# NF-κB-mediated degradation of the co-activator RIP140 regulates inflammatory response and contributes to endotoxin tolerance

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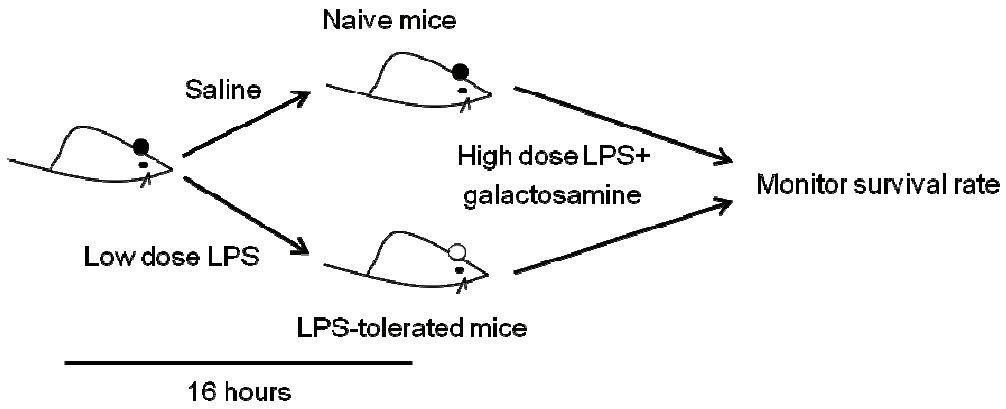
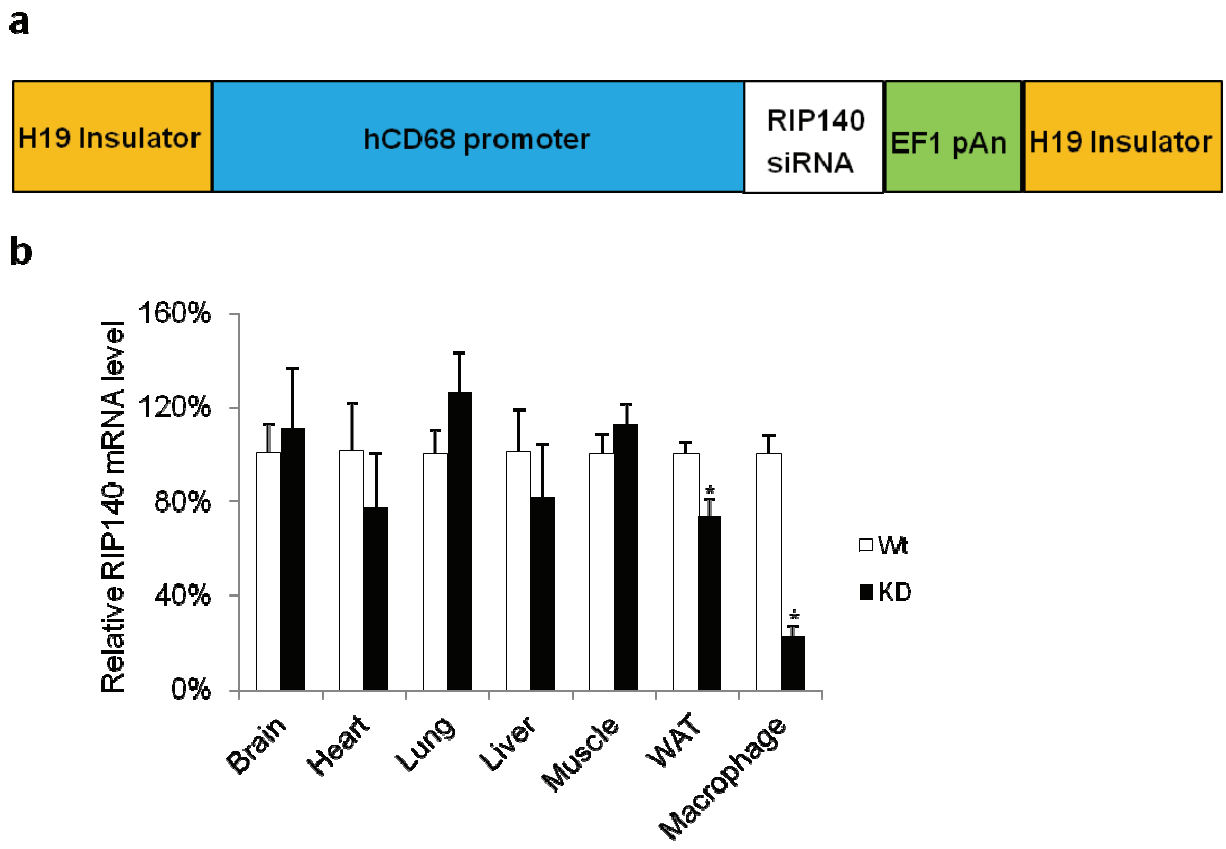
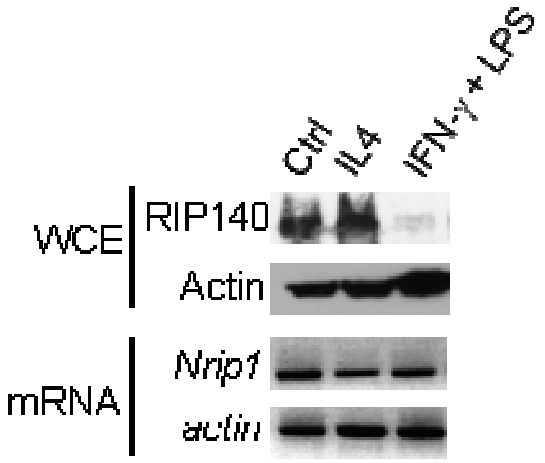


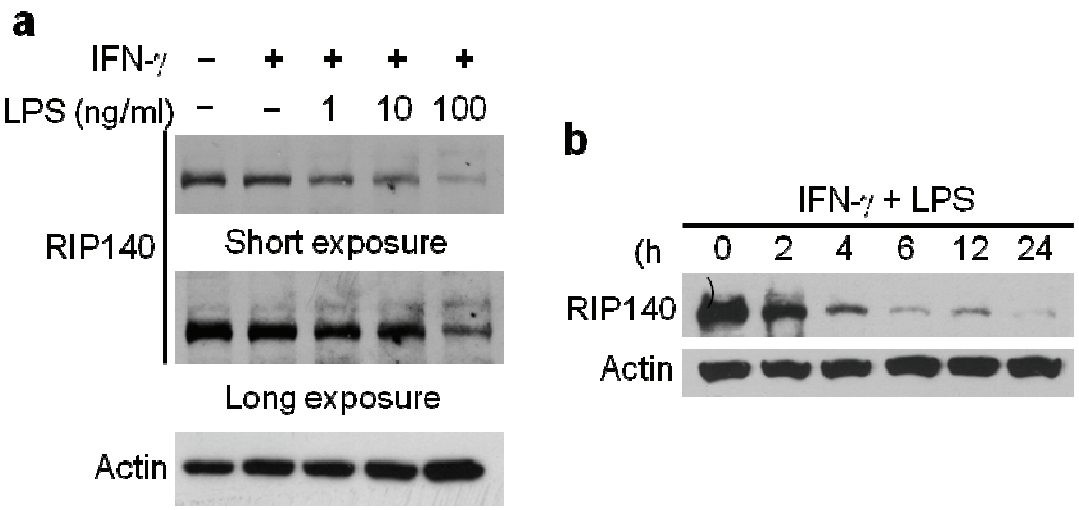

**Supplementary Figure 1.** Endotoxin tolerance *in vivo* model. A schematic diagram for the experimental design: mice were injected peritoneally with saline or LPS (0.1 μg/25 g body weight) for 16 h, followed by a lethal dose of LPS with D-galatosamine.



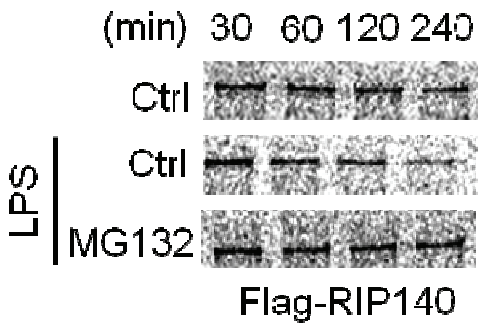

**Supplementary Figure 2.** Macrophage-specific knocking down of RIP140. (a) Illustration of transgenic vector. RIP140 siRNA was mimicked as endogenous miRNA and the expression was driven by human CD68 promoter. (b) The mRNA levels of RIP140 of indicated organs, tissues or primary peritoneal macrophages were measured by quantitative PCR. The expression level of RIP140 of indicated group from wild type mice was calculated as 100%. Result present in mean ± SD., n=3; *: $P < 0.05$.



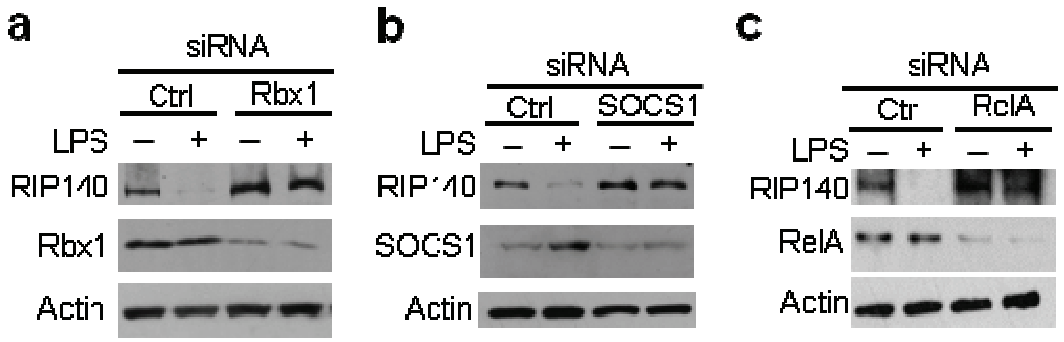

**Supplementary Figure 3.** M1, but not M2, stimulus reduces RIP140 level. Immunoblot and semi-quantitative PCR analyses of RIP140 protein and mRNA levels in Raw264.7 macrophages after stimulating with the vehicle (Ctrl), M1 stimulus (LPS plus IFN-γ) or M2 stimulus (IL-4) for 24 h.



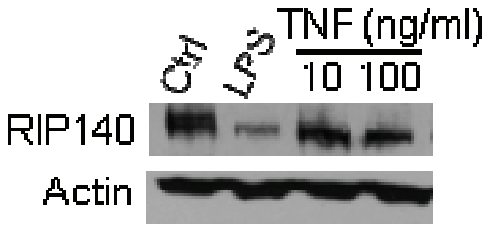

**Supplementary Figure 4.** LPS down-regulates RIP140 expression in a dose- and time-dependent manner. (a) Raw264.7 macrophages were treated with indicated treatments for 24 h. RIP140 and actin levels were analyzed by immunoblot. (b) Raw264.7 macrophages were co-treated with LPS plus IFN=γ for indicated duration. Cells were collected and the expression levels of indicated proteins were examined by immunoblot.



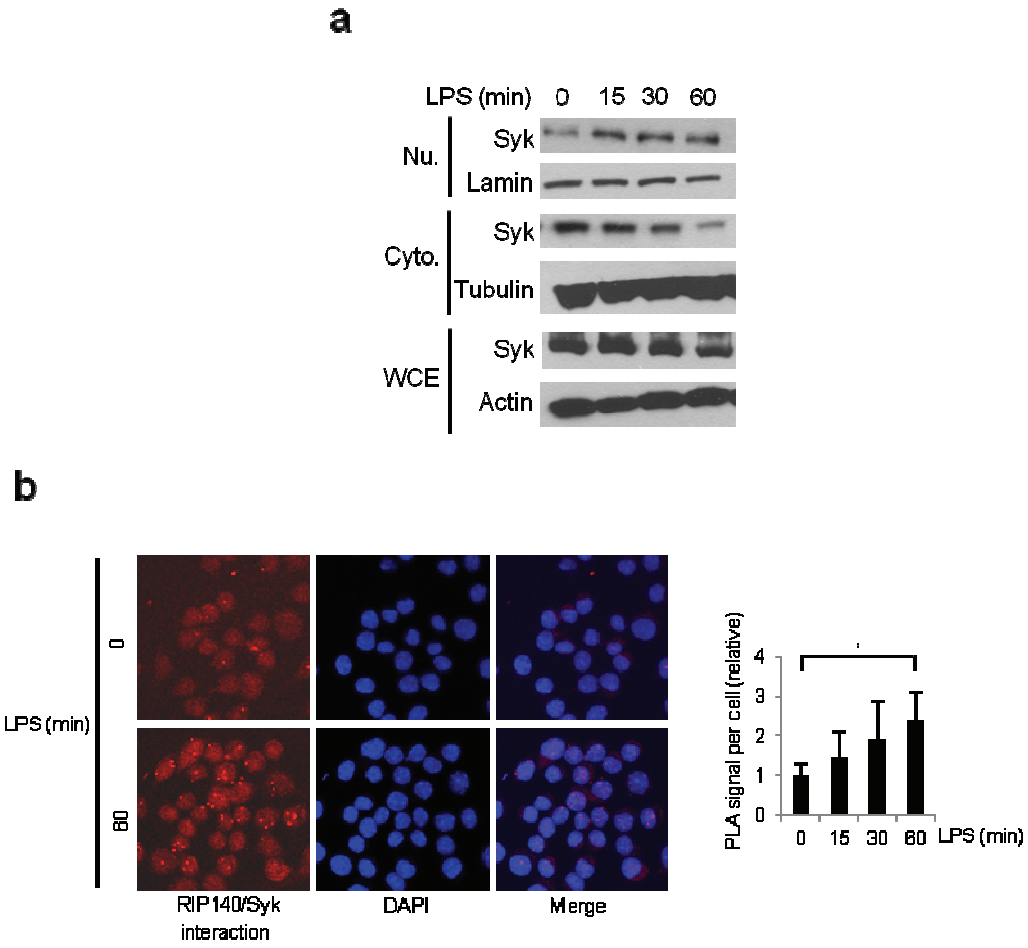

**Supplementary Figure 5.** LPS promotes proteasome-mediated RIP140 degradation in macrophages. Raw264.7 macrophages were transfected with Flag-tagged wild type RIP140. Cells were then cultured in the media containing $^{35}$S-labeled methionine and cysteine. After 16 h, cells were washed by normal culture media and then treated control vehicle or LPS in the absence or presence of MG132 (5 μg/ml) for indicated duration. 300 μg cell lysate of each sample was used for immunoprecipitation of anti-Flag. Immunoprecipitates were then subjected into SDS-PAGE and Flag-RIP140 levels were determined.



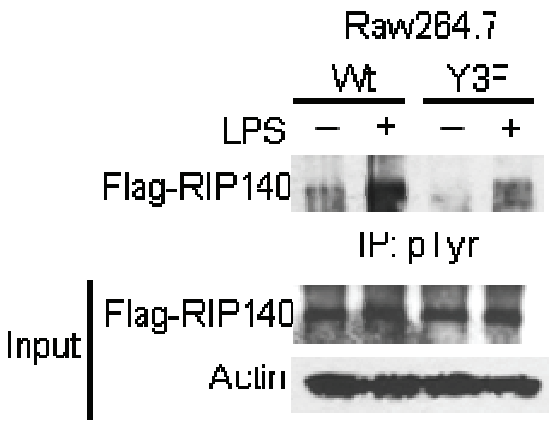

**Supplementary Figure 6.** Targeting SOCS1, Rbx1 or Syk diminished LPS-triggered reduction of RIP140 in Raw264.7 macrophages. Raw264.7 cells were transfected with control siRNA or indicated siRNA for 48 h. Cells were then treated with or without 100 ng/ml LPS for 24 h and whole cell lysates were collected for immunoblots of indicated proteins. Silencing of (a) SOCS1, (b) Rbx1 or (c) Syk blocked LPS-mediated reduction of RIP140.



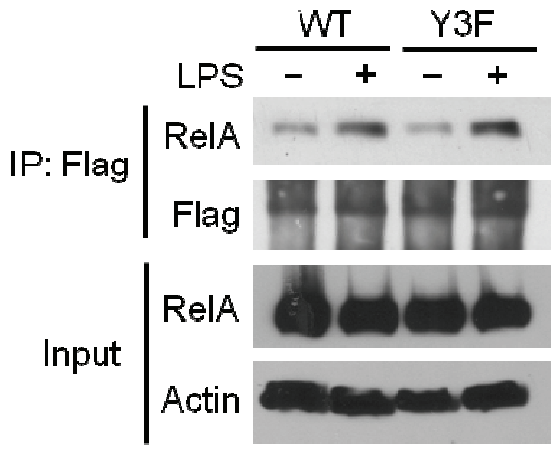

**Supplementary Figure 7.** TNF fail to reduce RIP140 protein level in Raw264.7 macrophages. The expression of RIP140 in Raw264.7 macrophages after 24 h treatments as indicated was determined by immunoblot analyses of RIP140 and actin.



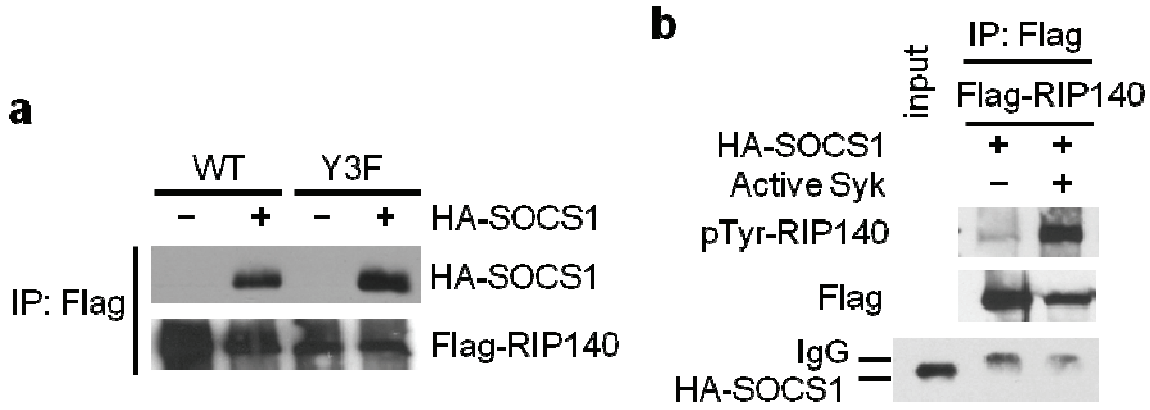

**Supplementary Figure 8.** LPS facilitates nuclear accumulation of Syk and the interaction of RIP140 with Syk in Raw264.7 macrophages. (a) Raw264.7 macrophages were treated with 100 ng/ml LPS for different duration. Whole cell extract (WCE), nuclear (Nu.) and cytoplasmic (Cyto.) fractions were collected and subjected into SDS-PAGEs for immunoblotting of indicated proteins. Actin, lamin and tubulin were used for loading controls. (b) LPS-facilitated interaction of RIP140 with Syk in the nuclei was determined by proximal ligation assay. DAPI shows nuclear staining. Right: a quantitative result for interaction signal intensity. Results are presented in mean ± SD. ; *: $P < 0.05$.



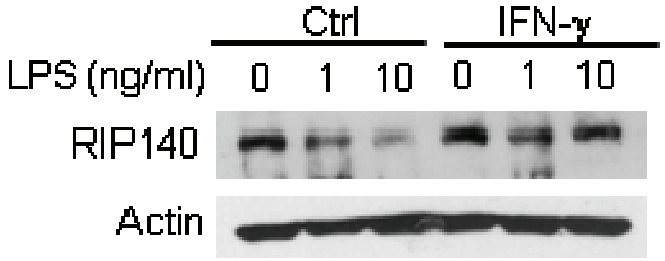

**Supplementary Figure 9.** LPS facilitates tyrosine phosphorylation of RIP140 on Y364,418,436 in Raw264.7 macrophages. Raw264.7 macrophages were transfected with wild type RIP140 (Wt) or tyrosine mutant RIP140 (Y3F) and then treated with or without LPS for 1 h.



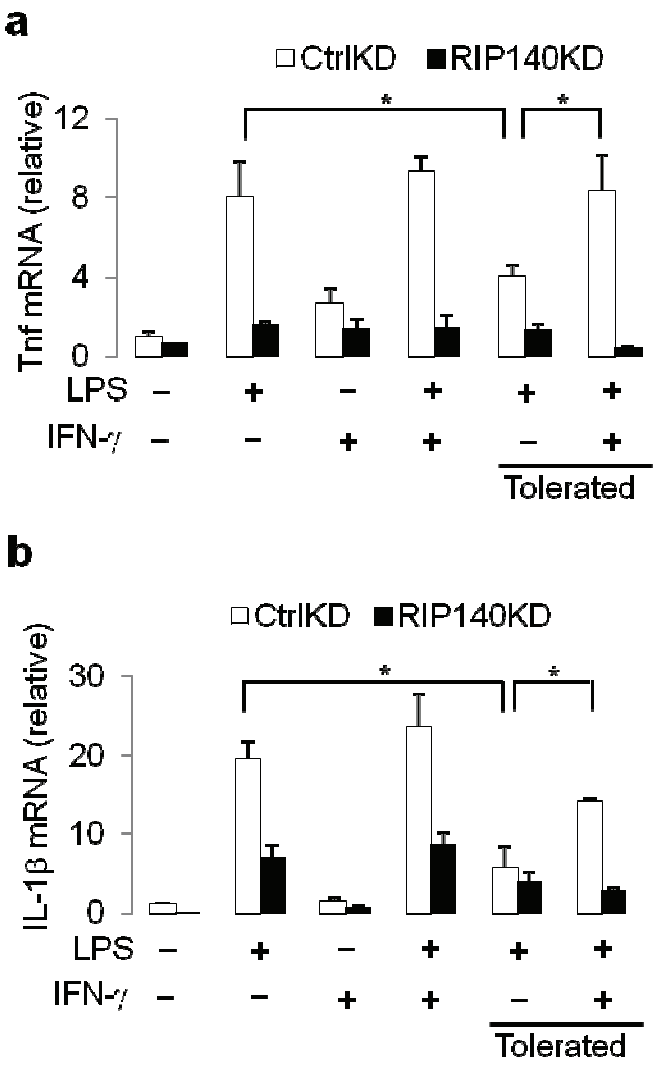

**Supplementary Figure 10.** LPS-stimulated interaction of RIP140 with RelA does not require tyrosine phosphorylation on Y364,418,436 of RIP140. Raw264.7 macrophages were transfected with Flag-tagged wild type RIP140 (Wt) or tyrosine mutant form of RIP140 (Y3F). Cells were then treated with or without LPS for 2 h. Cell lysates were for co-immunoprecipitation of Flag-RIP140. RelA and Flag-RIP140 levels in immunoprecipitates were determined by immunoblot. The levels of RelA and actin in inputs were determined by immunblot.



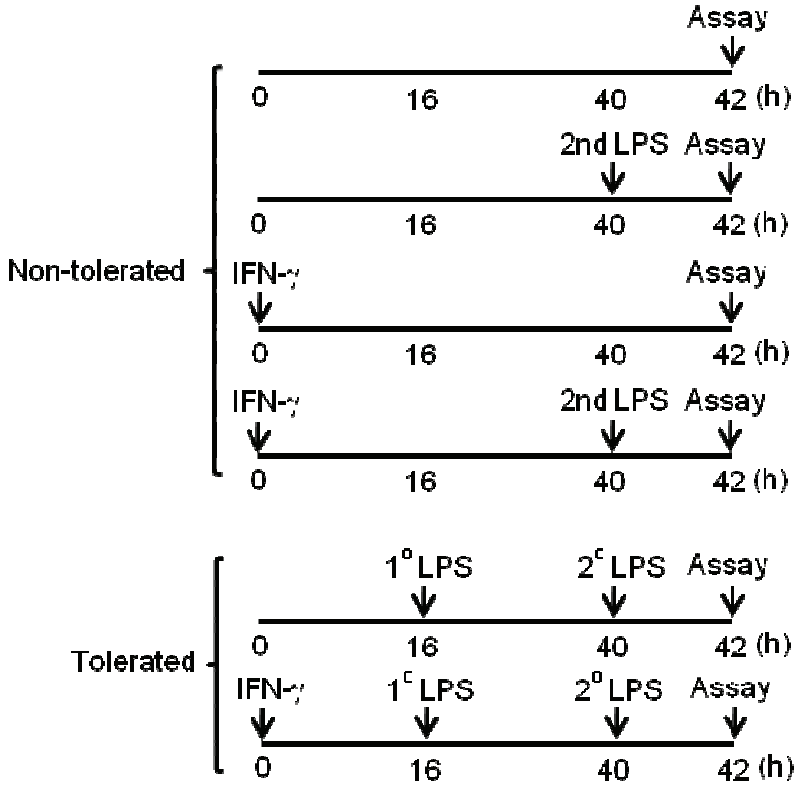

**Supplementary Figure 11.** SOCS1 associates with RIP140 does not require Syk-mediated tyrosine phosphorylations on RIP140. (a) 293T cells were transfected with Flag-tagged wild type (Wt) or tyrosine mutant form (Y3F) of RIP140 with control vector or HA-tagged SOCS1. After lysis, cell lysates were immunopreciptated with anti-Flag-agarose beads and immunoprecipitates were immunoblotted with anti-Flag to detect Flag-RIP140 or anti-HA to detect HA-SOCS1. (b) Cell lysates from 293T cell over-expressed Falg-tagged wild type RIP140 were immunoprecipitated by anti-Flag agarose. Immunoprecipitates were incubated with or without active Syk in kinase reaction buffer for 1 h. After washing, reacted immunoprecipitates were then incubated with in vitro translated HA-SOCS1 for 16 h to examine direct interaction. Afetr washing, the precipitates were subjected into SDS-PAGE and immunblots of anti-phospho-tyrosine, andti-Flag and anti-HA were performed to examine the levels of phosphor-Flag-RIP140, Flag-RIP140 and HA-SOCS1 in these reacted immunoprecipitates.



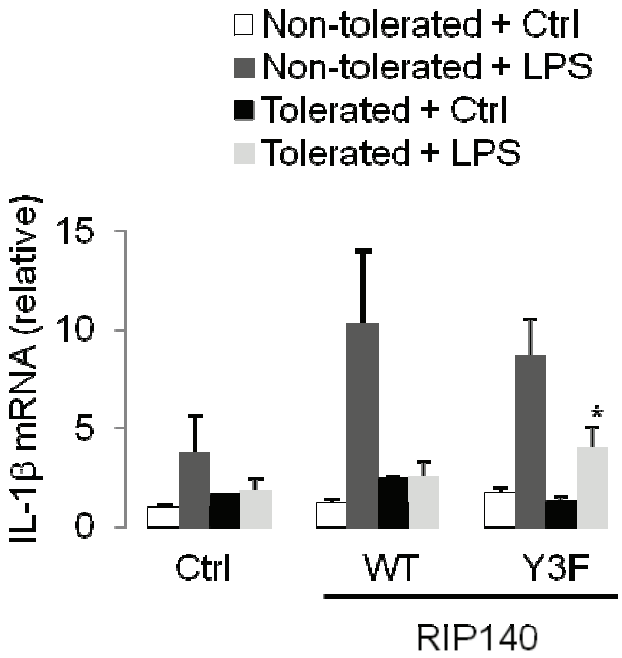

**Supplementary Figure 12.** IFN-γ prevent LPS-induced RIP140 degradation in Raw264.7 macrophages. Pre-treatment of IFN-γ in primary peritoneal macrophages prevents LPS-facilitated degradation of RIP140. Cells were pre-treated with or without IFN-γ for 16 h and then challenged with LPS for another 6 h.



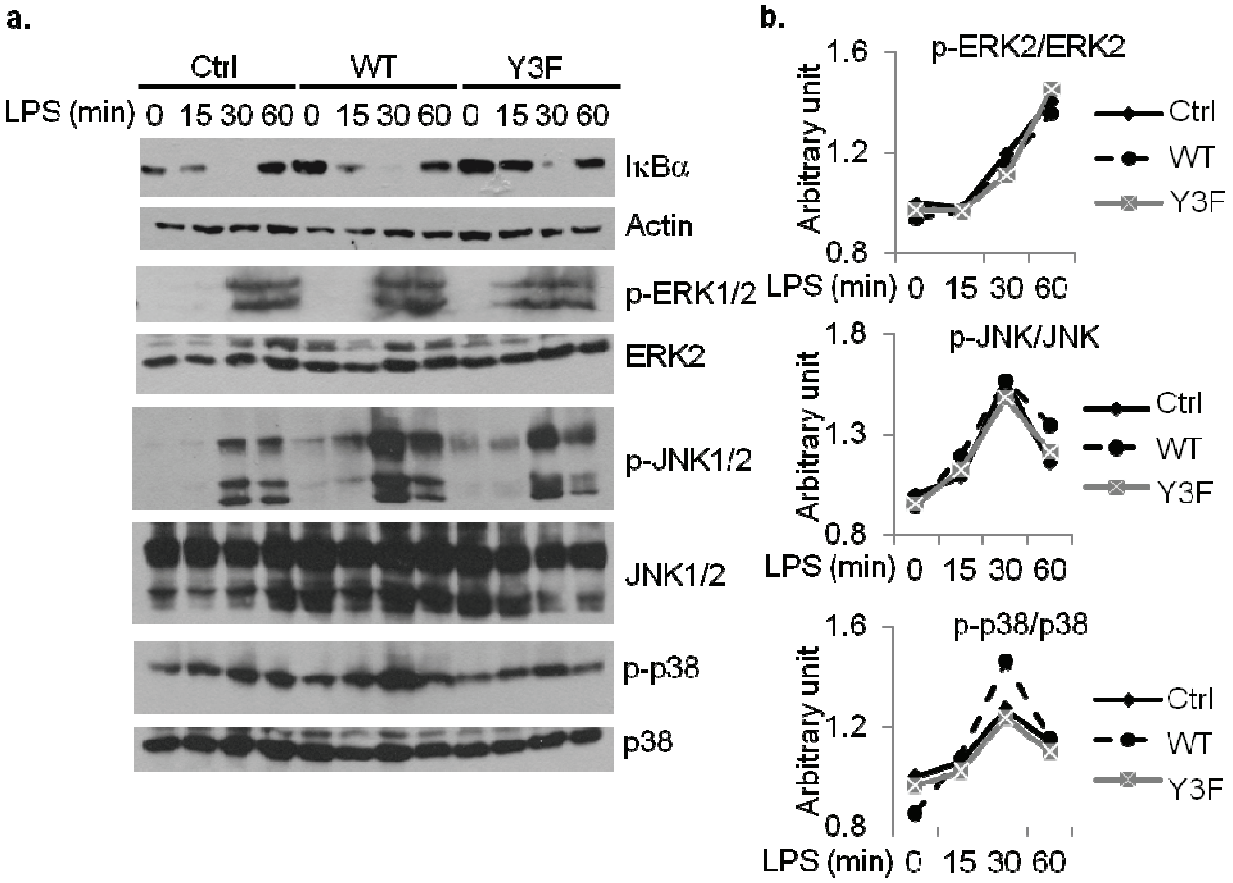

**Supplementary Figure 13.** IFN-γ fails to prevent endotoxin tolerance in RIP140-silencing macrophages. Raw264.7 macrophages were treated with or without IFNγ for 16 h, followed by control treatment or LPS challenge. After 24 h, cells were then re-challenged with LPS for another 2 h. The mRNA levels of (a) TNFα and (b) IL-1β from Raw264.7 macrophages were measured by quantitative PCR. Result present in mean ± SD., n=3; *: $P < 0.05$.



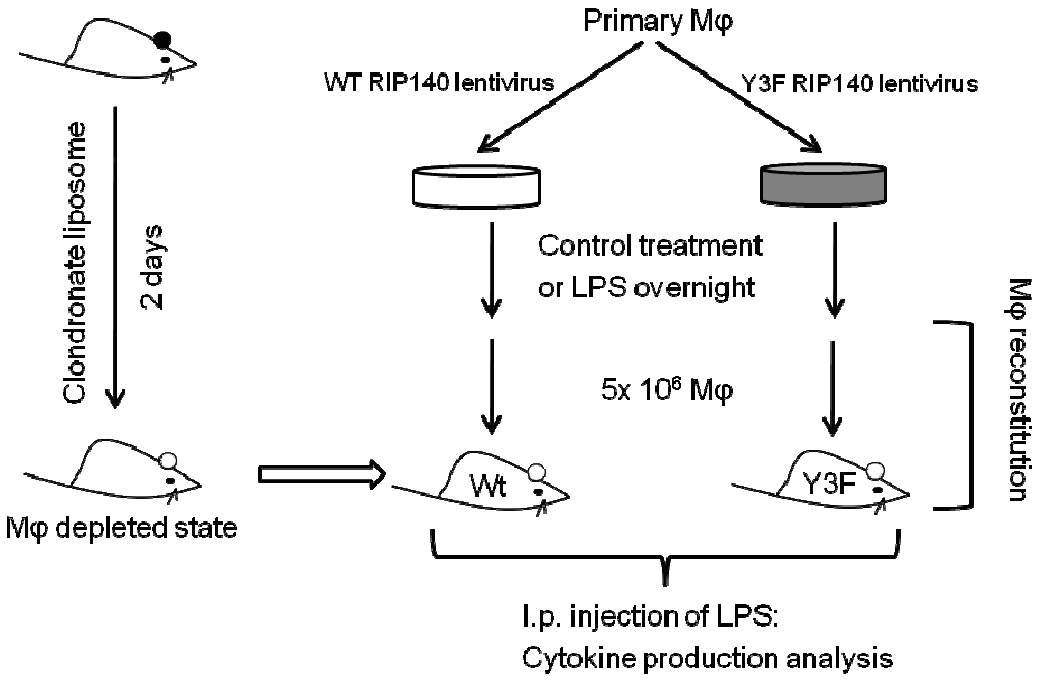

**Supplementary Figure 14.** Experimental design for IFN-γ effect on endotoxin tolerance. A schematic diagram for experimental design: macrophages were treated with or without IFNγ for 16 h, followed by control treatment or LPS challenge. After 24 h, cells were then re-challenged with LPS.




**Supplementary Figure 15.** Degradation of RIP140 involves in the establishment of endotoxin tolerance on IL-1β production. Raw264.7 macrophages were transfected with control vector (Ctrl), Flag-tagged wild type RIP140 (Wt) or tyrosine mutant RIP140 (Y3F). Cells were treated with or without LPS for 16 h to become non-tolerated or tolerated states, respective. After 24 h, cells were then challenged with LPS for another 2 h. mRNA levels of IL-1β were determined by quantitative PCR. Result present in mean ± SD., n=3; *: $P < 0.05$ as compared to control treatment of tolerated group.




**Supplementary Figure 16.** RIP140 does not affect proinflammaotry signaling in endotoxin tolerance state in macrophages. Raw264.7 macrophages were transfected with control vector (Ctrl), Flag-tagged wild type RIP140 (Wt) or tyrosine mutant RIP140 (Y3F). Cells were treated with or without LPS for 16 h to become tolerated states. After 24 h, cells were then challenged with LPS for various durations. Indicated protein levels were determined by immunoblot analyses of whole cell lysates.




**Supplementary Figure 17.** Non-degradable RIP140 prevents endotoxin tolerance in macrophage reconstitution model. A schematic diagram for macrophage reconstitution experiment. Macrophage depletion was achieved by injecting animals with clondronate liposome. Primary peritoneal macrophages were isolated from normal mice and transduced by lentivirus containing the indicated form of RIP140. These macrophages were induced into a non-tolerated or tolerated state *in vitro*, and injected into the macrophage-depleted mice.

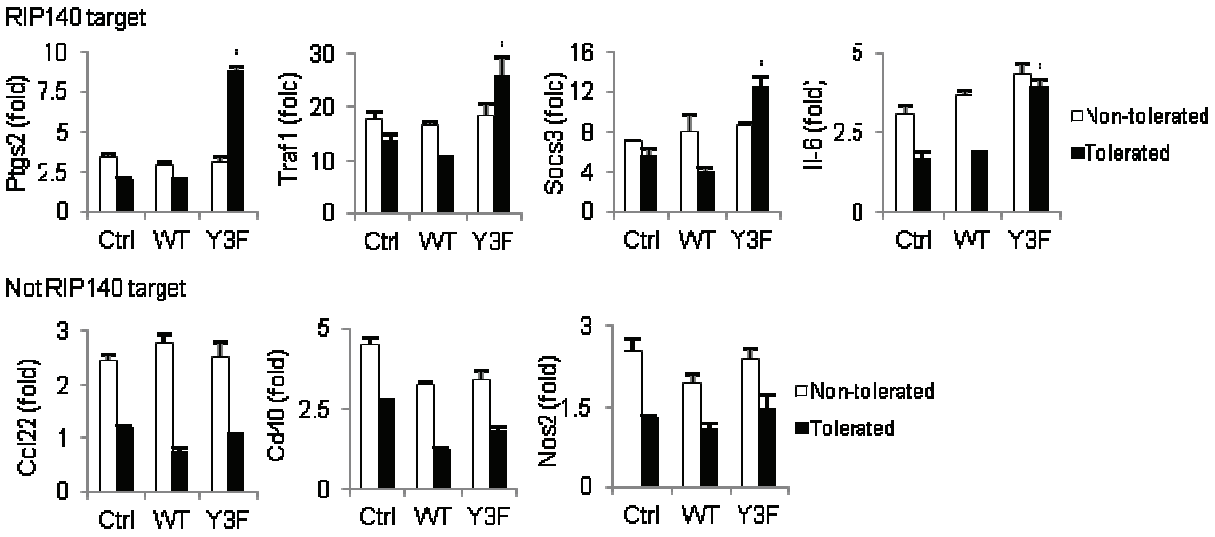
## Tolerated genes regulated by RIP140

| Symbol | Gene name |
|---|---|
| IL1B | Interleukin 1 beta |
| TNF | Tumor necrosis factor |
| IL6 | Interleukin 6 |
| TNFSF4 | Tumor necrosis factor (ligand) superfamily 4 |
| TRAF1 | TNF receptor associated factor 1 |
| SOCS3 | Suppressor of cytokine signaling 3 |
| PTGS2 | Prostaglandin-endoperoxide synthase 2 |
| COL18A1 | Collagen, type XVIII, alpha 1 |
| SELP | Selectin, platelet |

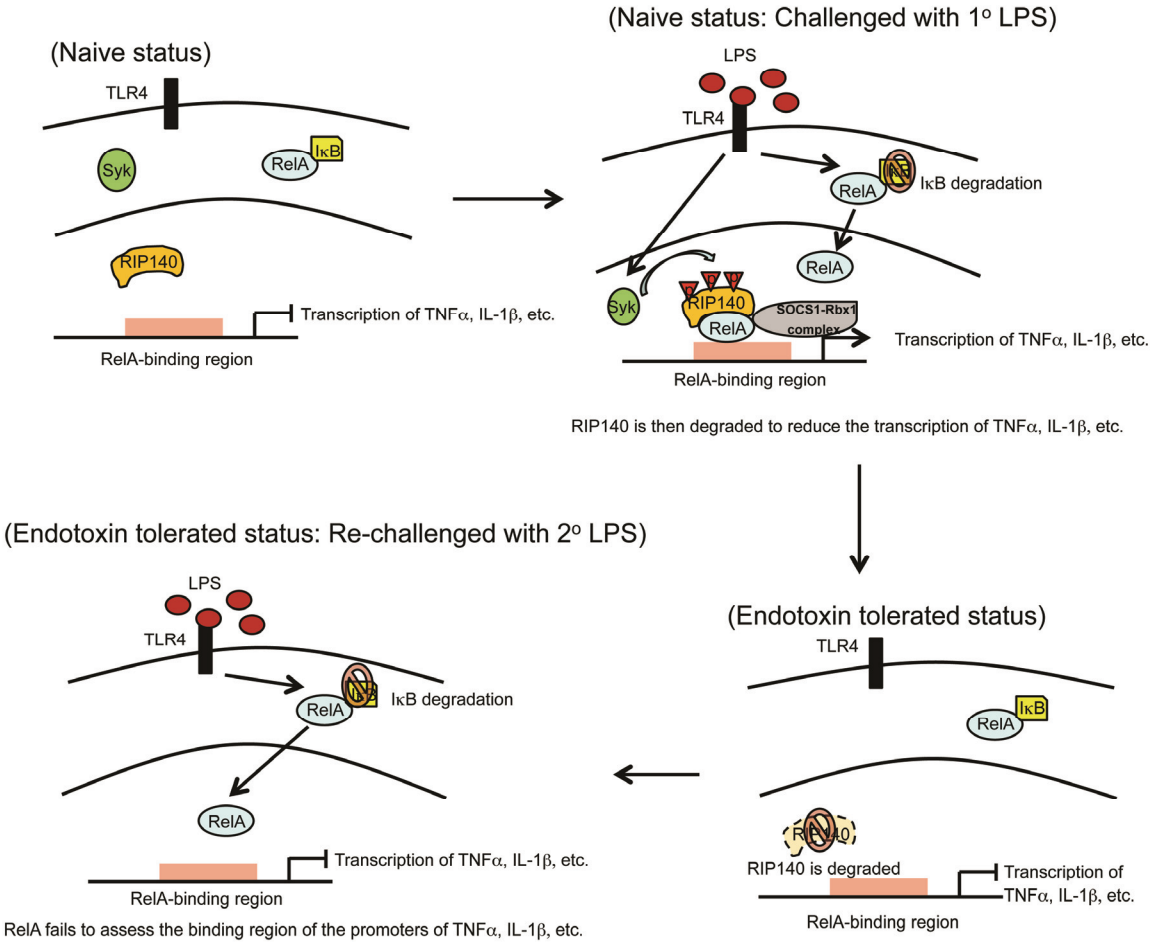
## Non-tolerated genes regulated by RIP140

| Symbol | Gene name |
|---|---|
| THBS1 | Thrombospondin 1 |

**Supplementary Figure 18.** Most proinflammatory genes affected by RIP140 are tolerated genes. This table is an organized list for comparison of genes affected by RIP140 with tolerated and non-tolerated genes categorized by Medzhitov's group. Nine genes affected by RIP140 are tolerated genes and only one gene, *Thbs1*, affected by RIP140 is non-tolerated.




**Supplementary Figure 19.** RIP140 degradation contribute to endotoxin tolerance in Raw264.7 macrophages. Real-time PCR analysis of mRNA of indicated genes in non-tolerated or tolerated Raw264.7 macrophages transfected with the control vector (Ctrl), wild type RIP140 (Wt) or non-degradable RIP140 (Y3F). Relative folds of mRNA levels after the second stimulation with LPS were determined. Results are presented in mean ± SD., n=3; *: $P < 0.05$ as compared to the non-tolerated+LPS group.




**Supplementary Figure 20.** A model for RIP140 degradation during the establishment of ET. Following the exposure of macrophages to LPS, RelA translocates into the nucleus and interacts with RIP140 to activate transcription of TNFα and IL-1β, and Syk phosphorylates RIP140 on Tyr364/418/436. The interaction of RelA with RIP140 further recruits SOCS1-Rbx1 E3 ligase to RelA-RIP140 complex on specific chromatin targets. In cooperation with Syk-mediated phosphorylation, SOCS1-Rbx1 E3 ligase promotes RIP140 polyubiquitination and subsequent degradation, which in turn reduces proinflammatory cytokine production and promotes the establishment of ET on specific genes. When these macrophages are challenged with the second LPS, although RelA still translocates into the nucleus, it fails to activate promoters of TNFα and IL-1β because of the lack of specific coactivator RIP140 for these genes.