

Supplemental Material for:

Attenuation of HOIL-1L ligase activity promotes systemic autoimmune disorders by augmenting linear ubiquitin signaling

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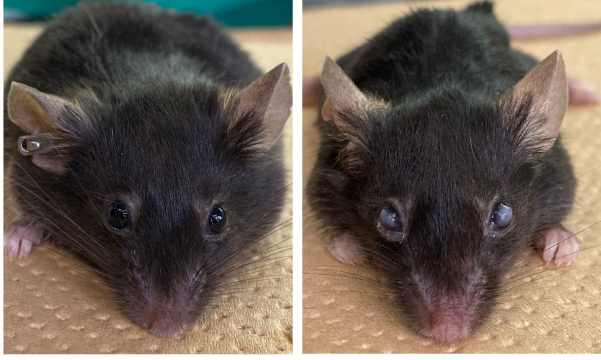
Supplemental Figure 3

Supplemental Figure 4

Supplemental Figure 5

Supplemental Figure 6

A

HOIL-1L^{+/+}HOIL-1L^{ΔRING1/ΔRING1}

7 months old

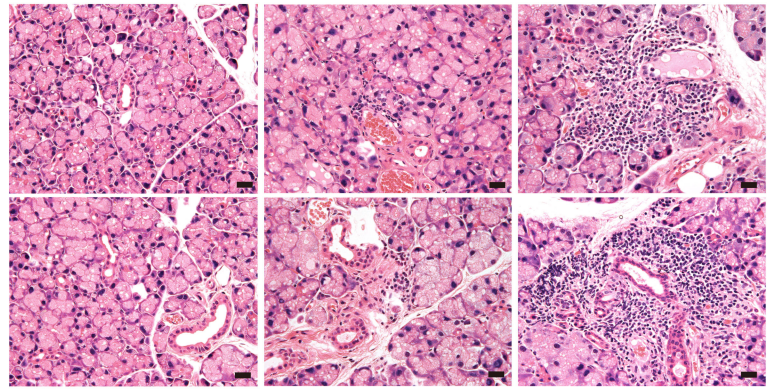
B

HOIL-1L^{+/+}HOIL-1L^{ΔRING1/+}HOIL-1L^{ΔRING1/ΔRING1}

#1

H&E

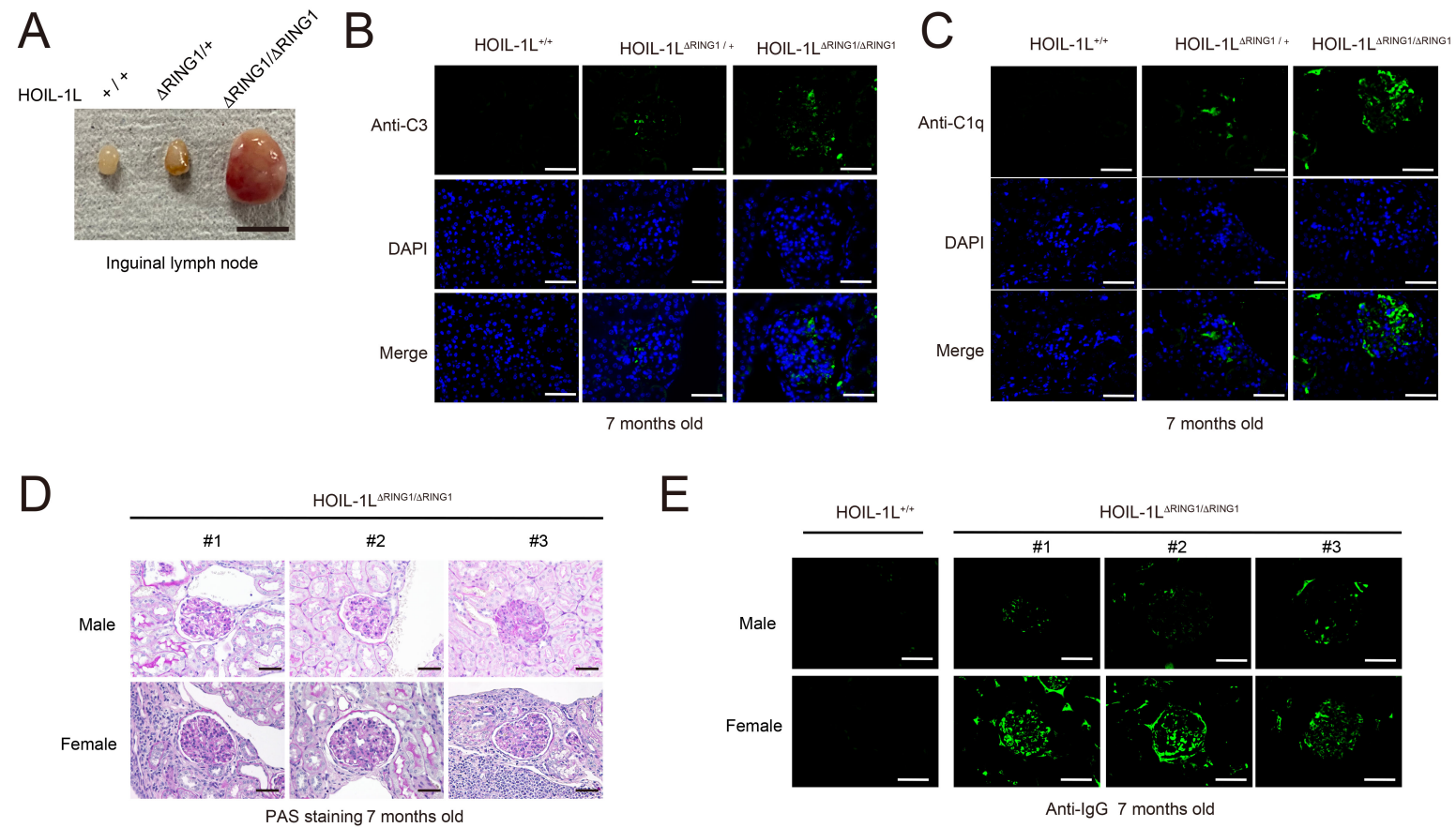
#2



Lacrimal gland 7 months old

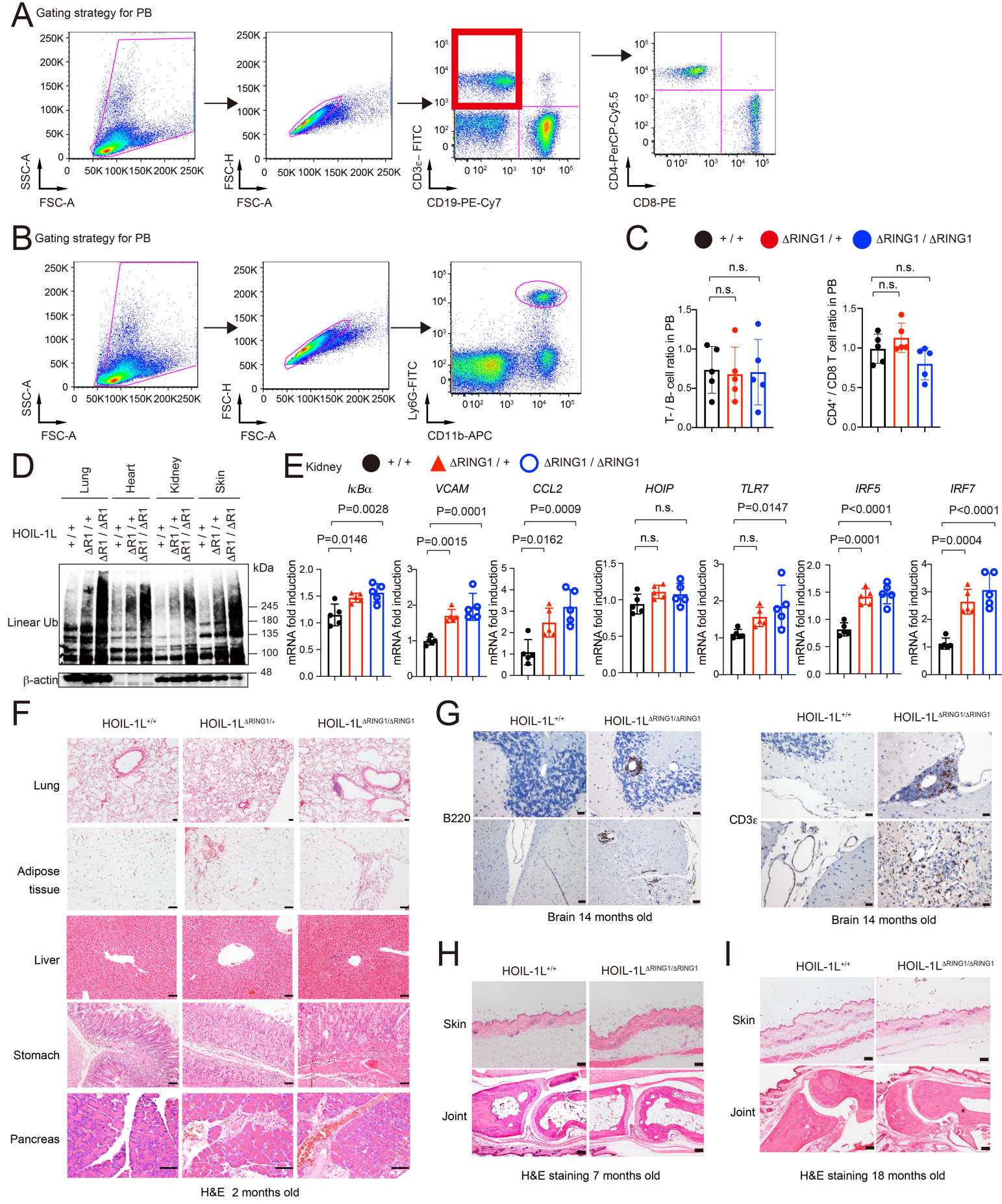
Supplemental Figure 1. HOIL-1L Δ RING1 mice show SS-like symptoms.

(A) Macroscopic pictures of female littermate mice of the indicated genotypes (7 months old). (B) Histological analysis (H&E staining) of lacrimal gland sections obtained from 7-month-old littermate mice of the indicated genotype. Scale bars, 20 μ m.



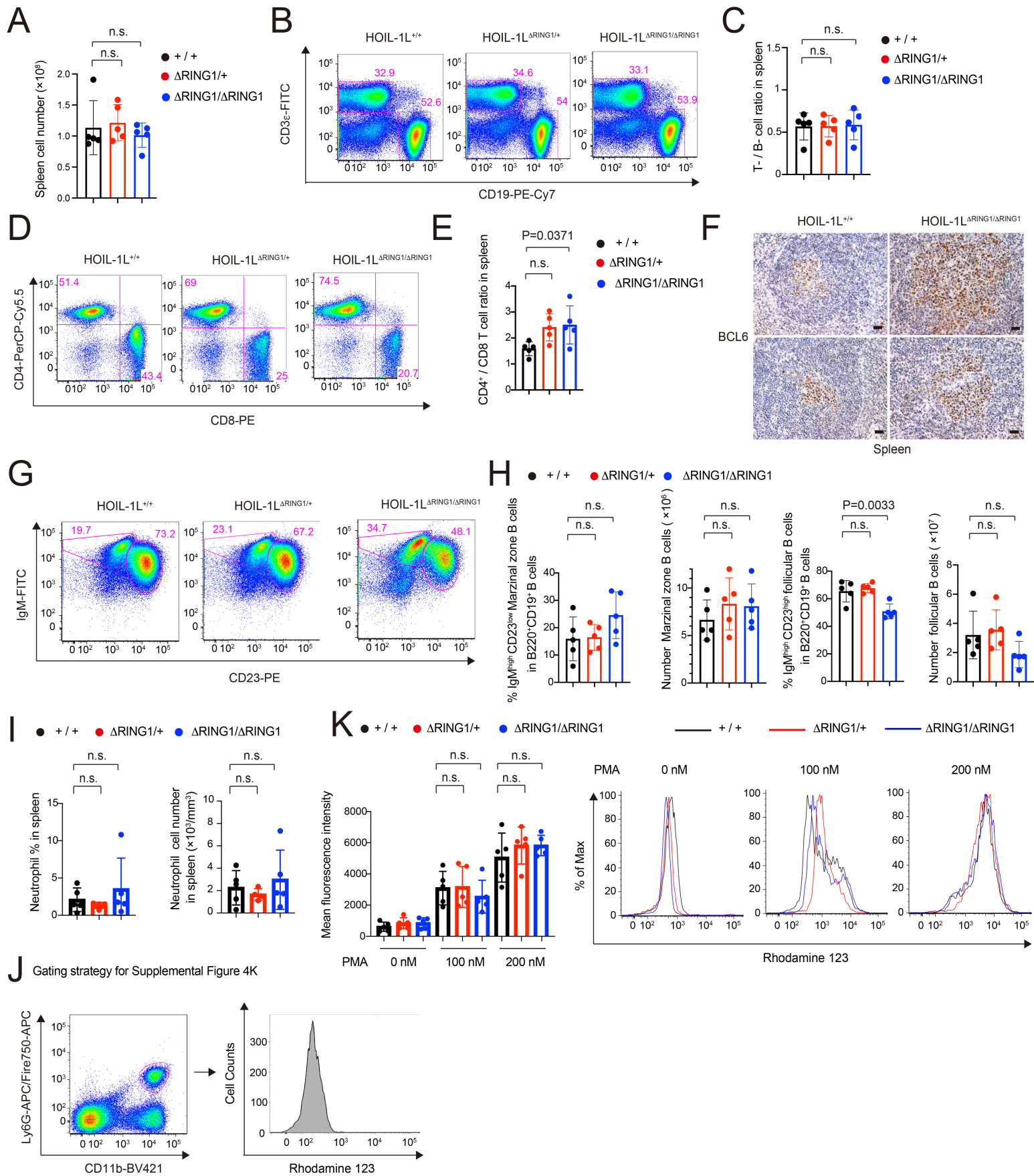
Supplemental Figure 2. HOIL-1L Δ RING1 mice developed lupus-like nephritis

(A) Macroscopic picture of the inguinal lymph nodes from the indicated female littermates (8 months old). Scale bars, 5 mm. (B and C) Histological analysis was performed by immunostaining kidney sections from 7-month-old littermate female mice of the indicated genotype to detect C3 (B) and C1q (C) (nuclei were stained with DAPI). Scale bars, 40 μ m. (D) Histological analysis (PAS staining) of kidneys from 7-month-old female and male mice of the indicated genotype. Scale bars, 20 μ m. n = 3 of each sex. (E) Immunostaining of kidney sections from 7-month-old mice of the indicated genotype to detect IgG. Scale bars, 50 μ m.



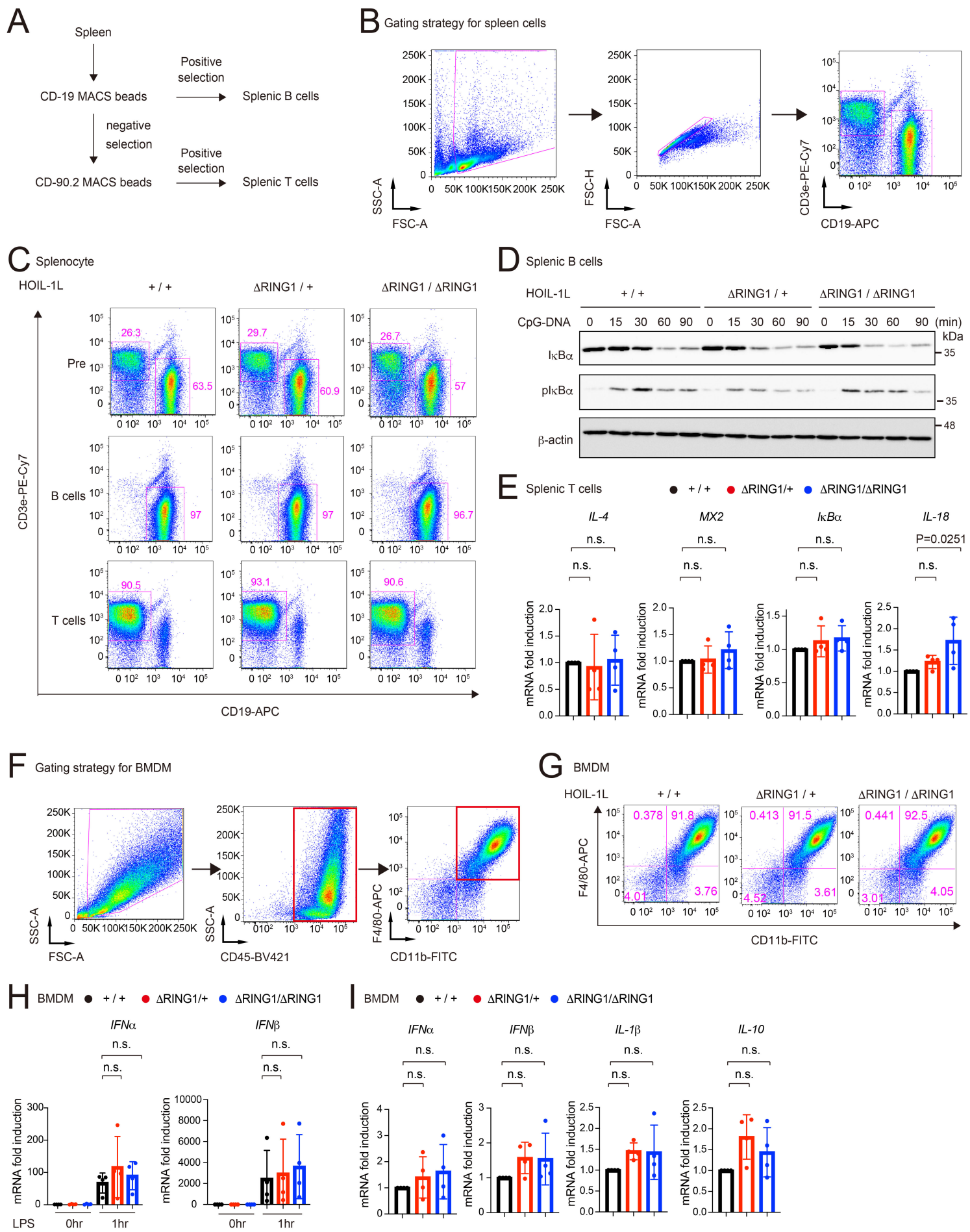
Supplemental Figure 3. HOIL-1L Δ RING1 mice show characteristic features of SLE

(**A and B**) Gating strategies used to identify lymphocytes (**A**) and neutrophils (**B**) in the peripheral blood (PB) of mice. (**C**) Quantification of leukocytes in the serum of 7–8-month-old female mice of the indicated genotype: T/B ratio and CD4/8 ratio. Data are expressed as the mean $\pm$ S.D. *P*-values were calculated by one-way ANOVA, followed by Dunnett's multiple comparisons test. n.s.: $p > 0.05$; $n = 5$ per group. (**D**) Lysates of organs from 7-month-old littermate female mice of the indicated genotype were subjected to immunoblotting to detect linear ubiquitin. (**E**) Quantitative PCR analysis of kidney tissue from 3–4-month-old female mice of the indicated genotype. Data are expressed as the mean $\pm$ S.D. *P*-values were calculated by one-way ANOVA, followed by Dunnett's multiple comparisons test. n.s.: $p > 0.05$. $n = 5$ per group. (**F**) H&E staining of multiple organs from 2-month-old female littermate mice of the indicated genotype. Scale bars, 50 μ m. (**G**) Acquisition of macroscopic images, immunostaining for B220 and CD3 ϵ in the brains of littermate (14-month-old) female mice of the indicated genotype. Scale bars, 50 μ m. (**H and I**) Histological analysis, performed by H&E staining, of skin and joint sections from 7-month-old mice (**H**), and from 18-month-old mice (**I**). Littermate female mice of the indicated genotype. Scale bars, 100 μ m (**H and I**).



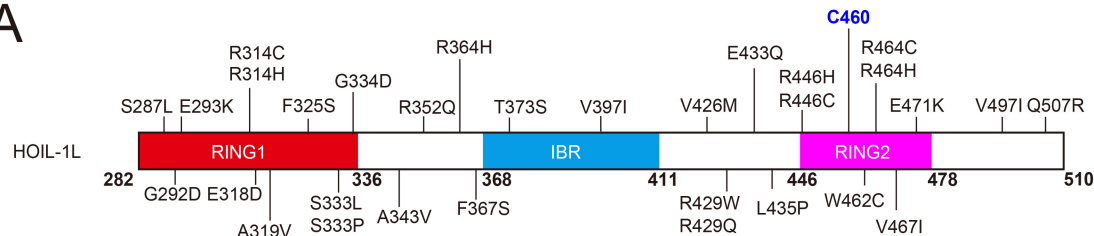
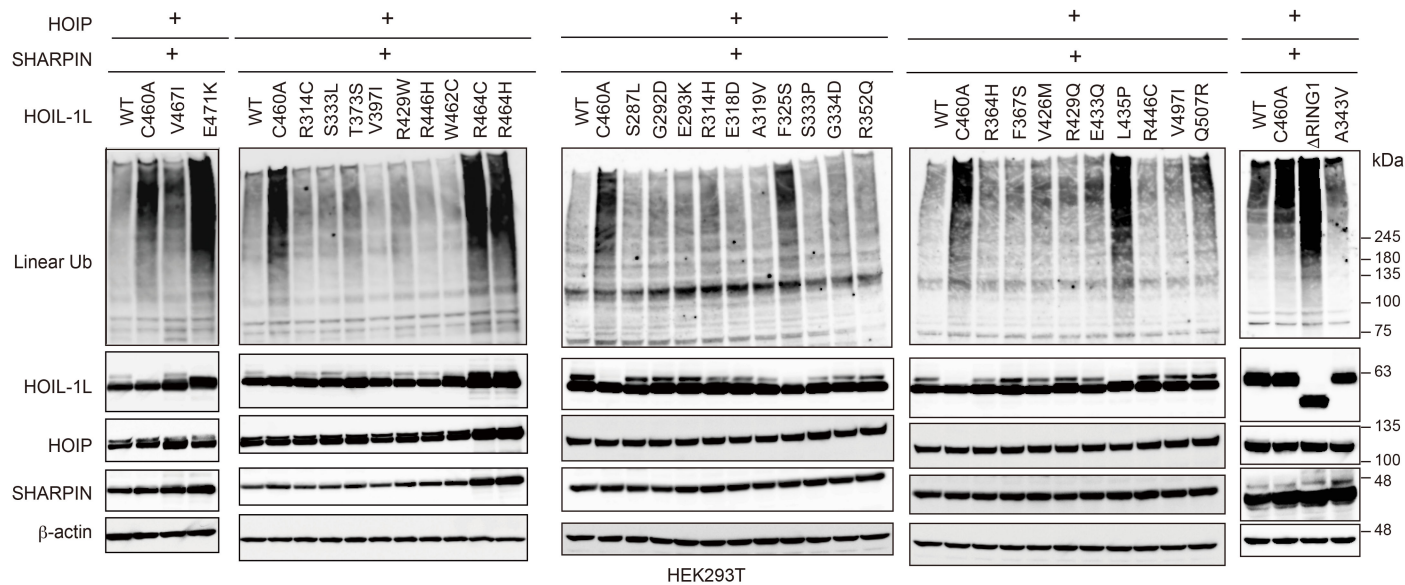
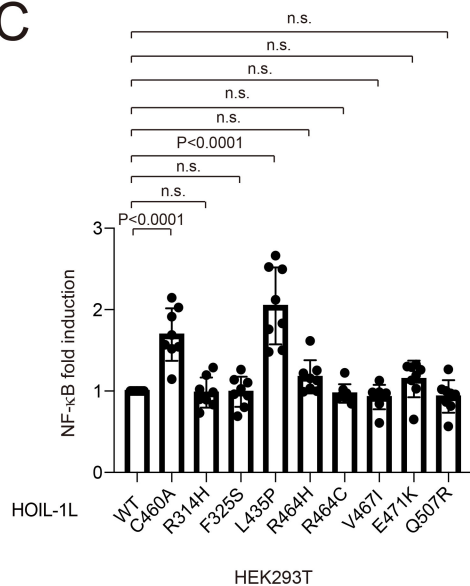
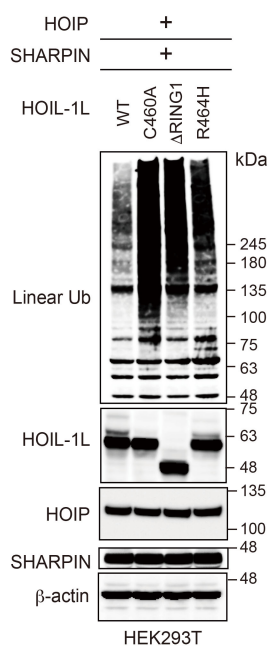
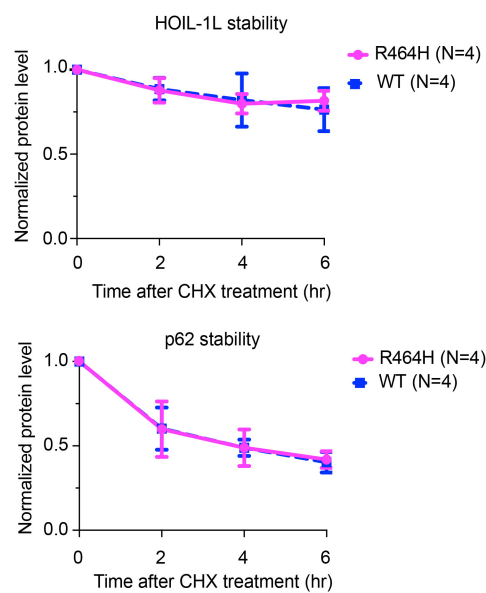
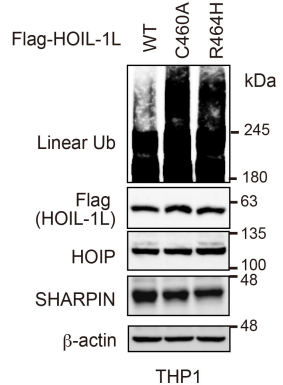
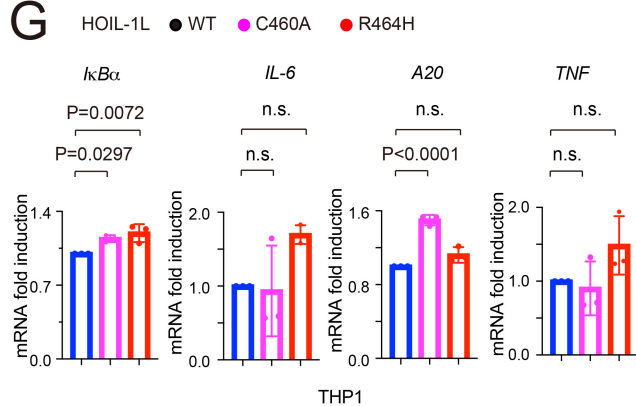
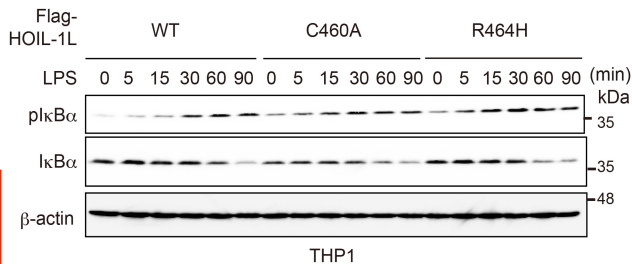
Supplemental Figure 4. Aged HOIL-1L Δ RING1 mice show activation of B and T lymphocytes.

(A) Cell counts in the spleen of 7–8-month-old female mice of the indicated genotype ($n = 5$ per group). (B–E and G–K) Representative flow cytometry plots, and quantification of splenocytes, from 7–8-month-old female mice of the indicated genotype. (B and C) T/B ratio, T cells ($CD3\epsilon^+CD19^-$), and B cells ($CD3\epsilon^-CD19^+$). (D and E) CD4/8 ratio within the T lymphocyte population. (G and H) Percentage and number of marginal zone B cells ($IgM^{high}CD23^{low}$) and follicular B cells ($IgM^{high}CD23^{high}$). (I) Percentage and number of splenic neutrophils. (J) Gating strategies used in Supplementary Figure 4K to identify neutrophils in peripheral blood. (K) Dihydrorhodamine (DHR) flow cytometry analysis of ROS production by neutrophils in peripheral blood of 7-month-old female mice of the indicated genotypes in the presence/absence of PMA (0 nM, 100 nM, and 200 nM). (F) Macroscopic images showing immunostaining for BCL6 in the spleen of littermate 7-month-old female mice of the indicated genotype. Scale bars, 20 μ m. (A, C, E, H, I and K) Data are expressed as the mean $\pm$ S.D. ($n = 4$ -5 per group). *P*-values were calculated by one-way ANOVA, followed by Dunnett's multiple comparisons test (A, C, E, H and I) and by Tukey's multiple comparisons test (K). n.s.: $p > 0.05$.



Supplemental Figure 5. Activation of signaling pathways in HOIL-1L Δ RING1 mice.

(A) Flow chart showing isolation of primary B and T lymphocytes from the spleen by magnetic activated cell sorting (MACS). (B) Gating strategies used to identify B cells and T cells in the spleen. (C) Purity of splenic B and T lymphocytes isolated from the spleen of 12-week-old female littermates of the indicated genotype. Cells were isolated by MACS and analyzed by flow cytometry. (D) Lysates of primary splenic B cells isolated from 12-week-old female mice of the indicated genotype and stimulated with CpG-DNA (100 nM). Cells were probed as indicated. (E) Quantitative PCR analysis of splenic T lymphocytes isolated from 10–12-week-old female mice of the indicated genotype. Data are expressed as the mean ($n = 4$) $\pm$ S.D. (F) Gating strategies used to identify BMDMs. (G) Purity of BMDMs from 12-week-old female littermates of the indicated genotype, as analyzed by flow cytometry. (H and I) Quantitative PCR analysis of BMDMs from 10–12-week-old female mice of the indicated genotype. BMDMs were treated with or without LPS (10 ng ml^{-1}) for the indicated times, followed by qPCR (H). Data are expressed as the mean ($n = 4$) $\pm$ S.D. For each target, data sets of H (0 hr) and I are the same. (E, H, and I) *P*-values were calculated by one-way ANOVA, followed by Dunnett's multiple comparisons test. n.s.: $p > 0.05$.

A**B****C****D****E****F****G****H**

Supplemental Figure 6. A HOIL-1L R464H variant is associated with a higher risk of SLE.

(A) Schematic representation of HOIL-1L, and the location of SNPs residing in the RING1-IBR-RING2 domains of HOIL-1L. (B and D) Expression of LUBAC components and the amounts of linear ubiquitin chains in HEK293T cells transfected with the indicated expression plasmids. (C) NF- κ B activation in HEK293T cells transfected with the indicated expression plasmids and 5 $\times$ NF- κ B luciferase reporters was measured in a luciferase assay. Data are expressed as the mean ($n = 8$) $\pm$ S.D. *P*-values were calculated by one-way ANOVA, followed by Dunnett's multiple comparisons test. (E) Stability of HOIL-1L and p62 in LUBAC TKO MEFs expressing the indicated proteins and treated with CHX (20 μ g ml⁻¹) for the indicated times. Data are expressed as the mean ($n = 4$) $\pm$ S.D. (F) Lysates from THP-1 cells stably expressing the indicated HOIL-1L proteins were probed as indicated. (G) QPCR analysis of THP-1 cells stably expressing the indicated HOIL-1L proteins. Mean values ($n = 3$) $\pm$ S.D. are shown, and *P*-values were calculated by one-way ANOVA, followed by Dunnett's multiple comparisons test. (H) THP-1 cells stably expressing the indicated HOIL-1L proteins were treated with LPS (20 μ g ml⁻¹) for the indicated times and then assessed by immunoblotting with the indicated antibodies. (C and G) n.s.: $p > 0.05$.