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Supplementary Materials for

Substrate-specific recognition of IKKs mediated by USP16 facilitates autoimmune inflammation

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Other Supplementary Material for this manuscript includes the following:

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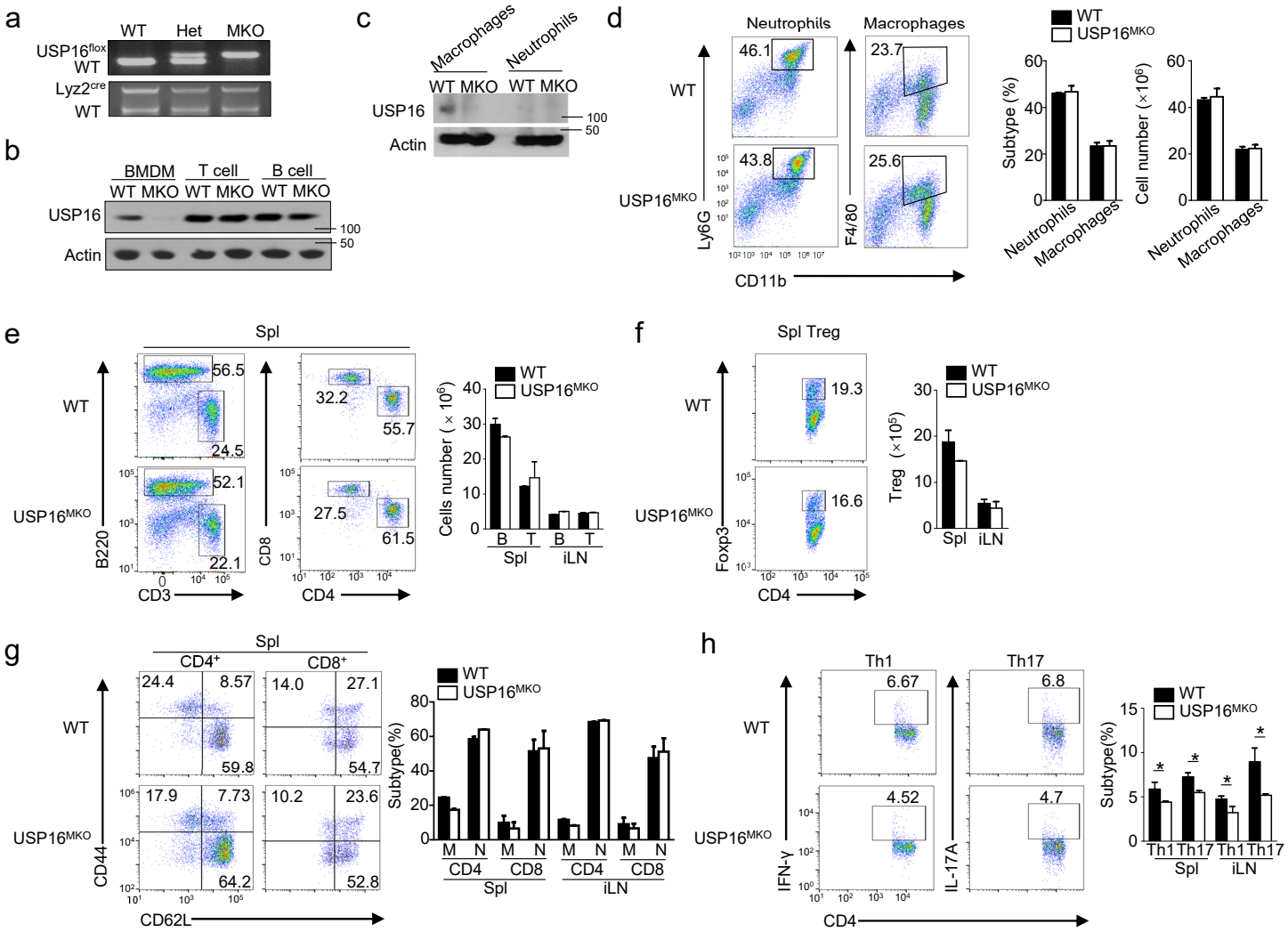
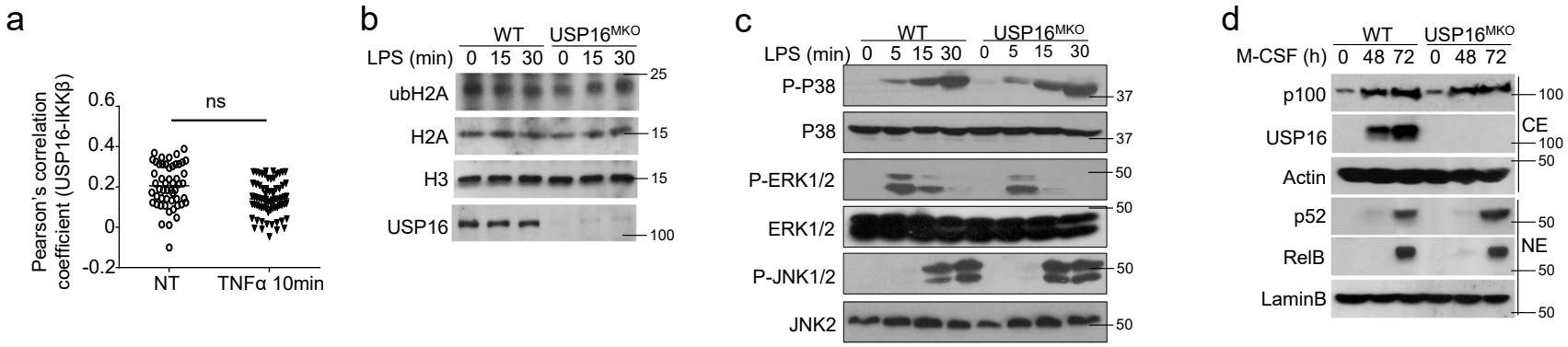
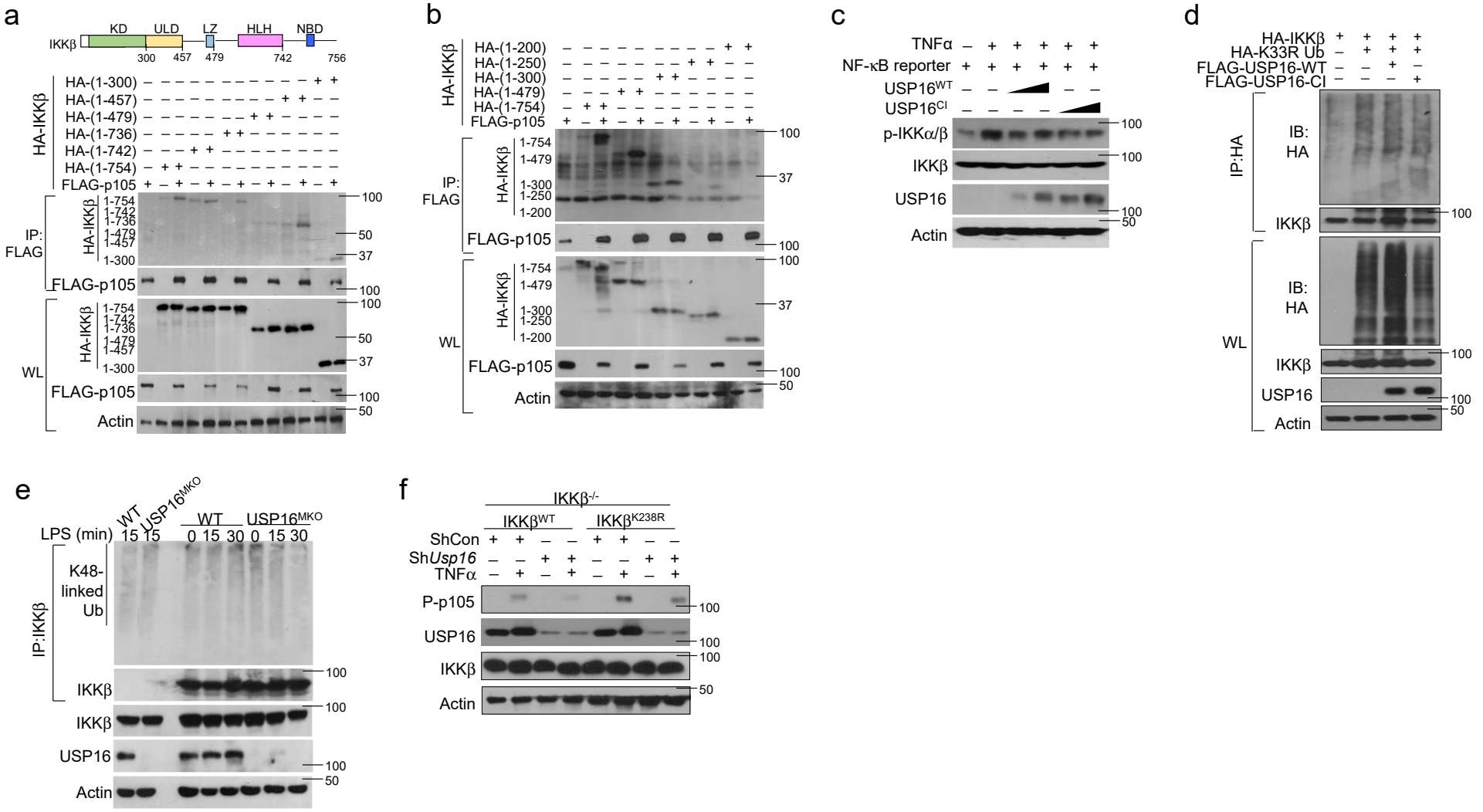


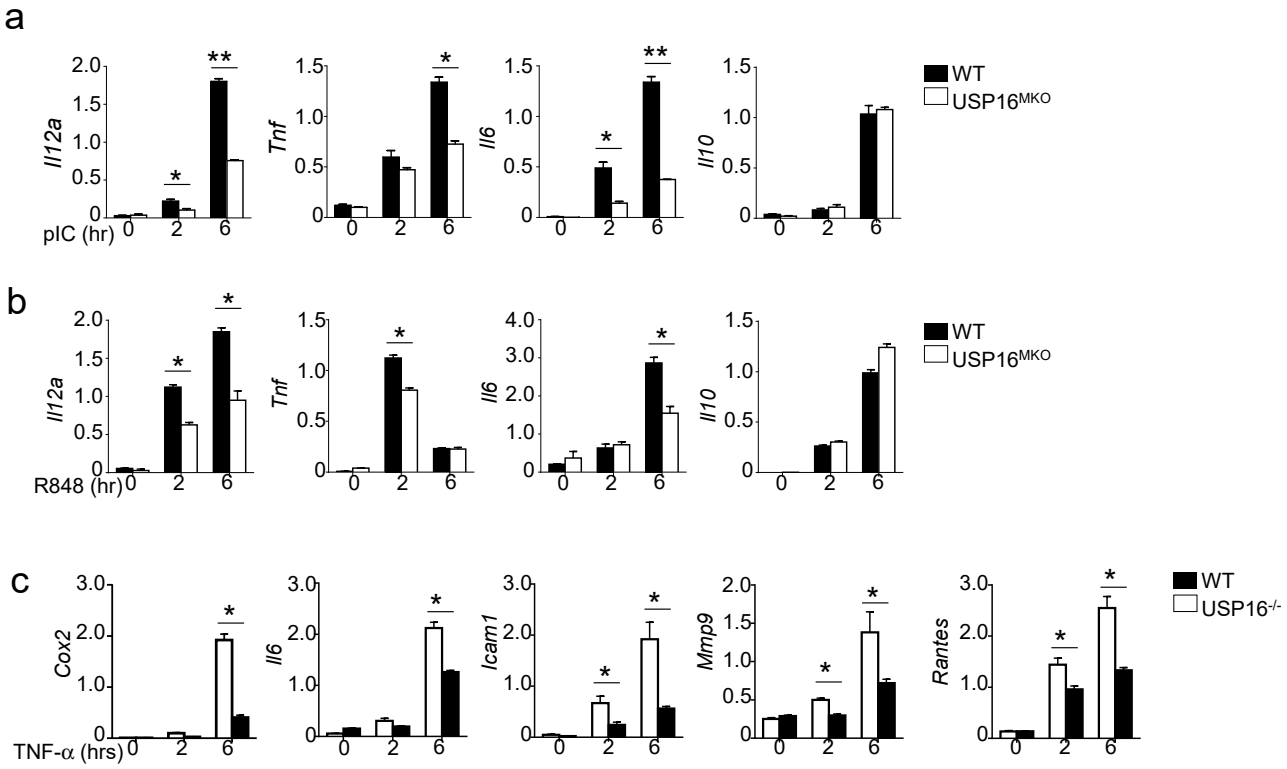
Supplementary Figure 1 USP16 did not affect the development and homeostasis of the immune system. (**a**) Genotyping PCR of WT (USP16$^{+/+}$*Lyz2*-Cre) and myeloid-conditional USP16-knockout (USP16^{MKO}, USP16$^{fl/fl}$*Lyz2*-Cre) mice. (**b**) IB assays showing specific ablation of USP16 in the M-CSF-induced BMDMs of USP16^{MKO} mice. (c) IB assays showing the levels of USP16 in the macrophages and neutrophils isolated from the bone marrow (BM) of WT and USP16^{MKO} mice. (d) Flow cytometry analysis of macrophages (CD11b^{+}F4/80^{+}) and neutrophils (CD11b+Ly6G^{+}) in BM from 6- to 8-week-old WT and USP16^{MKO} mice (n=3). (**e-h**) Flow cytometry analysis of the absolute numbers of different immune cells in the spleen (Spl) (**e**) and of Treg cells (**f**) and naïve and memory T cells (**g**) from WT or USP16^{MKO} mice (n=3). (**h**) Flow cytometric analysis of the percentages of IFN-γ– and IL-17–producing CD4^{+} T cells in the Spls of WT and USP16^{MKO} mice. All FACS data are presented in a representative plot and summary graph of the subpopulation percentages. All data are representative of three independent experiments. The bars and error bars show the means±SEMs. Significance was determined by the two-tailed Student's t test. *P < 0.05.



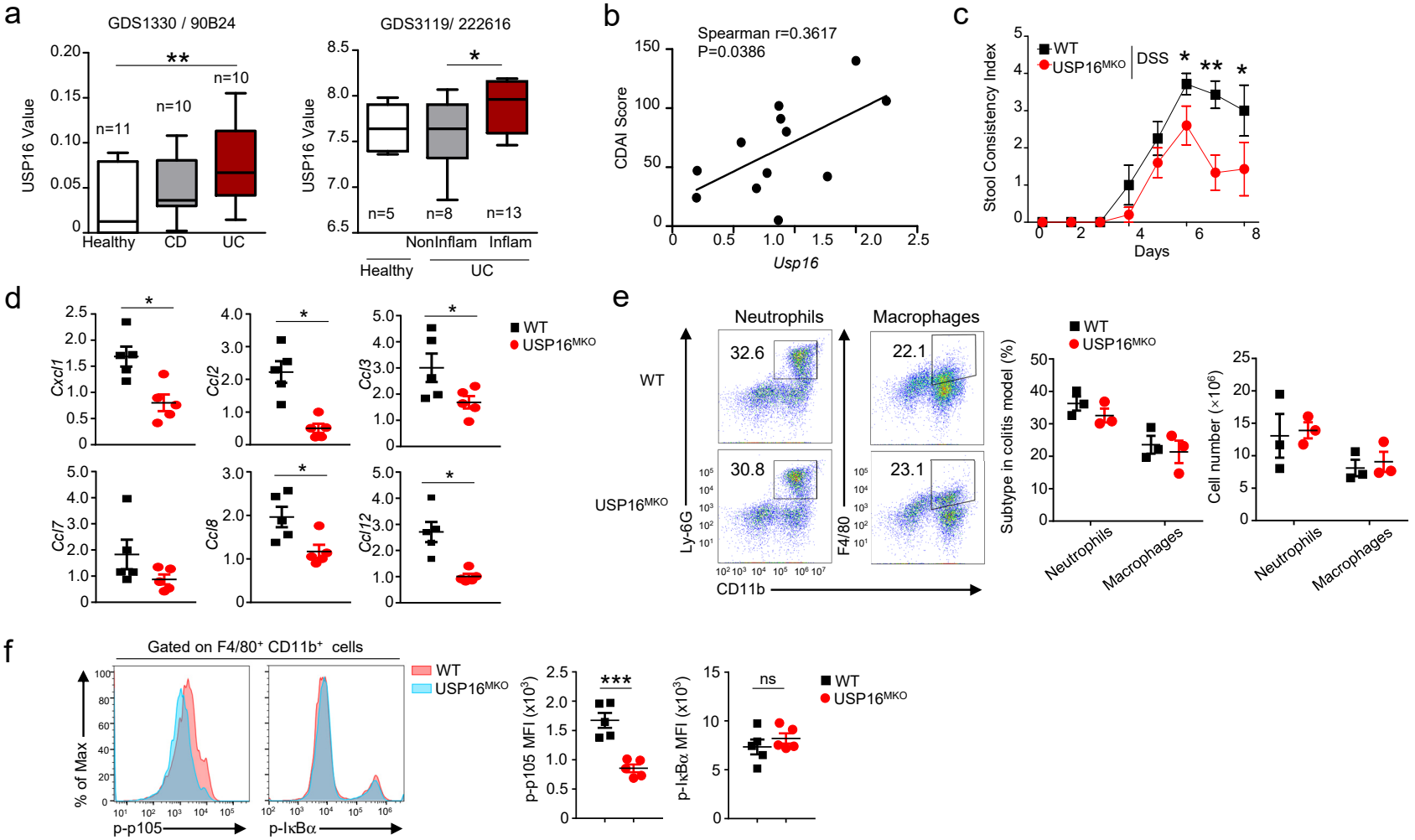
Supplementary Figure 2 USP16 has no effect on the chromatin level and noncanonical NF-κB activation. (**a**) Confocal microscopy analysis of IKKb and USP16 in the WT BMDMs from **Figure 2c**. Quantification of IKKβ/USP16 colocalization was performed as described in the Materials and Methods using Pearson's correlation coefficient (n=50). (**b**) Immunoblot (IB) analysis of lys119-monoubiquitinated H2A (UbH2A), total H2A, and H3 (loading control) in lysates of WT and USP16-deficient macrophages stimulated with LPS as indicated. (**c**) The phosphorylation of MAPKs in whole-cell lysates of WT and USP16-deficient BMDMs was measured by IB analysis. (**d**) Immunoblot analysis of the indicated proteins in the cytoplasmic (CE) and nuclear (NE) extracts of WT or USP16^{MKO} bone marrow cells stimulated with M-CSF (50 ng/ml) at the indicated time points. The data are representative of at least three independent experiments.



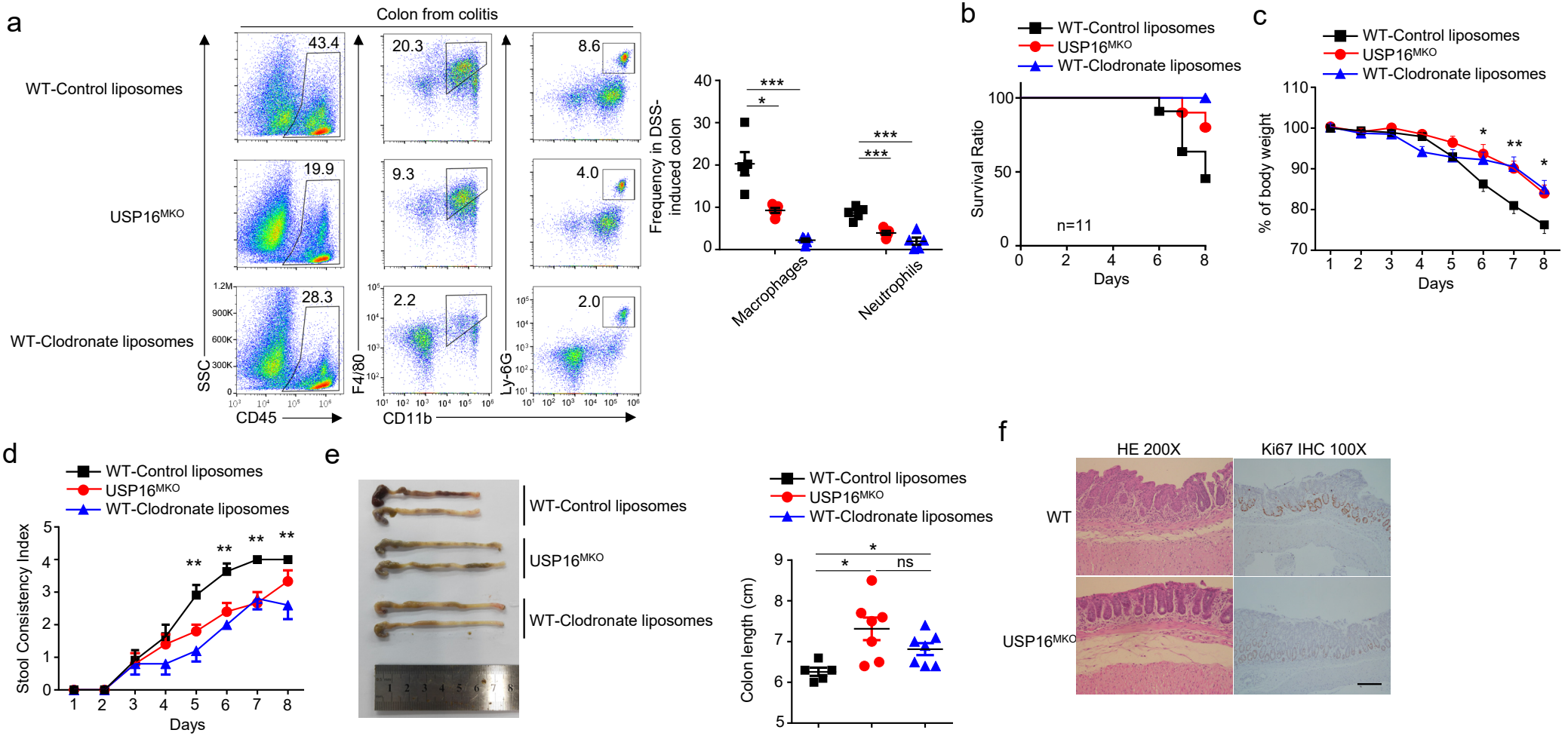
Supplementary Figure 3 USP16 specifically regulates the ubiquitination of IKKβ. (**a-b**) HEK293T cells were transfected with the indicated plasmids. The associations between p105 and various truncation mutants of IKKβ (**a**) or its kinase domain (**b**) were detected through the indicated IP and IB analyses. (**c**) HEK293T cells were transfected with an NF-κB-luciferase reporter plasmid in the presence (+) or absence (–) of the indicated empty vector or expression plasmids. Protein lysates were also subjected to direct IB. (**d**) HEK293T cells were transfected with K33R along with the indicated expression plasmids. FLAG-tagged IKKβ was isolated by IP, and the ubiquitination level was then detected by IB. (**e**) IKKβ was isolated by immunoprecipitation (IP) (under denaturing conditions) from whole-cell lysates of WT and USP16-deficient BMDMs treated with MG132, and then subjected to IB assays using anti-K48 linked ubiquitin (top panels). Protein lysates were also subjected to direct IB (bottom panels). (**f**) IKKβ-deficient MEFs were reconstituted with $IKK\beta^{WT}$ or $IKK\beta^{K238R}$, and then Usp16 was knocked down by shRNA. IB analysis of the phosphorylation of p105 under TNF-α (50 ng/ml) stimulation was performed as indicated. The data are representative of at least three independent experiments.



Supplementary Figure 4 USP16 deficiency inhibits proinflammatory cytokines. (**a-b**) qRT-PCR analysis of mRNA (vertical axes) in WT or USP16-deficient BMDMs unstimulated (0 hr) or stimulated for 2 or 6 hr with pIC (200 μg/ml; **a**) or R848 (1 μg/ml; **b**). (**c**) qRT-PCR analysis of the indicated genes in WT or USP16-deficient MEFs stimulated for 2 or 6 hr with TNF-α (50 ng/ml). All qRT-PCR data are presented as the fold induction relative to the *Actb* mRNA level. The data are presented as the means ± SEMs and are representative of at least three independent experiments. The statistical analysis results show the variations among experimental replicates. Two-tailed unpaired t-tests were performed. *P < 0.05, **P < 0.05.



Supplementary Figure 5 USP16 is associated with the onset of colitis. (**a**) Box plot of USP16 expression in the patients with CD or UC (using public data set GDS1330; n = 10, left). Box plot of USP16 mRNA level in the different area from the patients with UC (using public data set GDS3119, Right). (**b**) Spearman correlation analysis of USP16 mRNA levels in colon macrophages and CD activity index (CDAI)scores. (**c**) WT and USP16^{MKO} mice were treated with 3% DSS described as in **main Figure 6d**. Stool Consistency Index of DSS-treated WT and USP16^{MKO} mice. (**d**) qRT-PCR assay of chemokine levels in colon tissue of DSS-treated WT and USP16^{MKO} mice. All qRT-PCR data are presented as the fold induction relative to the *Actb* mRNA level. (**e**) Flow cytometry analysis of macrophages (CD11b^{+}F4/80^{+}) and neutrophils (CD11b^{+}Ly6G^{+}) in bone marrow (BM) from WT and USP16^{MKO} mice in DSS-induced colitis model (n=3). FACS data are presented in a representative plot and summary graph of the subpopulation percentages. (**f**) Phosphorylation (p-) of p105 and IκBα in macrophages from the colons of DSS-induced WT and USP16^{MKO} mice (n=5), assessed by flow cytometry (gated on CD11b^{+}F4/80^{+} cells). The data are presented as the mean ± SEM values and representative of at least three independent experiments. Statistical analyses represent variations in experimental replicates. Two-tailed unpaired t-tests were performed. *P < 0.05; **, P < 0.01; ***, P < 0.005.



Supplementary Figure 6 USP16 in macrophages is required for the onset of colitis. (a-e) Six- to eight-week-old weeks WT mice were i.v. injected with clodronate liposomes for three times every other day. WT and USP16^{MKO} mice were treated with 3.5% (**b**) or 3% (**a** and **c-e**) DSS (in drinking water) for 5 continuous days and then supplied with normal drinking water. (**a**) FACS analysis results for the total immune cells (CD45$^+$), macrophages (CD11b$^+$F4/80$^+$) and neutrophils (CD11b$^+$Ly6G$^+$), presented in a representative plot for multiple mice treated with clodronate liposomes (n=5). Survival rates (**b**), body weight loss (**c**), stool consistency index (**d**) and colon length (**e**) of DSS-treated WT and USP16^{MKO} mice. (**f**) Representative images of Ki-67 staining of colon tissues of DSS-treated WT and USP16^{MKO} mice (Scale bar: 20μM). Photo credits: Jian-shuai Yu and Yu Zhang (Zhejiang University). The data are presented as the means ± SEMs and are representative of at least three independent experiments. The statistical analysis results show the variations among experimental replicates. Two-tailed unpaired t-tests were performed. *P < 0.05, **P < 0.01; ***P < 0.005.

Supplementary Table 1 The gene-specific PCR primers. The forward and reverse sequences of the primers used for the qPCR assay. All of primers were designed by Oligo 7 v7.56 based on the mouse genes.