Supplementary Information

Kinase Suppressor of Ras-1 Protects against *Pseudomonas aeruginosa* Infections

Yang Zhang,1,3 Xiang Li,1,3 Alexander Carpinteiro,1 Jeremy A. Goettel2, Matthias Soddemann,1 and Erich Gulbins1

1Department of Molecular Biology, University of Duisburg-Essen, Hufelandstrasse 55, 45122 Essen, Germany.
2Department of Cell and Developmental Biology, Vanderbilt University School of Medicine and Monroe Carell Jr. Children's Hospital at Vanderbilt, Nashville, Tennessee 37232-0696, USA.
3These authors contributed equally to this work.

Correspondence should be addressed to:
Dr. Erich Gulbins, Department of Molecular Biology, University of Duisburg-Essen, Hufelandstrasse 55, 45122 Essen, Germany, Tel: 49-201-723-3118, Fax: 49-201-723-5974, e-mail: erich.gulbins@uni-due.de
Supplementary note 1:
The slow and inefficient elimination of the bacteria in Ksr1-deficient mice (see Fig. 1b) indicates that not all defense mechanisms in the lung depend on Ksr1. In fact, our findings show that the production of reactive oxygen species does not differ in the lungs of Ksr1-deficient and wild-type mice upon infection with *P. aeruginosa* (not shown).

Supplementary note 2:
To address whether the Ksr1-mediated iNOS activation is a general mechanism or is restricted to intracellular bacteria, we determined the activity of iNOS and the formation of NO in LPS-treated bone marrow-derived macrophages from wild-type and Ksr1-deficient mice by the Griess reaction (kit from Invitrogen), which determines the formation of nitrite, a stable product of NO. The cells were left untreated or treated overnight with *E. coli* LPS (100 ng ml$^{-1}$). Upon treatment with LPS, the release of NO from Ksr1-deficient macrophages (from 0.21 ± 0.05 µM to 2.65 ± 0.49 µM) was not significantly different from the release of NO from wild-type macrophages (from 0.16 ± 0.04 µM to 4.12 ± 0.55 µM) (mean ± SE, n = 4, p = 0.1). These findings indicate that Ksr1 plays a differential role in iNOS activation induced by LPS and by *P. aeruginosa* and suggest some specificity for the role of Ksr1 in *P. aeruginosa* infection of macrophages and neutrophils.

Supplementary note 3:
The assay was performed with a saturated concentration of substrates, i.e. the concentration of [³H]-L-arginine is 100-fold higher than K$_m$ of iNOS. Under these conditions the V$_{max}$ of iNOS activity is determined by the conversion rate of L-arginine (pmol h$^{-1}$ per mg protein) during 1 h of incubation. The activity is 25.5 pmol h$^{-1}$ per mg of protein for recombinant iNOS.
Supplementary Figure 1

**IL-1β concentrations in the lung after *P. aeruginosa* infection**

We measured the release of cytokines other than TNF-α in the lung after *P. aeruginosa* infection. Similar to the results for TNF-α (Fig. 1c), IL-1β was markedly increased in both wild-type and Ksr1-deficient mice 6 h after infection (Supplementary Fig. 1). After 20 h, IL-1β concentrations in the lungs of wild-type mice returned to control levels, while IL-1β remained high in the lungs of Ksr1-deficient mice 20 h after infections.

Supplementary Fig. 1: Ksr1 deficiency results in uncontrolled IL-1β release in the lung upon pulmonary *P. aeruginosa* infection. Data are means and standard deviations of six independent experiments. Asterisks indicate significant differences compared to the respective wild-type mice (*P < 0.05, **P < 0.005).
Supplementary Figure 2

Effect of neutralizing antibodies to TNF-α

TNF-α is crucial for the innate resistance to *P. aeruginosa* infection. The clearance of *P. aeruginosa* is defective in TNF-α knockout mice and in mice treated with pharmacological inhibitors of TNF-α, as well as in bone marrow-derived macrophages from mice deficient in TNF-α. However, although a moderate cytokine release is necessary for the elimination of *P. aeruginosa*, an uncontrolled and excessive release of TNF-α results in a cytokine storm and, eventually, death. In the present study, levels of cytokines, including TNF-α, in the lung remained very high in infected, Ksr1-deficient mice, a finding indicating that the defect in bacterial clearance in the lung is not caused by reduced TNF-α expression. Rather, the uncontrolled release of TNF-α in Ksr1-deficient mice, which occurs as the result of impaired bacterial clearance, contributes to a cytokine storm, septic shock, and death. This notion is supported by the finding that the death of Ksr1-deficient mice was prevented by treating the animals with neutralizing antibodies to TNF-α (Supplementary Fig. 2a), although the number of bacteria in the lung 20 h after infection was still very high in Ksr1-deficient mice treated with the neutralizing antibodies to TNF-α (Supplementary Fig. 2b). Those mice slowly and relatively inefficiently eliminated the bacteria by as long as 48 h after infection. All Ksr1-deficient mice that did not receive antibodies to TNF-α were already dead 48 h post infection and no CFU could be determined at this time point for this group. This finding indicates that Ksr1-deficient mice died of a cytokine storm caused by the impairment of bacterial clearance from the lung; this outcome was prevented by neutralizing antibody to TNF-α. However, these antibodies had no effect on bacterial clearance in the lungs of Ksr1-deficient mice 20 h after infection (Supplementary Fig. 2b). The prevention of a cytokine storm permits the mice to slowly kill *P. aeruginosa* in the lung after 48 h by Ksr1-independent pathways that are much less efficient and much slower than the pathway controlled by Ksr1.
Supplementary Fig. 2: Effect of antibodies to TNF-α on survival and bacterial clearance in the lung upon pulmonary *P. aeruginosa* infection. Ksr1-deficient mice were treated with or without antibodies to TNF-α (anti-TNF-α; i.p., 2 µg g⁻¹) 6 h after intranasal infection with *P. aeruginosa*. Antibody to TNF-α rescued the mice (a), but had no effect on clearance of *P. aeruginosa* in the lung at 20 h and 24 h after infection (b). Bacterial numbers in the lung slowly declined in Ksr1-deficient mice treated with neutralizing antibody to TNF-α up to 48 h (b). Data in a are presented as Kaplan-Meyer curves from three independent experiments with three mice per group (*, P < 0.05; log-rank test). Data in b are means and standard deviations of six independent experiments.

References


**Supplementary Figure 3**

*Fig. 3: Ksr1-siRNA efficiently down-regulates Ksr1 expression in J774 macrophages.* The efficiency of siRNA transfection in cultured J774 macrophages was determined by immunoblot analysis of Ksr1 expression 24 h after transfection. The blots and images are representative of three independent experiments.

**Supplementary Figure 4**

*Fig. 4: Ksr1 deficiency does not affect P. aeruginosa-induced up-regulation of iNOS in neutrophils.* The expression of iNOS in PMNs were determined by immunoblot analysis using iNOS-specific antibody after 2 h infection with *P. aeruginosa* (MOI = 100). The blots and images are representative of three independent experiments.
**Supplementary Figure 5**

Supplementary Fig. 5 DETA-NONOate inhibits the effect of L-NAME, which increases the mortality of wild-type mice when compared to saline-treated wild-type mice. Data are means and standard deviations of six independent experiments (Kaplan-Meyer curves; *, $P < 0.05$; log-rank test).
Supplementary Fig. 6 DETA-NONOate blocks the inhibitory effects of a specific iNOS inhibitor, 1400W, on bacterial killing in (a) wild-type macrophages and (b) PMNs. (c) 1400W sensitized wild-type mice to pulmonary *P. aeruginosa* infection, which was reversed by DETA-NONOate. Data in a–c are presented as means ± standard deviations from four independent experiments (a, b; ***, *P* < 0.001) and as Kaplan-Meyer curves with eight mice per group (c; *, *P* < 0.05; log-rank test).
Supplementary Methods:

**Cytokine levels and bacterial and neutrophil counts in the lung.** Mice were infected as described above for the indicated time. Lung tissue was collected from infected or uninfected mice and was immediately shock-frozen in liquid nitrogen. Frozen tissue was cut into small pieces, transferred into HEPES/saline (H/S; 132 mM NaCl, 20 mM HEPES [pH 7.4], 5 mM KCl, 1 mM CaCl₂, 0.7 mM MgCl₂, 0.8 mM MgSO₄), and homogenized in a Dounce potter. IL-1β and TNF-α concentrations were determined in the supernatants after centrifugation by using commercial ELISA kits (R&D Systems) according to the manufacturer’s instructions. For bacterial counts, the lung pellets were resuspended in H/S buffer after infection, cut into small pieces, mixed with 5 mg ml⁻¹ saponin, and incubated for 10 min at 37 °C for the release of internalized bacteria. The samples were centrifuged at 1,200 × g for 10 min, resuspended in PBS, applied for serial dilution, and plated on TSB agar plates, whereupon CFUs were counted. To count neutrophils in bronchoalveolar lavage fluid, we inserted a small catheter into the trachea, injected 1 ml of PBS into the lung, and recovered the fluid by very gentle suction. This procedure was repeated 10 times, and the total neutrophil count was determined by flow cytometry (BD Biosciences) with antibody to Gr1.

**Immunohistochemistry.** Mice were euthanized, and blood was cleared from the pulmonary circulation by perfusion through the right ventricle with 10 ml PBS (pH 7.4, 4 °C) at 20 cm H₂O. The trachea was cannulated, and the lungs were inflated with 4% paraformaldehyde (PFA) for 10 minutes at 25 cm H₂O. The lungs were subsequently removed, fixed in 4% PFA for 24 hours, and embedded in paraffin for sectioning at a thickness of 6 µm. The sections were stained with hematoxylin and eosin (H&E).

**Isolation of alveolar macrophages from mice.** Lung alveolar macrophages were isolated from bronchoalveolar lavage fluid. After the mice were euthanized, a small catheter was inserted into the trachea, and 1 ml of PBS was injected into the lung and recovered by very gentle suction. The lung was lavaged with a total of 15 ml ice-cold PBS in 20 aliquots (0.75 ml per aliquot). Approximately 0.5 to 1 × 10⁶ cells per mouse were consistently obtained. Cells were pelleted by centrifugation at 300 × g for 15 min,
resuspended and cultured for 1 hour in RPMI-1640 (Gibco-Life Technologies) with 1 mM HEPES (pH 7.4) in 24-well plates at a density of $10^5$ cells for each well. Alveolar macrophages are extremely adhesive cells. Therefore, after other blood cells were washed off, a pure cell culture was obtained in which more than 99% of cells were macrophages, as confirmed by flow cytometry after staining with FITC-coupled antibody to CD11b (BD Biosciences).

**Preparation of peripheral neutrophils.** Blood was collected from anesthetized mice by cardiac puncture using a 5 ml syringe and 22-gauge needle with 5 units of heparin. Peripheral neutrophils were then isolated using a discontinuous density gradient composed of two solutions of a radiopaque medium of differential density (Histopaque 1077 and 1119, Sigma). Briefly, 3 ml Histopaque 1119 were added to a 15 ml tube and then 3 ml Histopaque 1077 were gently loaded on top of the 1119 layer followed by careful addition of 1 ml peripheral blood over the upper gradient. Samples were centrifuged for 30 min at 700 $\times g$ at room temperature without using a brake. Neutrophils were collected from the interface of Histopaque 1077 and 1119 layers and transferred to a 15-ml tube. Cells were washed three times with 10 ml PBS, centrifuged for 5 min at 200 $\times g$ at room temperature and resuspended in DMEM supplemented with 10% mouse serum.

**Bacterial killing capability and phagocytosis assays.** To determine the bactericidal capability of macrophages, we cultured macrophages in 96-well plates ($10^5$ cells per well) and infected them with *P. aeruginosa* strain ATCC 27853 opsonized in 5% mouse serum (MOI = 1). Phagocytosis of *P. aeruginosa* ATCC 27853 by macrophages was synchronized by centrifugation at 300 $\times g$ for 5 min, followed by infection for 30 min at 37 °C. To remove extracellular and attached bacteria, we gently washed the cells three times with sterile PBS. To test whether the washing steps were sufficient to remove all extracellular bacteria, we counted the CFUs in the presence or absence of polymixin (100 µg ml$^{-1}$). The cells were then lysed in saponin (5 mg ml$^{-1}$) for 5 min to release intracellular bacteria. Aliquots of the lysates were plated on LB-agar plates, and the CFUs were counted after overnight growth at 37 °C. These studies revealed no difference
in the numbers of CFUs if we added or omitted polymyxin, a finding indicating that the washing steps removed all extracellular bacteria. This result permitted us to perform antibiotic-free studies to determine the intracellular killing of the bacteria, as previously described.\textsuperscript{1,2} Briefly, to measure intracellular killing in the absence of antibiotics, we incubated the macrophages for an additional 0 or 60 min at 37 °C in the presence or absence of the indicated inhibitors. After infection, macrophages were incubated with 20 µl saponin (5 mg ml\textsuperscript{-1}) for 5 min for the release of intracellular bacteria. Then 100 µl tryptose phosphate broth and 15 µl MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in PBS (5 mg ml\textsuperscript{-1}) were added to each well, and the cells were incubated at 37 °C for 4 h. Reactions were stopped by adding 100 µl isopropanol with 0.04 M HCl, and absorbance was measured at 570 nm with a microplate reader (BMG Labtech, Offenburg, Germany). To determine the relationship between absorbance and CFU, we incubated 10\textsuperscript{3} to 10\textsuperscript{5} CFUs \textit{P. aeruginosa} ATCC 27853 with 100 µl tryptose phosphate broth and 20 µl MTT in PBS (5 mg ml\textsuperscript{-1}) without macrophages for 4 h. Macrophage killing efficiency was indicated as the percentage of CFU of samples 60 min after infection over the CFU of samples 0 min after infection.

To determine the bactericidal capability of polymorphonuclear leukocytes (PMNs; isolated from blood as described above), we mixed 10\textsuperscript{5} PMNs with \textit{P. aeruginosa} (MOI = 1) in 96-well plates and incubated the mixture at 37 °C for 90 min with shaking. Samples with \textit{P. aeruginosa} only were used as controls. Incubations were terminated with saponin (5 mg ml\textsuperscript{-1}), and the number of bacterial CFUs was determined as described above. PMN killing efficiency was indicated as the CFU percentage of samples mixed with both PMN and \textit{P. aeruginosa} over that of samples with \textit{P. aeruginosa} only. As indicated, PMNs were pretreated with aminoguanidine (AG, 100 µM), 1400W (100 µM), DETA-NONOate (100 µM) or L-NAME (100 µM) for 20 min.

The phagocytotic capability of Ksr1-deficient macrophages was examined by internalization of \textit{P. aeruginosa}, FITC-zymosans, and latex beads. FITC-zymosans (Molecular Probes Inc.) were opsonized with BioParticle opsonizing reagent (Molecular Probes Inc.), as instructed by the manufacturer. Macrophages were then incubated with \textit{P. aeruginosa} (MOI = 100), FITC-zymosans (20 zymosans per cell), or latex beads (20 beads per cell). Phagocytosis was synchronized by spinning at 300 × g for 1 min, and
Macrophages were incubated for 30 min at 37 °C. Macrophages were washed twice with PBS and fixed with 2% PFA in PBS (pH 7.3) for 15 min at room temperature. Internalization of *P. aeruginosa* was determined by cellular crystal violet staining. To determine internalization of FITC-zymosans and latex beads, we mounted macrophages on glass coverslips with Moviol and examined them by confocal microscopy.

**Immunoblot analyses.** Cells were lysed in SDS-sample buffer (62.5 mM Tris[pH 6.8], 10% glycerol, 2% SDS, 0.04% bromphenol blue, and 5% 2-mercaptoethanol), boiled, and sonicated (3 times, 10 s each) on ice to break chromosomal DNA and to decrease sample viscosity. Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The blots were blocked in 4% BSA in Tris buffered saline supplemented with 0.1% Tween 20 and incubated overnight at 4 °C with the designated primary antibodies. Blots were labeled with alkaline phosphatase-coupled secondary antibodies (Santa Cruz Biotechnology Inc.) and developed using the Tropix system.

**RNA interference.** Small interference RNAs for mouse Ksr1 (Santa Cruz Biotech Inc.) and scramble siRNAs (Invitrogen) are commercially available. For transfection of each 10⁵ cells, 250 ng of siRNAs and 1 μl Lipofectamine Reagent (Invitrogen) were used. The siRNAs were introduced into J774 macrophages according to manufacturer’s instructions. Cells were used for experiments 24 h after transfection.

**Immunoprecipitations.** Macrophages (2 × 10⁷ J774 cells) were infected for 2 h and lysed in 1 ml ice-cold lysis buffer containing 30 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, and complete inhibitors of proteases (Roche) and phosphatases (Sigma).³ Samples were centrifuged at 14,000 × g for 5 min, and the supernatants were incubated for 4 h at 4 °C with 2 μg of mouse monoclonal antibody to Ksr1 or antibody to iNOS (BD Biosciences) or rabbit polyclonal antibody to Hsp90 (Cell Signaling). Immunocomplexes were incubated for 1 h with protein G plus agarose (Santa Cruz Biotechnologies) and were then washed three times with lysis buffer. Immunoprecipitates were resolved in 10% reducing SDS-PAGE gel, transferred to nitrocellulose membranes and blotted with the indicated antibodies (1:1,000). To detect
serine phosphorylation of Ksr1, we incubated membranes with antibodies against phosphoserine (1:1,000 dilution, Sigma). Corresponding control IgGs were used for immunoprecipitation studies, and neither Ksr1, iNOS, nor Hsp90 was detected in these control immunoprecipitates.

**Fluorescence microscopy.** Macrophages were infected, fixed, permeabilized, and blocked as described. The cells were then incubated for 45 min with mouse monoclonal FITC-conjugated antibody to iNOS (BD Biosciences, 0.5 µg ml⁻¹) and Alexa-555-conjugated antibody to Ksr1 (0.5 µg ml⁻¹) antibodies. We prepared Alexa-555-conjugated antibody to Ksr1 by using the Zenon mouse IgG labeling kit (Molecular Probes Inc.), as instructed by the manufacturer. Control experiments were performed with Alexa-555-conjugated irrelevant IgG. The cells were washed three times with PBS and stained with guinea pig antibody to *P. aeruginosa* (Biotrend, 50 ng ml⁻¹). The cells were washed three times with PBS and labeled with Cy5-conjugated antibody to guinea pig F(ab')₂ fragments (Jackson ImmunoResearch Laboratories, 0.5 µg ml⁻¹). After fluorescence labeling, cells were fixed again and mounted on glass coverslips with Moviol. Cells were examined with a Leica TCS-SP2 confocal microscope equipped with a 100 × oil lens, and images were analyzed with Leica LCS software (Leica Microsystems).

**Bone marrow transplantation.** Ksr1 chimeras were generated as previously described. Briefly, 4-week-old mice were irradiated for depletion of bone marrow; the bone marrow was then reconstituted by transplantation. Ten weeks after bone marrow transplantation, mice were intranasally infected with 5 × 10⁸ CFU *P. aeruginosa* ATCC 27853 and observed for survival for 7 days.

**References:**

