Supplementary Materials for

CircCDC42 encoded CDC42-165aa regulates macrophage pyroptosis in *Klebsiella pneumoniae* infection through pyrin inflammasome activation

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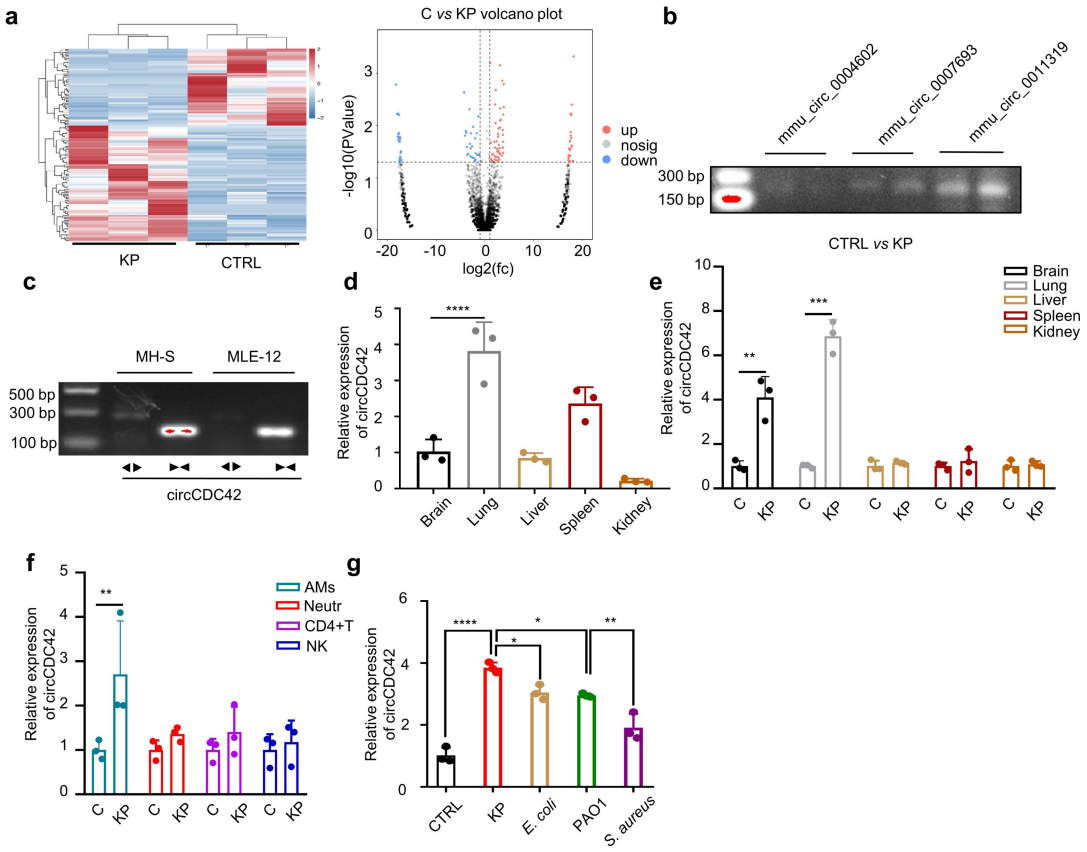
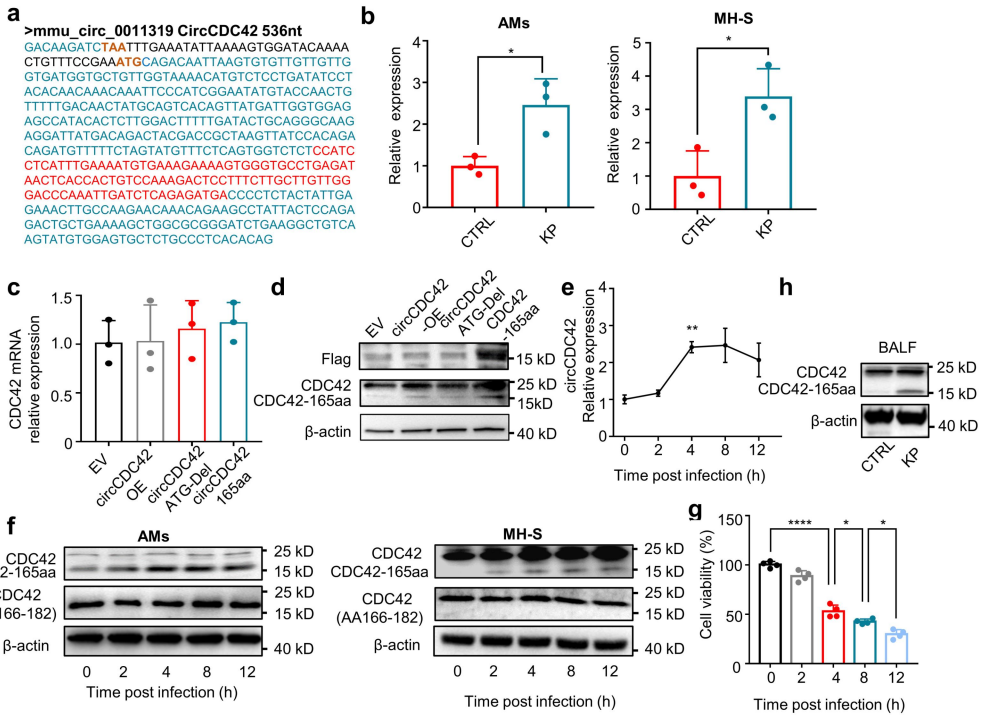


Supplementary Figure 1. CircRNAs screening in vivo and vitro after KP infection (Related to Figure 1)

a A total of 3 pairs of MH-S cell infected with or without KP (MOI of 20:1) were subjected to RNA sequencing (RNA-seq). Heatmap and volcano plot for microarray analysis of differentially expressed circRNAs in MH-S cells. **b** MH-S cells were infected with KP at an MOI of 20:1 for 4 h. cDNAs from MH-S were used as templates to amplify circRNAs using divergent primers. Related to Figure 1b (n = 3 biological replicates). **c** PCR products using circCDC42 divergent primers from MH-S and MLE-12 were analyzed by agarose gel electrophoresis (n = 3 biological replicates). **d** Expression levels of circCDC42 in the different tissues from C57BL/6J mice were examined by RT-qPCR (n = 3 biological replicates). **e** C57BL/6J mice were challenged intranasally with 5×10^6 colony forming units (CFU) of KP. Expression levels of circCDC42 in mice were examined by RT-qPCR (n = 3 biological replicates). **f** Expression levels of circCDC42 in the different cells were examined by RT-qPCR upon KP infection (n = 3 biological replicates). **g** Expression

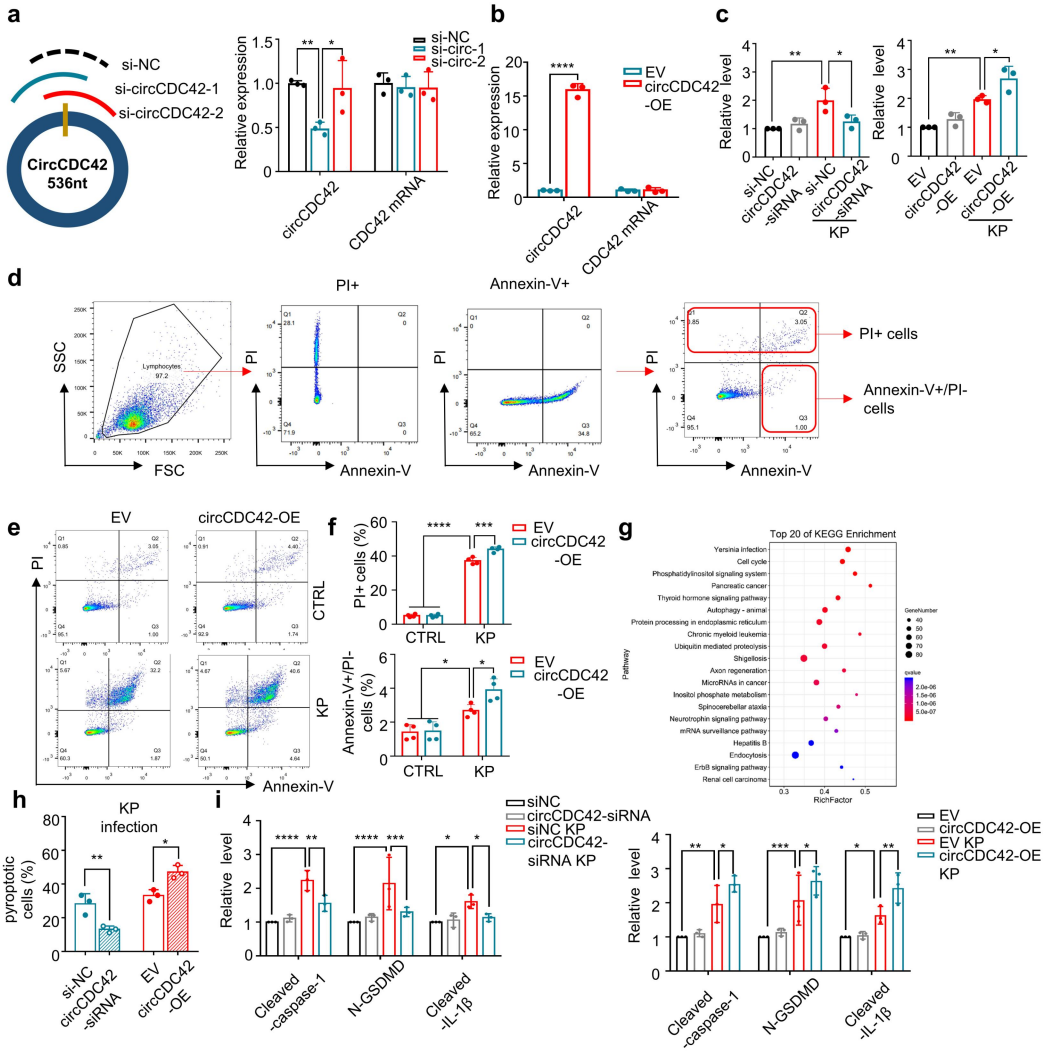
levels of circCDC42 in mouse lungs were examined by RT-qPCR upon indicated pathogen (5×10^6 CFU) infection (n = 3 biological replicates). Data are shown as means ± SD. **d, g** One-way ANOVA with Tukey test. **e, f** Two-way ANOVA with Bonferroni's test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.



Supplementary Figure 2. Identification the unique CDC42-165aa sequence in circCDC42 (Related to Figure 2)

a The sequences of the putative ORF and IRES (red) are shown. Related to Figure 2a. **b** Primary AMs and MH-S cells were infected with KP at an MOI of 20 : 1 for 4 h. Relative expression of CDC42-165aa expression in AMs (left panel) and MH-S (right panel) as in Figure 2d (n = 3 biological replicates). **c** MH-S cells were treated with circCDC42 ATG delete plasmid, circCDC42 or CDC42-165aa plasmid. The linear CDC42 mRNA level was detected by RT-qPCR (n = 3 biological replicates). **d** Expression of CDC42-165aa-Flag and CDC42-165aa in the indicated MH-S were detected by immunoblotting (n = 3 biological replicates). **e** MH-S cells were infected with KP at an MOI of 20 : 1, and the level of circCDC42 was detected by RT-qPCR at the indicated time points (n = 3 biological replicates). **f** Primary AMs (left panel) and MH-S cells (right panel) were infected with KP at an MOI of 20 : 1. Expression of CDC42 and CDC42-165aa proteins in cells for indicated time point were detected by immunoblotting using CDC42 antibody (Affinity, DF6322, AA51-74) or CDC42 antibody (Santa Cruz, SC-8401, AA166-182) (n = 3 biological replicates). **g** MH-S cells were infected with KP at an MOI of 20 : 1 for indicated time point, and cell viability was measured by MTT assay (n = 4 biological replicates). **h**

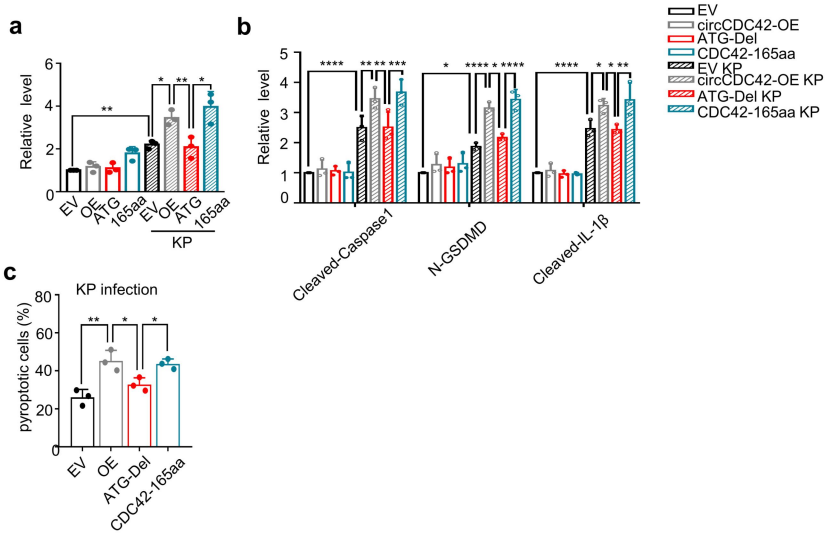
C57BL/6J mice were challenged intranasally with 5×10^6 CFU of KP for 24 h. Expression levels of CDC42-165aa in AMs isolated from BALF of mice with KP infection for 24 h were examined by immunoblotting (n = 3 biological replicates). Data are shown as means ± SD. **b** Unpaired two-tailed t-test. **c, e, g** One-way ANOVA with Tukey test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.



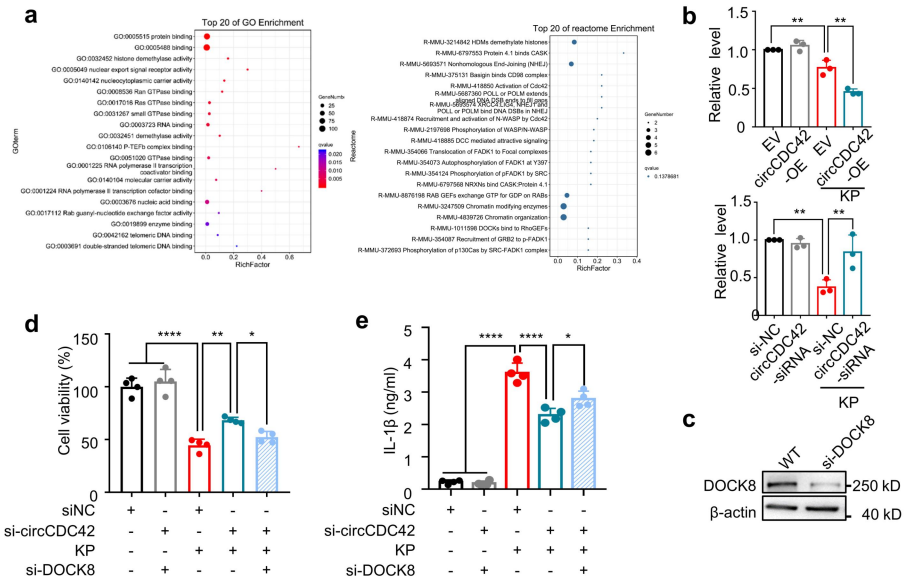
Supplementary Figure 3. Related to Figure 3.

a Left, illustration of the circCDC42 junction-specific FISH probe and circCDC42 siRNA target site. Right, MH-S cells were transfected with siNC or circCDC42 siRNA, circCDC42 and linear CDC42 mRNA level were detected by RT-qPCR (n = 3 biological replicates). **b** MH-S cells were transfected with empty vector (EV) or circCDC42 overexpreesion (OE) plasmid, respectively for 24 h. circCDC42 and linear CDC42 mRNA level were detected by RT-qPCR (n = 3 biological replicates). **c** The indicated MH-S cell swere infected with KP at an MOI of 20:1 for 4 h. Relative amounts of CDC42-165aa expression in MH-S cells treated as

in Figure 3a (n = 3 biological replicates). **d** Annexin-FITC and PI staining of MH-S cells. The gating strategy for the MH-S cells (Annexin V+PI^- cells and PI^+ cells). **e** Annexin-FITC and PI staining of MH-S cells subjected to KP infection with or without overexpreesion (OE) plasmid treatment. Representative FACS plots are shown (n = 3 biological replicates). **f** A summary of the percentage of Annexin V+PI^- cells and PI^+ cells among dead cells as in Supplementary Figure 3d (n = 3 biological replicates). **g** Enrichment of differentially expressed genes in different pathways by KEGG analysis. **h** MH-S cells were transfected with EV or circCDC42-OE plasmids, respectively for 24 h, then infected with KP at an MOI of 20:1 for 4 h. The percentage of pyroptotic cells in Figure 3d was calculated (n = 3 biological replicates). **i** MH-S cells were transfected with siNC or circCDC42 siRNA, EV or circCDC42-OE plasmid, respectively for 24 h. The cells were then infected with KP at an MOI of 20:1 for 4 h. Relative level of Cleaved-Caspase-1, N-GSDMD and Cleaved-IL-1β in MH-S cells treated as indicated in Figure 3f (n = 3 biological replicates). Data are shown as means ± SD. **a, b, c, f, h, i** Two-way ANOVA with Bonferroni's test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

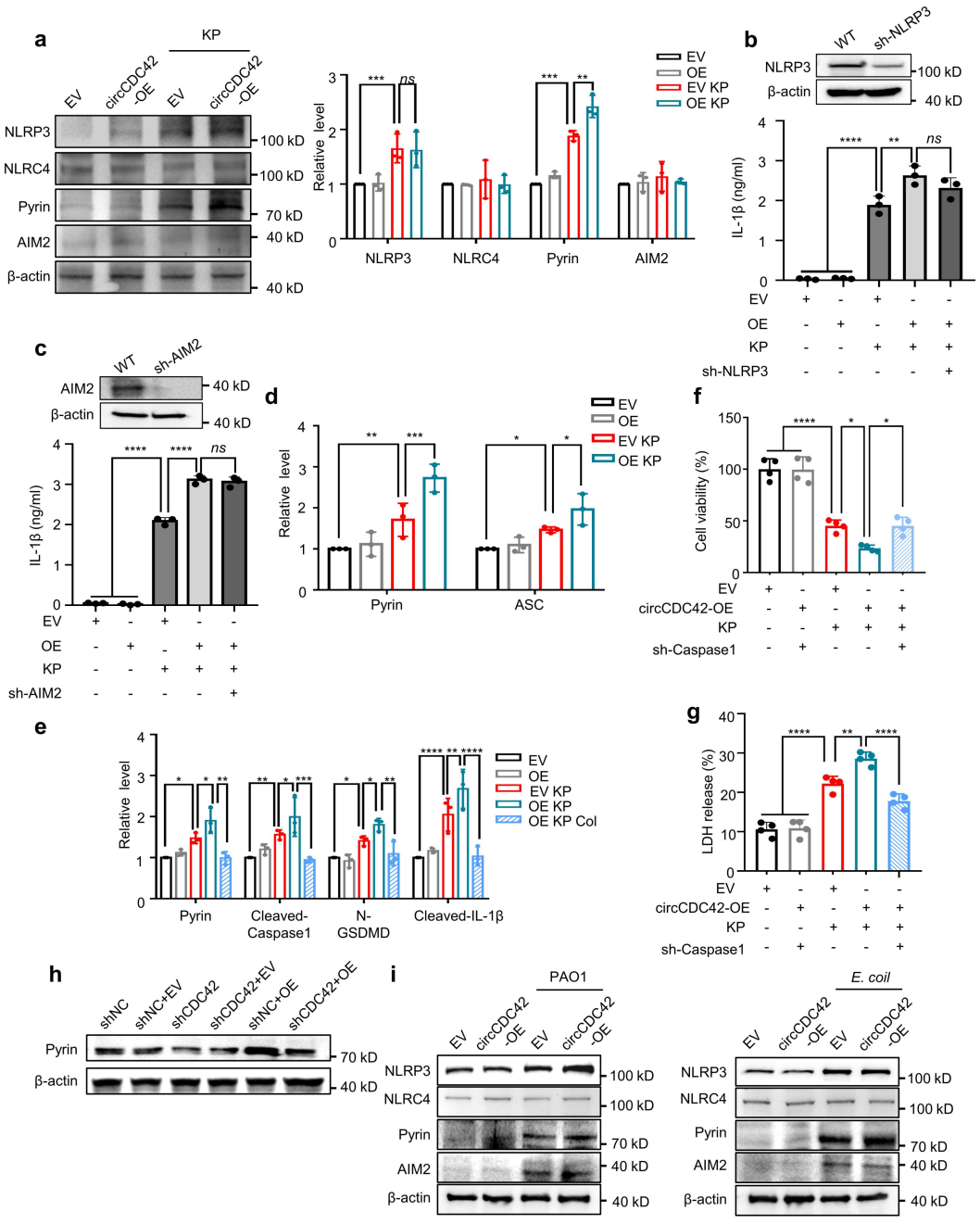


Supplementary Figure 4. Related to Figure 4 MH-S cells were transfected with EV, circCDC42-OE plasmid, circCDC42 ATG-Del plasmid, or CDC42-165aa plasmid, respectively for 24 h. **a** Relative level of CDC42-165aa expression in MH-S cells treated as indicated in Figure 4a (n = 3 biological replicates). **b** The percentage of pyroptotic cells in Figure 4c was calculated (n = 3 biological replicates). **c** Relative level of cleaved-Caspase-1, N-GSDMD and cleaved-IL-1β in MH-S cells treated as indicated in Figure 4e (n = 3 biological replicates). Data are shown as means ± SD. **a, c** Two-way ANOVA with Bonferroni's test. **b** One-way ANOVA with Tukey test. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.



Supplementary Figure 5. Related to Figure 5

a A total of 3 pairs of MH-S cell samples infected with or without KP (MOI of 20 : 1) were subjected to RNA sequencing (RNA-seq). Enrichment of differentially expressed genes by Reactome and GO analysis. **b** MH-S cells were transfected with empty EV, circCDC42-OE plasmid, circCDC42 ATG-Del plasmid, or CDC42-165aa plasmid, respectively for 24 h. Cells were then infected with KP at an MOI of 20 : 1 for 4 h. Relative level of activated GTPases CDC42 in MH-S cells treated as indicated in Figure 5e (n = 3 biological replicates). **c** MH-S cells were treated with or without DOCK8 siRNA for 24 h. DOCK8 levels in MH-S cells were detected by western blotting (n = 3 biological replicates). **d** The indicated MH-S cells were infected with or without KP (MOI of 20 : 1) for 4 h, and cell viability was measured by MTT assay (n = 4 biological replicates). **e** The secretion of IL-1β in cell supernatant after KP infection (MOI of 20 : 1) for 4 h was detected by ELISA (n = 4 biological replicates). Data are shown as means ± SD. **b, d, e** One-way ANOVA with Tukey test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

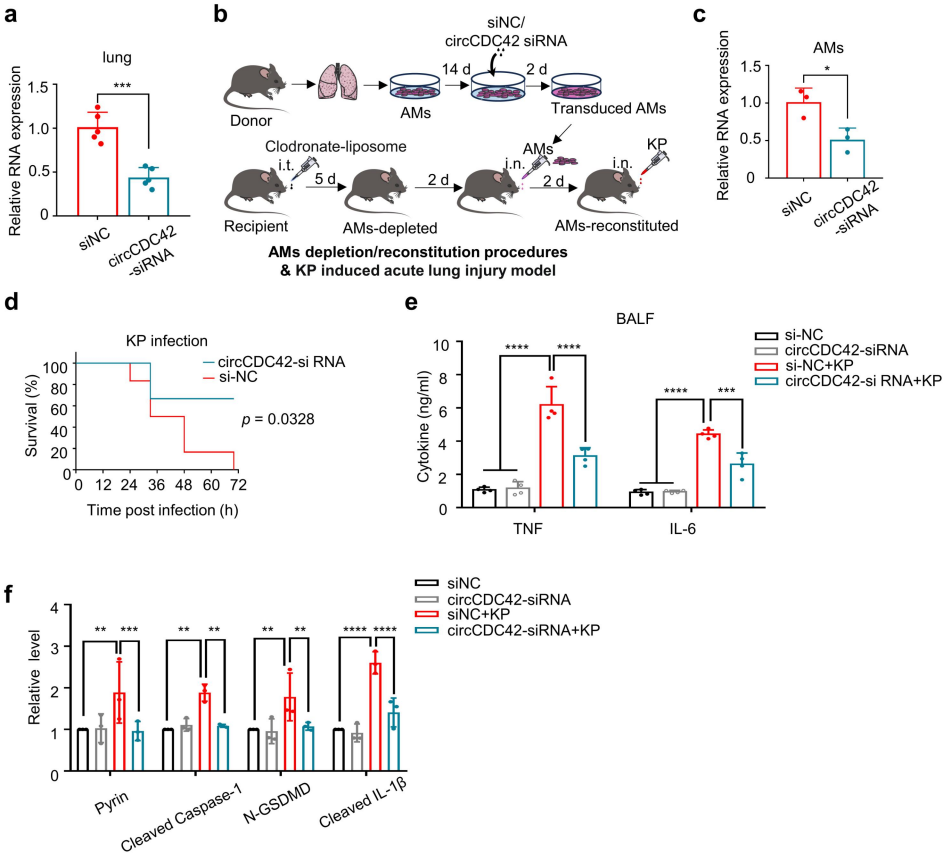


Supplementary Figure 6. Related to Figure 6

a MH-S cells were transfected with EV or circCDC42-OE plasmid, respectively for 24 h, then infected with KP at an MOI of 20 : 1 for 4 h. Left, expression of NLRP3, NLRC4, Pyrin, and AIM2 in MH-S cells were detected by immunoblotting. Right, relative levels were quantified by ImageJ (n = 3 biological replicates). **b** MH-S cells were treated with EV, circCDC42 -OE plasmid, or NLRP3 shRNA as indicated for 24

h, then infected with KP (MOI of 20 : 1, 4 h) or not. Western Blotting showing NLRP3 expression in MH-S cells. The secretion of IL-1β in cell supernatant was detected by ELISA (n = 3 biological replicates). **c** MH-S cells were treated with EV, circCDC42 -OE plasmid, or AIM2 shRNA as indicated for 24 h, then infected with KP (MOI of 20:1, 4 h) or not. AIM2 level in MH-S cells was detected by western blotting. The secretion of IL-1β in cell supernatant was detected by ELISA (n = 3 biological replicates). **d** The expression level of Pyrin bound to ASC was assessed using optical density and presented as fold change to EV group, as shown in Figure 6a (n = 3 biological replicates). **e** Relative level of Pyrin, cleaved-Caspase-1, N-GSDMD, and cleaved-IL-1β in MH-S cells treated as indicated, shown as in Figure 6e (n = 3 biological replicates). **f** MH-S cells were treated with EV, circCDC42 -OE plasmid, or Caspase-1 shRNA for 24 h, then infected with or without KP (MOI of 20 : 1) for 4 h. The cell viability was measured by MTT assay (n = 5 biological replicates). **g** LDH release was measured from MH-S cells infected with or without KP (MOI of 20:1) for 4 h , and presented as percent of medium activity compared to cell lysate from cells (n = 4 biological replicates). **h** MH-S cells were treated with EV, circCDC42 -OE plasmid, or CDC42 shRNA as indicated for 24 h, then infected with KP (MOI of 20:1) for 4 h. Pyrin level in MH-S cells was detected by western blotting. (n = 3 biological replicates). **i** Protein expression of NLRP3, NLRC4, Pyin, and AIM2 in MH-S cells transfected with indicated plasmid with PAO1 or *E. coli* infection (MOI of 20 : 1) for 4 h or not (n = 3 biological replicates). Data are shown as means ± SD. **a, d, e** Two-way ANOVA with Bonferroni's test. **b, f, g** One-way ANOVA with Tukey test. *p

< 0.05; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$, *ns* no significant difference.



Supplementary Figure 7. Related to Figure 7

a Moues model of KP infection was established as Figure 7a shown. RT-qPCR analysis of circCDC42 expression in lungs from KP-infected siNC- and circCDC42-siRNA- injected mice (n = 5). **b** AMs depletion/reconstitution procedures and KP induced acute lung injury model. **c** RT-qPCR analysis of circCDC42 expression in AMs from BLAF in Figure S7b mice (n = 3 biological replicates). **d** Kaplan-Meier survival curves of KP-infected siNC- and circCDC42 siRNA-AMs Recipient mice (n = 6). Survival was determined up to 72 h ($p = 0.0328$). **e** Expression levels of TNF and IL-6 in BLAF from KP-infected siNC- and circCDC42 siRNA-AMs Recipient mice were determined by ELISA (n = 4 biological replicates). **f** Relative level of Pyrin, cleaved-Caspase-1, N-GSDMD, and cleaved-IL-1β in AMs

from KP-infected siNC- and circCDC42 siRNA-AMs Recipient mice, as shown in Figure 7h (n = 3 biological replicates). Data are shown as means ± SD. **a, c** Unpaired two-tailed t-teste. Log-rank (Mantel-Cox) test. **e, f** Two-way ANOVA with Bonferroni's test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Supplementary Table 1. Key Resources

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Caspase-1 (E2Z1C) Rabbit mAb (1:1000)	Cell Signaling Technology	#24232
Cleaved Gasdermin D (Gly277) Antibody (1:1000)	Cell Signaling Technology	#34667
NLRP3 (D4D8T) Rabbit mAb (1:1000)	Cell Signaling Technology	#15101;
IL-1β (3A6) Mouse mAb (1:1000)	abcam	#ab234437
ASC/TMS1/PYCARD Antibody (B-3) (1:1000)	Santa Cruz	#sc-514414
Anti-Pyrin antibody (1:1000)	abcam	#ab195975
AIM2 Polyclonal antibody (1:1000)	Proteintech	#20590-1-AP
DOCK8 Polyclonal antibody (1:1000)	Proteintech	#11622-1-AP
Beta Actin Monoclonal antibod (1:5000)	Proteintech	#66009-1-Ig
Cdc42 Antibody (B-8)（166-182aa） (1:1000)	Santa Cruz	#sc-8401
m-IgG3 BP-HRP (1:1000)	Santa Cruz	#sc-533670
CDC42 Rabbit Polyclonal Antibody (1:1000)	Affinity Biosciences	#DF6322
ANTI-FLAG mouse Monoclonal antibody mouse (1:1000)	Sigma-Aldrich	#F1804-50UG
NLRC4 Rabbit pAb (A7382) (1:1000)	ABclonal	#A7382
CoraLite488-conjugated Goat Anti-Mouse IgG(H+L) (IF 1:100)	Proteintech	#SA00013-1
CoraLite594-conjugated Goat Anti-Rabbit IgG(H+L) (IF 1:100)	Proteintech	#SA00013-4
HRP-conjugated Affinipure Goat Anti-Mouse IgG(H+L) (WB 1:5000)	Proteintech	#SA00001-1
HRP-conjugated Affinipure Goat Anti-Rabbit IgG(H+L) (WB 1:5000)	Proteintech	#SA00001-2
CD170 (Siglec F) mAb (IF 1:100)	Thermo Fisher Scientific	#14-1702-82
Critical Commercial Assays		
Mouse TNF-alpha ELISA Kit	Proteintech	#KE10002
Mouse IL-6 ELISA Kit	Proteintech	#KE10007
Enhanced BCA Protein Assay Kit	Beyotime	#P0010
Lacate Dehydrogenase(LDH) Activity Assay Kit	Solarbio	#BC0685
Ultra-High Sensitivity ECL Kit	MCE	#HY-K1005
Cdc42 Pull-down Activation Assay	Cytoskeleton	# BK034
The Dual-Luciferase Reporter Assay System	Promega	#E1910

Supplementary Table 2. The list of primer sequences used in the study

Name	Sequence (5' to 3')
For quantitative real-time PCR	
circCDC42-F (divergent)	ATGTGGAGTGCTCTGCCCTC
circCDC42-R(divergent)	TCCTCTTGCCCTGCAGTATCAA
circCDC42-F (convergent)	GGATTATGACAGACTACGACCGCTAAG
circCDC42 -R(convergent)	GGCAGAGCACTCCACATACTTGAC
circRanbp9-F	ACCCGGCAAAAGCAGCACTG
circRanbp9-R	AAGCGGTCTATCTGGGCCTG
circSgms1-F	TGGGCGTTTTCTATTTGCGA
circSgms1-R	ACACTATCCCTCCTTGGCTG
IL-6-F	TTCTTGGGACTGATGCTGGT
IL-6-R	CTGTGAAGTCTCCTCTCCGG
TNF-F	TCAGGCGATCTTCCCATCTC
TNF-R	AAGTGGGATGGTTGGTAGGG
IL-1β-F	CGTTCCCATTAGACAGCTGC
IL-1β-R	TCAGCTCATATGGGTCCGAC
GAPDH-F (divergent)	GCTGAGTATGTCGTGGAGTCT
GAPDH-R (divergent)	GGCAGCCCTGGTGACCAGGCGC
GAPDH-F (convergent)	GAGCGAGACCCCACTAACAT
GAPDH-R (convergent)	CCCTTCCACAATGCCAAAGTT
U6-F	TGCTCGCTTCGGCAGCACAT
U6-R	CTTGCGCAGGGGCCATGCTA
Oligonucleotides for FISH	
circCDC42 FISH	Cy3-AGATCTTGTCCTGTGTGAGGGCAG-Cy3
For SiRNA/SHRNA sequences	
circCDC42-siRNA-1	AUCUUGUCCUGUGUGAGGGCATT
circCDC42-siRNA-2	AAUUAGAUCUUGUCCUGUGUGTT
si NC	UUCUCCGAACGUGUCACGUTT
si-Dock8	GGCTTGTACGAGACGGTTAAT
sh-AIM2	TCCCAGGATTAGTAAACTGAA
sh-Caspase1	CCATGTTGGATCAGATCAACT
sh-NLRC4	CAGAAATTGAAGCCCTGATAA
sh-NC	CCCGAACGGCACGTT