Supplementary Information

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I. Supplementary Materials and Methods

Macrophages

Macrophages obtained by peritoneal lavage 5 days after mice were injected intraperitoneally with 4% thioglycollate were cultured in the high glucose version of Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, 10 mM HEPES, 10 mM pyruvate, 10 mM L-glutamine, 50 μg/mL penicillin, and 50 μg/mL streptomycin. Bone marrow cells were cultured for 7 days in 30% M-CSF conditioned medium. For infection, adherent macrophages were cultured overnight in 50 ng/mL ultra-pure LPS, washed with antibiotic-free medium, and infected at a multiplicity of infection of 50 (30 for *F. tularensis*). Plates were spun for 15 min at 850 x g and then incubated at 37°C, 5% CO_2 for 1 (*S. typhimurium*), 2.5 (*L. monocytogenes*), 3 (*S. aureus*) or 5 (*F. tularensis*) h. With the exception of *S. typhimurium* infections, gentamicin (20 ug/mL) was added to cultures 90 min after infection. For immunoprecipitation and Western blot analyses, macrophages were lysed in buffer A [10 mM phosphate pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM DTT, Complete™ protease inhibitor cocktail (Roche)]. Culture supernatants were mixed 1:1 with 2x buffer A.

Bacteria

F. tularensis ssp. *novicida*, strain U112 was grown overnight with aeration in Trypticase Soy Broth supplemented with 0.2% cysteine. *S. typhimurium* SL1344 was grown in high salt Luria broth standing at 37°C overnight, *S. aureus* in BHI broth standing at 37°C, 5% CO_2 overnight. *L. monocytogenes* wild-type 10403s and listeriolysin O mutant DPL2161 strains were grown standing in BHI at 25°C.

II. Supplemental Figure Legends

Supplementary Figure 1. Generation of *Cias1*$^{-/-}$mice. (**a**) Exons 1, 2, and 3 (closed boxes) were replaced with a neomycin resistance cassette. Exon 3 encodes the initiating methionine (ATG) and the entire pyrin domain of cryopyrin. Exons 1 and 2 encode the 5' UTR. An additional 155 base pairs upstream of exon 1 also were deleted. C57BL/6 C2 embryonic stem (ES) cells were electroporated with the targeting vector and two independent *Cias1*$^{+/-}$clones (7G5 and 9B5) were identified. Chimeric mice were generated from each ES clone and backcrossed to C57BL/6N mice. Mouse strains 7G5 and 9B5 gave similar results. Mice analyzed were 6-20 weeks old. (**b**) Southern blot analysis of the offspring from *Cias1*$^{+/-}$intercrosses. *EcoRI*-digested tail DNA was hybridized with the probe indicated in (a) to yield a 10.9 kb wild-type and an 8.1 kb *cryopyrin* mutant band. (**c**) RT-PCR analysis of Cryopyrin mRNA expression in thioglycollate-elicited peritoneal macrophages. RNA was isolated from freshly harvested macrophages or macrophages cultured with LPS (200 ng/ml) for 6 h. The following intron-spanning primers were used for RT-PCR: Cryopyrin forward primer sm512 (binding within exon 4: 5' TAT GGT ATG CCA GGA GGA CAG CC) and reverse primer sm513 (binding within exon 5: 5' TCT GCT AGA CTC CTT GGC GTC C) amplified a 507 bp cDNA fragment (upper panel); Cryopyrin forward primer sm455 (binding within exon 3: 5' ATG ACG AGT GTC CGT TGC AAG C) and reverse primer sm457 (binding with exon 5: 5' CTG TTG AGG TCC ACA CTC TCA CCT AGA C) amplified a 485 bp cDNA fragment (middle panel); Control HPRT forward primer (5' GCT GGT GAA AAG GAC CTC T) and reverse primer (5' CAC AGG ACT AGA ACA

CCT GC) amplified a 249 bp cDNA fragment (lower panel). Amplification of the housekeeping gene HPRT was used to check the integrity of the RNA templates.

Supplementary Figure 2. Nigericin or maitotoxin alone does not promote IL-1β release. IL-1β secretion by wild-type (WT) macrophages treated with ultra-pure LPS and/or nigericin (**a**) or ultra-pure LPS and/or maitotoxin (**b**). Bars represent the mean ± standard deviation of triplicate wells.

Supplementary Figure 3. Generation of *Nod2*$^{-/-}$ mice. (**a**) Genomic sequence encoding exon 3 was replaced with a PGK-neo cassette. In addition, stop codons in all 3 reading frames were introduced after the exon 3 splice acceptor site. The targeting construct was electroporated into 129 R1 ES cells and chimeric mice were generated from two independent ES cell clones (9C4 and 14C4). *Nod2*$^{-/-}$ mice were backcrossed to C57BL/6N for up to 8 generations. Mouse strains 9C4 and 14C4 gave similar results. (**b**) Southern blot analysis of the offspring from *Nod2*$^{+/-}$ intercrosses. *Nhe* I-digested tail DNA was hybridized with the probe indicated in (a) to yield a 9.0 kb wild-type (WT) and a 4.0 kb *nod2* mutant band. (**c**) Immunoprecipitation and Western blot analysis of NOD2 in thioglycollate-elicited peritoneal macrophages from WT and *Nod2*$^{-/-}$ mice. Immunoprecipitations were performed with rabbit polyclonal antibodies raised against the two N-terminal CARDs of mouse Nod2 (Genentech) and Western blots were performed using a hamster monoclonal antibody raised against the leucine rich repeats of mouse NOD2 (clone 10G1, Genentech). H.C. = heavy chain

Supplementary Figure 4. Macrophage death after infection by *S. typhimurium*, *F. tularensis*, *S. aureus*, or *L. monocytogenes*. Macrophages were seeded in 96-well plates at 5 x 10^4 cells per well and incubated overnight with 50 ng/mL ultra-pure LPS. Cells were then infected with the indicated bacteria (as described in the Supplementary Methods) and cell death was quantified with a CytoTox96 LDH-release kit (Promega) after 1 (*S. typhimurium)*, 5 (*F. tularensis)*, 3 (*S. aureus)*, or 2.5 (*L. monocytogenes)* h. Percent cell death was quantified as: [(LDH released from infected cells – LDH released from untreated cells)/(LDH released from detergent-lysed cells – LDH released from untreated cells)]*100. Bars represent the mean ± standard deviation of 3 mice of each genotype. Results are representative of 4-5 independent experiments.

Supplementary Figure 5. *S. aureus* alpha, beta, and gamma hemolysins are not required for IL-1β secretion from infected macrophages. Wild-type ($Cias1^{+/+}$), $Cias1^{-/-}$ and $Asc^{-/-}$ macrophages were infected with WT *S. aureus* (strain 8325-4; ref. 1) or mutants deficient in alpha (strain DU1090; ref. 2), beta (strain DU5719; ref. 3), or gamma hemolysin (strain DU5942; ref. 4) and IL-1β secretion was determined after 3 h. Bars represent the mean ± standard deviation of triplicate wells. Results are representative of 3 independent experiments.

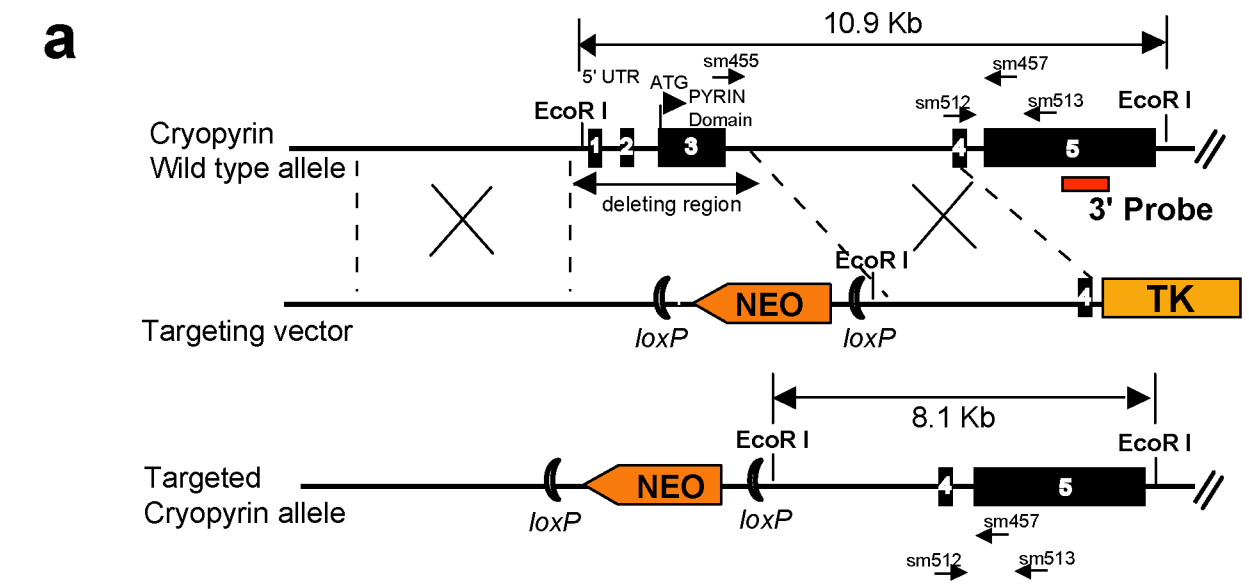
1. Novick, R. Properties of a cryptic high-frequency transducing phage in S*taphylococcus aureus*. *Virology* **33**:155–166. (1967).

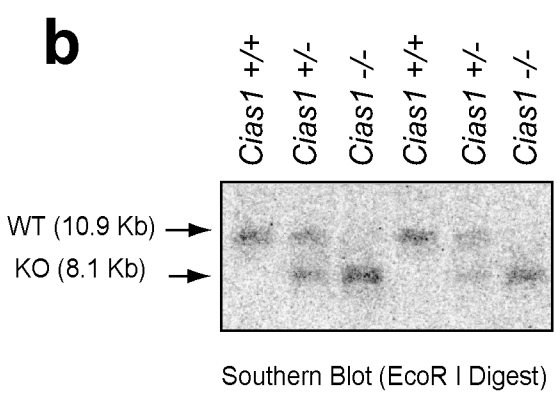
2. O'Reilly, M. De Azavedo, J. C. S. Kennedy,S. Foster, T.J. Inactivation of the alpha-hemolysin gene of *Staphylococcus aureus* 8325-4 by site directed mutagenesis and studies on the expression of hemolysins. *Microb. Pathog*. **1**:125–138. (1986).

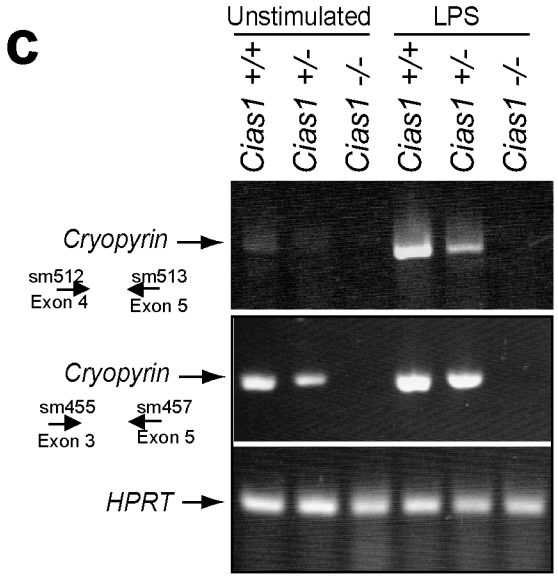
3. Bramley, A. J. Patel, A.H. O'Reilly, M. Foster, R. Foster, T.J. Roles of alpha-toxin and beta-toxin in virulence of *Staphylococcus aureus* for the mouse mammary gland. *Infect. Immun*. **57**:2489–2494. (1989).

4. Nilsson, I. Hartford, O. Foster, T.J. Tarkowskii, A. Alpha-toxin and gamma-toxin jointly promote *Staphylococcus aureus* virulence in murine septic arthritis. *Infect. Immun*. **67**:1045–1049. (1999).

Supplementary Figure 6. Macrophages infected with *S. aureus* or *F. tularensis* do not require pretreatment with LPS to secrete IL-1β. Macrophages from wild-type (*Cias1*$^{+/+}$), *Cias1*$^{-/-}$, or *Asc*$^{-/-}$ mice were infected with *S. aureus* or *F. tularensis* with (left panel) or without (right panel) overnight pretreatment with 50 ng/mL ultra-pure LPS. IL-1β released at 5 (*F. tularensis)* or 3 (*S. aureus)* h post-infection was measured by ELISA. Bars represent the mean ± standard deviation of 3 mice of each genotype.







Supplementary Figure 1
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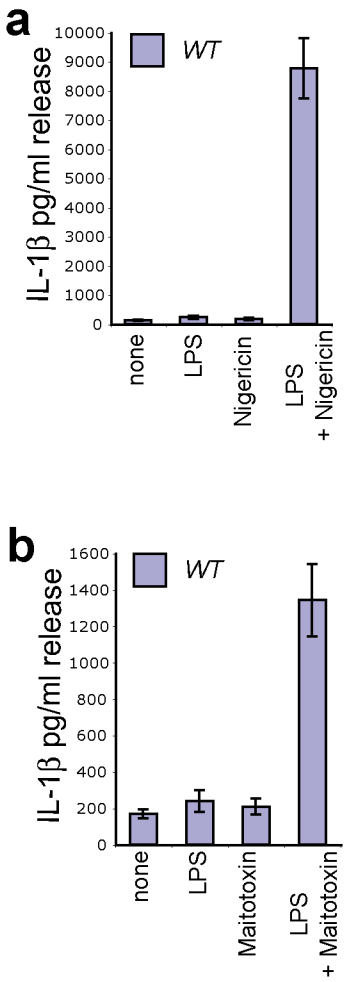

Supplementary Figure 2
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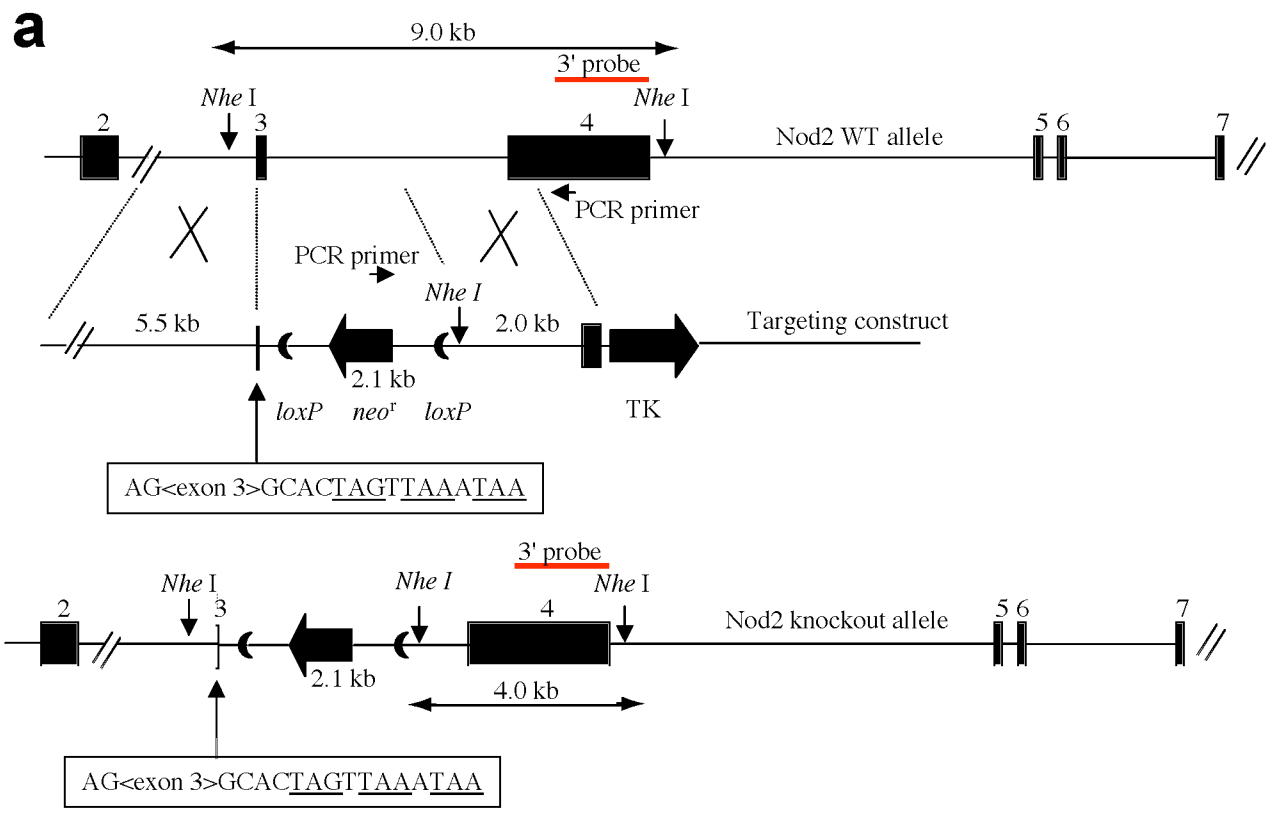
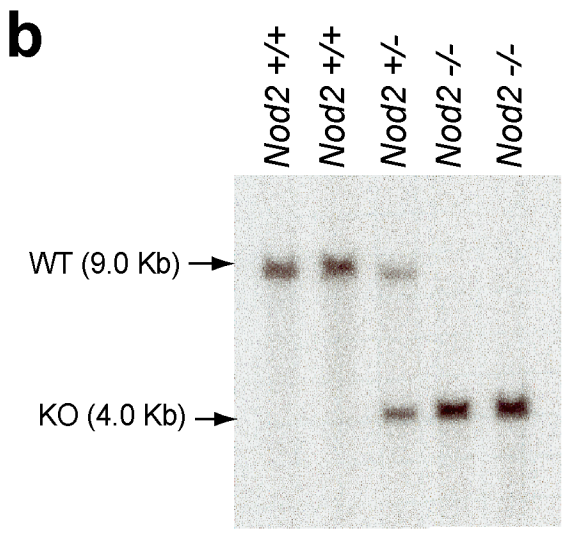
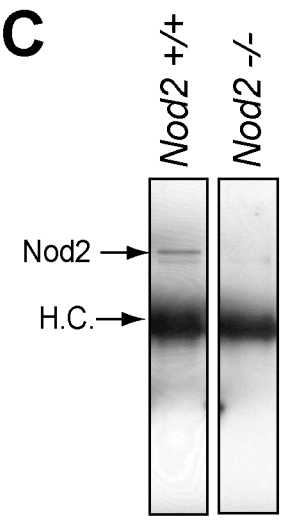




Southern Blot
Nhe 1 digestion



Western Blot

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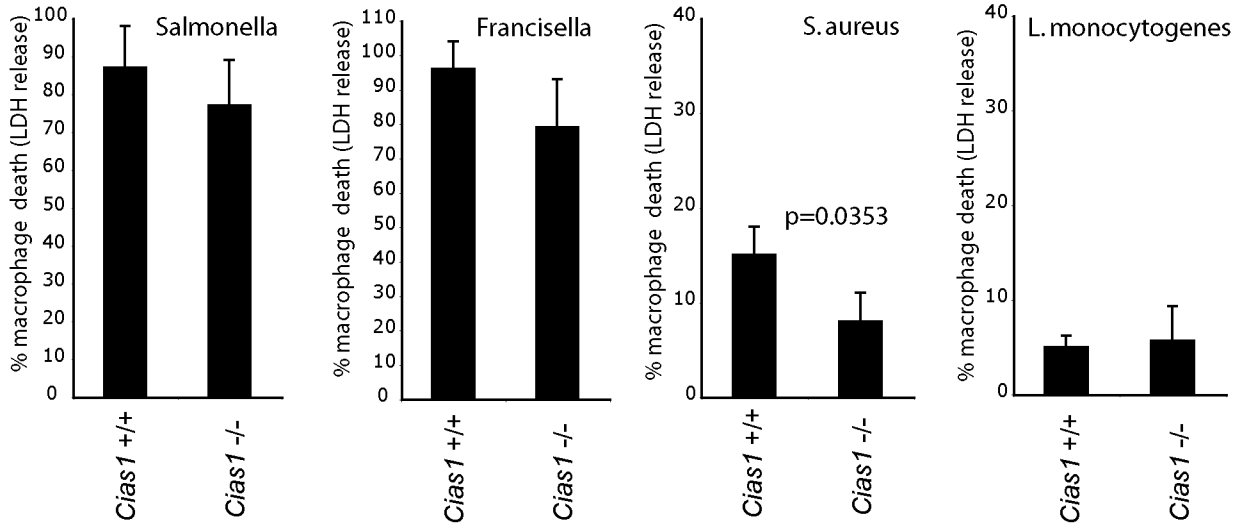

Supplementary Figure 4
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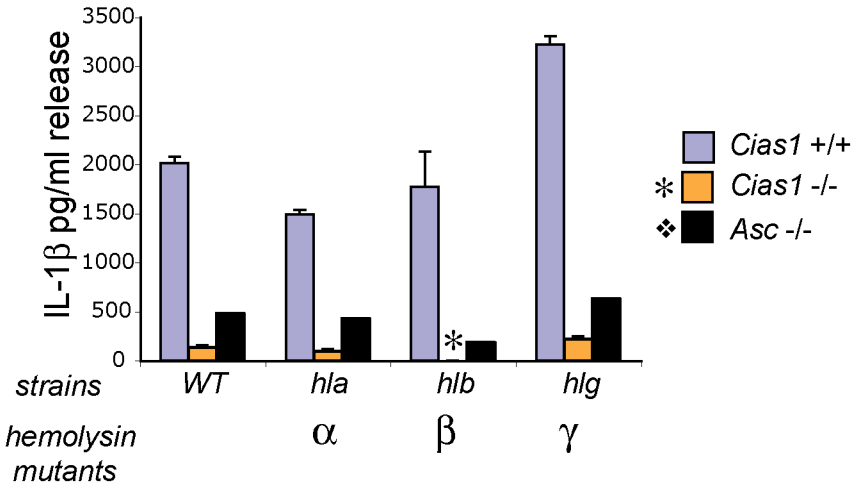

Supplementary Figure 5
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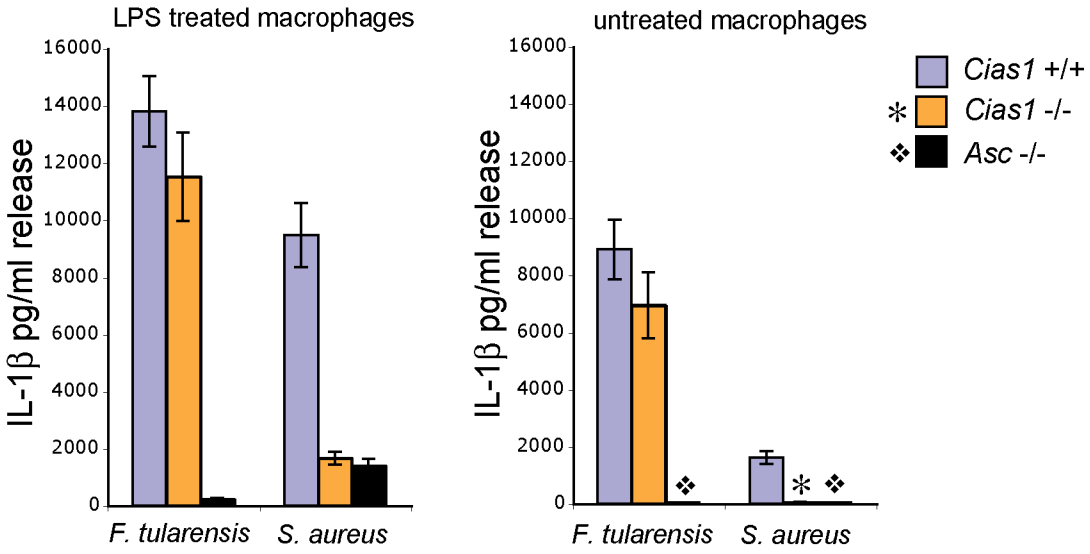

Supplementary Figure 6
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