Title: Myeloid TBK1 signaling contributes to the immune response to influenza

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ONLINE DATA SUPPLEMENT

Supplemental Online Methods

Cell culture – To generate macrophages, bone marrow was flushed from mouse femurs onto non-tissue culture treated plates and grown in media containing 20% L929-conditioned media and 10% FCS. Nonadherent cells were removed after 24 hours and media was changed at day 4 and day 6. After day 7 cells were washed into RPMI supplemented with 10% FCS and 1% penicillin-streptomycin and glutamine. Alveolar macrophages were isolated by bronchoalveolar lavage with PBS/EDTA from euthanized mice and checked for purity by flow cytometry for CD64 and F4/80. For western blot experiments, macrophages from mice of the same genotype were pooled to achieve sufficient numbers. WT and TBK1 KO MEFs were a kind gift of Dr. Tony Polervino (Amgen). LPS (E. coli O55:B5 ultrapure) and poly(I:C) were from Invitrogen.

RNA isolation and analysis of gene expression – tissue culture and sorted cells were lysed in QIAzol lysis reagent (Qiagen). Lungs were snap frozen in liquid N_2 at the time of harvest and homogenized in QIAzol. RNA was purified using the RNA Easy kit (Qiagen) and reverse transcribed with Superscript III RT (Thermo Fisher) or iScript (Bio-Rad). cDNA was amplified by qPCR using TaqMan Universal Master Mix II (Invitrogen) or SSo Advance Supermix (Bio-Rad) on an ABI 7500 Fast or ABI OneStepPlus thermocycler with Taqman probes (Applied Biosystems, Thermo Fisher). Samples included a reference 18S RNA probe, and gene expression was quantitated by $2^{-\Delta\Delta Ct}$ method(36). Taqman probes (Applied Biosystems/Thermo Fisher) are described in Supplemental Methods Table 1. IAV M2 gene was quantitated by RT-qPCR using Syber Green (Thermo Fisher) and previously described primers(37). For *in vivo* experiments, fold induction was calculated relative to an average of 3 separate day 0 lung samples per

genotype. For data in Figure 3C and Figure 7C, qPCR was performed with Sybr Green (Thermo Fisher) using primer pair sets derived from the Massachusetts General Hospital Primer Bank.

Viral titering -- Lung viral levels were determined by $TCID_{50}$ method on MDCK cells. Lungs were snap frozen in liquid N_2 at time of harvest. For analysis, lungs were thawed on ice and homogenized in ice cold PBS and $TCID_{50}$ was measured by serial dilution on MDCK cells.

Immunoblotting and antibodies – Cells were lysed in complete RIPA buffer and lysates were resolved by SDS-PAGE. Prior to western blotting, lysates were transferred to nitrocellulose membranes. For phospho-specific antibodies, membranes were blocked with 1%/BSA in TBST prior to probing. Antibodies are listed in the Supplemental Methods Table 2.

Flow cytometry and sorting – Lungs were digested by intratracheal instillation via a 20-gauge catheter with 1 mL of 5 mg/mL collagenase I (Worthington Biochemical Corp) and 0.25 mg/mL DNase I (Sigma) prepared in RPMI media (Life Technologies) prior to instilling 0.5 mL of 1% (wt/vol) low melting agarose (Invitrogen) similar to previous descriptions(34). Lungs were incubated at 37°C for 30 minutes and then minced, triturated through an 18-gauge needle, and filtered through a 100 µM filter prior to RBC lysis and staining. Single cell suspensions were suspended in buffer (PBS/1.6% BSA, Sigma) and total cell count determined by hemocytometer. Cells ($1.0\text{-}1.5 \times 10^6$) were blocked with rat anti-mouse FcγRIII/II receptor (CD16/32; BD Biosciences) for 5-10 minutes at 4° C to prevent non-specific antibody binding. After Fc blocking, cells were surface stained for 15-20 minutes at room temperature. Unbound antibody was washed away and samples were fixed with 1% paraformaldehyde. Flow cytometry was performed using a Cytoflex flow cytometer (Beckman Coulter, Brea, CA) and analyzed using

CytExpert (Beckman Coulter) software. Antibodies are described in Supplemental Methods Table 3. Cell sorting was performed using a BD FACSAria II. AM, IM and InfM subpopulations were isolated based on expression patterns described above. Purity was confirmed by post-sort cytometry and was over 95% in all experiments.

Supplemental Methods Table 1: RT qPCR probesets for gene expression studies

Gene	Thermo Taqman probe number
IFNα4	mm00833969_s1
IFNβ1	mm00439552_s1
TNFα	mm00443258_m1
IL6	mm00515153_m1
IFIT1	mm00446190_m1
Mx1	mm00487796_m1
CXCL10	mm00445235_m1
18S	4310893E
CCL5/RANTES	mm01302427_m1
IL10	mm00439614_m1
CCL2	mm00441242_m1
CCL3	mm00441259_m1
CCL4	mm00443111_m1
IL12a	mm00434165_m1
CCL20	mm0044228_m1

Gene	Primer pair sequence
ActB	GATGTATGAAGGCTTTGGGTC TGTGCACTTTTATTGGTCTC
IL6	GTCAGGGGTGGTTATTGCAT AGTGAGGAACAAGCCAGAGC
CXCL10	GCCGTCATTTTCTGCCTCA CGTCCTTGCGAGAGGGATC
Mx1	GACCATAGGGGTCTTGACCAA AGACTTGCTCTTTCTGAAAAGCC

TNFα	CCCTCACACTCAGATCATCTTCT GCTACGACGTGGGCTACAG
CCL2	TGGCTCAGCCAGATGCAGT TTGGGATCATCTTGCTGGTG
CCL4	TCTTGCTCGTGGCTGCCT GGGAGGGTCAGAGCCCA
CCL5	CAAGTGCTCCAATCTTGCAGTC TTCTCTGGGTTGGCACACAC
IFNβ1	CAGCTCCAAGAAAGGACGAAC GGCAGTGTAACTCTTCTGCAT

Supplemental Methods Table 2: Western blot antibodies

Antibody	Manufacturer	Catalog number
anti-TBK1	Cell Signaling	3013, 3054
anti-TBK1	Abcam	ab40676
anti-phospho TBK1 pS172	Cell Signaling	5843
anti-IKKϵ	Cell Signaling	2690
anti-IRF3	Cell Signaling	4947
anti-phospho IRF3 pS396	Cell Signaling	4962, 4302
anti-STING	Cell Signaling	13647
anti-p38	Cell Signaling	9212
anti-phospho p38 pT180/pY183	Cell Signaling	4511
anti-p65 RelA	Cell Signaling	8242
anti-phospho p65 pS536	Cell Signaling	3033
anti-histone H3	Cell Signaling	4499
anti-ISG15	Cell Signaling	2743
anti-beta actin	Sigma	A5441

Supplemental Methods Table 3: Flow cytometry antibodies

Antibody	Manufacturer	Catalog number
anti-CD45 FITC	BioLegend	103108
anti-Siglec F PE	BD Pharmingen	552126
anti-CD8 PE-CF594	BioLegend	100762
anti-γΔ TCR PerCP-Cy5.5	BioLegend	118118
anti-CD64 PE-Cy7	BioLegend	139314
anti-CD4 AlexaFluor 700	BioLegend	100430
anti-CD11b APC-Cy7	BioLegend	101225
anti-Ly6C BV421	BD Horizon	562727
anti-NK1.1 BV785	BioLegend	108749
anti-CD11c BV605	BioLegend	117333
anti-Ly6G APC	BioLegend	127613

Supplemental Figure 1: After intratracheal administration, M-TBK1 mice have a less severe host response to influenza than WT mice. (A) Survival and **(B)** weight curve for mice infected intratracheally with 800 EID PR8 IAV (366 $TCID_{50}$) influenza PR8. The amount of virus is similar in the two genotypes, when measured by RT-PCR to identify H1N1 genomes (C) or by plaque-forming units (D).

Supplemental Figure 2: Flow cytometry gating of lung digest cells. Representative flow cytometry gating and analysis of cells from single cell lung digests of mice infected intranasally with influenza. Immune cell populations were identified as indicated.

Supplemental Figure 3: M-TBK1 KO mice do not have altered immune cell populations in the blood or spleen. Flow cytometry of (A) blood and (B) spleen from WT or M-TBK1 KO mice showing immune cell populations as a percent of CD45+ cells. N = 5 mice per genotype per group and two independent experiments.

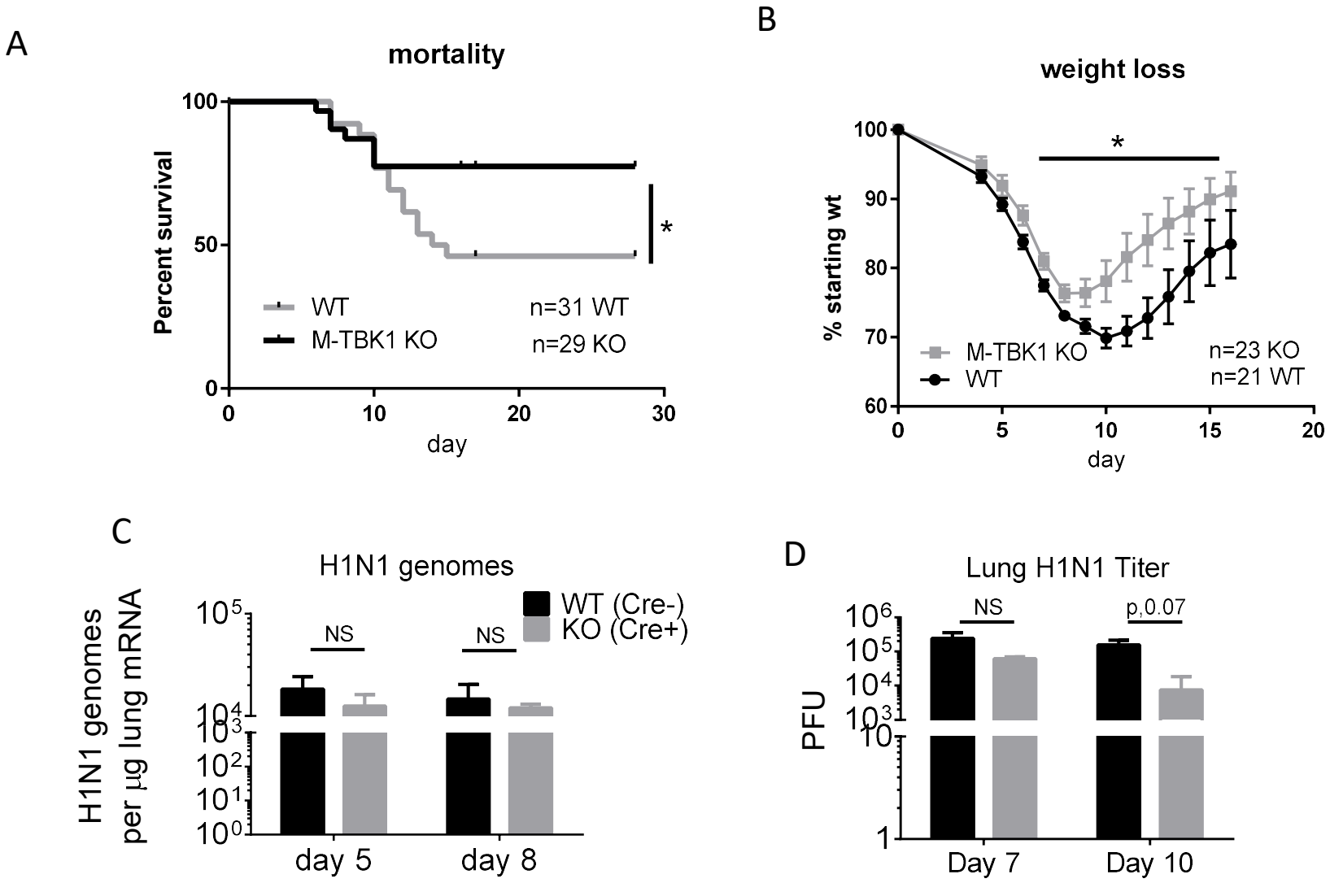
Supplemental Figure 4: Cytokine protein expression from M-TBK1 KO BMMs infected with IAV *in vitro* (A) ELISA of supernatants from BMM infected with IAV (MOI 1) for 24 hours. Uninfected and mock infected cultures showed negligible cytokine expression and no difference between WT and KO samples and are not shown. **(B) BMM but not AM upregulate TBK1 mRNA after 6h of IAV infection.** RT-qPCR of TBK1 from BMM or AM infected *in vitro* with IAV. Error bars represent technical triplicates and two independent experiments.

Supplemental Figure 5: Type 1 IFN expression in BAL (A) ELISA of IFNα and IFNβ in BAL fluid of lungs harvested at days 3 and 6 post-infection. N=3-8 mice per point and two separate experiments analyzed by Mann-Whitney u test.

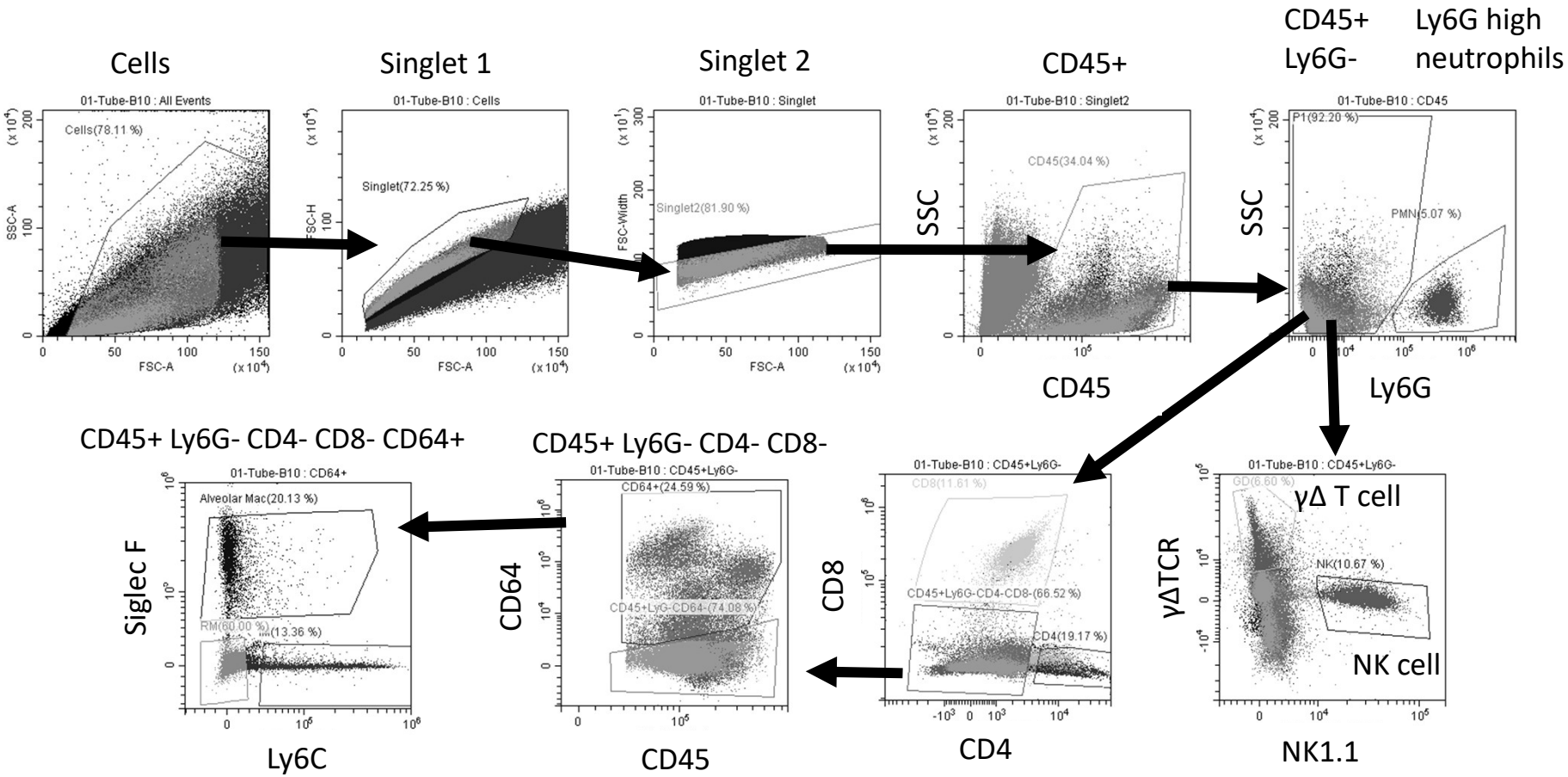
Supplemental Figure 6: RT-qPCR of (A) AM or (B) BMM infected with MOI 1 PR8 IAV *in vitro* for 6 hours or 6 hours and 24 hours. Data were normalized to the 18S mRNA and expressed as fold change compared to uninfected WT by ΔΔCt method. N = 3 per group and two independent experiments.

Supplemental Figure 7: Gene expression in macrophage subpopulations isolated from IAV-infected lungs. Macrophage subpopulations were isolated by FACS from lung digests at day 4 p.i. and subjected to RT-qPCR of indicated genes. mRNA levels were measured by qRT-PCR and normalized to ActB or to 18S by ΔΔCt. Data reflect 3 pooled mice analyzed in technical triplicate.

Supplemental Figure 1

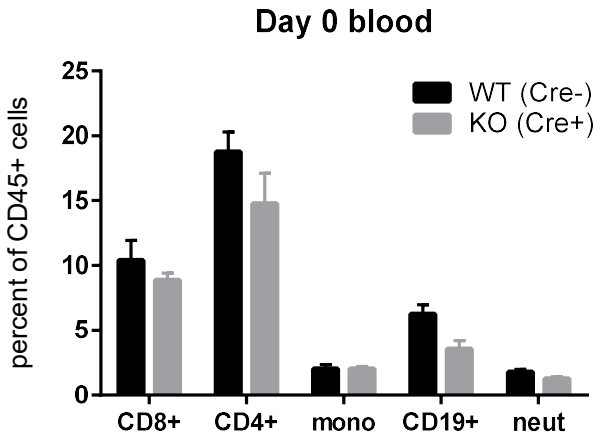


Supplemental figure 2



Supplemental figure 3

A



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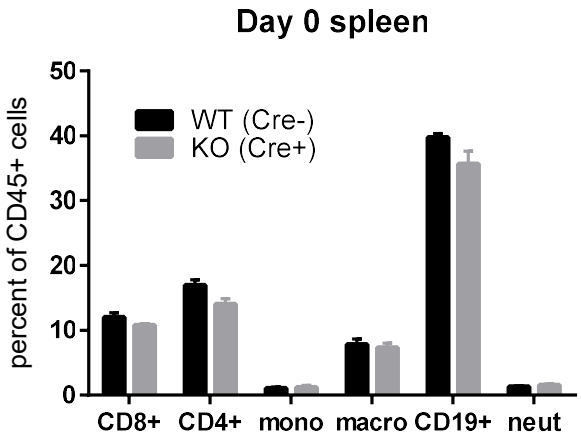
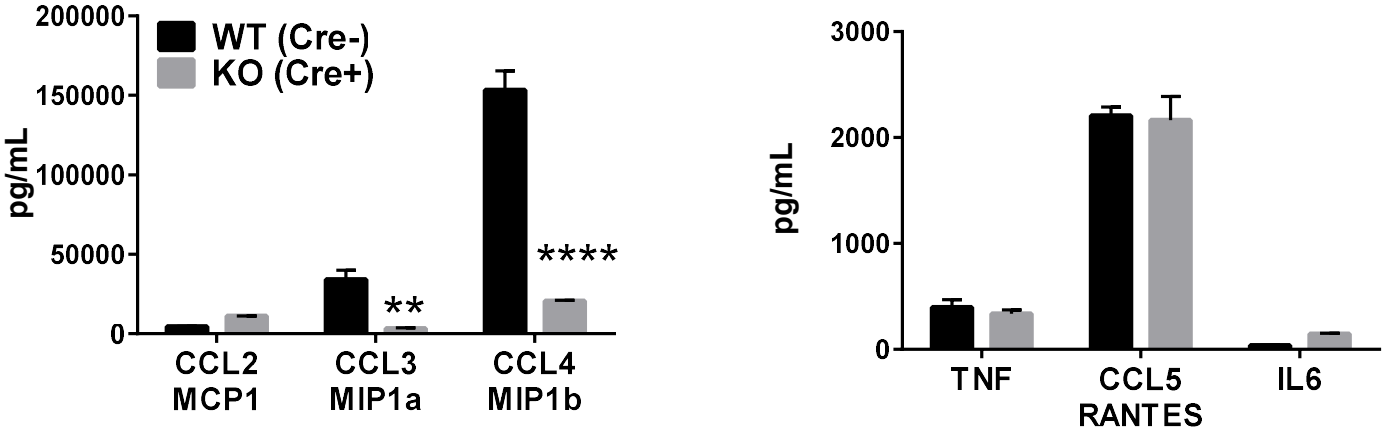


Supplemental Figure 4

A



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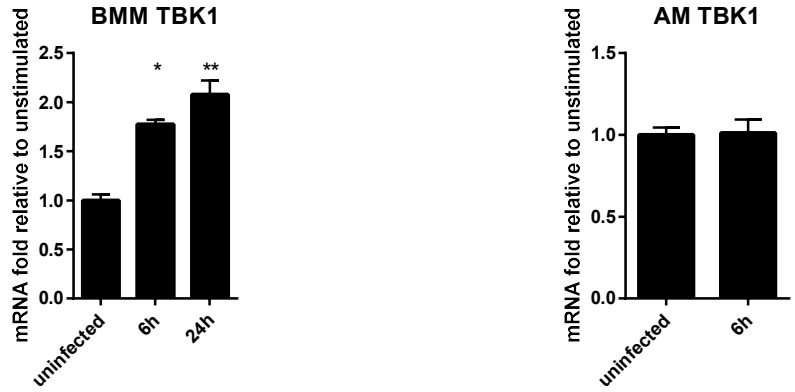
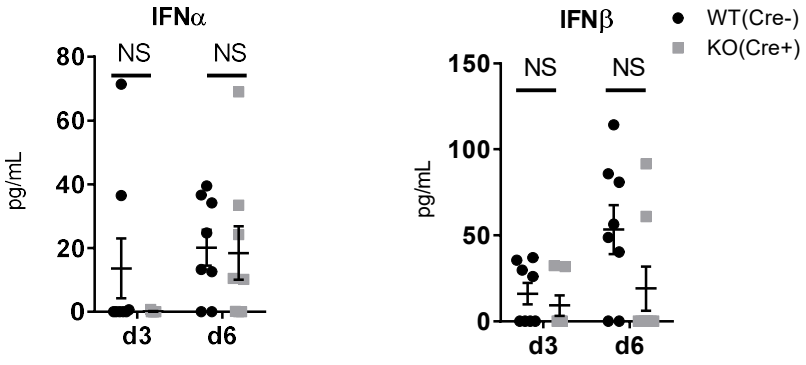


Supplemental Figure 5



Supplemental Figure 6

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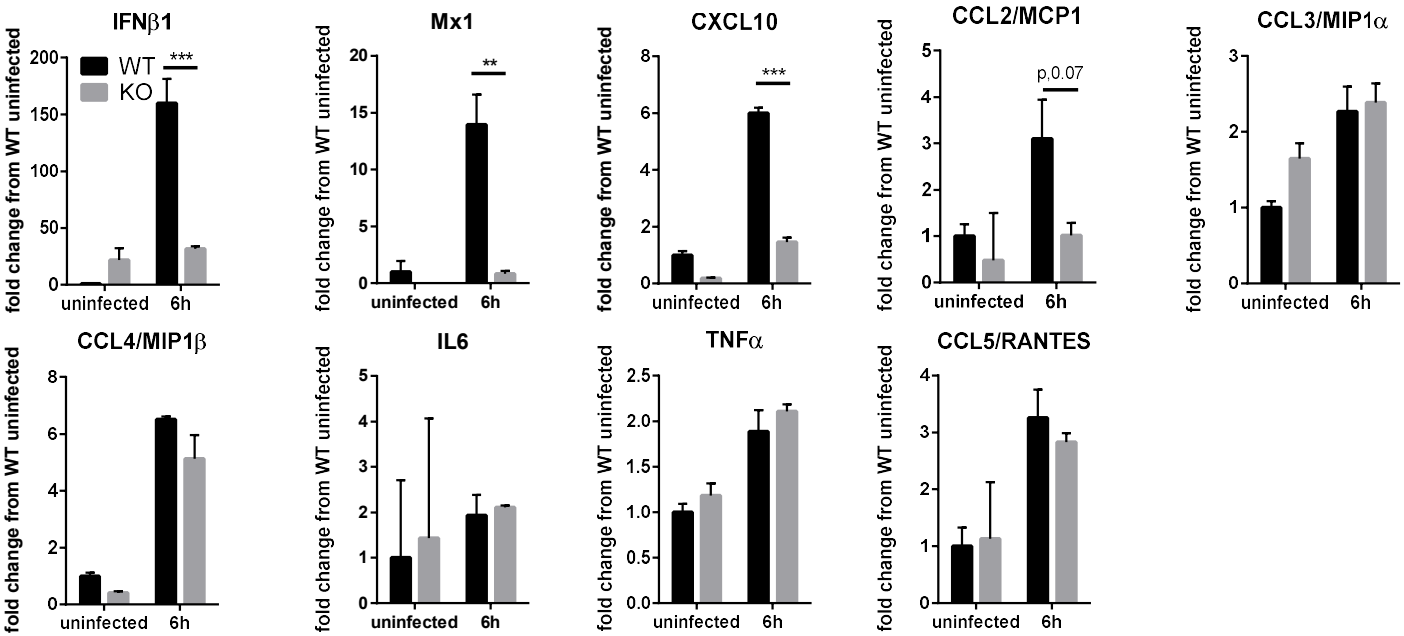

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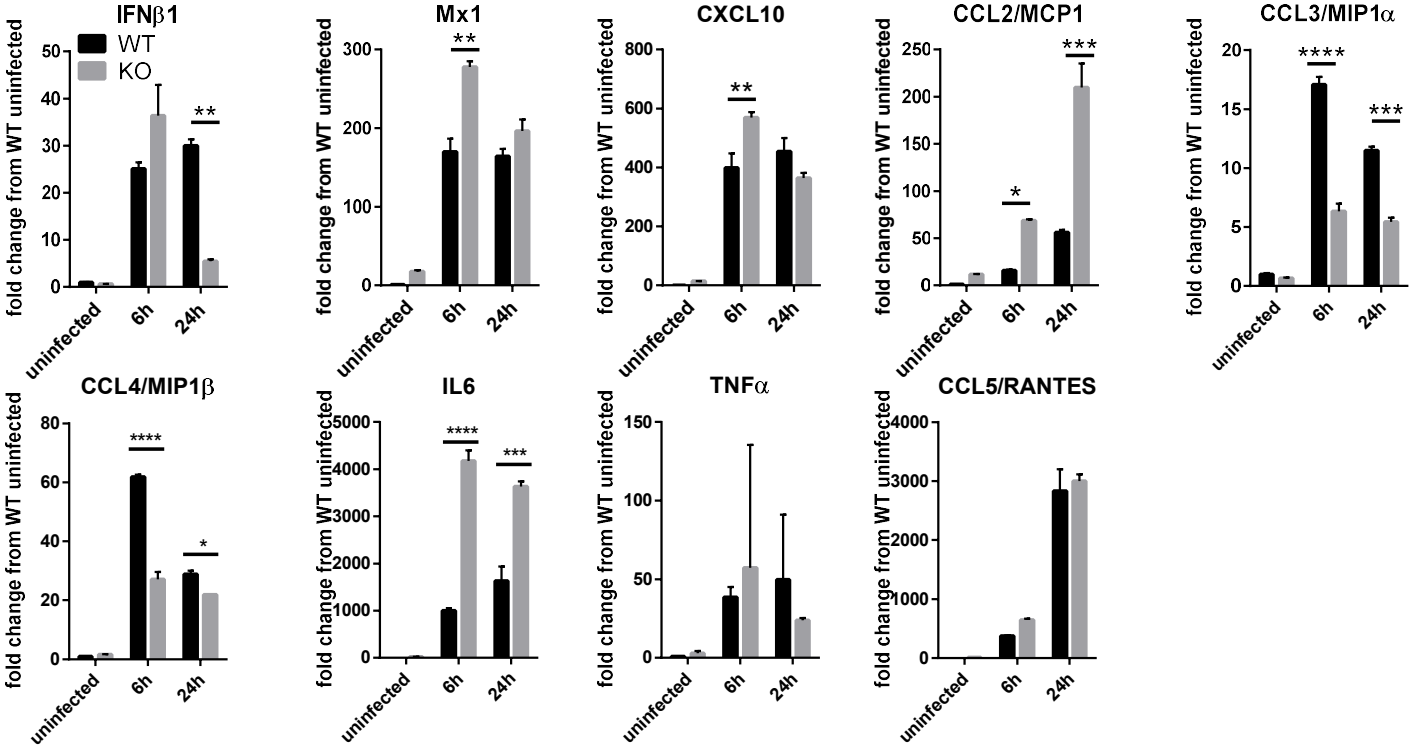

Supplemental
Figure 7