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Key Role for Scavenger Receptor B-I in the Integrative Physiology of Host Defense during Bacterial Pneumonia

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Abstract

Scavenger receptor B-I (SR-BI) is a multirecognition receptor that regulates cholesterol trafficking and cardiovascular inflammation. Although it is expressed by neutrophils (PMNs) and lung-resident cells, no role for SR-BI has been defined in pulmonary immunity. Herein, we report that, compared to SR-BI$^{+/+}$ counterparts, SR-BI$^{-/-}$ mice suffer markedly increased mortality during bacterial pneumonia associated with higher bacterial burden in lung and blood, deficient induction of the stress glucocorticoid corticosterone, higher serum cytokines, and increased organ injury. SR-BI$^{-/-}$ mice had significantly increased PMN recruitment and cytokine production in the infected airspace. This was associated with defective hematopoietic cell-dependent clearance of lipopolysaccharide from the airspace and increased cytokine production by SR-BI$^{-/-}$ macrophages. Corticosterone replacement normalized alveolar neutrophilia but not alveolar cytokines, bacterial burden, or mortality, suggesting that adrenal insufficiency derepresses PMN trafficking to the SR-BI$^{-/-}$ airway in a cytokine-independent manner. Despite enhanced alveolar neutrophilia, SR-BI$^{-/-}$ mice displayed impaired phagocytic killing. Bone marrow chimeras revealed this defect to be independent of the dyslipidemia and adrenal insufficiency of SR-BI$^{-/-}$ mice. During infection, SR-BI$^{-/-}$ PMNs displayed deficient oxidant production and CD11b externalization, and increased surface L-selectin, suggesting defective activation. Taken together, SR-BI coordinates several steps in the integrated neutrophilic host defense response to pneumonia.

Introduction

Community-acquired pneumonia is a serious condition that results when bacteria invade the distal airspaces and induce an innate immune response.[1] Despite medical advances,

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pneumonia remains the most common cause of severe sepsis and the leading cause of death by infection.[2] Although the lung is often considered in isolation during pneumonia, the pulmonary innate immune response arises from integrated communications between lung-resident cells and other organs including the bone marrow and adrenal glands. While adrenal function correlates with pneumonia severity,[3] its functional contribution to pulmonary host defense is undefined. Improved understanding of the molecular regulation that coordinates the integrated host response during pneumonia is needed in order to advance insight into why some patients develop bacteremia and septic shock while others do not.

Scavenger receptors (SRs) are a widely expressed family of pattern recognition receptors, categorized into 8 classes (A-H) by domain architecture. SRs bind a very broad array of ligands, both endogenous (e.g., oxidized lipids, amyloid) and exogenous (e.g., pathogens, particulates), and have previously been implicated in several diseases, including atherosclerosis and Alzheimer's disease.[4] While roles for class A SRs, including MARCO and SR-A, have been identified in the lung's response to pathogens and particulates, little is known about the role of other SR classes in pulmonary biology.[5-7]

SR class B type I (SR-BI) has been mostly studied in vascular biology as it mediates selective uptake of cholesterol ester from high density lipoprotein (HDL) by hepatocytes and endothelium, regulating serum HDL and atherogenesis.[8] However, HDL exerts wide-ranging actions, including suppression of leukocyte function,[9] and SR-BI is expressed by several tissues that collaborate in host defense. Indeed, suggesting the potential for a role in the integrated response of tissues to environmental stress, SR-BI expression by several cell types has recently been shown to regulate the LPS response (macrophages),[10] clearance of plasma LPS (hepatocytes),[11] chemotaxis (neutrophils [PMNs]),[9] and stress glucocorticoid production (adrenal glands).[12] The significance of these findings to humans is suggested by recent reports that SR-BI polymorphisms are associated with adrenal insufficiency, serum HDL, and atherosclerosis,[13, 14] and serum HDL correlates with pneumonia severity in patients.[15, 16]

In addition to expression on alveolar macrophages (AM), SR-BI is also expressed by alveolar epithelial (AE) cells, where it mediates vitamin E uptake.[17] However, no further role for SR-BI has been defined in the lung. Herein, we report that SR-BI is critical to survival during bacterial pneumonia. SR-BI$^{-/-}$ mice recruit increased PMNs to the infected lung, associated with defective clearance of LPS from the airspace and increased alveolar cytokines. Alveolar neutrophilia is also promoted by adrenal insufficiency, as correction of glucocorticoid deficiency in infected SR-BI$^{-/-}$ mice normalizes airspace PMNs. However, despite increased airspace PMNs, SR-BI$^{-/-}$ mice suffer marked bacteremia, cytokine storm, and organ injury that derives, in part, from a dramatic defect in phagocytic killing. Taken together, we report an essential new role for SR-BI in the integrated physiologic response to pneumonia and in phagocyte antimicrobial function.

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RESULTS

SR-BI deficiency compromises host defense during bacterial pneumonia

SR-BI has largely been studied in vascular biology due to its regulation of plasma HDL levels and endothelial responsiveness to HDL.[18] Given that HDL suppresses leukocyte pro-inflammatory functions[9] and SR-BI is expressed in the lung, we hypothesized that SR-BI-null mice would have an altered host defense response to bacterial pneumonia. To address this, SR-BI-sufficient (SR-BI$^{+/+}$) and -deficient (SR-BI$^{-/-}$) mice were dosed intratracheally (i.t.) with the clinically relevant Gram-negative bacterium *K. pneumoniae*, and mortality was monitored for 10 days. Remarkably, at an inoculum that was approximately an LD50 in SR-BI$^{+/+}$ mice, all SR-BI$^{-/-}$ mice were dead by 48h post-inoculation (p.i.)(Figure 1a).

Pneumonia can cause mortality through lung injury and consequent respiratory failure. In support of exacerbated acute lung injury, BALF protein, a surrogate measure of microvascular integrity in the lung,[19] was higher in infected SR-BI$^{-/-}$ mice than in controls (Figure 1b). Histopathologic evaluation also revealed more severe neutrophilic inflammation in the lungs of infected SR-BI$^{-/-}$ mice than SR-BI$^{+/+}$ counterparts (Figure 1c and Supplementary Figure 1). Bacteremia, arising from extrapulmonary dissemination of pathogens, is common in pneumonia, and can lead to septic shock through dysregulated induction of the systemic innate immune response.[2, 20] Compared to controls, infected SR-BI$^{-/-}$ mice had ~3-fold higher bacteria in lung tissue 24h p.i. (Figure 1d), indicating defective pathogen clearance in the lung. More remarkably, at 24h p.i., SR-BI$^{-/-}$ mice had a ~2 log increase in bacteria in the blood (Figure 1e) and a nearly 3-log increase in bacteria in the liver (Figure 1f) compared to controls, suggesting more profound failure of host defense in the extrapulmonary compartment. Consistent with a robust sepsis syndrome, SR-BI$^{-/-}$ mice had much higher serum cytokines (TNFα, G-CSF, CXCL2) than controls (Figure 1g), as well as higher serum lactate dehydrogenase (Figure 1h), a marker of cell death denoting organ injury.

SR-BI deficiency increases innate immune responses in the infected airspace

We next sought to determine if the innate immune response was defective in the SR-BI-null airway. Alveolar cytokines recruit PMNs into the airspace where they are pivotal bactericidal cells in the host response to infection.[2] SR-BI$^{-/-}$ mice had increased PMNs in the airspace 24h p.i. when compared to SR-BI$^{+/+}$ controls (Figure 2a). BALF of infected SR-BI$^{-/-}$ mice also had higher levels of TNFα, a cytokine of established importance to PMN recruitment,[21] and CXCL5/LIX, an AE-derived chemokine reported to play a key role in attraction of PMNs to the infected airspace (Figure 2b).[22] By contrast, several other cyto-/chemokines (G-CSF, CXCL1, CXCL2, IL-6) were induced to equivalent levels in SR-BI$^{+/+}$ and SR-BI$^{-/-}$ BALF, suggesting that SR-BI has selective effects on innate immune responses in the infected airspace. BALF and serum IL-17 were also equivalent between SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice 24h post-infection (data not shown).

SR-BI$^{-/-}$ mice have increased and sustained airway immune responses to inhaled LPS

Despite the higher alveolar neutrophilia in SR-BI$^{-/-}$ lungs at 24h p.i., bacteria were also increased in SR-BI$^{-/-}$ lungs at this time point (Figure 1), thus confounding conclusions as to

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cause vs. effect, and also obscuring the degree to which BALF cytokines derive from airway-resident vs. recruited immune cells. In order to examine the pulmonary innate immune response with more precise kinetics, we conducted a 30 minute exposure of SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice to aerosolized lipopolysaccharide (LPS), the major immunostimulatory glycolipid of the outer cell wall of Gram-negative bacteria. In response to inhaled LPS, SR-BI$^{-/-}$ mice had enhanced airspace recruitment of leukocytes (Figure 3a, Supplementary Figure 2) and PMNs specifically (Figure 3b). Increased alveolar neutrophilia was seen by 8h post-exposure, and was sustained out to 48h. Similar to the case with *K. pneumoniae*, SR-BI$^{-/-}$ mice had elevations in BALF TNFα and CXCL5, but not other cyto-/chemokines including IL-17 2h after LPS exposure, when compared to controls (Figure 3c, data not shown). This time point largely reflects cytokine production by resident alveolar cells as it precedes substantial recruitment of PMNs into the airspace (Figure 3b). At 8h after LPS, TNFα remained relatively elevated in SR-BI$^{-/-}$ BALF, but G-CSF and IL-6 were elevated compared to controls (Figure 3d). Sustained alveolar cytokines and neutrophilia in SR-BI$^{-/-}$ mice suggested to us the possibility that SR-BI might mediate clearance of LPS from the airspace.

Hematopoietic cell SR-BI mediates LPS clearance and regulates TLR responses in the airway

SR-BI is reported to mediate cellular uptake of LPS, and to play a role in clearance of LPS from plasma;[11, 23, 24] thus, SR-BI$^{-/-}$ mice have sustained induction of serum cytokines during endotoxemia due to delayed LPS clearance by the liver.[11] No reports, to our knowledge, have addressed how/whether LPS is cleared by the lung. In order to address this, BALF LPS was quantified by limulus amebocyte lysate assay at various time points following LPS inhalation. Compared to controls, SR-BI$^{-/-}$ mice had equivalent BALF LPS 2h following exposure, but significantly higher BALF LPS 8 and 24h after exposure (Figure 4a). In control experiments, an LPS standard was spiked *ex vivo* into cell-free BALF collected from naive SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice. Equal levels of LPS were detected in the two BALF types, verifying that SR-BI$^{+/+}$ and SR-BI$^{-/-}$ BALF have equivalent LPS neutralization capacity (data not shown).

Speculating that SR-BI expressed by airway-resident macrophages (i.e., AMs) might mediate LPS clearance from the airspace, we transferred SR-BI$^{+/+}$ or SR-BI$^{-/-}$ bone marrow to lethally irradiated SR-BI$^{+/+}$ recipients in order to generate mice chimeric for SR-BI expression in hematopoietic cells. Mice with isolated hematopoietic cell SR-BI deficiency (i.e., receiving SR6 BI$^{-/-}$ marrow) had higher BALF LPS 24h post-LPS inhalation than SR-BI-sufficient counterparts (Figure 4b), confirming that SR-BI expression by bone marrow-derived cells mediates LPS clearance from the airway. In order to address this further, peritoneal elicited macrophages from SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice were incubated with biotin-labeled LPS to assess intracellular uptake of LPS. Consistent with a role for macrophage SR-BI in LPS clearance from the airway, SR-BI$^{-/-}$ macrophages displayed a significant reduction in both surface-bound LPS and in the sum of surface-bound plus internalized LPS (Figure 4c).

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LPS is internalized by leukocytes after activation of TLR4 on the cell surface;[24] however, whether mass internalization of LPS serves as an activation or detoxification pathway remains unclear. Despite their reduced uptake of LPS, SR-BI$^{-/-}$ macrophages were hyper-responsive to LPS, producing elevated levels of TNFα (Figure 4d). Interestingly, SR-BI$^{-/-}$ macrophages also produced elevated TNFα after ligation of TLR2 (Pam3CSK4) – like TLR4, a plasmalemmal TLR – whereas ligation of the endosomal receptors TLR3 (polyI:C) and TLR9 (CpG DNA) induced equivalent TNFα in SR-BI$^{+/+}$ and SR-BI$^{-/-}$ macrophages (Figure 4d). Consistent with their heightened responsiveness to LPS, SR-BI$^{-/-}$ macrophages displayed increased cell-surface TLR4 (Figure 4e).

Taken together, these findings suggest an important role for hematopoietic cell, and potentially macrophage, SR-BI in clearance of LPS from the airway and in suppression of the innate cytokines that drive alveolar neutrophilia. Indeed, suggesting the importance of hematopoietic cell SR-BI to regulation of alveolar neutrophilia *in vivo*, chimeric mice with isolated deficiency of SR-BI in hematopoietic cells had higher airspace neutrophils 24h post-LPS inhalation than SR-BI-sufficient counterparts (Figure 4f).

SR-BI deficiency unveils a role for adrenal function in pulmonary innate immunity

Having found that airway-infected SR-BI$^{-/-}$ mice appear to die from failure of host defense, we next sought an explanation. SR-BI$^{-/-}$ mice reportedly display stress-induced adrenal insufficiency due to absence of HDL-mediated, SR-BI-dependent delivery of cholesterol, the precursor for steroid hormones, to the adrenals.[12] Although systemic glucocorticoids are well known to regulate airway and systemic inflammation *in vivo*,[3] the role of native adrenal function in pulmonary host defense remains undefined.

We next addressed the possibility that adrenal insufficiency might contribute to the pneumonia phenotype of the SR-BI-null mouse. Though SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice had comparable serum corticosterone in the naïve state, 24h after *K. pneumoniae*, SR-BI$^{+/+}$ mice had a significant increase in corticosterone that was absent in SR-BI$^{-/-}$ mice (Figure 5a), indicating adrenal insufficiency in the latter strain. We established conditions for i.p. corticosterone supplementation, commenced before induction of pneumonia, that achieved equivalent serum levels in infected SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice (Figure 5b). Corticosterone replacement induced a modest rightward shift in the survival curves of both strains during bacterial pneumonia, but did not rescue the increased mortality of SR-BI$^{-/-}$ mice (Figure 5c), nor the increased bacterial burden in the airspace or blood of SR-BI$^{-/-}$ mice (Figure 5d-e). Corticosterone replacement did not normalize the augmented BALF levels of TNFα or CXCL5 of SR-BI$^{-/-}$ mice (Figure 5f-g), suggesting, consistent with our *ex vivo* macrophage LPS exposure studies, that increased induction of cytokines in the SR-BI$^{-/-}$ airway does not simply arise due to disinhibition from glucocorticoids. Interestingly, despite the failure of corticosterone supplementation to normalize cytokines in the infected SR-BI$^{-/-}$ airway, it abolished the relative alveolar neutrophilia of SR-BI$^{-/-}$ mice (Figure 5h). Taken together, these studies suggest that, while adrenal insufficiency does not underlie the increased mortality or pathogen dissemination of SR-BI$^{-/-}$ mice during pneumonia, it does impact the innate immune response of the SR-BI$^{-/-}$ airway by disinhibiting alveolar neutrophilia through a cytokine-independent mechanism.

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SR-BI$^{-/-}$ mice have impaired clearance of bacteremia

Given the marked increase in bacterial burden in the blood of SR-BI$^{-/-}$ mice, we next pursued the possibility that defective clearance of pathogens in the bloodstream, once seeded from the lung, might underlie the severe failure of host defense in this strain. In order to address this directly, we bypassed the lung, and injected *K. pneumoniae* intravenously. Bacterial burden in both the blood and the liver were significantly higher in SR-BI$^{-/-}$ mice than SR-BI$^{+/+}$ controls 4h after intravenous inoculation (Figure 6a-b), confirming defective pathogen clearance in the blood *in vivo*. PMNs play a key role in bacterial killing in the bloodstream.[25] Excluding relative neutropenia as a cause for defective clearance of bacteremia in SR-BI$^{-/-}$ mice, circulating PMNs (as well as lymphocytes, monocytes, and eosinophils) were equivalent in number between naïve SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice (Figure 6c). Moreover, the two strains mounted a similar increase in circulating PMNs 24h following i.t. *K. pneumoniae* (Figure 6d), suggesting an intact bone marrow response to lung infection. The normal circulating PMN numbers and augmented alveolar PMN numbers in SR-BI$^{-/-}$ mice, taken together with our finding of defective bacterial clearance in these two compartments, suggested that PMN bacterial killing function might be defective in SR-BI$^{-/-}$ mice.

SR-BI deficiency impairs phagocytic killing

SR-BI is expressed by human PMNs and mediates the suppressive effect of HDL on CD11b display and chemotaxis by these cells.[9] In order to confirm SR-BI expression by murine PMNs, bone marrow PMNs were purified by Percoll gradient and further FACS-sorted for Ly6G^{+}CD11b^{+}F4/80^{neg}7AAD^{neg}, yielding a population that was >95% PMNs, with no detectable contamination by (F4/80^{+}) macrophages (Figure 7a). Purified WT PMNs did express SR-BI message (Ct value, 25.8), whereas no expression was detected in SR-BI$^{-/-}$ PMNs (Figure 7b). However, PMN expression of SR-BI was considerably lower than that in either macrophages or dendritic cells derived from murine bone marrow (Figure 7c).

Pursuing the effect of SR-BI deficiency upon PMN host defense functions, we found that SR-BI$^{-/-}$ PMNs were defective at phagocytosis of fluor-labelled bacterial bioparticles *ex vivo* (Figure 7d). In order to better model phagocytic killing as it occurs at inflammatory foci *in vivo*,[19] *K. pneumoniae* was next injected i.p. into SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice 4h after thioglycollate elicitation of PMNs to the peritoneum; peritoneal cells (~80% PMNs for both genotypes) were then harvested 30 min post-peritoneal infection and assayed for intracellular bacterial killing during a time course *ex vivo*. This model allows for precisely timed examination of the initial PMN-bacterial encounter as it occurs *in vivo*. As shown in Figure 7e, SR-BI$^{-/-}$ phagocytes had significantly lower intracellular *K. pneumoniae* CFUs than SR-BI$^{+/+}$ counterparts at time 0 (i.e., immediately upon harvest from the inoculated peritoneum), consistent with reduced bacterial internalization. More strikingly, whereas SR-BI$^{+/+}$ phagocytes, as expected, displayed a time-dependent intracellular killing curve (i.e., reduction of intracellular CFUs), SR-BI$^{-/-}$ phagocytes displayed a time-dependent increase in intracellular CFUs (i.e., effectively, an intracellular bacterial growth curve), indicating a marked defect in intracellular killing of bacteria.

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In order to more specifically address killing function of SR-BI$^{-/-}$ macrophages, we repeated the killing assay, but this time injected *K. pneumoniae* i.p. 96h post-thioglycollate, near the peak of peritoneal macrophage accumulation. As shown in Figure 7f, SR-BI$^{-/-}$ macrophages had reduced CFUs at time-0, consistent with defective phagocytosis. However, SR-BI$^{+/+}$ and SR-BI$^{-/-}$ macrophages reduced intracellular CFUs roughly equivalently, suggesting that SR-BI$^{-/-}$ macrophages have intact killing function. The inflammatory milieu of the peritoneum 4h and 96h post-thioglycollate is very different, and direct comparisons between killing assays at the two time points must thus be performed with caution. Nonetheless, taken together, these findings may suggest that SR-BI$^{-/-}$ PMNs account for the marked phagocytic killing deficit seen in the 4h post-thioglycollate assay.

SR-BI$^{-/-}$ mice have elevated plasma levels of enlarged HDL particles due to defective clearance of HDL by the liver.[8] Both HDL and glucocorticoids can suppress PMN functions.[9, 26] Thus, we next sought to determine whether or not the killing impairment observed in phagocytes of SR-BI$^{-/-}$ mice is secondary to the SR-BI$^{-/-}$ plasma microenvironment. The HDL and adrenal phenotypes of the SR-BI$^{-/-}$ mice both derive from SR-BI deficiency in radioresistant cells (hepatocytes and adrenocortical cells, respectively). Thus, as expected, lethally irradiated SR-BI$^{+/+}$ recipient mice transplanted with either SR-BI$^{+/+}$ or SR-BI$^{-/-}$ bone marrow had equivalent serum cholesterol and equivalent plasma corticosterone after induction of peritonitis (Supplementary Figure 3). When phagocytic killing function was compared between these two chimeras 4h post thioglycollate, phagocytes from mice with isolated hematopoietic cell SR-BI deficiency again displayed a profound defect in intracellular killing (Figure 7g), suggesting that PMN dysfunction in the SR-BI-null mouse is not secondary to HDL excess (i.e., acting through a receptor other than SR-BI). Taking a genetic approach to further address whether excess HDL drives the host defense phenotype of SR-BI$^{-/-}$ mice during pneumonia, we infected HDL-deficient apoA-I$^{-/-}$ mice with i.t. *K. pneumoniae*, aiming to see if they would display a phenotype opposite that of SR-BI$^{-/-}$ mice. Rather, apoA-I$^{+/+}$ and apoA-I$^{-/-}$ mice had comparable survival and alveolar neutrophilia 24h after infection, whereas, like SR-BI$^{-/-}$ mice, apoA-I$^{-/-}$ mice had a significant increase over WT counterparts in systemic bacterial burden, as assessed by splenic CFUs (Supplementary Figure 4). Taken together, these results indicate that HDL may impact subphenotypes of host defense during bacterial pneumonia, but that the profound sepsis and PMN killing deficit observed in SR-BI$^{-/-}$ mice does not arise from HDL excess.

SR-BI deficiency decreases neutrophil antimicrobial function

PMNs at the site of infection internalize pathogens and then kill them by oxidant-dependent and –independent mechanisms. Bacteria are internalized into the PMN phagosome which then matures, as NADPH oxidase-dependent reactive oxygen species (ROS) are released into it. In order to address the effect of SR-BI deficiency upon PMN bacterial killing mechanisms, intracellular ROS (iROS) were measured by flow cytometry in both alveolar and peripheral blood PMNs 24h after i.t. *K. pneumoniae*, a time point at which infected SR-BI$^{-/-}$ mice had much higher bacterial stimulus in both locations (Figure 1). Despite the higher bacterial burden, SR6 BI$^{-/-}$ PMNs in both locations had significantly lower iROS signal than SR-BI$^{+/+}$ controls (Figure 8a-b). Moreover, consistent with lower activation/

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maturation status of SR-BI$^{-/-}$ PMNs during infection, circulating SR-BI$^{-/-}$ PMNs had significantly higher surface display of CD62L/L-selectin (Figure 8c), consistent with lesser L-selectin shedding, and lower surface display of the activation marker CD11b (Figure 8d).

DISCUSSION

Pneumonia is a serious condition that arises when bacteria invade the lower airway and induce an innate immune response.[2, 20] As the host antimicrobial response is not restricted to cells of the airspace in isolation, and pneumonia often kills through complications of the systemic host response (i.e., severe sepsis), advances in pneumonia management will require an improved understanding of the integrated physiology of host defense.

In this report, we show that SR-BI, a SR heretofore largely studied in vascular biology in its role as a receptor for HDL, is pivotal to host survival during bacterial pneumonia, and plays an important role in integrating the responses of several cell types both within and outside the lung. We propose that this arises from expression of SR-BI by several distinct, but interacting cell types in the host, impacting both cell-intrinsic functions (e.g., macrophage TLR responses, adrenocortical stress hormone synthesis, PMN bacterial killing) and intercellular communications across organs that govern the host response (e.g., corticosteroid regulation of PMN trafficking to the lung). While some of these roles of SR-BI are HDL-dependent (adrenal stress hormone synthesis[12]), we show that others (i.e., phagocytic killing) are independent, in part, of the plasma HDL excess phenotype of SR-BI deficiency. In the end, several of the consequences of SR-BI deletion conspire to amplify alveolar neutrophilia, including enhanced alveolar cytokine production, reduced alveolar LPS clearance, and adrenal insufficiency. Despite this, impaired phagocytic killing enhances bacterial overgrowth in the lung (and bloodstream), providing yet further stimulus for alveolar neutrophilia.

Adrenal insufficiency worsens outcomes in human sepsis.[3] Supplemental glucocorticoids have been tested in the therapy of a wide array of diseases, including acute lung injury and pneumonia.[27] In our study, corticosterone supplementation normalized the relative alveolar neutrophilia of infected SR-BI$^{-/-}$ mice without correcting the elevated BALF levels of TNFα or CXCL5. This suggests that the endogenous stress corticosterone ‘burst’ that occurs in WT mice (and is absent in SR-BI$^{-/-}$ mice) shortly after infection represses alveolar accumulation of PMNs in a manner not simply due to attenuation of alveolar cytokines. We speculate that this may result from a direct repressive effect on PMN migration, as glucocorticoids inhibit PMN chemotaxis.[26] Derepressed PMN migration may explain why the relative increase in alveolar neutrophilia in SR-BI$^{-/-}$ mice was out of proportion to the modest and selective increase in alveolar cytokines. Given that the BALF cytokine increases were modest, transient (CXCL5 was normalized by 8h post-LPS [Fig. 3d]), and dissociable from the airspace neutrophilia, we posit that derepression of PMN migration by glucocorticoid deficiency may be the major driver of alveolar neutrophilia in the SR-BI$^{-/-}$ mouse.

Although SRs recognize partially overlapping sets of ligands (e.g., SR-A, CD36, and SR-BI all recognize oxidized LDL), critical differences among SRs have been identified, indicating

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that they are not functionally redundant. To date, SRs studied in lung biology have largely been of class A. SR-A$^{-/-}$ mice display enhanced pulmonary clearance of both *Cryptococcus* and *Pneumocystis*,[5, 6] whereas MARCO$^{-/-}$ mice have impaired clearance of *S. pneumoniae*.[7] Unlike our findings for SR-BI$^{-/-}$ mice, mice deficient in LOX1, a class E SR, display reduced lung neutrophilia after LPS exposure.[28] SR-BI has been little studied in the context of infection beyond its well-described role in cellular internalization of hepatitis C virus.[29] One report identified SR-BI as a receptor for *M. tuberculosis* but found no impact of SR-BI deletion on *M. tuberculosis* pneumonia *in vivo*,[30] whereas two other reports have identified a role for SR-BI in *Plasmodium* infection of the liver.[31]

Parallel to the diverse roles of different SRs in pulmonary immunity, important differences among SRs have been identified in relation to TLR signaling at the cellular level. Similar to our finding for SR-BI$^{-/-}$ macrophages, SR-A$^{-/-}$ macrophages produce increased TNFα in response to LPS.[6] On the other hand, CD36 promotes cellular responses to both LPS and TLR2 ligands.[32, 33] It will be of interest in future studies to discern the mechanism of enhanced TLR signaling in SR-BI knockout cells. Possible mechanisms include: i) deletion of a cellular ligand disposal (i.e., uptake) pathway that normally tempers TLR responses by competing for (i.e., clearing) extracellular ligand; ii) absence of tonic suppressive effects of HDL on the cell; iii) alterations in plasma membrane lipids; and/or iv) other metabolic alterations in the macrophage. While the increased cell-surface TLR4 on SR-BI$^{-/-}$ macrophages may conceivably contribute to their heightened LPS response, caution is warranted in gauging the physiologic significance of this finding.

The AE plays an important role in the innate immune response during bacterial pneumonia, producing key cytokines and chemokines such as GM-CSF and CXCL5 that recruit PMNs to the airspace.[20] AE cells are thought to be the solitary source of CXCL5 in the lung,[22] producing this chemoattractant in a NF-κB-dependent manner in response to LPS or *S. pneumoniae*.[34] Our finding that SR-BI$^{-/-}$ mice have elevated BALF CXCL5 implicates altered AE function. SR-BI is expressed by AE cells, wherein it is reported to mediate HDL-dependent uptake by AE cells of the antioxidant vitamin E.[17] SR-BI deficiency in AEs may conceivably enhance their direct response to LPS, as observed in macrophages. Alternatively, higher CXCL5 induction could be an indirect consequence of higher airspace TNFα, which is reported to synergize with IL-17 to promote CXCL5 production by the AE.[35] As NF-κB activation and pro-inflammatory cytokine induction are promoted by oxidative stress in AEs,[36] increased CXCL5 induction could also result in part from vitamin E deficiency.

A notable finding of our report is that SR-BI regulates phagocytic killing of bacteria. SR-BI$^{-/-}$ mice displayed defective bacterial clearance both in the lung and bloodstream. While our peritoneal phagocytic killing assay has the advantage of allowing precisely timed examination of the initial PMN-pathogen encounter in the *in vivo* context, we cannot strictly exclude contributions from phagocytes other than PMNs to our results (e.g., peritoneal macrophages). Nonetheless, we provide evidence of intact killing function in SR-BI$^{-/-}$ macrophages, and show that the bacterial killing deficit is associated with defects in PMN generation of oxidants, as well as in additional signs of PMN activation (L-selectin shedding, CD11b display). As both SR-A and LIMP2 are reported to regulate

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phagolysosomal fusion,[38, 39] we speculate that SR-BI may possibly regulate steps in post-internalization processing of microbes. PMN SR-BI was also recently reported to mediate vitamin E entry into PMNs,[40] and vitamin E supplementation enhances bacterial killing by PMNs.[41] Thus, it is possible that functional deficits in SR-BI$^{-/-}$ PMNs may derive, in part, from vitamin E deficiency. While the changes in CD11b and CD62L may suggest a less mature/activated PMN phenotype in SR-BI$^{-/-}$ mice, we cannot exclude the possibility that this is secondary to accelerated release of immature PMNs from the SR-BI$^{-/-}$ bone marrow due to exacerbated sepsis.

We are unaware of any past reports that have defined mechanisms by which LPS is cleared from the airspace. Our finding that hematopoietic cell, and, likely, macrophage SR-BI mediates LPS clearance from the airspace is noteworthy, although future studies outside the scope of the present report will be required to define the physiologic significance of this finding in different settings. Given that LPS was cleared by 48h post-exposure in the SR-BI$^{-/-}$ mouse, and BALF cytokines were only modestly and selectively increased in the SR-BI$^{-/-}$ airway, we speculate that defective LPS clearance was not the major driver of increased alveolar neutrophilia in our studies. However, LPS clearance could play a role not only in termination of pro-inflammatory signaling, but also in LPS tolerance in the lung and in regulation of disease phenotypes driven by chronic or recurrent pulmonary exposure to LPS, such as bronchiectasis and byssinosis.

As aging[43] as well as several environmental exposures (cigarette smoke,[44] ozone,[43] LPS[24]) downregulate SR-BI, it is possible that environmental modulation of SR-BI is a common immune-modifying event in the human lung. Conversely, there are several common systemic exposures/conditions reported to upregulate SR-BI, including statins,[45] vitamin E,[40] nifedipine,[46] aspirin,[47] and diabetes mellitus.[48] Finally, of interest, a growing number of genetic variants of SR-BI in humans have been described that phenocopy several findings in SR-BI$^{-/-}$ mice.[13, 14] Taken together, this suggests that genetic and environmental modulation of SR-BI may be a common modifier of immune responses in humans.

The recognition that SR-BI and HDL regulate pulmonary host defense now raises new and exciting questions, both fundamental and translational, about lung biology. Given that additional HDL cargo beyond vitamin E, such as microRNAs,[49] are also delivered into cells via SR-BI, it will be of interest to examine the extent of additional circulating immunomodulatory molecules for which SR-BI may serve as a gateway to the lung. The composition of HDL is now known to be altered in several common disease states that have been associated with increased risk for lung disease, including obesity and diabetes mellitus.[49] Future studies are warranted to define whether modifications of HDL are communicated to, and modify, the lung via SR-BI, and whether these interactions can be manipulated to therapeutic benefit.

Materials and Methods

Reagents

Escherichia coli 0111:B4 LPS, penicillin, and streptomycin were from Sigma (St. Louis, MO). *K. pneumoniae* 43816 (serotype 2), DMEM, and FBS were from American Type

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Culture Collection (Rockville, MD). The Limulus amebocyte lysate assay was from Lonza (Basel, Switzerland).

Mice

C57BL/6J (SR-BI$^{+/+}$), B6;129S2-Scarb1^{tm1Kri}/J (SR-BI$^{-/-}$), and B6.129P2-Apoa1^{tm1Unc}/J (apoA-I$^{-/-}$) male mice, 7-10 weeks old and weighing 18-22g, were used and were from Jackson Laboratories (Bar Harbor, ME) and bred in house. ApoA-I$^{-/-}$ mice were backcrossed 10 generations onto a C57/BL/6J background. SR-BI$^{-/-}$ mice were backcrossed >6 generations onto C57BL/6J before use. Experiments conducted using both littermate SR-BI$^{+/+}$ and commercial SR-BI$^{+/+}$ (C57BL/6J) controls confirmed very similar responses (Supplementary Figure 2). All experiments were performed in accordance with the Animal Welfare Act and the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals after review by the Animal Care and Use Committee of the NIEHS.

In vivo exposures

Exposure to aerosolized LPS (300 μg/ml, 30 min) was as previously described.[19] *K. pneumoniae* was delivered to the lung by oropharyngeal aspiration (200-2000 CFU/50 μl) or i.v. into the retro-orbital venous plexus (7×10^4 CFUs in 200 μl PBS) during isoflourane anesthesia. In some experiments, corticosterone (10 mg/kg) or vehicle was injected i.p. 1hr preceding other exposures and daily thereafter, as previously reported.[50]

Bronchoalveolar lavage fluid (BALF) collection and analysis

BALF was collected immediately following sacrifice and cell counts performed as described.[19] Protein analysis was performed using Bradford assay (Bio-Rad, Hercules, CA). Endotoxin levels in BALF were measured by Limulus amebocyte lysate assay (Lonza, Basel, Switzerland).

PMN and macrophage harvests and culture

Mature murine bone marrow PMNs were isolated from mouse femurs and tibias by discontinuous Percoll gradient centrifugation as previously reported.[19] This preparation was then sorted by a FACSAriaII (Becton Dickinson, Franklin Lakes, NJ) based on live (7AAD^{neg}), Ly6G^{+}, and F4/80^{6} to yield a population that was >95% pure with 0% F4/80^{+} cells (Figure 7A). Peritoneal PMNs were harvested by peritoneal lavage 4h after i.p. Brewer's thioglycollate (2 ml, 4%). Peritoneal exudate macrophages were harvested by peritoneal lavage 96h after i.p. Brewer's thioglycollate and then plated for 24h before stimulation. Macrophages were stimulated with media, Pam3CSK4 (Invivogen, San Diego, CA; 100ng/ml), Poly I:C (Invivogen, 10μg/ml), LPS (List Biologicals, Campbell, CA; 10ng/ml), or CpG oligodeoxynucleotide (Invivogen; 10Qg /ml) for 24h and cell supernatants harvested for cytokine analysis.

Bactericidal assays

Lung, spleen, and liver were homogenized in PBS, and serial dilutions plated on tryptic soy agar for bacterial quantification, as described.[19] Blood was collected from the right ventricle and similarly plated. Intracellular killing capacity of PMNs against i.p.-injected *K.*

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pneumoniae was quantified as reported.[19] In brief, mice received 2 ml of 4% thioglycollate i.p., followed 4h or 96h later by 1×10^8 CFU *K. pneumoniae* i.p. Peritoneal leukocytes were collected 30 minutes later by lavage (HBSS with 100 μg/ml gentamicin), washed, and then cells (1×10^6) were incubated (37°C) for varying durations, followed by lysis (0.1% Triton-X-100) for intracellular CFU quantification by plating. Morphologic analysis of cytospins confirmed that SR-BI$^{+/+}$ and SR-BI$^{-/-}$ lavages contained equal absolute and relative numbers (≥80% of lavage cells) of PMNs.

Peripheral blood leukocyte typing and enumeration

Blood samples were analyzed using the HEMAVET 1700 hematology analyzer (Drew Scientific, Inc.). Manual WBC differential counts were reported and smear estimates used for confirmation.

Statistical analysis

Analysis was performed using GraphPad Prism statistical software (San Diego, CA). Data are represented as mean ± SEM. Two-tailed student's t test was applied for comparisons of two groups, and ANOVA for comparisons of >2 groups. Survival was evaluated by log rank test. For all tests, $p<0.05$ was considered significant.

Additional methods describing cytokine analysis, RNA isolation and qPCR, flow cytometry, phagocytosis assay, LPS binding and uptake, serum and plasma analysis, generation of bone marrow chimeras, and histopathological evaluation are included in the supplemental methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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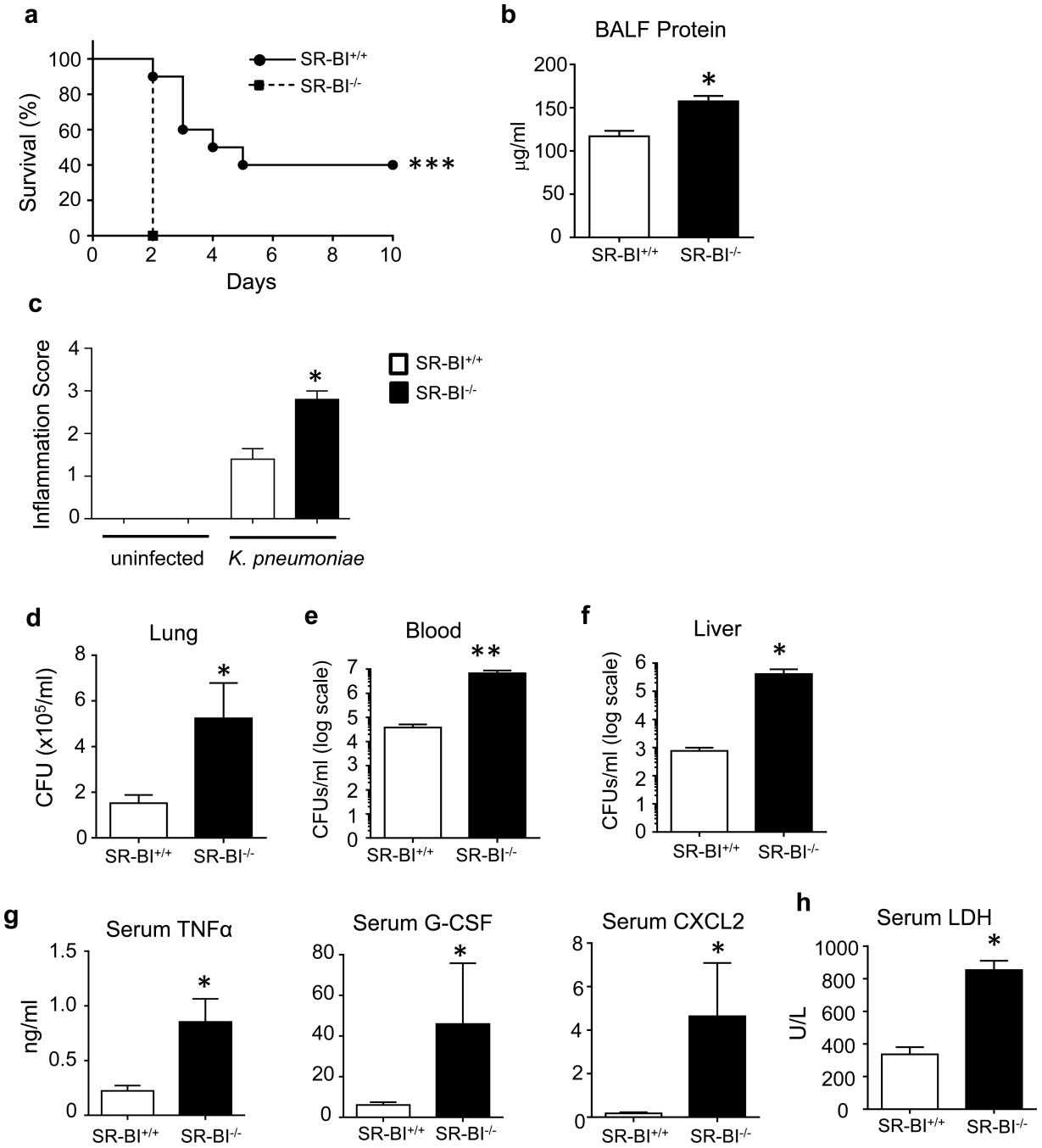

Figure 1. SR-BI$^{-/-}$ mice have a failure of host defense after pulmonary infection with *K. pneumoniae*

a) SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice (n=10/group) were inoculated i.t. with 200 CFUs of *K. pneumoniae*, and survival was monitored. P<0.0001 by log-rank test. **(b-h)** SR-BI$^{+/+}$ and SR6 BI$^{-/-}$ mice were inoculated i.t. with 2000 CFUs of *K. pneumoniae*, and necropsied 24h post- inoculation. BALF total protein was quantified by Bradford assay **(b)**, lung inflammation was scored **(c)**, and bacterial CFUs in lung **(d)**, blood **(e)**, and liver **(f)** were quantified. Serum levels of TNF-α, G-CSF, and CXCL2 **(g)**; and LDH **(h)** were also

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quantified. Data shown in panels b and d-h is representative of two independent experiments involving N=5-8/genotype. Panel c derives from one experiment of N=8/genotype. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

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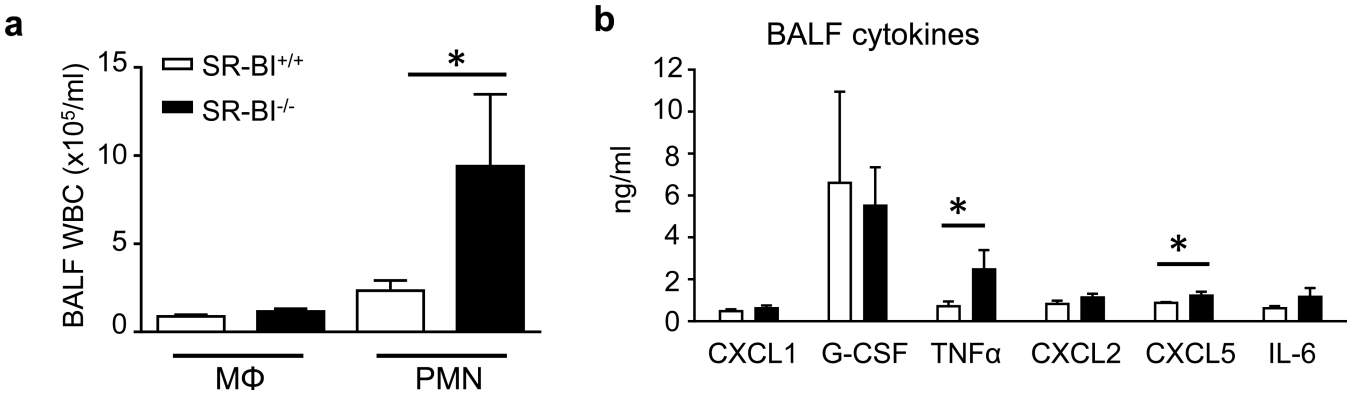

Figure 2. The pulmonary response to *K. pneumoniae* infection is enhanced in SR-BI$^{-/-}$ mice

SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice were inoculated i.t. with 2000 CFUs of *K. pneumoniae*, and necropsied 24h post-inoculation. **a)** BALF macrophages (Mϕ) and neutrophils (PMN) were counted. **b)** BALF levels of CXCL1, TNFα, G-CSF, CXCL2, and IL-6 were quantified by Bioplex assay and CXCL5 by ELISA. Data shown is representative of two independent experiments involving N=5-8 mice/genotype. *, $p<0.05$.

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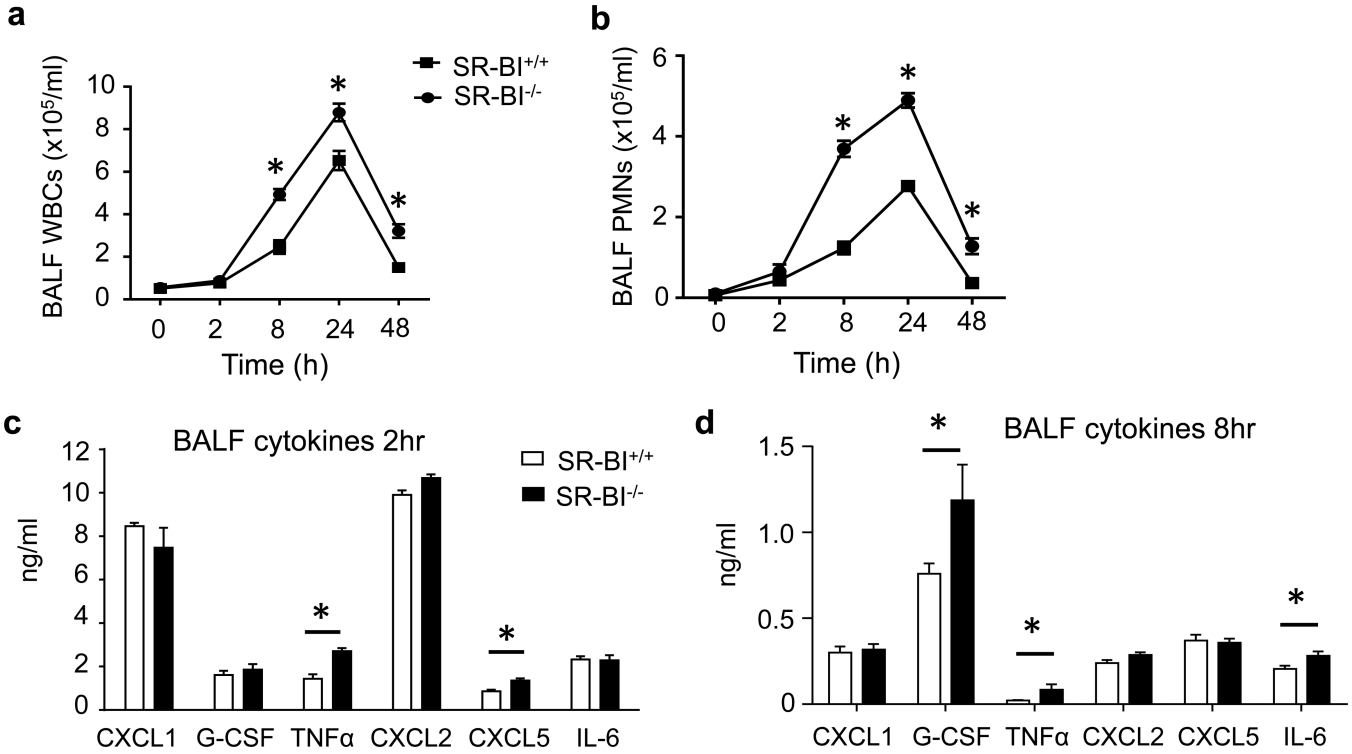

Figure 3. The pulmonary response to inhaled LPS is increased in SR-BI$^{-/-}$ mice

SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice were challenged with aerosolized LPS. BALF total leukocytes (WBCs) **(a)** and neutrophils (PMN) **(b)** were counted at 2, 8, 24, and 48hrs post-exposure. **(c-d)** BALF CXCL1, TNFα, G-CSF, CXCL2, and IL-6 protein was quantified by Bioplex assay and CXCL5 by ELISA at 2h **(c)** and 8h **(d)** after post-exposure. Data shown is representative of two independent experiments involving N=5-8 mice/genotype/timepoint. *, $p<0.05$.

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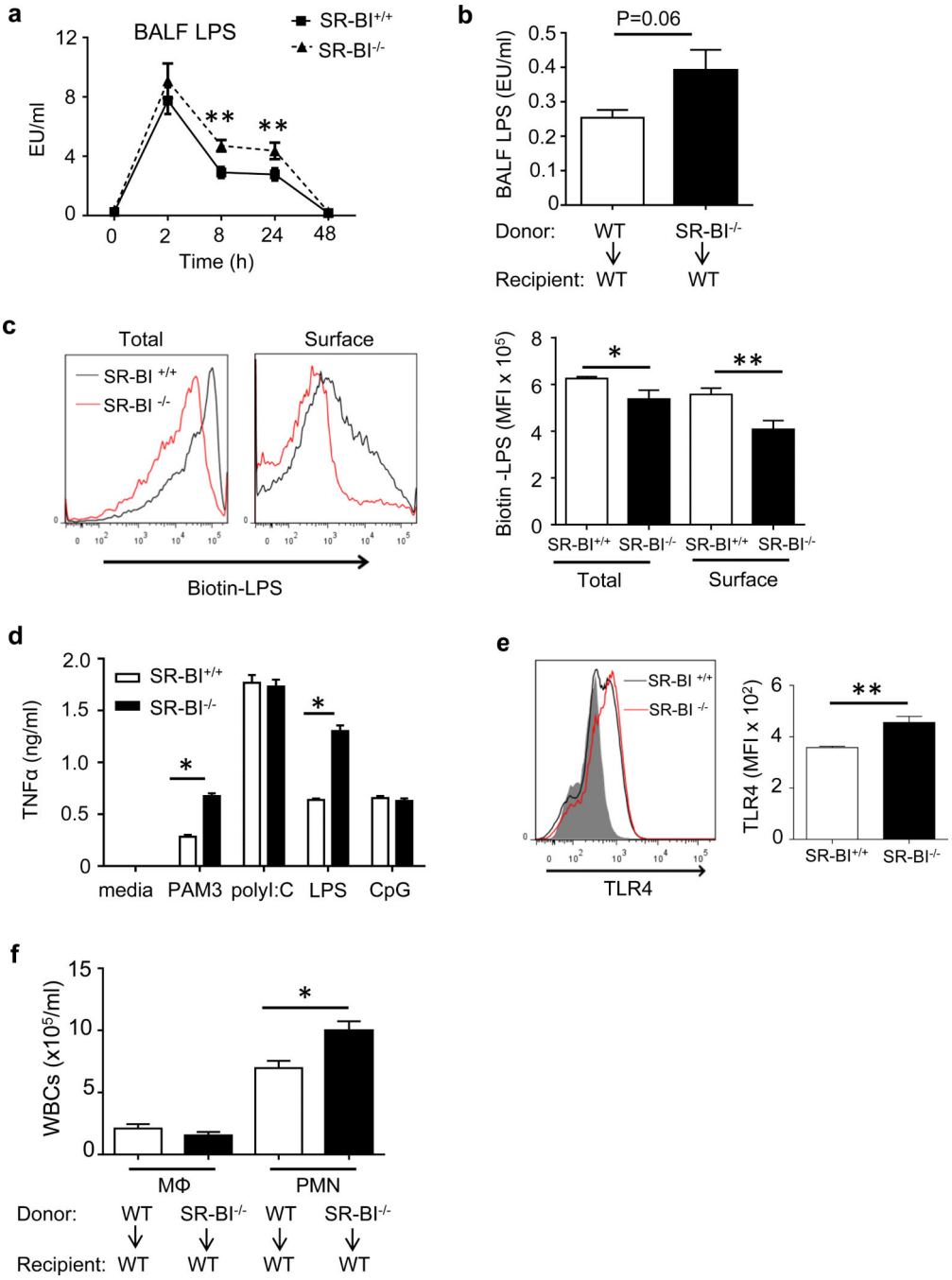

Figure 4. Decreased clearance of, and enhanced responsiveness to LPS in the SR-BI$^{-/-}$ airway

a) SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice (N=5-8/group) were challenged with aerosolized LPS (300 μg/ml, 30 min) and LPS clearance from the airspace at various time points post-exposure was measured by LAL assay on the BALF. **b)** SR-BI bone marrow chimeras, generated by bone marrow reconstitution of lethally irradiated SR-BI$^{+/+}$ (WT) recipients with bone marrow from WT or SR-BI$^{-/-}$ mice, were exposed to aerosolized LPS. Mice (N=7/genotype) were sacrificed 24h post-exposure for LAL measurement of BALF LPS. **c)** Peritoneal exudate macrophages from SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice were incubated with

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biotin-labeled LPS and both surface-bound and total (internalized plus surface-bound) LPS were quantified by flow cytometry. Histograms are shown at left and pooled and quantified mean fluorescence intensity (MFI) data at right. N=6/genotype. **d)** SR-BI$^{+/+}$ and SR-BI$^{-/-}$ peritoneal exudate macrophages were incubated with media, Pam3CSK4 (100ng/ml), Poly I:C (10μg/ml), LPS (10ng/ml), or CpG oligodeoxynucleotide (10Qg/ml) for 24h and TNFα was measured in cell supernatants by ELISA. **e)** TLR4 cell-surface display on SR-BI$^{+/+}$ and SR-BI$^{-/-}$ peritoneal elicited macrophages was quantified by flow cytometry. Histograms are shown at left (shaded trace is unstained cells) and pooled and quantified mean fluorescence intensity (MFI) data at right. N=3 mice/genotype run in technical duplicate. **, $p<0.01$. **f)** SR-BI bone marrow chimeras as in panel B (N=7/genotype), were exposed to aerosolized LPS and BALF macrophages (Mϕ) and neutrophils (PMN) were counted 24h post-exposure. Data shown is representative of two independent experiments. *, $p<0.05$; **, $p<0.01$.

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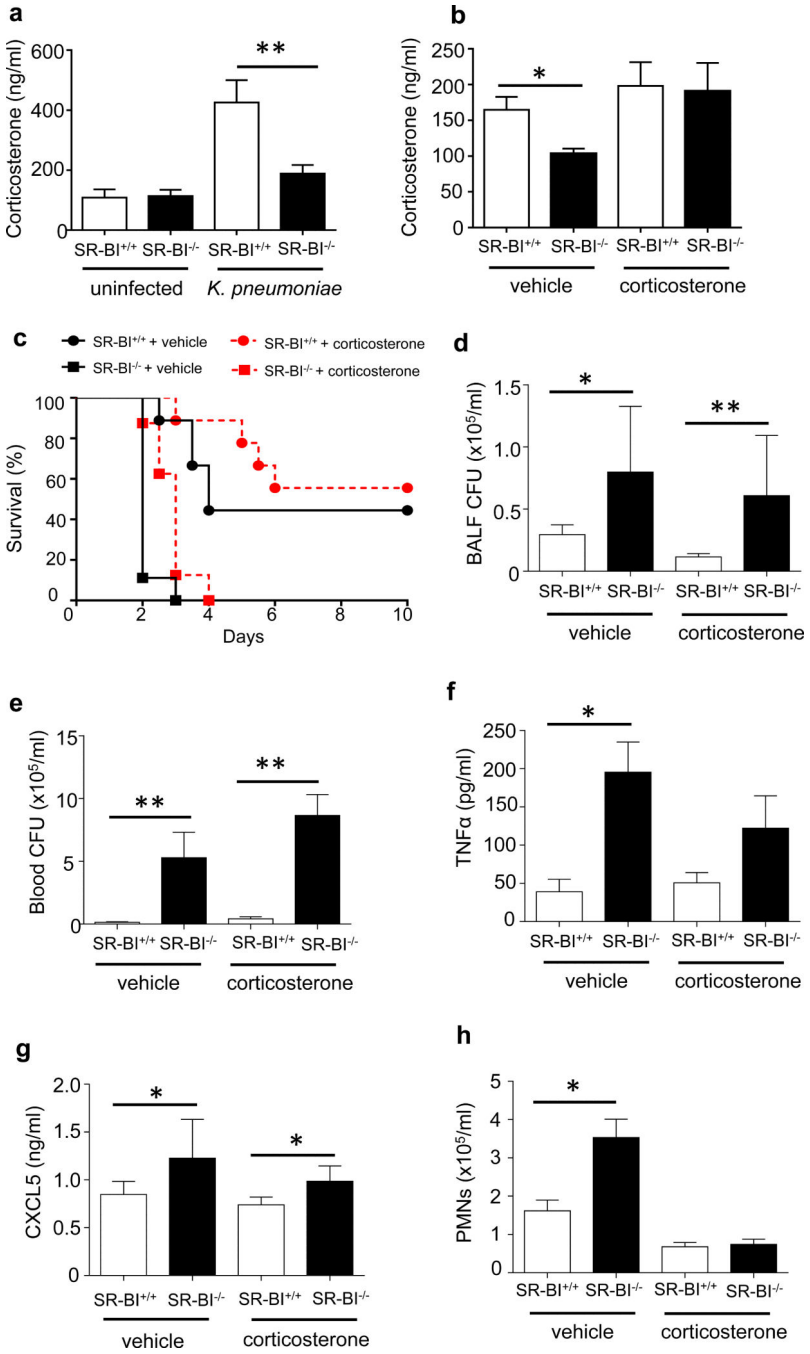

Figure 5. SR-BI$^{-/-}$ mice reveal a role for adrenal function in regulation of alveolar neutrophilia during pneumonia

a) Serum corticosterone was measured in SR-BI$^{+/+}$ and SR6 BI$^{-/-}$ mice at baseline and 24h post-infection with *K. pneumoniae* (2000 CFUs). N=5-8/genotype. **b)** SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice were dosed i.p. with vehicle (DMSO/corn oil) or corticosterone (10mg/kg) 1h before i.t. *K. pneumoniae* (2000 CFUs). Serum corticosterone levels were measured 24h post-inoculation. **c)** SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice (N=10/group) were dosed daily with i.p. vehicle or corticosterone (10mg/kg) starting 1h before i.t. *K. pneumoniae* (200 CFUs) and

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survival was monitored. **(d-e)** Bacterial CFUs in lung homogenates **(d)** and blood **(e)** were quantified 24h post-i.t. *K. pneumoniae* in mice pre-treated i.p. with vehicle or corticosterone. **(f-h)** BALF TNFα **(f)**, CXCL5 **(g)**, and PMNs **(h)** were quantified 24h post-i.t. *K. pneumoniae* in mice pre-treated with i.p. vehicle or corticosterone. Data shown is representative of one experiment. *, $p<0.05$; **, $p<0.01$.

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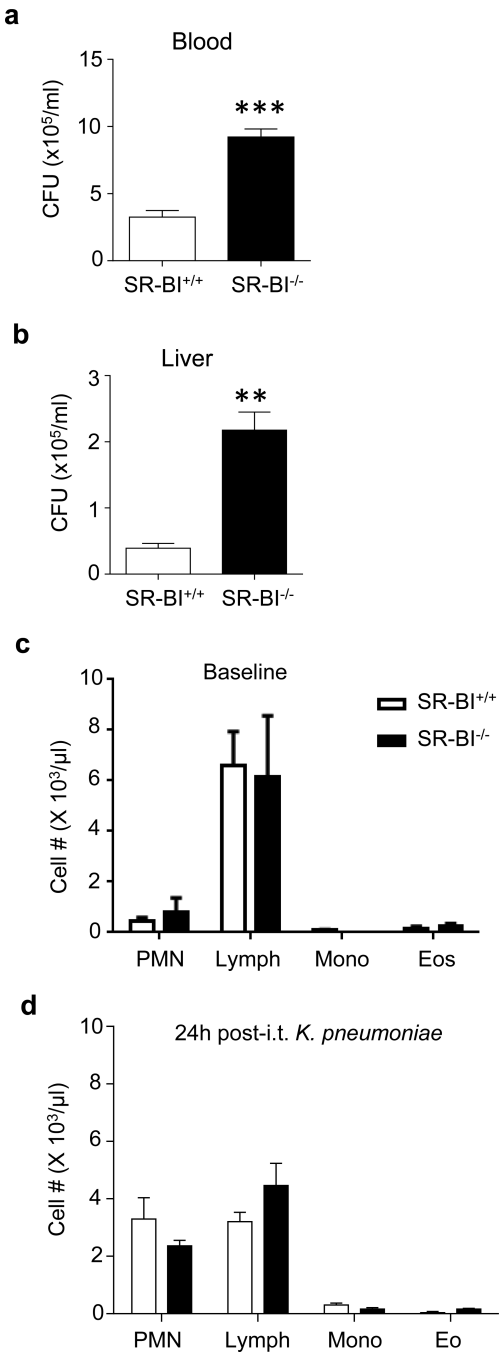

Figure 6. SR-BI$^{-/-}$ mice have decreased clearance of bacteremia

SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice (N=5-8/group) were inoculated i.v. with 70,000 CFUs of *K. pneumoniae*. Bacterial CFUs in blood **(a)** and liver homogenates **(b)** were quantified 4h post-inoculation. **(c-d)** Peripheral blood leukocyte and differential counts in SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice (N=567/group) at baseline **(c)** or 24h post-i.t. *K. pneumoniae* (2000 CFUs) **(d).** Data shown is representative of one experiment. **, p<0.01; ***, p<0.001.

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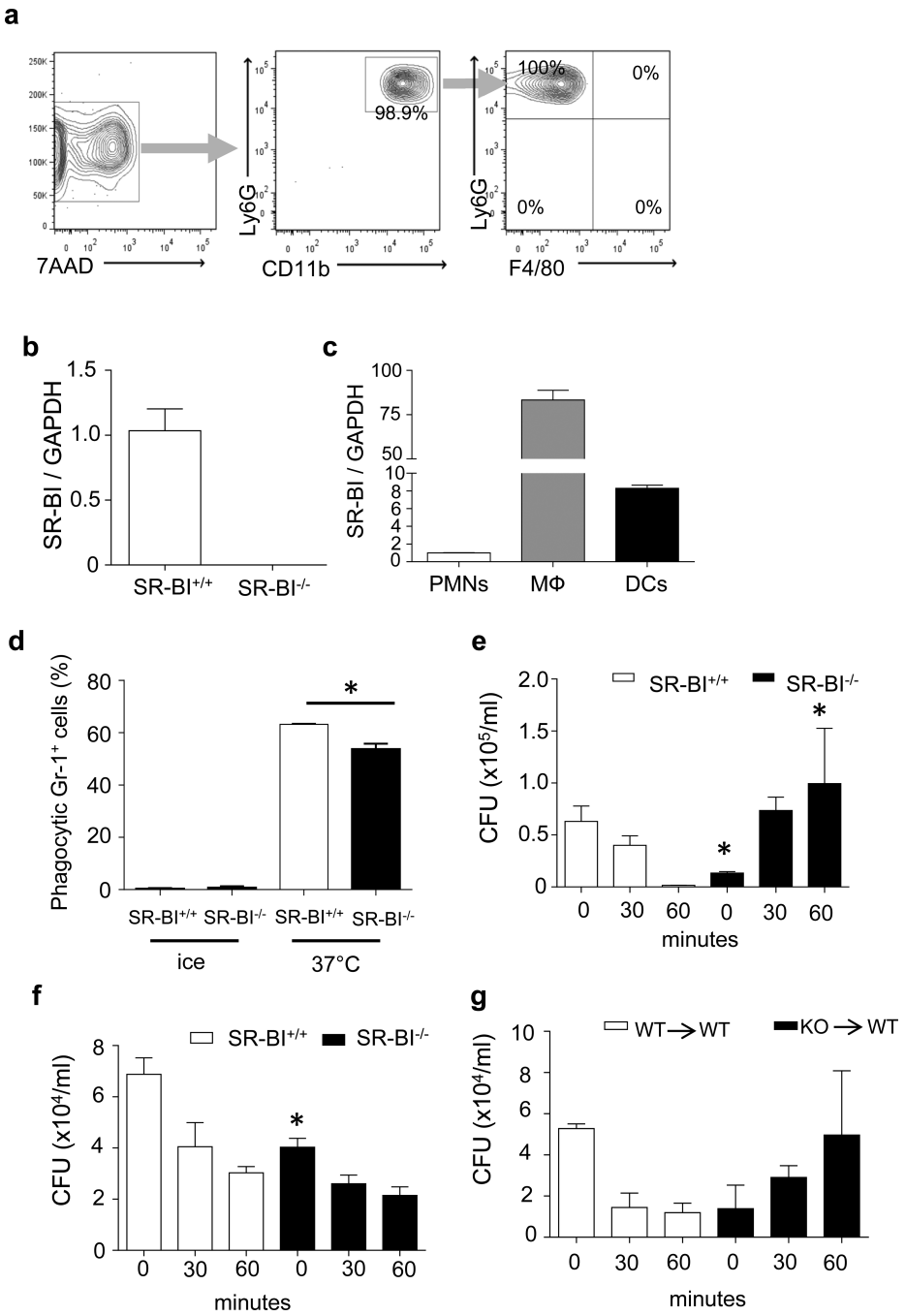

Figure 7. SR-BI$^{-/-}$ neutrophils display decreased phagocytosis and intracellular killing of bacteria

(a) Bone marrow neutrophils (PMNs) were isolated by Percoll gradient from SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice and further purified by FACS sorting for Ly6G^{+} CD11b^{+}7AAD^{-} F4/80^{-}. Representative plots indicating FACS scheme and post-sort purity are shown. **(b-c)** SR-BI expression relative to GAPDH (real-time PCR) in purified bone marrow PMNs from SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice **(b)**, and in SR-BI$^{+/+}$ bone marrow PMNs relative to bone marrow-derived macrophages (Mϕ) and bone marrow-derived dendritic cells (DCs) **(c)**. **d)**

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Phagocytosis of *E. coli* bioparticles by SR-BI$^{+/+}$ and SR-BI$^{-/-}$ peritoneal-elicited Gr-1^{+} PMNs was assessed (N=4-6 mice/condition). **(e-f)** SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice were injected i.p. with thioglycollate, followed 4h **(e)** or 96h **(f)** later by i.p. *K. pneumoniae* (1×10^{8} CFUs). Peritoneal cells were lavaged (HBSS with 100 μg/ml gentamicin) 30 min later, washed, and then incubated (37°C) for varying durations, followed by lysis for intracellular CFU quantification. N=5-8/genotype/time point. **g)** Phagocytic intracellular killing was tested as in panel e, except that bone marrow chimeric mice (SR-BI$^{+/+}$ recipients reconstituted with SR-BI$^{+/+}$ or SR-BI$^{-/-}$ bone marrow) were used (N=2-4/genotype/time point). Data shown is representative of one experiment. *, $p<0.05$.

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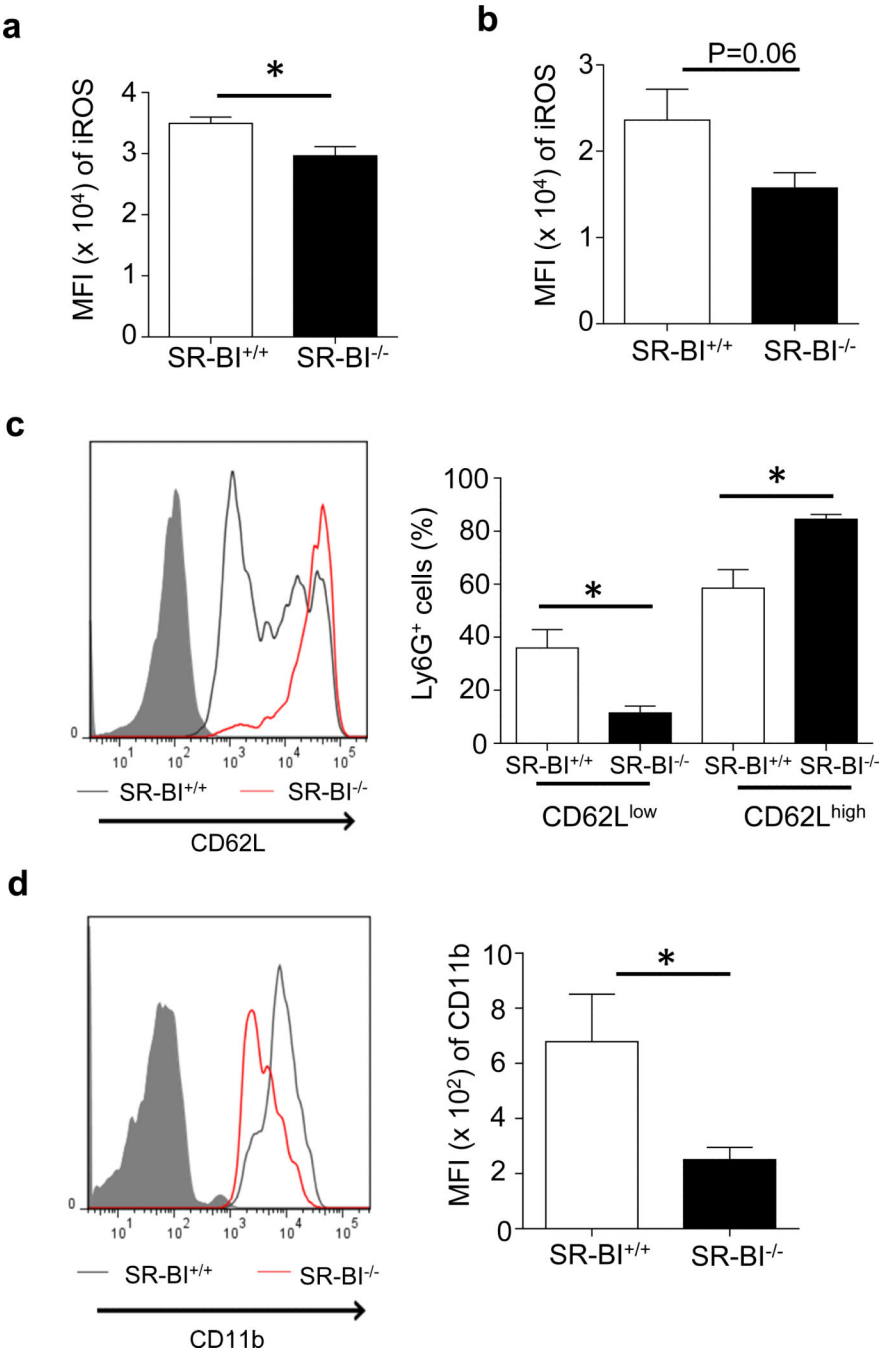

Figure 8. Infected SR-BI$^{-/-}$ mice display deficient neutrophil antimicrobial functions *in vivo*

SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice (N=5-8/group) were inoculated i.t. with 2000 CFUs of *K. pneumoniae*, and necropsied 24h post inoculation. Intracellular reactive oxygen species (iROS) generation was quantified in Ly6G^{+} BALF neutrophils (PMNs) **(a)** and blood PMNs **(b)** by flow cytometry (N=5/genotype). **c)** Left panel displays representative flow cytometry histograms of CD62L on blood PMNs of *K. pneumoniae*-infected SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice. Shaded trace is isotype control, black line is SR-BI$^{+/+}$, and red line is SR-BI$^{-/-}$. Mean fluorescence intensity (MFI) (right panel) of CD62L on Ly6G^{+} blood PMNs. **d)** Left panel

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displays representative flow cytometry histograms of CD11b on blood PMNs of *K. pneumoniae*-infected SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice. Shaded trace is isotype control, black line is SR-BI $^{+/+}$, and red line is SR-BI $^{-/-}$. Mean fluorescence intensity (MFI) (right panel) of CD11b on Ly6G^{+} blood PMNs. Data shown is representative of one experiment. *, $p<0.05$.