Supplementary Figure legends

Suppl. Fig. 1 Anti-endotoxin efficiency of tilapia hepcidin (TH)1-5, TH2-2, TH2-3 and human hepcidin. (A) Raw264.7 cells were stimulated with lipopolysaccharide (LPS) at 10 ng/ml or LPS co-treated with either TH1-5, TH2-2, or TH2-3 at 100 μg/ml, and tumor necrosis factor (TNF)-α secretion into the medium was measured by an ELISA at 1, 2, 4, and 24 h of treatment. (B) Control and 10 ng/ml LPS in the first two column sets. In the next 3 column sets, TH1-5, TH2-2, and Th2-3 at 100 μg/ml were pretreated for 30 min followed by LPS addition. (C) Control and 10 ng/ml LPS are in the first two column sets. TH1-5, TH2-2, and TH2-3 post-treated 30 min after LPS. (D) Influence of human hepcidin in TNF-α regulation of LPS-stimulated cells. (E) TNF-α release in cells treated with the antimicrobial peptide alone. Total immunoglobulin G (IgG) in the medium was estimated by an ELISA, and TNF-α secretion was converted to relative percent expression to IgG. Each bar represents the mean values of three to five independent experiments (n=3~5), and the error bar represents the standard error of the mean (SEM). Values statistically differing at p<0.05 according to one-way ANOVA are indicated by '*'.

Suppl. Fig. 2 Tilapia hepcidin (TH)2-3 modulates lipopolysaccharide (LPS)-stimulated gene expression under a microarray. RAW264.7 cells were treated with 10 ng/ml LPS, 100 μg/ml TH2-3, or TH2-3 with 10 ng/ml LPS (pre- and post-treatments) for 24 h. The cDNA isolated from total RNA was analyzed by a mouse 44k oligo microarray, and genes with multiples of change of 1.5 and p<0.05 (n=2) were used in Pathway Studio 6.2. (A) Differentially expressed genes between [TH2-3] vs. [TH2-3+LPS (Post)] conditions. Genes showing direct interactions within the entity list were mapped using Pathway Studio 6.2 with respect to gene regulation under Adriane ontology. Genes were color-coded green for downregulated and red for upregulated expression. (B) Selective proinflammatory-associated genes up- and downregulated by TH2-3 under [TH2-3] vs. [TH2-3+LPS (Post)] treatment conditions were manually selected and are displayed. (C) Differentially expressed genes between [LPS] vs. [TH2-3+LPS (Pre)] conditions. Genes showing direct interactions within the entity list were mapped using Pathway Studio 6.2 with respect to gene regulation.

Suppl. Fig. 3 (A-F) Real-time PCR expression of selected genes in RAW264.7 cells treated with a cyclooxygenase (COX)-2 inhibitor. Multiples of change (Y-axis, linear/log scale) for each gene were normalized to GAPDH and are relative to the gene expression in unstimulated cells at each time point (set to 1) using the comparative Ct method. (G-H) Secretion of tumor necrosis factor (TNF)-α relative to immunoglobulin G (IgG) in the culture medium at 24 h after treatment was measured by an ELISA. The TNF-α percentage expression over IgG was calculated, and results are displayed in the figure. Each value in the line graph represents the mean of three independent experiments (n=3), and the error bar represents the standard error of the mean (SEM). Values statistically differing at p<0.05 according to one-way ANOVA are indicated by '*'.

Suppl. Fig. 4 Effects of various inhibitors on lipopolysaccharide (LPS)-stimulated RAW264.7 cells in the presence and absence of tilapia hepcidin (TH)2-3. Overnight-cultured RAW264.7 cells were treated with 10 ng/ml of lipopolysaccharide (LPS) and/or 100 μg/ml of TH2-3 in the presence and absence of various phosphatase and kinase inhibitors in serum-free medium for 4 h. Secretions of tumor necrosis factor (TNF)-α and IgG in the supernatants of control, LPS, TH2-3t, and LPS co-treated with TH2-3 cells were assayed by an ELISA. The TNF-α percentage expression over IgG was calculated, and results are displayed in the figure. Each value in the line graph represents the mean of three independent experiments (n=3), and the error bar represents the standard error of the mean (SEM). Values statistically differing at p<0.05 according to one-way ANOVA are indicated by '*'.

Suppl. Fig. 5 Flow cytometric analysis of NF-κB proteins p65 and NF-κB2 levels in the presence and absence of tilapia hepcidin (TH)2-3 in lipopolysaccharide (LPS)-stimulated RAW264.7 nuclei and whole cells. (A) Nuclei were isolated at the end of treatment, and stained with p65-AF488- or NF-kB2-AF488-conjugated primary antibodies and 10 μg/ml of propidium iodide (PI). Then cells were analyzed by BD FACScaliber, and the shift in the florescence intensity is displayed in the figure. Results shown are representative of three independent experiment. (B) p65-cy3 and NF-kB2-cy3 expression in RAW264.7 whole cells after intracellular staining.

Suppl. Fig. 6 Secretion of tumor necrosis factor (TNF)-α relative to immunoglobulin G (IgG) in the culture medium of RAW264.7 cells (non-OE), RAW264.7 cells overexpressing

COX-2, and treated with TH2-3, LPS, or LPS together with TH2-3 (Pre-, Co-, and Post-treated conditions) for 24 h, and TNF-α and IgG were measured by an ELISA. The TNF-α percentage expression over IgG was calculated, and results are displayed. Each bar represents the mean values of three independent experiments (n=3), and the error bar represents the standard error of the mean (SEM). Values statistically differing at p<0.05 according to one-way ANOVA are indicated by '*'.

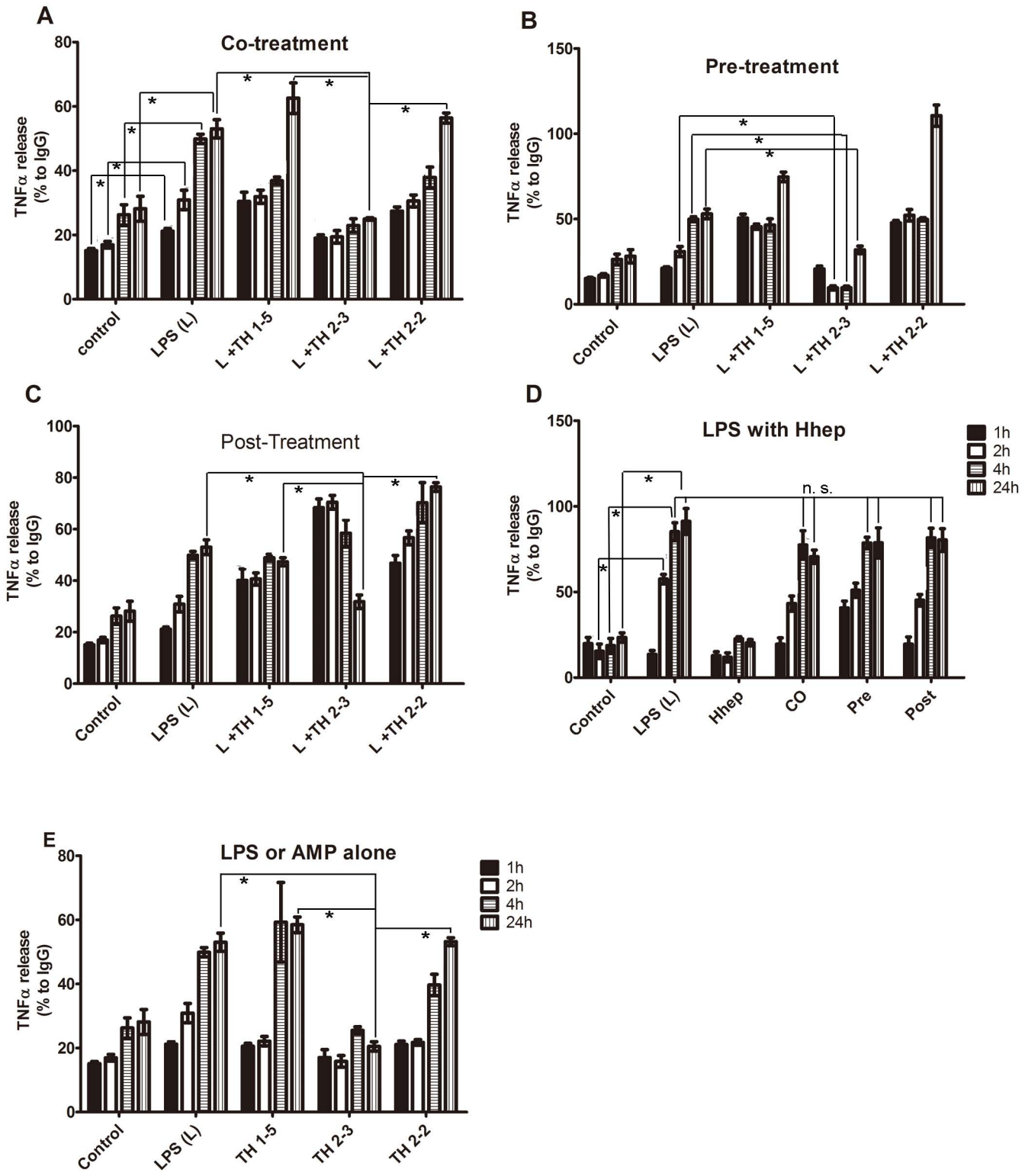
Suppl. Table. 1 List of primers used for real-time PCR

Suppl. Data. 1 (A) Differential gene expressions between tilapia hepcidin (TH)2-3 vs. post- (LPS for 0.5 h + TH2-3) treatments in 24 h-treated samples. (B) Differential gene expression between LPS vs. pre- (TH2-3 for 0.5 h + LPS) treatment in 24 h-treated samples. (C) Expression of cytokines in 24 h-treated RAW264.7 cells under a cytokine array

Suppl. Table. 1 List of primers used for real-time PCR

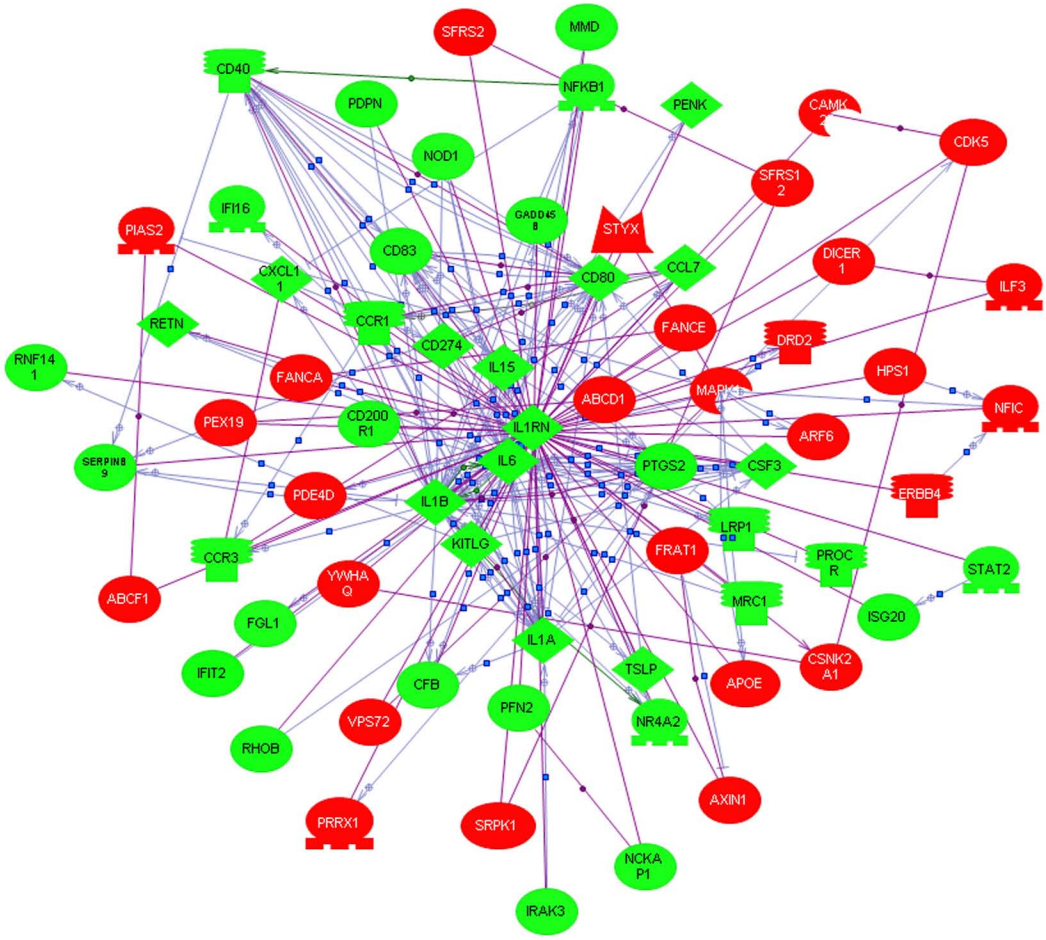
Gene	Forward	Reverse
TNF-α	GAACTGGCAGAAGAGGCACT	GGTCTGGGCCATAGAACTGA
COX-2	TCCATTGACCAGAGCAGAGA	TCTGGACGAGGTTTTTCCAC
IL-1α	TGTGAAATGCCACCTTTTGA	TGAGTGATACTGCCTGCCTG
IL-1β	CGTCAGGCAGAAGTTTGTCA	TTAGAGTCGTCTCCTCCCGA
IL-6	CCGGAGAGGAGACTTCACAG	CAGAATTGCCATTGCACAAC
NF-κBiZ	TATCGGGTGACACAGTTGGA	TGAATGGACTTCCCCTTCAG
SIAT1	TCGAGACCACGTGAATGTGT	TTAAACCTCAGGACCGCATC
PTGER2	TCTCGCAGGAGAGGAGAGAG	TAAAAACCGAAGAGCTCGGA
PTGES	TTTCTGCTCTGCAGCACACT	TCCACATCTGGGTCACTCCT
PDE4D	ATCTAGGATGTCCTGGCCCT	CTCCAATTCTCCAGGCATGT
IL-23α	CCAGCGGGACATATGAATCT	AGGCTCCCCTTTGAAGATGT
GAPDH	GGCATTGCTCTCAATGACAA	TGTGAGGGAGATGCTCAGTG

Supplementary figure 1



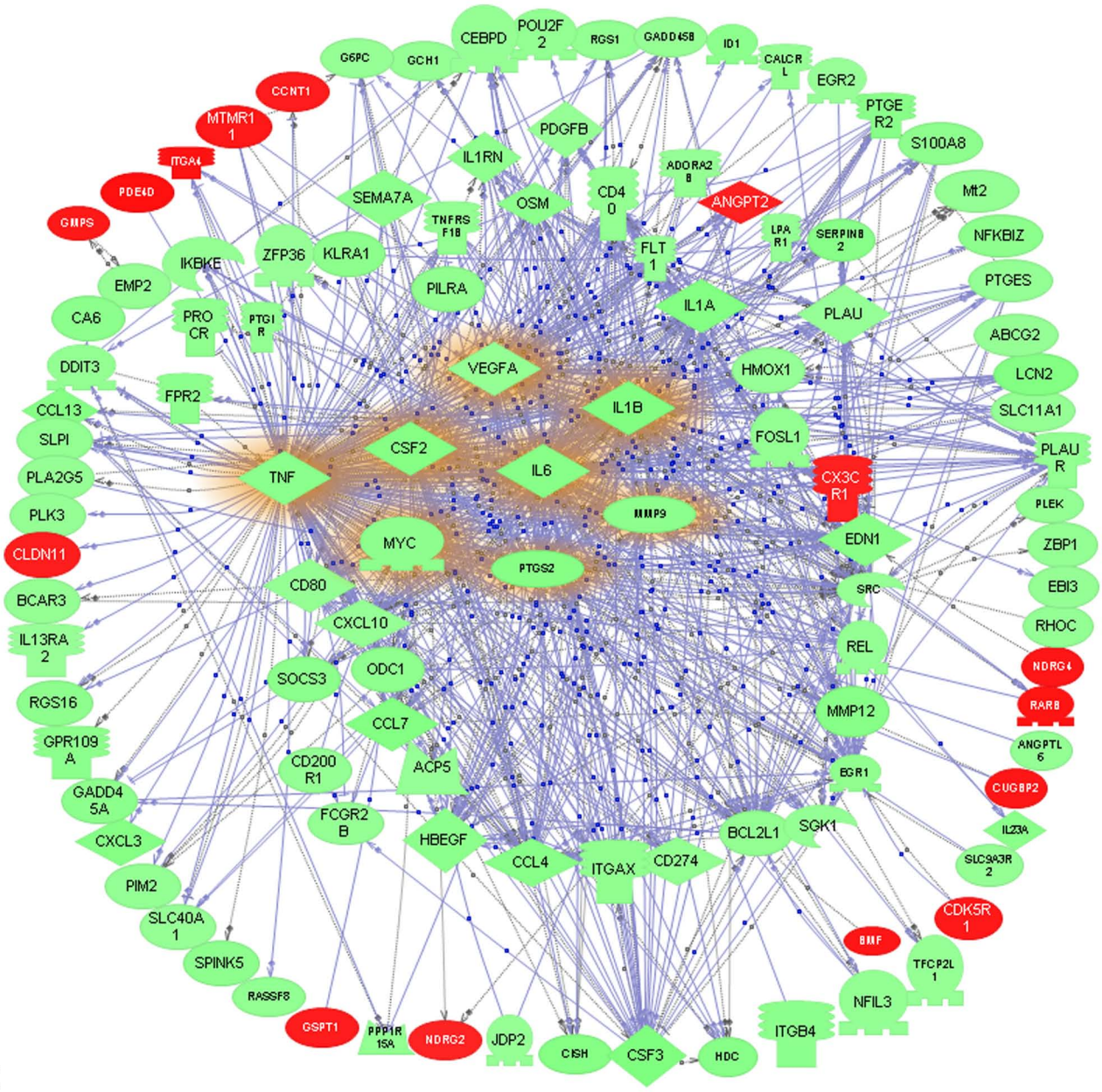
Supplementary figure 2

A



Supplementary figure 2

B



C

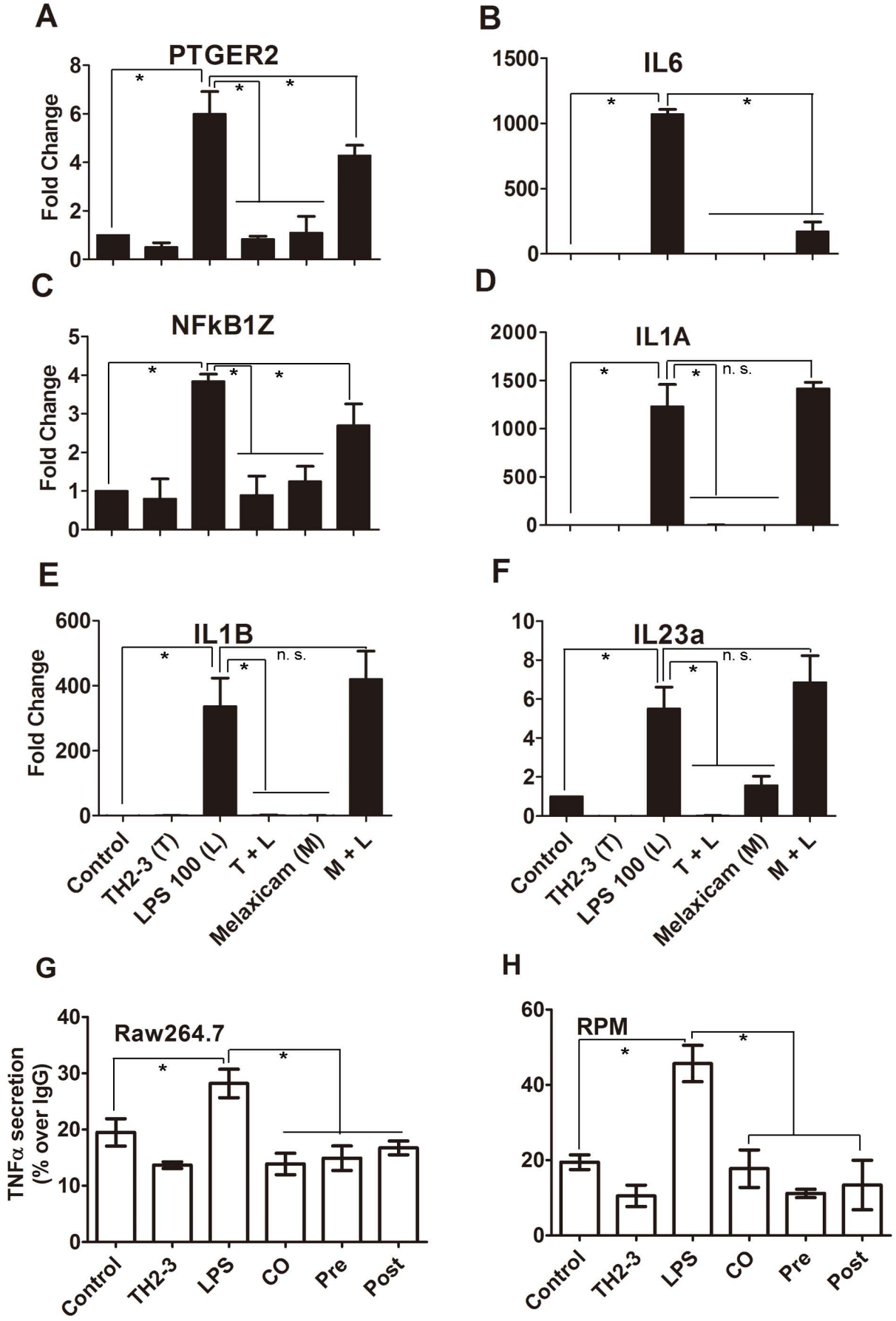
Genes Down regulated by TH2-3

TNF-α, PTGS2, PTGIR, PTGES, PTGER2, NFKB1Z, IL6, IL23A, IL1RN, IL1b, IL1A, ID1, CXCL3, CXCL10, CSF3, CSF2, cREL, cMYC, CD80, CCL7, CCL4, CCL13, BCL2L1, TNFSRF1B, SOCS3, SGK1, RGS16, MT2, MMP9, MMP12.

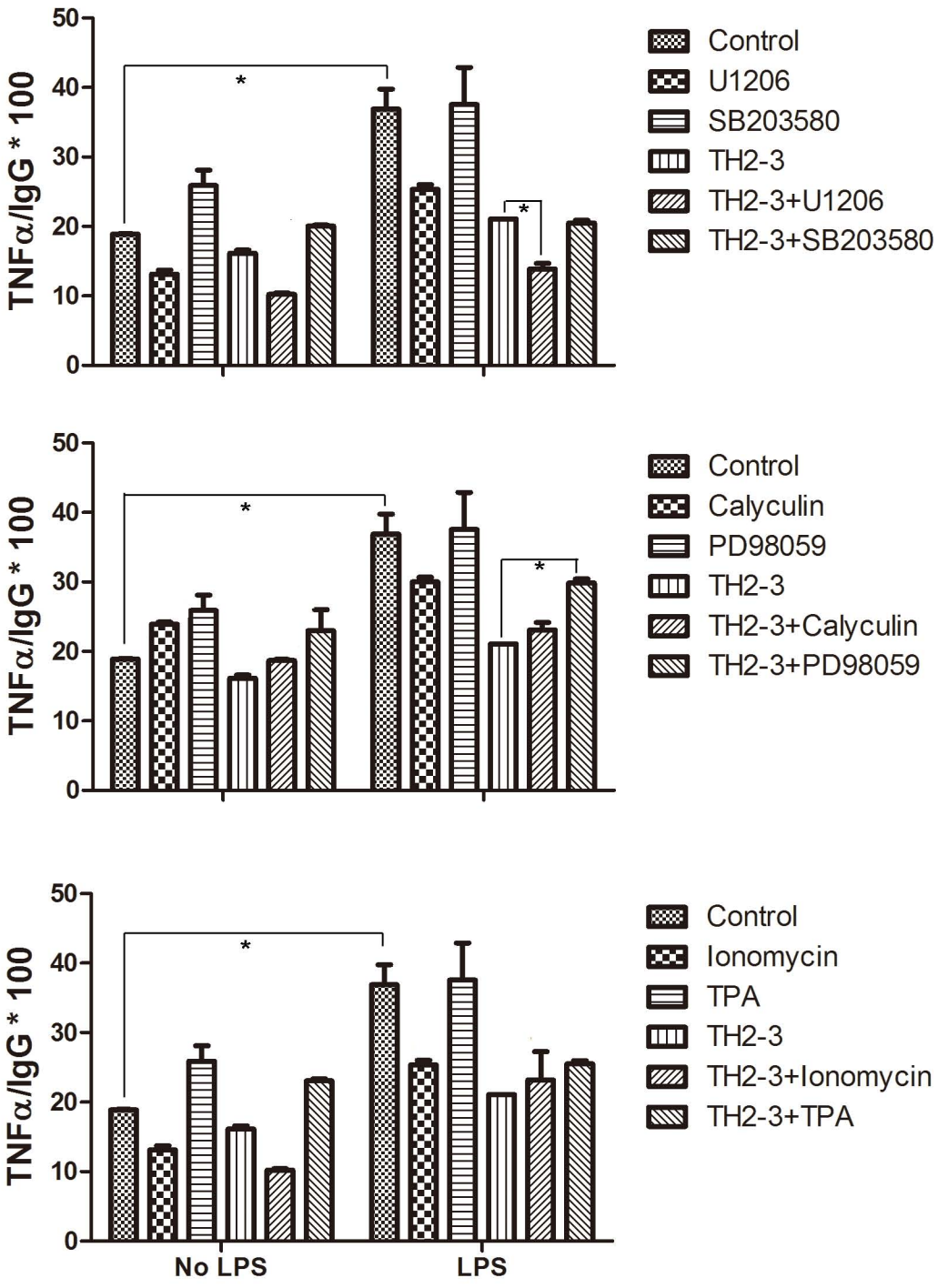
Genes Up regulated by TH2-3

PDE4D, NDRG4, NDRG2, MTMR11, ITGA4, GMPS, GLDN11, CXCR1, CUGBP2, BMF, ANGPT2, RARB.

Supplementary figure 3

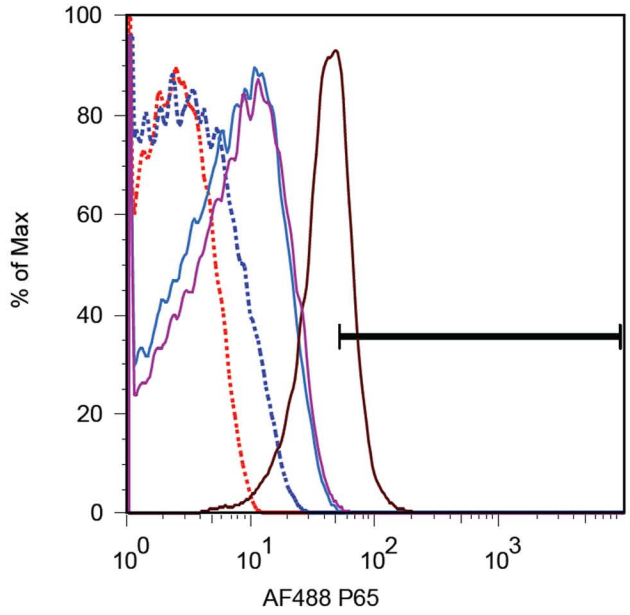


Supplementary figure 4



Supplementary figure 5

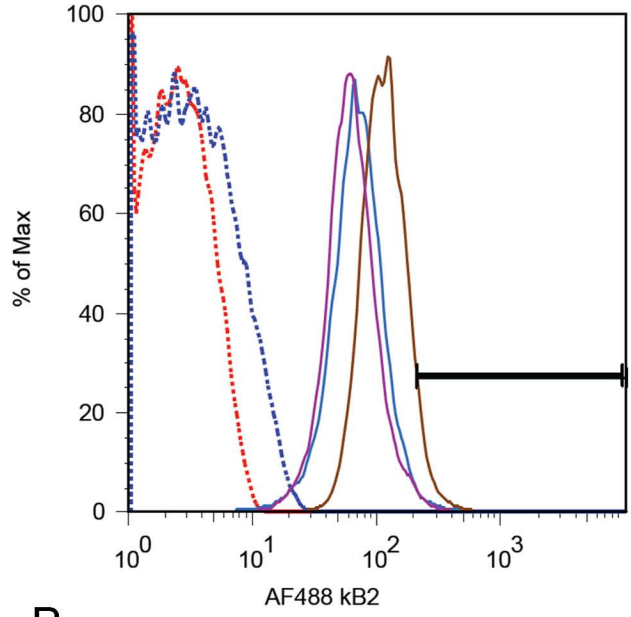
A



p65

Sample Name
P65 AF488 PI L+T.022
P65 AF488 PI LPS.021
P65 AF488 PI CTRL.020
isotype + PI .013
unstain.001

Samples	Mean	Freq. of
unstain.001		
PI only.012	3.52	
isotype + PI .013	3.55	
P65 AF488 PI CTRL.020	10.92	0.12%
P65 AF488 PI LPS.021	44.49	31.88%
P65 AF488 PI L+T.022	9.28	0.12%
No value	No value	No value
No value	No value	No value

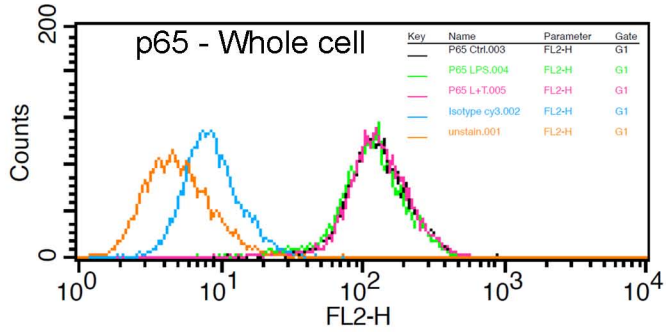


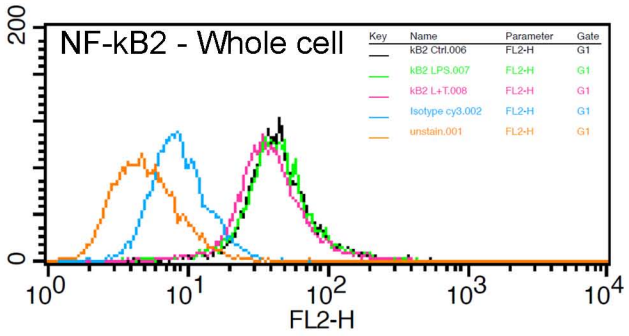
NF-kB2 (kB2)

Sample Name
kB2 AF488 PI L+T.019
kB2 AF488 PI LPS.018
kB2 AF488 PI CTRL.017
isotype + PI .013
unstain.001

Samples	Mean (FL1-H),	Freq. of
unstain.001		
PI only.012	3.52	
isotype + PI .013	3.55	
kB2 AF488 PI CTRL.017	77.41	1.59%
kB2 AF488 PI LPS.018	124.09	7.08%
kB2 AF488 PI L+T.019	70.07	1.40%
No value	No value	No value
No value	No value	No value

B





Supplementary figure 6