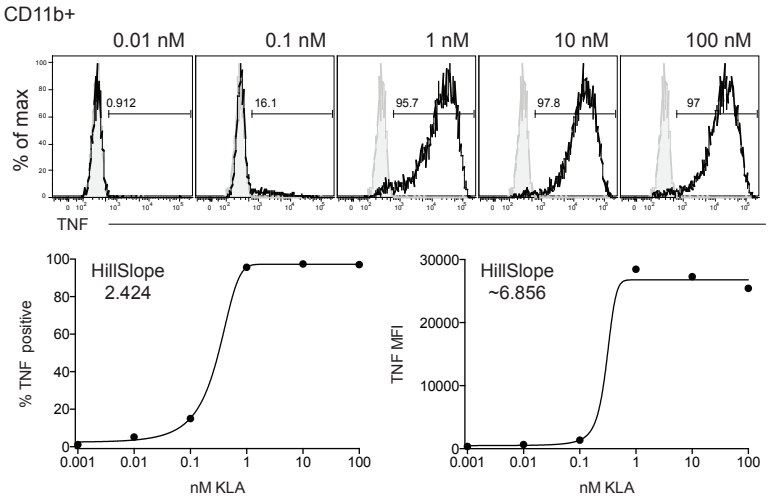


Figure S1. TLR4 ligand concentration dependent TNF production, related to Figure 1. Cells were isolated from the peritoneal cavity of C57BL/6 mice and stimulated *in vitro* with the indicated concentration of KLA for 4 hours in the presense of BFA, before TNF was measured by intracellular flow cytometry. Data are representative of 2 independent experiments.

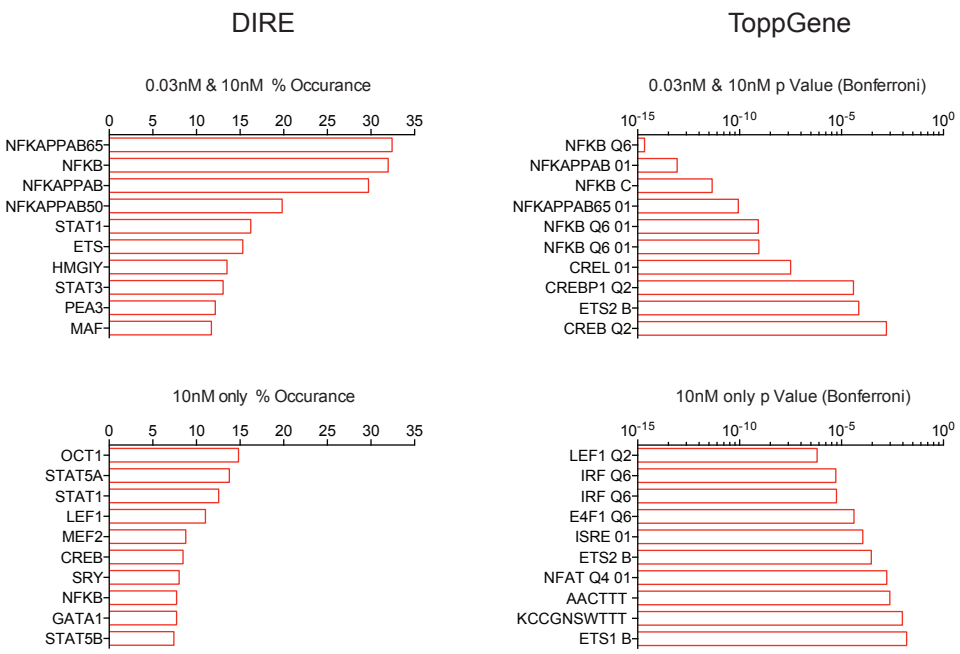


Figure S2. NF-kB motif occurance and enrichement significance in genes induced by sub-threshold concentrations of TLR4 ligand, related to Figure 3. BMDM were stimulated with KLA and gene expression was assessed using RNAseq. Transcription factor motif enrichment analysis using DiRE or ToppGene for the indicated sets of genes, upregulated by both 0.03nM and 10nM KLA or exclusively by 10nM (log2 fold change > 0.5 and 1% FDR). RNAseq was performed on pooled RNA from 3 biological replicates, for 2 independent experiments

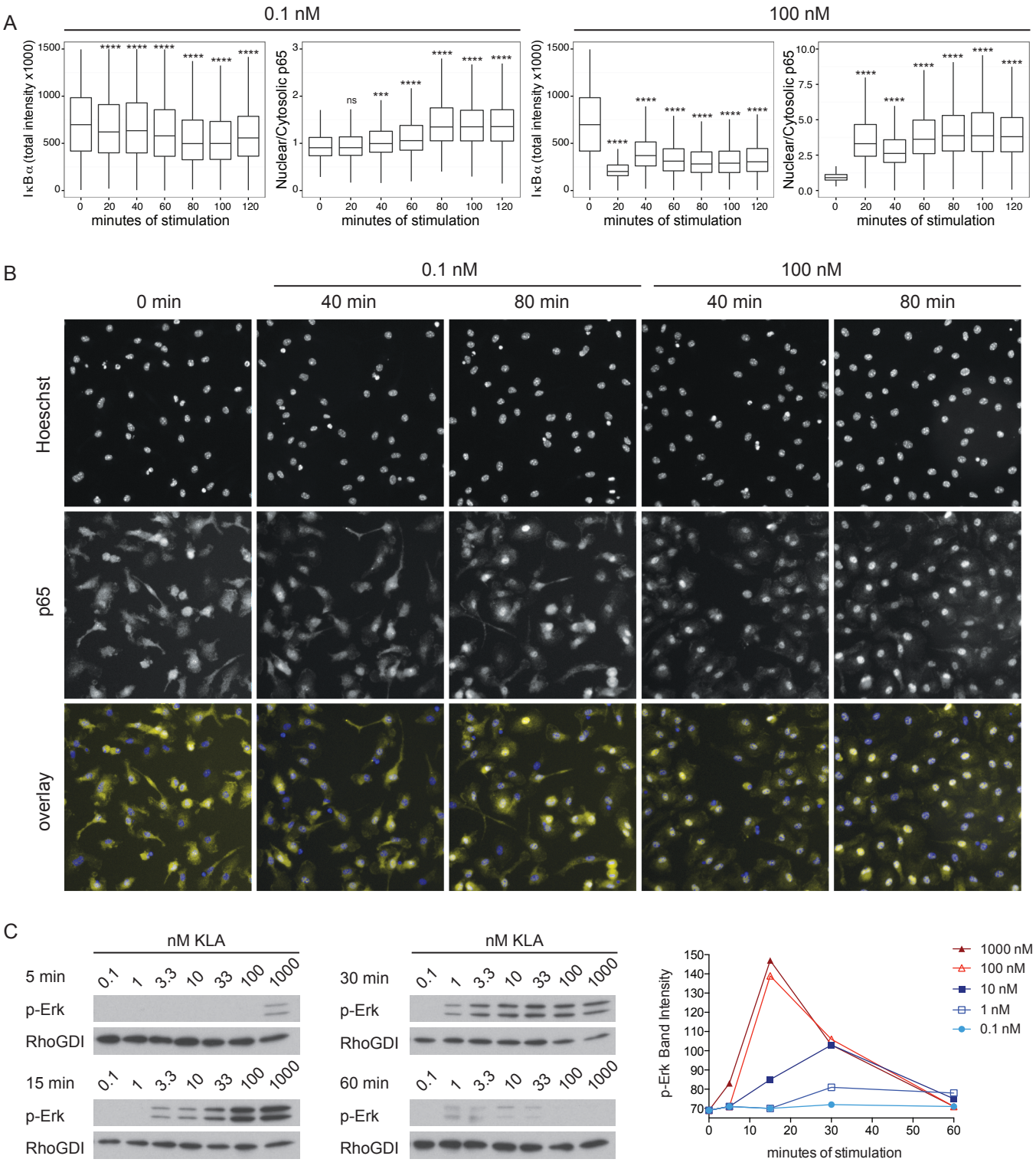


Figure S3. NF-kB signaling occurs at TLR4 ligand concentrations below those yielding MAPK activation, related to Figure 4. BMDM were stimulated with the indicated concentration of KLA. (A,B) BMDM were fixed at the indicated times, stained, and analyzed by high-content imaging. (A) IkBa intensity and p65 localization are plotted for individual cells from 10 fields in two duplicate wells. Boxes represent 25th to 75th percentiles, whiskers represent the highest or lowest value that is within 3/2 of the interquartile range from the boundaries of the box. Stars represent statistical significance based on ordinary one-way ANOVA, versus control (*** p≤0.001, **** p≤0.0001). (B) Representative imaging fields from select time points in (A). (C) BMDM were lysed and Erk phosphoyrlation was detected by Western blotting. RhoGDI was used as a loading control. Data are representative of two or more independent experiments.

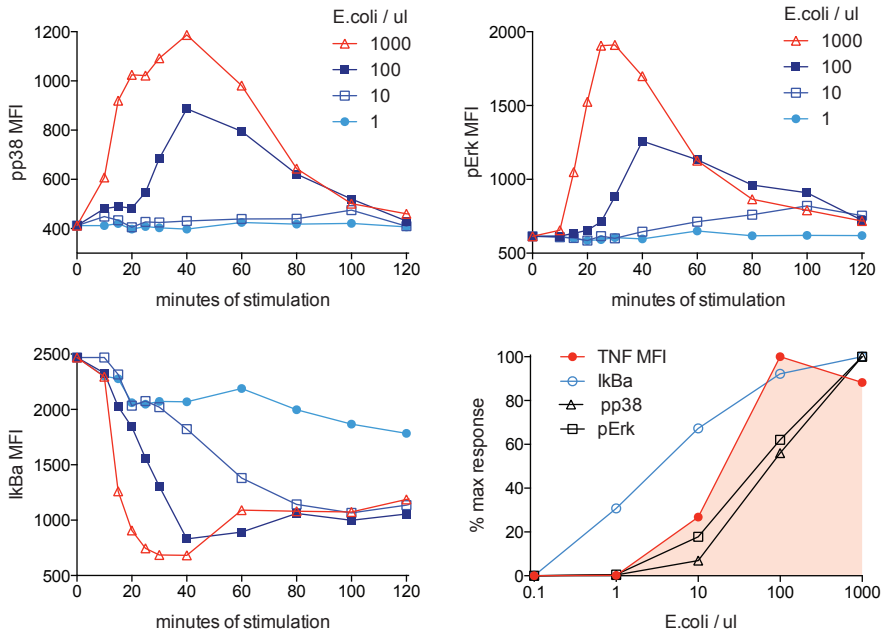


Figure S4. Divergent signaling thresholds in response to *E. coli*, related to Figure 4. BMDM were stimulated with the indicated concentration of *E. coli* K-12 strain BioParticles (invitrogen). The area under the curve for each concentration of ligand was extracted from time course data of IκBα degradation, p38 phosphorylation, or Erk phosphorylation and plotted over the dose response for TNF production. Data are representative of 2 independent experiments.

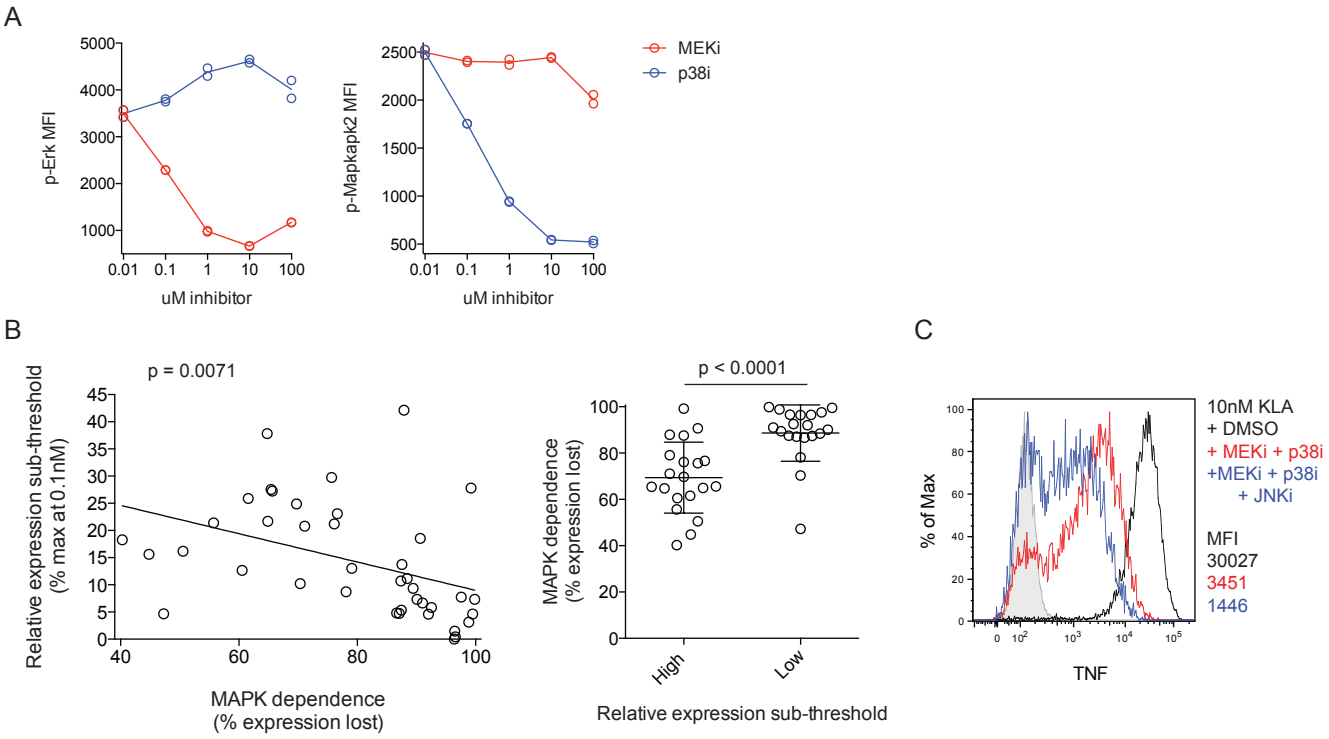


Figure S5. Optimization of MAPK inhibition, related to Figure 5. (A) BMDM were treated with the indicated concentraion of inhibitor for 30 minutes and then stimulated with 100nM KLA for and additional 30 minutes before fixation and analysis of signaling intermediates by flow cytometry. Erk phosphorylation is used to assess MEK activity, while Mapkapk2 phosphorylation reflects p38 activity. Datapoints represent duplicate wells. (B) As described in Figure 5, BMDM were stimulated with KLA and mRNA expression for 40 TLR-induced genes was measured using qPCR. MAPK dependence was calculated based on the percentage of induction lost, comparing the 10nM fold change at peak timepoint (1h or 2h), with and without MAPK inhibition: MEK1/2 inhibitor U0126 (MEKi, 10uM), p38 inhibitor SB203580 (p38i, 10uM), andJNK1/2/3 inhibitor SP600125 (JNKi 10uM). The MAPK dependence for the 40 genes assessed is shown as compared to relative induction at 0.1nM for each gene. Statistical significance for the slope of the regression line was determined using an F test and groups were compared using an unpaired t test, for the left and right plot respectively. (C) BMDM were treated with the indicated inhibitors for 30 minutes and then stimulated with 10nM KLA for 5 hours in the presense of brefeldin A. TNF was measured by intracellular cytokine staining and flow cytometry.

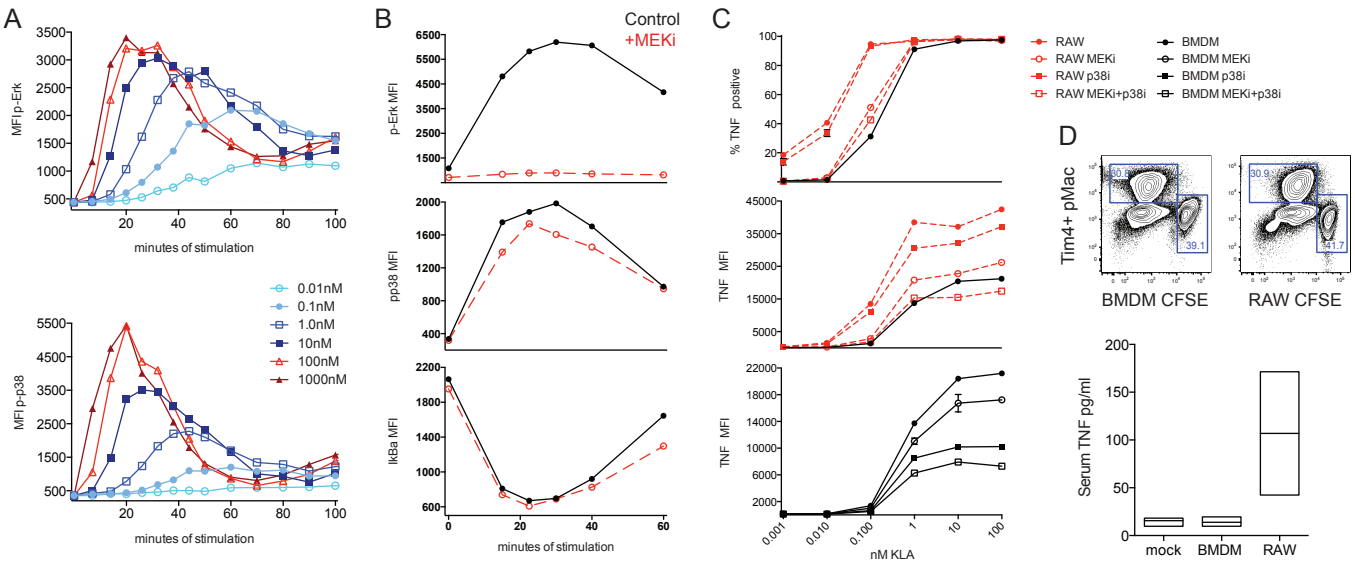


Figure S6. The MEK/Erk pathway mediates aberrant TNF production in RAW cells, while both Erk and p38 responses scale TNF production at high ligand concentrations, related to Figure 6. (A) RAW cells were stimulated with the indicated concentration of KLA and phospho-Erk and phospho-p38 were assessed by flow cytometry. (B) RAW cells were stimulated with 100nM KLA for the indicated number of minutes, with or without 10uM U0126 (MEKi) added 30 minutes prior to stimulation, and signaling intermediates were analyzed by flow cytometry. MEK inhibitor treated or untreated RAW cells are represented by red open circles or black closed circles, respectively. (C) RAW and BMDM were stimulated with the indicated concentration of KLA for 4 hours, with or without addition of MAPK inhibitors (MEKi and/or p38i) and TNF was measured by flow cytometry. Signaling and inhibitor data are representative of 2 or more independent expreiments. (D) C57BL/6 mice were injected i.p. with $2.5x10^6$ CFSE labeled BMDM or RAW cells just prior to administration of 1ng KLA, and peritoneal macrophages and serum TNF were quantified 1h later. RAW cell transfer results are comparable to the 3 independent experiments shown in Figure 6. BMDM versus mock transfer represent 3 mice per group.

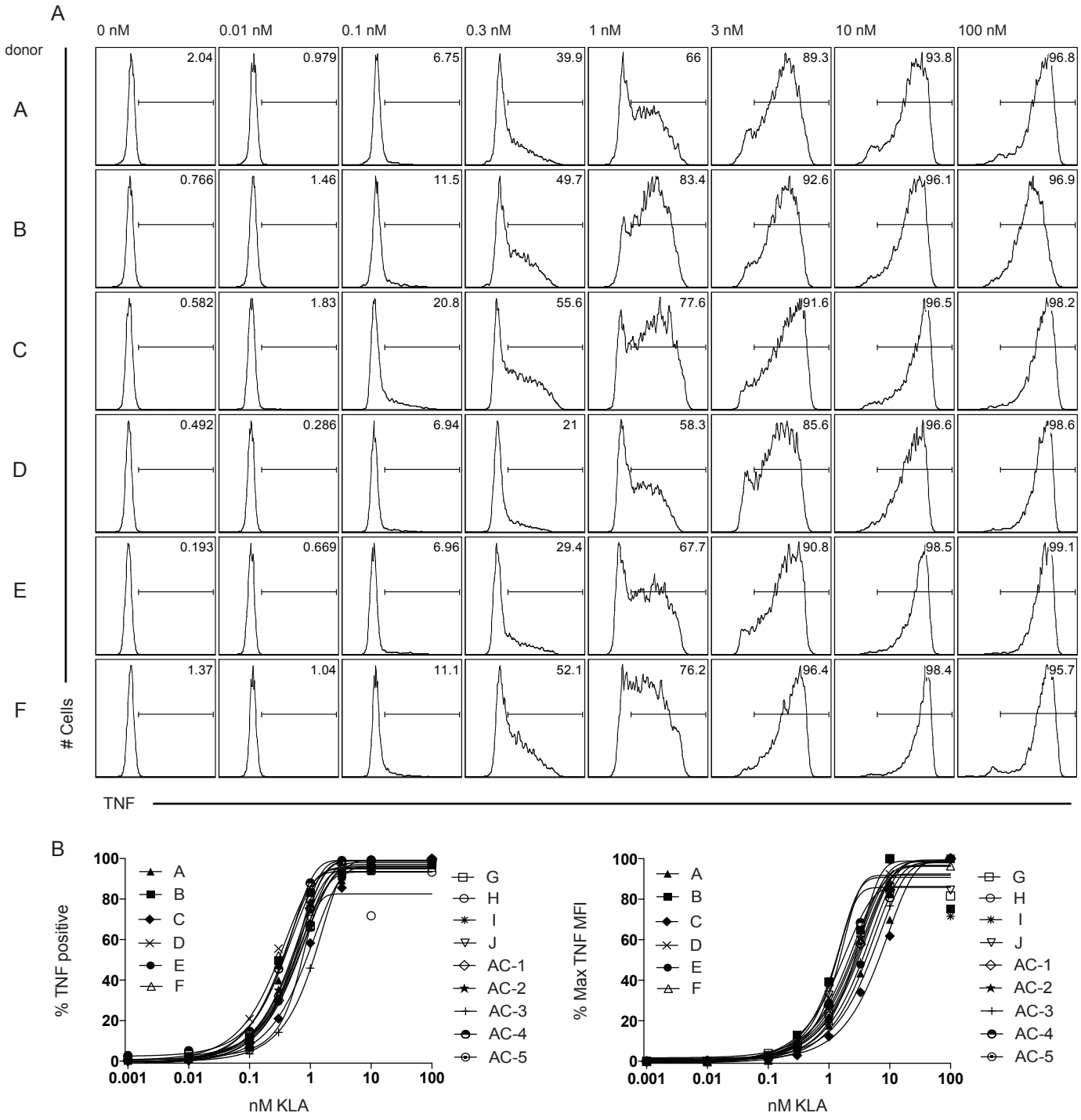


Figure S7. Highly conserved TLR4 induced TNF dose responses in human macrophages, related to Figure 7. (A) Monocyte derived macrophages from 6 independent healthy donors were stimulated with TLR4 ligand KLA for 4 hours in the presence of BFA and subsequently stained for TNF. (B) Pooled data, including the 6 donors shown in (A) and Figure 7, with 9 additional donors, from a total of 3 independent experiments.

## Supplemental Experimental Procedures

### Mouse macrophages and *in vitro* treatments

Mice were maintained in specific-pathogen-free conditions and all procedures were approved by the NIAID Animal Care and Use Committee (National Institutes of Health, Bethesda, MD). Bone marrow progenitors isolated from C57BL/6 mice or Balb/c (Jackson Laboratories) were differentiated into BMDM during a 6-day culture in complete Dulbecco's modified Eagle's medium (10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-Glutamine, 20 mM HEPES) supplemented with 30ng/ml recombinant mouse M-CSF (R&D systems), added at days 0 and 3. RAW 264.7 cells purchased from ATCC were also maintained in complete DMEM. BMDM or RAW cells were plated in 48 well plates one day prior to stimulation, at which time 10x the TLR4 ligand Kdo2-Lipid A (KLA, Avanti Polar Lipids) was added to the final concentration indicated. Ligand addition was staggered so that all time points were harvested at the same time (termed time zero). In some experiments Brefeldin A (BD Golgi plug) was added 30 minutes after KLA, while MEK inhibitor U0126 (10uM Cell Signaling Technology) or p38 inhibitor SB203580 (10uM Cell Signaling Technology) was added 30 minutes prior to KLA.

### Human monocyte isolation and macrophage differentiation

Peripheral blood was obtained by leukopheresis from male healthy donors by the NIH Blood Bank (Department of Transfusion Medicine, National Institutes of Health Clinical Center) with informed consent. Mononuclear cells from de-identified samples were isolated using low endotoxin Ficoll-Paque PLUS gradient centrifugation (GE Healthcare Life Sciences). Monocytes were enriched to <98% CD14+CD16- using the Dynabeads Untouched Human Monocytes negative isolation kit (Life Technologies). Isolated monocytes were frozen in 10% DMSO (Sigma), 40% human autologous plasma

(obtained from the upper layer of Ficoll isolation) and 50% X-vivo 15 media (Lonza). Prior to experiments, frozen monocyte vials were quickly thawed and plated in 48 well plates in X-vivo 15 media containing 100 ng/ml recombinant human M-CSF (R&D Systems). On day 3 and day 6 of differentiation, culture medium was replaced by fresh X-vivo 15 containing 100 ng/mL M-CSF. Cells were stimulated on day 7 as in mouse macrophage experiments, described above. For samples labeled "AC", frozen PBMC from random healthy donors (including two female donors) were purchased from AllCells, and cells were differentiated in to macrophages, as detailed for frozen monocytes.

***In vivo* TLR stimulation**

C57BL/6 mice or Balb/c (Jackson Laboratories) between 7 and 8 weeks of age were injected intraperitoneally (i.p.) with the indicated dose of Kdo2-Lipid A (KLA, Avanti Polar Lipids), diluted in PBS at a volume of 400ul, or 200ul when cells were also injected. Mice were sacrificed at 1h or 2h after ligand injection, cells were lavaged from the peritoneal cavity with cold PBS containing 4% FBS and 2mM EDTA, and blood was collected from the inferior vena cava. In some experiments RAW 264.7 macrophages were labeled with 5uM CFSE (Invitrogen) and $2.5x10^6$ cells were injected i.p. just prior to administration of ligand. Blood was allowed to coagulate for 1 hour and serum was separated by centrifugation. Serum TNF was detected using cytometric bead array (BD Biosciences), which was collected on a BD Fortessa and analyzed in FlowJo.

**Protein detection by flow cytometry**

After the indicated period of stimulation, cells were fixed by addition of paraformaldehyde to cell cultures at a final concentration of 1.6%, followed by a 10-minute incubation at room temperature (RT). After one wash with PBS 1% FBS, cells were gently removed

from plates for staining. Intracellular cytokine samples were processed using BD Cytofix/Cytoperm reagents, as directed, blocked using 5% goat serum and Fc receptor specific antibody (24G2 for mouse, or BioLegend Human TruStain FcX), and stained with anti-TNF (BioLegend). Signaling samples were permeabilized using ice cold MeOH for 1-18 hours at -20C, blocked using 5% goat serum and Fc receptor specific antibody, and stained for 1h at RT with anti-IκBα (Cell Signaling Technology L35A5), anti-Erk (phospho-Thr202/Tyr204, Cell Signaling Technology D13.14.4E or 197G2), and/or anti-p38 (phospho-Thr180/Try182, BD 36/p38). Data were collected on a BD Fortessa, analyzed in FlowJo.

**Protein detection by high content imaging and Western blotting**

For imaging experiments, cells were fixed by addition of paraformaldehyde to cell cultures at a final concentration of 1.6% for 10-minutes at RT. Cells were blocked in 5% goat serum and 0.3% Triton X-100, and subsequently stained overnight at 4°C with anti-IκBα (Cell Signaling Technology L35A5) or anti-p65 (Santa Cruz Technology sc-109), followed by an appropriate Alexa-Fluor conjugated secondary antibodies (Molecular Probes, Invitrogen). Antibody incubations were performed in the presence of 1% BSA and 0.3% Triton X-100. Images were collected on a CellInsight NXT and analyzed in Thermo Scientific HCS Studio. For Western blotting, cells were lysed on ice (10mM Tris-HCl, 140mM NaCl, 2nM EDTA, 10% NP40 lysis buffer containing Roche PhosSTOP and cOmplete ULTRA phosphatase and protease inhibitors) and boiled with SDS reducing sample buffer. Samples were electrophoresed on 4–20% Novex® Tris-Glycine Gels (Invitrogen), transferred to nitrocellulose (Bio-Rad), and probed with anti-Erk (phospho-Thr202/Tyr204, Cell Signaling Technology D13.14.4E) and anti-RhoGDI (Cell Signaling Technology), followed by HRP-conjugated secondary antibodies. Blots were developed

with SuperSignal Chemiluminescence Substrate (Thermo Scientific) and imaged using a Fujifilm LAS-4000. Bands were quantified using ImageJ.

**RNAseq library preparation and data analysis**

RNA was prepared as described above. RNA quality was confirmed by Agilent Bioanalyzer. RNA samples from triplicate wells per treatment were pooled and 500 ng of total RNA was used to prepare RNA sequencing libraries using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, Massachusetts) with Poly A selection module (New England Biolabs). Final libraries were sequenced on the Illumina Nextseq platform (Illumina, San Diego, California) using V1 reagents to a read depth of 20 to 25 million paired-end, 75 base-pair reads. Reads were mapped to the *M.musculus* UCSC mm9 genome assembly using Bowtie2 version 2.2.2 (Langmead and Salzberg, 2012). Counting of reads mapping to each gene was performed using *featureCounts* from the Subread package (Liao et al., 2014). Differential expression analysis was carried out using DESeq2 (Love et al., 2014) package for the R statistical platform (R version 3.1.3). Heatmaps were produced using GENE-E (Broad Institute). Regulatory element analysis was performed using DiRE (Gotea and Ovcharenko, 2008). Upregulated gene lists for DiRE, Venn diagrams and heatmaps, were derived using a DESeq2-calculated log2 fold change cutoff of >0.5 and Benjamini-Hochberg procedure false discovery rate (FDR) of 1%. As the background gene list for DiRE analysis, all genes used as in the featureCount mapping of reads to genes were used. RNAseq data are available in GEO (accession number: GSE70510).