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Neutrophil depletion causes a fatal defect in murine pulmonary *Staphylococcus aureus* clearance

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Abstract

Background—*Staphylococcus aureus* is the most common cause of healthcare-associated pneumonia. Despite the significant morbidity and mortality associated with the disease, animal models of *S. aureus* pneumonia are rare.

Materials and Methods—We examined the pathogenicity of four different strains of *S. aureus* (both methicillin-sensitive and resistant as well as Panton-Valentine leukocidin positive and negative) in four strains of immunocompetent inbred and outbred mice (FVB/N, C57Bl/6, Balb/c, ND4, n=148). The immunologic basis for the development of murine S. *aureus* pneumonia was then determined by selectively depleting neutrophils, lymphocytes, or pulmonary macrophages prior to the onset of infection. An additional cohort of animals was rendered immunosuppressed by induction of abdominal sepsis via cecal ligation and puncture 2, 4 or 7 days prior to the onset of pneumonia.

Results—Nearly all immunocompetent mice survived, regardless of which strain of S. *aureus* was used or which strain of mouse was infected. Among animals with immune depletion or prior immunosuppression, survival was decreased only following neutrophil depletion (26% vs. 90% alive at 7 days, $p<0.0001$). Compared to immunocompetent animals, neutrophil-depleted mice with *S. aureus* pneumonia had delayed pulmonary bacterial clearance at 16 and 40 hours but had no difference in levels of bacteremia. Neutrophil-depleted mice also had elevated levels of pulmonary MCP-1 (822 pg/ml vs. 150 pg/ml, $p<0.05$). In contrast, pulmonary histologic appearance was similar in both groups as was dry/wet lung weight.

Conclusions—These results suggest that neutrophils play a critical role in the host response to *S. aureus* pneumonia, and the survival differences observed in neutrophil-depleted mice are associated with alterations in bacterial clearance and pulmonary cytokine response.

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Keywords

Pneumonia; sepsis; *Staphylococcus aureus*; bacteria; infection; neutrophil; MCP-1; murine; model; survival

INTRODUCTION

S. aureus is the most common cause of healthcare-associated, hospital-acquired, and ventilator-associated pneumonia in the United States (1). More than 50% of *S. aureus* infections in the intensive care unit are caused by methicillin resistant (MRSA) strains (2;3). *S. aureus* infections are especially important in surgical patients as they can cause soft tissue infections and bacteremia in addition to pneumonia (4). *S. aureus* infections have become more challenging to treat recently as increasingly resistant strains have gained in dominance, including strains found in the community (5;6) and those which contain the highly virulent Panton-Valentine leukocidin (PVL) (7).

Compared to other common and lethal microbes, there are relatively few animal models of *S. aureus* pneumonia. Initial descriptions of a mouse model of *S. aureus* pneumonia demonstrated that 6×10^8 colony forming units (CFU) of bacteria had to be inoculated to cause lethality (8). However, bacteria did not replicate *in vivo* at this dose, and the mortality observed may have been related to toxicity of bacterial cell components rather than active infection. Broad-spectrum immunosuppression with systemic cyclophosphamide and impairment of mucociliary clearance with intranasal formalin also allow for development of *S. aureus* pneumonia, independent of bacterial toxin production (9). Injection of PVL positive MRSA causes a rapidly fatal (20% survive 24 hours) necrotizing pneumonia in immunocompetent mice via transcription of genes coding for secreted and cell wall-anchored proteins including lung inflammatory factor staphylococcal protein A (10). Additionally, intranasal inoculation of $4–8 \times 10^8$ (but not 8×10^7) CFU of *S. aureus* Newman, a human clinical isolate, causes a rapidly fatal model of pneumonia in immunocompetent C57Bl/6 mice with evidence of bacterial growth *in vivo* associated with production of sortase A (11). Intranasal injection of 2×10^8 CFUs *S. aureus* also causes early pneumonia in neonatal mice, which is dependent on the *agr* and *sar A* loci, but survival in this model beyond 24 hours has not been described (12).

In order to develop a strategy for treatment of *S. aureus* pneumonia, it is critical to understand both pathogen-related and host-related elements that lead to morbidity and mortality. Currently, treatment of *S. aureus* pneumonia primarily consists of targeted therapy in the form of antibiotics. However, when antibiotics fail, treatment becomes non-specific (such as supplemental oxygen) and is independent of the host response to the pathogen, often with resultant poor outcomes. While pathogen-related factors accounting for *S. aureus* virulence in animal models are beginning to be understood, the factors underlying the varied host response to this organism are less well-defined. We therefore used a series of cell-specific and generalized immune depletion strategies to determine what is responsible for resistance to *S. aureus* infection in a variety of mouse strains and examined the mechanisms through which survival may be mediated in pneumonia caused by this organism.

MATERIALS AND METHODS

Bacteria

Strains of *S. aureus* used included 292, 295, 301, and 313, all of which were isolated from patients in the BJC HealthCare system (St. Louis, MO). Further description of the strains is as follows: 292 (MSSA, multilocus sequence type 45), 295 (MRSA, PVL negative, multilocus

sequence type 8, SCC IV), 313 (MRSA, PVL negative, multilocus sequence type 5, SCC II), and 301 (MRSA, PVL positive, multilocus sequence type 8, SCC IV). Bacteria were maintained at −80°C as frozen stock cultures. They were then cultured on blood agar medium for 24 hours prior to inoculation into trypticase soy broth which was then incubated overnight. Cells were then centrifuged, washed twice in sterile PBS and resuspended twice for 10 minutes at 6000g in sterile 0.9% NaCl. The inoculum was then adjusted to an absorbance of 0.5 at 600nm.

Pneumonia model

Surgeries were performed on six to twelve week-old FVB/N, ND4 (Harlan BioProducts, Indianapolis, IN), C57Bl/6, BALB/c, or Rag-$1^{-/-}$ mice (Jackson Laboratory, Bar Harbor, ME) that lack mature lymphocytes (13). FVB/N, C57Bl/6 and BABL/c mice were chosen since they are commonly used inbred strains while ND4 mice were chosen since they are a commonly used outbred strain. Midline cervical incision was performed under isoflurane anesthesia using a technique previously described using *Pseudomonas aeruginosa* (14;15). Each animal received an intratracheal injection of 40 μL of a solution containing *S. aureus* after which the mouse was held vertically for 10 seconds to enhance delivery into the lung. The final density of the inoculum was 5×10^8 CFU/ml as determined by serial dilution and colony counts, corresponding to a dose of 2×10^7 CFU/injection. Sham-operated mice were handled identically except they received 40 μL of 0.9% NaCl only. Mice were either sacrificed at pre-determined timepoints (for functional studies) or assessed daily for survival for 7 days. Of note, animals were assessed daily for the presence of wound infections although none were detected. Mice were maintained on an alternating 12-hr light-dark schedule in a pathogen-free environment and received standard mouse feed *ad libitum.* All studies complied with National Institutes of Health laboratory animals use guidelines and were approved by the Washington University Animal Studies Committee.

“Two-hit” model

Death following sepsis frequently results from a combination of two insults. Frequently, neither is sufficient to decrease survival. However, the first “hit” results in immunosuppression of the host and the second insult (which ordinarily is not lethal) results in a substantial decrease in survival. Published “two hit” models using cecal ligation and puncture (CLP), a model of polymicrobial intra-abdominal infection, followed by pneumonia with different organisms demonstrate the time window where animals are maximally susceptible to the second insult vary widely (16–18). In this study, FVB/N mice underwent CLP by the method of Baker et al. with a single 29 gauge needle puncture (19). Mice were given 1ml of 0.9% NaCl subcutaneously at the time of operation for fluid resuscitation, as well as antibiotics in the form of a single dose of 1mg imipenem 6 hours after operation. Seven day survival of CLP alone or CLP followed by sham pneumonia (intratracheal injection of 0.9% NaCl) ranged from 0–15% depending on length of time between CLP and sham pneumonia (p=ns). Mice then had intratracheal instillation of *S. aureus* 2, 4 or 7 days following CLP.

Cellular depletion

Neutrophil depletion was performed in FVB/N mice which received intraperitoneal injections on two successive days prior to surgery of 1 mL of rabbit anti-mouse PMN polyclonal antibody (Accurate Chemical & Scientific, Westbury, NY) diluted 1:10 in 0.9% NaCl (20). Neutrophil depletion was verified by manual differential of leukocytes on a smear with Wright’s stain performed in a blinded fashion demonstrating an absolute neutrophil count of less than 300/mm^3.

Pulmonary macrophage depletion was performed in FVB/N mice via administration of liposomes composed of phosphatidylcholine and cholesterol containing PBS-dissolved

dichloromethylene diphosphonate (clodronate, Boehringer Mannheim, Mannheim, Germany) 48 hours prior to *S. aureus* challenge as previously described (21). Since there was no difference in survival in any inbred or outbred strains of mice given *S. aureus*, the decision to use FVB/N mice for depletion and "two hit" experiments was due to our laboratory's extensive use of this strain in the past (14;15).

Cultures and cytokine analysis

FVB/N animals were anesthetized with ketamine/xylazine either 16 or 40 hours following injection of *S. aureus*. These timepoints were chosen to sample animals a) before any animals died and b) shortly before most neutrophil-depleted animals died. Blood was taken from the inferior vena cava through a midline laparotomy. Bronchoalveolar lavage (BAL) fluid was obtained by cannulation of the trachea followed by lavaging the lungs with 1ml of 0.9% NaCl. Blood and BAL cultures were serially diluted and grown overnight at 37° C on blood agar plates. Growth was identified 24 hours after plating. Log transformation of calculated colony counts was then used for further analysis (22). In a different cohort of mice, BAL fluid was also characterized for cytokine analysis using a commercially available kit via cytometric bead array (Mouse Inflammation Kit, which tests for MCP-1, IFN-γ, TNF-α, IL-6, IL-10 and IL-12, Cat. # 552364 BD Biosciences, San Jose, CA). Following obtaining blood and BAL samples, animals were sacrificed while under anesthesia.

Histology

Neutrophil-depleted and WT mice were sacrificed 16 or 40 hours after instillation of *S. aureus*. Lungs were then harvested, fixed in formalin and stained with hematoxylin and eosin. Slides were evaluated by a pathologist (MJD) who was blinded to sample identity for both severity and distribution of pneumonia using a subjective grading scale developed for this study. Scores were from 0–4 (no abnormality to most severe pneumonia) and 0–3 (no abnormality to most widespread pneumonia).

Lung Weights

A different cohort of neutrophil-depleted and WT mice were sacrificed 40 hours after instillation of *S. aureus*, and lungs were harvested. A "wet" weight was immediately obtained. Lungs were then dried in a 60 degree oven for 7 days and reweighed (23). The ratio of dry/wet weight was then calculated.

Statistics

Group survival differences were analyzed using the chi square test. Blood and bronchoalveolar lavage data were compared by Mann-Whitney test. Data analysis was performed using Prism 3.0 (GraphPad Software, San Diego, CA). A p value <0.05 was considered to be statistically significant.

RESULTS

S. aureus pneumonia does not affect survival in WT inbred or outbred mice, regardless of whether bacteria are methicillin sensitive or resistant or carry PVL

Intratracheal instillation of *S. aureus* was performed on FVB/N, C57Bl/6, Balb/C and ND4 mice. A total of 148 animals from these four strains of mice were injected with four strains of *S. aureus* (Table 1A). When stratified by mouse genetic background independent of which strain of *S. aureus* was given, the percentage of animals alive at 7 days was 97% in FVB/N animals (37/38), 100% in C57Bl/6 animals (30/30), 93% in BALB/c animals (28/30), and 96% in ND4 animals (48/50, Fig 1A). When stratified by which strain of *S. aureus* was given independent of which type of mouse was infected, the percentage of animals alive was 100%

with 292 (10/10), 98% with 295 (46/47), 93% with 313 (42/45), and 98% with 301 (45/46, Fig 1B). Of note, *S. aureus* strains 295 and 301 were used to infect all four genetic strains of mice (range 5–15 mice/group) while strain 292 was used exclusively in FVB/N mice.

S. aureus pneumonia does not affect survival following CLP in a "two-hit" model of sepsis

Mice subjected to a minimally lethal model of CLP subsequently received an intratracheal injection of strain 313 *S. aureus* 2, 4, or 7 days after the onset of intra-abdominal sepsis (n=134 total, Table 1B). Animals that were inoculated 2 days following CLP had similar survival (76% vs. 86%) compared to mice subjected to CLP followed by sham pneumonia ($p>0.05$, Figure 2). All animals survived when *S. aureus* was introduced 4 or 7 days after CLP.

Neutrophil depletion, but not lymphocyte or pulmonary macrophage depletion, decreases *S. aureus* pneumonia-induced survival

Neutrophil-depleted FVB/N mice had decreased survival, with only 26% alive 7 days following intratracheal instillation of S. *aureus* strain 313. This was significantly different than immunocompetent littermate mice given the same infection (90% alive at 7 days, $p<0.0001$, Fig. 3, Table 1B). Of note, neutrophil depletion itself had no impact on survival in neutrophil-depleted mice subjected to sham pneumonia. In contrast, 92% of Rag-$1^{-/-}$ mice (n=14) were alive 7 days following *S. aureus* pneumonia, as were all pulmonary macrophage depleted mice (n=10). Of note, the decision to use strain 313 in this experiment and subsequent experiments is because MRSA is more lethal than MSSA, and the majority of *S. aureus* strains causing clinical disease are PVL negative.

Comparison of neutrophil-depleted and immunocompetent mice subjected to *S. aureus* pneumonia

Having determined that there was a marked survival difference between neutrophil-depleted and immunocompetent animals given *S. aureus* pneumonia, mice from each group were compared to identify functional differences that might explain the survival difference after instillation of strain 313.

BAL cultures were taken in each group 16 or 40 hours post pneumonia (n=6/group/timepoint), to examine bacterial clearance before significant mortality occurred in either group. Culture results demonstrated significantly higher levels of bacteria in the lungs of neutrophil-depleted mice at both 16 and 40 hours ($p<0.05$ at both timepoints, Fig. 4). In contrast, no significant differences were identified in systemic blood cultures (n=6/group/timepoint). Cultures at 16 hours demonstrated only a single neutrophil-depleted animal had trace levels of bacteremia while the other 11 animals had no detectable bacteria in their bloodstream. At 40 hours, 3/6 animals in each group had trace levels of bacteremia (ranging from 10 to 50 CFU/ml), regardless of whether they were immunocompetent (p=ns).

Cytokine levels in BAL fluid were also measured 40 hours following *S. aureus* pneumonia (n=7/group, Table 2). Levels of Monocyte chemotactic protein-1 (MCP-1) were significantly higher in neutrophil-depleted mice. There was also a non-statistically significant trend ($p=0.07$ and 0.09) toward higher levels of IFN-γ and Interleukin (IL)-6 in neutrophil-depleted mice as well. Of note, neither IL-10 nor IL-12 was detectable in BAL fluid in any animals given *S. aureus* pneumonia.

Despite the differences in bacterial clearance and pulmonary cytokines, there were no gross differences in pulmonary histologic appearance (either severity or distribution of pneumonia) between neutrophil-depleted and immunocompetent mice subjected to *S. aureus* pneumonia (Fig. 5). Additionally, the mean dry lung weight of neutrophil-depleted mice subjected to

pneumonia was 22.4% of fresh weight, while this value was 22.9% in immunocompetent mice with the same insult (n=7–8, p=ns).

DISCUSSION

Our study demonstrates that neutrophils are critical in mediating survival following intratracheal injection of *S. aureus* in mice, whereas the absence of lymphocytes or pulmonary macrophages as well as immunosuppression via prior sepsis, is insufficient to cause lethality following intratracheal *S. aureus* inoculation. The decreased survival in neutrophil-depleted animals is associated with decreased pulmonary bacterial clearance and increased pulmonary cytokine secretion.

Recently published studies have focused predominantly on bacterial components necessary to induce pneumonia in immunocompetent animals (10;11). We were unable to induce a lethal infection in immunocompetent mice despite using four strains of *S. aureus* that were MSSA or MRSA, PVL negative or positive and had three distinct multilocus sequence types. This suggests that the factors necessary to produce a lethal infection in immunocompetent mice are not straightforward, as altering a number of variables in our study did not lead to this result.

In contrast, these results expand our understanding of the host response to *S. aureus* pneumonia, a topic on which there have been fewer published studies than those examining pathogen-specific mechanisms, although it is known that overexpression of elafin, an anti-elastase/antimicrobial molecule, improves clearance of *S. aureus* in a non-lethal model of pneumonia (24). Since the host response to infection is drastically different depending on which genetic background is used, we used multiple strains of animals in these studies (25). Our findings indicate that murine resistance to *S. aureus* pneumonia is not simply a case of differing response depending on the animals genetic background, since similar results were seen with both inbred and outbred murine strains and regardless of whether they are Th1 or Th2 predominant (26). However, neutrophils appear to be critical to the development of lethal *S. aureus* pneumonia while lymphocytes and pulmonary macrophages do not appear to play a major role in mediating host survival. It has previously been shown that immune suppression with cyclophosphamide converts a fully survivable *S. aureus* insult into one that is uniformly lethal (9). Cyclophosphamide is a common chemotherapeutic agent that is also used for treatment of autoimmune disorders. While the drug is well-known to cause neutropenia (27), it has a number of unrelated side effects as well (28–30). As such, it was unclear if the neutropenia associated with cyclophosphamide was responsible for the development of murine pneumonia. By using a neutrophil-specific antibody, we were able to determine that neutrophil depletion is sufficient for the development of lethal *S. aureus* pneumonia. Further, the lack of effect seen with cellular depletion studies using Rag-$1^{-/-}$ mice and liposomes directed against pulmonary macrophages allows a more focused understanding of the cell types responsible for mediating survival in *S. aureus* pneumonia.

It was somewhat surprising that the “two hit” model failed to result in substantial lethality. The approach of inducing immunosuppression via nonlethal CLP followed by pneumonia is clinically relevant and has been demonstrated to work with multiple organisms (16–18). A similar approach of using two sublethal injuries has yielded substantial lethality following the second “hit” in other models of critical illness as well (31). Although no survival studies have been performed using *S. aureus* pneumonia in “two hit” models, bacterial clearance is decreased if *S. aureus* is given after prior infection with respiratory syncytial virus (32). We did not examine bacterial clearance in any model other than neutrophil-depletion reasoning that delayed clearance in a model where all animals survived would be of limited interest. However, based upon the results of our immune depletion studies, we would predict that the “two hit” model of sepsis would not cause a marked delay in bacterial clearance since CLP is

notable for lymphopenia caused by apoptosis (33) without accompanying neutropenia, whereas only neutrophil-depletion resulted in decreased survival following intratracheal injection of *S. aureus.*

Our results also indicate some potential mechanisms through which host response is altered via neutrophil-depletion to yield lethal *S. aureus* pneumonia. Pulmonary bacterial clearance is decreased at both 16 and 40 hours compared to immunocompetent mice. This defect is compartmentalized since there is scant bacteremia in either immunocompetent or neutrophil-depleted mice at either timepoint, despite the fact that most of the latter mice die 2 days following intratracheal injection of *S. aureus*. Additionally, there is a markedly greater pulmonary cytokine response in neutrophil-depleted animals. MCP-1 levels are more than 5-fold greater, and there are non-significant trends towards increased levels of IL-6 and IFN-γ as well. Interestingly, the difference in survival was not associated with histological differences in pneumonia, as both histologic severity and distribution of pneumonia was similar in neutrophil-depleted and immunocompetent animals. It is difficult to know why histology was not associated with survival. It is possible that this means that survival is mediated, at least in part, by systemic factors. Although levels of bacteremia were similar between neutrophil-depleted and immunocompetent animals at 16 and 40 hours, this does not rule out a role for systemic factors not directly associated with bacterial burden. Alternatively, there might have been differences in oxygenation or ventilation between the groups that would not have been identified simply by examining lung histology and dry/wet weight.

The role MCP-1 plays in the pathophysiology of pneumonia is complex and appears to be organism-specific. While no studies correlating *S. aureus* pneumonia and MCP-1 have been performed in mice, this has been examined in a number of related models. Pulmonary MCP-1 is increased following administration of the superantigen staphylococcal enterotoxin B; however, this is unrelated to IFN-γ, since MCP-1 levels are similar in control and IFN-γ-knockout mice (34). *Pseudomonas aeruginosa* pneumonia increases MCP-1 levels, associated with increased numbers of alveolar macrophages. Anti-MCP-1 reduces alveolar macrophages and hepatocyte growth factor levels in BAL fluid and increases lung injury while administration of MCP-1 has the opposite effect and attenuates lung injury (35). In contrast, MCP-1 levels are elevated in idiopathic pneumonia following allogeneic bone marrow transplantation and neutralization of MCP-1 results in decreased lung injury (36). Additionally, while MCP-1 levels are elevated following *Streptococcus pneumoniae* pneumonia, these are correlated to bacterial load but do not appear to have functional significance since MCP-1 knockout mice are indistinguishable from control mice with regard to inflammatory response or lethality (37). There is less data surrounding the role of MCP-1 following pneumonia in neutrophil-depleted animals. However, neutropenic rats have similar levels of MCP-1 as immunocompetent rats following intratracheal injection of lipopolysaccharide despite an abrogation of both early and late increases in pulmonary monocyte/macrophages seen in immunocompetent animals (38).

This study has a number of limitations. Primarily, while our results lead to a clearer understanding of the host response to *S. aureus* pneumonia, it is unclear what relevance they have toward disease in humans. *S. aureus* pneumonia is more common in immunocompromised patients than immunocompetent patients; however, the disease can clearly be acquired in a community setting. We were unable to induce lethal pneumonia in immunocompetent mice regardless of which strain of bacteria or mice were used. This suggests that simply injecting MRSA is insufficient to cause severe pneumonia in mice, and even the addition of PVL, which has severe virulence in patients, does not guarantee a lethal model. Further, generalized immunosuppression via prior CLP, a clinically relevant model of sepsis, failed to induce lethality when followed by intratracheal injection of *S. aureus*. However, since we did not perform an extensive dose response curve, our results may simply have been due to injection

of an insufficient number of bacteria to cause lethal pneumonia (i.e. the inoculum used might have been sufficient to decrease survival in a subset of immunosuppressed animals but a higher dose might have shown the same effect in immunocompetent or animals with generalized immunosuppression although it should be noted that injections of 5.2×10^7 or 7.6×10^7 CFU were insufficient to decrease survival). Additionally, BAL cytokine samples were obtained at 40 hours. This timepoint was chosen since it was shortly before neutrophil-depleted mice died. It is possible that looking at earlier timepoints would have yielded additional mechanistic insights. Also, the finding that MCP-1 levels are elevated following intratracheal injection of *S. aureus* in neutrophil-depleted mice is associative and knockout or antibody experiments would have to be performed to demonstrate its functional significance.

Despite these limitations, these results represent an advance in our understanding of the host response to murine *S. aureus* pneumonia. Survival is mediated by neutrophil depletion, but not by lymphocytes or pulmonary macrophages, and decreased survival is associated with a defect in pulmonary (but not systemic) bacterial clearance and elevated MCP-1. Further understanding of the host response to *S. aureus* pneumonia may prove as important as understanding the microbial components responsible for infection, and future research should target both pathogen and host responses to this increasingly common and lethal infection.

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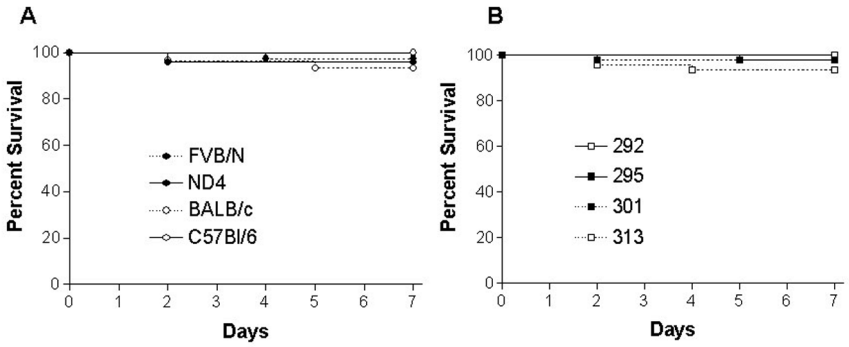

FIG. 1.

Percentage of animals alive at 7 days by genetic background or bacterial strain of mice that received intratracheal injection of *S. aureus.* Regardless of whether mice (n=148) were (A) inbred or outbred or received (B) MSSA or MRSA (PVL positive or negative), essentially all immunocompetent animals survived seven days.

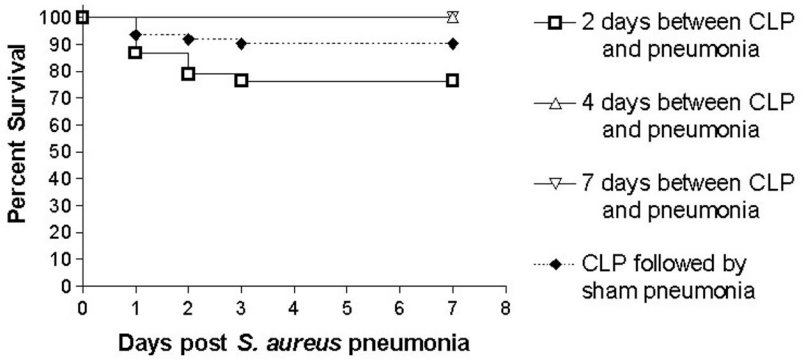

FIG. 2.

Percentage of animals alive at 7 days in mice subjected to "two hit" model of sepsis. Animals were subjected to CLP followed by intratracheal injection of *S. aureus* a variable number of days later. Regardless of interval between CLP and *S. aureus*, survival was statistically similar to animals subjected to CLP followed by sham pneumonia.

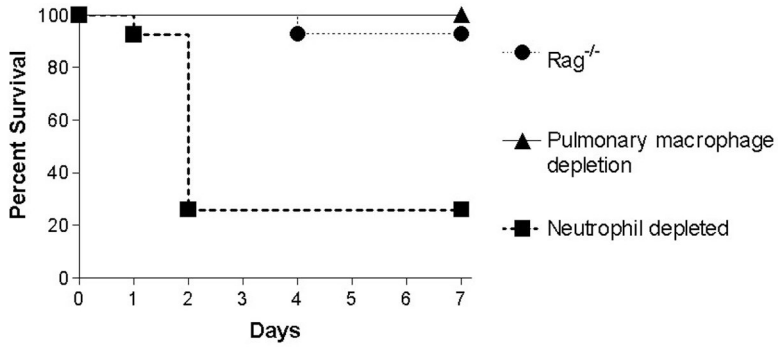

FIG. 3.

Percentage of animals alive at 7 days of immunodepleted animals that had intratracheal injection of *S. aureus.* Mice that had neutrophil depletion via polyclonal antibody (n=27) had decreased survival within two days while lymphocyte-deficient Rag-$1^{-/-}$ mice (n=14) and animals that had pulmonary macrophage depletion (n=10) essentially all survived ($p<0.0001$).

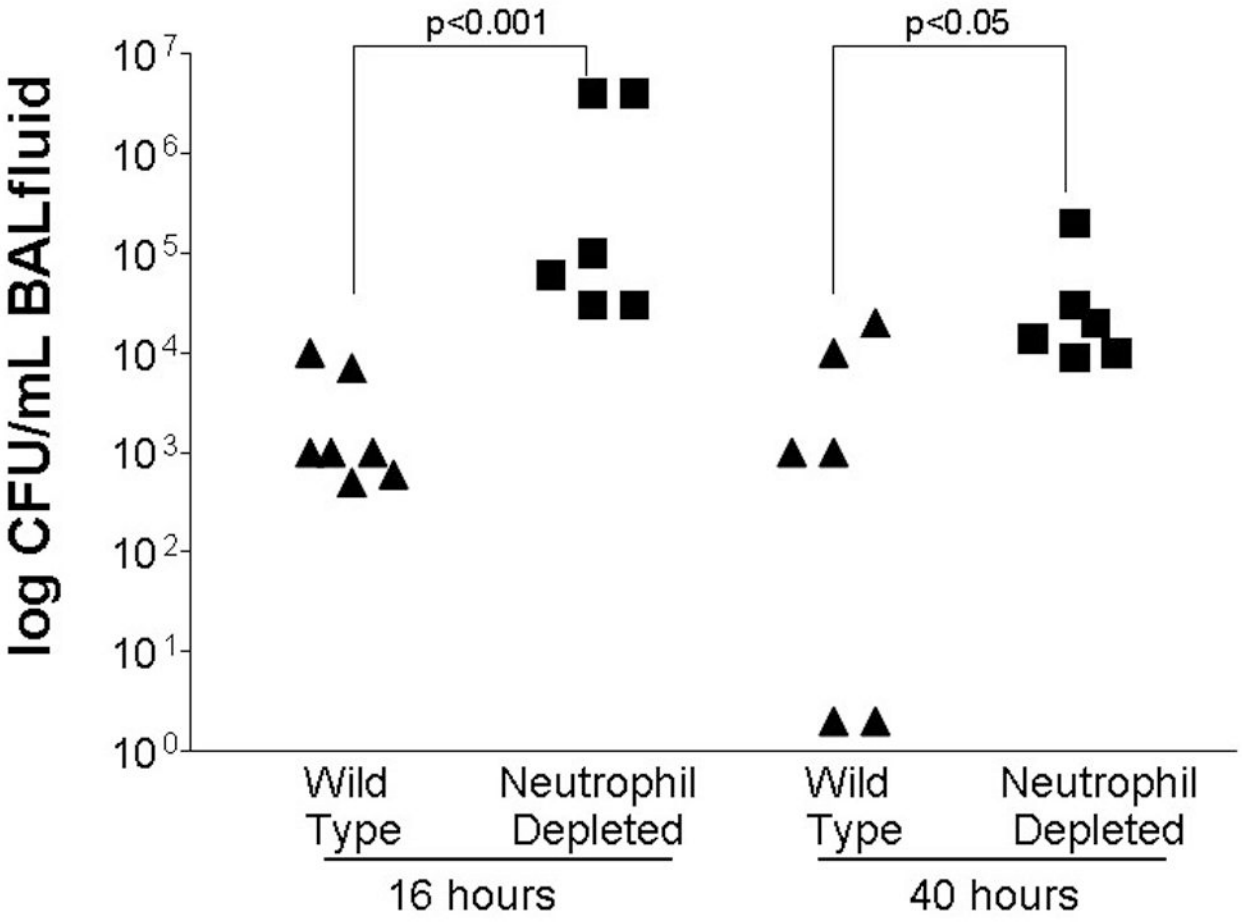

FIG. 4.

Bacterial clearance in neutrophil-depleted and immunocompetent ("wild type") mice following intratracheal injection of *S. aureus* (n=6–7 animals/group/timepoint). Neutrophil-depleted mice had higher concentrations of bacteria in BAL fluid at both 16 and 40 hours. Data has been log transformed for presentation.

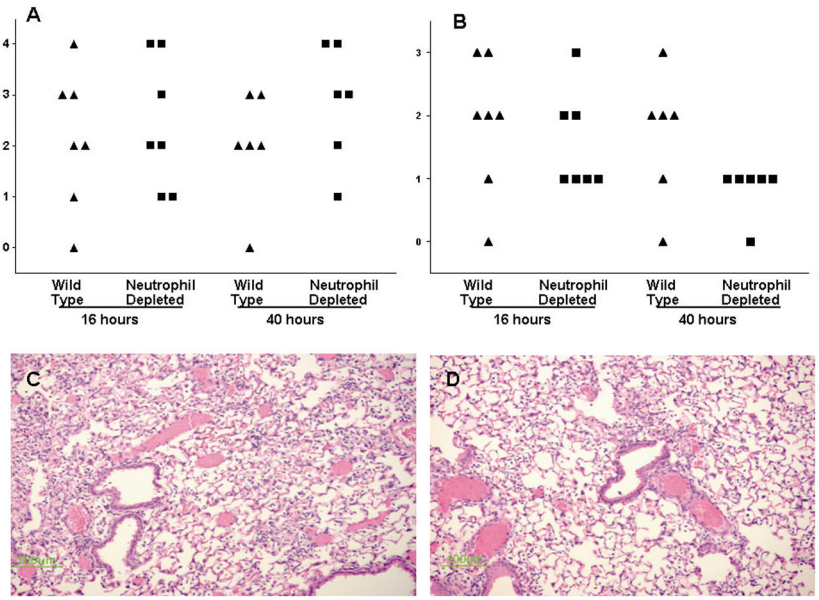

FIG. 5.

Histologic severity and distribution of pneumonia in neutrophil-depleted and immunocompetent ("wild type") mice following intratracheal injection of *S. aureus* (n=6–7 animals/group/timepoint). Marked interanimal variation was seen in both severity (A) and anatomic distribution (B) without significant differences seen between neutrophil-depleted or immunocompetent animals ($p>0.05$). Severity was graded on a 1–4 scale, with 1 representing normal lung and 4 representing severe pneumonia. Distribution was graded on a 1–3 scale with 1 representing no pneumonia and 3 representing diffuse pneumonia. Representative micrographs of immunocompetent (C) and neutrophil-depleted (D) mice with grade 3 severity pneumonia 16 hours after intratracheal injection of *S. aureus* demonstrate similar histologic appearance.

TABLE 1

Table 1A Survival by mouse strain and bacterial strain

Mouse Strain	Bacterial Strain	Survival
FVB/N	292	100% (10/10)
	295	100% (10/10)
	301	100% (8/8)
	313	90% (9/10)
ND4	295	100% (10/10)
	301	100% (5/5)
	313	94% (33/35)
Balb/c	295	93% (14/15)
	301	93% (14/15)
C57Bl/6	295	100% (12/12)
	301	100% (18/18)

Table 1B Survival in depletion and "two hit" experiments

Mouse Model	Strain/Experiment	Survival
Rag-1$^{-/-}$	313	100% (6/6)
	301	88% (7/8)
Pulmonary macrophage depletion	313	100% (10/10)
FVB/N PMN depleted	313	26% (7/27)
FVB/N WT	313	96% (23/24)
Two-Hit	313 2 days after CLP	76% (29/38)
	Sham 2 days after CLP	86% (24/28)
	313 4 days after CLP	100% (18/18)
	Sham 4 days after CLP	100% (17/17)
	313 7 days after CLP	100% (17/17)
	Sham 7 days after CLP	88% (14/16)

TABLE 2

Cytokine levels in pulmonary BAL fluid

Cytokine	Immunocompetent (SEM)	Neutrophil-depleted (SEM)	p value
TNF-α (pg/ml)	199 (53)	249 (57)	0.62
IFN-λ (pg/ml)	15 (26)	67 (4)	0.07
MCP-1 (pg/ml)	150 (225)	822 (48)	**0.007**
IL-6 (pg/ml)	33 (13)	63 (9)	0.09