

Kinase suppressor of Ras-1 protects against pulmonary *Pseudomonas aeruginosa* infections

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Pseudomonas aeruginosa is a Gram-negative pathogen that causes severe infections in immunocompromised individuals and individuals with cystic fibrosis or chronic obstructive pulmonary disease (COPD). Here we show that kinase suppressor of Ras-1 (Ksr1)-deficient mice are highly susceptible to pulmonary *P. aeruginosa* infection accompanied by uncontrolled pulmonary cytokine release, sepsis and death, whereas wild-type mice clear the infection. Ksr1 recruits and assembles inducible nitric oxide (NO) synthase (iNOS) and heat shock protein-90 (Hsp90) to enhance iNOS activity and to release NO upon infection. Ksr1 deficiency prevents lung alveolar macrophages and neutrophils from activating iNOS, producing NO and killing bacteria. Restoring NO production restores the bactericidal capability of Ksr1-deficient lung alveolar macrophages and neutrophils and rescues Ksr1-deficient mice from *P. aeruginosa* infection. Our findings suggest that Ksr1 functions as a previously unknown scaffold that enhances iNOS activity and is therefore crucial for the pulmonary response to *P. aeruginosa* infections.

P. aeruginosa is a ubiquitous Gram-negative and opportunistic pathogen that causes severe respiratory tract and systemic infections, especially in immunocompromised individuals, individuals with cystic fibrosis, healthcare-associated pneumonia and ventilator-associated pneumonia in intensive care units^{1–4}. Mortality rates associated with ventilator-associated pneumonia range from 33% to 72%. Chronic airway infections with *P. aeruginosa* are also common among people with advanced-stage COPD⁵. Thus, it is crucial to define the molecular mechanisms that determine the host defense against pulmonary *P. aeruginosa* infections.

In this study, we investigate the function of Ksr1 in the response to *P. aeruginosa* infections of the lung. Ksr1, a ceramide-activated, proline-directed serine-threonine kinase, has been shown to directly phosphorylate and activate Raf1 (refs. 6,7). Ksr1 also functions as a scaffold for the Raf-1–mitogen-activated protein kinase kinase (Mek)–extracellular signal-regulated kinase (Erk)–mitogen-activated protein kinase (Mapk) pathway⁸. Genetic deficiency of Ksr1 results in defects in Erk–Mapk signaling⁹, antigen-triggered T cell proliferation¹⁰, formation of hair follicles¹¹ and Ras-dependent tumor formation^{11,12}.

Here we investigate the role of Ksr1 in phagocytes during bacterial pneumonia^{13,14}. Alveolar macrophages, the resident mononuclear phagocytes in the respiratory tract, are part of the first line of cell-mediated defenses against inhaled organisms^{15,16}. Neutrophils infiltrate from the peripheral blood to the site of infection in the lung to clear bacteria. We found that Ksr1 is necessary for the control of pulmonary infections with *P. aeruginosa*. Ksr1-deficient mice fail to clear *P. aeruginosa* from the lung, rapidly experience sepsis and die, whereas wild-type mice resist the infection. Ksr1 serves to assemble iNOS and Hsp90 and, thus, to activate iNOS. Ksr1-deficient alveolar macrophages and neutrophils fail to activate iNOS and kill

P. aeruginosa. DETA-NONOate, an NO donor, restores the bactericidal capability of Ksr1-deficient alveolar macrophages and neutrophils, thereby protecting Ksr1-deficient mice from mortality induced by pulmonary *P. aeruginosa* infections. These findings demonstrate that the interaction of Ksr1, Hsp90, and iNOS is a crucial component of the innate host response during pulmonary *P. aeruginosa* infection.

RESULTS

Ksr1 protects mice from pulmonary *P. aeruginosa* infection

To determine whether Ksr1 has a role in the response to pulmonary *P. aeruginosa* infection, we infected Ksr1-deficient and wild-type mice intranasally with 5×10^8 colony-forming units (CFU) of *P. aeruginosa* strain American Type Culture Collection (ATCC) 27853. Wild-type mice cleared the infection within 1 to 2 d and survived. In contrast, Ksr1-deficient mice were highly sensitive to the infection and died as early as 22 h after infection (Fig. 1a). This mortality was associated with uncontrolled bacterial growth and release of cytokines in the lung (Fig. 1b,c, Supplementary Fig. 1 and Supplementary Note 1). The bacterial load and cytokine abundance in the lungs paralleled the recruitment of neutrophils into the bronchoalveolar lavage fluid (Fig. 1d). H&E staining of lung sections confirmed the destruction of lung alveolar spaces and the infiltration of neutrophils in Ksr1-deficient mice 20 h after infection with *P. aeruginosa* (Fig. 1e). As a consequence of impaired bacterial clearance, Ksr1-deficient mice died of sepsis, as indicated by high bacterial numbers in the spleen (Fig. 1f and Supplementary Fig. 2). Taken together, our findings suggest that Ksr1 is key for resistance to *P. aeruginosa* and for clearance of the pathogen from the respiratory tract. The failure of Ksr1-deficient mice to clear the infection results in sepsis, cytokine storm and death.

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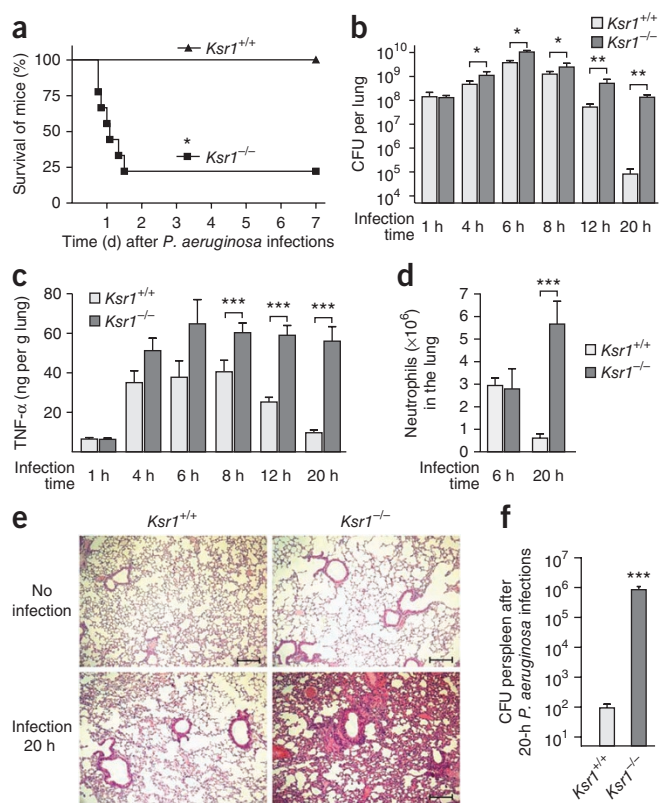


Figure 1 Ksr1 deficiency results in hypersusceptibility to pulmonary *P. aeruginosa* infection. (a–e) Displayed are the mortality rates after infection of wild-type and Ksr1-deficient mice with 5×10^8 CFU of *P. aeruginosa* strain ATCC 27853 (a), the number of the bacteria in the lungs (b), the release of TNF- α in the lungs (c), the neutrophil counts in the bronchoalveolar lavage fluid (d) and H&E staining of lungs before and 20 h after pulmonary *P. aeruginosa* infection (e). (f) CFU counts in the spleens of wild-type and Ksr1-deficient mice 20 h after pulmonary infection. Scale bar, 80 μ m. Data in a are presented as Kaplan-Meier curves from three independent experiments with three mice per group (* $P < 0.05$; log-rank test). Data in b–f are means \pm s.d. from six independent experiments or are representative of six independent experiments (* $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$).

(Fig. 2a). In contrast, *P. aeruginosa* survived and replicated in Ksr1-deficient macrophages (Fig. 2a). We observed a very similar deficiency in killing capability for Ksr1-deficient neutrophils; freshly isolated Ksr1-deficient peripheral neutrophils only partially cleared *P. aeruginosa* and failed to kill the bacteria, whereas neutrophils from wild-type mice efficiently killed *P. aeruginosa* (Fig. 2a). These findings indicate that Ksr1 deficiency impairs the ability of macrophages and neutrophils to kill *P. aeruginosa*. This defect is not caused by the failure of Ksr1-deficient macrophages to internalize *P. aeruginosa*, latex beads or FITC-zymosan (Fig. 2b).

Recent studies have shown that Ksr1 regulates the Erk-Mapk signaling pathway⁸. However, the Mek inhibitor U0126 did not alter the killing of *P. aeruginosa* by wild-type macrophages (Fig. 2c), although it inhibited *P. aeruginosa*-induced Erk1/2 phosphorylation (Fig. 2c). Furthermore, the early nuclear translocation of nuclear factor- κ B and the immediate (but not the later) release of cytokines (tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) and KC) did not differ between wild-type and Ksr1-deficient macrophages after infection with *P. aeruginosa* (Fig. 2d,e). Taken together, these findings indicate that the known pathways initiated by Ksr1, and the Erk pathway in particular, do not mediate defective bacterial clearance in Ksr1-deficient mice.

Ksr1 is required for bactericidal activity of phagocytes

Alveolar macrophages are a major part of the first line of innate host defense to clear bacteria in the early stages of infection. To determine the function of Ksr1 in macrophages, we used a bactericidal killing assay to measure the *ex vivo* clearance of intracellular *P. aeruginosa* by alveolar macrophages. Freshly isolated alveolar macrophages from wild-type mice efficiently killed *P. aeruginosa* 60 min after internalization

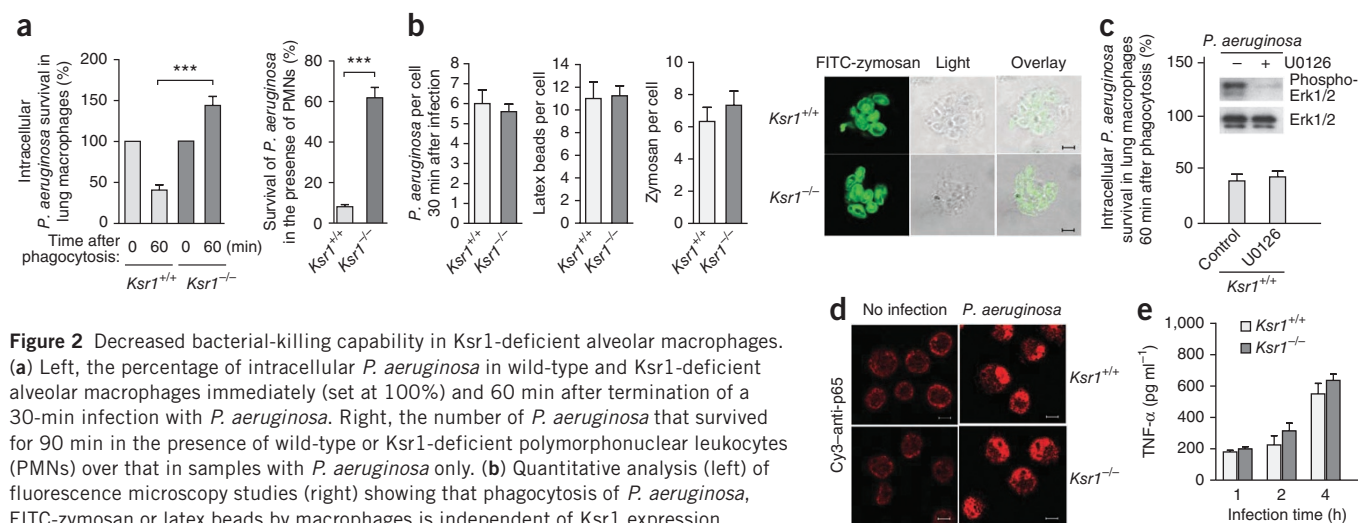


Figure 2 Decreased bacterial-killing capability in Ksr1-deficient alveolar macrophages. (a) Left, the percentage of intracellular *P. aeruginosa* in wild-type and Ksr1-deficient alveolar macrophages immediately (set at 100%) and 60 min after termination of a 30-min infection with *P. aeruginosa*. Right, the number of *P. aeruginosa* that survived for 90 min in the presence of wild-type or Ksr1-deficient polymorphonuclear leukocytes (PMNs) over that in samples with *P. aeruginosa* only. (b) Quantitative analysis (left) of fluorescence microscopy studies (right) showing that phagocytosis of *P. aeruginosa*, FITC-zymosan or latex beads by macrophages is independent of Ksr1 expression. Scale bar, 2.5 μ m. (c) Shown is the influence of Erk1/2 inhibitor U0126 on survival of intracellular *P. aeruginosa* in wild-type macrophages, given as percentage of bacteria 60 min after a 30-min infection compared to the number immediately after termination of the 30-min infection. Phosphorylation and expression of Erk1/2 after infection is shown in the immunoblot insets. (d) Confocal microscopy to determine nuclear localization of the p65 subunit of nuclear factor- κ B. (e) TNF- α release measured after short-term infection with *P. aeruginosa* in wild-type and Ksr1-deficient macrophages. Scale bar, 5 μ m. Images in b and d are representative of three separate experiments. Data are presented as means \pm s.d. of four independent experiments. Asterisks indicate significant differences from results obtained from wild-type cells at the respective time points (*** $P < 0.001$).

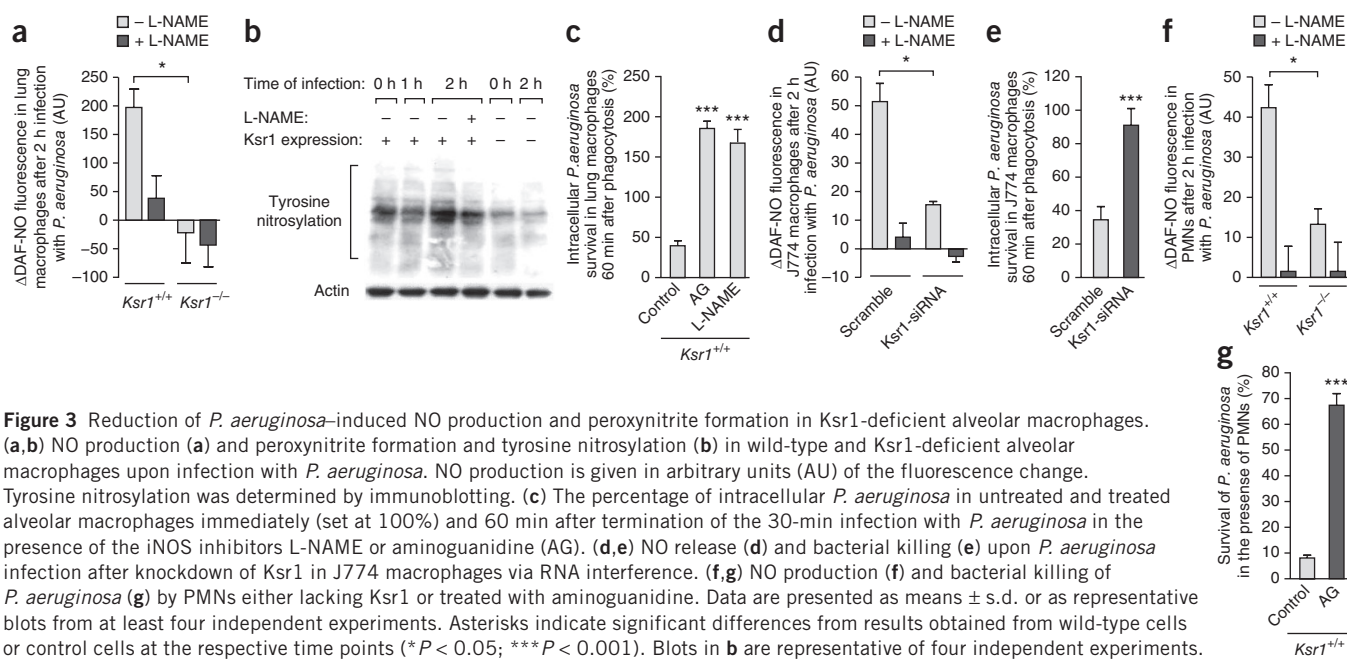


Figure 3 Reduction of *P. aeruginosa*-induced NO production and peroxynitrite formation in Ksr1-deficient alveolar macrophages. (a,b) NO production (a) and peroxynitrite formation and tyrosine nitrosylation (b) in wild-type and Ksr1-deficient alveolar macrophages upon infection with *P. aeruginosa*. NO production is given in arbitrary units (AU) of the fluorescence change. Tyrosine nitrosylation was determined by immunoblotting. (c) The percentage of intracellular *P. aeruginosa* in untreated and treated alveolar macrophages immediately (set at 100%) and 60 min after termination of the 30-min infection with *P. aeruginosa* in the presence of the iNOS inhibitors L-NAME or aminoguanidine (AG). (d,e) NO release (d) and bacterial killing (e) upon *P. aeruginosa* infection after knockdown of Ksr1 in J774 macrophages via RNA interference. (f,g) NO production (f) and bacterial killing of *P. aeruginosa* (g) by PMNs either lacking Ksr1 or treated with aminoguanidine. Data are presented as means \pm s.d. or as representative blots from at least four independent experiments. Asterisks indicate significant differences from results obtained from wild-type cells or control cells at the respective time points (* $P < 0.05$; *** $P < 0.001$). Blots in b are representative of four independent experiments.

Ksr1 mediates the formation of NO and peroxynitrite

To define how Ksr1 mediates the host defense, we determined whether Ksr1 is involved in the production of reactive nitrogen, a key pathway in innate immune responses¹⁷. All three isoforms of NOS (endothelial NOS, neuronal NOS and iNOS) are expressed in the lung, but only iNOS is expressed after inflammatory stimulation, is more potent than the other two isoforms in generating NO and produces relatively large, micromolar quantities of NO in a Ca^{2+} -independent manner. Knocking out the gene encoding iNOS eliminates the bactericidal activity of alveolar macrophages and neutrophils against *P. aeruginosa*^{18,19}. Therefore, we examined whether Ksr1 deficiency disrupts NO production in Ksr1-deficient macrophages and neutrophils upon infection. We determined intracellular NO production by measuring DAFFM-NO (see Online Methods) fluorescence with flow cytometry analysis. Infection with *P. aeruginosa* greatly increased DAFFM-NO fluorescence, a finding that indicates a release of NO in wild-type macrophages; this release was prevented by the NOS inhibitor L-NAME. In contrast, DAFFM-NO fluorescence did not change in Ksr1-deficient macrophages, indicating that Ksr1-deficient macrophages failed to produce NO when infected with *P. aeruginosa* (Fig. 3a and Supplementary Note 2).

NO rapidly reacts with superoxide to form peroxynitrite, which is more toxic to bacteria than NO itself. Therefore, we measured the level of protein tyrosine nitrosylation to determine whether Ksr1 deficiency affects peroxynitrite formation. Infecting wild-type cells with *P. aeruginosa* resulted in marked tyrosine nitrosylation of cellular proteins, a result that was abrogated in Ksr1-deficient macrophages (Fig. 3b). Inhibiting iNOS with the iNOS inhibitors aminoguanidine or L-NAME increased the intracellular survival of *P. aeruginosa* in wild-type alveolar macrophages (Fig. 3c). Similarly, knockdown of Ksr1 via RNA interference in J774 macrophages, a mouse lung macrophage-like cell line, inhibited NO production (Fig. 3d) and impaired the ability of these cells to kill *P. aeruginosa* (Fig. 3e). The efficiency of knockdown was determined by measuring the protein expression of Ksr1 in J774 macrophages (Supplementary Fig. 3). Likewise, Ksr1-deficient peripheral neutrophils failed to produce NO upon *P. aeruginosa* infection (Fig. 3f), and aminoguanidine abolished the killing of *P. aeruginosa* by wild-type neutrophils

(Fig. 3g). These findings indicate the key role of Ksr1 in triggering the formation and release of NO and peroxynitrite by macrophages and neutrophils to kill *P. aeruginosa*.

Ksr1 associates with and activates iNOS

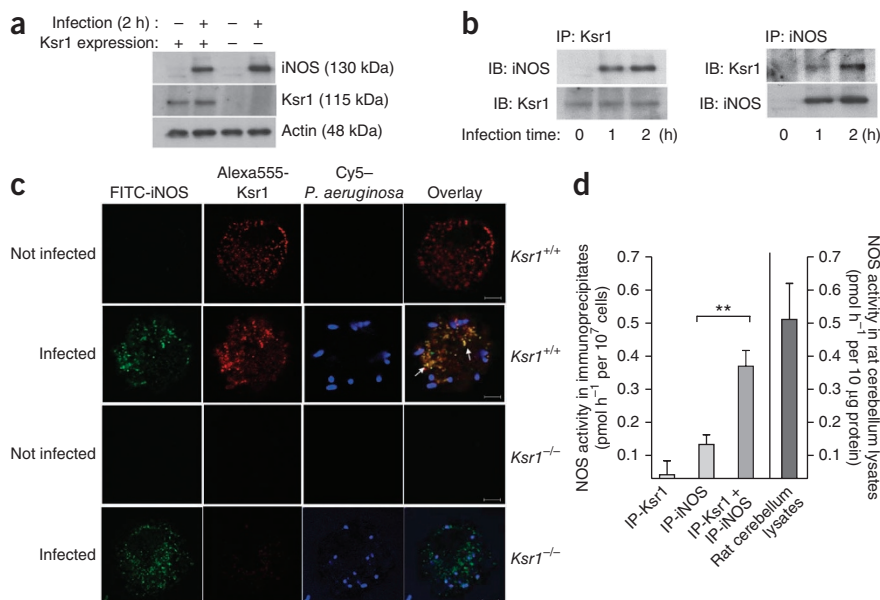
The differential NO production noted above may be the result of reduced iNOS expression in Ksr1-deficient cells. However, wild-type and Ksr1-deficient macrophages did not differ in *P. aeruginosa*-induced iNOS expression upon infection (Fig. 4a). Infection also had no effect on Ksr1 expression (Fig. 4a). Similar results were found for neutrophils (Supplementary Fig. 4). Next, we immunoprecipitated Ksr1 or iNOS and examined whether iNOS physically interacts with Ksr1. *P. aeruginosa* infection induced an association between Ksr1 and iNOS (Fig. 4b) that was absent before infection. Fluorescence microscopy studies confirmed a colocalization of iNOS and Ksr1 in wild-type alveolar macrophages after infection with *P. aeruginosa* (Fig. 4c). However, neither iNOS nor Ksr1 colocalized with *P. aeruginosa*-containing phagosomes in wild-type alveolar macrophages (Fig. 4c), suggesting that the generation of NO may not occur in or be limited to phagosomal membranes.

To determine whether Ksr1 enhances NOS activity upon infection *in vivo*, we immunoprecipitated Ksr1 from uninfected wild-type macrophages and iNOS from *P. aeruginosa*-infected, Ksr1-deficient macrophages. We then incubated the immunoprecipitates of iNOS with or without the immunoprecipitates of Ksr1, and we determined iNOS activity by measuring the conversion of L-[³H]-arginine to L-[³H]-citrulline in the absence of Ca^{2+} , finding that Ksr1 markedly enhances iNOS activity (Fig. 4d). These results indicate that Ksr1 stimulates iNOS after *P. aeruginosa* infection and support the existence of a previously unknown pathway controlled by Ksr1 during bacterial infections.

Ksr1 forms a complex with iNOS and Hsp90

To identify the mechanism of iNOS regulation by Ksr1, we examined whether Ksr1 and iNOS directly interact with each other and whether Ksr1 functions as a scaffold protein²⁰ or whether the kinase domain in Ksr1 is required for interaction with iNOS²¹. We used recombinant Flag-tagged Ksr1 (Flag-WT-Ksr1) and the kinase-inactive form of Ksr1 (Flag-KI-Ksr1), which we expressed in *Escherichia coli*

Figure 4 Ksr1 anchors and enhances the activity of iNOS. **(a)** Expression of iNOS in wild-type and Ksr1-deficient alveolar macrophages before and after *P. aeruginosa* infections, as determined by immunoblotting. **(b,c)** Coimmunoprecipitation (IP) **(b)** and confocal microscopy **(c)** experiments indicate that Ksr1 in macrophages interacts with iNOS 2 h after infection with *P. aeruginosa*. Immunoprecipitates were analyzed by immunoblotting (IB) with the corresponding antibodies to Ksr1 (left) or iNOS (right). Aliquots of the immunoprecipitates were blotted with the same antibody used for immunoprecipitation as a control for loading of similar amounts of protein. Arrows in **c** indicate the sites of colocalization of Ksr1 and iNOS in the confocal microscopy studies. Scale bar, 2.5 μm . **(d)** The activity of iNOS as measured in Ksr1 and iNOS immunoprecipitates and upon incubation of Ksr1 immunoprecipitates from wild-type cells (IP-Ksr1) with iNOS immunoprecipitates (IP-iNOS) from Ksr1-deficient macrophages infected with *P. aeruginosa*. Data are representative of four independent experiments or are presented as means \pm s.d. from four independent experiments (** $P < 0.005$).



and purified with agarose beads conjugated to antibody specific for Flag. We incubated the immobilized recombinant Ksr1 proteins with recombinant iNOS proteins and found that both the wild-type and the kinase-inactive forms of Ksr1 interact with recombinant iNOS (Fig. 5a). Furthermore, recombinant iNOS immobilized by agarose beads conjugated to iNOS-specific antibody also bound recombinant Ksr1 and kinase-inactive Ksr1 expressed in bacterial lysates (Fig. 5a). This finding indicates that iNOS directly binds Ksr1 and that this binding is independent of the kinase activity of Ksr1. The association of recombinant Ksr1 or kinase-inactive Ksr1 with recombinant iNOS did not increase the serine or threonine phosphorylation of iNOS, nor did it increase *P. aeruginosa* phosphorylation of serine in Ksr1 (data not shown). Collectively, these results indicate that Ksr1 anchors iNOS independently of its kinase activity.

To further investigate this notion, we examined the activity of recombinant iNOS upon binding to Ksr1 and found that neither recombinant Ksr1 nor kinase-inactive Ksr1 altered iNOS activity (Fig. 5b).

This finding suggests that the association of Ksr1 with iNOS alone is not sufficient for the enhancement of iNOS activity and that other factors are necessary for the stimulation of iNOS complexed to Ksr1. Previous findings indicated that Hsp90 couples with iNOS and regulates its activity in macrophages upon stimulation with lipopolysaccharide (LPS)^{22,23}. Therefore, we examined whether Hsp90 is present in Ksr1 immunoprecipitates obtained from macrophages. The results demonstrate a constitutive binding of Hsp90 to Ksr1 (Fig. 5c) and indicate the formation of a multiprotein complex consisting of Ksr1, iNOS and Hsp90 upon infection. In accordance, recombinant Hsp90 stimulated the activity of recombinant iNOS (Fig. 5b), and geldanamycin, an inhibitor of Hsp90, prevented NO production in macrophages upon *P. aeruginosa* infection (Fig. 5d). Adding recombinant Ksr1 to iNOS and Hsp90 produced only a slight additional increase in iNOS activity, indicating that the interaction of Hsp90 with iNOS is sufficient for activating iNOS and that Ksr1 acts *in vivo* by recruiting and assembling Hsp90 and iNOS (Fig. 5b and Supplementary Note 3).

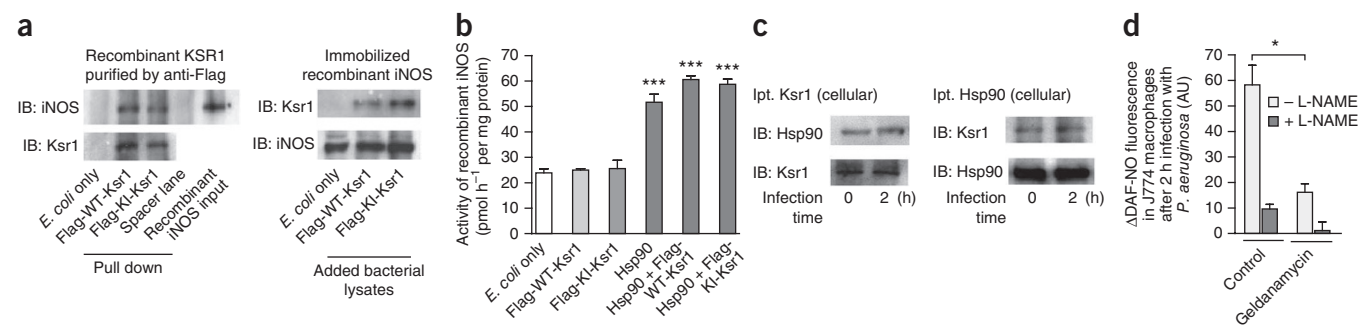


Figure 5 Ksr1 assembles Hsp90 and iNOS to activate iNOS. **(a)** Left, immobilized, recombinant Flag-tagged Ksr1 or kinase-inactive Ksr1 interact with soluble iNOS, as revealed by immunoblotting with antibodies to iNOS. Recombinant iNOS alone was loaded as control. Right, immobilized, recombinant iNOS binds Flag-tagged Ksr1 or kinase-inactive Ksr1 expressed in bacterial lysates. The blots were developed with Ksr1-specific antibodies. The bottom blots show the loading controls. **(b)** Measurements of the activity of recombinant iNOS showing a stimulation by Hsp90 alone and a maximal augmentation by recombinant Ksr1 or kinase-inactive Ksr1. **(c)** Ksr1 (left) or Hsp90 (right) immunoprecipitates from infected or noninfected macrophages immunoblotted with Hsp90-specific or Ksr1-specific antibodies, respectively. The bottom blots are loading controls. **(d)** NO release determined in geldanamycin-treated, L-NAME-treated or untreated control J774 macrophages by measuring DAFFM-NO fluorescence. The blots and images in **a** and **c** are representative of four independent experiments. Data in **b** and **d** are means \pm s.d. from three independent experiments (* $P < 0.05$; *** $P < 0.001$).

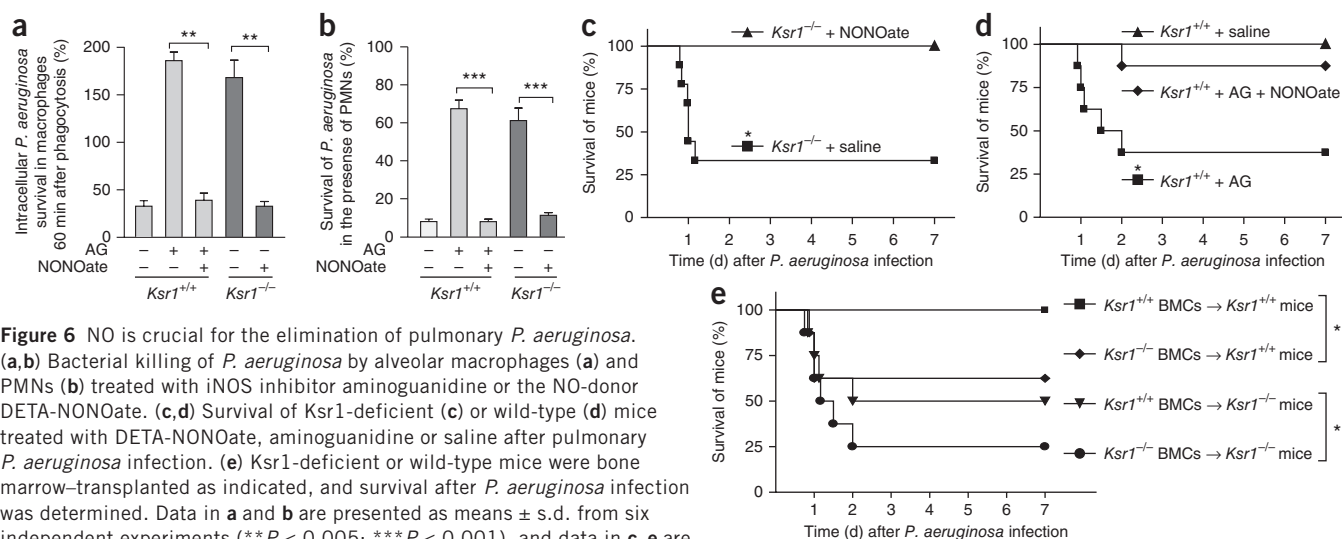


Figure 6 NO is crucial for the elimination of pulmonary *P. aeruginosa*. (a,b) Bacterial killing of *P. aeruginosa* by alveolar macrophages (a) and PMNs (b) treated with iNOS inhibitor aminoguanidine or the NO-donor DETA-NONOate. (c,d) Survival of *Ksr1*-deficient (c) or wild-type (d) mice treated with DETA-NONOate, aminoguanidine or saline after pulmonary *P. aeruginosa* infection. (e) *Ksr1*-deficient or wild-type mice were bone marrow-transplanted as indicated, and survival after *P. aeruginosa* infection was determined. Data in a and b are presented as means \pm s.d. from six independent experiments (** $P < 0.005$; *** $P < 0.001$), and data in c–e are presented as Kaplan-Meier curves (* $P < 0.05$; log-rank test).

These findings indicate that infection of macrophages and neutrophils with *P. aeruginosa* results in the recruitment of iNOS and Hsp90 to Ksr1, a recruitment that is necessary for iNOS activation.

NO protects *Ksr1*-deficient mice from *P. aeruginosa* infection

To confirm that failure to release NO during infection contributes to the defect in the host defense of *Ksr1*-deficient mice against *P. aeruginosa*, we determined whether exogenous supplementation of NO by NO donors enhances the bactericidal capability of *Ksr1*-deficient macrophages and neutrophils and protects *Ksr1*-deficient mice from death upon pulmonary *P. aeruginosa* infection. DETA-NONOate, an NO donor with a half-life time of 22 h at 37 °C, restored the bacterial killing capability of *Ksr1*-deficient alveolar macrophages or wild-type macrophages that were treated with aminoguanidine (Fig. 6a). We found similar results for neutrophils (Fig. 6b). *In vivo*, DETA-NONOate rescued *Ksr1*-deficient mice infected with *P. aeruginosa* (Fig. 6c). Treatment with aminoguanidine prevented bacterial clearance in wild-type mice and finally resulted in sepsis and death; treating the mice with aminoguanidine plus DETA-NONOate substantially increased their survival rates after infection (Fig. 6d). We obtained similar results experiments using L-NAME and 1400W, a more specific inhibitor of iNOS (Supplementary Figs. 5 and 6). These findings indicate that NO release by DETA-NONOate restores the reduced bacterial killing capability of alveolar macrophages and neutrophils in *Ksr1*-deficient mice and protects these mice from *P. aeruginosa*-induced death.

To examine whether the lethality of *P. aeruginosa* infection to *Ksr1*-deficient mice and the inability of these mice to clear the bacterium are entirely attributed to the absence of Ksr1 in cells of the immune system, we transplanted bone marrow cells (BMCs) from wild-type mice into *Ksr1*-deficient mice, and vice versa. The mortality rates for wild-type mice with *P. aeruginosa* infection increased when the mice were transplanted with *Ksr1*-deficient BMCs, whereas the mortality rates for infected *Ksr1*-deficient mice decreased when the mice were transplanted with wild-type BMCs (Fig. 6e). These data confirm that *Ksr1* expression in immune cells is required for the pulmonary defense against *P. aeruginosa*. However, transplanting wild-type BMCs into *Ksr1*-deficient mice does not fully restore the resistance to pulmonary *P. aeruginosa* infection in these mice (Fig. 6e), a finding suggesting that *Ksr1* deficiency may also affect the response of nonimmune cells (for example, bronchial epithelial cells) to *P. aeruginosa*.

DISCUSSION

In this study, we found that the hypersusceptibility of *Ksr1*-deficient mice to pulmonary *P. aeruginosa* infection is caused by the inability of *Ksr1*-deficient lung macrophages and neutrophils to clear the bacteria. *Ksr1* functions as a scaffold for iNOS and Hsp90; this scaffold stimulates iNOS activity during *P. aeruginosa* infection. *Ksr1* deficiency results in a failure to form the multiprotein complex with iNOS and Hsp90 and, thus, in the failure to activate iNOS, release NO and form more toxic reactive nitrogen species, such as peroxynitrite. Exogenous NO restores the bacterial killing capability of *Ksr1*-deficient lung phagocytes and increases the survival rates of infected *Ksr1*-deficient mice. These studies provide strong evidence that *Ksr1* protects against pulmonary *P. aeruginosa* infection through a previously unknown scaffolding action and the iNOS-NO pathway.

NO is released during infection with *P. aeruginosa*²⁴ and upregulates proapoptotic CD95; this upregulation is blocked by inhibition of NO release^{24,25}. These studies link *Ksr1* through NO to CD95 and the induction of cell death by CD95 in *P. aeruginosa* infection. Mice lacking CD95 have been found to be hypersusceptible to infection with *P. aeruginosa* and were unable to clear the bacteria²⁶. This lack of clearance resulted in a generalized, primarily pulmonary infection, sepsis and death, a pattern similar to the phenotype of *Ksr1*-deficient mice. However, the exact role of CD95 in NO-mediated killing upon *P. aeruginosa* infection requires definition.

Previous studies have shown that *Salmonella* species activate iNOS in macrophages but exclude iNOS from phagosomes, resulting in survival and multiplication of the pathogen within macrophages^{27,28}. It was suggested that *Salmonella* uses the type III secretion system encoded by *Salmonella* pathogenicity island 2 to translocate effector proteins that prevent the colocalization of phagosomes containing iNOS and *Salmonella*²⁷. Moreover, it was also suggested that NO and peroxynitrite are involved less in killing intracellular *Salmonella* and more in autotoxicity²⁸. Although our findings show that iNOS does not colocalize with intracellular *P. aeruginosa* in either wild-type or *Ksr1*-deficient macrophages after infection, they also clearly indicate that iNOS and *Ksr1* are required for killing these bacteria. This requirement could be explained by the diffusion of NO into phagosomes, where it directly kills *P. aeruginosa* even if iNOS is excluded from phagosomes containing *P. aeruginosa*. These observations also indicate that the iNOS system is differentially regulated by various pathogens.

iNOS has been considered to be usually constitutively active, and its activity was believed to be regulated primarily at the transcriptional level²⁹. However, in macrophages, Hsp90 physically interacts with iNOS upon induction with LPS, IFN- γ or both, and the overexpression of Hsp90 enhances iNOS-mediated NO production²². This finding is consistent with our results suggesting the formation of a multiprotein complex consisting of Ksr1, Hsp90 and iNOS upon *P. aeruginosa* infection. We further found that Ksr1 does not directly stimulate iNOS, as indicated in the experiments with recombinant proteins. Thus, Ksr1 serves as a scaffold to facilitate the activation of iNOS, which is mediated upon recruitment of Hsp90 to the Ksr1-iNOS complex.

The function of KSR1 in human disorders is presently undefined. Ksr1-deficient mice are prone to chronic colitis³⁰, but they also resist the induction of arthritis³¹. To our knowledge, the function of Ksr1 in bacterial infections has not been previously identified. Our studies have identified a major role of this protein in bacterial infections and have shown a link between Ksr1 and the regulation of bacterial pneumonia and sepsis. It is possible that KSR1 functions not only in acute pulmonary *P. aeruginosa* infections but also in other diseases and in infections with other pathogens. For instance, epithelial cells from individuals with cystic fibrosis, which is caused by mutations in the cystic fibrosis transmembrane conductance regulator that result in chronic pneumonia, fail to produce NO when stimulated with LPS. This lack of NO production correlates with the failure of individuals with cystic fibrosis to clear *P. aeruginosa* from their lungs. However, whether dysregulation of KSR1 in cells deficient in the cystic fibrosis transmembrane conductance regulator is involved in the impaired NO response of these cells to infection remains to be determined. In summary, Ksr1 protects against pulmonary *P. aeruginosa* infections through a unique scaffold interaction with Hsp90 and iNOS, and this scaffolding is necessary for killing *P. aeruginosa*.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturemedicine/>.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

Y.Z. performed the internalization assays, confocal and fluorescence microscopy, immunoprecipitation studies, flow cytometry analysis and protein overexpression and iNOS activity assays. X.L. conducted the mouse infection experiments, determined cytokine abundance in the lung and performed the bacterial-killing assay and immunoblot analysis. A.C. performed bone marrow transplantation and determined the neutrophil count. M.S. characterized the genotypes of Ksr1-deficient mice. J.A.G. cloned recombinant Ksr proteins. Y.Z., X.L. and E.G. designed the experiments and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

The Animal Care and Use Committee of the Bezirksregierung Düsseldorf approved all procedures performed on mice.

Infection experiments. Bacteria from glycerol stocks were grown overnight on tryptic soy agar plates at 37 °C, resuspended in tryptic soy broth (TSB) an absorbance of 0.25 at 550 nm, shaken at 125 r.p.m. for 1 h at 37 °C, and collected during the early logarithmic growth phase by pelleting and resuspension in fresh TSB. For *in vivo* infection, Ksr1-deficient mice (backcrossed to C3H background for 12 generations; kindly provided by Dr. R. Kolesnick) and syngenic wild-type mice were briefly anesthetized with ether, and 5×10^8 CFU of the laboratory strain *P. aeruginosa* ATCC 27853 were injected into the nose as previously described²⁶. If indicated, 1 h before infection the mice were given intraperitoneal injections of the iNOS inhibitors aminoguanidine (100 mg per kg body weight), L-NAME (100 mg per kg body weight) or 1400W (20 mg per kg body weight) and/or the NO donor DETA-NONOate (10 mg per kg body weight). Mice were either observed for 7 d (as shown in **Fig. 1a** and **Fig. 6c–e**) or killed at the indicated time points (as shown in **Fig. 1b–d,f**). For *in vitro* infection experiments, cells were maintained in buffered RPMI-1640 medium with 1 mM HEPES during infection and were inoculated with *P. aeruginosa* at bacteria-to-host ratio of 100 in the experiments shown in **Figures 2b–e, 3a,b,d,f, 4a–d** and **5d** and of 1 in **Figures 2a, 3c,g** and **6a,b**. Synchronous infection conditions and an enhanced bacterium-host cell interaction were achieved by a 2-min centrifugation (350g) of the bacteria onto the cells. The end of the centrifugation was defined as the starting point of infection. For bacterial killing assays, the method of *in vitro* infection was modified as described in the **Supplementary Methods**. If required for the experiment, we added 100 μM L-NAME, aminoguanidine, 1400W or DETA-NONOate or 10 μM U0126 or geldanamycin.

NO production and peroxynitrite formation. NO production was determined by FACS analysis with 4-amino-5-methylamino-2',7'-difluorofluorescein (DAFFM) as a probe. DAFFM is nonfluorescent; however, it reacts with NO to form a fluorescent compound, DAFFM-NO (excitation/emission: 490 nm/525 nm). We loaded macrophages with DAFFM-DA (5 μM, DAFFM linked with a diacetate group, Molecular Probes) for 30 min at 37 °C and washed them with PBS to remove extracellular DAFFM-DA. Cells were further incubated at 37 °C for 10 min so that intracellular esterases could completely remove the diacetate group from DAFFM-DA. Cells were then infected with *P. aeruginosa* or left uninfected for 2 h in the presence or absence of iNOS inhibitors and then fixed with 2% (wt/vol) paraformaldehyde in PBS (pH 7.2). DAFFM-NO fluorescence intensity (*F*) was analyzed by FACS. Relative NO production induced by *P. aeruginosa*, defined as $\Delta F_{[PA]}$, was calculated by the following

formula: $\Delta F_{[PA]} = F_{[PA]} - F_{[No\ infection]}$ (arbitrary units). Fluorescence intensity of the L-NAME-treated group was calculated as follows: $\Delta F_{[PA + L-NAME]} = F_{[PA + L-NAME]} - F_{[L-NAME\ only]}$. Peroxynitrite formation was detected by immunoblot analysis of tyrosine nitrosylation in cell lysates with a rabbit antibody to nitrotyrosine (1 in 1,000 dilution, Molecular Probes).

Activity assay of iNOS immunoprecipitates. We infected 1×10^7 Ksr1-deficient, bone marrow-derived macrophages for 2 h with *P. aeruginosa*. Ksr1 immunoprecipitates were prepared from uninfected wild-type, bone marrow-derived macrophages. The NOS activity of iNOS immunoprecipitates was determined in the presence or absence of Ksr1 immunoprecipitates by measuring the conversion of L-[³H]-arginine (50.6 μCi μmol⁻¹, PerkinElmer) to L-[³H]-citrulline in the absence of Ca²⁺. A NOS assay kit (Calbiochem) was used according to the manufacturer's instructions.

Recombinant proteins. To obtain recombinant Ksr1 proteins, we used N-terminal Flag-tagged constructs of *Ksr1* encoded by the plasmids pET30a-WT-Ksr1 or pET30a-KI-Ksr1. These plasmids contain the 2.7-kb gene *Ksr1* encoding the wild-type or the kinase-inactive *Ksr1*. The dual mutation sites (D683A and D700A) in the putative kinase domain of pET30a-KI-Ksr1 were confirmed by nucleotide sequencing. We expressed the proteins in *E. coli* BL21 by induction with isopropyl β-D-1-thiogalactopyranoside (0.1 mM) at 25 °C. The bacteria were lysed in 50 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 10 mg ml⁻¹ lysozyme (Sigma), and recombinant Flag-WT-Ksr1 and Flag-KI-Ksr1 were purified with anti-Flag M2 affinity gels (Sigma) according to the manufacturer's instructions. The purity of the preparations was controlled by Coomassie and silver staining of SDS-PAGE gels. Recombinant iNOS (Sigma) and Hsp90 (Biotrend) were purchased from commercial sources.

Statistical analyses. Data are expressed as arithmetic means ± s.d. One-way analysis of variance (ANOVA) was used to test between-group and within-group differences; pairwise comparisons were made with Student's *t* test. The data were normally distributed and therefore met the assumptions of ANOVA and Student's *t* test. Kaplan-Meier survival curves were plotted and analyzed with the log-rank test. Statistical significance was set at the level of $\alpha = 0.05$. All data were obtained from independent measurements. The GraphPad Prism statistical software program (GraphPad Software) was used for the analyses.

Additional methods. Details for measuring cytokine levels and bacterial and neutrophil counts in the lung, bacterial killing capability and phagocytosis assays, fluorescence microscopy and bone marrow transplantation are described in the **Supplementary Methods**.