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Supplemental information

The ubiquitin ligase HOIL-1L regulates immune responses by interacting with linear ubiquitin chains

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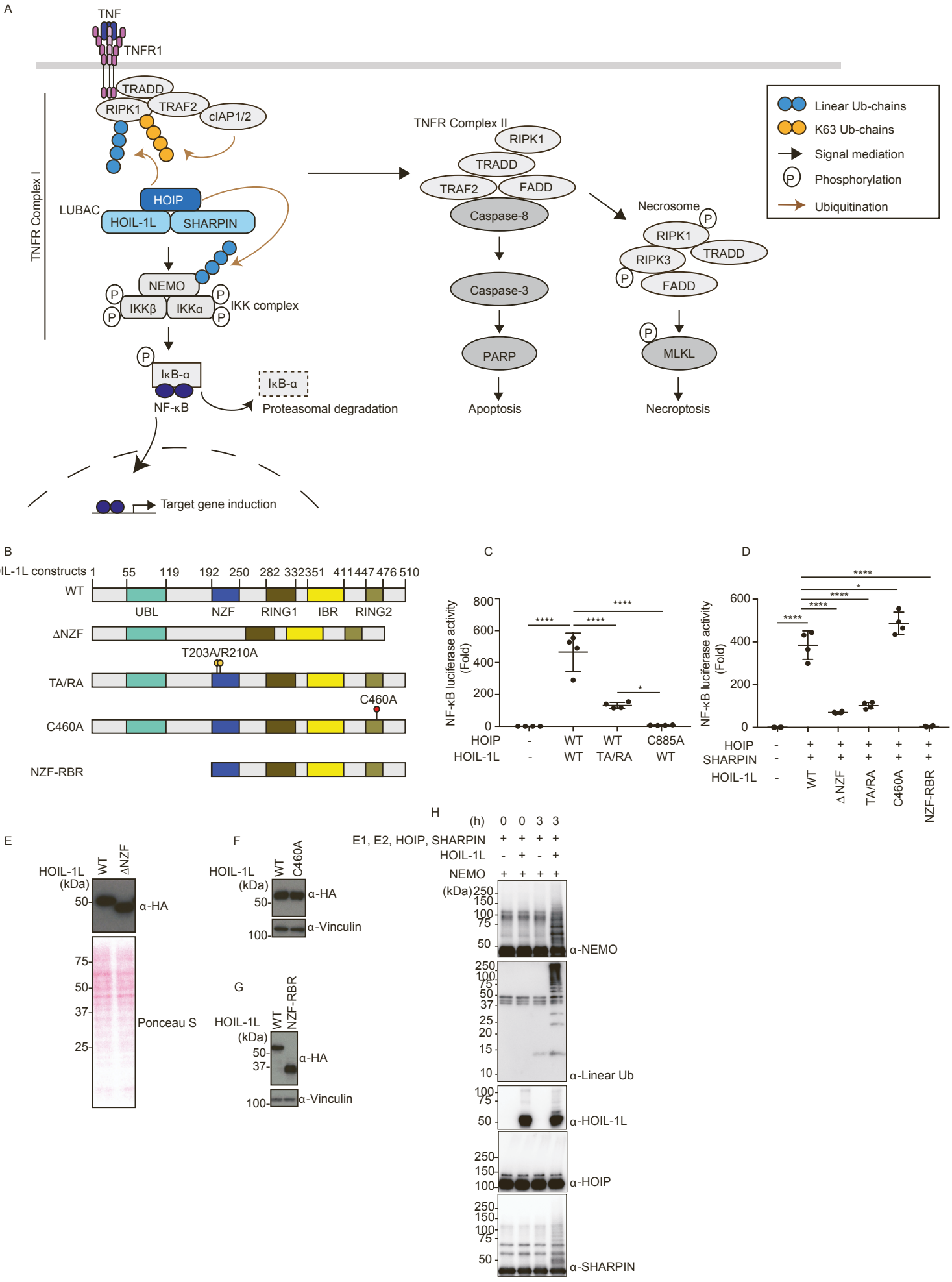
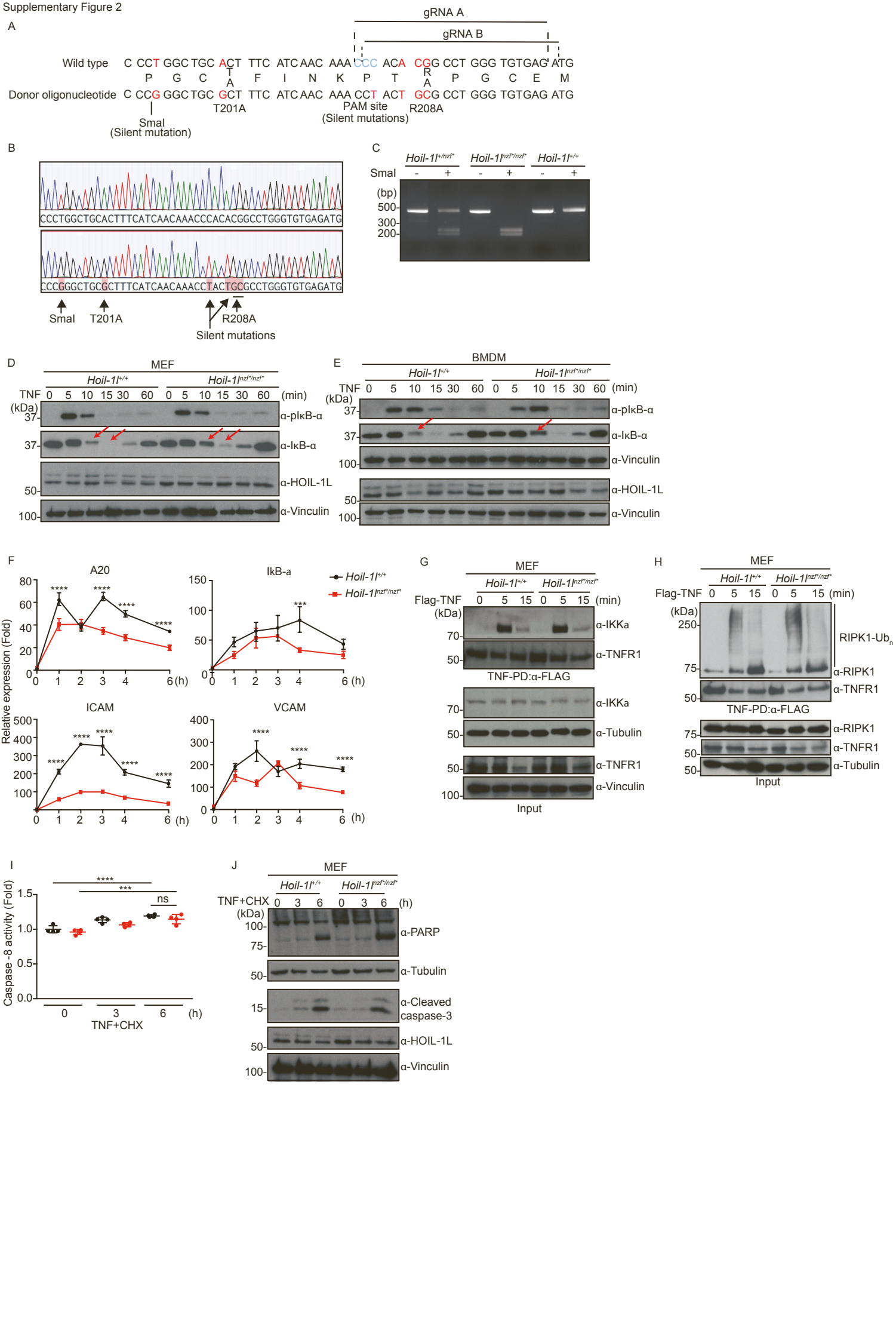
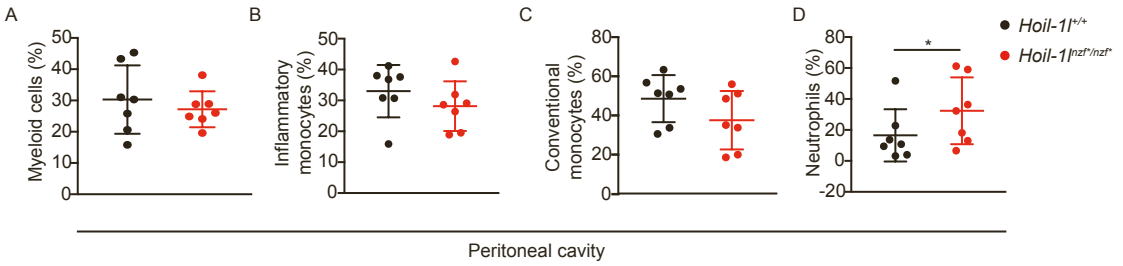
Supplementary Figure 1



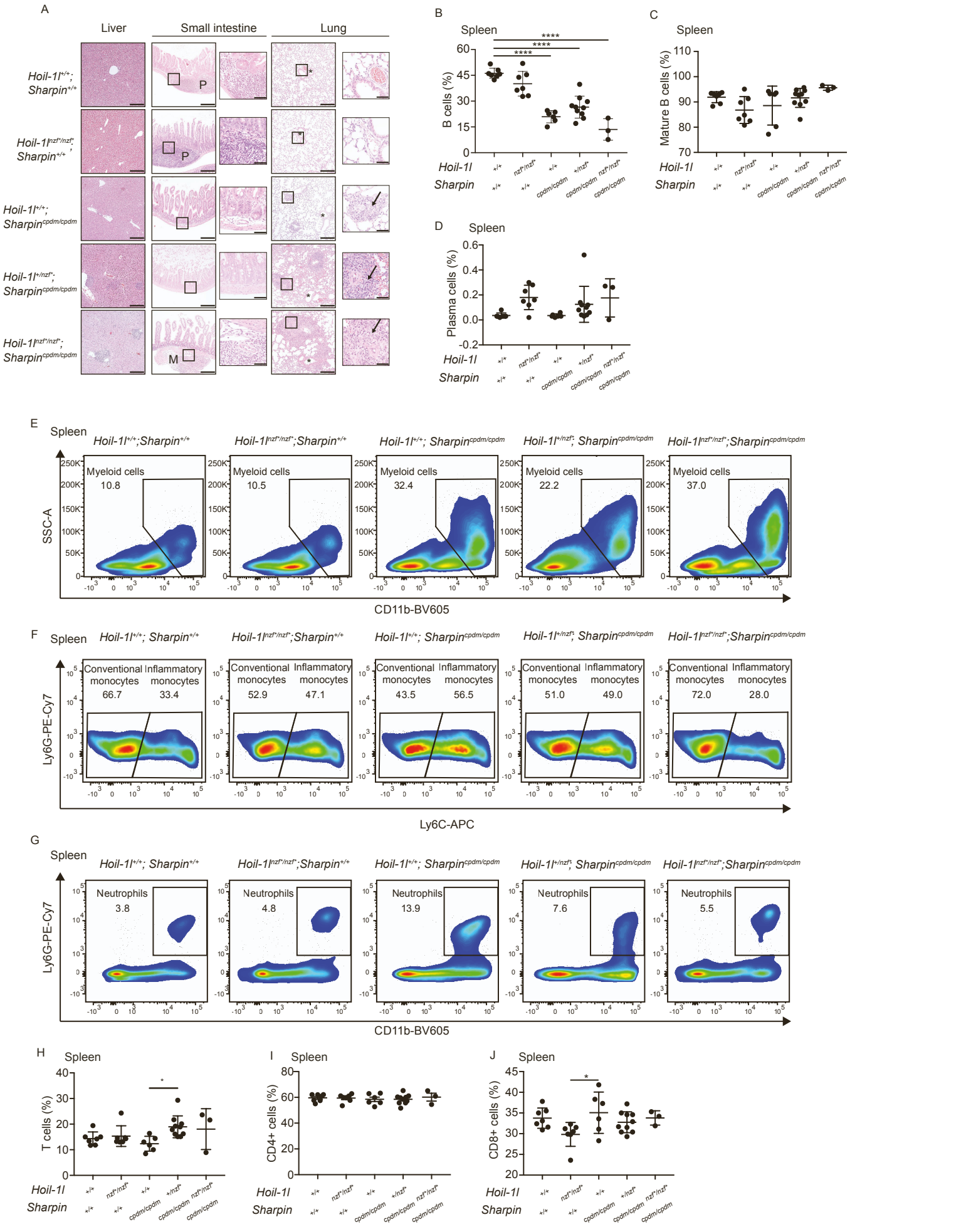
Supplementary Figure 2



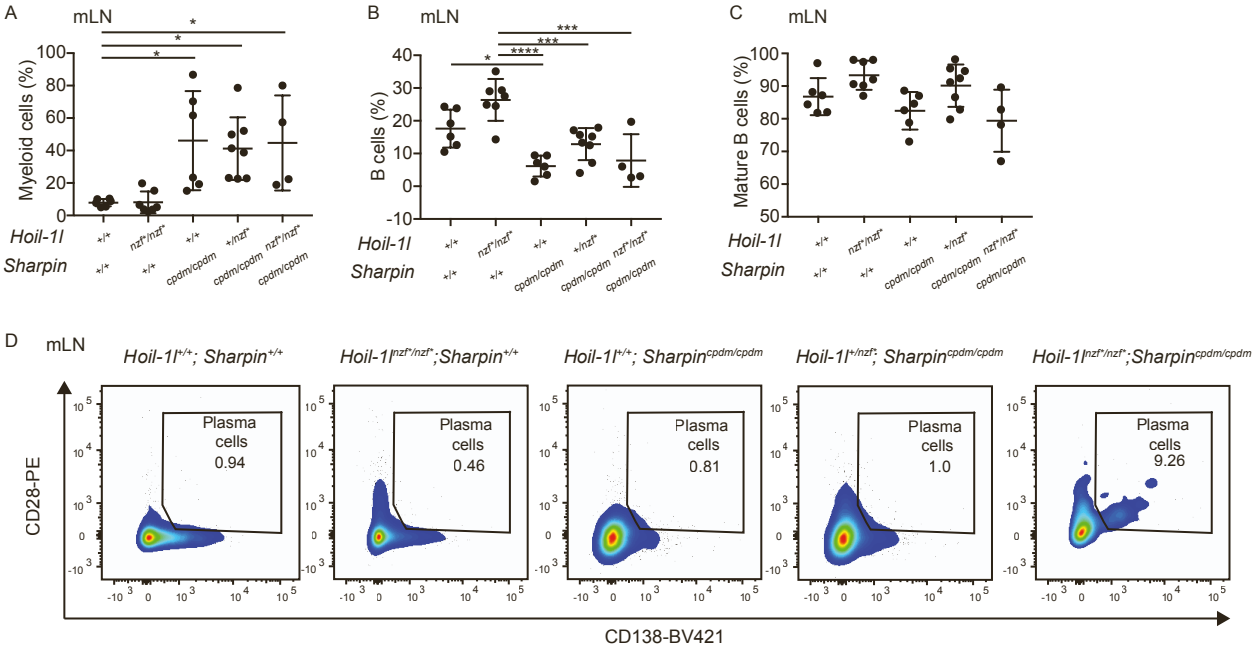
Supplementary Figure 3



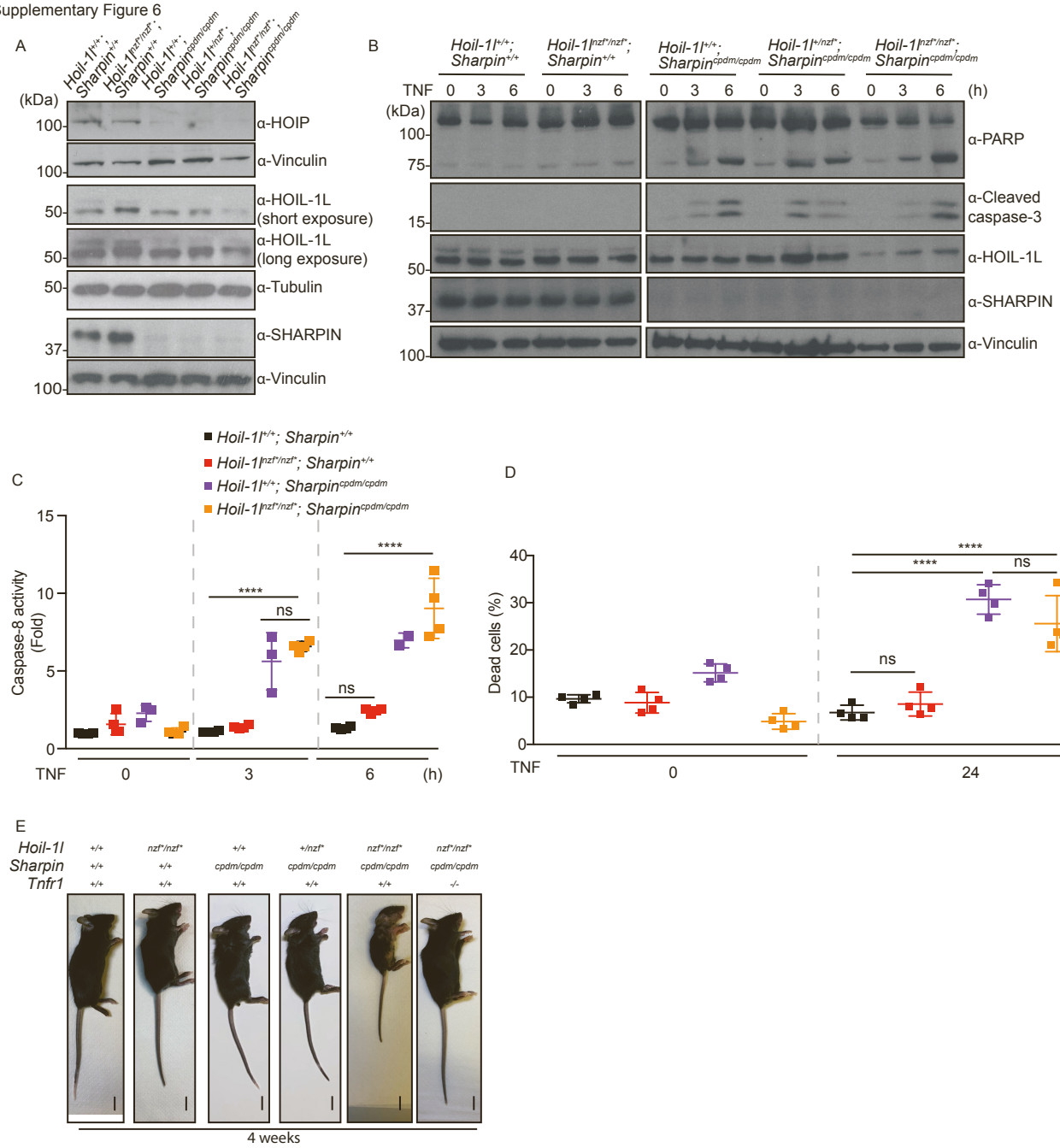
Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 1, related to Figure 1. Thr203 and Ala210 of HOIL-1L-NZF are important to mediate interaction with linear ubiquitin chains and to activate the NF-κB pathway.

A. Schematic of the TNF-TNFR1 signaling pathway.

B.Schematic representation of the human HOIL-1L constructs used in the study.

C. NF-κB reporter assay using lysates of HEK293T cells co-transfected with NF-κB reporter and Renilla-expression plasmids with Myc-HOIP (WT or C885A) and HOIL-1L-HA (WT or TA/RA) plasmids.

D. NF-κB reporter assay using lysates of HEK293T cells co-transfected with NF-κB reporter and Renilla-expression plasmids with Myc-HOIP (WT), Flag-SHARPIN (WT) and HOIL-1L-HA (WT or the indicated mutants) plasmids.

E-G. Immunoblotting to detect expression levels of HOIL-1L-ΔNZF-HA (a mutant lacking the linear ubiquitin chain-binding domain), HOIL-1L-C460A-HA (an inactive mutant) and HOIL-1L-NZF-RBR-HA overexpressed in HEK293T cells. α-HA antibody was used to detect the HOIL-1L constructs and ponceau staining (E) or α-Vinculin antibody (F-G) were used to monitor the loading amount.

H. In vitro ubiquitination assays using the recombinant proteins of ubiquitin (Ub), Ube1 (E1), E2 (UbcH7), HOIP, HOIL-1L WT and NEMO. The different components were incubated at 37°C for 3 hours. Linear ubiquitin chain formation and modifications of NEMO, HOIP HOIL-1L and SHARPIN were detected by immunoblotting with the indicated antibodies.

Data information. (C-H) Data are representative of at least three independent experiments. (C and D) Data are represented as mean ±SD, ANOVA, n=4, * p-value ≤0.05, ****p-value ≤0.0001.

Supplementary Figure 2, related to Figure 2. The linear ubiquitin chain recognition of HOIL-1L contributes to TNF-induced NF-κB dependent gene induction.

A. Targeting strategy to generate the *Hoil-1l*$^{T201A;R208A/T201A;R208A}$ (*Hoil-1l*$^{nzf*/nzf*}$) mice by CRISPR/Cas9. Cas9 mRNA, gRNA and a donor single-stranded oligonucleotide were injected into the cytosol of zygotes. Threonine 201 and Arginine 208 were mutated to Alanine respectively. For genotyping purposes, a silent mutation generating a SmaI restriction site was introduced. Two additional silent mutations were introduced to avoid re-editing of the DNA by Cas9 after homology-directed repair.

B. Sanger Sequencing results corresponding to the targeted region in WT (upper panel) and *Hoil-1l*$^{nzf*/nzf*}$ (lower panel) mice.

C. Genotyping results of *Hoil-1*$^{+/+}$, *Hoil-1l*$^{+/nzf*}$ and *Hoil-1l*$^{nzf*/nzf*}$ mice. PCR products of genomic DNA isolated from the respective mice were digested with SmaI. *Hoil-1*$^{+/+}$ has no digested alleles, *Hoil-1l*$^{+/nzf*}$ has one allele digested, and *Hoil-1l*$^{nzf*/nzf*}$ has both alleles digested.

D and E. Immunoblotting to assess phosphorylation and degradation of IκB-α upon TNF treatment (20ng/ml) for the indicated time points in MEFs (D) and BMDMs (E) derived from *Hoil-1*$^{+/+}$ and *Hoil-1l*$^{nzf*/nzf*}$ mice.

F. mRNA levels of NF-κB targets (A20, IκB-α, ICAM and VCAM) determined by qRT-PCR in *Hoil-1l*$^{+/+}$ and *Hoil-1l*$^{nzf*/nzf*}$ MEFs treated with mTNF (20 ng/ml) for the indicated timepoints. Normalization to β-actin.

G and H. Immunoblotting to assess TNFR Complex I formation in *Hoil-1l*$^{+/+}$ and *Hoil-1l*$^{nzf*/nzf*}$ MEFs treated with hTNF (100 ng/ml). Total cell lysates (Input) and immunoprecipitates (IP: α-FLAG) were subjected to SDS-PAGE. Recruitment of IKKα (G) and RIPK1 (H) was monitored by immunoblotting. α-Tubulin antibody was used to monitor loading amount.

I. Caspase-8 activity in *Hoil-1l*$^{+/+}$ and *Hoil-1l*$^{nzf*/nzf*}$ MEFs treated with mouse TNF (100ng/ml) and cycloheximide (CHX) (1ug/ml) for the indicated timepoints.

J. Immunoblotting to detect levels of TNF-induced cleaved-PARP and cleaved-caspase3 in *Hoil1l*$^{+/+}$ and *Hoil-1l*$^{nzf*/nzf*}$ MEFs treated with mTNF (100ng/ml) and cycloheximide (CHX) (1ug/ml) for the indicated timepoints. α-Tubulin and α-Vinculin antibodies were used to monitor loading amount.

Data information. Data are representative of at least three independent experiments. (F, I) Data are represented as mean ±SD, ANOVA **p-value ≤0.01, ****p-value ≤0.0001. (F) n=3, (I) n=4.

Supplementary Figure 3, related to Figure 4. Normal levels of myeloid cell populations are observed in *Hoil-1l*$^{nzf*/nzf*}$ mice after LPS-induced shock.

A-D. Frequencies of the indicated immune cell types determined by flow cytometry in the peritoneal exudate of *Hoil-1l*$^{+/+}$ (n=7) and *Hoil-1l*$^{nzf*/nzf*}$ (n=7) mice subjected to an LPS challenge for 12 hours. Myeloid cells are defined as CD11b^{+}, inflammatory monocytes as CD11c^{-} CD11b^{+} Ly6G^{-} Ly6C^{+}, conventional monocytes as CD11c^{-} CD11b^{+} Ly6G^{-} Ly6C^{-} and neutrophils as CD11b^{hi} Ly6G^{+}. Frequencies are calculated based on viable CD45^{+} cells (**A, D**) or viable CD45^{+} CD11b^{+} cells (**B, C**)

Data information. Data are represented as mean ±SD, ANOVA, *p-value ≤0.05.

Supplementary Figure 4, related to Figure 5. *Hoil-1l*$^{nzf*/nzf*}$*; Sharpin*$^{cpdm/cpdm}$ mice show systemic inflammation and a distinctive immune cell composition in the spleen.

A. H&E staining of liver, small intestine and lung from 4-week-old mice of the indicated genotypes. P is Peyer's patches. M indicates site of mesenteric inflammation. * indicates a bronchiole. Scale bar: 200μm.

B-D. Percentage of viable B cells ($CD19^+$) out of viable $CD45^+$ cells (**B**), percentage of mature B cells ($CD45^+$ $CD19^+$ $CD93^-$) out of viable $CD45^+$ $CD19^+$ B cells (**C**), percentage of plasma cells ($CD138^+$ $CD28^+$) out of viable $B220^+$ Lin^- B cells (**D**) in the spleen of 4-week-old mice from the indicated genotypes examined by flowcytometry. The lineage (Lin) cocktail consists of TCRβ, DX5, CD11b and CD23.

E. Representative flow cytometry plots for myeloid cells ($CD11b^+$) out of viable $CD45^+$ cells in the spleen from 4-week-old mice of the indicated genotypes.

F. Representative flow cytometry plots for inflammatory monocytes ($CD11c^-$ $Ly6G^-$ $Ly6C^+$) and conventional monocytes ($CD11c^-$ $Ly6G^-$ $Ly6C^-$) out of viable $CD45^+$ $CD11b^+$ cells in the spleen from 4-week-old mice of the indicated genotypes.

G. Representative flow cytometry plots for neutrophils ($Ly6G^+$ $CD11b^{hi}$) out of viable $CD45^+$ cells in the spleen from 4-week-old mice of the indicated genotypes.

H-J. Percentage of viable T cells ($TCR\beta^+$) out of viable $CD45^+$ cells (**H**), percentage of $CD4^+$ T cells out of viable $TCR\beta^+$ T cells (**I**), percentage of $CD8^+$ T cells out of viable $TCR\beta^+$ T cells (**J**) in the spleen from 4-week-old mice of the indicated genotypes examined by flow cytometry.

Data information. Data are represented as mean ±SD, ANOVA, *p-value ≤0.05, ****p-value ≤0.0001. *Hoil-1l*$^{+/+}$*; Sharpin*$^{+/+}$ (n=7), *Hoil-1l*$^{nzf*/nzf*}$*; Sharpin*$^{+/+}$ (n=7), *Hoil-1l*$^{+/+}$*; Sharpin*$^{cpdm/cpdm}$ (n=6), *Hoil-1l*$^{+/nzf*}$*; Sharpin*$^{cpdm/cpdm}$ (n=10), *Hoil-1l*$^{nzf*/nzf*}$*; Sharpin*$^{cpdm/cpdm}$ (n=3).

Supplementary Figure 5, related to Figure 5. *Hoil-1l*$^{nzf*/nzf*}$*; Sharpin*$^{cpdm/cpdm}$ mice show systemic inflammation and a distinctive immune cell composition in the mLN.

A-C. Frequency of viable myeloid cells (viable CD45$^+$ CD11b$^+$ cells) (**A**), B cells (CD19$^+$) out of viable CD45$^+$(**B**), mature B cells (CD19$^+$ CD93$^-$) out of viable CD45$^+$ CD19$^+$ (**C**) in the mLN from 4-week-old mice of the indicated genotypes examined by flow cytometry.

D. Representative flow cytometry plots for plasma cells (CD138$^+$ CD28$^+$) out of viable B220$^+$ Lin$^-$ cells in the mesenteric lymph nodes (mLN) from 4-week-old mice of the indicated genotypes. The lineage (Lin) cocktail consists of TCRβ, DX5, CD11b and CD23.

Data information. Data are represented as mean ±SD, ANOVA, *p-value ≤0.05, ***p-value ≤0.001, ****p-value ≤0.0001. *Hoil-1l*$^{+/+}$*; Sharpin*$^{+/+}$ (n=6), *Hoil-1l*$^{nzf*/nzf*}$*; Sharpin*$^{+/+}$ (n=7), *Hoil-1l*$^{+/+}$*; Sharpin*$^{cpdm/cpdm}$ (n=6), *Hoil-1l*$^{+/nzf*}$*; Sharpin*$^{cpdm/cpdm}$ (n=8), *Hoil-1l*$^{nzf*/nzf*}$*; Sharpin*$^{cpdm/cpdm}$ (n=4).

Supplementary Figure 6, related to Figure 6. *Hoil-1l*$^{nzf*/nzf*}$*; Sharpin*$^{cpdm/cpdm}$ MEFs are not further sensitized to TNF-induced cell death in comparison to *Sharpin*$^{cpdm/cpdm}$ MEFs .

A. Immunoblotting to assess the expression levels of LUBAC components in MEFs from the indicated genotypes.

B. Immunoblotting to detect levels of TNF-induced cleaved-PARP and cleaved-caspase3 in MEFs from the indicated genotypes treated with mTNF (100 ng/ml).

C. Caspase-8 activity in MEFs from the indicated genotypes treated with mouse TNF (100 ng/ml) for the indicated timepoints.

D. Percentage of eFluor780 (viability dye) positive MEFs (Dead cells) from the indicated genotypes after 24 hours of mTNF (100 ng/ml) stimulation.

E. Gross appearance picture of 4-week-old mice from the indicated genotypes. Scale bar: 10 mm.

Data information. Data are representative of at least three independent experiments (A-D). Data are represented as mean ±SD, ANOVA, *p-value ≤0.05, ***p-value ≤0.001, ****p-value ≤0.0001. (C and D) *Hoil-1l*$^{+/+}$*; Sharpin*$^{+/+}$ (n=4), *Hoil-1l*$^{nzf*/nzf*}$*; Sharpin*$^{+/+}$ (n=4), *Hoil-1l*$^{+/+}$*; Sharpin*$^{cpdm/cpdm}$ (n=4), *Hoil-1l*$^{nzf*/nzf*}$*; Sharpin*$^{cpdm/cpdm}$ (n=4).