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Supplemental Information

Anthrax Toxin Induces Macrophage Death by p38 MAPK Inhibition but Leads to Inflammasome Activation via ATP Leakage

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Inventory of Supplementary Information

- Figure S1, related to Figure 1- shows caspase-1, Nod2, Nalp1 and Nalp3 mRNA in *B. anthracis* infected WT and *Ripk2*$^{-/-}$ macrophages.
- Figure S2, related to Figure 2- shows *B. anthracis*-induced inflammasome activation and IL-1β production in *C57BL/6* macrophages.
- Figure S3, related to Figure 4- shows IL-1β production from mouse macrophages by concomitant treatment of ΔLF *B. anthracis* and ATP.
- Figure S4, related to Figure 5- shows *B. anthracis*-induced ATP release.
- Figure S5, related to Figure 6- shows the protective role of IL-1β in *B. anthracis* infection.
- Table S1, related to figure 5- summarizes the time points at which the different parameters examined in the study were measured.
- Supplemental Experimental Procedures
- Supplemental References

Figure S1

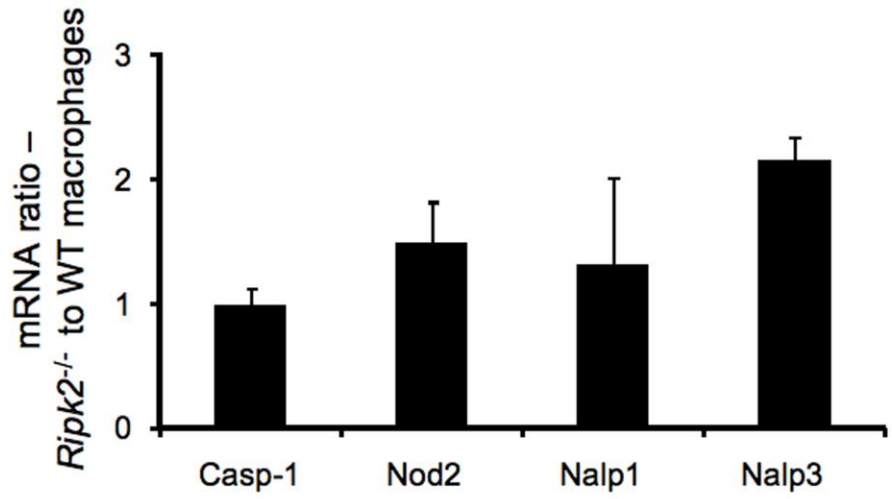
mRNA ratio –
Ripk2-/- to WT macrophages
3
2
1
0
Casp-1
Nod2
Nalp1
Nalp3

Figure S2

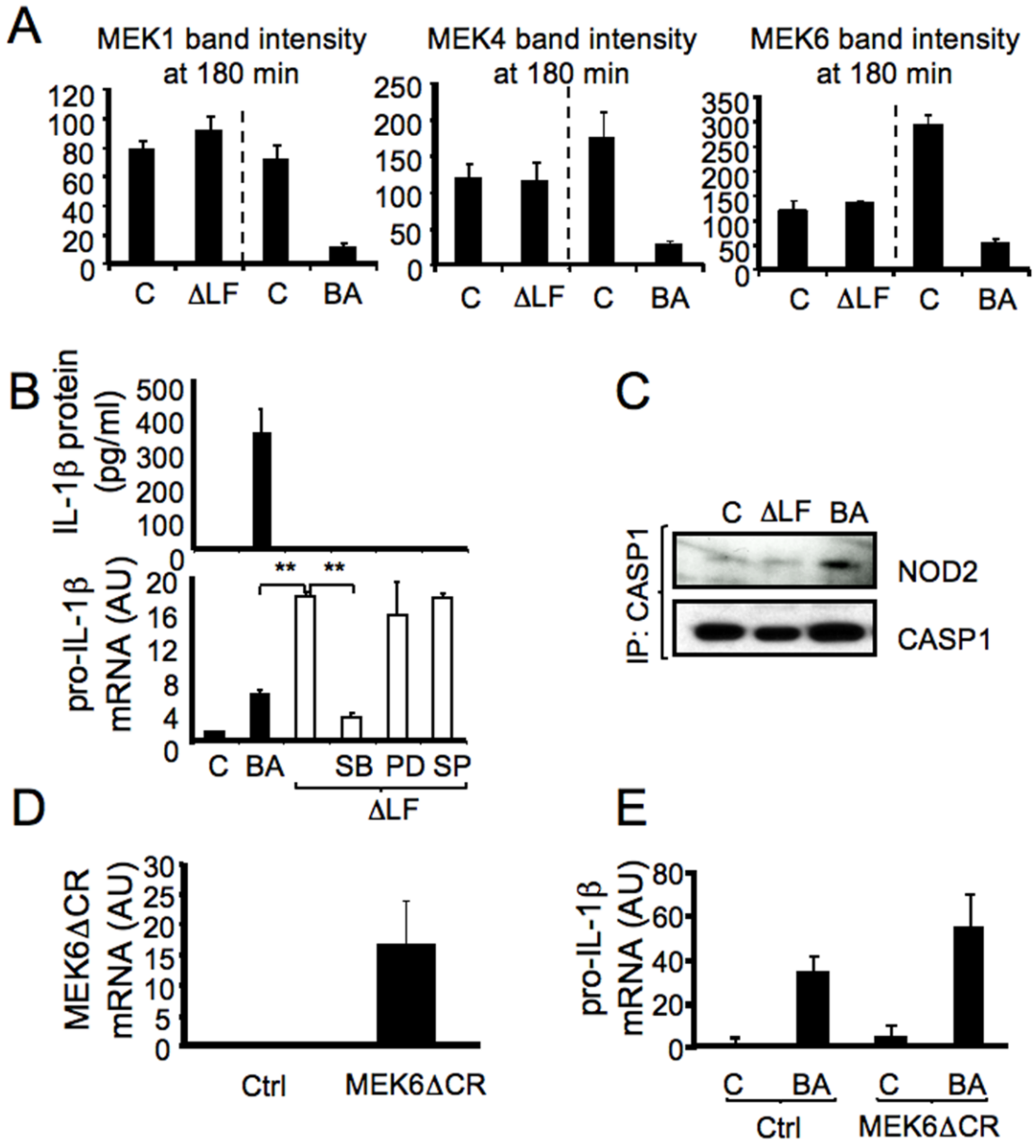
A
MEK1 band intensity at 180 min
MEK4 band intensity at 180 min
MEK6 band intensity at 180 min
C ΔLF C BA
B
IL-1β protein (pg/ml)
pro-IL-1β mRNA (AU)
**
**
C BA SB PD SP
ΔLF
C
C ΔLF BA
IP: CASP1
NOD2
CASP1
D
MEK6ΔCR mRNA (AU)
Ctrl
MEK6ΔCR
E
pro-IL-1β mRNA (AU)
C BA C BA
Ctrl
MEK6ΔCR

Figure S3

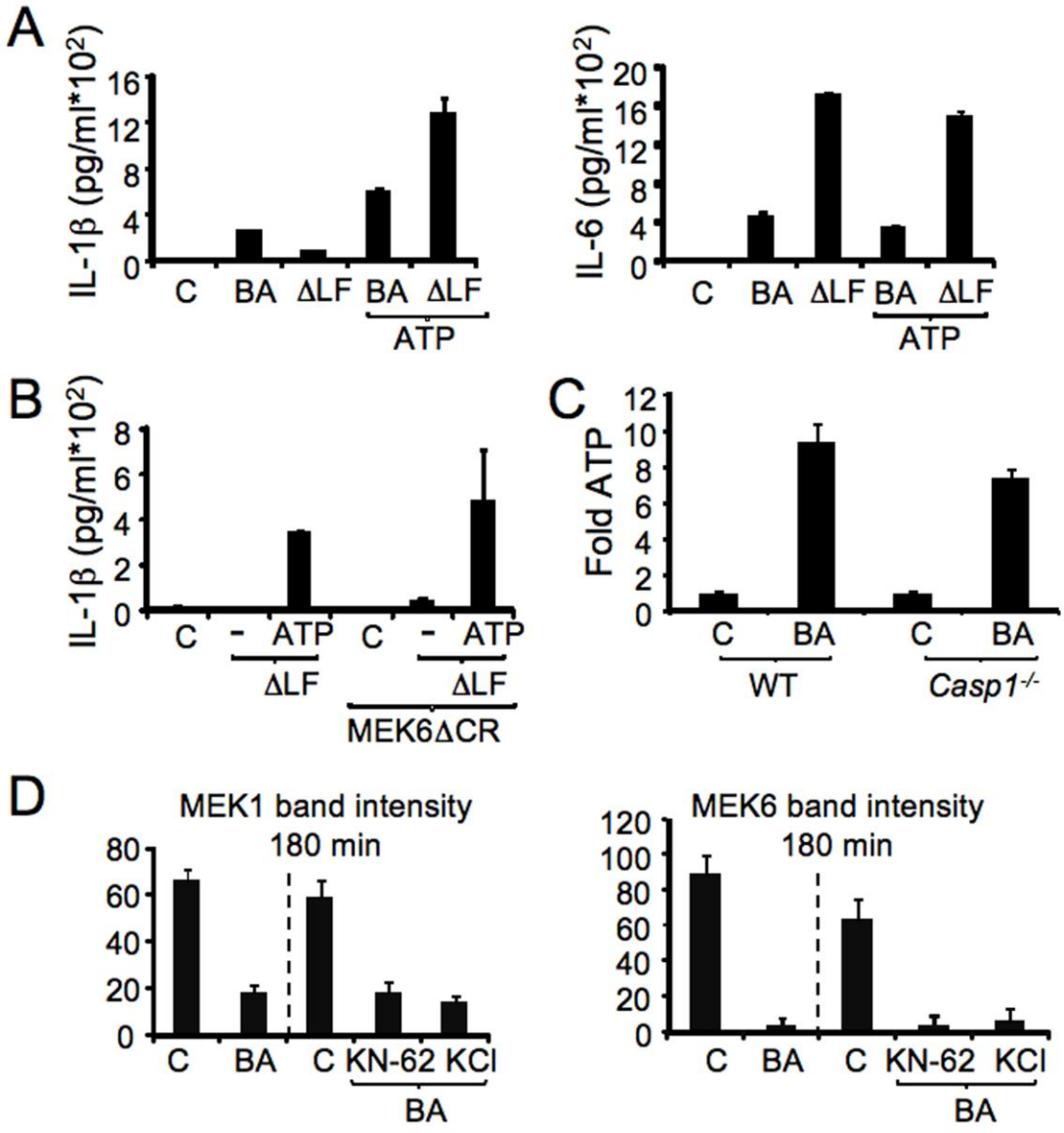
A
IL-1β (pg/ml*10²)
16
12
8
4
0
C
BA
ΔLF
BA
ΔLF
ATP
IL-6 (pg/ml*10²)
20
16
12
8
4
0
C
BA
ΔLF
BA
ΔLF
ATP
B
IL-1β (pg/ml*10²)
8
6
4
2
0
C
-
ATP
C
-
ATP
ΔLF
ΔLF
MEK6ΔCR
C
Fold ATP
12
10
8
6
4
2
0
C
BA
C
BA
WT
Casp1-/-
D
MEK1 band intensity
180 min
80
60
40
20
0
C
BA
C
KN-62
KCl
BA
MEK6 band intensity
180 min
120
100
80
60
40
20
0
C
BA
C
KN-62
KCl
BA

Figure S4

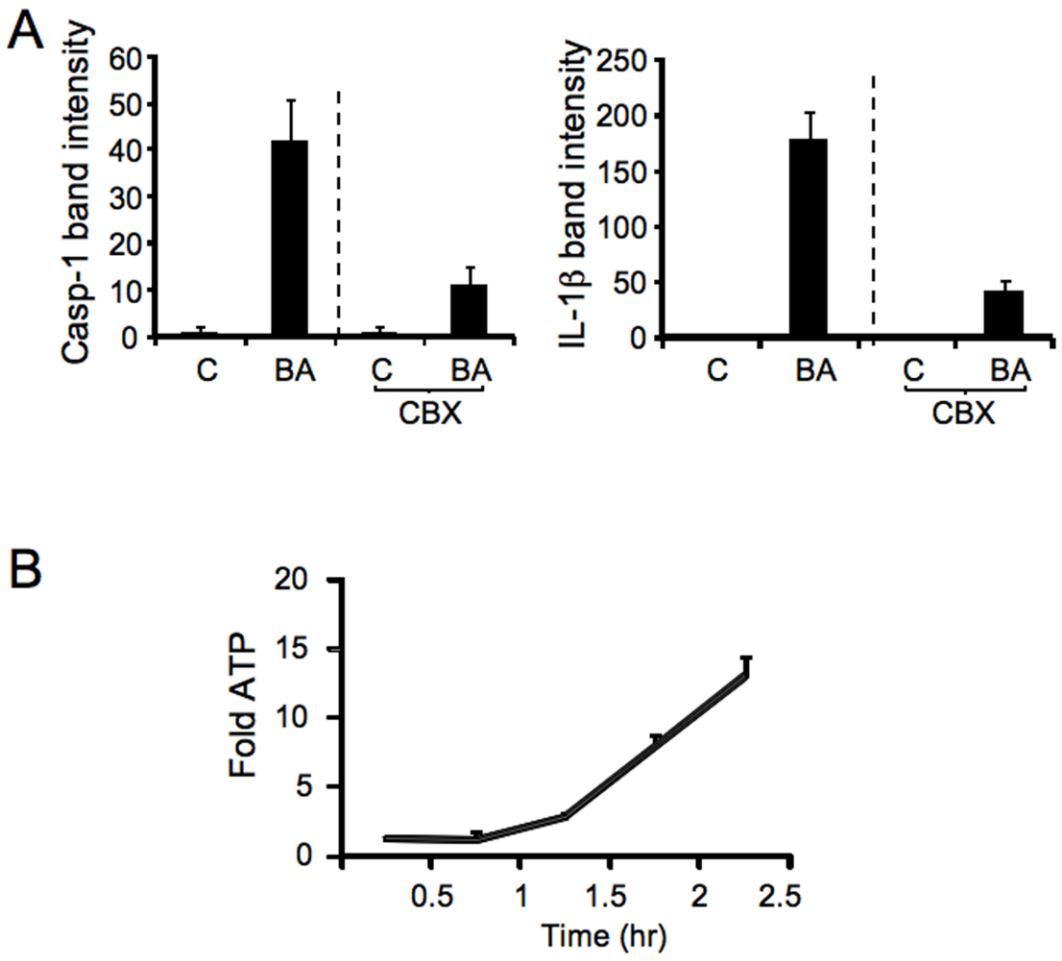
A
Casp-1 band intensity
60
50
40
30
20
10
0
C
BA
C
BA
CBX
IL-1β band intensity
250
200
150
100
50
0
C
BA
C
BA
CBX
B
Fold ATP
20
15
10
5
0
0.5
1
1.5
2
2.5
Time (hr)

Figure S5

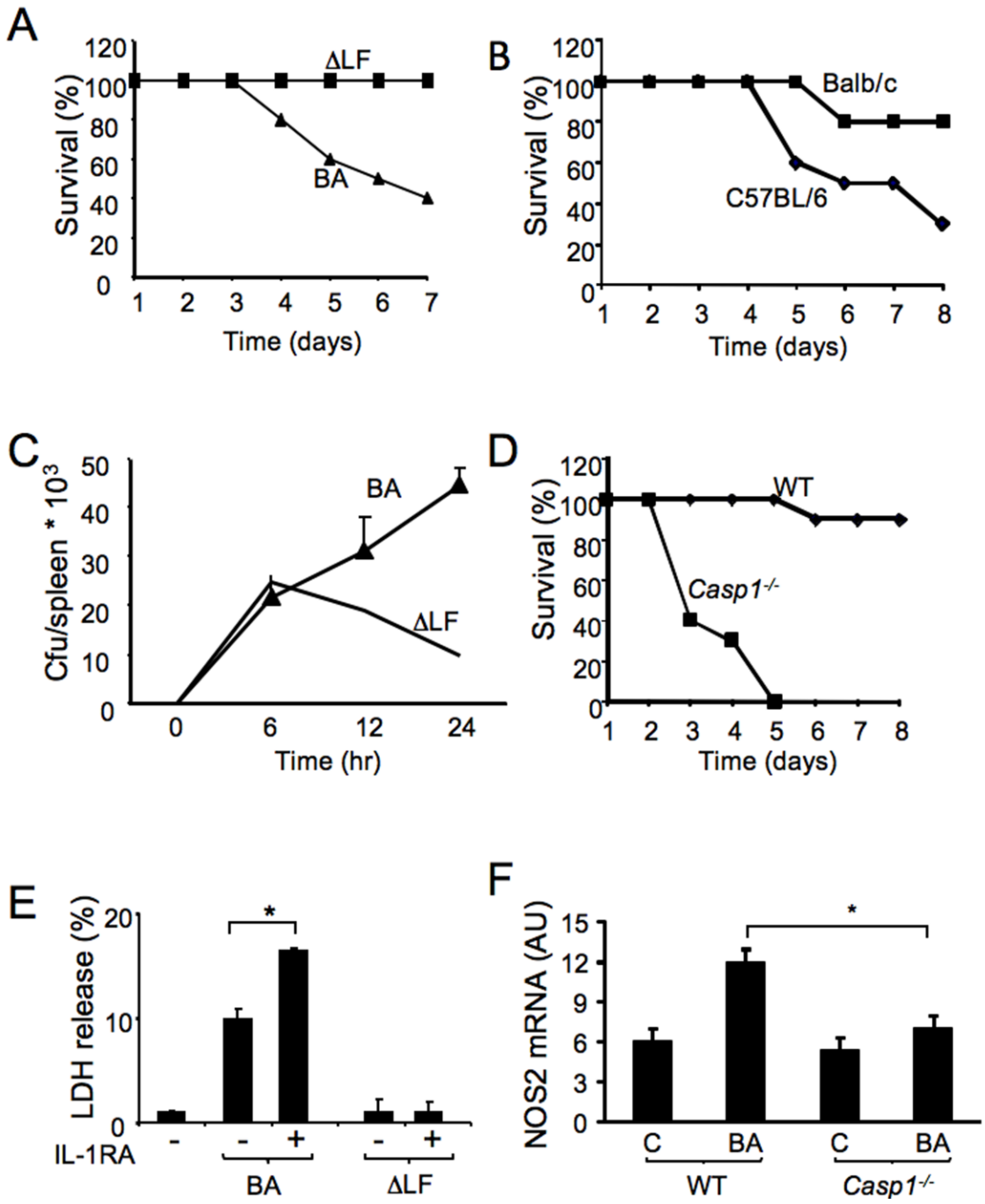
A
Survival (%)
120
100
80
60
40
20
0
ΔLF
BA
1 2 3 4 5 6 7
Time (days)
B
Survival (%)
Balb/c
C57BL/6
1 2 3 4 5 6 7 8
Time (days)
C
Cfu/spleen * 10^3
50
40
30
20
10
0
BA
ΔLF
0 6 12 24
Time (hr)
D
Survival (%)
WT
Casp1-/-
1 2 3 4 5 6 7 8
Time (days)
E
LDH release (%)
20
10
0
*
IL-1RA
- - + - +
BA
ΔLF
F
NOS2 mRNA (AU)
15
12
9
6
3
0
*
C BA C BA
WT
Casp1-/-

Table S1

Time post infection	Measurements and events
0-2 hrs	Disappearance of MEK and inactivation of p38-signaling
0-2 hrs	Inactivation of AKT-signaling
0-2 hrs	Connexin dephosphorylation
0-3 hrs	ATP release
0-5 hrs	Cell death
0-3 hrs	Inflammasome assembly
8-12 hrs	Caspase-1 and IL-1β release into culture supernatant

LEGENDS FOR SUPPLEMENTAL FIGURES

Figure S1: *B. anthracis*-infected WT and *Ripk2*$^{-/-}$ macrophages express similar amounts of mRNAs for different inflammasome components.

WT and *Ripk2*$^{-/-}$ macrophages were infected with WT *B. anthracis* and 3 hr later, the cellular content of the indicated mRNAs was analyzed by Q-RT-PCR. Results were normalized to the amount of GAPDH mRNA and are presented as ratio of relative mRNA content in *Ripk2*$^{-/-}$ to WT macrophages. This experiment was repeated twice and the results of one representative experiment done in triplicates are shown.

Figure S2: *B. anthracis* induction of inflammasome activation and IL-1β release from infected macrophages depends on LF.

(A) The results of 2-3 immunoblots, similar to the one shown in Figure 2A, were quantified using Image J software and mean values were determined.

(B) Mouse macrophages were infected with WT or ΔLF *B. anthracis* in the absence or presence of 10 μM of the p38 inhibitor SB202190 (SB), 20 μM of ERK inhibitor PD98059 (PD), or 40 μM of the JNK inhibitor SP600125 (SP). Cellular content of pro-IL-1β mRNA was analyzed by Q-RT-PCR and IL-1β protein secretion to culture supernatants was determined by ELISA at 2 and 12 hr post-infection, respectively. Results are means ± SD, **$p < 0.01$, denote significant differences between the groups. This experiment was repeated 3 times and the results of one representative experiment done in triplicates are shown.

(C) Mouse macrophages were infected with WT or ΔLF *B. anthracis* and 1.5 hr later, macrophage lysates were prepared and immunoprecipitated (i.p.) with caspase-1 antibody. Presence of the

indicated proteins in the immunoprecipitates was examined by immunoblotting. This experiment was repeated twice with similar results.

(D) RAW264.7 macrophages were transfected with MEK6ΔCR or empty vector. The MEK6ΔCR mRNA was analyzed by Q-RT-PCR using primers that specifically recognize mutant MEK6ΔCR but not WT MEK6 mRNA.

(E) RAW264.7 macrophages were transfected with MEK6ΔCR or empty vector. Transfected cells were left uninfected or infected with *B. anthracis* and 2 hr later the cellular content of pro-IL-1β mRNA was analyzed by Q-RT-PCR. This experiment was repeated twice and the results of one representative experiment done in triplicates are shown.

Figure S3: LF-deficient *B. anthracis* can induce p38-independent IL-1β release upon addition of exogenous ATP.

(A) Macrophages from WT mice were infected with ΔLF *B. anthracis* and 6 hr later were pulsed with 5 mM ATP for 20 min. Culture supernatants were collected after 4 hr and analyzed for cytokine secretion by ELISA. This experiment was repeated 3 times and the results of one representative experiment done in triplicates are shown.

(B) RAW264.7 cells expressing MEK6ΔCR or empty vector were infected with ΔLF *B. anthracis* and exogenous ATP was added as above. After 4 hr, cytokine secretion was measured by ELISA. This experiment was repeated 2 times and the results of one representative experiment done in triplicates are shown.

(C) WT and *Casp1*$^{-/-}$ macrophages were infected with WT *B. anthracis* and 2 hr later ATP release to culture supernatants was measured using an ATP detection kit. This experiment was repeated twice and the results of one representative experiment done in triplicates are shown.

(D) The results of 2-3 immunoblots, similar to the one shown in Figure 4F, were quantified using Image J software and mean values were determined.

Figure S4: *B. anthracis* induced ATP release.

(A) The results of 2-3 immunoblots, similar to the one shown in Figure 5C, were quantified using Image J software and mean values were determined.

(B) Mice macrophages were infected with WT *B. anthracis* and at the indicated times, ATP in the culture supernatants was measured using an ATP detection kit. This experiment was repeated 3 times and the results of one representative experiment done in triplicates are shown.

Figure S5: *C57BL/6* mice are susceptible to *B. anthracis* infection.

(A) *C57BL/6* mice were infected with WT (BA) or ΔLF *B. anthracis* (2×10^6 cfu/mouse, n=5-8 mice per group). Mouse survival was analyzed over 7 days post-infection.

(B) *C57BL/6* and *Balb/c* mice were infected with WT *B. anthracis* (2×10^6 cfu/mouse, n=5-8 mice per group). Mouse survival was analyzed over 8 days post-infection.

(C) *C57BL/6* mice were infected as above and at the indicated times, intrasplenic bacterial counts were determined.

(D) WT and *Casp1*$^{-/-}$ mice were infected with WT *B. anthracis* (0.5×10^6 cfu/mouse, n=5-8 mice per group). Mouse survival was analyzed over 8 days post-infection.

(E) Mouse macrophages were infected with WT (BA) or ΔLF *B. anthracis* in the absence or presence of Anakinra (IL-1RA, 15 μg/ml) and after 4 hr, LDH release into culture supernatants was determined. Results are means ± SD, *$p < 0.05$, denote significant differences between the groups. This experiment was repeated twice and the results of one representative experiment done in triplicates are shown.

(F) WT and *Casp1*$^{-/-}$ macrophages were infected with WT *B. anthracis* and 4 hr later, the cellular content of the indicated mRNAs was analyzed by Q-RT-PCR. Results were normalized to the amount of GAPDH mRNA and are means ± SD, *$p < 0.05$ denote significant differences between the groups. This experiment was repeated 3 times and the results of one representative experiment done in triplicates are shown.

Table S1: Summary of time points used for analysis of various parameters.

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Mice and reagents

Nod2$^{-/-}$, *caspase-1*$^{-/-}$, *Ripk2*$^{-/-}$ and *Nlrpc4*$^{-/-}$ mice were described (Chin et al., 2002; Kobayashi et al., 2005; Kuida et al., 1995; Mariathasan et al., 2004), and were all in the *C57BL/6* background. WT *C57BL/6* mice from Charles River Laboratories were bred and maintained in the UCSD vivarium. Fluorescent-labeled antibodies for flow cytometry were from eBioscience. Immunoblot analysis was carried out with antibodies recognizing: Caspase-1 (p10), MEK6 and p-connexin-43(S279/282) from Santa Cruz, IL-1β, connexin-43, phospho-p38(Thr180/Tyr182), phospho-JNK(Thr183/Tyr185), phospho-ERK(p44/42) from Cell Signaling. KN-62 and carbenoxolone were purchased from Promega and Sigma, respectively.

Analysis of gene expression, cytokine secretion and cell signaling

RNA was extracted using RNeasy Plus kit (BioMiga) and reverse transcribed using an iScript kit (Bio-Rad). Real-time PCR was performed using SYBR green (Bio-Rad) on a Bio-Rad iQ5 machine. Expression data were normalized to GAPDH mRNA. Primer sequences are available upon request. Whole cell extracts were separated by SDS-polyacrylamide gel electrophoresis, transferred to Immobilon membranes (Millipore) and analyzed by immunoblotting. Cytokines were quantitated using ELISA kits from BD.

Co-immunoprecipitation

Macrophage lysates were precleared with anti rabbit IgG immuprecipitation beads (True blot, eBioscience) for 1 hr following manufacturer's recommendations to reduce non-specific binding. Immunoprecipitation was performed with caspase-1 antibody by overnight incubation at 4°C. Immuprecipitation beads were added for 2 hr at 4°C and the co-precipitated proteins were recovered according to manufacturer's recommendation and analyzed by immunoblotting with the following antibodies: NOD2, IPAF and ASC from Genentech, caspase-1 (p10) and RIP2 from Santa Cruz, and NALP1 and NALP3 from Abcam.

Cytokine analysis by immunoblotting

Culture supernatants were mixed with 6.1N tri-chloroacetic acid solution (10:1) and incubated overnight at 4°C to allow protein precipitation. Samples were centrifuged at 14K rpm for 30 min and the pellets were washed with ice cold acetone. After heat drying, protein pellets were resuspended in Laemmli sample buffer, separated by SDS PAGE and analyzed by immunoblotting with the following antibodies: caspase-1 (p10) from Santa Cruz and IL-1β (p17) from Cell Signaling.

Transfections and Retroviral infection

293T cells and RAW264.7 cells were transfected using Lipofectamine 2000 (Invitrogen) and SuperFect (Qiagen), respectively, according to manufacturer's instructions. Retroviruses were prepared in 293T cells and culture supernatants containing retroviruses were used for infection of macrophages. Myr-AKT expression was assayed at 72 hr post-viral infection by immunoblotting.

Bacterial infections

For ex vivo infection, early log phase WT or ΔLF *B. anthracis* Sterne strain were added to mouse macrophages at MOI = 2. After 1 hr, gentamicin (50 μg/ml) was added to kill extracellular bacteria and culture supernatants were collected and analyzed for cytokines and activated caspase-1 8 hr after infection. For in vivo infections, mice were injected intraperitoneally with 2×10^6 cfu of early log phase bacteria. Mice were evaluated at different time points for viability and cell death by in situ TUNEL assay.

Bacterial killing assay

Macrophages were infected with WT or ΔLF *B. anthracis* as above. After 45 min, bacteria were washed off and fresh medium containing gentamycin was added for 20 min, after which it was replaced with antibiotic-free medium. Cells were incubated for the indicated times, after which they were lysed with 0.025% Triton X-100 and intracellular bacteria were collected, serially diluted and plated on Todd-Hewitt agar plates for determination of bacterial cfu.

ATP release

ATP in culture supernatants was determined using a kit and a protocol from Promega with little modifications. In brief, mouse macrophages were infected with bacteria in RPMI medium containing 0.1% FBS. Supernatants were collected at the indicated times and spun at high speed for 5 min to pellet and exclude any cellular or bacterial debris. The pellets were removed and the supernatants were used for measurement of ATP. Under conditions of high cell death, this protocol may not work as leakage of apyrases may hydrolyze the secreted ATP.

shRNA-mediated gene silencing. Oligonucleotides (sequences available upon request) corresponding to connexin-43-specific shRNA were cloned into pLSLPw (lentivirus vector) and

lentiviruses were prepared as described (Hsu et al., 2008). RAW264.7 cells were infected with the lentiviruses and cultured for 48 hr before infection with *B. anthracis*.

Statistical analysis

To analyze differences in mean values between groups, a two-tailed unpaired Student's t test was used.

SUPPLEMENTAL REFERENCES

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