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Supplemental Information

A *Batf3*/*Nlrp3*/IL-18 Axis Promotes Natural Killer Cell IL-10 Production during *Listeria monocytogenes* Infection

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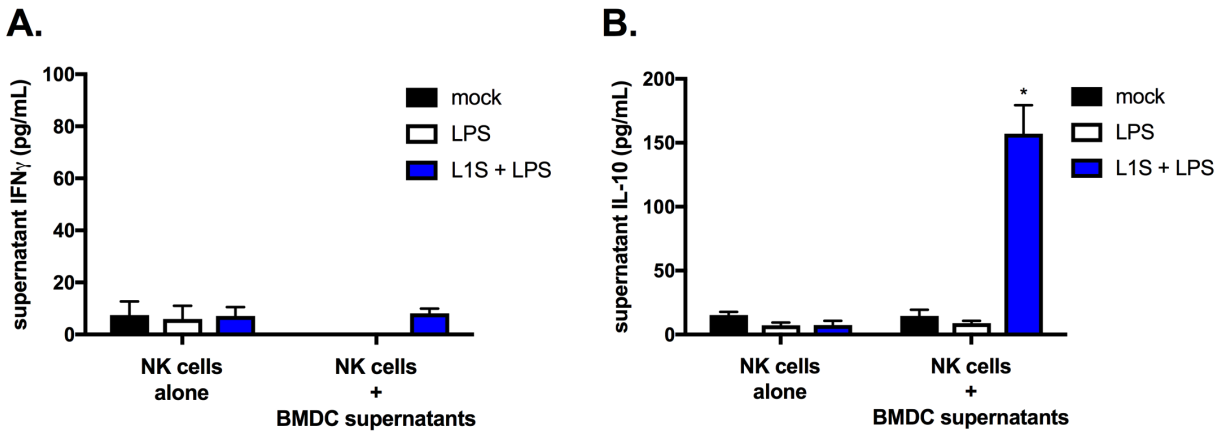

Figure S1. Accessory cell secreted factor(s) are required for NK cell IL-10. *Related to Figure 1.*

(**A-B**) Supernatant cytokines detected from NK cell cultures at 24 h (IFNγ, A) or 72 h (IL-10, B) post-stimulation with LPS or L1S+LPS directly (NK cells alone) or exposure to filtered supernatants collected 1 h post-stimulation of B6.*il10*$^{-/-}$ BMDCs with LPS or L1S+LPS (NK cells + BMDC supernatants). Data were pooled from three independent experiments and are displayed as mean ± SEM with *p<.05 as measured by *t*-test.

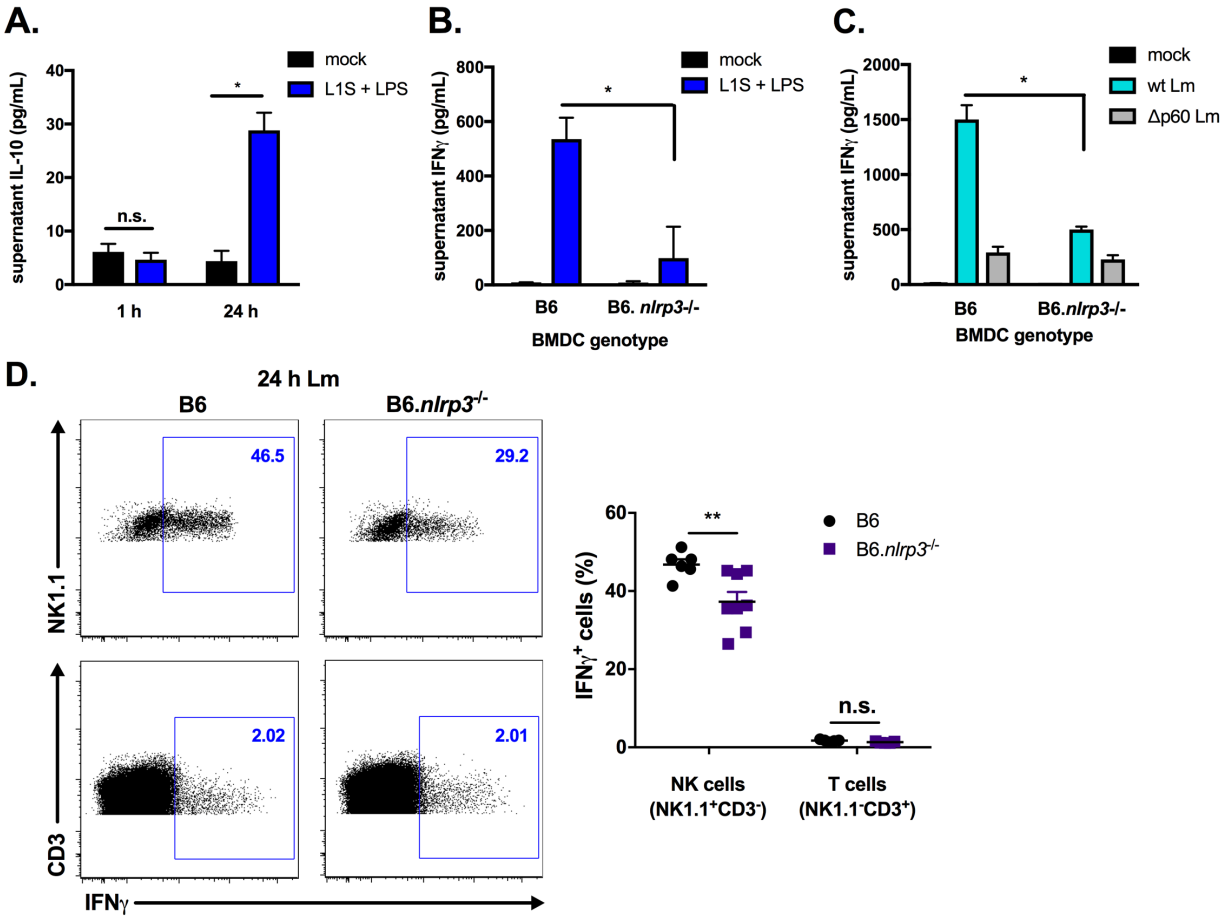

Figure S2. NLRP3 is required for Lm-induced NK cell IFNγ. *Related to Figure 2.*

(**A**) B6 BMDC cell culture supernatant IL-10 detected at indicated time points post-stimulation with L1S+LPS (n = 3 independent experiments pooled). (**B-C**) Supernatant IFNγ detected from NK cell cultures at 24 h post co-culture with B6 or B6.*nlrp3*$^{-/-}$ BMDCs stimulated with L1S+LPS (B) or infected with WT or Δp60 Lm (C), (n = 3 independent experiments pooled). (**D**) Intracellular IFNγ produced by NK1.1^{+}CD3^{-} cells (NK cells) or NK1.1^{-}CD3^{+} cells (T cells) detected from mice at 24 hpi with 10^4 Lm i.v. (n = 2 experiments with 3-5 mice per group). Data are displayed as mean ± SEM with *p<.05, **p<.01 as measured by t-test.

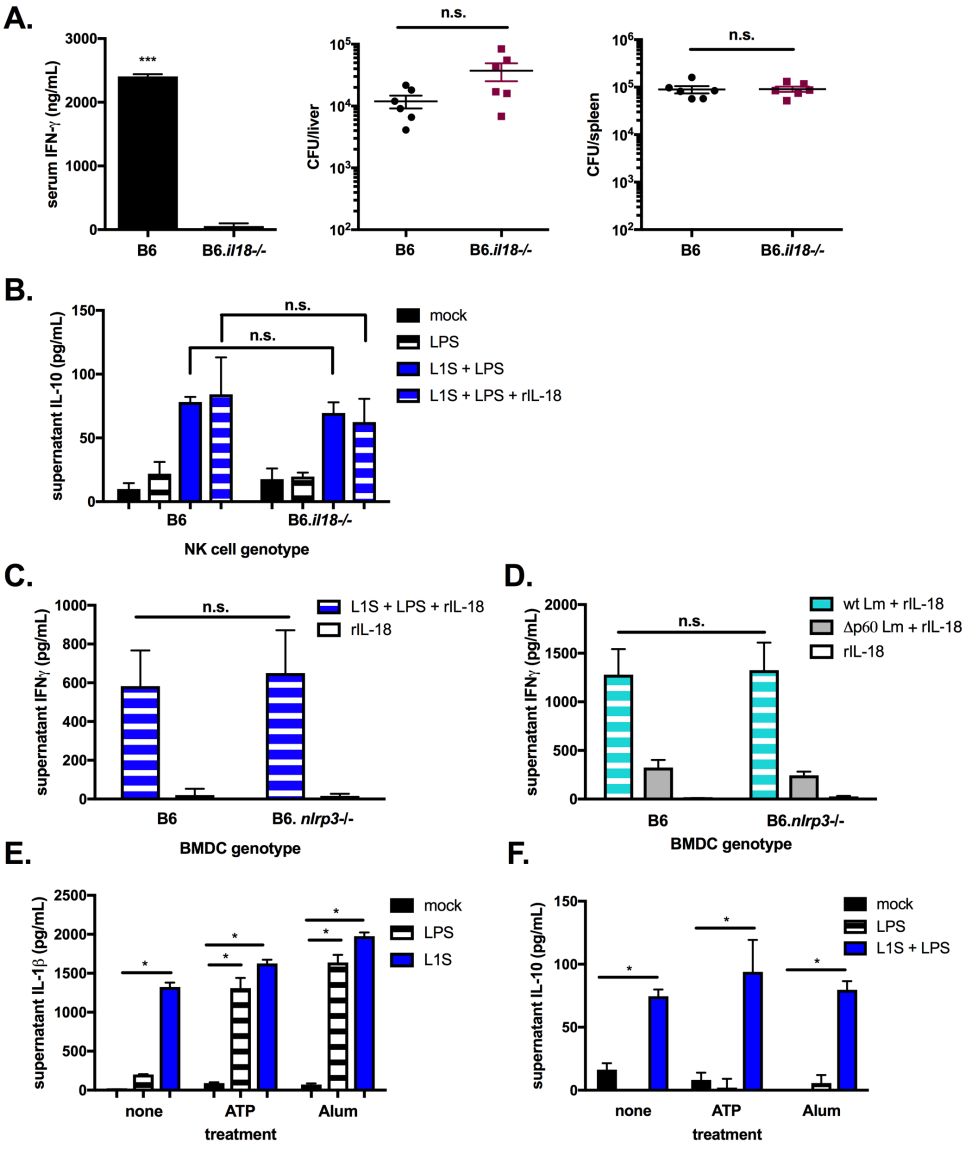

Figure S3. The contribution of IL-18 to Lm-induced IFNγ. *Related to Figure 3.*

(A) Serum IFNγ and Lm burdens per organ from B6 or B6.*il18*$^{-/-}$ mice sacrificed 24 hpi with 10^4 Lm i.v. ($n = 2$ experiments with 3-5 mice per group). (**B**) Supernatant IL-10 detected from B6 or B6.*il18*$^{-/-}$ NK cells at 72 h post exposure to filtered supernatants collected 1 h post-stimulation of B6 BMDCs with L1S+LPS ± 50 pg/mL rIL-18 added to NK cell cultures ($n = 3$ independent experiments pooled). (**C-D**) Supernatant IFNγ detected from NK cell cultures at 24 h post co-culture with B6 or B6.*nlrp3*$^{-/-}$ BMDCs stimulated with L1S+LPS (C) or infected with WT or Δp60 Lm (D) + 50 pg/mL rIL-18 added to co-cultures ($n = 3$ independent experiments pooled). (**E**) Supernatant IL-1β detected at 24 h from BMDC cell cultures post-stimulation with ATP ± LPS (3 h LPS pre-stimulation and 30 min ATP stimulation) or Alum ± LPS (3 h LPS pre-stimulation and 1 h Alum stimulation) ± L1S ($n = 3$ independent experiments pooled). (**F**) Supernatant IL-10 detected from NK cells at 72 h following exposure to filtered supernatants collected from B6 BMDCs stimulated with ATP ± LPS or Alum ± LPS ± L1S at 1 h post (final) stimulation ($n = 3$ independent experiments pooled). Data are displayed as mean ± SEM with *p<.05, ***p<.001 as measured by *t*-test.

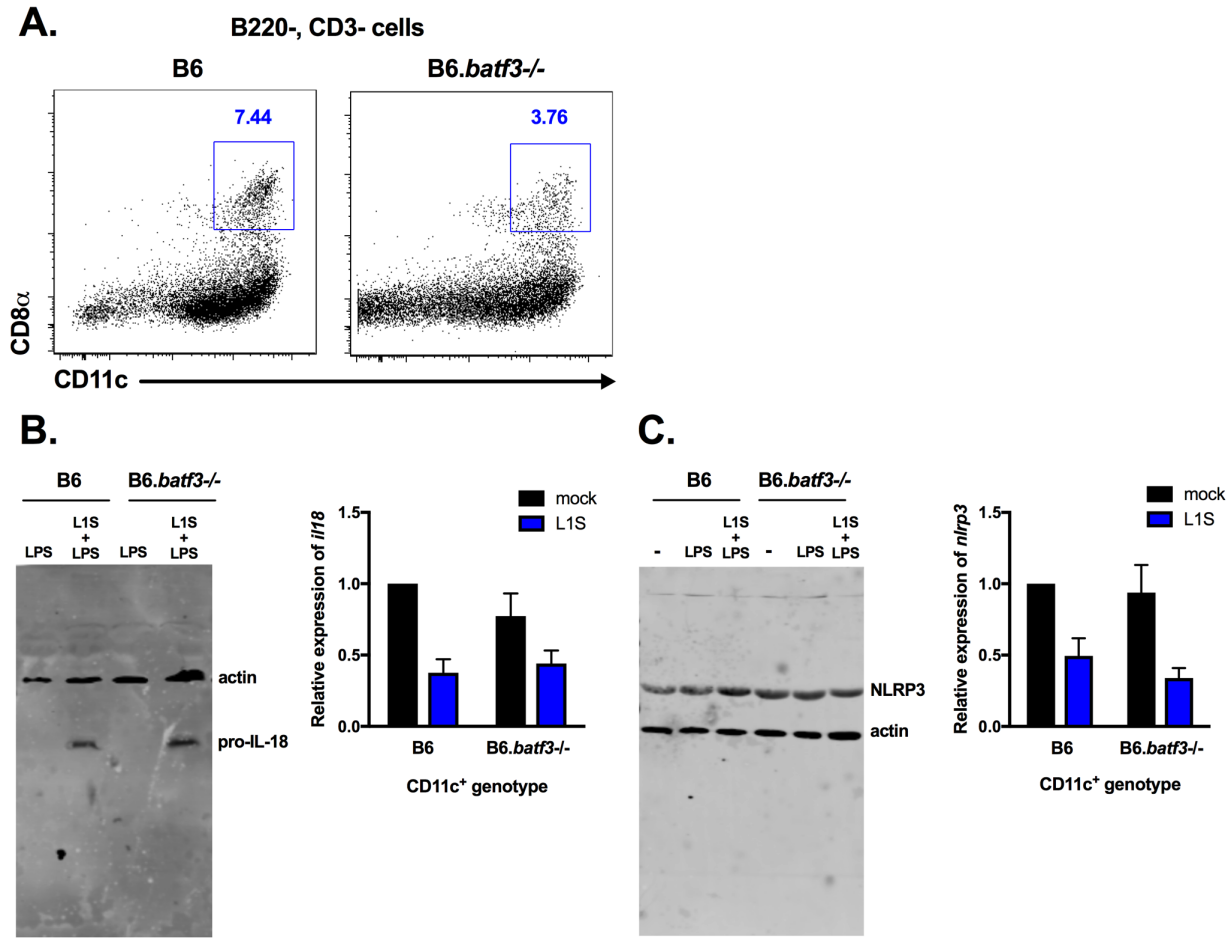

Figure S4. IL-18 and NLRP3 are expressed in *Batf3*-deficient DCs. *Related to Figure 5.*

(A) CD8α^{+}CD11c^{+} cells detected by flow cytometry from B220^{-}CD3^{-} gate of purified CD11c^{+} cells from B6 or B6.*batf3*$^{-/-}$ mice (representative from $n = 3$ experiments). (**B-C**) Western blots of cell lysate pro-IL-18 protein (and actin protein loading control) and relative expression of *il18* detected by qRT-PCR (B) or cell lysate NLRP3 protein (and actin protein loading control) and relative expression of *nrlp3* (C) from CD11c^{+} cells purified from B6 versus B6.*batf3*-/- mice stimulated with L1S+LPS for 24 h (representative from $n = 3$ experiments).

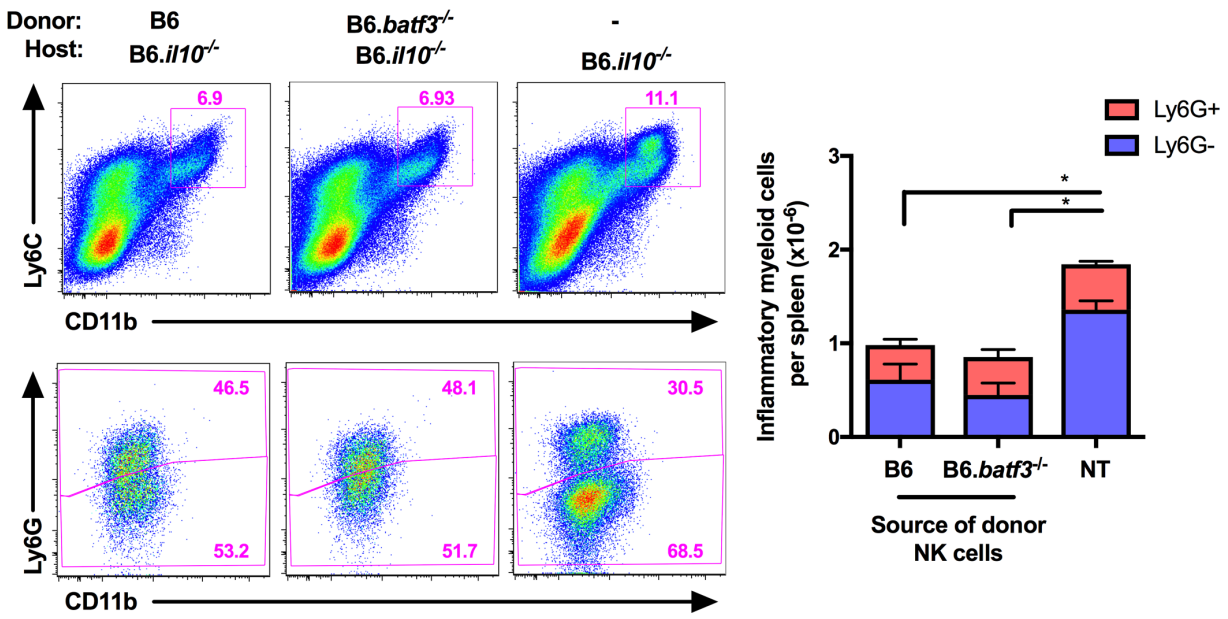

Figure S5. Non-NK Batf3-dependent cells limit inflammatory myeloid cell recruitment. *Related to Figure 6.*

$Ly6C^{+}CD11b^{+}$ inflammatory myeloid cells and $Ly6G^{+/-}CD11c^{+}$ cells within the inflammatory myeloid cell gate detected by flow cytometry from the spleens of B6.*il10*$^{-/-}$ recipient mice at 96 hpi with 10^4 Lm i.v. following transfer of donor purified splenic NK cells from B6 or B6.*batf3*$^{-/-}$ mice at 24 hpi. NT = no transfer (n = 3 experiments with 3-5 mice per group). Data are displayed as mean ± SEM with *p<.05 as measured by *t*-test.

Supplemental Experimental Procedures

Co-cultures

For co-culture experiments, BMDCs were cultured 6 days in GM-CSF and 3 x 10^5 BMDCs (>90% $CD11c^+$) were plated per well overnight in 24 well plates. For infections, log phase WT or Δp60 Lm were added at a multiplicity of one bacterium per BMDC. One hour later, cells were washed and gentamycin was added at 10 μg/mL. For L1S stimulations, BMDCs were activated for one hour by treatment with 10 ng/mL LPS (L8274 Sigma-Aldrich) and 30 μg/mL purified L1S protein. Purified splenic NK cells were added to cultures 2 h after Lm or L1S+LPS treatments at a ratio of 1:10 (NK cells:BMDCs). Supernatants were harvested for analysis of IFNß, IL-18, IL-6, IL-12p70, IFNγ and IL-10 at indicated time points using commercial ELISAs (BD Biosciences or eBioscience).

Cell stimulations

Cytokines and inhibitors were added to co-cultures or filtered supernatants at the time of NK cell addition. For cytokine stimulations, 50 ng/mL of rIL-12, rIL-2, or rIL-18 (BioLegend) were added as indicated. To inhibit STAT4, lisofylline (Sigma-Aldrich) was added at a concentration of 80 mM. To inhibit IL-12R, purified anti-IL-12R (CD212, BD Pharmingen) was added at a concentration of 50 ug/mL. To inhibit IL-12p70, anti-IL-12p70 (R2-9A5, BioXcell) was added at a concentration of 1 ug/mL. For ATP and Alum BMDC stimulations, 3 x 10^5 cells BMDCs were plated overnight and pre-stimulated with 10 ng/mL LPS for 3 h, followed by 5 mM ATP for 30 min (Sigma-Aldrich) or 300 μg/mL Alum for 1 h (Sigma-Aldrich). Supernatants were collected 24 h post-stimulation for cytokine detection.

Flow cytometry

Single cell suspensions were stained for flow cytometry analysis. Spleens and livers were harvested into media containing 1 mg/mL type 4 collagenase (Worthington) in HBSS plus cations (Invitrogen). Organs were incubated for 30 min at 37ºC and single cell suspensions were created by passage through a 70 μM strainer. Livers were re-suspended in 40% Percoll in HBSS minus cations, then underlayed with 60% Percoll. Gradients were centrifuged 20 min at 1625 x g. Leukocytes were collected from the interface using a Pasteur pipet and diluted in RPMI with 5% FBS. Cells were pelleted and re-suspended in HBSS plus cations (Sigma-Aldrich). Cells from the blood were harvested in HBSS plus cations and heparin (Sigma-Aldrich). Red blood cells were removed from all cell preparations by treatment with RBC lysis buffer (0.15 M NH_4Cl, 10 mM $KHC0_3$, 0.1 mM Na_2EDTA, pH 7.4).

Anti-CD16/32 (2.4G2 hybridoma supernatant) was added to single cell suspensions and incubated for 30 min to block Fc receptors. Cells were pelleted and staining was completed in FACS buffer (1% BSA, 0.01% NaN3, PBS). Antibodies for staining included anti-CD3 (clone 1452C11), NK1.1 (clone PK136), CD8α (clone 53-6), CD11c (N418), B220 (clone RA3-6B2), IL-18R1 (clone BG/IL18RA), CD45.1 (clone A20), CD45.2 (clone 104), and IFNγ (clone XMG1.2). All antibodies were purchased from eBioScience or BioLegend. Following surface staining, cells were fixed with 2% paraformaldehyde. For intracellular staining, cells were permeabilized using 1 mg/mL saponin treatment. Prior to intracellular staining cells were incubated in RP10 media (RPMI 1640, Sigma, 10% FBS, 1% L-glutamine, 1% Sodium Pyruvate, 1% Penicillin, 1% Streptomycin and 0.1% ß-ME) with Brefeldin A (BD Biosciences). To amplify the IL-10 GFP signal in samples from B6.*tiger* mice, an intracellular stain was done using rabbit monoclonal anti-GFP followed by goat anti-rabbit IgG Alexa Fluor 488 (Life Technologies) as previously described (Tarrio et al., 2014). A minimum of 100,000 events per sample were collected using an LSRII (BD Biosciences). FlowJo software (Treestar) was used for data analysis.

Western Blotting

Protein levels of IL-18 and NLRP3 were detected by western blotting of equivalent cell lysates loaded onto a 10% SDS-PAGE gel following semi-dry transfer onto a nitrocellulose membrane and blocking with 5% milk in PBST (PBS + 0.5% Tween-20, Sigma) for 2 h. Membranes were probed with primary antibodies against IL-18 (clone 39-

3F, MBL), NLRP3/NALP3 (clone Cryo-2, Adipogen), and β-Actin (clone 8H10D10, Cell Signaling) followed by secondary antibodies against mouse IgG. Protein expression was detected on an Oyssey Scanner (LI-COR).

Quantitative Real-time PCR

Expression of *il18* and *nlrp3* was detected using a quantitative PCR machine (Bio-Rad) on cDNA samples prepared from RNA preparations using reverse transcriptase and an RNA extraction kit (Bio-Rad). Primers for transcript detection included il18F: ACTGTACAACCGCAGTAATACGG and il18R: AGTGAACATTACAGATTTATCCC (Kim et al., 2000) and nlrp3F: CCCTTGGAGACACAGGACTC and nlrp3R: GAGGCTGCAGTTGTCTAATTCC (He et al., 2012).

Supplemental References

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