

Title: Cyclical palmitoylation regulates TLR9 signalling and systemic autoimmunity in mice

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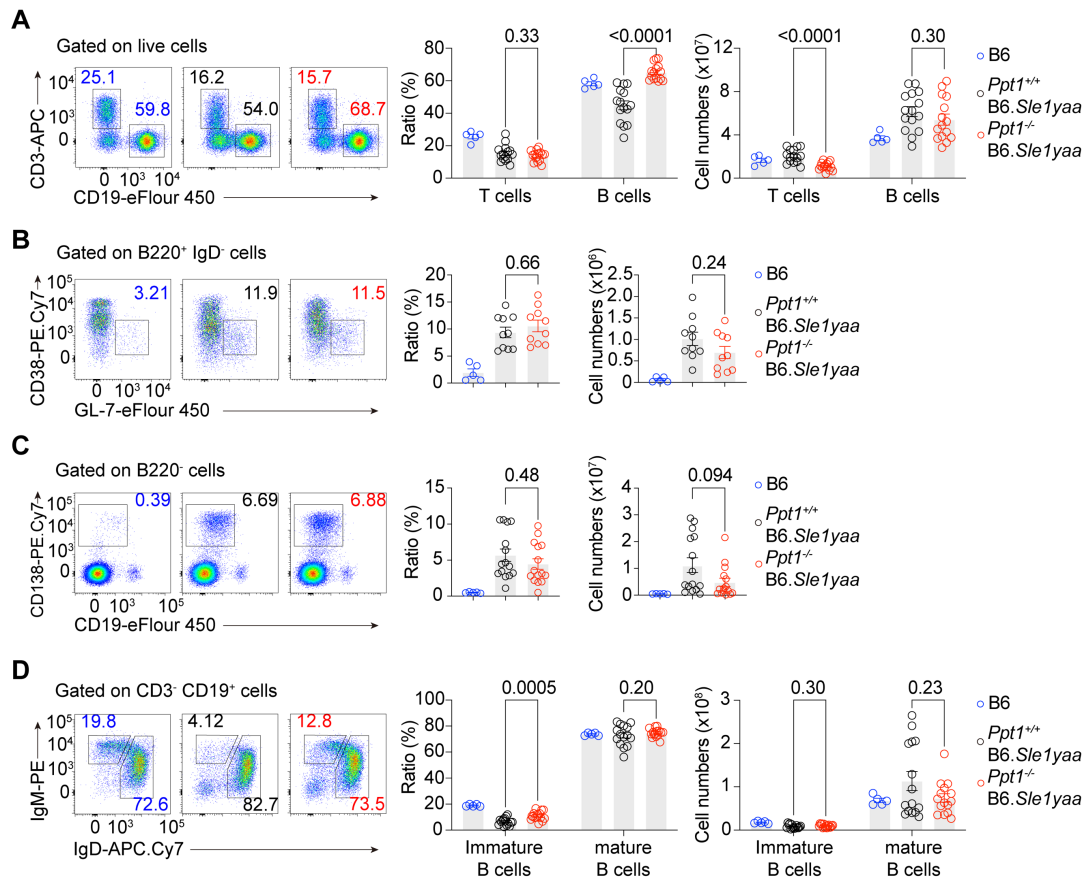
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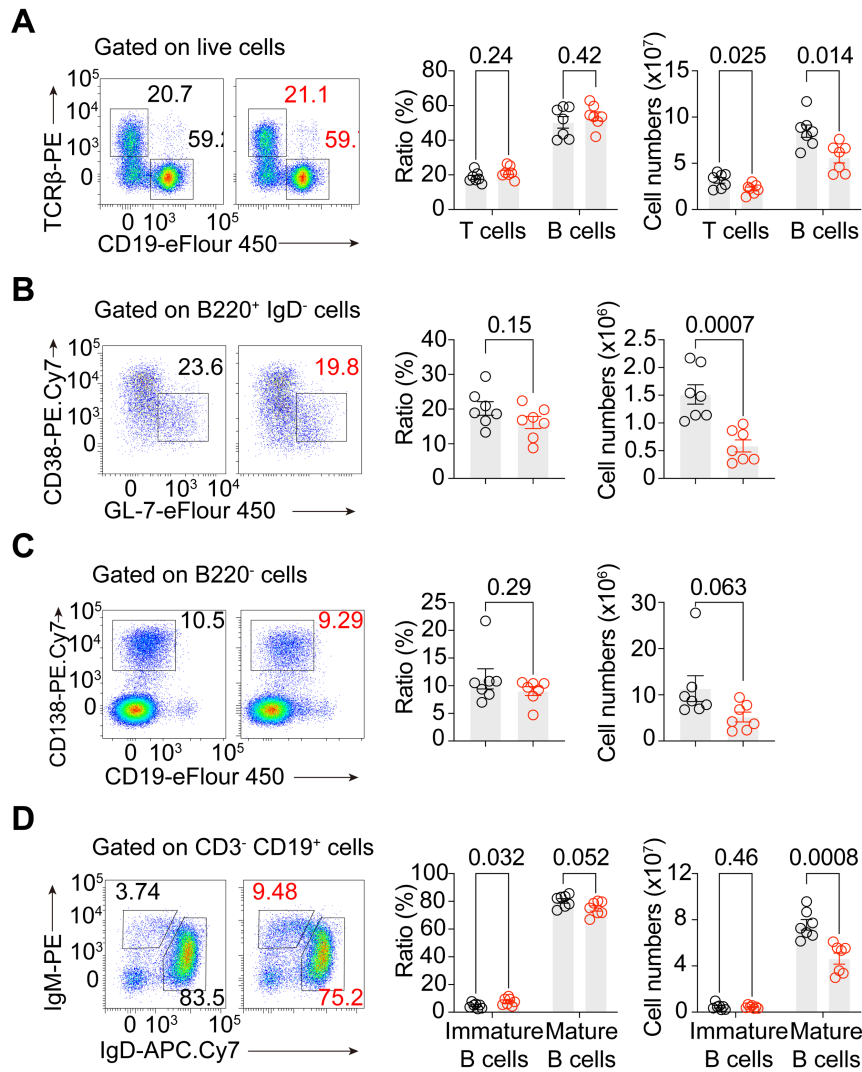
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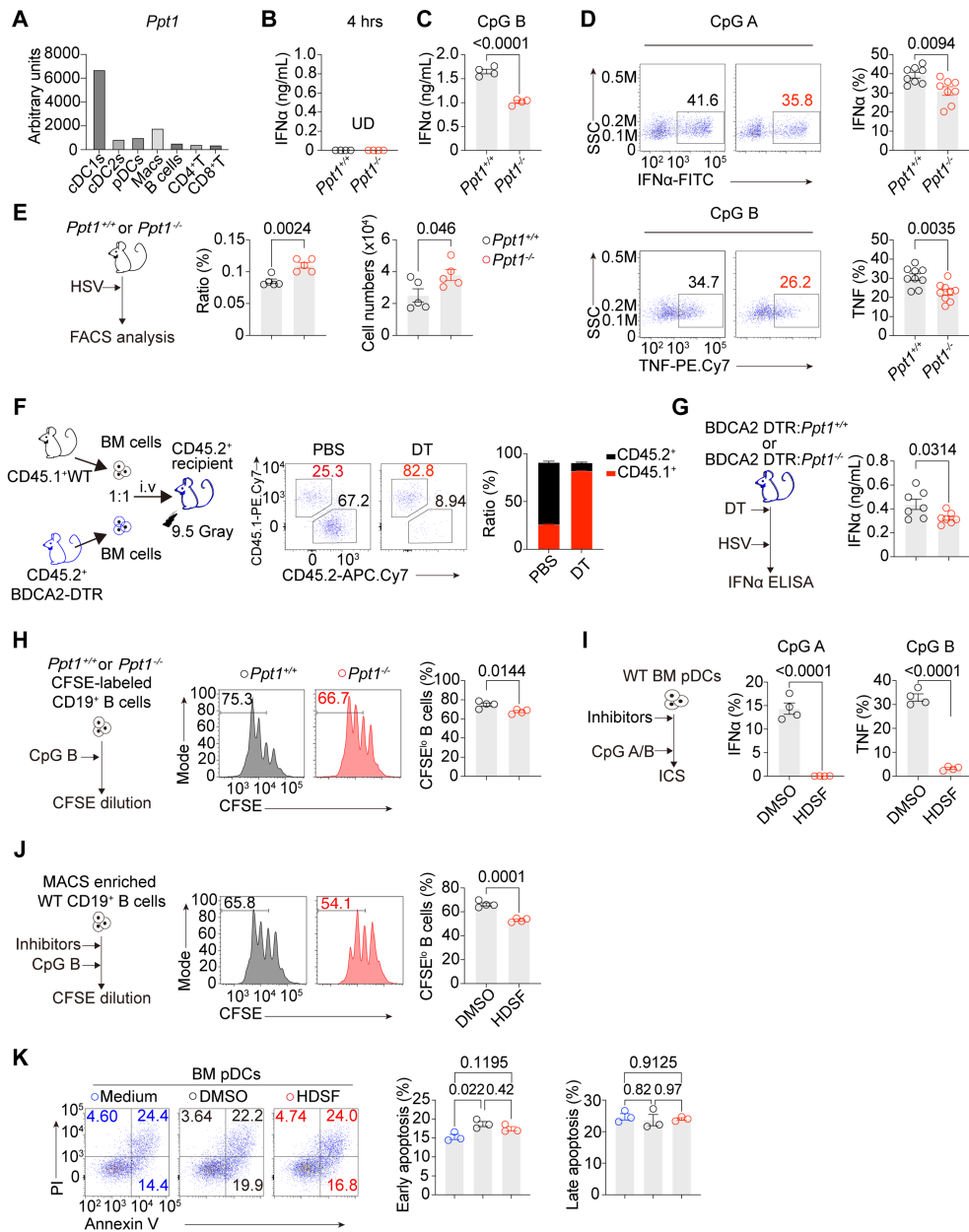
Supplementary Figure 1. The role of PPT1 in SLE pathogenesis.

C57BL/6J (B6), *Ppt1*^{+/+} B6.*Sle1yaa* and *Ppt1*^{-/-} B6.*Sle1yaa* mice were sacrificed at 16 weeks. Immune cell subsets in the spleen were analyzed by FACS. **(A)** Percentage and cell numbers of total T cells (CD3⁺) and total B cells (CD19⁺). Representative FACS plots (left), percentages (middle), and cell numbers (right) are shown (n=5 mice in the B6 group; n=15 mice in the *Ppt1*^{+/+} B6.*Sle1yaa* and *Ppt1*^{-/-} B6.*Sle1yaa* groups). **(B)** Percentage and cell numbers of CD38⁻ GL-7⁺ GC B cells in the splenic B-cell population. Representative FACS plots (left), percentages (middle), and cell numbers (right) are shown (n=5 mice in the B6 group; n=10 mice in the *Ppt1*^{+/+} B6.*Sle1yaa* and *Ppt1*^{-/-} B6.*Sle1yaa* groups). **(C)** Percentage and cell numbers of B220⁻ CD19⁻ CD138⁺ plasma B cells. Representative FACS plots (left), percentages (middle), and cell numbers (right) are shown (n=5 mice in the B6 group; n=15 mice in the *Ppt1*^{+/+} B6.*Sle1yaa* and *Ppt1*^{-/-} B6.*Sle1yaa* groups). **(D)** Fractions of IgM⁺ IgD⁻ immature B cells and IgM⁺ IgD⁺ mature B cells gated from splenic CD3⁻ CD19⁺ cell population. Representative FACS plots (left), percentages (middle), and cell numbers (right) are shown (n=5 mice in the B6 group; n=15 mice in the *Ppt1*^{+/+} B6.*Sle1yaa* and *Ppt1*^{-/-} B6.*Sle1yaa* groups). All data are representative of three **(A, C-D)** or two **(B)** independent experiments (mean $\pm$ SEM.; P values were calculated by two-way Student's t test).



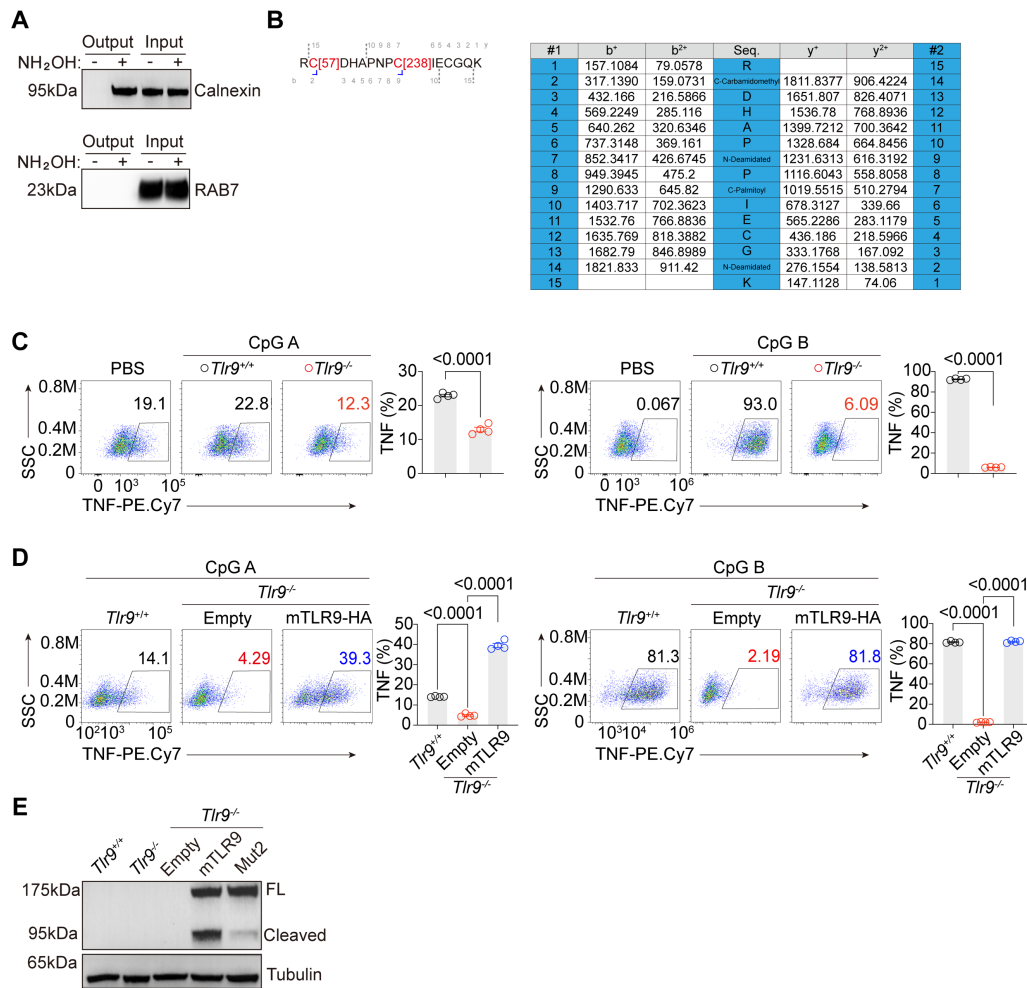
Supplementary Figure 2. Using PPT1 inhibitors to treat SLE mice.

B6.*Sle1yaa* mice were treated with DMSO or HDSF for 8 weeks. Immune cell subsets in the spleen were analyzed by FACS (n=7 mice per group for the rest of figure). **(A)** Percentage and cell numbers of total T cells (TCRβ⁺) and total B cells (CD19⁺). **(B)** Percentage and cell numbers of CD38⁻ GL-7⁺ GC B cells in the splenic B-cell population. **(C)** Percentage and cell numbers of B220⁻ CD19⁻ CD138⁺ plasma B cells. **(D)** Percentage and cell numbers of IgM⁺ IgD⁻ immature B cells and IgM⁺ IgD⁺ mature B cells gated from the splenic CD3⁻ CD19⁺ cell population. All data are representative of three independent experiments (mean ± SEM.; P values were calculated by two-way Student's t test).



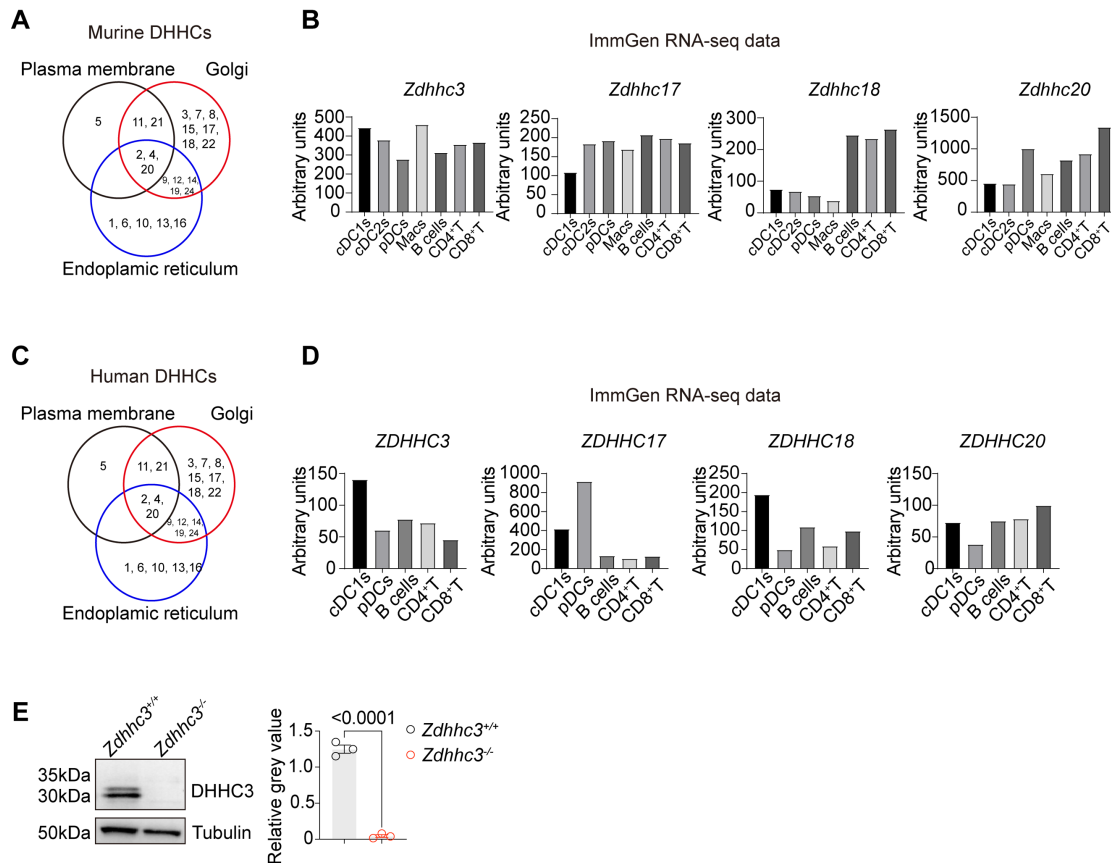
Supplementary Figure 3. Construction of *Ppt1*^{+/+} or *Ppt1*^{-/-} chimeras.

(A) Expression of *Ppt1* (in arbitrary unit) in the indicated populations reanalyzed from ImmGen gene microarray. (B-C) Sorted *Ppt1*^{+/+} or *Ppt1*^{-/-} BM pDCs were treated with CpG A for 4 hours (B) or CpG B overnight (C), IFNα production was measured by ELISA (n = 4 mice per group), UD: Undetectable. (D) *Ppt1*^{+/+} or *Ppt1*^{-/-} BM pDCs were treated with CpG A (left) or CpG B (right), and IFNα (left) and TNF (right) production was measured by intracellular staining (n = 8 mice per group for CpG A, n = 9 mice per group for CpG B). (E) *Ppt1*^{+/+} and *Ppt1*^{-/-} mice were infected with HSV (left). Spleen pDCs ratio (middle) and numbers (right) for HSV are shown (n=5 mice for HSV). (F) Generation of BDCA2-DTR:*Ppt1*^{+/+} and BDCA2-DTR:*Ppt1*^{-/-} mixed chimeras. An equal number of bone marrow cells from CD45.2⁺ BDCA2-DTR mice and CD45.1⁺ WT mice were injected into lethally irradiated CD45.2⁺ mice. After 8 weeks of restitution, the deletion rate of CD45.2⁺ pDCs in the spleen after DT treatment (right), as measured by flow cytometry (gated according to live B220⁺ CD11c^{int} SiglecH⁺ Bst-2⁺ cells in the spleen, n = 3 mice per group). (G) BDCA2-DTR:*Ppt1*^{+/+} and *Ppt1*^{-/-} mixed chimeras were injected with DT before infection (left). Serum IFNα ELISAs for HSV are shown (n=7 mice for HSV). (H) *Ppt1*^{+/+} or *Ppt1*^{-/-} B cell proliferation was measured after stimulation for 3 days with CpG B. (n=4 mice per group). (I) DMSO or HDSF pretreated WT BM pDCs were treated with CpG A or CpG B (left), and IFNα (middle) and TNF (right) production was measured by intracellular staining (n=4 mice per group). (J) DMSO or HDSF treated WT B cell proliferation was measured after stimulation for 3 days with CpG B. (n=4 mice per group). (K) BM pDCs apoptosis were analyzed by Annexin V and PI staining (n = 3 mice per group). The data are representative of three independent experiments (B-F, H-K), two (G) experiments (mean ± SEM.; P values were calculated by two-way Student's t test), or one independent experiment (F).



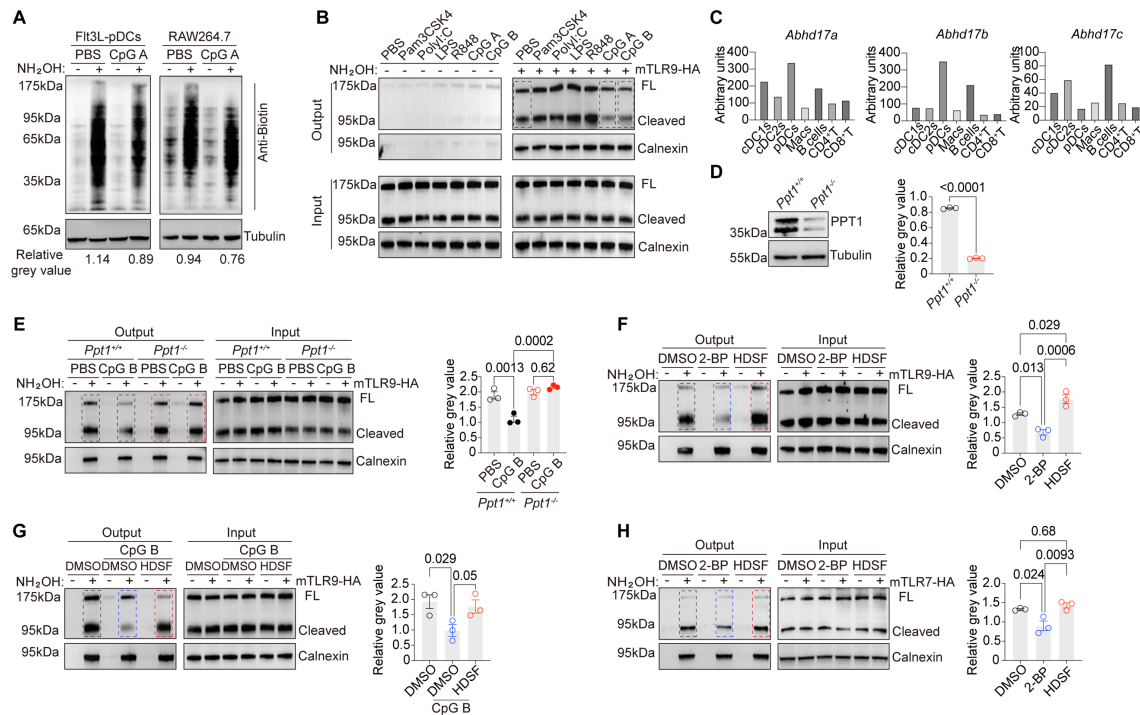
Supplementary Figure 4. Construction of the *Tlr9*^{-/-} *Tlr9*^{Mut2} cell line.

(A) Controls for the ABE assays. Calnexin (left) was used as the positive control, and RAB7 (right) was used as the negative control. (B) The value of b ion and y ion for selected peptide spectra for mTLR9 C258 and C265. (C) TNF production in *Tlr9*^{-/-} RAW264.7 cells. Cells were treated with CpG A (left) or CpG B (right) and then subjected to intracellular cytokine staining (n = 4 replicates from 3 independent experiments). (D) TNF production in *Tlr9*^{-/-} RAW264.7 cells overexpressing TLR9-HA. The cells were treated with CpG A (left) or CpG B (right) and then subjected intracellular cytokine staining (n = 4 replicates from 3 independent experiments). (E) Protein expression of mTLR9 or mTLR9 C258A+C265A (*Tlr9*^{Mut2}) mutants (Mut2) in the *Tlr9*^{-/-} RAW 264.7 cell line. The data are representative of at least five (A), three (C-E) independent experiments (mean ± SEM). P values were calculated by two-way Student's t test, (B) were obtained from one mass spectrometry run.



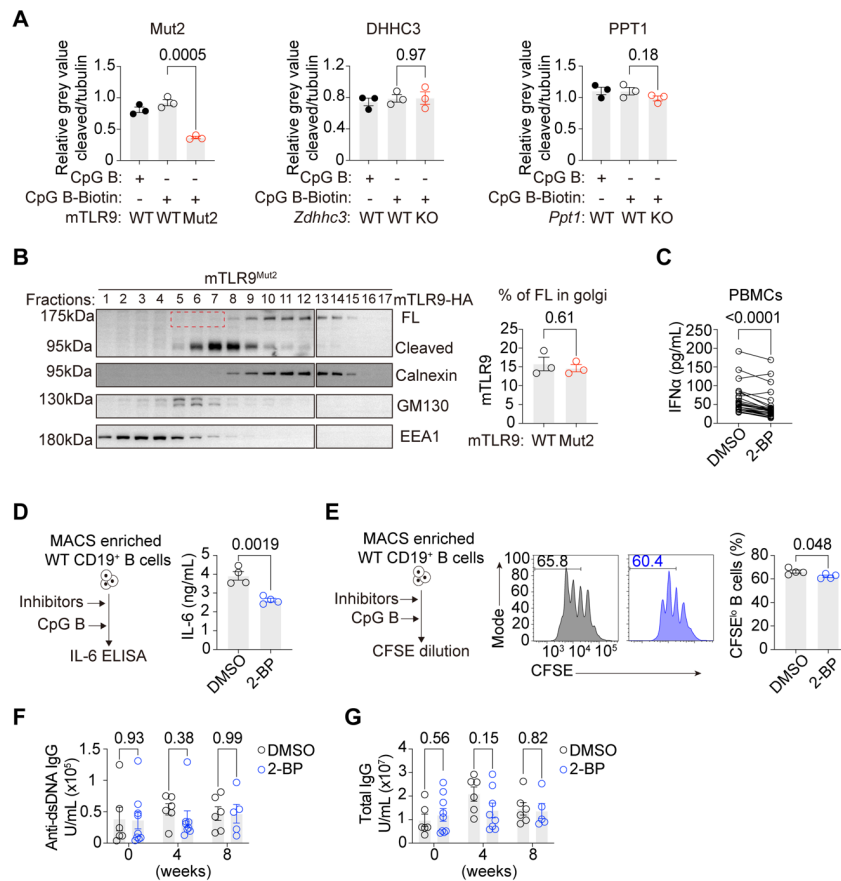
Supplementary Figure 5. Ubiquitous expression of Golgi DHHC proteins.

(A) Venn diagrams showing the intracellular locations of all murine DHHCs. (B) Murine *Zdhhc* expression in immune cells. RNA-seq datasets from ImmGen were downloaded, and the results are shown for the indicated populations using arbitrary units. (C) Venn diagrams showing the intracellular locations of all human DHHCs. (D) Human *ZDHHC* expression in immune cells. RNA-seq datasets from ImmGen were downloaded, and the results are shown for the indicated populations using arbitrary units. (E) Validation of DHHC3-deficient RAW264.7 cell lines by Western blotting (n=3 replicates). Data is representative of three independent experiments (mean $\pm$ SEM. P values were calculated by two-way Student's t test).



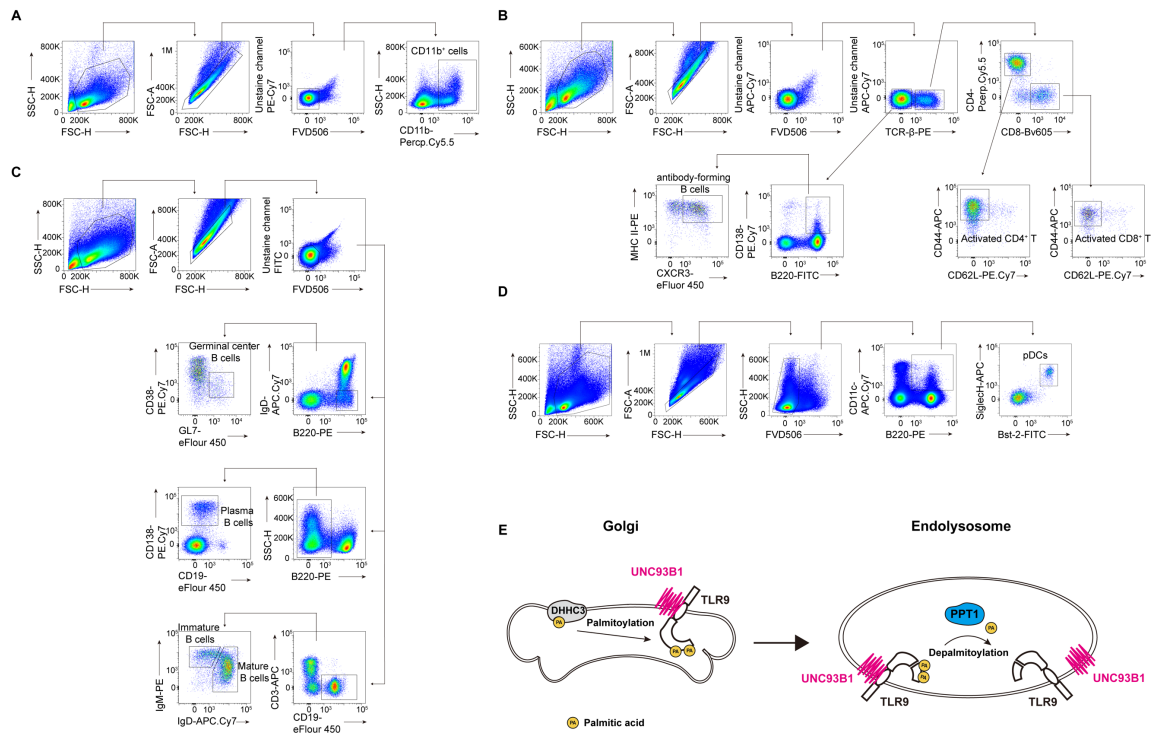
Supplementary Figure 6. Identification of PPT1 as a depalmitoylating enzyme.

(A) An ABE assay was performed to measure the total S-palmitoylation level in Flt3L-pDCs (left) and RAW264.7 cells (right) treated with CpG A. **(B)** mTLR9-HA was retrovirally transduced into RAW264.7 cells. The cells were treated with the indicated TLR agonists for 4 hours and then subjected to an ABE assay. A representative SDS-PAGE gel is shown. **(C)** Expression of *Abhd17a*, *Abhd17b*, *Abhd17c* (in arbitrary unit) in the indicated populations reanalyzed from ImmGen gene microarray. **(D)** Validation of PPT1-deficient RAW264.7 cell lines by Western blotting (n=3 replicates). **(E)** TLR9 palmitoylation in PPT1-deficient cell lines. PPT1 in RAW264.7 cells was knocked out by CRISPR/Cas9. The cells were then retrovirally transduced with mTLR9-HA. After CpG B activation for 4 hours, ABE assays were performed. A representative SDS-PAGE gel is shown on the left. The relative palmitoylation levels of mTLR9 shown on the right were quantified by ImageJ and calculated as a percentage of the WT PPT1 signal (n=3 replicates). **(F)** mTLR9 palmitoylation after inhibitor treatment. RAW264.7 cells overexpressing mTLR9-HA were incubated with DMSO, 2-BP or HDSF, and then, ABE assays were performed (n=3 replicates). **(G)** TLR9 palmitoylation status after PPT1 inhibition. mTLR9-HA was retrovirally transduced into RAW264.7 cells. The cells were pretreated with DMSO or HDSF overnight before the CpG B was added. An ABE assay was performed after 4 hours. A representative SDS-PAGE gel is shown on the left. mTLR9 S-palmitoylation was quantified and presented as the ratio of mTLR9 output to calnexin output on the right (n=3 replicates). **(H)** mTLR7 palmitoylation after inhibitor treatment. RAW264.7 cells overexpressing mTLR9-HA were incubated with DMSO, 2-BP or HDSF, and then, ABE assays were performed (n=3 replicates). Data are pooled from three **(B, D-H)** or two **(A)** independent experiments (mean $\pm$ SEM. P values were calculated by two-way Student's t test).



Supplementary Figure 7. The role of palmitoylation inhibitors in SLE pathogenesis.

(A) Quantification of the cleaved TLR9 in mTLR9^{Mut2} *Zdhhc3*^{-/-} or *Ppt1*^{-/-} input before pull down were shown. **(B)** Cell fractionation of *Tlr9*^{-/-} RAW264.7 cells transduced with mTLR9-HA or TLR9 C258A+C265A (mTLR9^{Mut2}) showing the distributions of TLR9 in the indicated organelle markers. A representative blot (left) and the ratio of the cleaved mTLR9 in endosome (right, quantified as the ratio of the boxed fractions to all fractions, n=3 replicates). **(C)** PBMCs of SLE patients were treated with DMSO or 2-BP overnight. After CpG A stimulation, IFNα levels was evaluated by ELISAs (n= 26 individuals for 2-BP/DMSO). **(D)** CD19⁺ B cells enriched from B6 mice spleen were treated with 2-BP (left). After CpG B stimulation, IL-6 levels were evaluated by ELISAs (n = 4 mice per group). **(E)** CD19⁺ B cells enriched from B6 mice spleen were labelled with CFSE. B cells proliferation was measured after 2-BP and CpG B stimulation for 3 days. **(F-G)** B6.*Slc1yaa* mice were treated with DMSO or 2-BP for 8 weeks. Serum was collected from each mouse group at the indicated timepoints, and anti-DNA antibody **(F)** and total IgG **(G)** levels were measured by ELISAs (n = 5 mice in the DMSO treatment group; n = 9 mice in the 2-BP treatment group). All data are representative of three independent experiments (mean ± SEM. P values were calculated by two-way Student's t test), except for those presented in **C**, which were pooled from six independent experiments (P values were calculated by two-tailed Wilcoxon matched-pair signed rank test).



Supplementary Figure 8. Flow cytometry gating strategies.

(A) Gating strategy to identify CD11b⁺ cells presented on Fig. 1G; Fig. 2H. **(B)** Gating strategy to identify activated CD4⁺ T cells presented on Fig. 1H; Fig. 2I and activated CD8⁺ T cells presented on Fig. 1I; Fig. 2J. Gating strategy to identify antibody-forming B cells presented on Fig. 1J; Fig. 2K. **(C)** Gating strategy to identify germinal center B cells presented on Supplementary Fig1. B; Supplementary Fig2. B. Gating strategy to identify plasma B cells presented on Supplementary Fig1. C; Supplementary Fig2. C. Gating strategy to identify immature and mature B cells presented on Supplementary Fig1. D; Supplementary Fig2. D. **(D)** Gating strategy to identify pDCs cells was used in Fig. 3C; Fig. 3F; Supplementary Fig3. B-F, I, K. **(E)** A schematic of the working model.