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Cecal ligation and puncture followed by MRSA pneumonia increases mortality in mice and blunts production of local and systemic cytokines

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Abstract

Mortality in the ICU frequently results from the synergistic effect of two temporally-distinct infections. This study examined the pathophysiology of a new model of intraabdominal sepsis followed by methicillin-resistant *Staphylococcus aureus* (MRSA) pneumonia. Mice underwent cecal ligation and puncture (CLP) or sham laparotomy followed three days later by an intratracheal injection of MRSA or saline. Both CLP/saline and sham/MRSA mice had 100% survival while animals with CLP followed by MRSA pneumonia had 67% seven-day survival. Animals subjected to CLP/MRSA had increased bronchoalveolar lavage (BAL) concentrations of MRSA compared to sham/MRSA animals. Animals subjected to sham/MRSA pneumonia had increased BAL levels of IL-6, TNF- α , and G-CSF compared to those given intratracheal saline while CLP/MRSA mice had a blunted local inflammatory response with markedly decreased cytokine levels. Similarly, animals subjected to CLP/saline had increased peritoneal lavage levels of IL-6 and IL-1 β compared to those subjected to sham laparotomy while this response was blunted in CLP/MRSA mice. Systemic cytokines were upregulated in both CLP/saline and sham/MRSA mice, and this was blunted by the combination of CLP/MRSA. In contrast, no synergistic effect on pneumonia severity, white blood cell count or lymphocyte apoptosis was identified in CLP/MRSA mice compared to animals with either insult in isolation. These results indicate that a clinically relevant model of CLP followed by MRSA pneumonia causes higher mortality than

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could have been predicted from studying either infection in isolation, and this was associated with a blunted local (pulmonary and peritoneal) and systemic inflammatory response and decreased ability to clear infection.

Keywords

Sepsis; two-hit; cecal ligation and puncture; pneumonia; *Staphylococcus aureus*; MRSA; cytokines; host response

INTRODUCTION

Sepsis is the leading cause of death in critically ill patients. More than 750,000 people develop the disease annually in the United States, with over 200,000 dying from the disease (1;2). Patients with sepsis initially have a proinflammatory state that shifts towards a prolonged period of immune suppression or immunoparalysis (3–6). This, in turn, increases susceptibility to a secondary infection.

The two-hit model of critical illness postulates that an initial insult can prime the host for a later insult that can lead to a synergistic response that is disproportionate to the severity of either insult (7). Thus two injuries (either infectious or non-infectious) that might be relatively innocuous in isolation can be fatal in patients if combined over a short time period in the ICU.

There are multiple two-hit animal models that mimic what might be seen in a surgical ICU. A common model utilized is cecal ligation and puncture (CLP), a mouse model of peritonitis, followed by pneumonia (3;8–10). The majority of published studies examining this use *Pseudomonas aeruginosa* pneumonia, a common gram negative nosocomial infection. While one study uses *Streptococcus pneumoniae* as a model of gram positive pneumonia, this organism is typically community acquired and has not been implicated as a significant cause of secondary pneumonia in ventilated patients. There is thus limited understanding of the host response to a common gram positive organism that causes nosocomial pneumonia following intraabdominal sepsis. This is important to understand in light of the fact that the host response to sepsis varies widely with the initiating organism, even if the ultimately mortality is similar (11).

Staphylococcus aureus is, by far, the most common cause of nosocomial pneumonia in the ICU (12). The majority of infections caused by this organism in the ICU are resistant, and the incidence of methicillin-resistant *Staphylococcus aureus* (MRSA) is becoming increasingly common (13). Nearly 100,000 people are diagnosed with invasive MRSA annually which is significant, since compared to methicillin-sensitive *Staphylococcus aureus*, MRSA is associated with increased costs and increased mortality (14;15).

The purpose of this study was to develop a two-hit model of CLP followed by MRSA pneumonia to mimic a situation frequently encountered in the surgical ICU and to understand potential mechanisms through which this combination of insults induces mortality that could not have been predicted by studying either variable in isolation.

MATERIALS AND METHODS

Animals

All studies were performed using 6 to 15 week-old FVB/N mice. All mice had free access to water and mouse chow and were kept on a strict 12 hour light-dark cycle. All studies were in

accordance with the National Institutes of Health laboratory animals use guidelines and were approved by the Washington University and Emory University Animal Studies Committees.

Sepsis models

Two models of sepsis were used – CLP and MRSA pneumonia. Animals first underwent either CLP or sham laparotomy (16). For animals that received CLP, a midline abdominal incision was made under isoflurane anesthesia. The cecum was exteriorized and ligated near the ileocecal junction and punctured once with a 30-gauge needle. The cecum was then replaced into the abdominal cavity, and the abdominal wall was closed in layers. Mice that underwent sham laparotomy were treated similarly except that the cecum was neither ligated nor punctured. All mice were treated with ceftriaxone (25 mg/kg) and metronidazole (12.5 mg/kg) via intraperitoneal injection three hours after surgery.

Unless otherwise specified, three days after either CLP or sham laparotomy, mice were inoculated with MRSA or saline via intratracheal injection to induce MRSA or sham pneumonia. Animals that received MRSA pneumonia were infected with strain 313, which was isolated from a patient in the BJC HealthCare system (St. Louis, MO). This strain is multilocus sequence type 5, *SCCmecII*, and is negative for the Pantone-Valentine leukocidin (17;18). An additional cohort of animals received heat killed MRSA. Animals received an intratracheal injection of 60µl of MRSA prepared at a final density of 0.7 at 600nm (7.8×10^7 CFU/injection) or an identical volume of saline. The rationale for choosing to induce pneumonia three days after CLP was to mimic the human condition where the risk of ventilator associated pneumonia begins to increase three days following intubation. While animals were not on ventilators in this study, there is no clear data on when risk of healthcare acquired pneumonia increases following peritonitis, so ventilator associated pneumonia was used both as a surrogate and because we wished to mimic the clinical scenario seen in the intensive care unit as closely as possible. While MRSA was given 3 days after CLP in the majority of experiments, an additional group of animals was given MRSA pneumonia 7 days following CLP. This was done both a) because of the still significant risk that patients have of developing pneumonia 7 days after a first hit and b) experimental data suggesting mice are susceptible to *Pseudomonas aeruginosa* pneumonia 3 days after nonlethal CLP but are no longer susceptible after 7 days (19).

In order to distinguish a synergistic effect resulting from the combination of CLP and MRSA compared to either insult alone, the experimental design had 4 groups: a) sham laparotomy followed by sham pneumonia (sham/saline), b) sham laparotomy followed by MRSA pneumonia (sham/MRSA), c) CLP followed by sham pneumonia (CLP/saline), and d) CLP followed by MRSA pneumonia (CLP/MRSA). The decision was made to refer to sham laparotomy as “sham” and sham pneumonia as “saline” to differentiate between the two distinct sham operations since 3 of 4 groups of mice were subjected to at least one sham surgery. Unless otherwise specified, all mice were sacrificed either 24 hours (mechanistic studies) or 7 days (survival studies) after intratracheal injection of MRSA or saline.

Bacterial Counts

Quantitative bacterial cultures were measured from both blood and bronchoalveolar lavage (BAL) samples. Whole blood was obtained retro-orbitally and BAL fluid was obtained by cannulating the trachea and lavaging the lungs with 1ml of sterile saline. Samples were serially diluted in sterile saline and grown on blood agar plates at 37°C. MRSA colonies were counted after 48 hours of incubation. Growth was calculated as colony forming units/ml and log transformation of these values was used for further analysis (20).

Cytokine analysis

Cytokines were measured from plasma, BAL, and peritoneal fluid using a cytometric bead array (Bio-plex Cytokine Pro assay, Biorad, Hercules, CA) according to manufacturer protocol. The bead array was designed to interrogate a number of pro- and anti-inflammatory cytokines that are commonly implicated in the pathophysiology of sepsis. In addition to the four cytokines reported in the results section, IL-10 and IL-13 were also measured in the plasma, BAL, and peritoneal fluid but are not reported since no differences were detected in these cytokines. All samples were measured in duplicate. Plasma was obtained by centrifuging whole blood obtained as above. BAL fluid was obtained as above. Peritoneal fluid was obtained by cannulating the peritoneal cavity and lavaging with 5ml of sterile saline.

Lung histology and weights

Pneumonia severity was evaluated on H&E-stained sections by a pathologist (ABF) blinded to sample identity to determine the severity and distribution of pneumonia. The percent of lung tissue with inflammation was first assessed. Next, an inflammation score ranging from 0 to 5 (0 = none, 1 = <5%, 2 = 5–10%, 3 = 10 – 19%, 4 = 20 – 49%, 5 = > 50%) was then calculated. Finally, a subjective analysis of each individual section was performed.

Lung edema was identified by measuring a wet to dry ratio (17). Both lungs were weighed immediately after organ harvest to determine the “wet” weight. Lungs were then dried for 48 hours at 80°C and reweighed at which time a wet to dry weight ratio was calculated.

Myeloperoxidase (MPO) activity

MPO activity was evaluated in whole lung tissue to assess neutrophil infiltration. Lungs were homogenized in 50mM sodium phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide, heated for 2 hours at 55°C, centrifuged, and then supernatants were collected. Substrate buffer containing O-dianisidine and 0.0005% hydrogen peroxide was then added, and MPO activity was measured at 460 nm wavelength over 6 minutes (Bio-Tek Instruments- μ Quant Microplate Spectrophotometer, Winooski, VT). MPO activity was calculated as optical density/minute per mg of lung tissue.

White blood counts

Whole blood was collected in tubes lined with EDTA to measure white blood cell counts. Blood was measured at three timepoints: a) prior to CLP or sham laparotomy, b) prior to MRSA or sham pneumonia, and c) 24 hours after MRSA or sham pneumonia. White blood count was performed on a HemaVet 950 hematology analyzer (Drew Scientific, Dallas, TX).

Lymphocyte apoptosis

Splenic lymphocyte apoptosis was quantified by both active caspase-3 and TUNEL assay using flow cytometry as previously described (21). Splenocytes were harvested 24 hours after MRSA or sham pneumonia. FITC-labeled antimouse CD3e (CD3e chain) and PE-Cy5-labeled CD45R/B220 (1:10; BD Pharmingen, Franklin Lakes, NJ) were used to identify T- and B-cell populations, respectively. Cells were fixed in 2% paraformaldehyde and permeabilized with 90% methanol. Apoptosis of individual cell populations was quantified using antibodies against active caspase-3 (1:100; Cell-Signaling Technology, Danvers, MA) and TUNEL (Phoenix Flow Apo-BrdU Kit, San Diego, CA). Flow cytometric analysis (50,000 events/sample) was performed after incubation in PE-labeled secondary antibody on FACScan (BD Biosciences; San Jose, CA) using gates set with appropriate controls.

Intestinal apoptosis and permeability

Intestinal epithelial apoptosis was identified and quantified in 100 well-oriented crypt-villus unit by both morphological and functional techniques (22). Nuclear fragmentation and cell shrinkage with condensed nuclei were used to identify morphological changes consistent with apoptosis in H&E-stained sections. Caspase-3 staining was used as a functional assessment of apoptosis (23). Paraffin-embedded sections were deparaffinized, rehydrated, and blocked for endogenous peroxidase activity by incubation in 3% H₂O₂ in methanol. Slides were placed in antigen decloaker solution (Biocare Medical) and heated for 45 minutes, blocked for 30 minutes with 20% goat serum (Vector Laboratories, Burlingame, CA) and then incubated with rabbit polyclonal anti-active caspase-3 (1:100 diluted in PBS; Cell Signaling Technology, Danvers, MA) overnight at 4°C. Sections were then incubated with goat anti-rabbit biotinylated secondary antibody (1:200 diluted in PBS; Vector Laboratories) for 30 minutes at room temperature and incubated for 30 minutes in Vectastain Elite avidin-biotin-peroxidase complex reagent (Vector Laboratories). Diaminobenzidine was used to develop sections and slides were counterstained with hematoxylin.

Permeability was measured *in vivo* to fluorescein isothiocyanate-dextran (FD-4, average molecular weight 4.4 kDa, Sigma, St. Louis, MO) (24). Mice were gavaged with 0.5 ml of 22 mg/ml FD4 in sterile PBS using a 20 gauge feeding needle 19 hours after MRSA or sham pneumonia. Five hours post-gavage, blood was collected and plasma was isolated by centrifuging for 20 minutes at 3,000 rpm. The concentration of FD4 in the plasma was determined by fluorospectrometry (NanoDrop 3300, Thermo Scientific, Wilmington, DE) with an excitation wavelength of 470 nm and an emission wavelength of 515 nm using serially diluted samples as standards.

Statistics

Two way comparisons were made using the Mann-Whitney test. Multiple group comparisons were made using one way ANOVA followed by Dunn's multiple comparison test. Survival studies were analyzed using the logrank test. All data were analyzed using the statistical software program Prism (GraphPad Software, San Diego, CA) and are presented as mean $\pm$ SEM. A p value less than 0.05 was considered to be statistically significant.

RESULTS

The combination of CLP followed by MRSA pneumonia leads to increased mortality compared to either insult in isolation

Mice were subjected to a) CLP followed 3 days later by sham pneumonia (CLP/saline), b) sham laparotomy followed 3 days later by MRSA pneumonia (sham/MRSA) or c) CLP followed 3 days later by MRSA pneumonia (CLP/MRSA). Both CLP and MRSA pneumonia injuries were nonlethal since CLP/saline and sham/MRSA mice had 100% survival. In contrast, only 67% of CLP/MRSA mice survived 7 days following pneumonia (Fig. 1A). To determine whether the length of time between CLP and the development of pneumonia impacted mortality, a different group of animals were subjected to either sham laparotomy followed 7 days later by MRSA pneumonia (sham/MRSA) or CLP followed 7 days later by MRSA pneumonia (CLP/MRSA). While sham/MRSA mice had 100% survival, 60% of CLP/MRSA mice survived 7 days following pneumonia (Fig. 1B). Since mortality was grossly similar regardless of whether MRSA was given 3 or 7 days following CLP, all subsequent experiments were performed giving MRSA or saline 3 days after CLP.

The combination of CLP followed by MRSA pneumonia causes decreased local bacterial clearance

BAL concentrations of MRSA were compared in mice that underwent sham/MRSA or CLP/MRSA. Mice subjected to CLP followed by MRSA pneumonia had higher colony counts of MRSA in BAL fluid than those subjected to sham laparotomy followed by MRSA pneumonia (Fig. 2). BAL cultures were not obtained in CLP/saline mice or sham/saline mice since there was no reason to believe animals subjected to sham pneumonia would have MRSA detectable in their BAL fluid. In contrast, there were no statistically significant differences between concentrations of MRSA detectable in the bloodstream of sham/MRSA and CLP/MRSA mice 6, 12 or 24 hours after induction of pneumonia ($p=0.42$, 0.13 , 1.0 for the timepoints respectively). Despite having 100% survival, there was a trend toward more bacteria being present in the blood of sham/MRSA mice at 6 and 12 hours (data not shown). MRSA was not detectable in blood cultures in either group 24 hours following MRSA pneumonia.

The combination of CLP followed by MRSA pneumonia blunts the local pulmonary inflammatory response compared to MRSA pneumonia alone

In order to determine if the decreased pulmonary clearance of MRSA was related to differences in cytokine production, BAL fluid was analyzed for several inflammatory mediators at 6, 12, and 24 hours after MRSA or sham pneumonia (Fig. 3). Sham/MRSA mice generally had marked increases in IL-6, G-CSF, TNF- α , and IL-1 β compared to animals given sham pneumonia regardless of whether they had CLP or sham laparotomy 3 days earlier independent of which timepoint was sampled. However, CLP/MRSA mice had a significantly blunted local inflammatory response compared to sham/MRSA mice with markedly diminished levels of IL-6 and G-CSF at 6 hours, all cytokines measured at 12 hours, and IL-6, G-CSF and TNF- α at 24 hours (Fig. 3). To determine whether heat-killed MRSA caused the same effect as live MRSA, an additional cohort of experiments was performed comparing IL-6, G-CSF, TNF- α , and IL-1 β in BAL fluid of mice given CLP/saline vs. mice given CLP/heat killed MRSA at 24 hours. No differences were identified for any cytokine (data not shown).

The combination of CLP followed by MRSA pneumonia does not alter the histologic severity of pulmonary pathology or lung edema compared to MRSA pneumonia alone

Sham/MRSA mice had a marked increase in pneumonia severity compared to animals given sham pneumonia regardless of whether they had CLP or sham laparotomy 3 days earlier (data not shown). However, the combination of CLP/MRSA did not have a synergistic effect, however, as there were no differences in either pneumonia severity or percent of lung tissue with inflammation between sham/MRSA or CLP/MRSA mice (Figure 4A, B). There were no differences in lung wet to dry weight ratio in any of the four experimental groups (Fig. 4C). Further, there was no difference in neutrophil infiltration as assayed by the MPO assay between sham/MRSA and CLP/MRSA mice (Fig. 4D). There were also no subjective difference in neutrophil or monocyte infiltration into lung tissue as judged by a pathologist blinded to sample identity between sham/MRSA and CLP/MRSA mice, nor was the location of infiltration (perivascular, peribronchial) different between the groups (data not shown).

The combination of CLP followed by MRSA pneumonia partially blunts the local peritoneal inflammatory response compared to CLP alone at 24 hours

Since animals undergoing CLP/MRSA have two local responses (first intraabdominal, second pulmonary), peritoneal cytokines were examined to determine if the abdominal cytokine response was similar to the pulmonary cytokine response. Peritoneal fluid was analyzed for the same inflammatory mediators at the same timepoints as above (Fig. 5).

CLP/saline mice yielded an increase in IL-6 and IL-1 β at 24 hours compared to animals given sham laparotomy regardless of whether they had MRSA pneumonia. In contrast, CLP/MRSA mice had a blunted local inflammatory response compared to CLP/saline mice with diminished levels of IL-6 and IL-1 β . Neither of these cytokines were affected at 6 or 12 hours. Neither G-CSF nor TNF- α were affected by the combination of CLP followed by MRSA pneumonia at any timepoint as levels of these cytokines were similar following CLP, regardless of whether an animal had MRSA or sham pneumonia (TNF- α levels were undetectable in all groups at 12 hours and were just above detection at 24 hours and are not shown).

The combination of CLP followed by MRSA pneumonia blunts the systemic inflammatory response compared to either CLP or MRSA pneumonia alone

To determine whether the systemic host response was similar to the local host responses, plasma cytokines were examined at 6, 12, or 24 hours following MRSA or sham pneumonia (Fig. 6). MRSA pneumonia alone (sham/MRSA) induced an upregulation of G-CSF, TNF- α and IL-6 at 12 and 24 hours. However, the combination of CLP followed by MRSA pneumonia blunted this response in G-CSF and TNF- α with a similar but not significant trend with IL-6. IL-1 β levels were also increased in sham/MRSA mice at 12 hours, which was blunted by the combination of CLP/MRSA. Cytokine levels were generally unaffected at 6 hours. To determine whether heat-killed MRSA caused the same effect as live MRSA, an additional cohort of experiments was performed comparing IL-6, G-CSF, TNF- α , and IL-1 β in plasma of mice given CLP/saline vs. mice given CLP/heat killed MRSA at 24 hours. No differences were identified for any cytokine (data not shown).

CLP or MRSA pneumonia each cause a decrease in white blood cell count, which is not affected by the combination of CLP followed by MRSA pneumonia

White blood cell (WBC) counts in sham/MRSA or CLP/MRSA mice were measured at 3 timepoints: a) prior to any manipulation (pre-sham laparotomy or CLP), b) three days after sham laparotomy or CLP, but just prior to MRSA pneumonia in order to determine the effects of CLP on the mice and the state of immune suppression prior to MRSA pneumonia, and c) 24 hours after MRSA pneumonia in order to study the response to MRSA inoculation and whether a pre-existing intraabdominal infection altered this response. Of note, WBC counts were not obtained in CLP/saline mice or sham/saline mice since there was no reason to believe that sham pneumonia would appreciably alter this parameter.

As might be expected, unmanipulated mice had similar WBC counts in the normal range, regardless of what injuries they would subsequently undergo (Fig. 7). Mice subjected to CLP had lower WBC counts than mice subjected to sham laparotomy three days after these insults. This was secondary to a decrease in absolute lymphocyte count which fell from 5600 ± 670 cells/ μ l in sham/MRSA mice to 2000 ± 310 cells/ μ l in CLP/MRSA mice ($p=0.0007$) without a change in absolute neutrophil count (1500 ± 350 cells/ μ l in sham/MRSA mice vs. 2200 ± 230 cells/ μ l, respectively, $p=0.2$). MRSA pneumonia also decreased WBC count 24 hours after induction of pneumonia in animals that had sham laparotomy three days earlier. However, MRSA pneumonia did not further diminish the WBC count in animals subjected to CLP three days earlier which also had similar absolute lymphocyte and neutrophil counts.

CLP causes increased splenic T- and B-lymphocyte apoptosis which is not affected by the combination of CLP followed by MRSA pneumonia

In light of the decreased total WBC count and absolute lymphocyte count seen above, splenic T- and B-lymphocyte apoptosis was quantitated using both active caspase-3 and TUNEL staining 24 hours after MRSA or sham pneumonia (Fig. 8). CLP alone induced an increase in both T- and B- lymphocyte apoptosis compared to either sham mice or those

with MRSA pneumonia. However, the addition of MRSA pneumonia three days after CLP did not further augment lymphocyte apoptosis as there were no significant differences in apoptosis levels between CLP/saline mice and CLP/MRSA mice.

Gut integrity is not affected by CLP, MRSA, or the combination of CLP followed by MRSA pneumonia

Gut integrity was assayed by both intestinal epithelial apoptosis and intestinal permeability. Neither gut apoptosis (Fig. 9A, B) nor permeability (Fig. 9C) was affected by CLP, MRSA pneumonia or the combination of CLP followed by MRSA pneumonia.

DISCUSSION

This study demonstrates that the combination of non-lethal peritonitis followed by non-lethal MRSA pneumonia causes increased mortality. This increased mortality is associated with decreased pulmonary bacterial clearance and a blunted local (BAL and peritoneal) and systemic cytokine response compared to either CLP or pneumonia alone. This two-hit model mimics a common clinical scenario in the surgical ICU where a patient has an intraabdominal infection and secondarily develops MRSA pneumonia (the most common cause of nosocomial pneumonia).

Our results expand upon an existing literature of two-hit models of critical illness, either of CLP followed by pneumonia (3;8–10) or sepsis associated with a traumatic or thermal injury (25–30). The fact that the combination of CLP followed by MRSA pneumonia increases mortality is similar to findings that CLP followed by either *Pseudomonas aeruginosa* or *Streptococcus pneumoniae* pneumonia 1–7 days later induces increased mortality, albeit at a variable rate (3;8;9). The finding that there is decreased pulmonary clearance of MRSA in CLP/MRSA mice compared to sham/MRSA mice is also consistent with published findings that pre-existing abdominal sepsis decreases pulmonary clearance of *Pseudomonas aeruginosa* from the lungs following induction of pneumonia. The finding that pneumonia severity and tissue infiltration of neutrophils and monocytes in CLP/MRSA mice and sham/MRSA mice was similar leads us to speculate that pulmonary bacterial clearance is different because cells that are recruited to the lung are dysfunctional. Of note, bacteria were not identified in this study in the bloodstream of any animal 24 hours after induction of MRSA pneumonia regardless of whether an animal had peritonitis three days earlier although bacteria were detectable at both 6 and 12 hours after induction of pneumonia. This is contrary to studies of CLP followed three to four days later by either *Pseudomonas aeruginosa* or *Streptococcus pneumoniae* which showed the organism that caused pneumonia was detectable in the bloodstream of all animals 24 hours after induction of pneumonia. This suggests that bacteria are cleared more rapidly in this model and that either mechanisms of mortality vary between two-hit models in part related to rapidity of bacterial clearance or that bacteremia is an epiphenomenon that has no impact on mortality.

A major finding of this study was the combination of CLP followed by MRSA pneumonia blunted the local cytokine response in both the lungs and the abdomen. These results suggest that a robust local inflammatory response is adaptive in the host's response to sepsis as both non-lethal CLP and MRSA pneumonia cause an upregulation of multiple cytokines in peritoneal fluid and BAL fluid respectively, but the more lethal combination of CLP followed by MRSA pneumonia was associated with a significant diminution of these cytokines in BAL fluid at all timepoints measured and to a lesser degree in peritoneal fluid at 24 hours. To the best of our knowledge, this is the first time the local cytokine response has been assayed following a two-hit model of CLP followed by pneumonia. The local findings are consistent with the finding that systemic cytokines are generally lower in CLP/MRSA mice at 12 and 24 hours than those subjected to a single septic insult alone.

suggesting that the lack of response following the combination of the two insults is maladaptive. We speculate that CLP induces local inflammatory factors that reach the lungs (either via the bloodstream or the mesenteric lymph) that are anti-inflammatory in nature which prevent an adaptive immune response to MRSA pneumonia. The exact nature and identification of these factors and/or how they downregulate pulmonary inflammation is outside the scope of this manuscript. This extends findings in two manuscripts by Muenzer et al that found no difference between animals subjected to CLP/saline and CLP/*Pseudomonas* except for an increase in the anti-inflammatory cytokine IL-10 in the latter group (3) as well as decreased IL-6 and MCP-1 compared to CLP or pneumonia in isolation (8). The reasons for these differences are unclear and may include (but are not limited to) factors related to the initiating microorganism (MRSA vs. *Pseudomonas*), the strain of mice used (FVB/N vs. ND4 mice), type of mice used (inbred vs. outbred), route of bacterial delivery (intratracheal vs. intranasal), and type of inhalational anesthesia used (halothane vs. isoflurane).

While the host inflammatory response may play a role in mediating mortality in mice subjected to CLP followed by MRSA pneumonia, this study identified a number of factors that do not appear to play a causative role in mortality induced by these two hits. First, while CLP induces T- and B- lymphocyte apoptosis, this was not augmented by MRSA pneumonia. This was surprising on two counts. First, MRSA pneumonia, in isolation, did not cause lymphocyte apoptosis. There is an extensive literature demonstrating that sepsis – including *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* pneumonia – induces lymphocyte apoptosis (31;32). While there is limited data on MRSA pneumonia inducing lymphocyte apoptosis, staphylococcal infections from other sources have been demonstrated to cause lymphocyte apoptosis (33). The fact that MRSA did not induce lymphocyte apoptosis may have been due to the fact that this was a relatively minor insult in isolation that caused neither mortality nor bacteremia or to the fact that mice are generally resistant to MRSA infections (17). The fact that there was no synergistic effect on lymphocyte apoptosis between CLP and MRSA when the combination of CLP and *Pseudomonas aeruginosa* pneumonia cause a disproportionate increase lymphocyte apoptosis over either insult in isolation (3;8) emphasizes the point that simply because mortality is increased in disparate models of sepsis does not mean it is mediated through similar mechanisms.

Another surprising result was the fact that neither intestinal apoptosis nor permeability was altered by CLP, MRSA or the combination. Our group and others have published multiple studies in both CLP and (non-MRSA) pneumonia demonstrating increases in sepsis-induced gut epithelial apoptosis and permeability (24;34;35). The finding that CLP did not induce detectable alterations in gut integrity may be due to the fact that the CLP model was not lethal in this study or that animals were examined four days after CLP (24 hours after MRSA or sham pneumonia) in this study while previous publications examined this 12–48 hours after the onset of sepsis. Alternatively, it is possible that MRSA simply does not alter intestinal integrity. Regardless of which explanation(s) is correct, the fact that there was no synergistic alteration in gut integrity following CLP/MRSA suggests the intestine does not have a significant role in mediating mortality from the combination of these two insults.

This study has a number of limitations. First, there are limitations inherent in our mouse model. While CLP is a widely used model of peritonitis, “source control” is not obtained since the infection is not drained. Source control is a mainstay of treatment of human sepsis, thus CLP is somewhat artificial by its very nature of allowing undrained infection to remain. Next, we are attempting to model the ICU scenario in which patients develop peritonitis and then develop nosocomial infection (frequently, ventilator associated pneumonia). Although MRSA can cause either hospital acquired pneumonia or ventilator associated pneumonia, the animals in this study were not intubated. Therefore, they did not have the changes in

local host defenses that patients have from having an endotracheal tube in place. Next, mice are highly resistant to infections with *Staphylococcus aureus* compared to humans secondary to the microorganism's preferential ability to take iron needed to proliferate from host hemoglobin in humans (36). Thus mice need to be given relatively high doses of MRSA in order to cause infection, and how this mimics disease in patients is unclear. Further, the data presented here do not clearly determine the mechanism that results in pre-existing peritonitis increasing the susceptibility to MRSA pneumonia. Additionally, outside of documenting lymphopenia, we did not examine immune function following CLP but prior to pneumonia so cannot definitively conclude that mice were immunocompromised prior to the induction of MRSA pneumonia. In addition, the results correlating blunted local and systemic host response with mortality are associative. Without further mechanistic studies, it is not possible to conclude that the decrease in cytokines seen in CLP/MRSA are responsible for the differences seen in mortality.

Despite these limitations, this is the first description of a clinically relevant model of intraabdominal sepsis followed by MRSA pneumonia, the most common cause of nosocomial pneumonia in the ICU. The abnormalities seen in the local and systemic response are distinct from other two-hit models of critical illness and suggest that initiating infections may induce mortality via different mechanisms. Further study is needed to understand whether the blunted inflammatory response is responsible for the mortality seen in this two-hit model.

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FIG. 1. Mortality in CLP/saline, sham/MRSA, CLP/MRSA mice

Mice were subjected to CLP or sham laparotomy, followed (a) three days later or (b) seven days later by MRSA pneumonia or sham pneumonia (saline). Animals were followed for survival for 7 days after sham or MRSA pneumonia. Both CLP/saline and sham/MRSA mice had 100% survival. A total of 67% of CLP/MRSA mice survived after the second insult when they were separated by three days ($n=10-33/\text{group}$, $p=0.02$), and a total of 60% of CLP/MRSA mice survived after the second insult when they were separated by seven days ($n=10/\text{group}$, $p=0.08$).

FIG. 2. MRSA concentration in BAL fluid in sham/MRSA, CLP/MRSA mice
Compared to sham operated mice that received MRSA pneumonia, mice that received CLP followed by MRSA pneumonia had increased MRSA in their BAL fluid 24 hours after induction of pneumonia. Data is log transformed for presentation ($p=0.04$, $n=5/\text{group}$).

FIG. 3. BAL cytokines in sham/MRSA, CLP/saline, CLP/MRSA mice

Mice that received sham laparotomy followed by MRSA pneumonia generally had increases in all cytokines measured compared to mice that received sham pneumonia regardless of whether they first received sham laparotomy or CLP. In contrast, mice that underwent CLP/MRSA generally had lower cytokine levels than those that underwent sham/MRSA 6, 12 or 24 hours earlier (n=4-11/group). *p<0.05 compared to sham/saline, CLP/saline. +p<0.05 compared to sham/MRSA.

FIG. 4. Pneumonia pathology, lung wet to dry ratio and MPO activity in sham/MRSA, CLP/saline, CLP/MRSA mice

The addition of CLP prior to MRSA pneumonia did not alter either pneumonia severity (A) or percent of lung with inflammation (B) compared to animals that received sham laparotomy prior to MRSA pneumonia (n=7–12/group). (C) No differences in wet to dry ratio were noted in any group regardless of whether they underwent CLP or MRSA pneumonia (n=4–8/group). (D) The addition of CLP prior to MRSA pneumonia did not alter MPO activity in the lungs compared to MRSA pneumonia alone (n=4–5/group, all analysis performed on samples collected 24 hours after MRSA or sham pneumonia).

FIG. 5. Peritoneal cytokines in sham/MRSA, CLP/saline, CLP/MRSA mice
 IL-6, IL-1 β and G-CSF were generally unaffected by MRSA, CLP or the combination at 6 or 12 hours after MRSA or sham pneumonia. At 24 hours, mice that received CLP followed by sham pneumonia had increases in IL-6 and IL-1 β , compared to mice that received sham laparotomy regardless of whether they subsequently received sham or MRSA pneumonia. In contrast, mice that underwent CLP/MRSA had lower IL-6 and IL-1 β levels than those that underwent CLP/saline * $p < 0.05$ compared to CLP/saline (n=4–8/group for all cytokines).

FIG. 6. Plasma cytokines in sham/MRSA, CLP/saline, CLP/MRSA mice

Mice that received MRSA pneumonia had increased in G-CSF, TNF- α , IL-6 and IL-1 β at 12 hours compared to mice that received sham pneumonia regardless of whether they had CLP or sham laparotomy 3 days earlier. This response was blunted for TNF- α and IL-1 β in mice that underwent CLP/MRSA. Similar results were seen at 24 hours in G-CSF and TNF- α . Cytokine levels were generally unaffected at 6 hours. * $p < 0.05$ compared to sham/MRSA (n=4–19/group for all cytokines)

FIG. 7. WBC counts in sham/MRSA and CLP/MRSA mice

Mice had similar WBC counts prior to any intervention. Mice subjected to CLP 3 days earlier (prior to MRSA pneumonia) had lower WBC counts compared to mice subjected to sham laparotomy. The induction of MRSA pneumonia 24 hours earlier also decreased WBC count compared to counts prior to the onset of pneumonia. However, the combination of CLP/MRSA did not cause a synergistic decrease in WBC count. * $p < 0.05$, $n = 5-10$ /group).

FIG. 8. Lymphocyte apoptosis in sham/MRSA, CLP/saline, CLP/MRSA mice

T- (A,B) and B- (C,D) lymphocyte apoptosis was increased in CLP/saline mice compared to those that underwent sham laparotomy regardless of whether they subsequently had MRSA pneumonia. Apoptosis was increased regardless of whether it was assayed by caspase-3 staining (A,C) or the TUNEL assay (B,D). The combination of CLP/MRSA did not increase lymphocyte apoptosis compared to CLP/sham alone. * $p < 0.05$ compared to sham/saline, sham/MRSA ($n = 4$ /group, all samples collected 24 hours after MRSA or sham pneumonia).

FIG. 9. Gut integrity in sham/MRSA, CLP/saline, CLP/MRSA mice

No significant differences were identified in either gut epithelial apoptosis (A,B, n=8–12/group) or permeability (C, n=4–8/group) regardless of whether mice underwent CLP, MRSA pneumonia or CLP/MRSA (all samples collected 24 hours after MRSA or sham pneumonia).