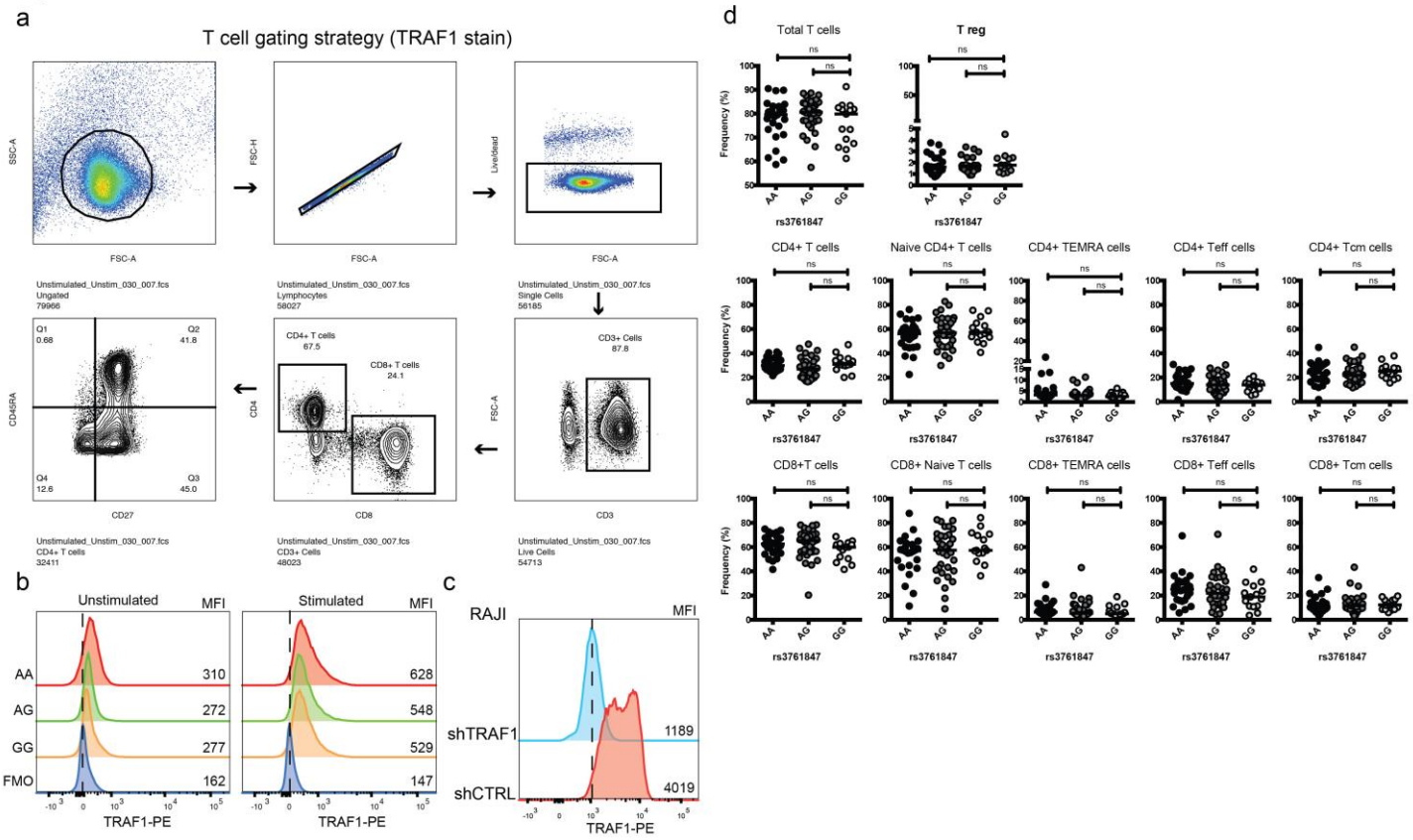
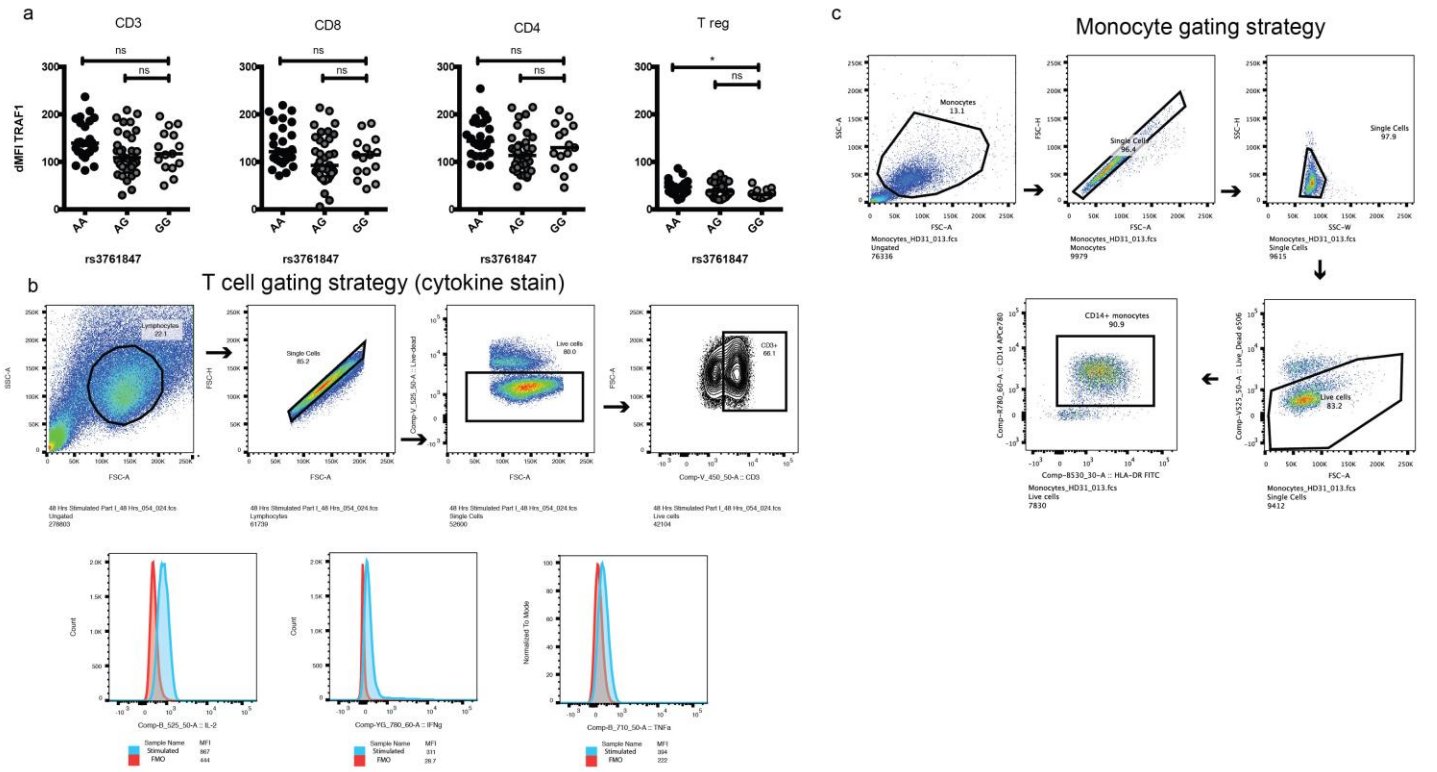



**Supplementary Figure 1**

Gating strategy for flow cytometric analysis of T cells and frequency of T cell subsets

(**a**) Gating strategy for the flow cytometry analysis performed on T cells from healthy human subjects' PBMCs for TRAF1 measurements in T cells, as in Fig. 1a. (**b**) Representative histograms showing TRAF1 expression in stimulated and unstimulated T cells from PBMCs of AA, AG and AG donors. (**c**) control knockdown (*shCTRL*) or *TRAF1* knockdown (*shTRAF1*) RAJI cells were used to confirm specificity of TRAF1 stain. (**d**) Frequency of the various subsets of T cells from donor PBMCs with the AA, AG or GG genotype were measured by flow cytometry using the antibodies listed in the methods section. n = 26, 36, 15 human subjects of each genotype AA, AG, GG respectively. Results are representative from 2 similar experiments. Statistical analysis was performed using a non-parametric (Mann Whitney) unpaired t-test.

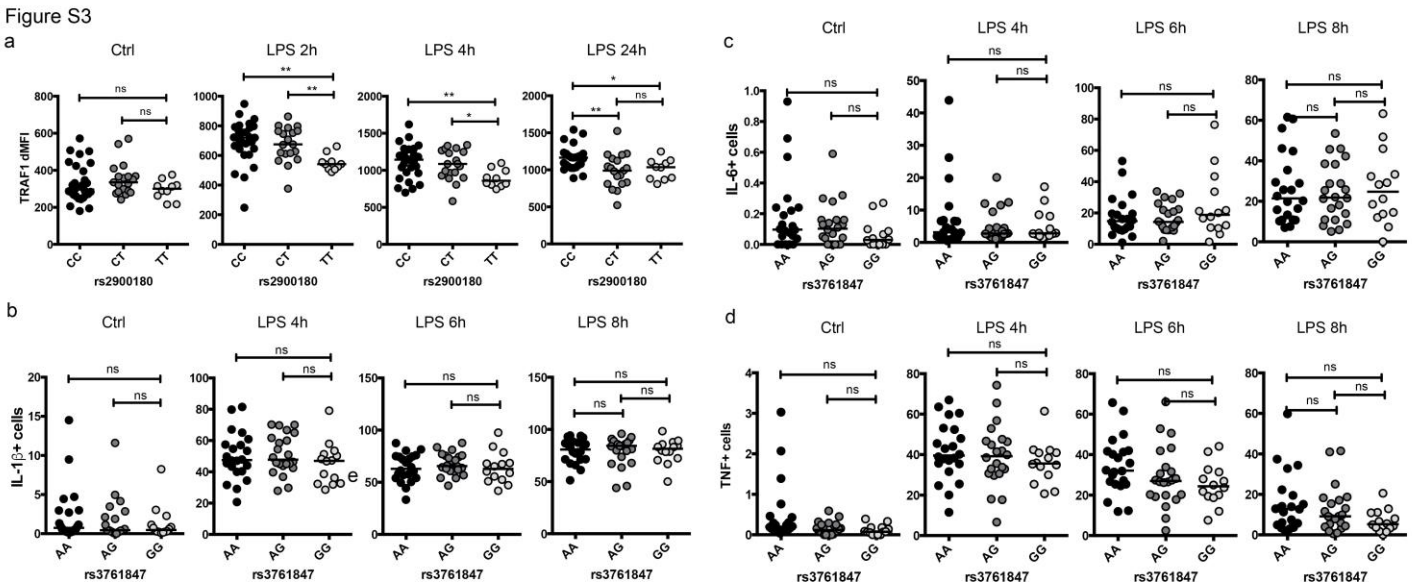
Figure S2




**Supplementary Figure 2**

TRAF1 protein levels in unstimulated T cells and gating strategies for T cell cytokines and monocytes

(**a**) TRAF1 levels were measured by flow cytometry using a PE labeled anti-hTRAF1 antibody in unstimulated T cells from donor PBMCs with the AA, AG or GG genotypes. Each symbol represents one donor; n = 26, 36, 15 human donors of each genotype AA, AG, GG, respectively. Results are representative of 2 similar experiments. Statistical analysis was performed using a non-parametric (Mann Whitney) unpaired t-test. * p <0.05 (**b**) Gating strategy for intracellular flow cytometry analysis performed on T cells from healthy human donor PBMCs for cytokine measurements, as in Fig. 1b. (**c**) Gating strategy for the flow cytometry analysis performed on purified monocytes from healthy human donor PBMCs, as in Fig. 2.

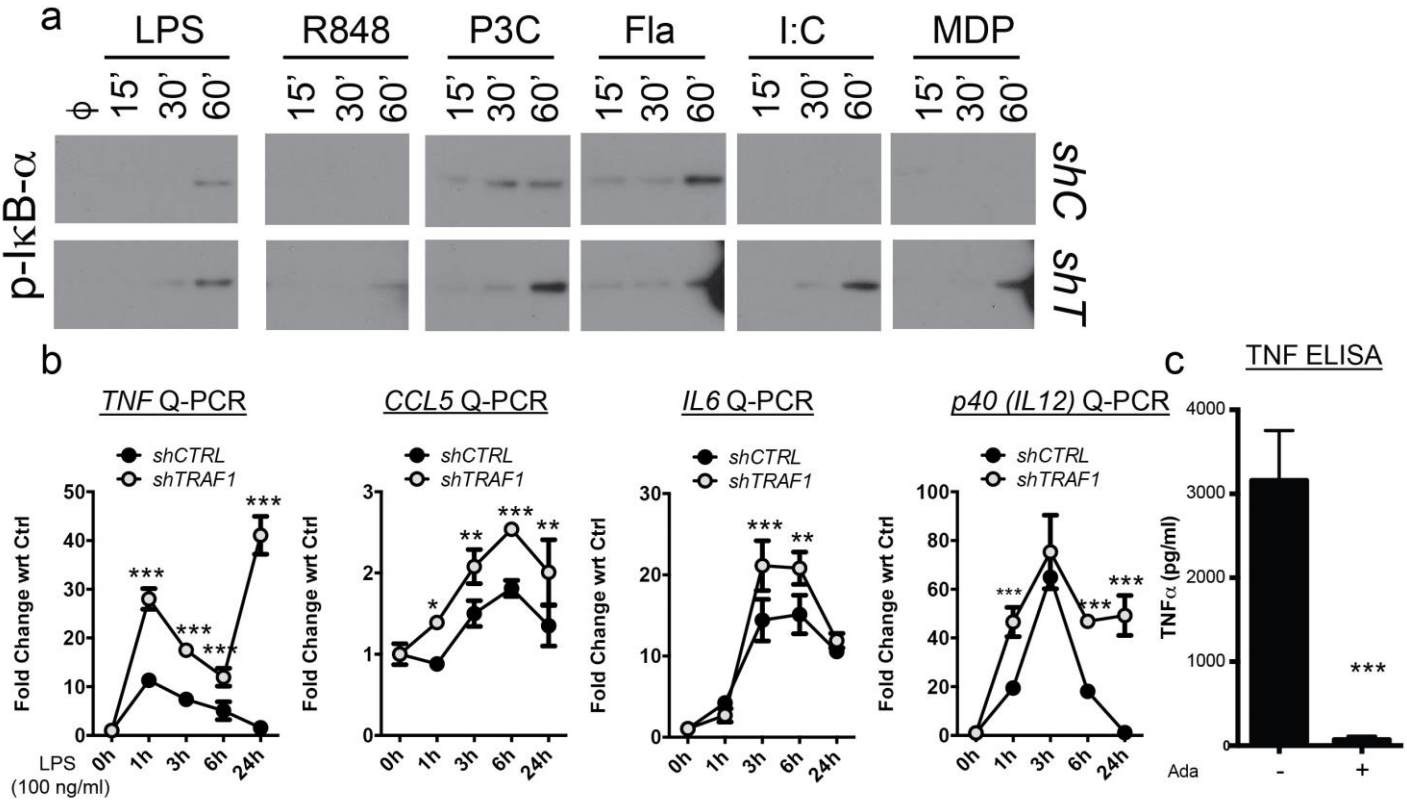



**Supplementary Figure 3**

Flow cytometric measurements of TRAF1 and cytokines from human PBMC monocytes

(**a**) Purified monocytes from healthy human subjects' PBMCs were treated or not with LPS (100 ng/ml for 2, 4 or 24 hrs), and TRAF1 levels were measured by flow cytometry using a PE-labeled anti-hTRAF1 antibody. (**a**) Graphical representation of the mean fluorescence intensities (dMFI = MFI of sample – MFI of FMO) of TRAF1 in purified monocytes of donors with the CC, CT and TT genotypes of the *TRAF1* SNP rs2900180. Each symbol represents one donor; n = 29, 19, 10 human donors of each genotype CC, CT, TT, respectively. (**b-d**) Purified monocytes from healthy human donor PBMCs were treated or not with LPS (100 ng/ml for 4, 6 or 8 hrs), and percentage of cytokine producing cells was assessed by flow cytometry using an (**b**) IL-1β antibody, IL-6 antibody (**c**) or TNF antibody (**d**). (b-d) Each symbol represents one donor; n = 22, 22, 14 human donors of each genotype AA, AG, GG, respectively. Results are representative of 2 similar experiments. (a-d) Statistical analysis was performed using a non-parametric (Mann Whitney) unpaired t-test. * $p < 0.05$ and ** $p < 0.01$

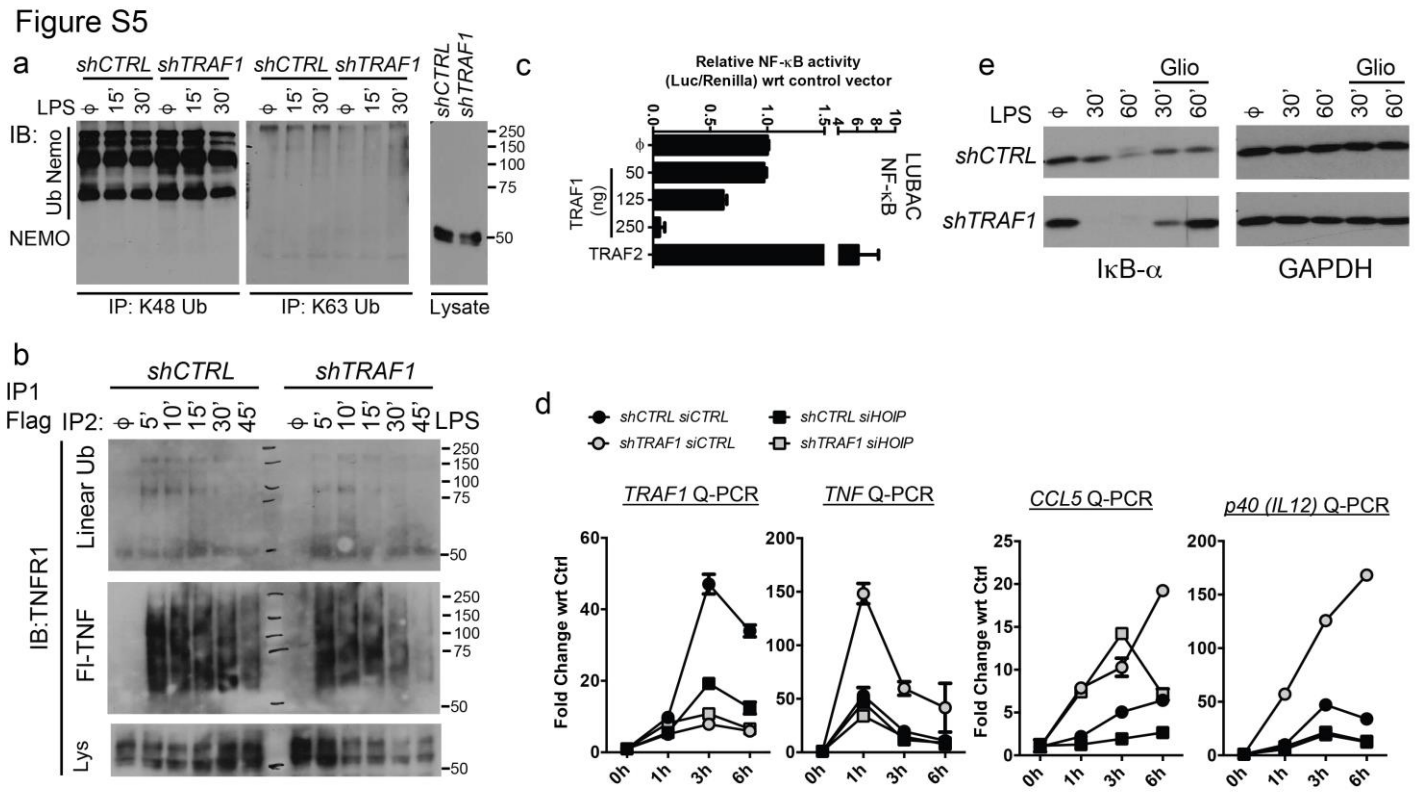
Figure S4




**Supplementary Figure 4**

*shTRAF1* THP-1 cells have enhanced NF-κB activation and express higher levels of inflammatory cytokines than *shCTRL* cells after TLR or NOD ligand treatment

(**a**) *shCTRL* (*shC*) and *shTRAF1* (*shT*) THP-1 cells were stimulated with Pam3CSK4 (P3C; 10 ng/ml), Flagellin (Fla; 0.5 μg/ml), Resiquimod (R848; 10 ng/ml), Poly(I:C) (I:C; 10 μg/ml), L18-muramyl dipeptide (MDP; 10 ng/ml) or LPS (100 ng/ml) for the indicated times and whole cells extracts (WCE) were immunoblotted for phospho-IκB-α (p-IκB-α). Blots are representative of two independent experiments (**b**) Gene expression of *TNF*, *CCL5*, *IL6* and *p40 (IL12)* was evaluated by real-time PCR (Q-PCR) in 100 ng/ml LPS stimulated *shCTRL* or *shTRAF1* THP-1 cells for the indicated times as in Fig. 4e. Results were normalized to GAPDH and reported as relative fold change w.r.t. untreated control. Graphs show the mean ± standard deviation of three independent experiments, which were compared using a 2 way ANOVA with multiple comparisons. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ (**c**) TNF levels were measured by Enzyme-linked immunosorbent assay (ELISA) in supernatants of anti-CD3/anti-CD28 stimulated T cells from 23 human donors PBMCs with or without co-treatment with 1 μg/ml Adalimumab (Ada) for 48 hrs. Graphs show the mean ± SEM. Statistical analysis was performed using a paired t-test. *** $p < 0.001$

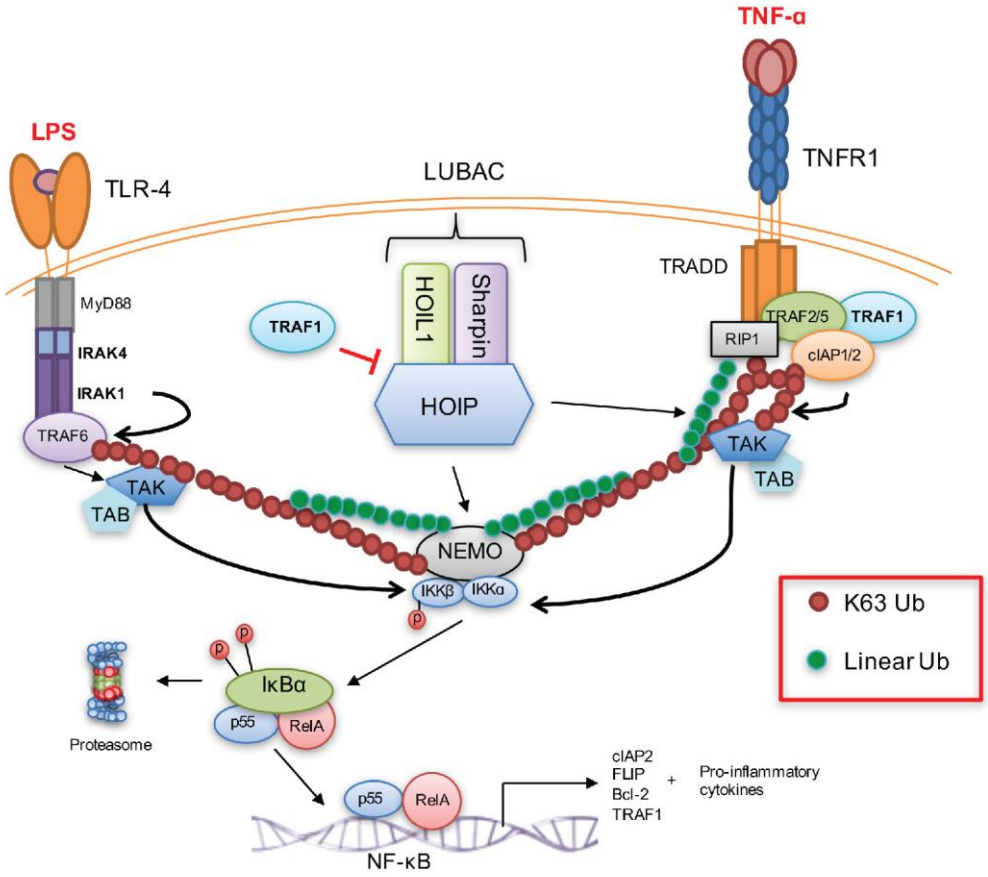



**Supplementary Figure 5**

Status of NEMO ubiquitination following LPS and TNF stimulation

(**a**) IP of endogenous Lys48 ubiquitin chains (K48 Ub) or Lys63 ubiquitin chains (K63 Ub) followed by immunoblotting for NEMO as in Fig. 6 panel c, with whole cell lysates, as a loading control, shown to the right. (**b**) Analysis of TNFR1-associated NEMO ubiquitination status and TNFR1 immunoprecipitates in Flag-TNF-treated *shCTRL* and *shTRAF1* THP-1 cells. Lysates were first immunoprecipitated with anti-Flag (IP1: Flag), dissociated and re-immunoprecipitated with anti-linear ubiquitin antibody (IP2: Linear Ub) or anti-K63 ubiquitin antibody (IP2: K63 Ub), and blotted for TNFR1. Whole cell lysates are shown in the bottom panel. (**c**) NF-κB firefly luciferase reporter was cotransfected into HEK293 FT cells with control Renilla luciferase and LUBAC (HOIP + HOIL-1). Some cells were additionally transfected with increasing amounts of *TRAF1* or with 125 ng of *TRAF2* expression vectors, as indicated. Luciferase activity was measured after 48 h. Graphs show the mean ± standard deviation of three independent experiments (**d**) Gene expression of *TRAF1*, *TNF*, *CCL5* and p40 *(IL12β)* was evaluated by real-time PCR (Q-PCR) in 100 ng/ml LPS stimulated *shCTRL* or *shTRAF1* THP-1 cells (treated with non-targeting siRNA (*siCTRL*) or HOIP targeting siRNA (*siHOIP*) for 48 hrs) for the indicated times as in panel a. Results were normalized to GAPDH and reported as relative fold change w.r.t. untreated control. Graphs show the mean ± standard deviation of three independent experiments, which were compared using a 2 way ANOVA with multiple comparisons. (**e**) LUBAC inhibition by gliotoxin reverses the enhanced NF-κB activation in *shTRAF1* THP-1 cells. WCE from control knockdown (s*hCTRL*) and TRAF1 knockdown (s*hTRAF1*) THP-1 cells treated with LPS (100 ng/ml) for the indicated times were immunoblotted for IκB-α (left panels) or as a loading control GAPDH (right panels). Some *shCTRL* THP-1 cells were pretreated (15') with 1 μM Gliotoxin. Blots are representative of 3 independent experiments.

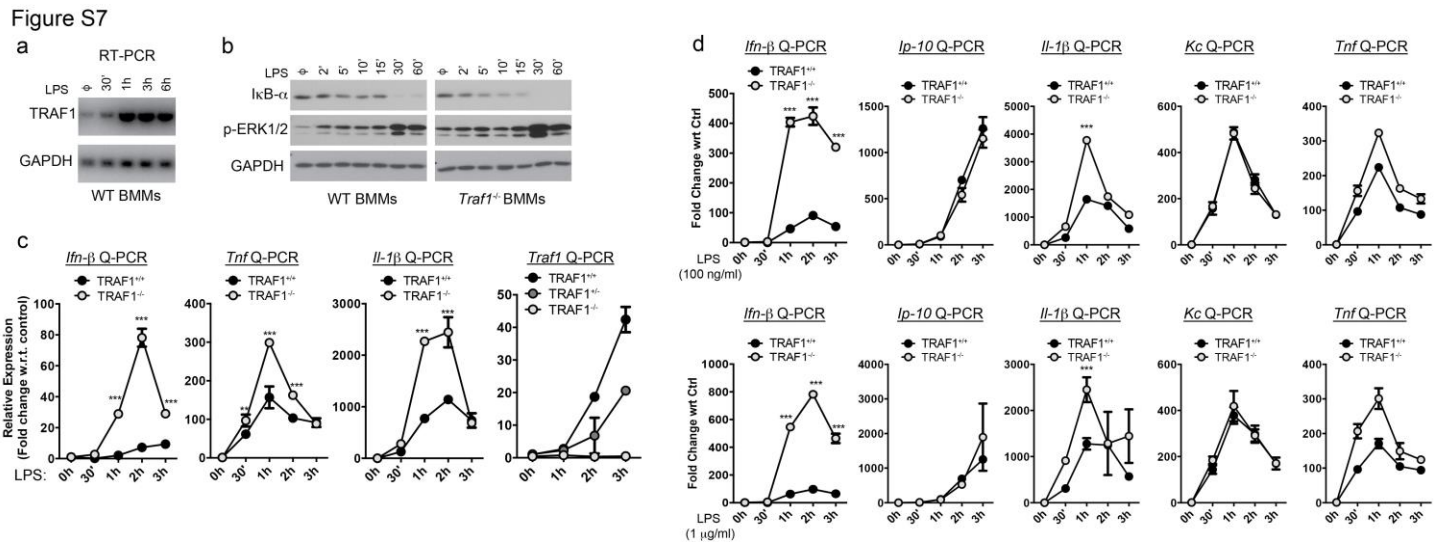
Figure S6




**Supplementary Figure 6**

Proposed mechanism for TRAF1 role in TLR and TNFR1 signaling

TRAF1 limits linear ubiquitination of NEMO in both the TLR and TNFR1 signaling pathways, but these effects in TNFR1 signaling are counterbalanced by the role of TRAF1 in recruitment of cIAP1 to enhance K63-linked polyubiquitination of NEMO.

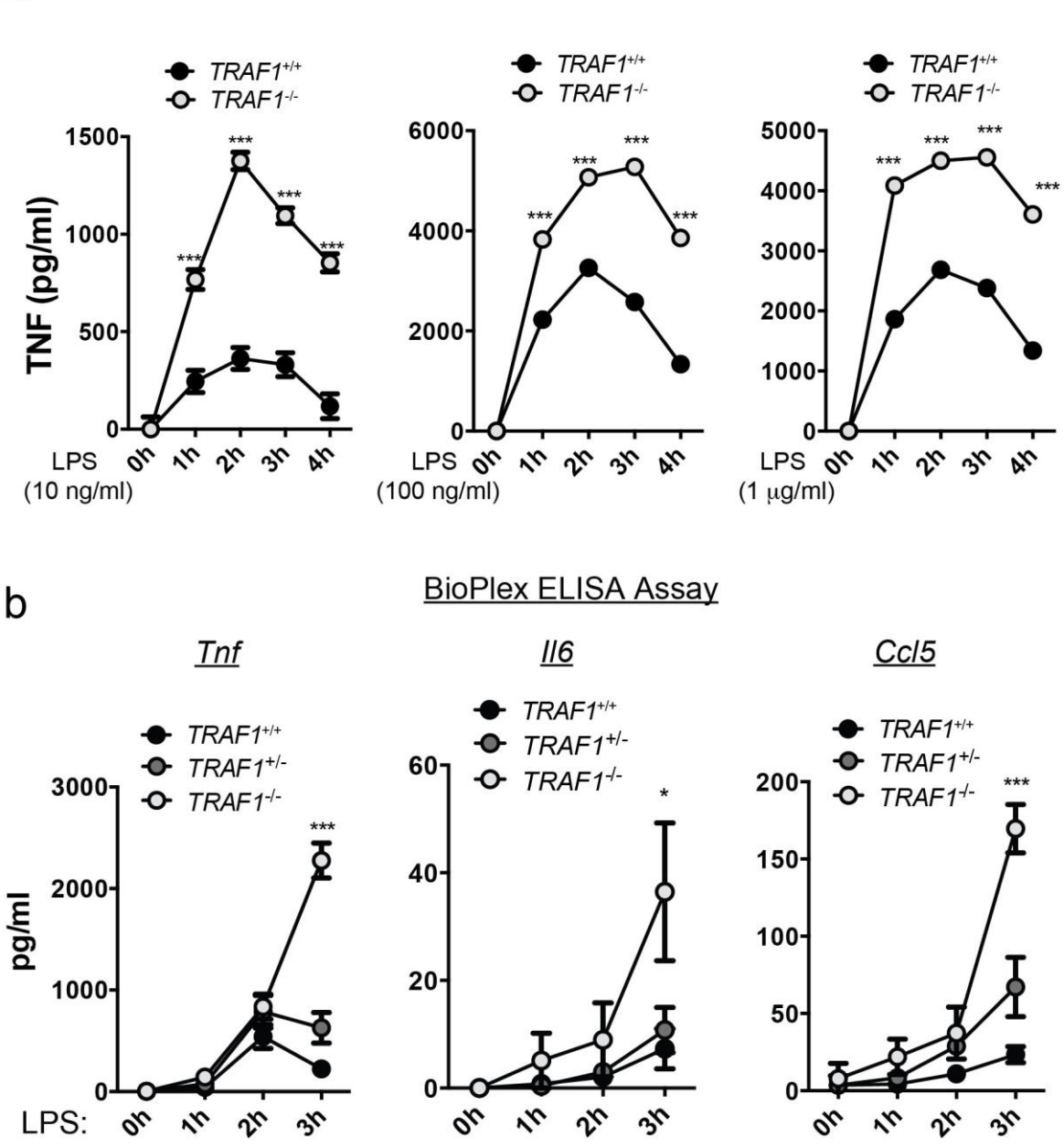



**Supplementary Figure 7**

Macrophages from *Traf1*$^{-/-}$ mice are hyper-responsive to TLR stimulation

(**a**) d5 bone marrow macrophages (BMMs) from C57BL/6NCrl (WT) mice were stimulated with 10 ng/ml LPS for the indicated times, and gene expression was assessed by reverse-transcriptase PCR (RT-PCR). (**b**) BMMs from WT (left) or *Traf1*$^{-/-}$ (right) littermate mice were stimulated with 10 ng/ml LPS for the indicated times, and whole cells extracts (WCE) were immunoblotted for total IκB-α (IκB-α), phospho-ERK1/2 (p-ERK1/2) and as a loading control GAPDH. Blots are representative of at least three independent experiments. (**c**) Gene expression of *Ifn*-β, *Tnf*, *Il1*-β and *Traf1* was evaluated by real-time PCR (Q-PCR) in LPS stimulated BMMs for the indicated times as in panel a (10ng/ml LPS). (**d**) d5 bone marrow macrophages (BMMs) from WT or *Traf1*$^{-/-}$ littermate mice were stimulated with 100 ng/ml or 1 μg/ml LPS for the indicated times, and gene expression of *Ifn*-β, *Ip*-10, *Tnf*, *Il-1*β and *Kc* was evaluated by real-time PCR (Q-PCR). (c-d) Results were normalized to GAPDH and reported as relative fold change w.r.t. untreated control. Graphs show the mean ± standard deviation of three independent experiments, which were compared using a 2 way ANOVA with multiple comparisons. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$

Figure S8




**Supplementary Figure 8**

*Traf1*$^{-/-}$ macrophages produce higher levels of cytokines than WT

(**a**) Tnf levels were measured by Enzyme-linked immunosorbent assay (ELISA) in supernatants from WT (*Traf1*$^{+/+}$) or KO (*Traf1*$^{-/-}$) BMMs treated with 10 ng/ml, 100 ng/ml or 1 μg/ml LPS for the indicated times. Graphs show the mean ± standard deviation of three independent experiments, which were compared using a 2 way ANOVA with multiple comparisons. *** $p < 0.001$ (**b**) The levels of *Tnf*, *Il6* and *Ccl5* secreted into the supernatants of LPS stimulated WT (*Traf1*$^{+/+}$) heterozygous (*Traf1*$^{+/-}$) or KO (*Traf1*$^{-/-}$) BMMs were measured using Bio-plex cytokine assay. Graphs show the mean ± standard deviation of three independent experiments, which were compared using unpaired t-test; * $p < 0.05$ and *** $p < 0.001$

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