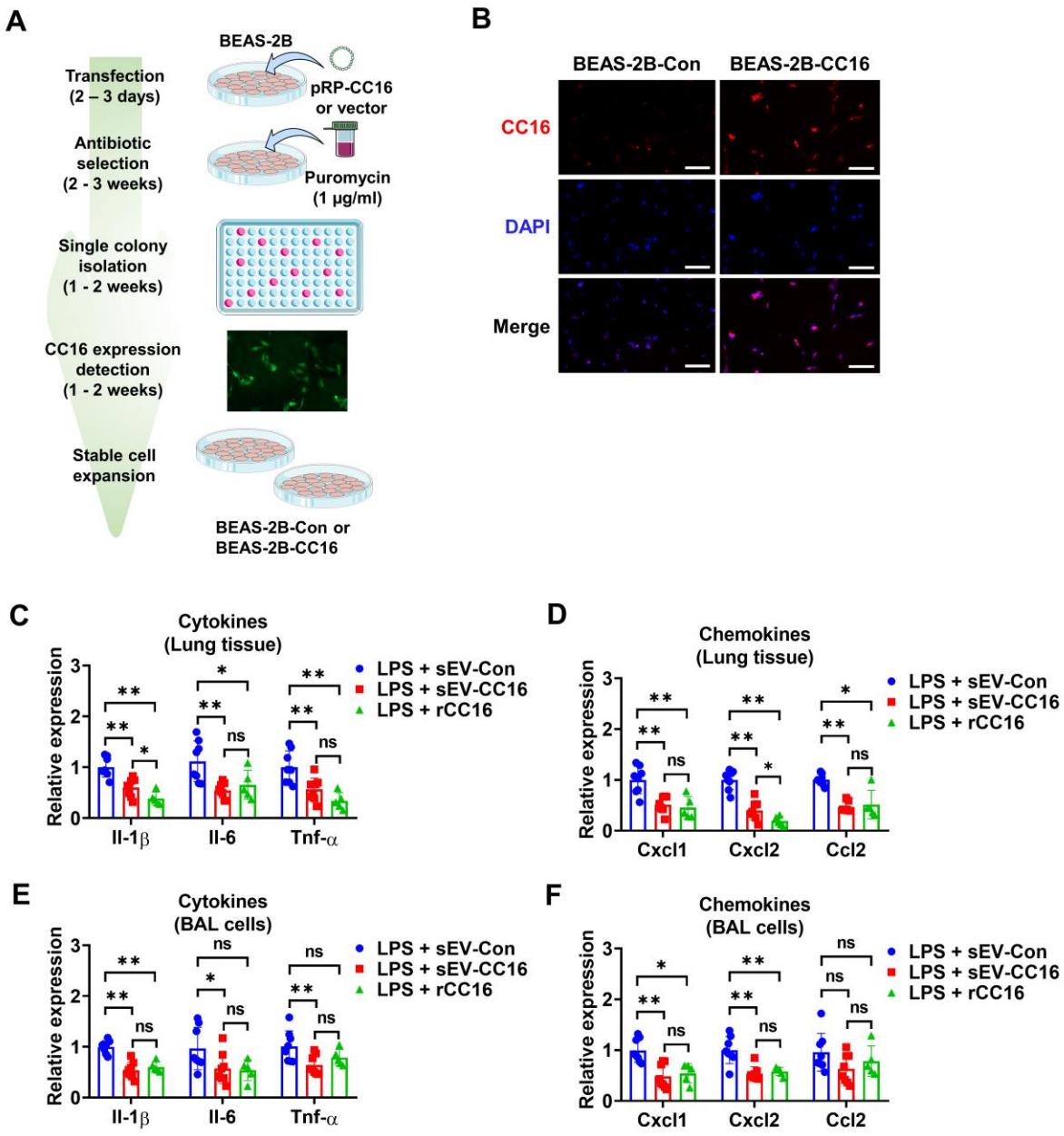

fig. S3. sEV-CC16 generated from BEAS-2B with stable expression of CC16 protein inhibits cytokine and chemokine gene expressions in vivo. (**A**) Schematic overview of generation of BEAS-2B with stable expression of CC16 protein. Electroporation-mediated transfection of pRP-CC16 or vector was conducted and stabilized the cells for 2 - 3 days. Stably transfected cells were selected using 1 μg/mL puromycin. Parts of the figure were drawn by using pictures from Servier Medical Art (http://smart.servier.com/), licensed under a Creative Commons Attribution 3.0

Unported License (https://creativecommons.org/licenses/by/3.0/). (**B**) CC16 staining was performed on BEAS-2B-Con and BEAS-2B-CC16 using an antibody against CC16. Scale bar = 100 μm. (**C-F**) LPS (1 μg in 50 μL PBS) was delivered into murine lungs (5 to 8 mice per group) via the i.t. route. After 3 h, mice were treated with 7.5×10^{10} (in 50 μL PBS) sEV-Con, sEV-CC16, or 50 μg (in 50 μL PBS) rCC16 via the i.t. route. Lung tissue and BAL were collected 24 h after sEV administration. mRNA levels of cytokines (**C**) and chemokines (Cxcl1, Cxcl2, and Ccl2) (**D**) in lung tissues were measured by RT-qPCR. mRNA levels of cytokines (Il-1β, Il-6, and Tnf-α) (**E**) and chemokines (**F**) (Cxcl1, Cxcl2, and Ccl2) in BAL cells were measured by RT-qPCR. Results represent mean ± SD and were analyzed by a one-way ANOVA followed by Tukey's HSD. ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$.

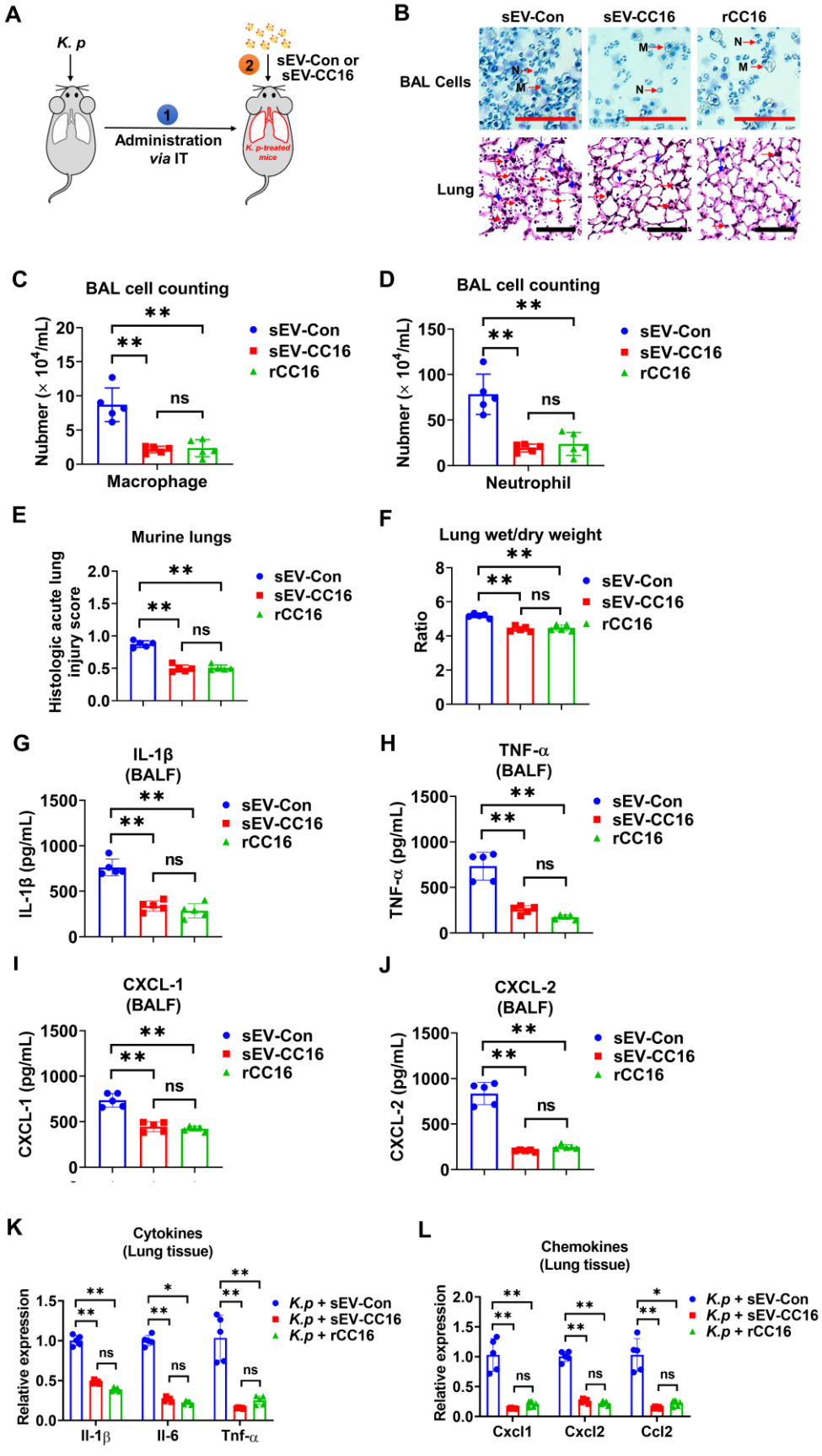
A
K. p
1
Administration
via IT
2
sEV-Con or
sEV-CC16
K. p-treated
mice
B
sEV-Con
sEV-CC16
rCC16
BAL Cells
Lung
M
N
C
BAL cell counting
Nubmer (× 10^4/mL)
Macrophage
sEV-Con
sEV-CC16
rCC16
**
ns
D
BAL cell counting
Nubmer (× 10^4/mL)
Neutrophil
E
Murine lungs
Histologic acute lung injury score
F
Lung wet/dry weight
Ratio
G
IL-1β (BALF)
IL-1β (pg/mL)
H
TNF-α (BALF)
TNF-α (pg/mL)
I
CXCL-1 (BALF)
CXCL-1 (pg/mL)
J
CXCL-2 (BALF)
CXCL-2 (pg/mL)
K
Cytokines
(Lung tissue)
Relative expression
Il-1β
Il-6
Tnf-α
K.p + sEV-Con
K.p + sEV-CC16
K.p + rCC16
*
L
Chemokines
(Lung tissue)
Cxcl1
Cxcl2
Ccl2

fig. S4. sEV-CC16 protects *K. p*-induced lung injury in mice. Mice (n = 5 per group) received 5×10^3 CFU of *K. p* in 50 μL PBS via i.t. After 3 h, mice were given sEV-Con (7.5×10^{10} in 50 μL PBS), sEV-CC16 (7.5×10^{10} in 50 μL PBS), or rCC16 (2 mg/kg body weight) via the i.t. route. Mice were sacrificed 24 h after sEVs treatment. Schematic illustration of delivery of sEV-Con and sEV-CC16 into *K. p*-pretreated mice (**A**). H&E staining was performed using BAL cells and lung sections. M: macrophage; N: neutrophil; Red arrows: neutrophils; Blue arrows: increased alveolar disruption with hyaline membranes. Scale bar =100 μm (**B**). (**C**-**D**) The number of macrophages (**C**) or neutrophils (**D**) from BALF was counted. Lung injury was scored based on histological images (n = 5 per group) (**E**). Lung wet to dry weight ratios were calculated (**F**). Protein levels of IL-1β (**G**), TNF-α (**H**), CXCL-1 (**I**), and CXCL-2 (**J**) in BALF were detected using ELISAs. mRNA levels of cytokines (Il-1β, Il-6, and Tnf-α) (**K**) and chemokines (Cxcl1, Cxcl2, and Ccl2) (**L**) in lung tissues were measured by RT-qPCR. The results were presented as mean ± SD. The data were analyzed by a one-way ANOVA followed by Tukey's HSD. ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$.

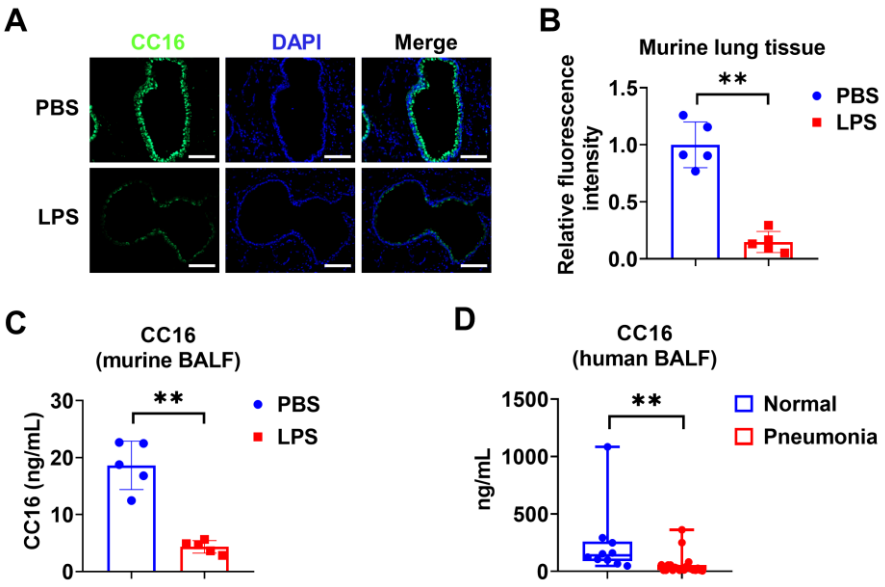

fig. S5. LPS-induced lung injury decreases CC16 level of lung and BALF. (**A-B**) Mice (n = 5 per group) received 1 μg LPS (in 50 μL PBS) via i.t. After 24 h, mice were sacrificed for immunofluorescence staining of lung tissue (**A**). Scale bar = 100 μm. Relative fluorescence intensity is measured by Image J software (**B**). (**C**) Protein level of CC16 in BALF without EVs was detected using ELISAs. (**D**) Protein level of CC16 in normal human (n = 10) BALF without EVs and pneumonia patients (n = 21) BALF without EVs is detected using ELISAs. The results presented as mean ± SD. In panels **B** and **C**, results are analyzed by two-tailed unpaired Student's t-test. In panel **D**, the boxes in the boxplots show the medians with 25th and 75th percentiles, and the whiskers show the Min and Max value. Data are analyzed using a Kruskal-Wallis one-way ANOVA followed by pairwise testing with Mann-Whitney U tests. **, $p < 0.01$.

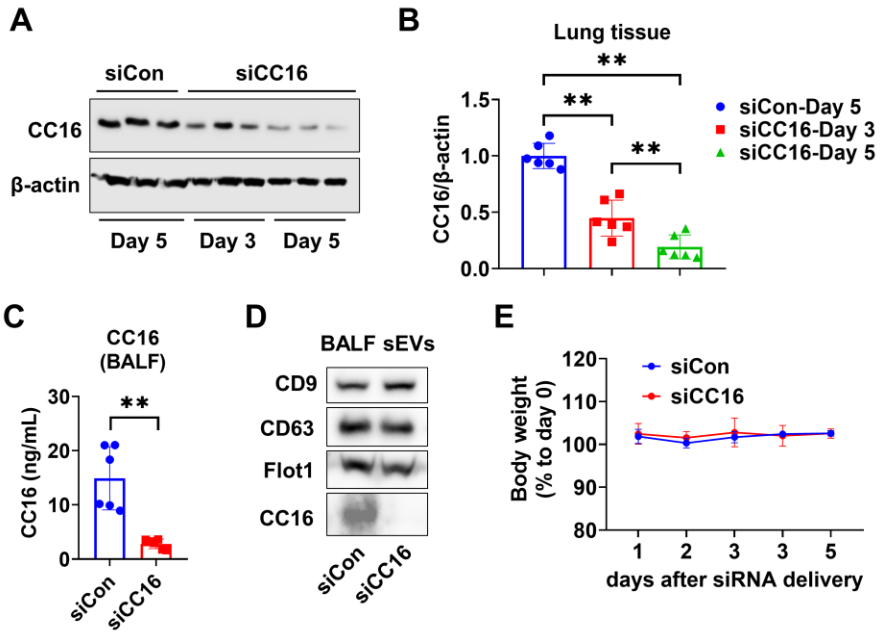

fig.S6. Characterization of CC16 knockdown mice and sEVs isolated from BALF. Mice (n = 6 per group) receive control siRNA (siCon) or CC16 siRNA (siCC16) via i.t. After 3 days and 5 days, mice were sacrificed. (**A**) Protein level of CC16 in the lung tissue is taken by western blot. β-actin is used as loading control. (**B**) Band intensity is measured by Image J software and CC16 expression level is normalized by β-actin. (**C**) Protein level of CC16 in BALF is detected using ELISAs. (**D**) sEVs are isolated from BALF 5 days after siCon or siCC16 treated mice. sEVs positive markers (CD9, CD63, and Flot1) and CC16 are detected in 100 μg sEVs protein using western blot. (**E**) Results are presented as mean ± SD. In panel **B**, Data are analyzed by one-way ANOVA followed by Tukey's HSD. In panels **C** and **E**, results are analyzed by two-tailed unpaired Student's t-test. **, $p < 0.01$.

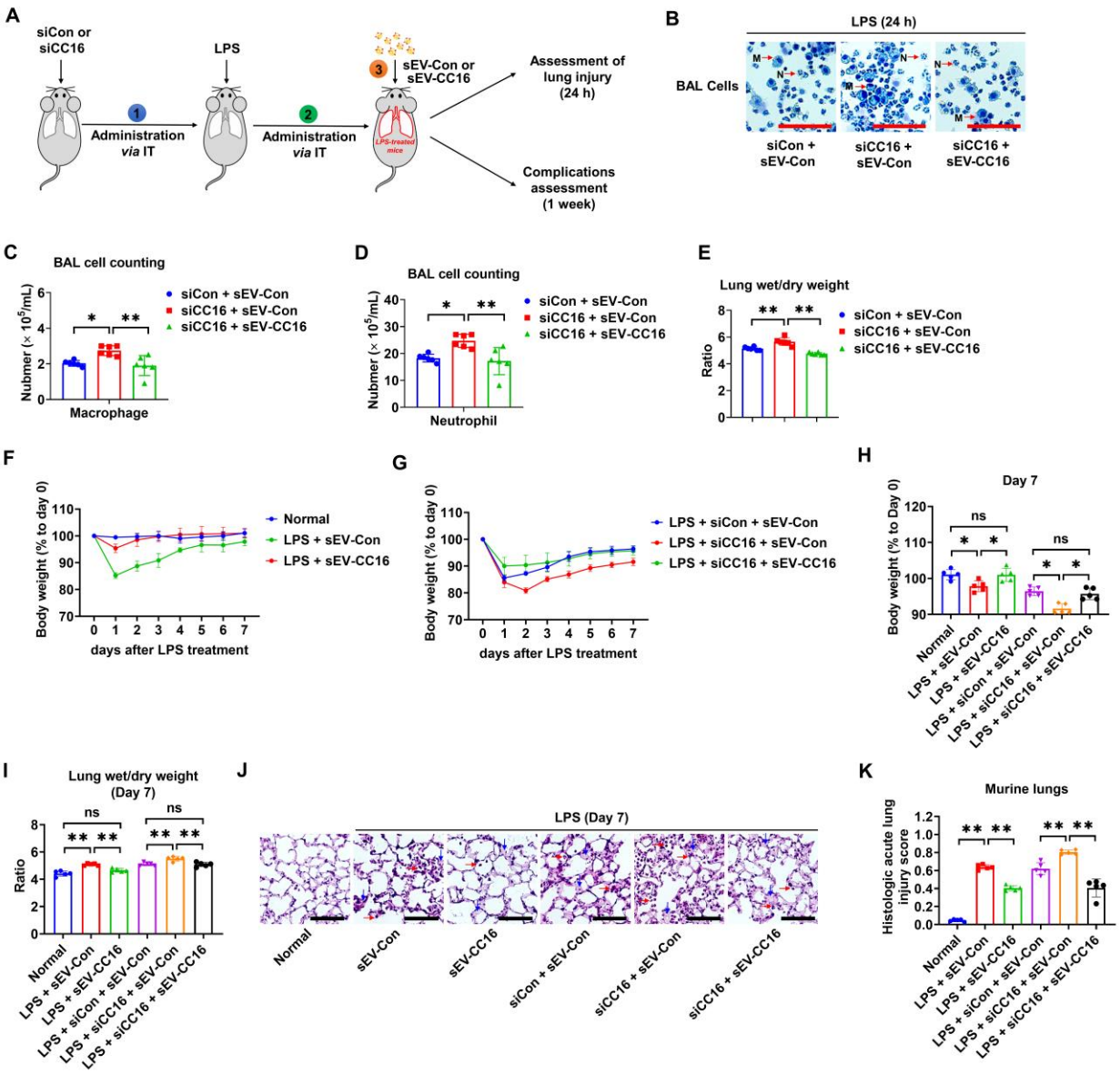

fig. S7. sEV-CC16 ameliorates LPS-induced lung injury without complications. Schematic illustration of delivery of sEV-Con and sEV-CC16 into LPS-pretreated mice after CC16 knockdown (**A**). Mice were intratracheally treated with CC16 siRNA and maintained for 5 days. After inducing knockdown, LPS treatment was applied, and sEV-Con or sEV-CC16 was treated 3 hours later. Mice were then maintained for 24 h (**B-E;** n = 6 per group) or 1 week (**F-K;** n = 5 per group). H&E staining of BAL cells. M: macrophage; N: neutrophil. Scale bar = 100 μm (**B**). The number of BALF macrophages (**C**) or neutrophils (**D**). Lung wet to dry weight ratios (**E**). Weight

change ratio of mice body was recorded after inducing CC16 knockdown (**F-H**). Lung wet-to-dry weight ratios on day 7 (**I**). H&E staining of lung sections (**J**). Red arrows: neutrophils; Blue arrows: alveolar disruption with hyaline membranes. Scale bar = 100 μm. Lung injury scored (**K**). Results represent mean ± SD and the data is analyzed by a one-way ANOVA followed by Tukey's HSD. ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$.

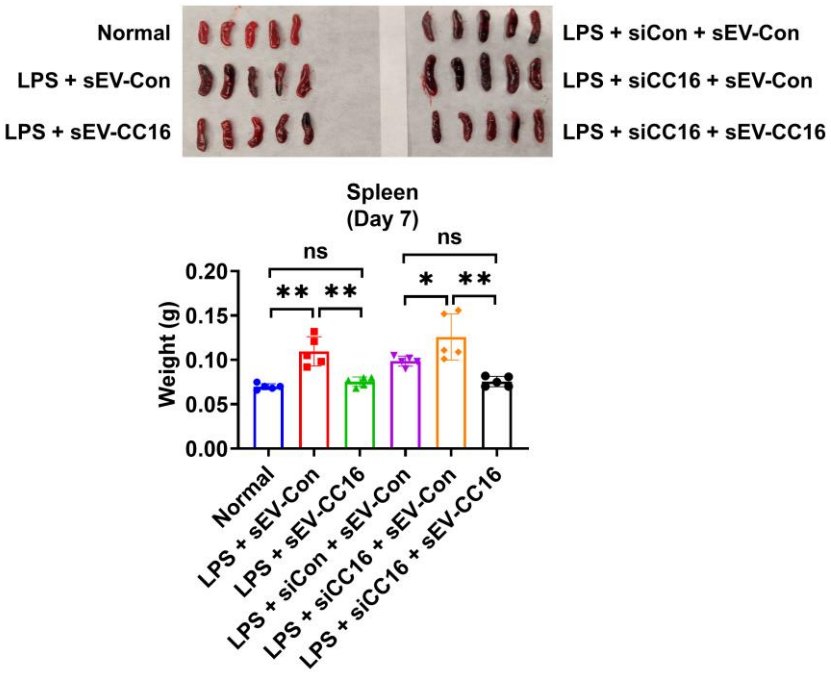

fig.S8. sEV-CC16 prevents enlargement of the spleen. Mice (n = 5 per group) receive control siRNA (siCon) or CC16 siRNA (siCC16) via i.t. After 5 days, received 1 μg LPS (in 50 μL PBS) via i.t. After 3 h, mice were given sEV-Con (7.5×10^{10} in 50 μL PBS) or sEV-CC16 (7.5×10^{10} in 50 μL PBS) via the i.t. route. After 1 week, the spleen was collected for image and weight change confirmation (A-B). Results are presented as mean ± SD and are analyzed by one-way ANOVA followed by Tukey's HSD. ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$.

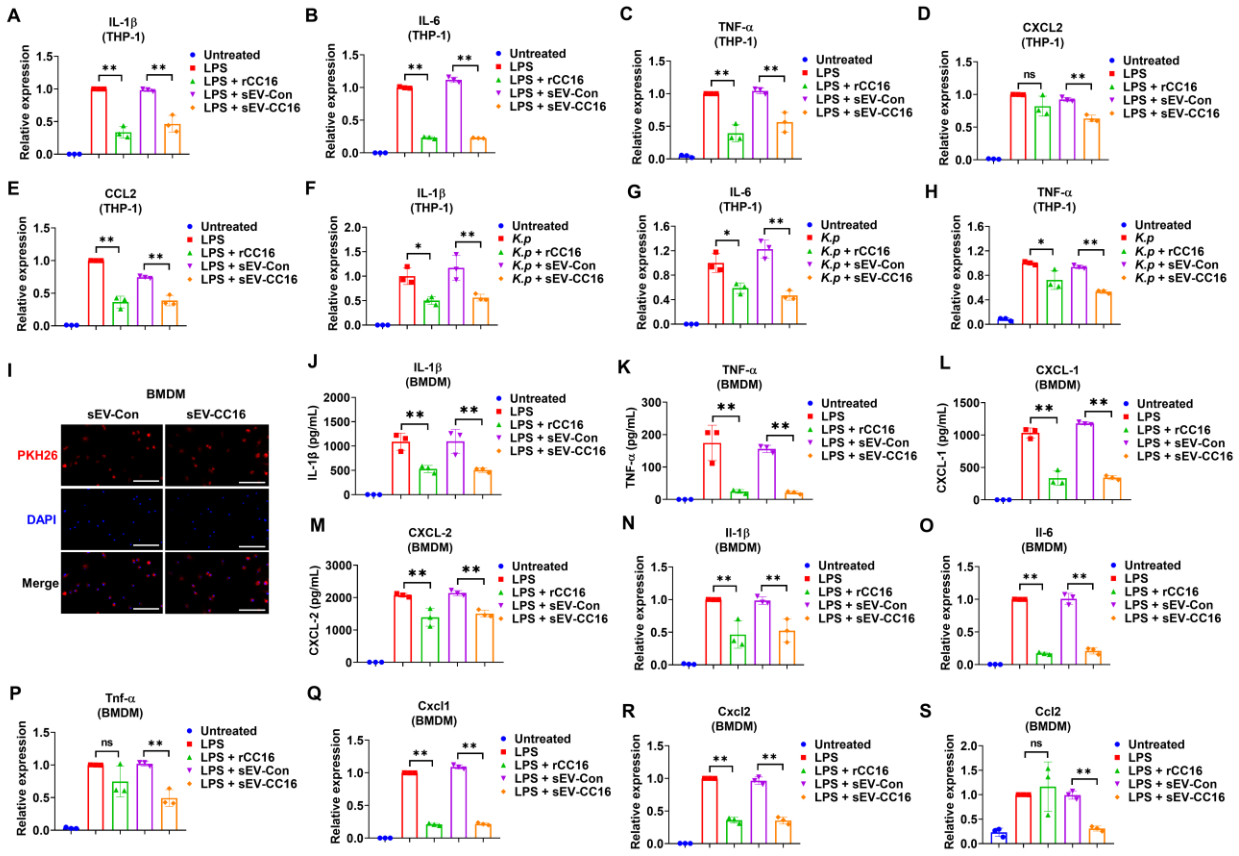

fig. S9. sEV-CC16 inhibits cytokine and chemokine expression in macrophage-like cells. (**A-E**) 100 ng/mL LPS-treated THP-1 cells were incubated with 5×10^8/mL sEV-Con, sEV-CC16, or 1 μg/mL rCC16 for 24 h. mRNA levels of cytokines (IL-1β, IL-6, and TNF-α) and chemokines (CXCL2 and CCL2) were measured by RT-qPCR (n = 3 per group). (**F-H**) THP-1 cells were infected with *K.p* (MOI 1:5) for 1 h and then were incubated with 5×10^8/mL sEV-Con, sEV-CC16, or 1 μg/mL rCC16 for 24 h. mRNA levels of IL-1β, IL-6, and TNF-α were measured by RT-qPCR (n = 3 per group). Results represent mean ± SD and were analyzed by a one-way ANOVA followed by Tukey's HSD. *, $p < 0.05$; **, $p < 0.01$. (**I-S**) 100 ng/mL LPS-treated BMDM were incubated with 5×108/mL sEV-Con, sEV-CC16, or 1 μg/mL rCC16 for 24 h. PKH26-labeled sEV-Con or sEV-CC16 were added to BMDM for 24 h and the internalization of sEV was observed using a fluorescence microscope. Scale bar = 100 μm (**I**). The secretion of cytokines (IL-1β and TNF-α)

and chemokines (CXCL-1 and CXCL-2) was measured by ELISA (n = 3 per group) (**J**-**M**). mRNA levels of cytokines (Il-1β, Il-6, and Tnf-α) and chemokines (Cxcl-1, Cxcl-2, and Ccl2) were determined by RT-qPCR (n = 3 per group) (**N**-**S**). Results represent mean ± SD and were analyzed by one-way ANOVA followed by Tukey's HSD. ns, $p > 0.05$; **, $p < 0.01$.

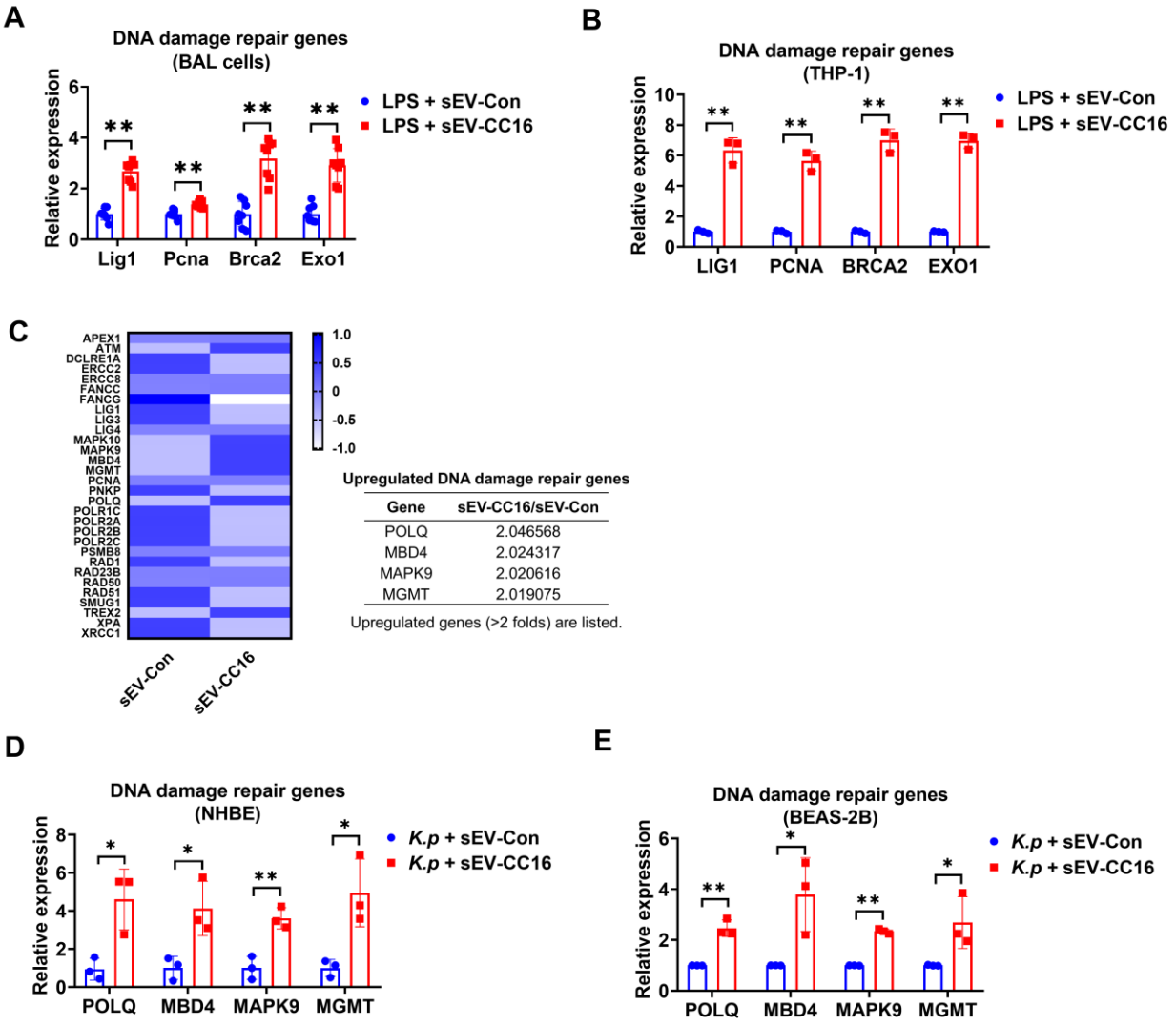

fig. S10. sEV-CC16 induces DNA damage response genes. (**A**) Mice received 1 μg LPS in (50 μL PBS) via the i.t. route. After 3 h, mice were treated with sEV-Con or sEV-CC16 via the i.t. route. Mice were sacrificed 24 h after sEV treatment. mRNA levels of Lig1, Pcna, Brca2, and Exo1 are determined by RT-qPCR in BAL cells (n = 8 per group). (**B**) 100 ng/mL LPS-treated THP-1 cells were incubated with 5×10^8/mL sEV-Con, sEV-CC16, or 1 μg/mL rCC16 for 24 h. DNA damage response genes were measured by RT-qPCR. (**C-E**) *K.p*-infected (MOI 1:5) NHBE or BEAS-2B were treated with 5×10^8/mL sEV-Con or sEV-CC16. mRNAs from treated NHBE were subject to TaqMan™ Array Human DNA Repair Mechanism analysis and upregulated genes

are listed (n = 3 per group) (**C**). Upregulated genes were confirmed using RT-qPCR in NHBE (**D**) and BEAS-2B cells (**E**). Results were represented as mean ± SD and analyzed by two-tailed unpaired Student's t-test. *, $p < 0.05$; **, $p < 0.01$.

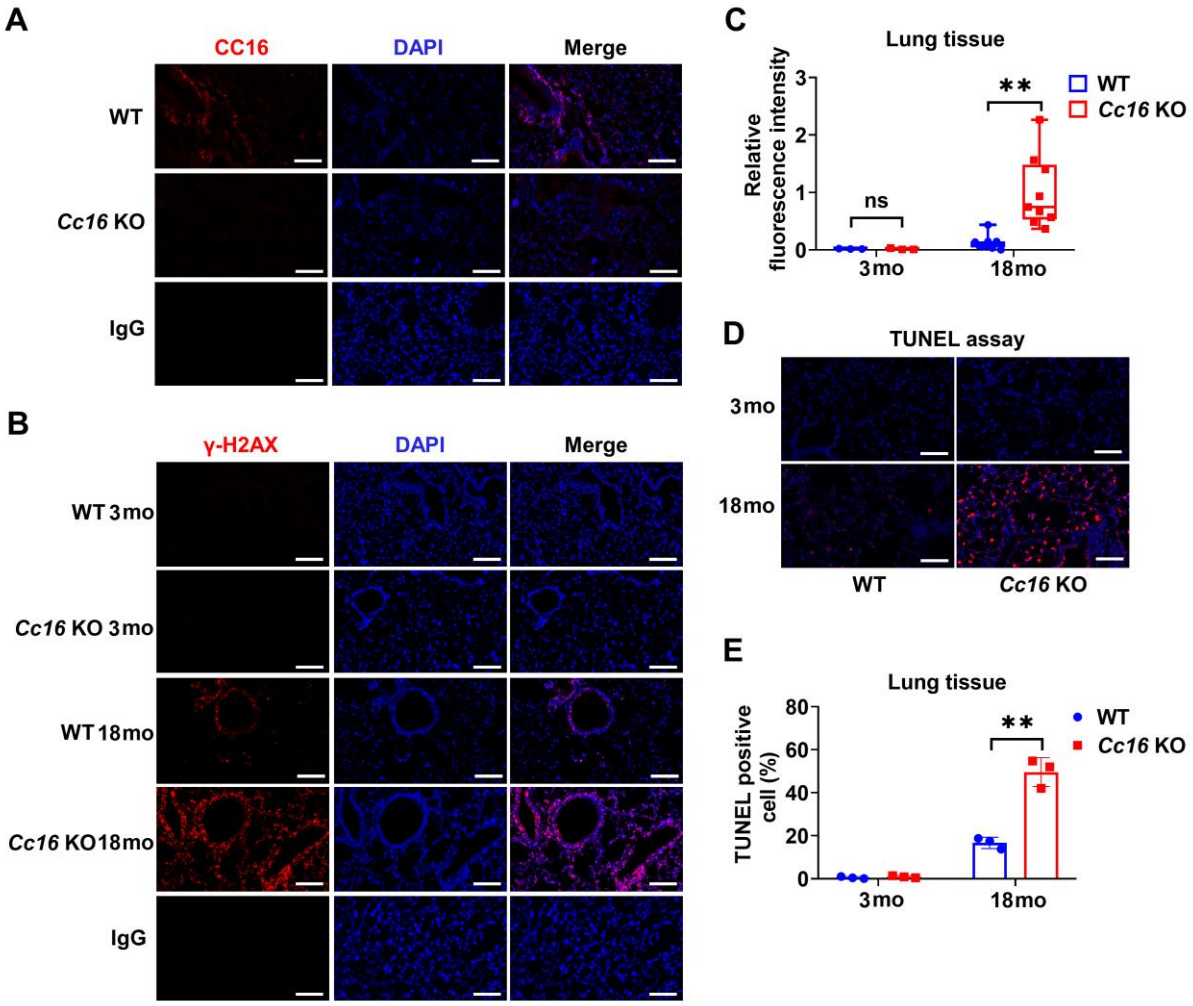

fig. S11. *Cc16* deficiency results in increased DNA damage in mice. Untreated WT mice and *Cc16* KO mice were maintained in normal condition for 3 months or 18 months. (**A**) Lung sections from 3-month-old WT and *Cc16* KO mice were stained using an antibody against CC16 (n = 3 per group). Non-immune rabbit IgG was used as a negative control. Scale bar = 100 μm. (**B**) Immunofluorescence staining was performed in lung sections from 3-month-old (n = 3 per group) or 18-month-old (n = 8-9 per group) WT and *Cc16* KO mice using an antibody against γ-H2AX. Lung sections from 18-month-old WT mice were stained with non-immune rabbit IgG. Scale bar = 100 μm. (**C**) Relative fluorescence intensity is measured by Image J software (n = 3 for 3-month-old WT and *Cc16* KO mice; n = 8 for 18-month-old WT mice; n = 9 for 18-month-old *Cc16* KO

mice). The boxes in the boxplots show the medians with 25th and 75th percentiles, and the whiskers show the Min and Max values. The data is analyzed using two-tailed unpaired Mann-Whitney U test. ns, $p > 0.05$; **, $p < 0.01$. (**D**) DNA damage in the lung was measured by TUNEL staining. Scale bar = 100 μm. (**E**) The percentage of TUNEL-positive cells is calculated (n = 3 per group). Results represented mean ± SD and are analyzed using two-tailed unpaired Student's t-test. ns, $p > 0.05$; **, $p < 0.01$.

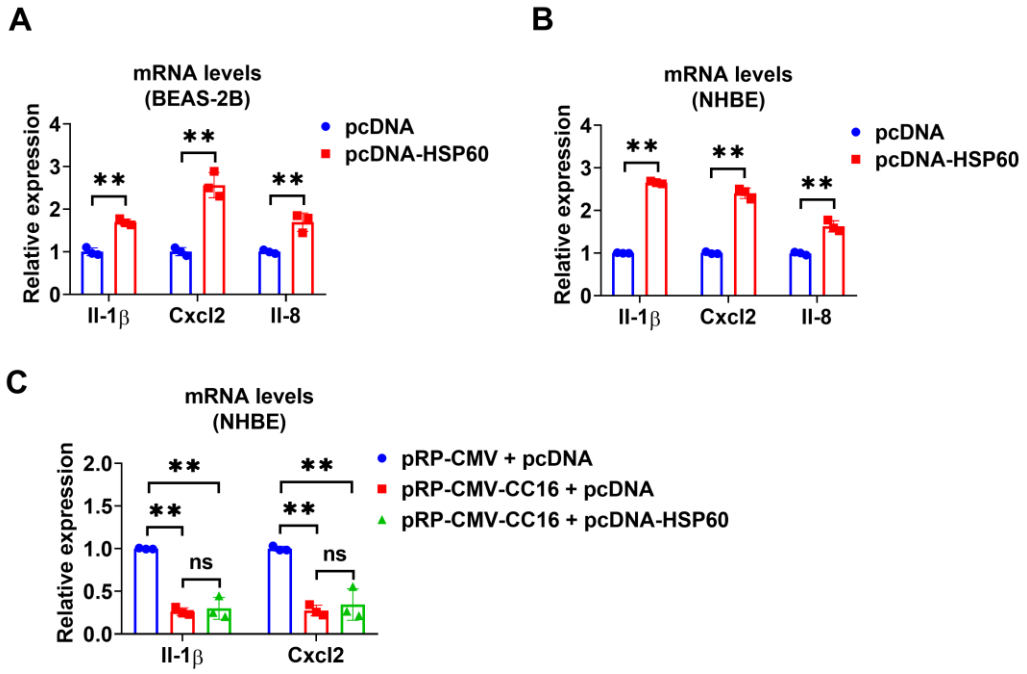

fig. S12. CC16 suppresses mRNA levels of cytokines through interaction with HSP60. (**A-C**) mRNA levels of IL-1β, CXCL2, and IL-8 are detected after transfection of HSP60 and/or CC16 plasmid as indicated. In panel **A-B**, results are mean ± SD of 3 independent experiments and analyzed by two-tailed unpaired Student's t-test. In panel **C**, the results of 3 independent experiments are represented as mean ± SD and analyzed by a one-way ANOVA followed by Tukey's HSD. ns, $p > 0.05$; **, $p < 0.01$.

Table S1 Clinical characteristics of study subjects

Characteristics	Normal (N = 10)	Pneumonia (N = 21)	*p*-Value
Age (years), mean (SD)	60.2 ± 17.7	55.7 ± 16.6	*N.S*
Number of males (%)	4 (40)	12 (57)	*N.S*

Detailed information

Sample #	Age (years)	Gender	Pneumonia	Source	Sample details
1	74	Male	No	BioIVT	Normal donor wihout diseases including COPD, Idiopathic Pulmonary Fibrosis and Cystic Fibrosis.
2	66	Female	No	BioIVT	Normal donor wihout diseases including COPD, Idiopathic Pulmonary Fibrosis and Cystic Fibrosis.
3	53	Male	No	BioIVT	Normal donor wihout diseases including COPD, Idiopathic Pulmonary Fibrosis and Cystic Fibrosis.
4	44	Female	No	BioIVT	Normal donor wihout diseases including COPD, Idiopathic Pulmonary Fibrosis and Cystic Fibrosis.
5	23	Female	No	BioIVT	Normal donor wihout diseases including COPD, Idiopathic Pulmonary Fibrosis and Cystic Fibrosis.
6	76	Female	No	BioIVT	Normal donor wihout diseases including COPD, Idiopathic Pulmonary Fibrosis and Cystic Fibrosis.
7	68	Male	No	BioIVT	Normal donor wihout diseases including COPD, Idiopathic Pulmonary Fibrosis and Cystic Fibrosis.
8	50	Female	No	BioIVT	Normal donor wihout diseases including COPD, Idiopathic Pulmonary Fibrosis and Cystic Fibrosis.
9	67	Female	No	BioIVT	Normal donor wihout diseases including COPD, Idiopathic Pulmonary Fibrosis and Cystic Fibrosis.
10	81	Male	No	BioIVT	Normal donor wihout diseases including COPD, Idiopathic Pulmonary Fibrosis and Cystic Fibrosis.
11	82	Male	Yes	Discovery Life	Pnuemonia positive (Escherichia coli)

				Sciences Inc	
12	55	Male	Yes	Discovery Life Sciences Inc	Pnuemonia positive (Staphylococcus lugdunensis, Methicillin resistant Staphylococcus aureus)
13	78	Male	Yes	Discovery Life Sciences Inc	Pnuemonia positive (Pseudomonas aeruginosa)
14	65	Female	Yes	Discovery Life Sciences Inc	Pnuemonia positive (Pseudomonas aeruginosa)
15	55	Female	Yes	Discovery Life Sciences Inc	Pnuemonia positive (Gram-negative bacilli)
16	19	Female	Yes	Discovery Life Sciences Inc	Pnuemonia positive (Providencia stuartii | Achrombacter xylosoxidans/ proteus mirabilis)
17	31	Male	Yes	Discovery Life Sciences Inc	Pnuemonia positive (Gram-negative bacilli)
18	42	Male	Yes	Discovery Life Sciences Inc	Pnuemonia positive (Gram-negative bacilli)
19	31	Male	Yes	Discovery Life Sciences Inc	Pnuemonia positive (Gram-negative bacilli)
20	45	Male	Yes	BioLINCC	ARDS positive, primary pneumonia
21	44	Male	Yes	BioLINCC	ARDS positive,primary aspiration, secondary pneumonia, secondart trauma
22	60	Female	Yes	BioLINCC	ARDS positive, primary pneumonia
23	71	Female	Yes	BioLINCC	ARDS positive,primary pneumonia
24	45	Male	Yes	BioLINCC	ARDS positive,primary pneumonia
25	71	Male	Yes	BioLINCC	ARDS positive, primary pneumonia
26	65	Female	Yes	BioLINCC	ARDS positive, primary sepsis site lung pleura, primary pneumonia
27	60	Female	Yes	BioLINCC	ARDS positive, primary pneumonia
28	71	Female	Yes	BioLINCC	ARDS positive, primary pneumonia
29	71	Male	Yes	BioLINCC	ARDS positive, primary pneumonia
30	44	Male	Yes	BioLINCC	ARDS positive,primary aspiration, secondary pneumonia, secondart trauma
31	65	Female	Yes	BioLINCC	ARDS positive,primary sepsis site lung pleura, primary pneumonia

Table S2 WikiPathway

Table S3 DNA oligo sequences

	Name	Forward primer (5' to 3')	Reverse primer (5' to 3')
qPCR primers	mouse IL-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
	mouse Il-1β	TGGACCTTCCAGGATGAGGACA	GTTCATCTCGGAGCCTGTAGTG
	mouse Tnf-α	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
	mouse Cxcl-1	CTGGGATTCACCTCAAGAACATC	CAGGGTCAAGGCAAGCCTC
	mouse Cxcl-2	CCAACCACCAGGCTACAGG	GCGTCACACTCAAGCTCTG
	mouse IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
	mouse Ccl2	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
	mouse Lig1	TTCTGAGCTGTGAAGGGGAG	GACGCTTTGGGAATCCTGATG
	mouse Pcna	TTTGAGGCACGCCTGATCC	GGAGACGTGAGACGAGTCCAT
	mouse Brca2	ATGCCCGTTGAATACAAAAGGA	ACCGTGGGGCTTATACTCAGA
	mouse Exo1	TGGCTGTGGATACCTACTGTT	ATCGGCTTGACCCCATAAGAC
	mouse Tbp	TCAAACCCAGAATTGTTCTCC	GGGGTAGATGTTTTCAAATGC
	human Il-1β	CCACAGACCTTCCAGGAGAATG	GTGCAGTTCAGTGATCGTACAGG
	human Tnf-α	CTCTTCTGCCTGCTGCACTTTG	ATGGGCTACAGGCTTGTCACTC
	human Cxcl-1	AGG GAA TTC ACC CCA AGA AC	ACT ATG GGG GAT GCA GGA TT
	human Cxcl-2	CCCATGGTTAAGAAAATCATCG	CTTCAGGAACAGCCACCAAT
	human Il-8	CTGTGTGAAGGTGCAGTTTTGCC	CTCAGCCCTCTTCAAAAACTTCTCC
	human IL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
	human Ccl2	CCCCAGTCACCTGCTGTTAT	TGGAATCCTGAACCCACTTC
	human Lig1	GCCCTGCTAAAGGCCAGAAG	CATGGGAGAGGTGTCAGAGAG
	human Pcna	CCTGCTGGGATATTAGCTCCA	CAGCGGTAGGTGTCGAAGC

	human Brca2	CACCCACCCTTAGTTCTACTGT	CCAATGTGGTCTTTGCAGCTAT
	human Exo1	CCTCGTGGCTCCCTATGAAG	AGGAGATCCGAGTCCTCTGTAA
	human Polq	CTGCGTCGGAGTGGGAAAC	CTGTAGGCTTGCATTCTCCTG
	human Mbd4	CCGTCACCTCTAGTGAGCG	GCAGAAGCGATGGGTTCTTGTA
	human Mapk9	GAAACTAAGCCGTCCTTTTCAGA	TCCAGCTCCATGTGAATAACCT
	human Mgmt	ACCGTTTGCGACTTGGTACTT	GGAGCTTTATTTCGTGCAGACC
	human Tbp	GATAAGAGAGCCACGAACCAC	GATAAGAGAGCCACGAACCAC
Cloning primers	human HSP60	CGGGGTACCATGCTTCGGTTACCCACAGT	CCGCTCGAGGAACATGCCACCTCCCATAC