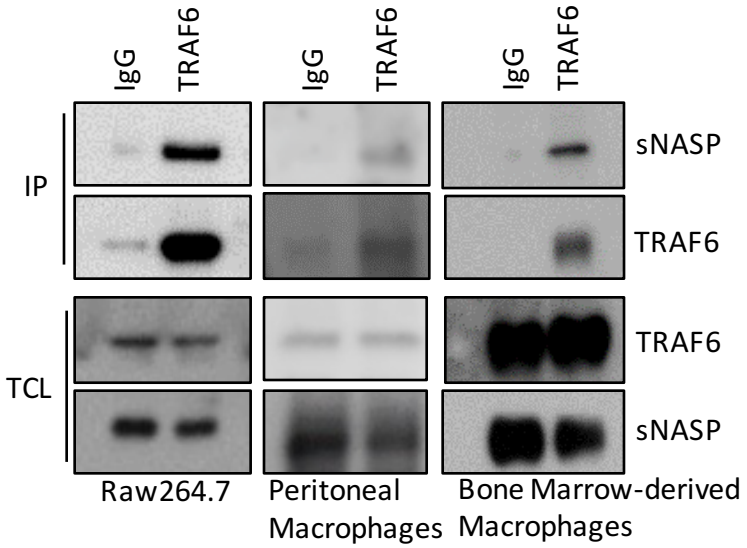
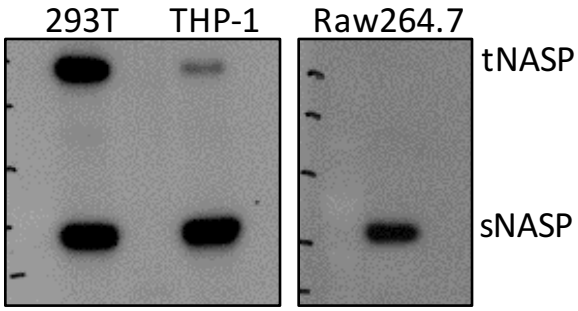
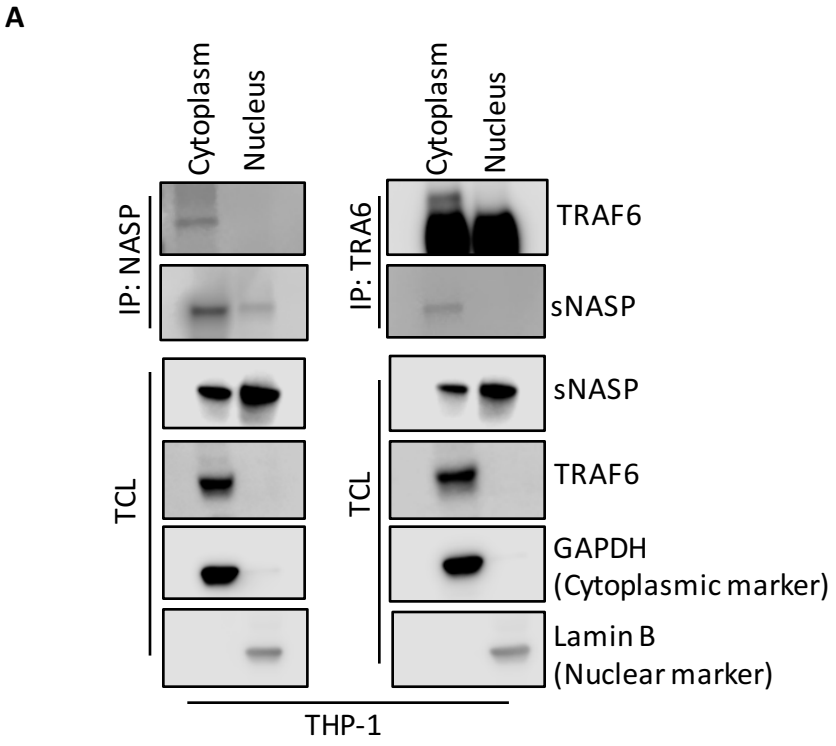
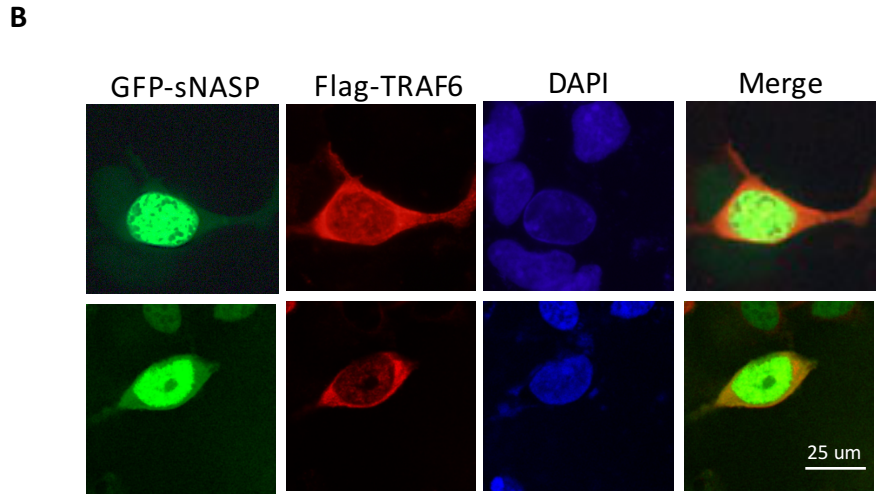


Supplemental Figure 1. Endogenous TRAF6 interacts with sNASP in unstimulated-mouse macrophage cells. Immunoprecipitation (IP) of endogenous TRAF6 (with anti-TRAF6) or IgG from RAW264.7, peritoneal macrophage, and bone marrow-derived macrophages, followed by immunoblot analysis (IB) with antibody to TRAF6 or NASP. Data are representative of at least three independent experiments.

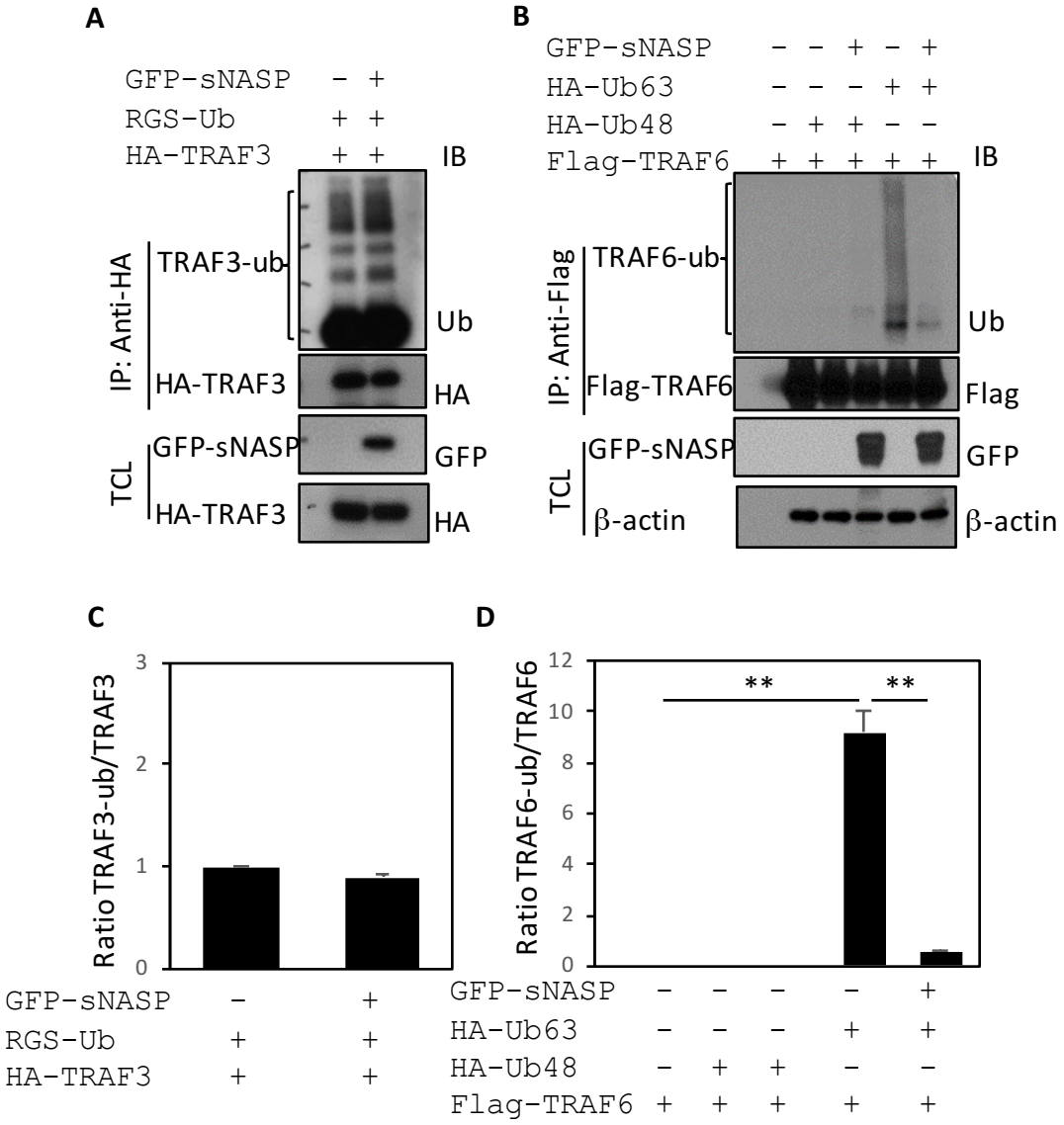


Supplemental Figure 2. Somatic NASP (sNASP) is highly expressed in human monocyte (THP-1) and mouse macrophage cell lines (Raw264.7), whereas testicular NASP (tNASP) is not. Whole-cell extracts from indicated cells were IB with anti-NASP. Data are representative of at least three independent experiments.

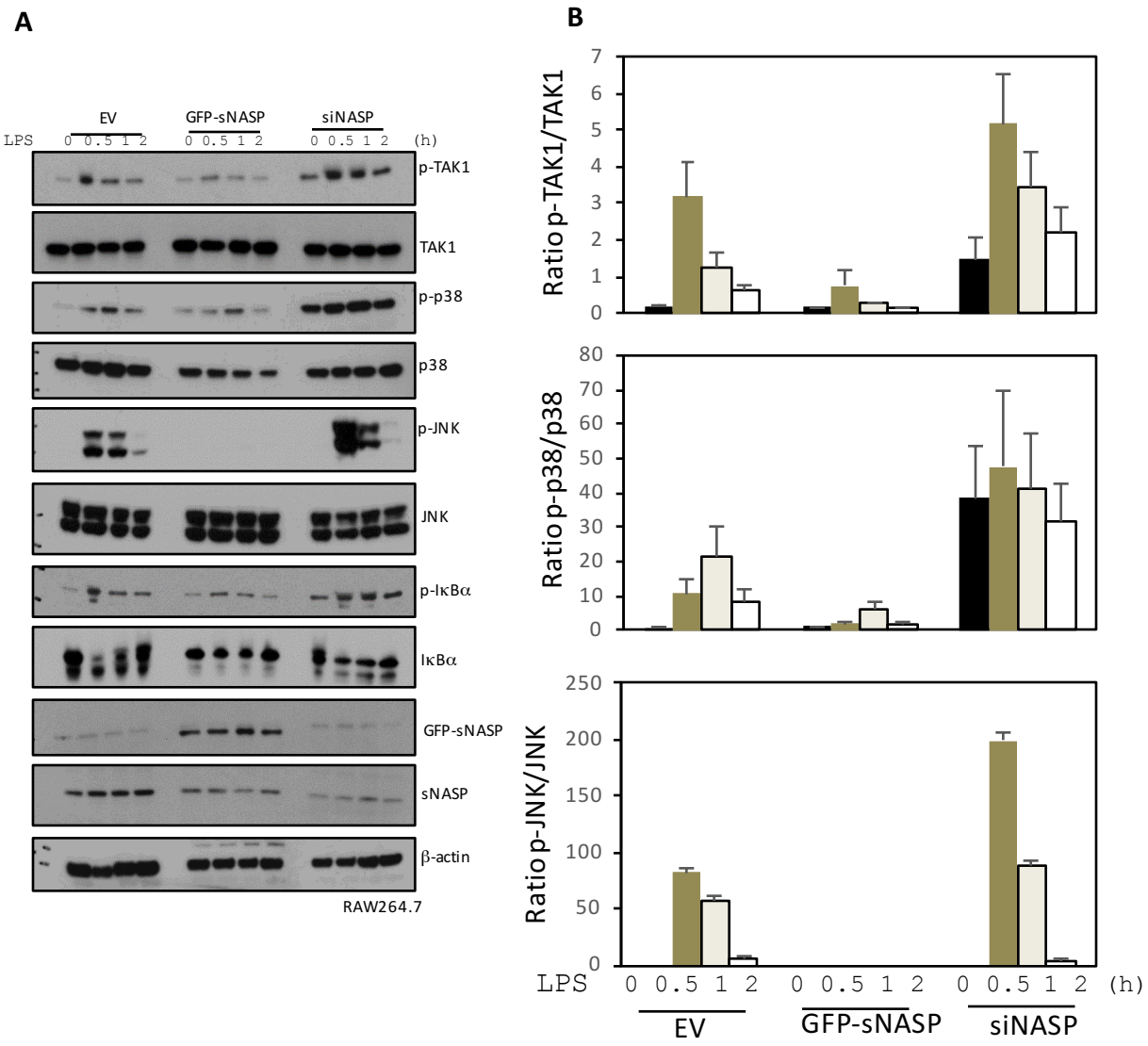




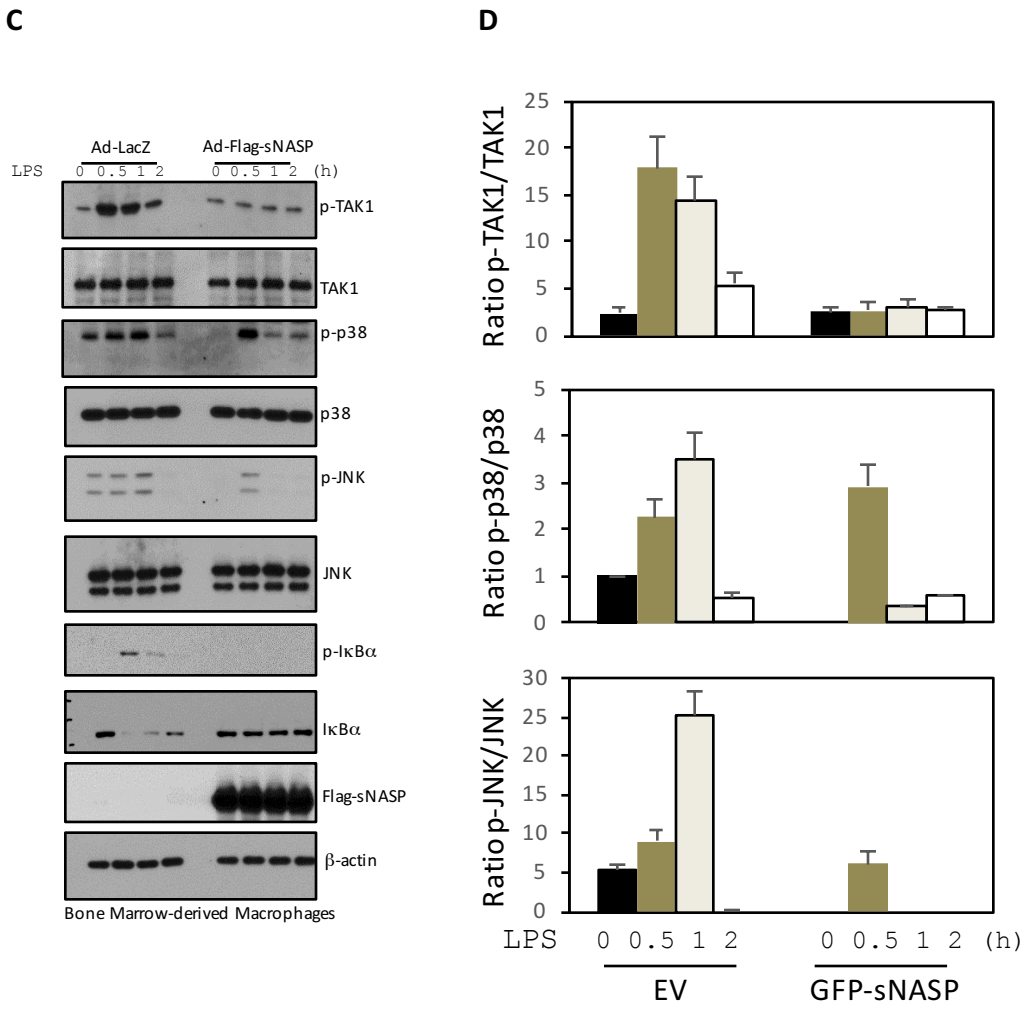
Supplemental Figure 3. TRAF6 binds to sNASP in the cytosol. (**A**) IB of TRAF6 and sNASP in cytoplasm and nuclear fractions from THP-1 cells following IP with NASP or TRAF6. TCL was IB with different antibodies as indicated. GAPDH serves as a cytoplasmic marker and Lamin B serves as nuclear marker. (**B**) Confocal microscopy of sNASP and TRAF6 colocalization in HEK293 cells transfected with GFP-tagged sNASP WT and Flag-TRAF6. Scale bar = 25 μm. Data are representative of at three independent experiments.



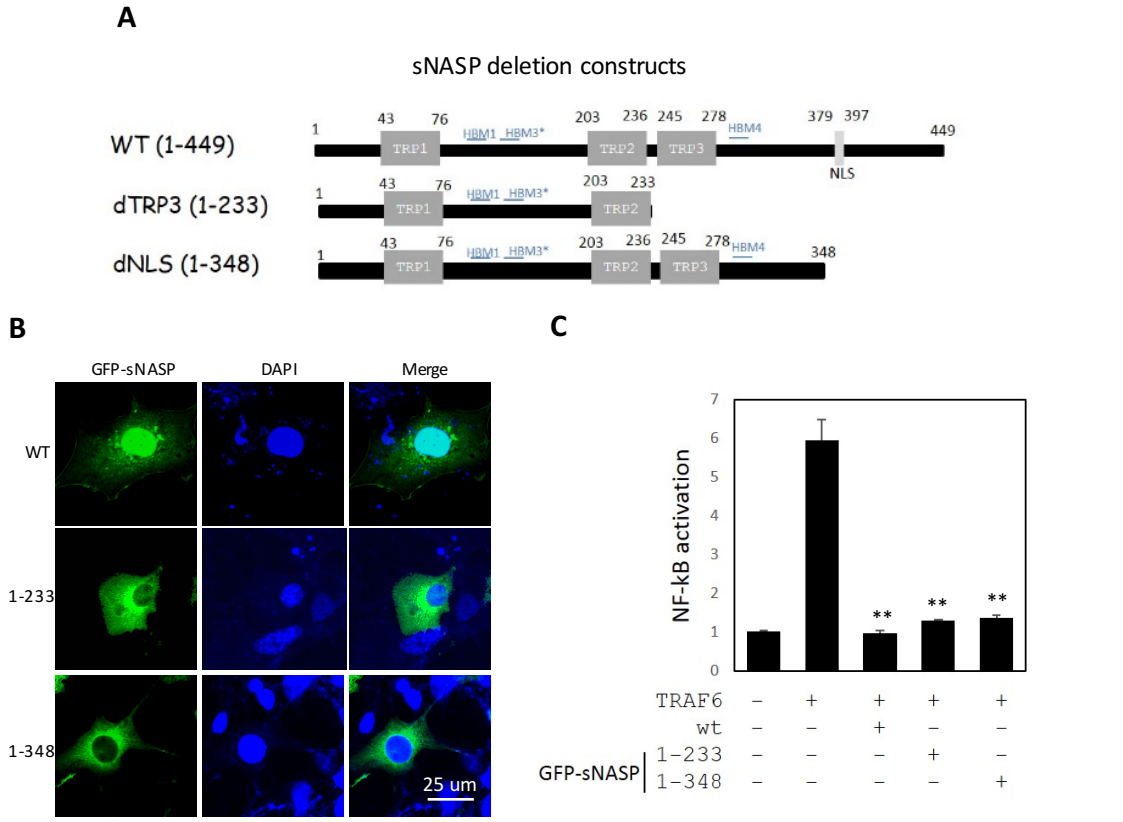
Supplemental Figure 4. sNASP blocks K63-linked ubiquitination of TRAF6, but not TRAF3 ubiquitination. (**A**) IB with anti-ubiquitin or anti-HA in HEK293 cells transfected with HA-tagged TRAF3 and RGS-tagged ubiquitin (RGS-Ub) in the presence (+) or absence (–) of vector encoding GFP-tagged sNASP, followed by IP with anti-HA. TCL was IB with indicated antibodies. (**B**) HEK293 cells were transfected with Flag-TRAF6, GFP-tagged sNASP, HA-tagged K48-linked ubiquitin (HA-Ub48), or K63-linked ubiquitin (HA-Ub63) as indicated and IB with anti-Flag or anti-ubiquitin, followed by IP with anti-Flag. TCL was IB with anti-GFP or anti-β-actin. (**C** and **D**) Western blot was quantified for (A) and (B). Data shown represent mean ± SEM. **P < 0.01 (Student's t-test). Data are representative of at least three independent experiments.



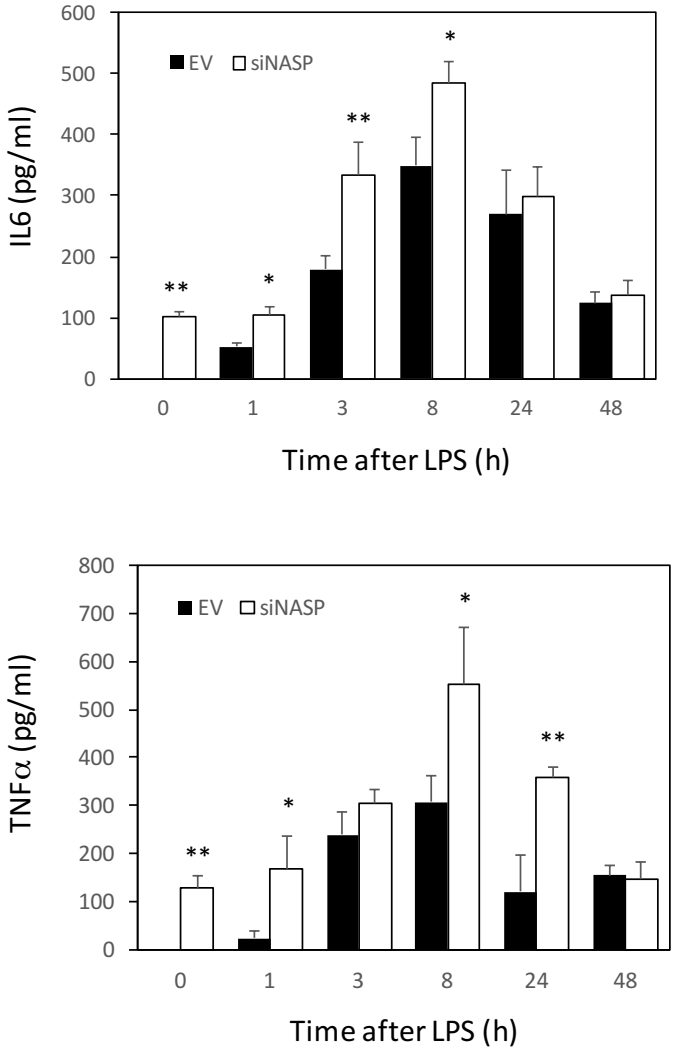
Supplemental Figure 5



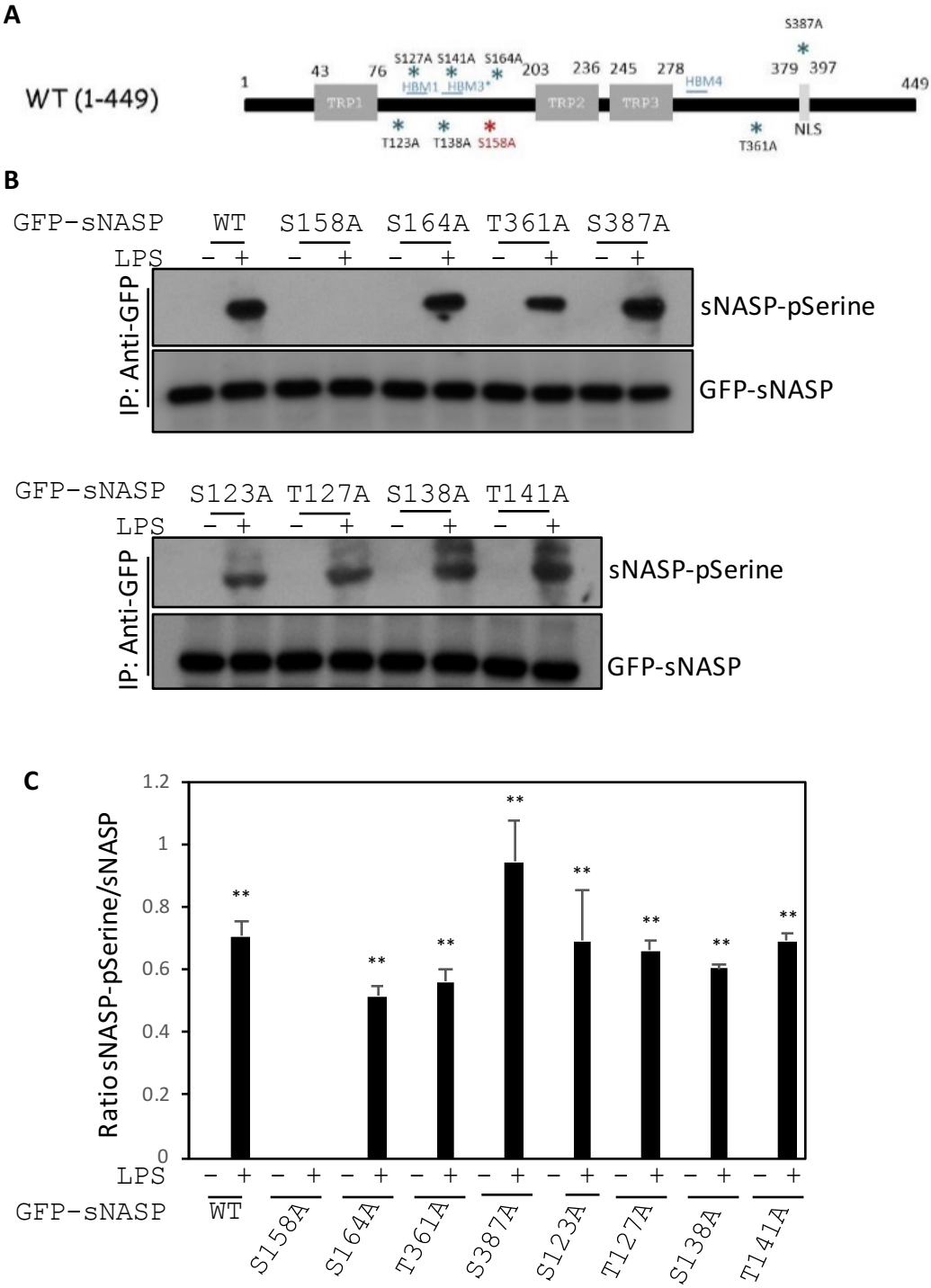
Supplemental Figure 5. sNASP inhibits kinase activation downstream of TRAF6. (**A**) IB of indicated antibodies in RAW264.7 cells transfected with empty vector (EV) or GFP-tagged sNASP or siNASP followed by LPS stimulation. (**C**) IB with indicated antibodies in BMDM cells transduced with control adenovirus (Ad-LacZ) or adenovirus carrying Flag-tagged sNASP (Ad-Flag-sNASP) followed by LPS stimulation. (**B** and **D**) Western blot was quantified for (A) and (C). Data are expressed as mean ± SE for each group. All experiments were repeated 3 times.



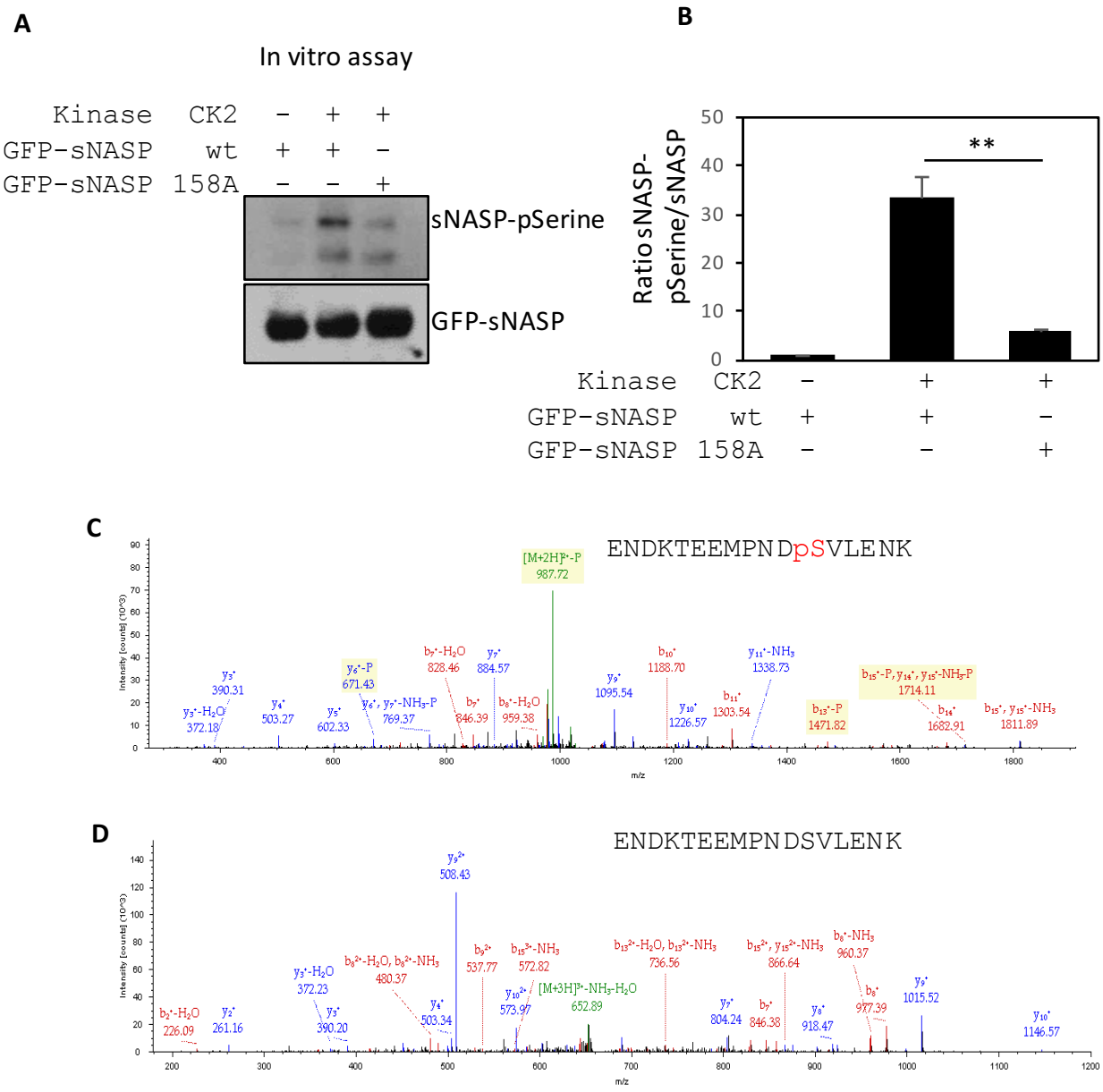
Supplemental Figure 6. NLS of sNASP is not required in inhibiting TRAF6-induced NF-kB activation. (**A**) A schematic showing structures in various sNASP deletion constructs. TRP, kinase domain; HBM, leucine zipper; NLS, nuclear localization signal. (**B**) Fluorescent microscopy of sNASP localization in HEK293 cells transfected with GFP-tagged sNASP WT or 1-233 or 1-348 deletion mutants. (**C**) Luciferase activity in HEK293 cells transfected with a luciferase reporter driven by NF-κB-responsive promoter, plus empty vector (EV) or vector encoding TRAF6 in the presence (+) or absence (–) of vector encoding GFP-tagged sNASP WT or 1-233 or 1-348 deletion; Luciferase value in the absence of TRAF6 was used to normalize the data. Data are expressed as mean ± SE for each group. **P < 0.01 (Student's t-test). All experiments were repeated 3 times. Scale bar = 25 μm.



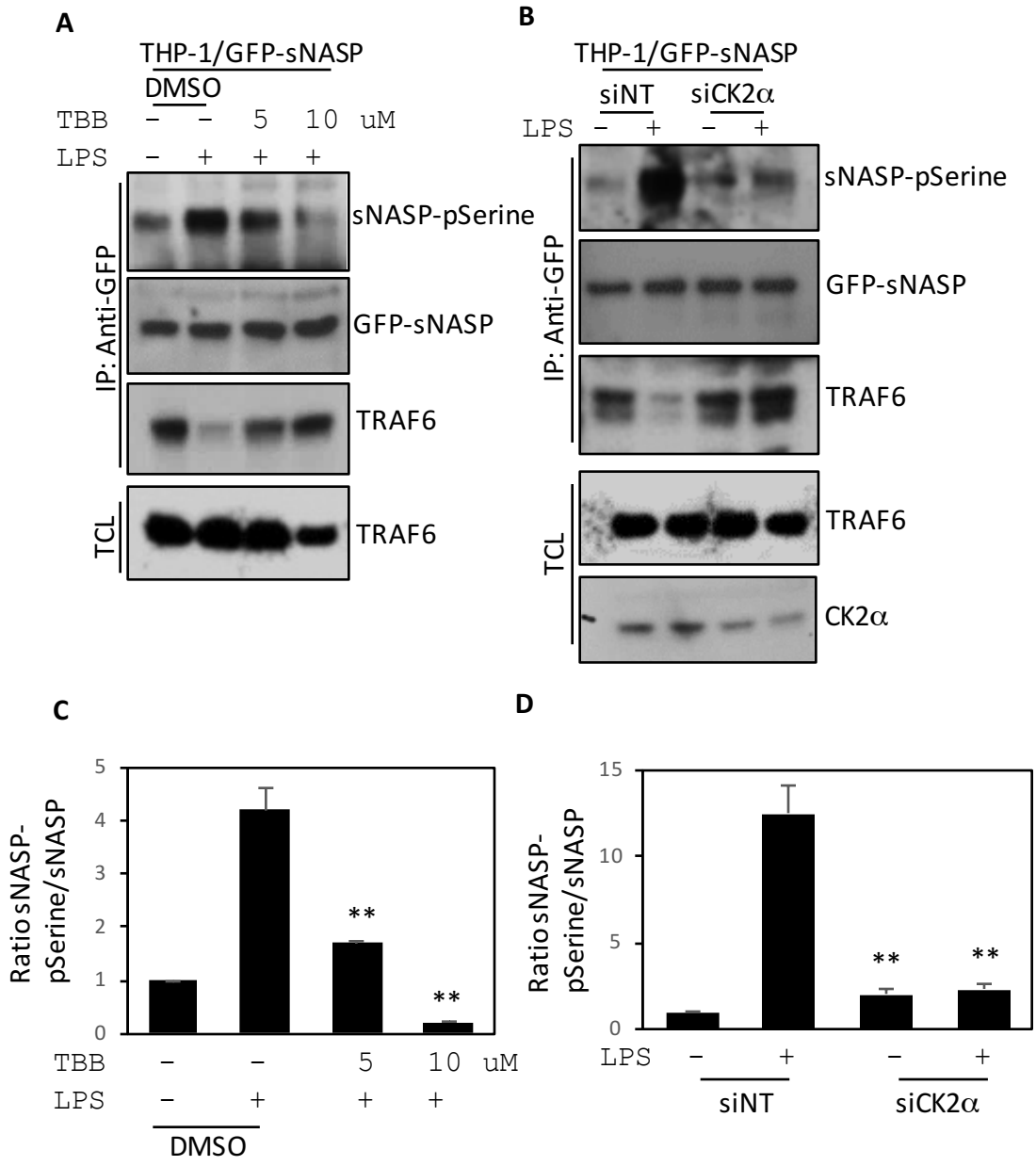
Supplemental Figure 7. sNASP regulates LPS-induced proinflammatory cytokines production. Production of TNFα and IL-6 in RAW264.7 cell lines transduced with empty vector (EV) or siNASP and stimulated with LPS; results were normalized to the expression of ACTB (encoding β-actin) and are presented relative to those of untreated cells. Data are expressed as mean ± SE for each group. *P < 0.05, **P < 0.01 (one-way ANOVA). All experiments were repeated 3 times.



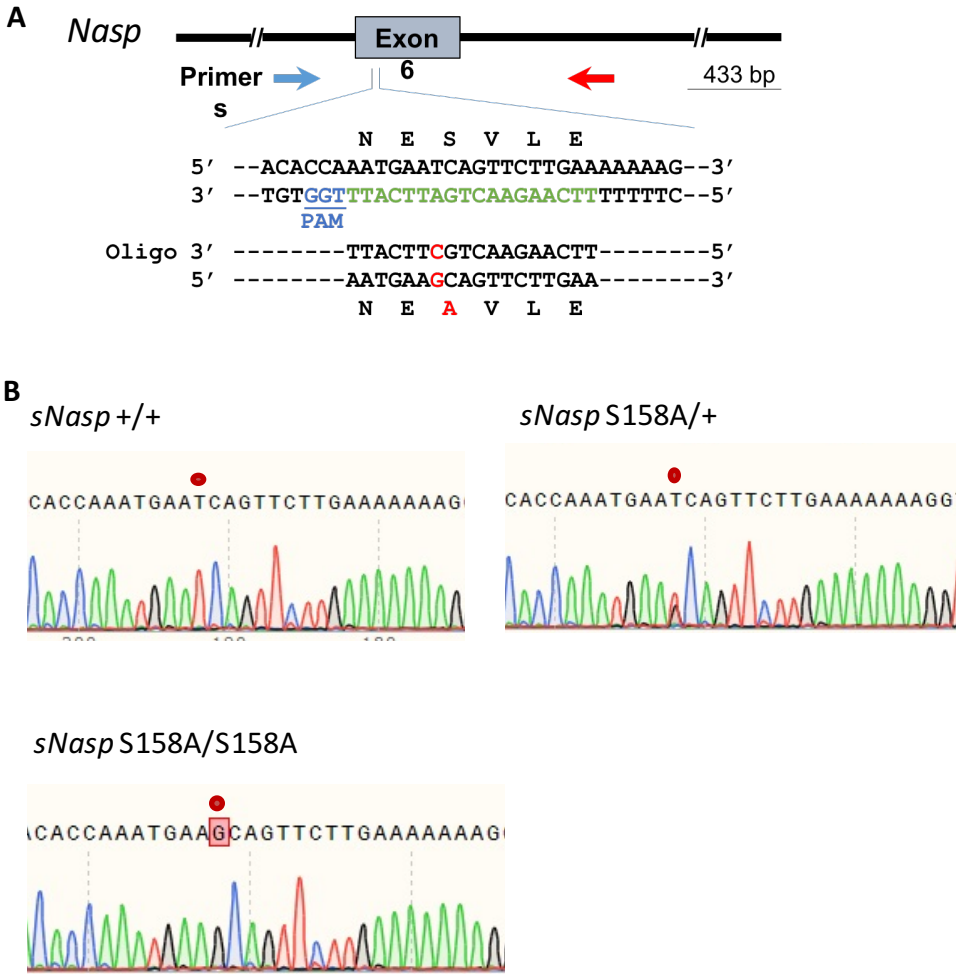
Supplemental Figure 8. Identification of phosphorylation sites on sNASP. (**A**) A schematic diagram showing protein structures of sNASP with potential phosphorylation sites. * Indicates serine or threonine residues mutate to alanine. TRP, kinase domain; HBM, leucine zipper; NLS, nuclear localization signal. (**B**) THP-1 cells transfected with GFP-tagged wild-type sNASP (WT) or serine/threonine mutants was stimulated with LPS, IP with anti-GFP, then IB with antibody to phosphorylated serine (p-S) or anti-GFP. (**C**) Western blot was quantified for (B). Data are expressed as mean ± SE for each group. **P < 0.01 (Student's t-test). All experiments were repeated 3 times.



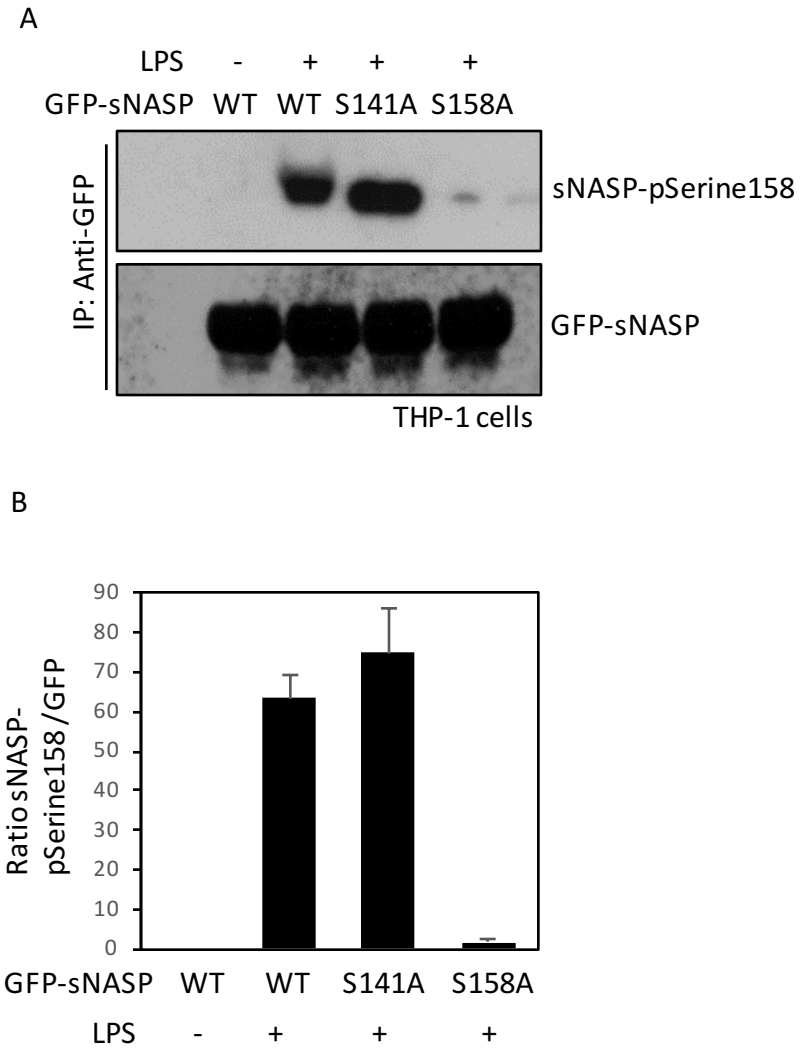
Supplemental Figure 9. Mass spectrometric (MS) analysis confirmed CK2-induced phosphorylation of sNASP on Ser158 site. (**A**) CK2 phosphorylates sNASP in vitro. IP of GFP-tagged sNASP wild-type or S158A mutant protein were incubated with CK2 kinase at 30 degree for 30 min in kinase buffer. The reaction products were IB with anti-phosphorylated serine (p-S) or anti-GFP antibody. (**B**) Western blot was quantified for (A). Data are expressed as mean ± SE for each group. **P < 0.01 (Student's t-test). All experiments were repeated 3 times. (**C** and **D**) An LC-MS/MS analysis identified sNASP phosphorylation sites by CK2 at Ser158.



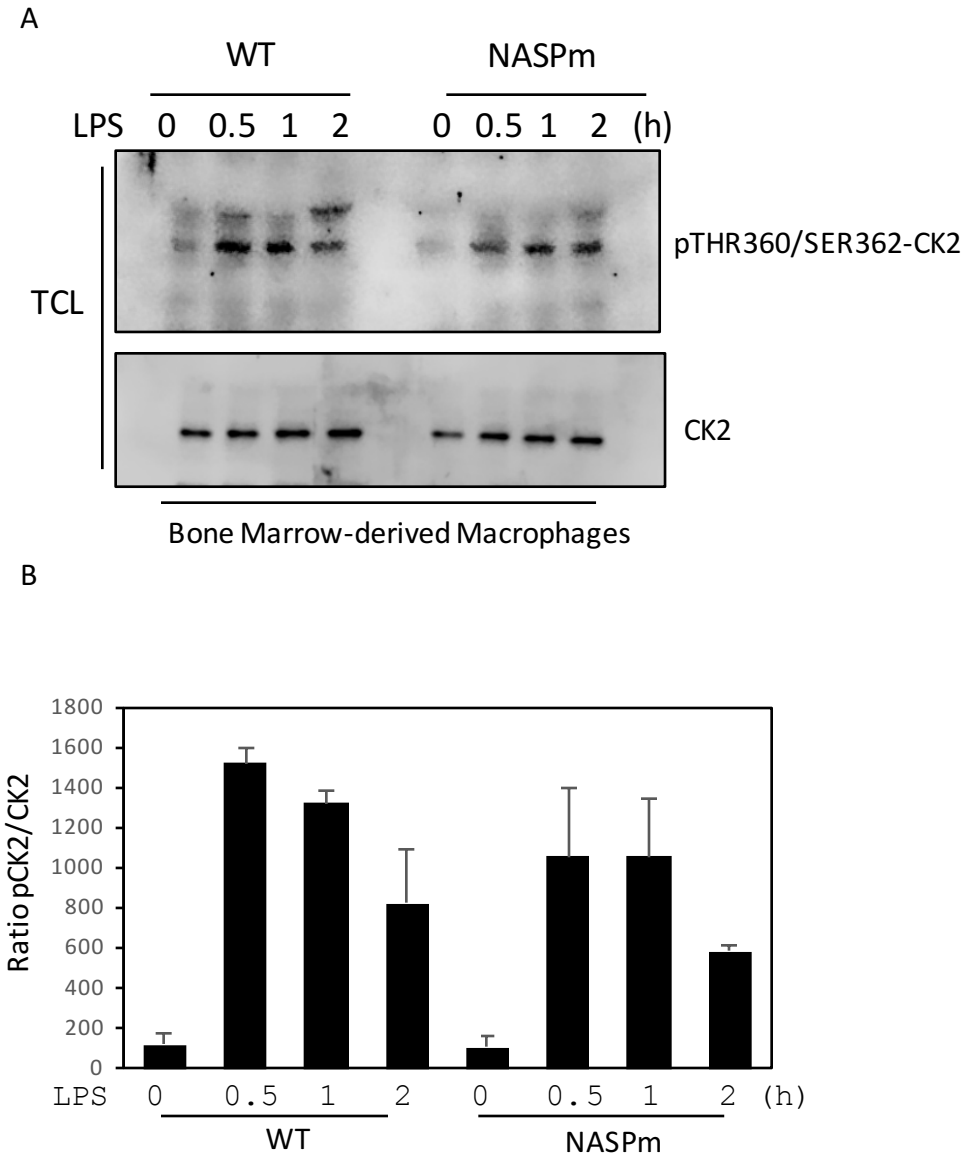
Supplemental Figure 10. CK2 regulates sNASP interaction with TRAF6. (**A**) THP-1 cells were stimulated with LPS in the absence (-) or presence (+) of TBB, assessed by IB with antibody to phosphorylated serine (p-S), GFP or TRAF6 after IP with anti-GFP. TCL was IB with anti-TRAF6. (**B**) THP-1 cells were stimulated with LPS in the presence of siNT or siCK2, assessed by IB with antibody to phosphorylated serine (p-S), GFP or TRAF6 after IP with anti-GFP. TCL was IB with anti-TRAF6 or anti-CK2α. (**C** and **D**) Western blot was quantified for (A) and (B). Data are expressed as mean ± SE for each group. **P < 0.01 (Student's t-test). All experiments were repeated 3 times.



Supplemental Figure 11. Generation of *sNasp* S158A KI mice by CRISPR/Cas9 system. (**A**) Schematic of the target region of the murine *sNasp* locus. The targeted site is located in exon 6. The sgRNA target sequence and PAM sequence are shown in green or blue, respectively. Mismatched bases in mutant oligonucleotides are labelled in red. Arrows indicate the location of the primers used for amplifying the fragment. (**B**) Representative *sNasp* genomic sequences from the founder mice (upper-left; wild-type *sNasp* mice, upper-right heterozygous *sNasp* S158A mice, bottom; homozygous *sNasp* S158A mice). The mutated nucleotide is shown in a red dot.



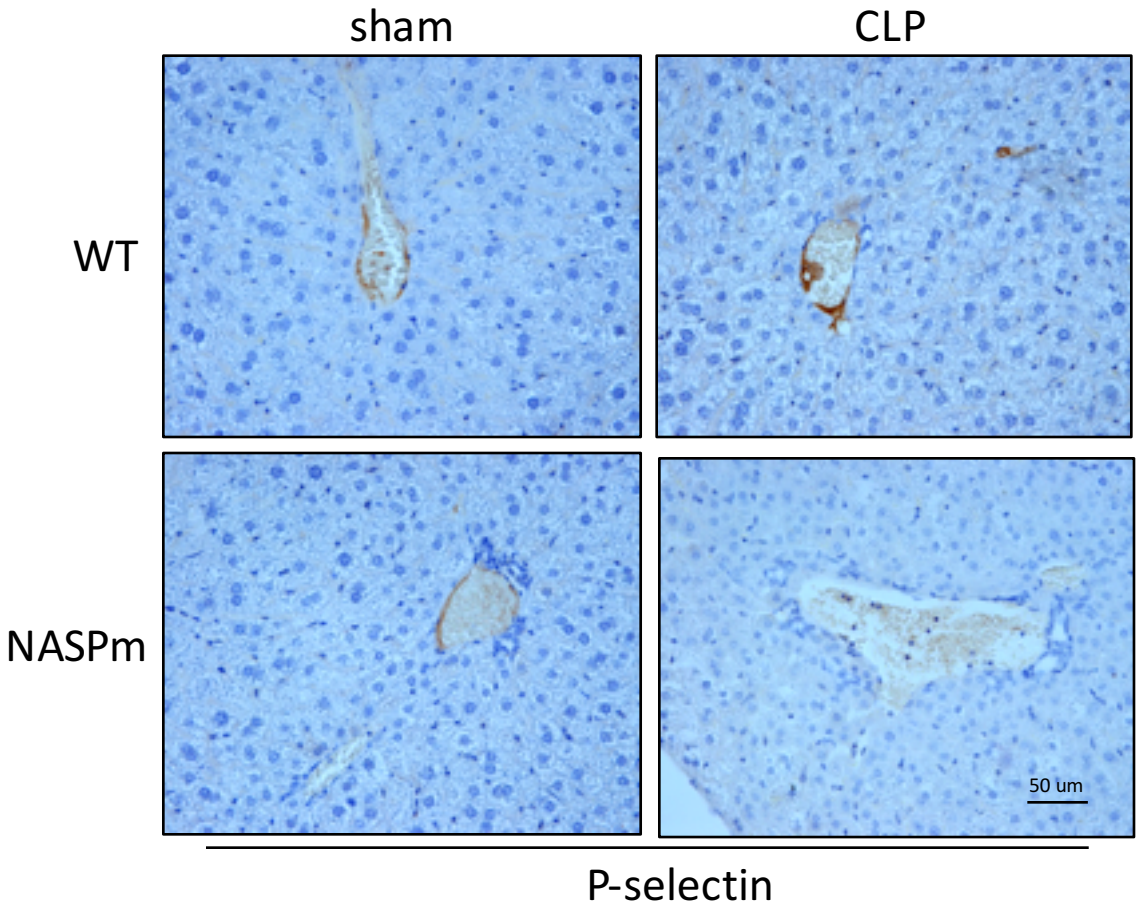
Supplemental Figure 12. Characterize the pSerine158-sNASP antibody. (**A**) THP-1 cells transfected with GFP-tagged wild-type sNASP (WT) or serine mutants was stimulated with LPS, IP with anti-GFP, then IB with antibody to pSerine158-sNASP or anti-GFP. (**B**) Western blot was quantified for (A). Data are expressed as mean ± SE for each group. All experiments were repeated 3 times.



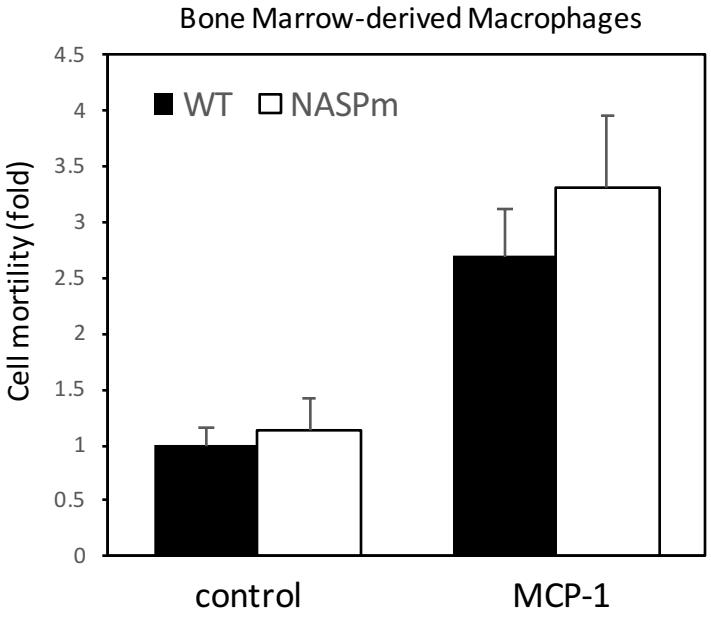
Supplemental Figure 13. LPS regulates CK2 activation. (**A**) BMDM cells from wild-type (WT) and NASP S158A-KI (NASPm) mice were stimulated with LPS and assessed by IB analysis with antibody to anti-pTHR360/SER362-CK2 or CK2. (**B**) Western blot was quantified for (A). Data are expressed as mean ± SE for each group. All experiments were repeated 3 times.

Complete Blood Counts with Differentials		
Peripheral Blood Counts	Genotype	
	WT (n = 8) (Mean ± SE)	NASPm (n = 8) (Mean ± SE)
Total WBC, cells/μl	6440 ± 1509	4053 ± 1203
Neutrophils, cells/μl	3742 ± 624 (58.11%)	1732 ± 343 (42.73%)
Lymphocytes, cells/μl	2504 ± 1308 (38.88%)	2232 ± 718 (55.07%)
Monocytes, cells/μl	16 ± 5 (0.25%)	10 ± 1 (0.24%)
Eosinophils, cells/μl	172 ± 57 (2.67%)	72 ± 38 (1.77%)
Basophils, cells/μl	6 ± 5.48 (0.09%)	7.5 ± 5 (0.19%)
RBC, X 10e6/μl	10.21 ± 0.63	10.28 ± 0.60

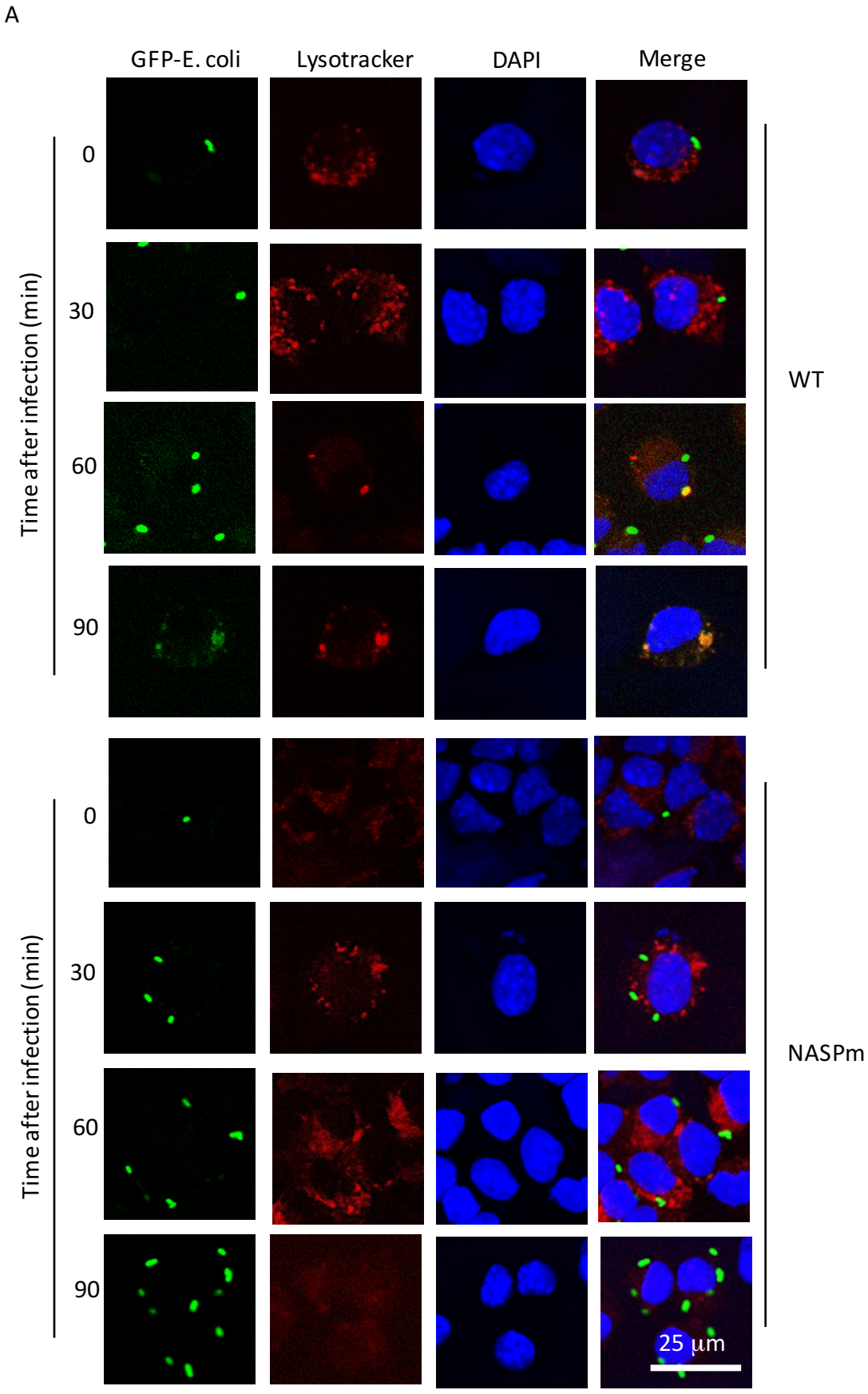
Supplemental Figure 14. Peripheral blood cell counts with differentials. The figure shows 2-fold decrease neutrophils and eosinophils in NASP S158A-KI (NASPm), compared to wild-type (WT) (n=8 per group). WBC, white blood cells; RBC, red blood cells. Data are expressed as mean ± SE for each group. All experiments were repeated 3 times.



Supplemental Figure 15. *sNasp* S158A-KI mice has defective on P-selectin expression in response to CLP. Immunohistochemisty staining for P-selectin in the liver isolated from wild-type (WT) and NASP S158A-KI (NASPm) mice following cecal ligation and puncture (CLP) (n=8 per group per experiment). Results represent at least 3 independent experiments. Scale bar = 50 μm.

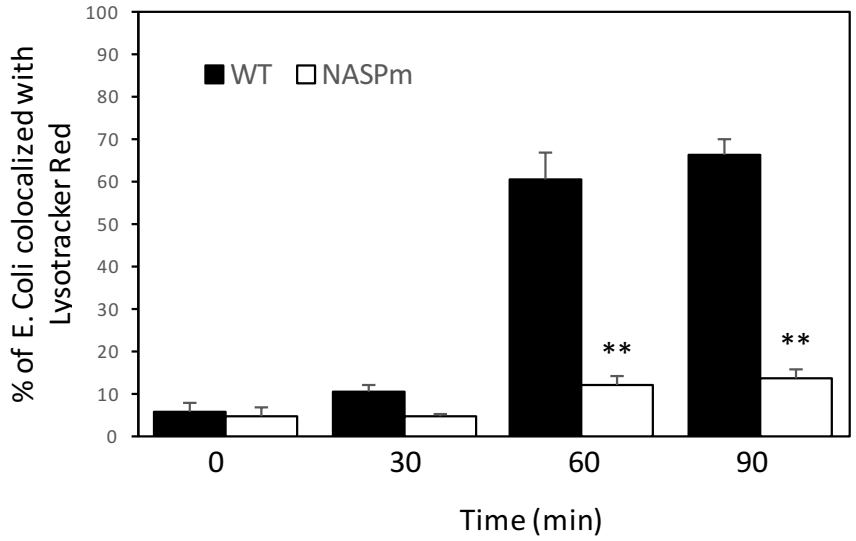


Supplemental Figure 16. *sNasp* S158A-KI macrophages did not affect monocyte chemotactic protein-1 (MCP-1) -induced macrophages migration. BMDM cells from wild-type (WT) and NASP S158A-KI (NASPm) mice were studied in Transwell with 20 ng/mL MCP-1 protein (experimental group) or without protein (control group) in the bottom chamber. All cells were seeded on the upper chamber. 20 h later, the migratory cells on the bottom chamber were stained and counted. Data are expressed as mean ± SE for each group. All experiments were repeated 3 times.

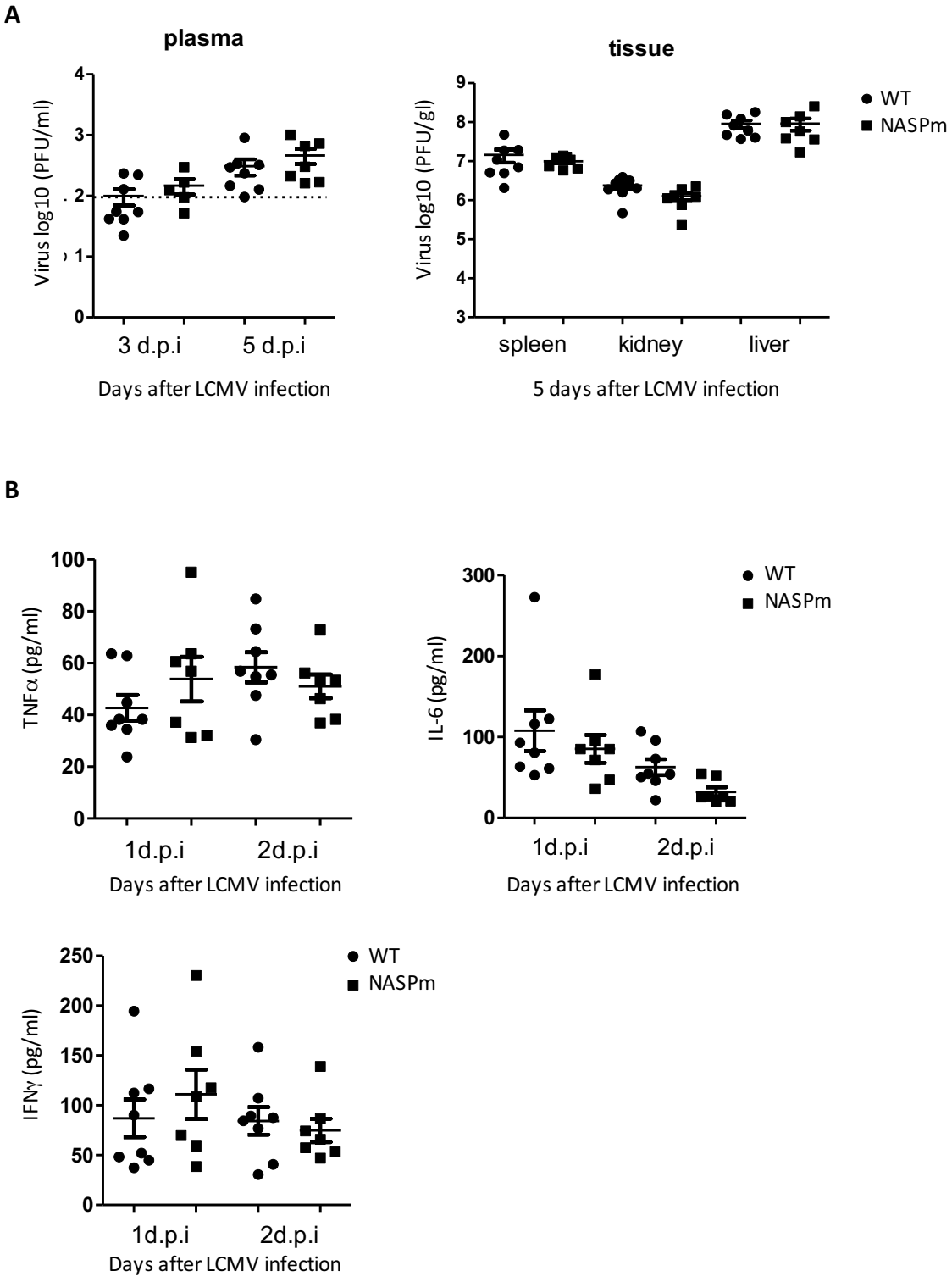


Supplemental Figure 17

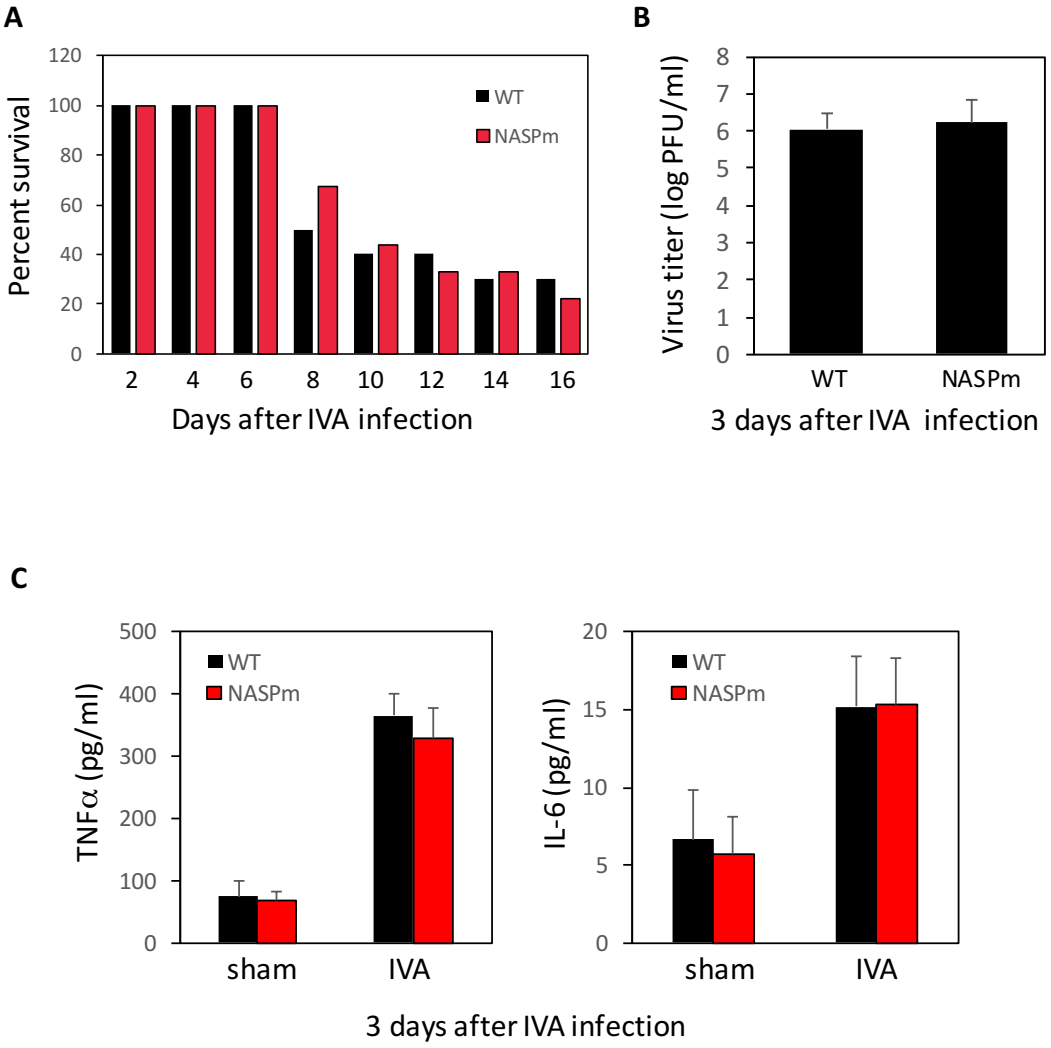
B



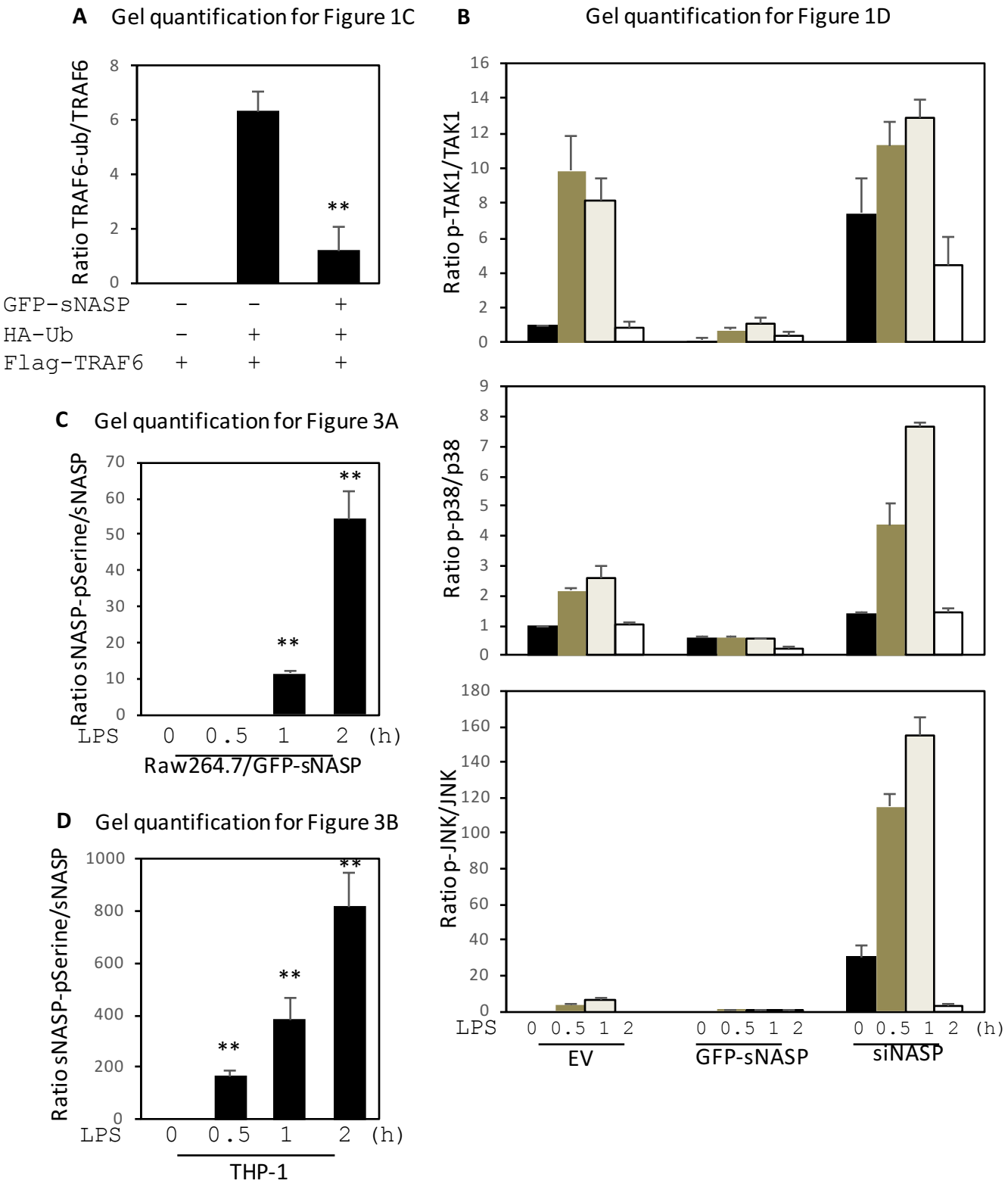
Supplemental Figure 17. *sNasp* S158A-KI macrophages are defective in phagolysosomal formation. (A) Confocal microscopy of wild-type (WT) and NASPm (NASPm) BMDMs treated with lysotracker (Red), imaged after infection for 0–90 min (above images) with GFP–E. coli (green), nuclei were counterstained with (blue). Scale bars=25 µm. (**B**) Data are expressed as mean ± SE for each group. **P < 0.01 (one-way ANOVA). All experiments were repeated 3 times.



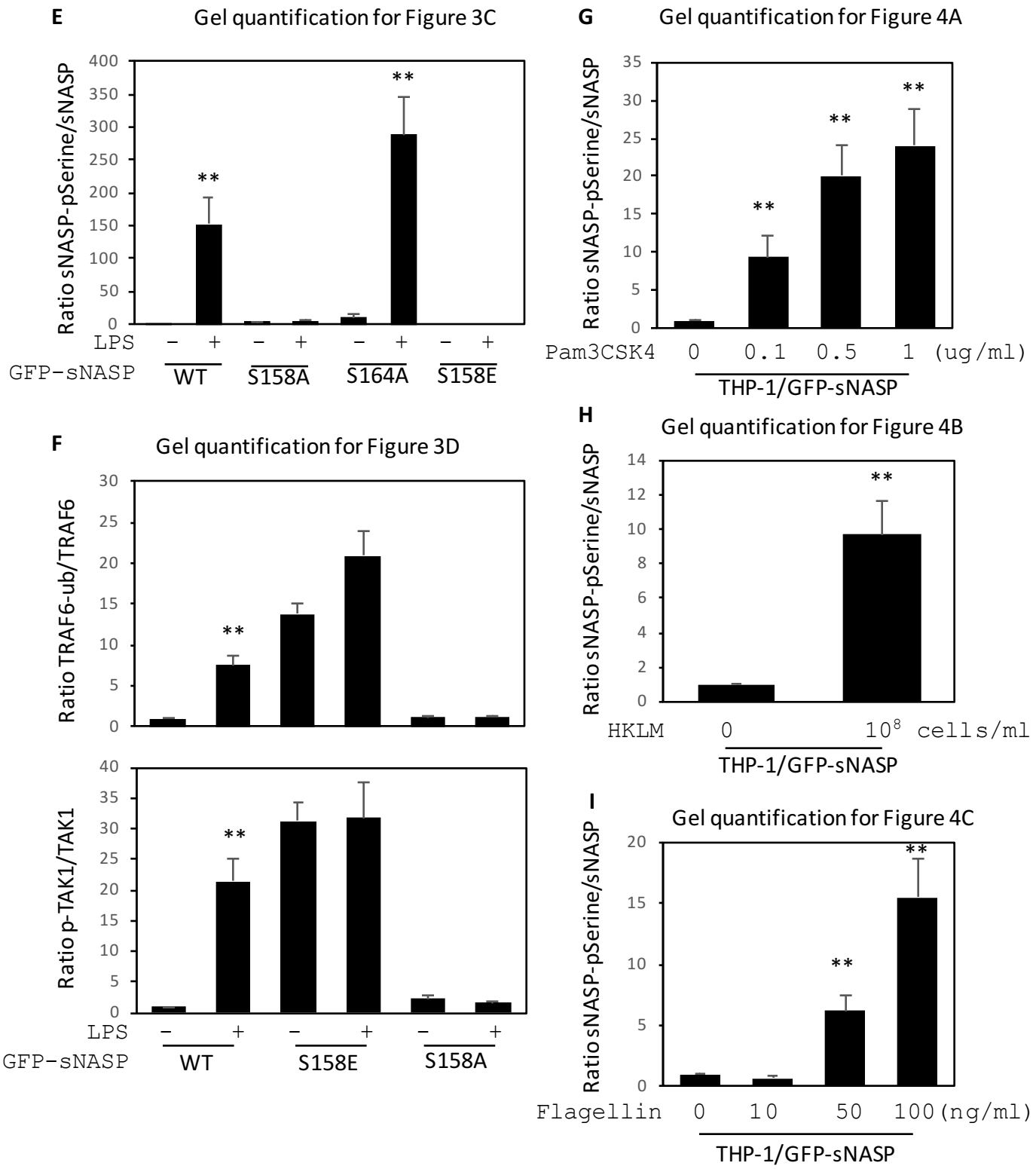
Supplemental Figure 18. Wild type and *sNasp* S158A-KI mice responded similarly to LCMV infection (**A**) Viral titers in plasma, spleen, kidney and liver of wild-type (WT) and NASP S158A-KI (NASPm) 3 days or 5 days post infection (d.p.i) were measured. Data were plotted as mean ± SEM. (**B**) WT and NASPm mice (n=10) were infected with LCMV (IP) and serum TNFα, IL-6 and IFNγ were measured by flow cytometer at 1 or 2 d.p.i. Data are expressed as mean ± SE for each group and representative of at least two independent experiments.



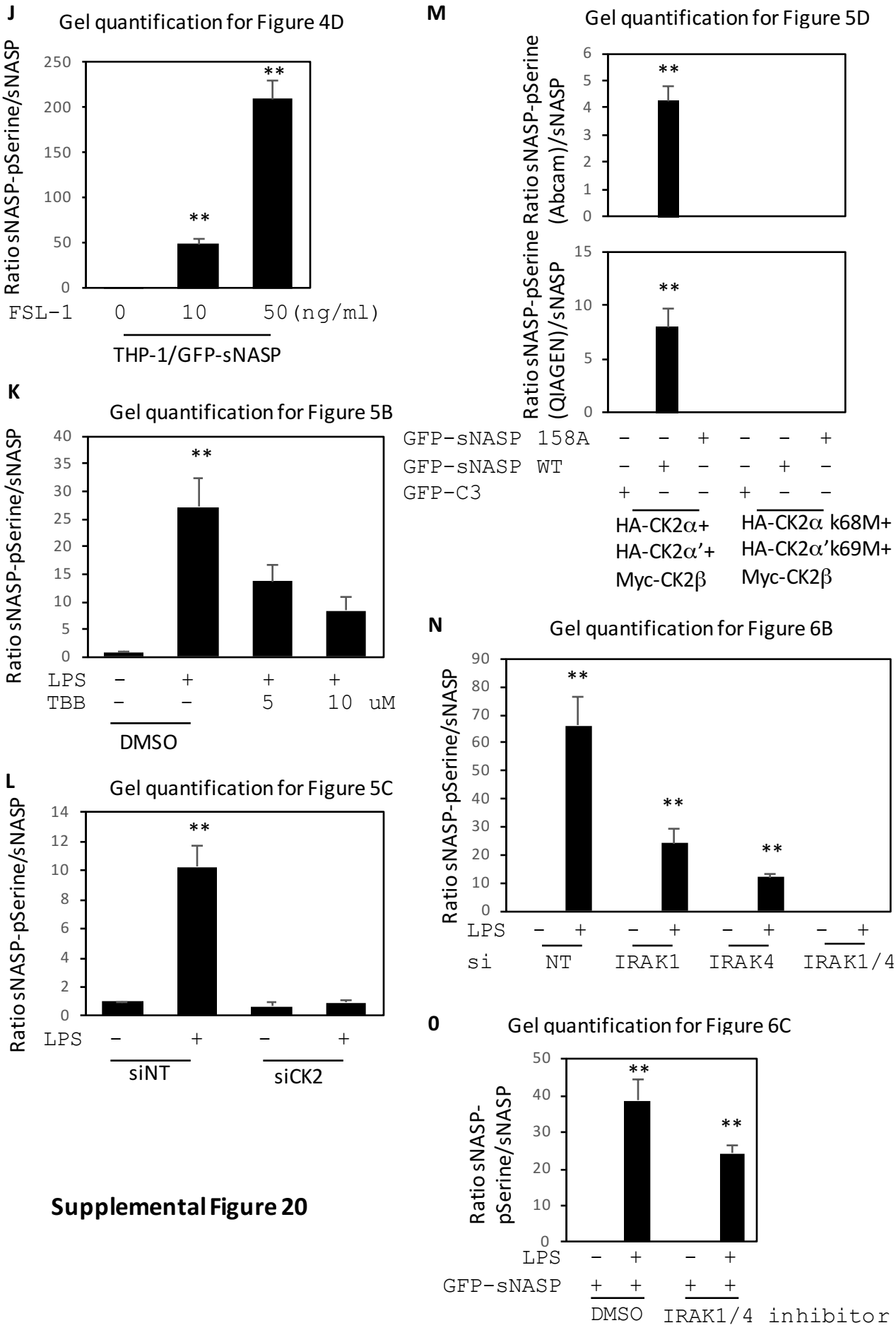
Supplemental Figure 19. Wild type and *sNasp* S158A-KI mice responded similarly to influenza A infection (**A**) Survival curves of wild-type (WT) and NASP S158A-KI (NASPm) mice were examined after influenza A virus (IVA) infection (n=10 per group per experiment). (**B-C**) Virus titers (**B**) and cytokines (**C**) in the lung of wild-type (WT) and sNasp S158A-KI (NASPm) mice were measured 3 days after sham or IVA treatment (n=5 per group per experiment). Data were representative of at least two independent experiments. Data represent mean ± SEM in panel **B** and **C**.



Supplemental Figure 20

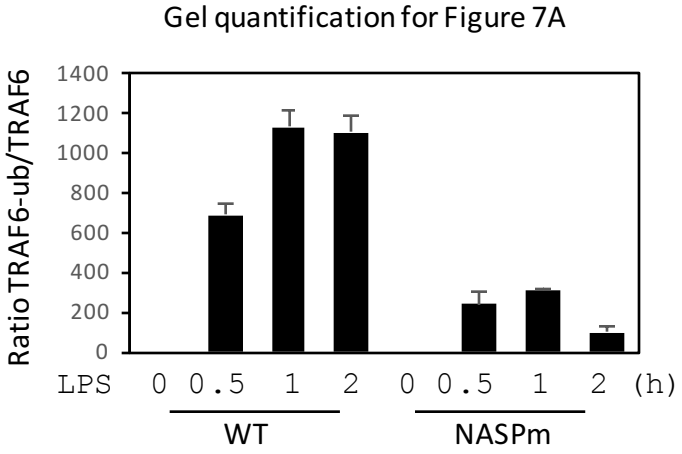


Supplemental Figure 20

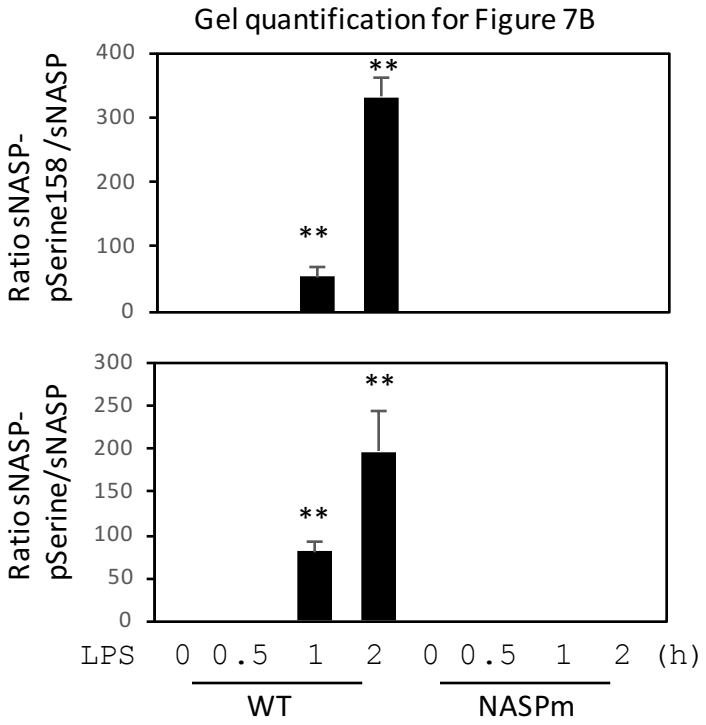


Supplemental Figure 20

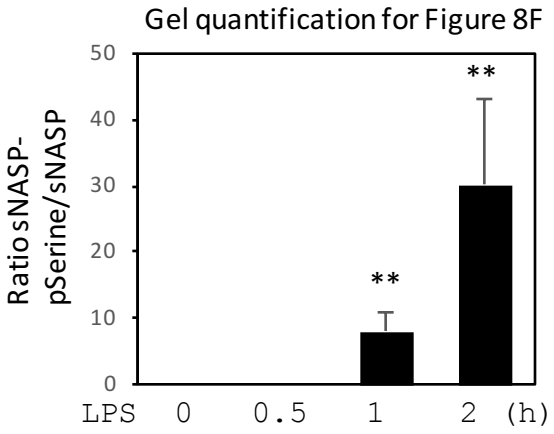
P



Q



R



Supplemental Figure 20. Western blot quantification. (**A**-**P**) Western blot was quantified for Figure 1C, 1D, 3A, 3B, 3C, 3D, 4A, 4B, 4C,4D, 5B, 5C, 5D, 6B, 7A, 7B and 8F. Signal values were normalized to unstimulated conditions (set at 1).Data are expressed as mean ± SE for each group. Signal values were compared to unstimulated conditions. **P < 0.01 (one-way ANOVA). Data represent at least 3 independent experiments.