

Supplemental Information

Type I Interferon Signaling Prevents Lethal Systemic Il-1 Signaling-Driven Hyperinflammation during Invasive Bacterial Infection of Soft Tissue

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Supplemental Figures

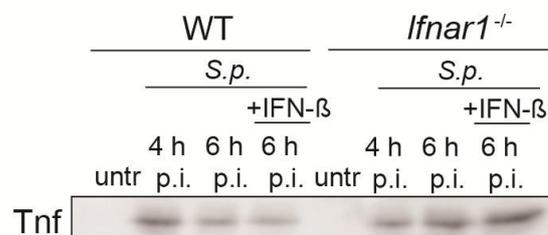


Figure S1 (relates to Figure 6B): Tnf cytokine in the supernatants of BMDMs infected with *S. pyogenes* is not reduced by IFN-I signaling

The Western blot shown in the Figure 6B (Il-1 β staining) was reprobed with Tnf antibody (goat anti-murine Tnf, R&D). The figure shows analysis of supernatants of WT and *Ifnar1^{-/-}* BMDMs treated as indicated in the figure.

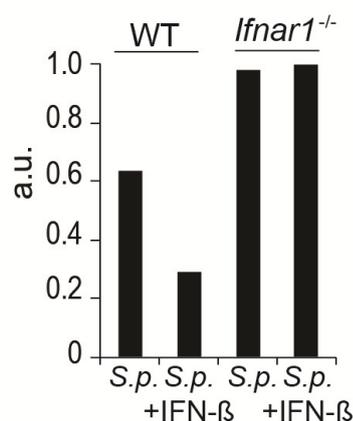


Figure S2 (relates to Figure 6C): Semiquantitative analysis of Western blot signals for pro-IL-1 β shown in Figure 6C

The pro-IL-1 β signals (enhanced chemiluminescence, ECL) in *S. pyogenes*-treated samples of the Western blot shown in the Figure 6C were measured using Image Lab (Bio-Rad) and normalized to tubulin signals in the same lane. a.u., arbitrary units

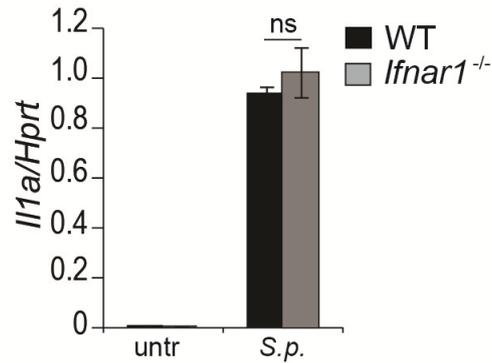


Figure S3 (relates to Figure 6F): *Il1a* mRNA induction by *S. pyogenes* is similar in WT and *Ifnar1*^{-/-} BMDMs

BMDMs from WT and *Ifnar1*^{-/-} mice were infected for 4 h with *S. p.* and *Il1a* mRNA levels were determined by qRT-PCR. Error bars represent mean with SD (n>3). Student's t-test: ns, not significant.

Supplementary Experimental Procedures

Mice

All mice were bred and kept under specific pathogen free (SPF) conditions according to recommendations of the Federation of European Laboratory Animal Science Association and were all on C57BL/6 background. *Ifnar1*^{-/-}, *Casp1*^{-/-}, *Casp11*^{-/-}, *Il1r1*^{-/-} and *Tlr13*^{-/-} mice have been described previously (Labow et al., 1997; Li et al., 1995; Li and Chen, 2012; Muller et al., 1994). *Ifnar1*^{fl/fl}-LysMCre and *Ifnar1*^{fl/fl}-CD11cCre mice were obtained by crossing *Ifnar1*^{fl/fl} mice (Kamphuis et al., 2006) with LysMCre (Clausen et al., 1999) and CD11cCre (Caton et al., 2007) mice, respectively. C57BL/6N wild type (WT) mice were purchased from Charles River Laboratories. *Ifnb*^{+/ $\Delta\beta$ -luc}, *Ifnb*^{+/ β -luc}-LysMCre, *Ifnb*^{+/ β -luc}-CD11cCre and *Ifnb*^{+/ β -luc}-CD4Cre reporter mice (Lienenklaus et al., 2009; Solodova et al., 2011). *Ifnb*^{+/ $\Delta\beta$ -luc}-*Irf3*^{-/-} and *Ifnb*^{+/ $\Delta\beta$ -luc}-*Irf5*^{-/-} reporter mice were obtained by crossing *Ifnb*^{+/ $\Delta\beta$ -luc} mice with *Irf3*^{-/-} (Sato et al., 2000) and *Irf5*^{-/-} mice (Takaoka et al., 2005), respectively. A mutation in *Dock2*, which spontaneously occurred in the *Irf5*^{-/-} mice thereby impairing the development subsets of immune cells (Purtha et al., 2012), was crossed out as described (Thackray et al., 2014). Both male and female mice were used in every experiment, and gender and age were matched between genotypes.

Cell Culture

For in vitro experiments primary bone marrow derived macrophages (BMDMs) and conventional dendritic cells (cDCs) were obtained from bone marrow of the femur and tibia of 7-10 weeks old mice. Macrophages were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (GIBCO) in the presence of L929 cell-derived CSF-1, as described (Gratz et al., 2011). Dendritic cells were cultivated for 7 days in DMEM supplemented with 10% FCS in the presence of X-6310 cell-derived GM-CSF as described (Gratz et al., 2011). For isolation of peritoneal neutrophils, 9% casein solution was intraperitoneally injected 12 and 3 h prior to isolation of peritoneal exudate. Neutrophils were purified from the exudate by discontinuous density gradient centrifugation as described (Luo and Dorf, 2001) and then kept in culture in RPMI medium supplemented with 10% FCS. Human CD14+ monocytes were enriched from Buffy coats provided by the Austrian Red Cross by negative selection with RosetteSep Human Monocyte Enrichment Cocktail followed by density gradient isolation with Lymphoprep (StemCell Technologies). Monocytes were allowed to recover over night in RPMI containing 10% fetal calf serum. For the generation of macrophages, monocytes were cultured for an additional 7 days in the presence of 50 ng/ml recombinant human M-CSF (Peprotech).

Bacterial Culture

The *Streptococcus pyogenes* serotype M1 strain ISS3348 (provided by Roberta Creti, Istituto Superiore di Sanita, Italy) was used in mouse infection experiments. For infection of cells with *S. pyogenes* and for isolation of *S. pyogenes* RNA the strain ISS3348 or the M1 serotype strain ATCC 700294 were used. *S. pyogenes* was grown at 37°C with 5% CO₂ without agitation in Todd-Hewitt-Broth (BD Biosciences) supplemented with 0.2% yeast extract (THY media) and on trypticase soy agar containing 5% sheep blood (Biomerieux). The cell growth was turbidimetrically monitored at 600 nm until mid-logarithmic phase (optical density 0.3) was reached.

Determination of Bacterial Loads

For determination of bacterial loads in the blood, livers, spleens and skin lesions, mice were anesthetized, and blood was withdrawn by puncturing the heart with a 25 G needle, followed by 1:1 dilution of blood in sterile ddH₂O to lyse blood cells and euthanasia of animals. Spleen, liver and skin lesions were removed aseptically, weighted, placed in 1 ml of sterile PBS followed by homogenization and plating of serial dilutions on blood agar plates. The bacterial load was calculated as colony-forming units (CFUs) per ml of blood or per gram of tissue.

Cytokine Measurement

Homogenates of infected and uninfected tissues were prepared as for determination of bacterial loads using PBS containing proteinase inhibitors (Complete protease inhibitor, Roche). Homogenates were freeze-thawed twice, centrifuged (4000 rpm, 10 min, 4°C) and diluted prior to measurement. Cytokine release during the air pouch infections was assessed in the collected exudates. Cytokines released by infected BMDMs, cDCs and neutrophils were determined using the cell supernatants. IL-1 β , IL-1 α , Tnf, IL-10, Cxcl1 levels were determined using DuoSET ELISA kits (R&D Systems), IFN- β levels were measured using the VeriKine-HS Mouse IFN- β Serum ELISA Kit (PBL Interferon, Biomedical Laboratories) according to the manufacturer's instructions.

Analysis of Supernatants and Cell Lysates by Western Blot

For the preparation of whole-cell extracts, cells were washed with ice-cold PBS and lysed using 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 30 mM NaPPi, 50 mM NaF, 2 mM EDTA, 1% Triton X-100, and 1x protease inhibitor (Roche). Extracts were cleared by centrifugation at 13,200 rpm and 4°C, mixed with 2 \times SDS-PAGE Laemmli sample buffer and boiled for 10 min. For analysis of secreted proteins, cell supernatants (900 μ l) were collected, mixed with 300 μ l ice-cold 6.1 M trichloroacetic acid solution and incubated 1 h on ice to allow protein precipitation. Samples were centrifuged at 10,000 \times g for 1 h at 4°C. Supernatants were discarded and pellets were washed three times with 1 ml ice-cold acetone and dissolved in 10 μ l 0.2 M NaOH, and mixed with 25 μ l 2 \times SDS-PAGE Laemmli sample buffer, followed by boiling for 5 min. Fifteen μ l sample was separated on 16.25%SDS-PAGE gel, electrotransferred to PVD membranes and probed with goat anti-IL-1 β (R&D Systems, Minneapolis, MN), rabbit anti-Caspase 1 (Millipore) and mouse anti-tubulin (Sigma). For the detection, the blots were incubated with horseradish peroxidase-conjugated secondary antibody and developed using an ECL system (Amersham, UK) according to the manufacturer's instructions.

Determination of Clinical Parameters

To assess organs tissue damage, levels of blood urea, creatinine, bilirubin, aspartate transaminase (AST), alanine transaminase (ALT) and glutamate dehydrogenase (GLDH) were determined in serum samples from blood collected by heart puncture of anesthetized mice. Tests were performed by InVitro GmbH, Vienna.

Histology Analysis

Mice were sacrificed, organs were aseptically removed and fixed in 4% paraformaldehyde for 24 hours, trimmed, routinely processed and embedded. 5 μ m-thick sections were stained

with Hematoxylin and Eosin (H&E) and evaluated by a European board-certified veterinary pathologist, in a blind-fashion. The qualitative and semiquantitative analysis included identification of histopathological changes in the spleen, liver, kidney, skin, and evaluation of the predominant leukocytic type in inflammatory infiltrate. The final result takes into account both the extent and distribution of lesions and as such it is an indicator of the overall gravity of the damage observed.

Isolation of *S. pyogenes* RNA, Ribosomal RNA Fractions and Mammalian RNA

Bacteria were grown to mid-log phase and lysed using 2% SDS and 1 mg/ml proteinase K (Sigma-Aldrich). For isolation of RNA, Trizol LS reagent (Invitrogen) was used, following by treatment with DNase (TURBO DNase, Ambion). Ribosomal RNA (rRNA) fractions were obtained by cutting out the respective bands after RNA separation on formaldehyde agarose gel and elution with the QIAquick Gel Extraction Kit 250 (Qiagen). Mammalian RNA was obtained from bone marrow derived macrophages using the Nucleospin II Kit (Macherey and Nagel) according to the manufacturer's protocol.

In vitro Infection with *S. pyogenes*

In vitro infections were carried out as described (Gratz et al., 2011). Briefly, BMDMs, cDCs or peritoneal neutrophils were seeded at 1×10^6 cells per 6 cm dish in media without antibiotics. Cells were then infected with *S. pyogenes* at a multiplicity of infection (MOI) of 100. After 30 min of incubation at 37°C, bacteria were killed by adding 60 µg/ml penicillin to the media and the cells were further incubated as indicated in figures. Human cells were infected with an MOI of 10 CFUs *S. pyogenes*; 2 h post infection, the culture medium was supplemented with 60 µg/mL penicillin G (Sigma).

Stimulation and Treatments of Cells

Lipopolysaccharide (LPS) from *Escherichia coli* (Sigma) was used at a concentration of 10 ng/ml. Where specified, cells were stimulated with IFN-β (500 U/ml, PBL Interferon Source) at the time of infection. In RNA decay experiments transcription was stopped by addition of actinomycin D (5 µg/ml, Sigma). For stimulation of cells with nucleic acids, transfection using DOTAP (Roche) was used as described (Fieber et al., 2015). Briefly, 1 µg of purified bacterial RNA, 5S rRNA, 16S rRNA, 23S rRNA, mammalian RNA, TLR13 ligand (oligoribonucleotide SA19 with the sequence 5'-GGACGGAAAGACCCCGUGG-3') (Oldenburg et al., 2012) or mutated TLR13 ligand (oligoribonucleotide 5'-GGACGGGAAGACCCCGUGG-3') (Oldenburg et al., 2012) were used to form complexes with 30 µl DOTAP followed by addition to the cells.

RNA Isolation, cDNA Synthesis and Quantitative Reverse Transcription PCR (qRT-PCR)

RNA was isolated from cells using the Nucleospin II kit (Macherey and Nagel) according to the protocol. Reverse transcription of 1 µg of RNA was carried out using oligo-(dT)18 as primer and Mu-MLV reverse transcriptase (Fermentas). qRT-PCR was run on a Realplex Mastercycler (Eppendorf) using the GoTaq qPCR Master Mix (Promega). Relative expression was calculated using the housekeeping gene *Hprt*. The following primers were used:

mHPRT-fwd 5'-GGATTTGAATCACGTTTGTGTCAT-3'

mHPRT-rev 5'-ACACCTGCTAATTTTACTGGCAA-3'

mTNF-fwd 5'-GATCGGTCCCCAAAGGGATG-3'

mTNF-rev 5'-CACTTGGTGGTTTGCTACGAC-3'

mIL-1 β -fwd 5'-AGATGAAGGGCTGCTTCCAAA-3'

mIL-1 β -rev 5'-AATGGGAACGTCACACACCA-3'

mPre-IL-1 β fwd 5'-GTCTTTCCCGTGGACCTTCC-3'

mPre-IL-1 β rev 5'-ACTTGAGGGGCGTTGAGTTC-3'

hGAPDH-fwd 5'-GAAGGTGAAGGTCGGAGTC-3'

hGAPDH-rev 5'-GAAGATGGTGATGGGATTTTC-3'

hIFNB1-fwd 5'- ATGACCAACAAGTGTCTCCTCC-3'

hIFNB1-rev 5'-GGAATCCAAGCAAGTTGTAGCTC-3'

hPre-IL1b-fwd GGTGACAGGGGAGGAGTAGT-3'

hPre-IL1b-rev 5'-GATGAGGTCCAAAGCGGGTA-3'

Supplemental References

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