Supplemental Information

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

Sarah E. Clark, Rebecca L. Schmidt, Daniel S. McDermott, and Laurel L. Lenz
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 10⁴ 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.ii18−/− mice sacrificed 24 hpi with 10⁴ Lm i.v. (n = 2 experiments with 3–5 mice per group). (B) Supernatant IL-10 detected from B6 or B6.ii18−/− 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 10⁴ 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 (R29A5, BioXcell) was added at a concentration of 1 ug/mL.

**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 underlayered 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 NH₄Cl, 10 mM KHC03, 0.1 mM Na₂EDTA, 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% β-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*: GAGGCTGAGTTGTCTAAATCC (He et al., 2012).

**Supplemental References**

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