USP3 deubiquitinates and stabilizes the adapter protein ASC to regulate inflammasome activation

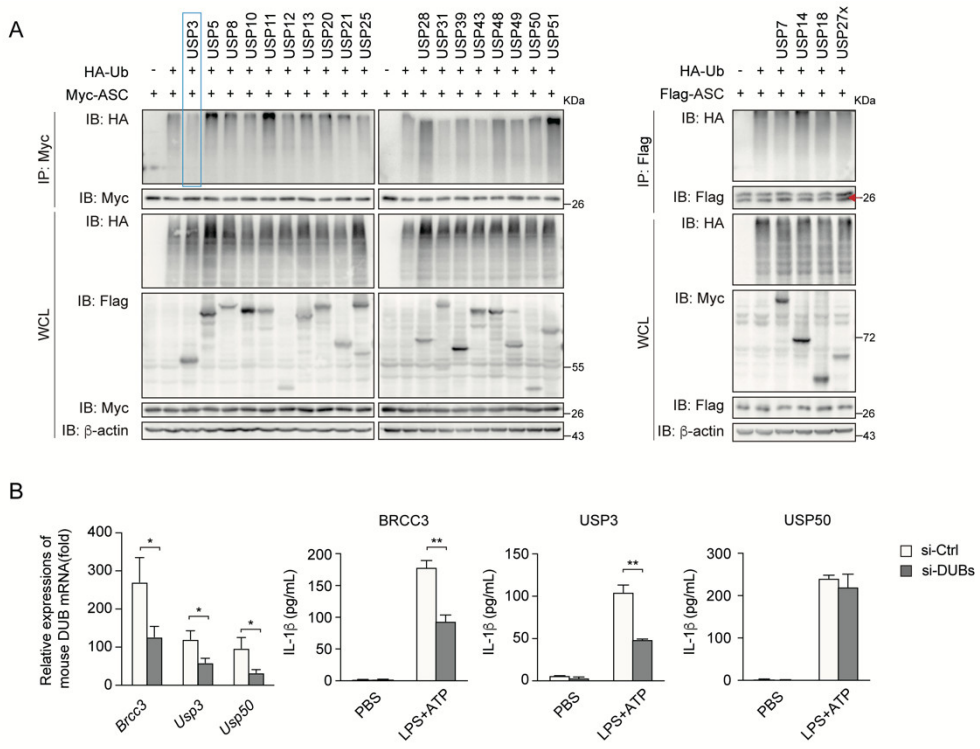
Wanxin Zhuang[1,2], Lei Zhang[1,2], Yi Zheng[1,2], Bingyu Liu[1,2], Chunhong Ma[1,2], Wei Zhao[1,3], Suxia Liu[1,2], Feng Liu[1,2,*], and Chengjiang Gao[1,2,*]

[1]Key Laboratory of Infection and Immunity of Shandong Province & Key Laboratory for Experimental Teratology of Ministry of Education, Shandong University, Jinan, Shandong 250012, P.R. China

[2]Department of Immunology, School of Biomedical Sciences, Shandong University, Jinan, Shandong 250012, P.R. China

[3]Department of Pathogenic Biology, School of Biomedical Sciences, Shandong University, Jinan, Shandong 250012, P. R. China

*Correspondence to: Dr. Chengjiang Gao (lead contact), E-mail: cgao@sdu.edu.cn
or Dr. Feng Liu, E-mail: liufeng2019@sdu.edu.cn

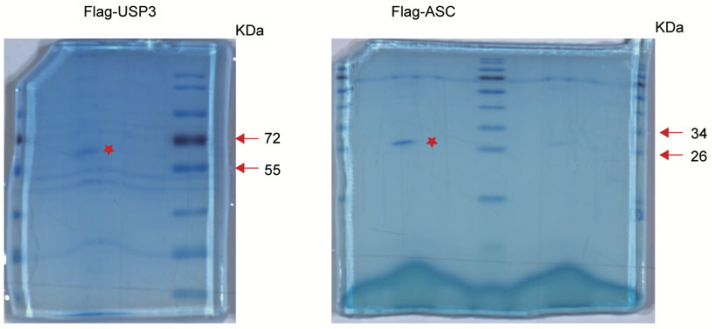


Supplementary Fig. 1 Identification of USP3 as a DUB of ASC.

A HEK293T cells were transfected with the indicated combinations of Myc or Flag-tagged ASC, HA-Ub, and Flag or Myc-tagged DUBs for 36 h, then the cell lysates were subjected to immunoprecipitation with anti-Myc or anti-Flag antibody and followed by immunoblot analysis.

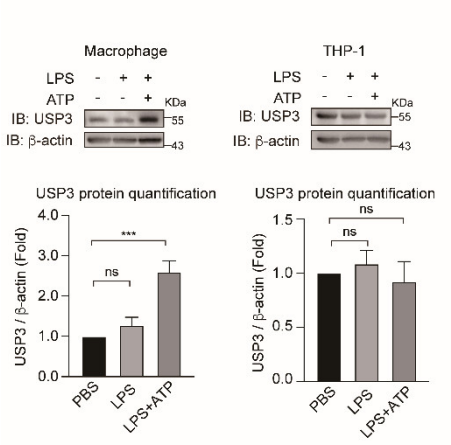
B ELISA analysis of IL-1β secretion from PMs silenced for various DUBs prior to stimulation with control medium (PBS) or LPS (8 h) plus ATP (30 min).

Data in **(B)** are from three independent experiments (n=3) and shown as mean + S.D., and other data are representative of three independent experiments with similar results. *$p < 0.05$, **$p< 0.01$, ***$p< 0.001$; two-tailed Student's *t* test. β-actin was used as loading control. Red arrow, IgG.



Supplementary Fig. 2 Coomassie blue staining of purified ASC and USP3

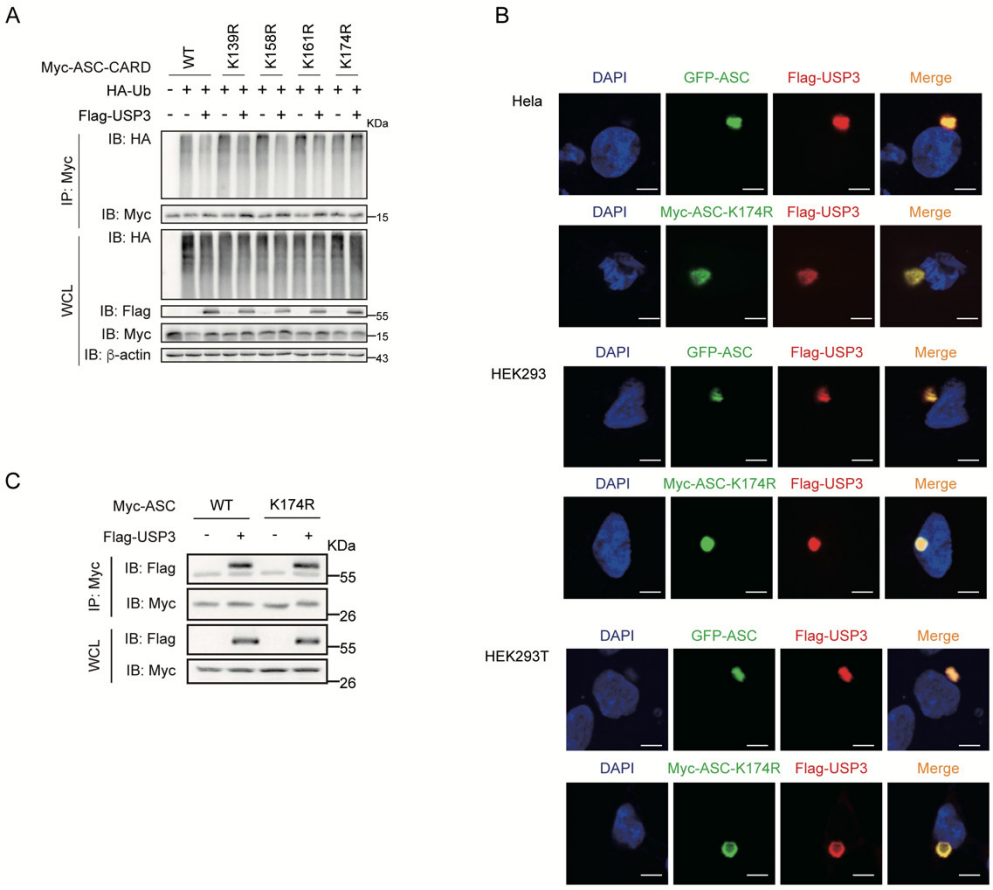
Red asterisk, indicated protein.



Supplementary Fig. 3 Immunoblot analysis of endogenous USP3 expression in PMs or THP-1 cells with inflammatory stimulation.

The cells were primed with LPS for 8 h and then stimulated with ATP for 30 min. The cell lysates were collected and detected by immunoblot. The band density was quantified using Image J software. Quantification of USP3 protein levels was normalized to β-actin.

Data in **Supplementary Fig. 3** are from three independent experiments (n=3) and shown as mean + S.D..*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; two-tailed Student's *t* test.



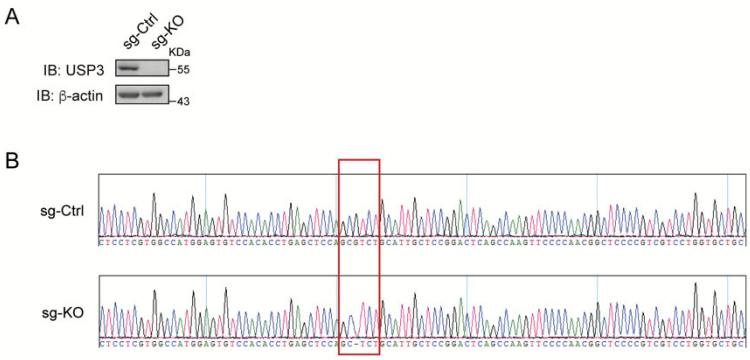
Supplementary Fig. 4 USP3 interacts with ASC-K174R and regulates its deubiquitination.

A Immunoprecipitation analysis of the ubiquitination of ASC-CARD and its lysine residue mutants in HEK293T cells transfected with Myc-ASC-CARD or its mutants and HA-Ub in the presence of Flag-USP3.

B Colocalization of Flag-USP3 (red) with GFP-ASC or Flag-ASC-K174R (green) in HEK293T cells, HEK293 cells, and Hela cells. Scale bar, 10 μm.

C The reciprocal immunoprecipitation analysis of the interaction between USP3 and ASC or its lysine residue mutant K174R. HEK293T cells were transfected with the indicated plasmids for 36 h. The cell lysates were subjected to immunoprecipitation with anti-Myc antibody and then analyzed by immunoblot..

Data in **Supplementary Fig. 4** are representative of three independent experiments with similar results.

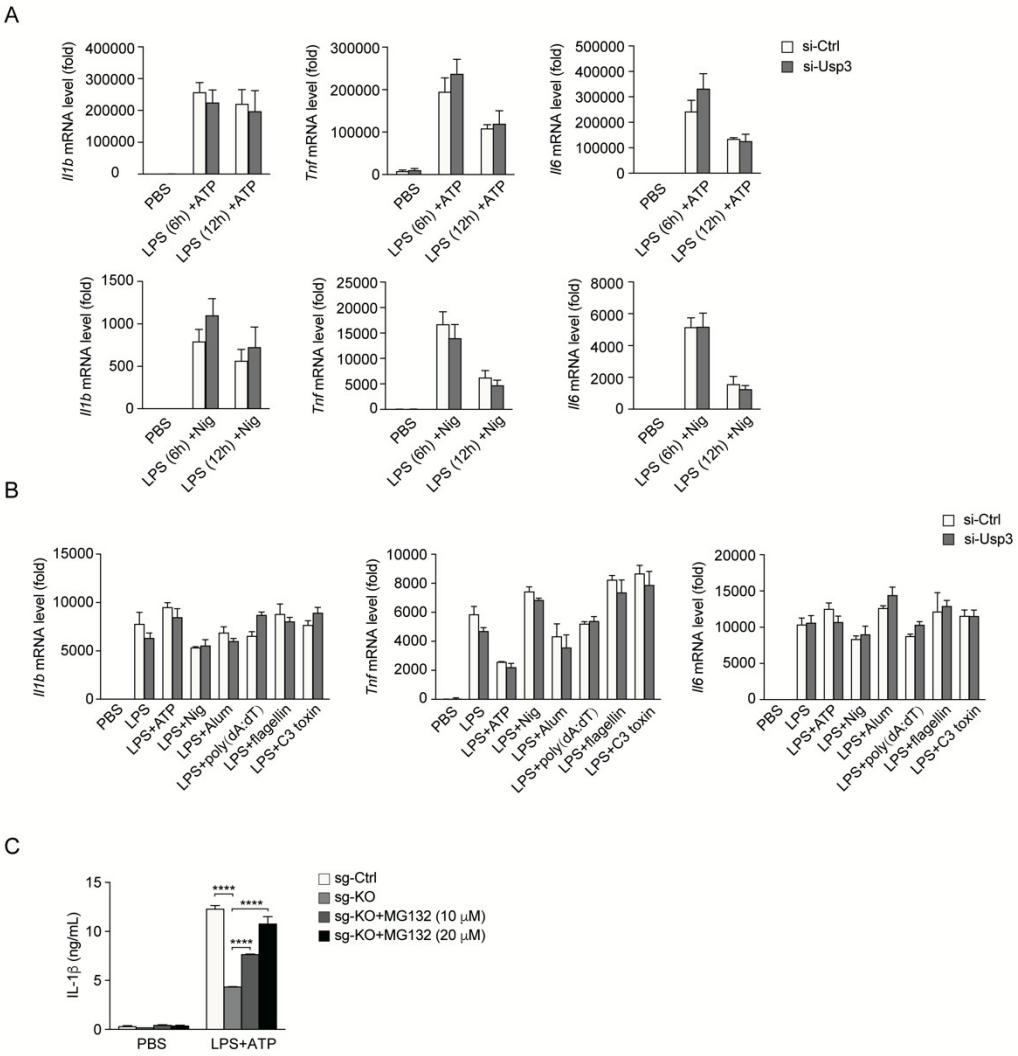


Supplementary Fig. 5 Construction of knockout cell lines

A Western blots of cell lysates from THP-1 cells engineered by CRISPR/Cas9 to induce *USP3* knockout (KO).

B The DNA sequencing results of the control and *USP3*-KO Clone.

Data in **(A)** are representative of three independent experiments with similar results.



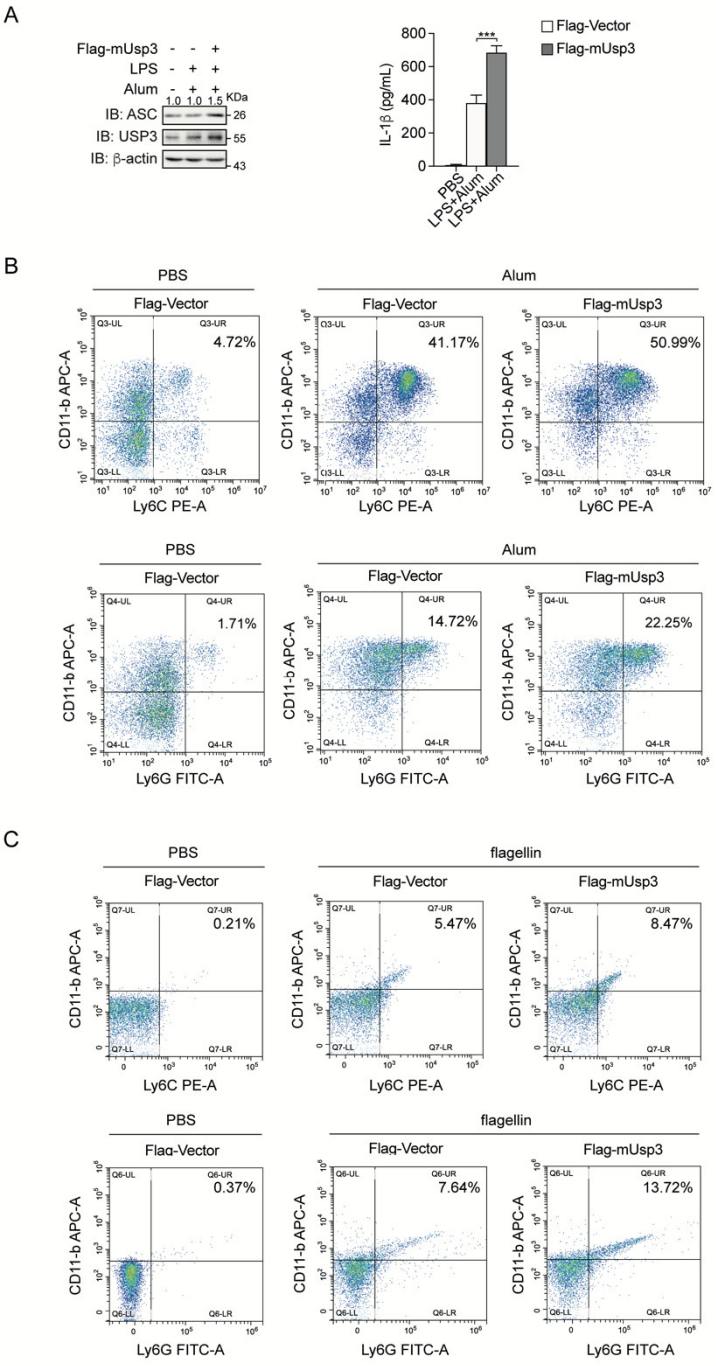
Supplementary Fig. 6 USP3 regulates the stability of ASC and IL-1β production in the NLRP3 inflammasome activation but not affects LPS-triggered transcription response.

A qRT-PCR for *Il1b*, *Tnf* and *Il6* gene expression in response to LPS priming for indicated times and ATP (30 min) or Nig (45 min) stimulation in PMs silenced for *Usp3*.

B qRT-PCR for *Il1b*, *Tnf* and *Il6* gene expression in response to LPS priming (8 h) and various stimulations (ATP (30 min), Nig (45 min), Alum (6 h), poly (dA: dT) (1 h), flagellin (1 h) or C3 toxin (6 h) in PMs silenced for *Usp3*.

C ELISA analysis of IL-1β secretion in WT , *USP3*-KO THP-1 cells and *USP3*- KO THP-1 cells treated with gradient amount of MG132. Cells were stimulated with LPS (8 h) plus ATP (30 min) treatment.

Data in (**Supplementary Fig. 6**) are from three independent experiments (n=3) and shown as mean + S.D.. **p* < 0.05, ***p*< 0.01, ****p*< 0.001; two-tailed Student's *t* test.



Supplementary Fig. 7 USP3 overexpression facilitates NLRP3 and NLRC4 inflammasome activation *in vivo*

A Immunoblot analysis of USP3 and ASC, and ELISA analysis of IL-1β in PMs from WT or USP3-overexpressed mice. PMs were stimulated with LPS (8 h) plus Alum (6 h) treatment.

B Flow cytometric analysis of peritoneal cell exudates from WT or USP3-overexpressed

mice intraperitoneally injected with Alum for 12 h. (mice: n = 5 for each group).

C Flow cytometric analysis of BALF from WT or USP3-overexpressed mice 12 h after isotonic saline or Flagellin intranasal instillation. (mice: n = 5 for each group).

Data in (**A**) are from three independent experiments (n=3) and shown as mean + S.D., and other data are representative of three independent experiments with similar results. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; two-tailed Student's *t* test. The band density of ASC in (**A**) was quantified using Image J software and the protein quantification was normalized with corresponding β-actin

Supplementary Table S1

Primers for RNA Quantification, Related to Experimental Procedures

Gene	Forward primer (5'-3')	Reverse primer (5' -3')
mouse Actb	CCACACCCGCCACCAGTTCG	TACAGCCCGGGGAGCATCGT
mouse Usp3	TCAATGGTGTTTCCCGCTCA	GCAGTTGACCTCGTTCTGGA
mouse Il6	ACAACCACGGCCTTCCCTAC	CATTTCCACGATTTCCCAGA
mouse Tnf	GCCACCACGCTCTTCTGTCT	TGAGGGTCTGGGCCATAGAAC
mouse Il1b	ACCTTCCAGGATGAGGACATGA	AACGTCACACACCAGCAGGTTA