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Supplementary Materials for

Ubiquitin-Specific Protease 25 Regulates TLR4-Dependent Innate Immune Responses Through Deubiquitination of the Adaptor Protein TRAF3

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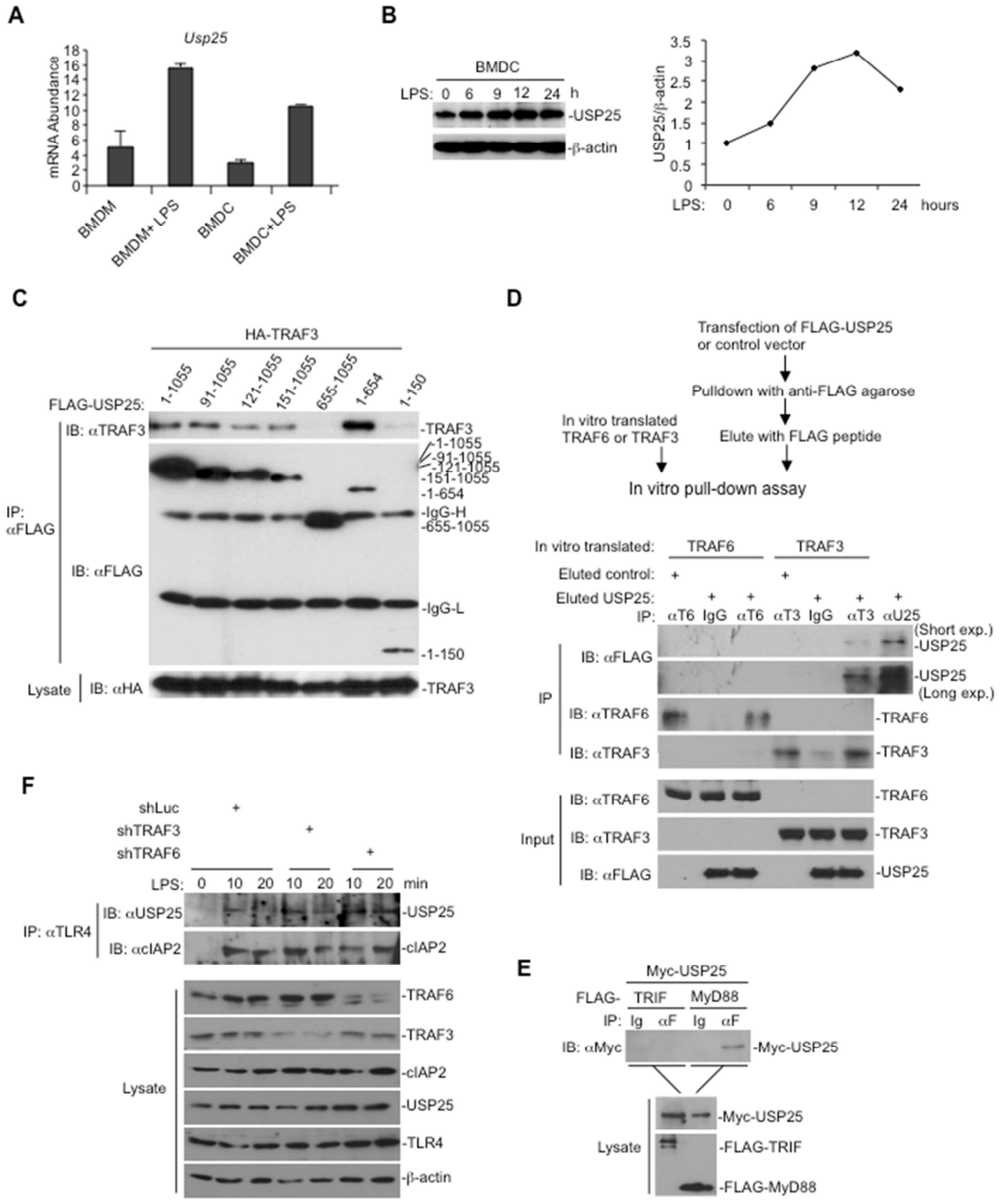

Fig. S1. USP25 interacts with TRAF3. (**A** and **B**) The abundance of USP25 is increased in response to LPS. (A) BMDMs and BMDCs were stimulated with LPS (1 μg/ml) for 8 hours before they were analyzed by real-time PCR to determine *Usp25* mRNA abundance. (B) BMDCs were stimulated with LPS (1 μg/ml) for the indicated time points before they were analyzed by Western blotting with anti-USP25 and anti-β-actin antibodies (left blots). The relative abundance of USP25 was quantified by densitometric analysis and normalized to that of β-actin (right graph). (**C**) Domain mapping of the

interaction between USP25 and TRAF3. HEK 293T cells were transfected with plasmids encoding the indicated proteins. Immunoprecipitation (IP) and Western blotting (IB) assays were performed twenty hours after transfection. (**D**) USP25 interacts with TRAF3 in vitro. In vitro pulldown assays were performed according to the strategy illustrated (upper scheme) with control IgG or with anti-TRAF3 (αT3) or anti-TRAF6 (αT6) antibodies. The immunoprecipitated samples and input material were subjected to Western blotting analysis with antibodies against the FLAG tag or the indicated proteins (lower blots). exp, exposure. (**E**) USP25 interacts with MyD88, but not TRIF. HEK 293T cells were cotransfected with plasmids encoding Myc-USP25 and either FLAG-TRIF or FLAG-MyD88. Twenty hours later, immunoprecipitations were performed with control IgG (Ig) or anti-FLAG antibody (αF), which were followed by Western blotting analysis with anti-Myc antibody. Protein abundances were determined by Western blotting analysis with anti-Myc or anti-FLAG antibodies. Data shown are representative of two independent experiments. (**F**) Knockdown of TRAF3 or TRAF6 has no effect on the recruitment of USP25 to TLR4. RAW264.7 cells were transduced with retrovirus expressing control shRNA (shLuc) or shRNAs specific for TRAF3 (shTRAF3) or TRAF6 (shTRAF6). Twenty hours later, the cells were selected with puromycin (1 μg/ml) for two days. The cells were then treated with LPS (10 μg/ml) for the indicated times after which they were subjected to immunoprecipitations and Western blotting analysis to detect the indicated proteins. Data are representative of at least three (A) or two independent (B to F) independent experiments.

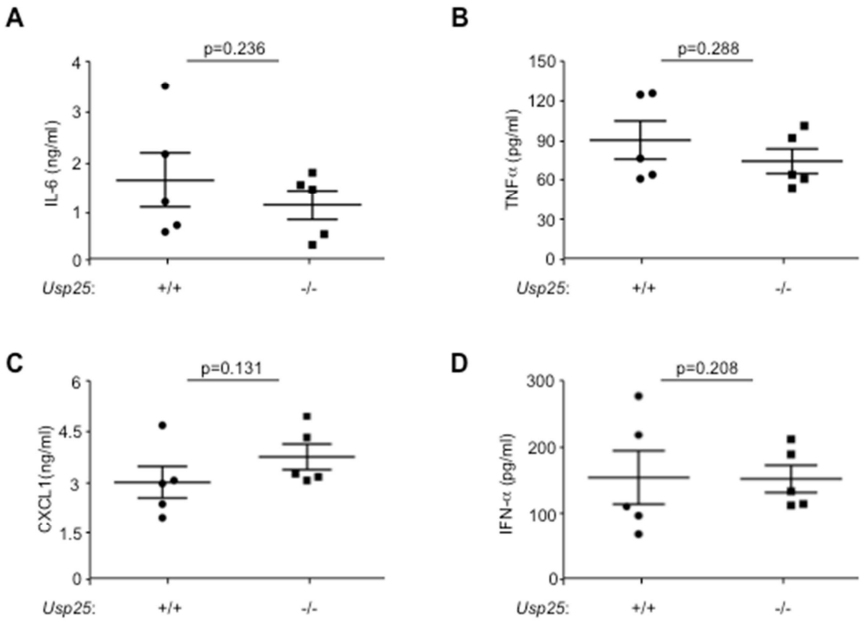

Fig. S2. Deficiency in USP25 does not affect poly(I:C)-stimulated production of cytokines in vivo. (**A** to **D**) Age- and sex-matched wild-type and *Usp25*$^{-/-}$ littermate mice were intraperitoneally injected with poly(I:C) (5 mg per kg body weight). Two and a half hours later, blood was obtained from the mice and the concentrations of (A) IL-6, (B) TNF-α, (C) CXCL1, and (D) IFN-α in the sera were determined by ELISA. Graphs show means ± SD (n = 5 mice per experiment, *t* test). Data are representative of two independent experiments.

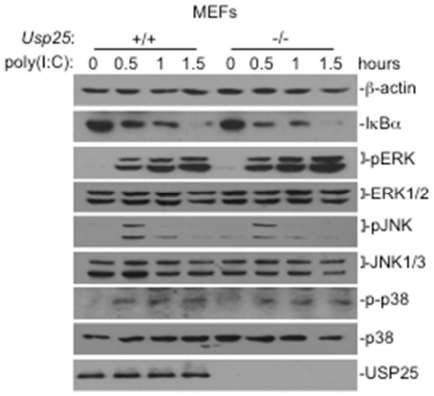

Fig. S3. USP25 does not regulate TLR3-dependent activation of NF-κB and MAPKs in MEFs. Wild-type and *Usp25*$^{-/-}$ MEFs were stimulated with poly(I:C) (50 μg/ml) for the indicated times and then were analyzed by Western blotting with antibodies against the indicated proteins. Data are representative of two independent experiments.

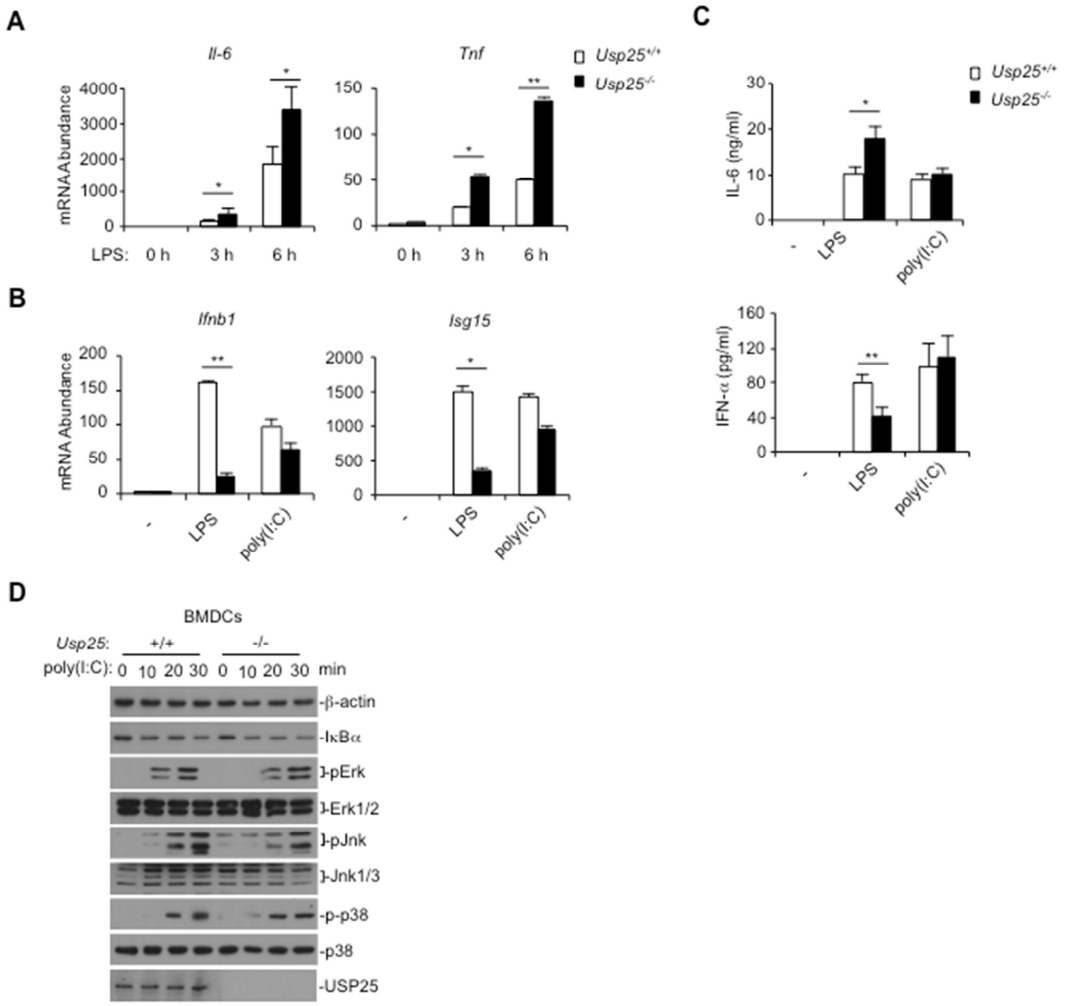

Fig. S4. Role of USP25 in wild-type and *Usp25*$^{-/-}$ BMDMs in response to LPS or poly(I:C). (**A**) USP25 inhibits the LPS-induced production of proinflammatory cytokines in BMDMs. Wild-type and *Usp25*$^{-/-}$ BMDMs were stimulated with LPS (1 μg/ml) for the indicated times before being analyzed by real-time PCR to determine the abundances of the indicated mRNAs. (**B**) USP25 is required for LPS-dependent, but not poly(I:C)-dependent, generation of type I IFNs. Wild-type and *Usp25*$^{-/-}$ BMDMs were stimulated with LPS (1 μg/ml) or poly(I:C) (10 μg/ml) for 6 hours before being analyzed by real-time PCR to determine the abundances of the indicated mRNAs. (**C**) USP25 differentially regulates LPS-induced production of proinflammatory cytokines and type I IFNs. Wild-type and *Usp25*$^{-/-}$ BMDMs were stimulated with LPS (0.1 μg/ml) for 12 hours. The supernatants were harvested to determine the concentrations of the indicated proteins by ELISA. (**D**) Wild-type and *Usp25*$^{-/-}$ BMDCs were stimulated with poly(I:C) (50 μg/ml) for the indicated times and then were analyzed by Western blotting with antibodies against the indicated proteins. Data are representative of three (A to C) or two (D) independent experiments. Graphs show means ± SD from three independent experiments. **P* < 0.05; ***P* < 0.01 by ANOVA.

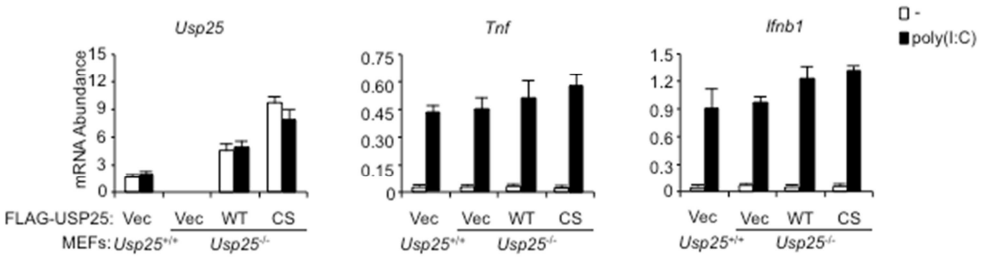

Fig. S5. Reconstitution of *Usp25*$^{-/-}$ MEFs with USP25 or USP25(C178S) has no effect on poly(I:C)-induced production of proinflammatory cytokines or type I IFNs. *Usp25*$^{-/-}$ MEFs were reconstituted with empty vector (Vec), USP25, or USP25(C178S) and then were stimulated with poly(I:C) (10 μg/ml) for 4 hours before being analyzed by real-time PCR to determine the abundances of the indicated mRNAs. Graphs show means ± SD from three independent experiments.

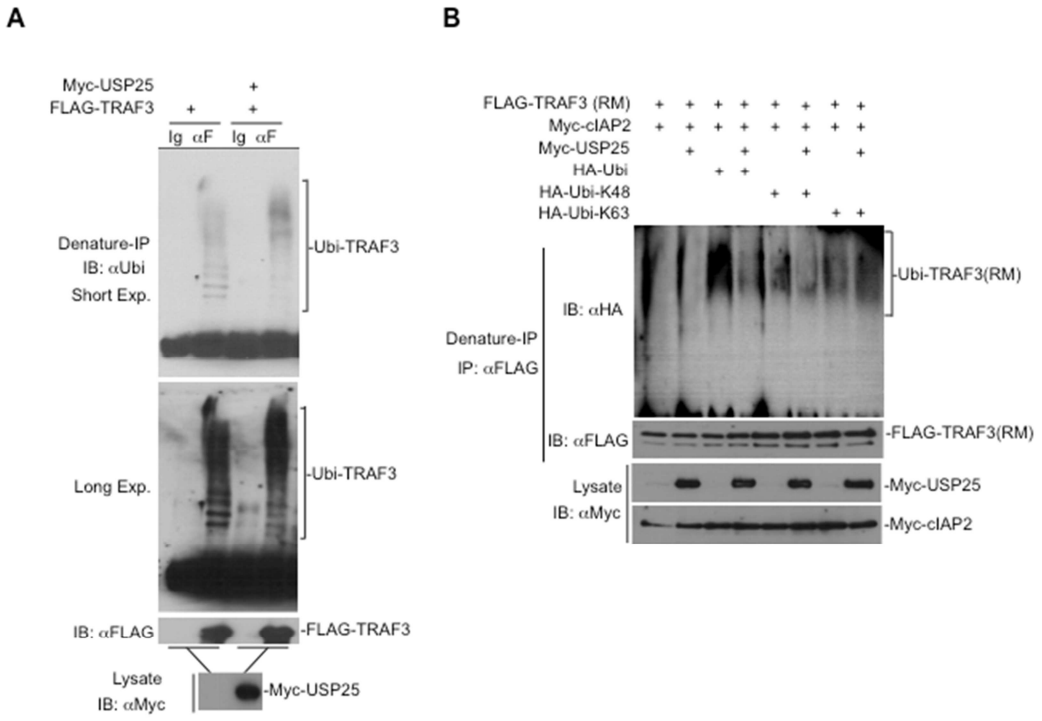

Fig. S6. USP25 removes K^{48}-linked ubiquitin chains from TRAF3(RM). (**A**) USP25 does not inhibit the self-ubiquitination of TRAF3. HEK 293T cells were transfected with plasmids encoding the indicated proteins. Twenty hours later, denaturing immunoprecipitation (Denature-IP) and Western blotting analysis were performed to determine the extent of ubiquitination of TRAF3. (**B**) USP25 removes cIAP2-mediated, K^{48}-linked ubiquitin conjugates from TRAF3(RM). HEK 293T cells were transfected with plasmids encoding the indicated proteins. Twenty hours later, denaturing immunoprecipitations and Western blotting assays were performed with antibodies against the indicated proteins. RM, RING-finger mutant. Data are representative of two independent experiments.

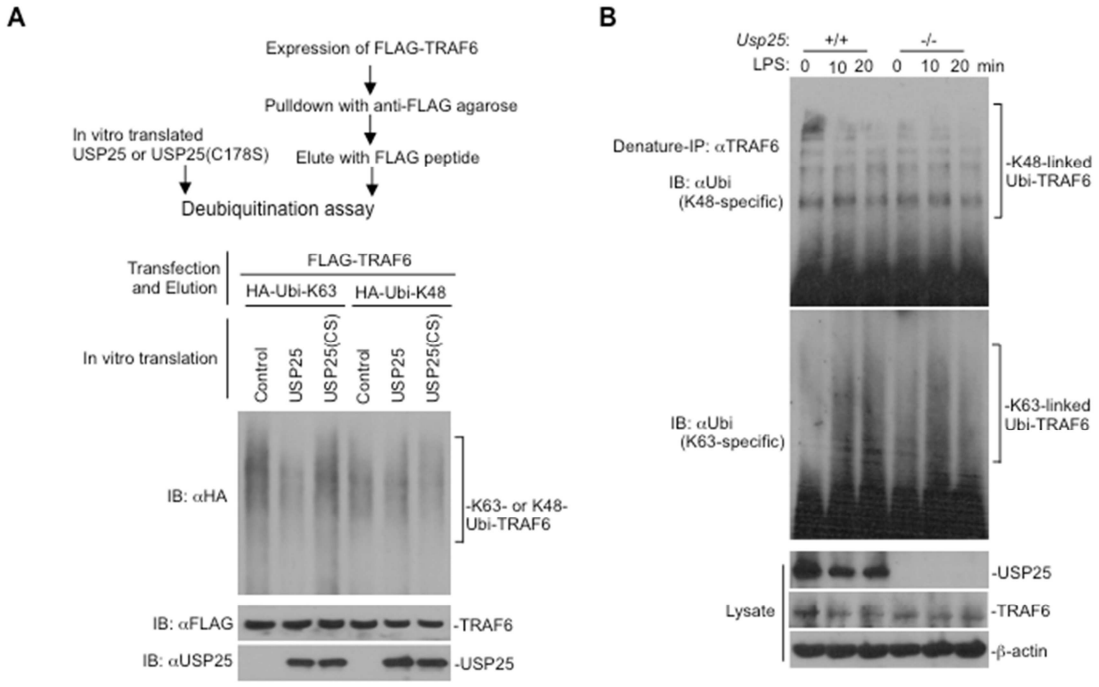

Fig. S7. Role of USP25 in the deubiquitination of TRAF6. (**A**) USP25 specifically removes K63-linked ubiquitin chains from TRAF6 in vitro. In vitro deubiquitination assays were performed as illustrated (top) with the indicated proteins incubated at 30°C for 2 hours and then at 16°C overnight before being analyzed by Western blotting with antibodies against HA, FLAG, or USP25. (**B**) USP25 does not regulate the LPS-stimulated ubiquitination of TRAF6. Wild-type and *Usp25*$^{-/-}$ BMDMs were stimulated with LPS (10 μg/ml) for the indicated times and then subjected to denaturing immunoprecipitation with anti-TRAF6 antibody. The immunoprecipitated samples were subjected to Western blotting analysis with antibodies specific for either K63-linked or K^{48}-linked ubiquitin chains. The abundances of the indicated proteins in the cell lysates were determined by Western blotting analysis with specific antibodies. USP25(CS), USP25(C178S) mutant. Data are representative of two independent experiments.