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RESEARCH ARTICLE

Inhibition of LpxC Protects Mice from Resistant *Acinetobacter baumannii* by Modulating Inflammation and Enhancing Phagocytosis

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ABSTRACT New treatments are needed for extensively drug-resistant (XDR) Gram-negative bacilli (GNB), such as *Acinetobacter baumannii*. Toll-like receptor 4 (TLR4) was previously reported to enhance bacterial clearance of GNB, including *A. baumannii*. However, here we have shown that 100% of wild-type mice versus 0% of TLR4-deficient mice died of septic shock due to *A. baumannii* infection, despite having similar tissue bacterial burdens. The strain lipopolysaccharide (LPS) content and TLR4 activation by extracted LPS did not correlate with *in vivo* virulence, nor did colistin resistance due to LPS phosphoethanolamine modification. However, more-virulent strains shed more LPS during growth than less-virulent strains, resulting in enhanced TLR4 activation. Due to the role of LPS in *A. baumannii* virulence, an LpxC inhibitor (which affects lipid A biosynthesis) antibiotic was tested. The LpxC inhibitor did not inhibit growth of the bacterium (MIC > 512 μg/ml) but suppressed *A. baumannii* LPS-mediated activation of TLR4. Treatment of infected mice with the LpxC inhibitor enhanced clearance of the bacteria by enhancing opsonophagocytic killing, reduced serum LPS concentrations and inflammation, and completely protected the mice from lethal infection. These results identify a previously unappreciated potential for the new class of LpxC inhibitor antibiotics to treat XDR *A. baumannii* infections. Furthermore, they have far-reaching implications for pathogenesis and treatment of infections caused by GNB and for the discovery of novel antibiotics not detected by standard *in vitro* screens.

IMPORTANCE Novel treatments are needed for infections caused by *Acinetobacter baumannii*, a Gram-negative bacterium that is extremely antibiotic resistant. The current study was undertaken to understand the immunopathogenesis of these infections, as a basis for defining novel treatments. The primary strain characteristic that differentiated virulent from less-virulent strains was shedding of Gram-negative lipopolysaccharide (LPS) during growth. A novel class of antibiotics, called LpxC inhibitors, block LPS synthesis, but these drugs do not demonstrate the ability to kill *A. baumannii* in vitro. We found that an LpxC inhibitor blocked the ability of bacteria to activate the sepsis cascade, enhanced opsonophagocytic killing of the bacteria, and protected mice from lethal infection. Thus, an entire new class of antibiotics which is already in development has heretofore-unrecognized potential to treat *A. baumannii* infections. Furthermore, standard antibiotic screens based on *in vitro* killing failed to detect this treatment potential of LpxC inhibitors for *A. baumannii* infections.

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Toll-like receptor 4 (TLR4) is an archetypal pattern recognition receptor for lipopolysaccharide (LPS) from Gram-negative bacilli (GNB) (1–3). In the absence of completely functional TLR4, both mice and humans are more susceptible to lethal infection caused by a broad array of pathogenic GNB, including enteric commensal organisms (e.g., *Klebsiella pneumoniae* and *Escherichia coli*), highly virulent nonenteric members of the *Enterobacteriaceae* (e.g., *Salmonella*), community Gram-negative pathogens (e.g., *Neisseria* and *Haemophilus*), and nonfermenting GNB that cause lethal nosocomial infections (e.g., *Pseudomonas*) (3–11).

Acinetobacter baumannii is a GNB that has emerged as one of the most common and highly antibiotic-resistant nosocomial pathogens in the United States and throughout the world (12–14). The majority of such infections are now extensively drug resistant (XDR) (i.e., resistant to carbapenems and all other antibiotics except colistin or tigecycline) (15–22), and they are increasingly nonsusceptible even to both colistin and tigecycline (12, 23–29). Such pandrug-resistant (PDR) *A. baumannii* infections are resistant to every U.S. Food and Drug Administration-approved antibiotic and are hence untreatable. Indeed, *A. baumannii* is one of the few bacterial pathogens that have become resistant to all available antibiotics.

With rising rates of resistance, *A. baumannii* infections threaten to become progressively more lethal. In a recent study of 13,796 patients in 1,265 intensive care units (ICUs) from 75 countries, *A. baumannii* was 1 of only 2 of the 19 microorganisms evaluated which were strongly linked ($P < 0.01$) to increased hospital mortality by multivariate logistic regression (30). Furthermore, the odds ratio for in-hospital mortality of *A. baumannii* infections was 1.53, the highest for all GNB and in the top three among all organisms. Infections caused by carbapenem-resistant, XDR *A. baumannii* are associated with longer hospitalization, greater health care costs, and higher mortality versus infections caused by carbapenem-susceptible strains (12, 19, 21, 24, 31–35). Bacteremia with sepsis syndrome is a common clinical syndrome in patients with these infections, and bloodstream infections caused by XDR *A. baumannii* caused >50 to 60% mortality rates (31 33, 34 36–38). Given their extreme resistance, rising frequency, and high mortality rates, defining fundamental host-pathogen interaction mechanisms for *A. baumannii* infections is critical to future development of novel small-molecule and biological inhibitors of disease.

A. baumannii expresses immune-reactive LPS on its cell surface (39). LPS from *A. baumannii* induces macrophage release of tumor necrosis factor (TNF) and interleukin 8 (IL-8) in a TLR4-dependent manner (40). *In vivo*, TLR4-deficient mice did not mediate an inflammatory response to intranasal *A. baumannii* LPS (41). Furthermore, TLR4-deficient mice had slower clearance of *A. baumannii* from lung parenchyma (41). Thus, the contemporary understanding maintains that *A. baumannii* LPS-induced signaling of TLR4 was critical for protecting the host against infection, as is true of many other GNB. However, the *in vivo* model used in this previous study was nonlethal, and the outcome measured was slower clearance of bacilli.

The current study defines the role of innate immune mechanisms and LPS stimulation during lethal *A. baumannii* infections. Surprisingly, TLR4-mutant mice were not susceptible to and were instead highly resistant to lethal infection caused by *A. baumannii*. The distinguishing characteristic of more- or less-virulent *A. baumannii* strains was the TLR4-stimulating activity of LPS shed during growth, rather than the content of LPS per bacillus or the intrinsic potency of TLR4-stimulating activity of extracted LPS. Finally, small-molecule antibiotic inhibition of LPS synthesis decreased TLR4 activation and protected mice from lethal infection even though the antibiotic did not kill the bacteria. These results have fundamental implications for pathogenesis of infections caused by GNB and for the discovery of novel therapeutics that are not detected in standard *in vitro* antibiotic screens and suggest new treatment strategies for XDR/PDR GNB infections.

RESULTS

***A. baumannii*-infected wild-type mice died of septic shock that did not occur in TLR4-mutant mice.** To determine the impact of TLR4 deficiency on survival, C3H/FeJ (wild-type) and C3H/HeJ (TLR4-defective mutant) mice were infected via the tail vein with a highly virulent strain of XDR *A. baumannii*, HUMC1 (42), or a second clinical isolate (and type strain), ATCC 17978, that is less virulent in mice (42). HUMC1 induced 100% fatal infection in wild-type mice but no mortality in TLR4-mutant mice (Fig. 1). ATCC 17978 was nonlethal in both mouse strains (Fig. 1). The same phenomenon was observed in TLR4-knockout (KO) mice and congenic C57BL/6 wild-type controls infected with HUMC1 (Fig. 1).

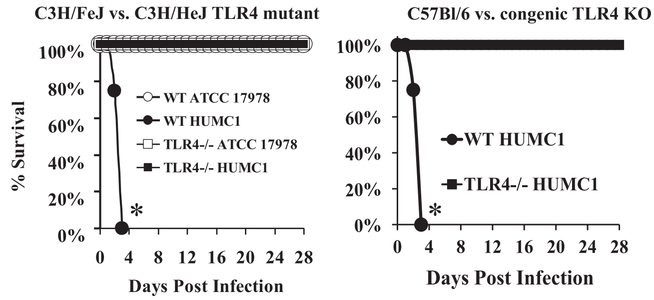

FIG 1 TLR4 is antiprotective against *A. baumannii* bloodstream infection. C3H/FeJ wild-type or C3H/HeJ TLR4-mutant mice ($n = 10$ mice per group, except for 9 mice in the wild-type HUMC1-infected group) or C57BL/6 or congenic TLR4-knockout (KO) mice were infected via the tail vein with 2×10^7 bacteria and followed for 28 days. All remaining mice at day 28 appeared clinically well. *, $P < 0.05$ versus results for all other groups.

Additional mice were infected to quantify sepsis biomarkers. The initial experiments demonstrated that a substantial number of control mice would begin to die on day 2 postinfection, making it impossible to accurately measure sepsis biomarkers after day 2. Prior to and on days 1 and 2 postinfection, rectal temperatures were measured between 8 and 9 AM using a digital thermometer (Physitemp, model BAT-12). Compared to the baseline, wild-type mice infected with *A. baumannii* HUMC1 became profoundly hypothermic during the first 2 days of infection, while TLR4-mutant mice maintained normal body temperatures (Fig. 2A). At day 2 postinfection (the day the control mice were anticipated to begin dying), wild-type mice infected with HUMC1 became profoundly acidemic (Fig. 2B) and had substantially higher levels of the serum proinflammatory cytokines, TNF and IL-6, and the counterregulatory, suppressive cytokine, IL-10, than did TLR4-mutant mice (Fig. 2C). In both mouse strains, *A. baumannii* ATCC 17978 induced lower levels of these cytokines, as well as gamma interferon (IFN-γ), than did HUMC1 (Fig. 2C).

Despite marked differences in sepsis biomarkers and survival, there were surprisingly small differences in tissue bacterial burdens during infection between wild-type and TLR4-mutant mice (Fig. 2D) *A. baumannii* HUMC1 infection resulted in trends to higher blood and tissue bacterial burdens in wild-type versus TLR4-mutant mice, but none of the differences were statistically significant. The lower-virulence *A. baumannii* strain ATCC 17978 resulted in lower bacterial burdens in blood and tissue than did HUMC1, but the ATCC 17978 bacterial burden was similar in wild-type versus TLR4-mutant mice. Thus, the difference in severity of infection between wild-type and TLR4-mutant mice was not related to alterations in clearance of the bacterial pathogen.

Histopathology revealed relatively normal parenchymal organs. To determine if lethal infection was the result of differences in organ invasion in HUMC1-infected mice, histopathology was performed. Surprisingly, all parenchymal organs evaluated from wild-type or TLR4-mutant mice infected with either HUMC1 or ATCC 17978 had preserved, normal architecture with no evidence of bacterial invasion or a host response to infection within the organs (Fig. 3A). In spleens of all groups, neutrophil influx into the perifollicular red pulp was seen (Fig 3A). In the lungs, no

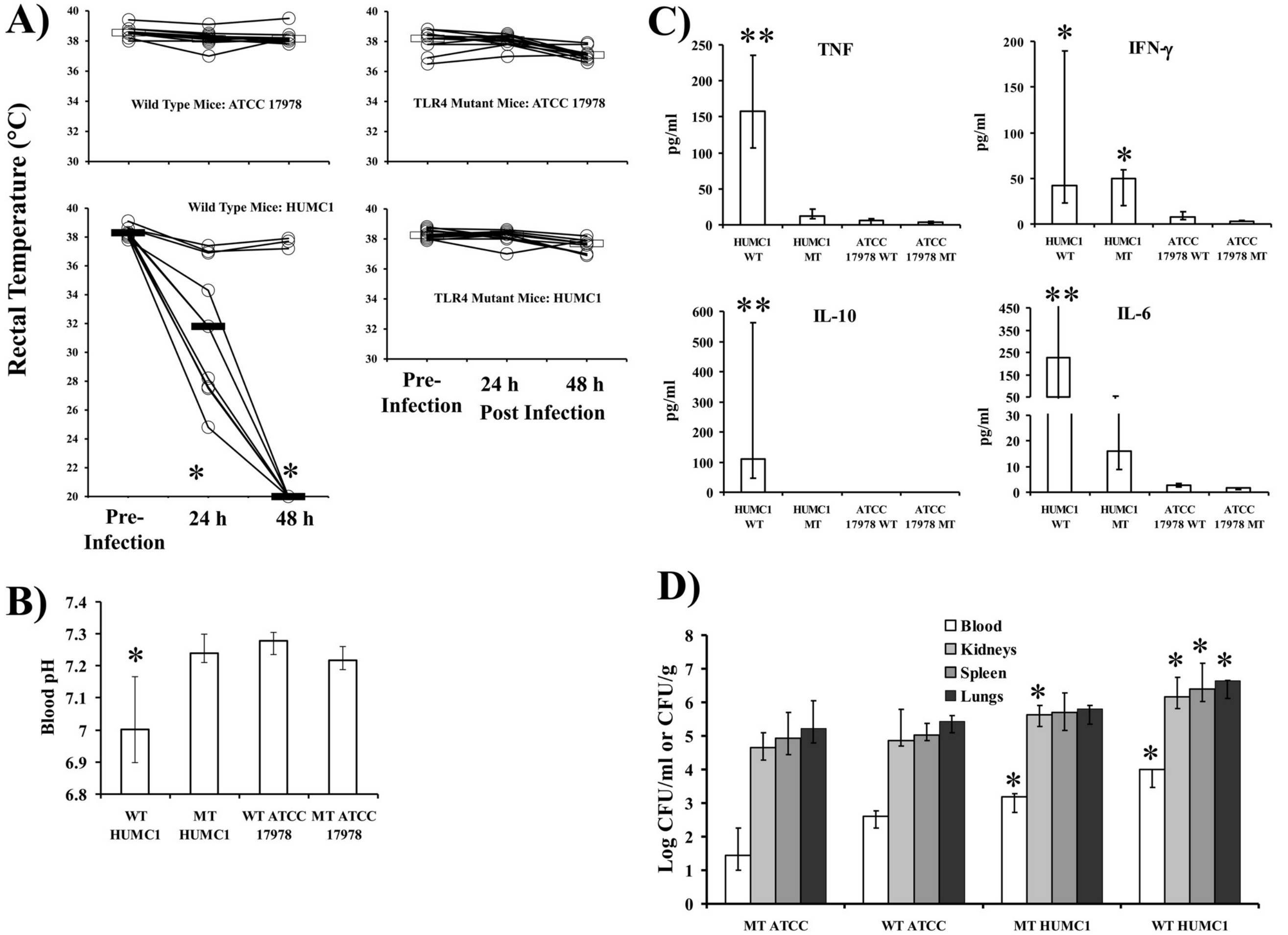

FIG 2 Lethally infected wild-type mice had septic shock, whereas TLR4-mutant mice did not. (A) Rectal temperatures taken from mice infected with *A. baumannii* HUMC1 or ATCC 17978 (the same mice in Fig. 1; $n = 10$ mice per group, except for 9 in the wild-type HUMC1-infected group). Temperatures were taken at the same time (8 to 9 AM) daily before and for 2 days after infection (wild-type mice began dying on day 2). *, $P < 0.05$ versus preinfection findings. (B) Blood pH at 48 h of infection was measured by using i-STAT cartridges from separate C3H/FeJ or C3H/HeJ (TLR4-mutant) mice infected with 2×10^7 *A. baumannii* HUMC1 or ATCC 17978 bacteria ($n = 16$ mice per group from 2 experiments, except for 8 mice for HUMC1-infected wild-type mice). *, $P < 0.05$ versus results for other groups. (C) Serum inflammatory and anti-inflammatory cytokine levels were measured at 48 h of infection in C3H/FeJ or C3H/HeJ (TLR4-mutant) mice. $n = 8$ mice per group. **, $P < 0.05$ versus results for all other groups; *, $P < 0.05$ versus results for ATCC 17978 groups. (D) Blood and tissue bacterial burden at 48 h of infection differed between mice infected with 2×10^7 HUMC1 and ATCC 17978 bacteria ($n = 12$ mice per group from 2 experiments) but not between mutant and wild-type mice. *, $P < 0.05$ versus results for mice infected with ATCC 17978. Median and interquartile ranges are graphed.

alveolar infiltrates were seen, but neutrophils were found in the capillaries, consistent with capillaritis typical of LPS-induced sepsis syndrome (Fig. 3A). The kidneys appeared histologically normal. The histopathological findings were remarkably similar to those seen in tissues that had been fixed during HUMC1 infection in diabetic BALB/c mice from experiments previously reported (42).

Immunohistochemistry was performed to localize the bacteria in the organs. Consistent with the finding of extensive neutrophil influx into the perifollicular red pulp, mice had extensive bacterial infiltration localized to the perifollicular red pulp of the spleen (Fig. 3B). Splenic lymph node follicles were spared. In the kidneys, scattered bacterial influx was found localized to capillaries surrounding renal tubules (Fig. 3B). Similarly, in the lung, bacteria were found in interstitial capillaries (Fig. 3B). In none of the organs was bacterial invasion into the parenchymal tissues found. Thus, *A. baumannii* did not appear to be capable of invasion of parenchymal organs during systemic infection.

LPS shedding distinguished more-virulent from less-virulent strains of *A. baumannii*. The protection against lethal infection afforded by mutant TLR4 and the localization of bacteria to capillaries consistent with sepsis syndrome implicated LPS as a primary pathogenesis factor of *A. baumannii*. A panel of clinical isolates of *A. baumannii* (Table 1) was compared for in vivo virulence so that more- and less-virulent strains could be compared for LPS bioactivity. Pilot studies demonstrated that five XDR clinical isolates (HUMC4, -5, -6, and -12) (Table 1) (42) were avirulent at the 2×10^7 inoculum at which the HUMC1 strain was 100% fatal. Thus, a higher inoculum (5×10^7) was tested, at which HUMC4, -5, -6, and -12 caused 100% fatal infection within 4 days, while ATCC 17978 remained nonlethal (Fig. 4A). Two colistin-resistant clinical isolates with defined LPS mutations causing

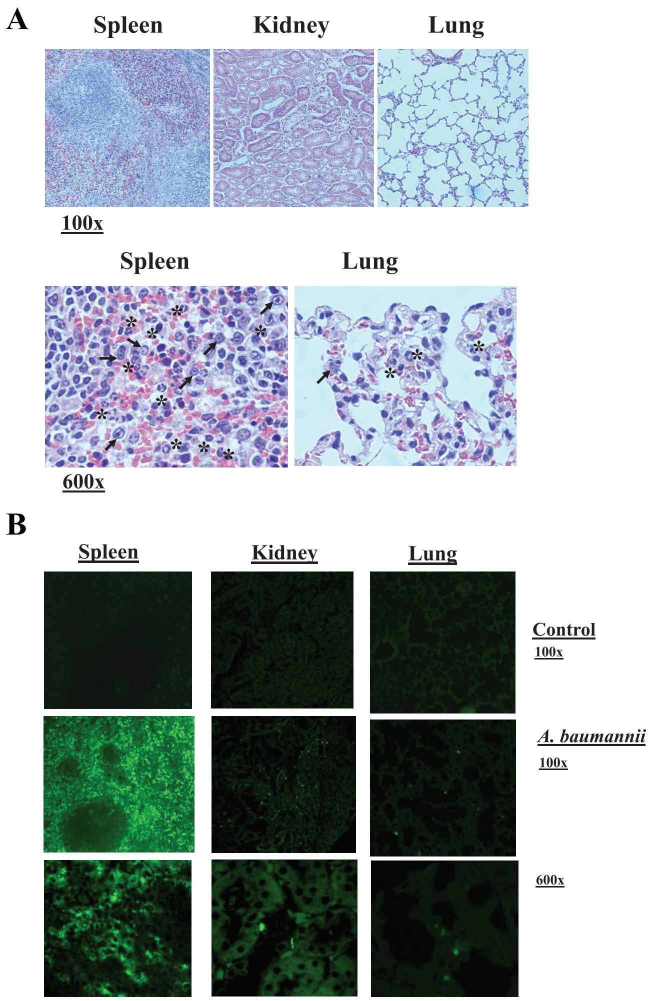

FIG 3 Histopathology and immunohistochemistry of *A. baumannii* during infection in mice. (A) Histopathology of spleens, lungs, and kidneys from C3H/FeJ mice given lethal infection with *A. baumannii* HUMC1 (2×10^7 inoculum) demonstrated normal parenchymal anatomy, with no evidence of bacterial invasion (100× power shown). At higher power (600×), the only abnormalities found were accumulation of neutrophils (asterisks), including pyknotic neutrophils (arrows) undergoing nucleolysis consistent with apoptosis, in the splenic perifollicular red pulp area and in the pulmonary capillaries, consistent with Gram-negative LPS-induced sepsis. The kidney appeared histopathologically normal at higher power (not shown). (B) Immunohistochemistry was used to localize *A. baumannii* in parenchymal organs. In the spleen, the bacteria accumulated in the perifollicular red pulp areas and spared lymph node follicles (note green spots surrounding dark follicles). In the kidneys, the organisms were found scatted in capillaries surrounding renal tubules, and there was no evidence of parenchymal organ invasion. In the lung, the organisms were found in capillaries in the interstitium, and again there was no evidence of alveolar or parenchymal invasion from the capillaries. The control was stained with normal mouse serum as the primary antibody.

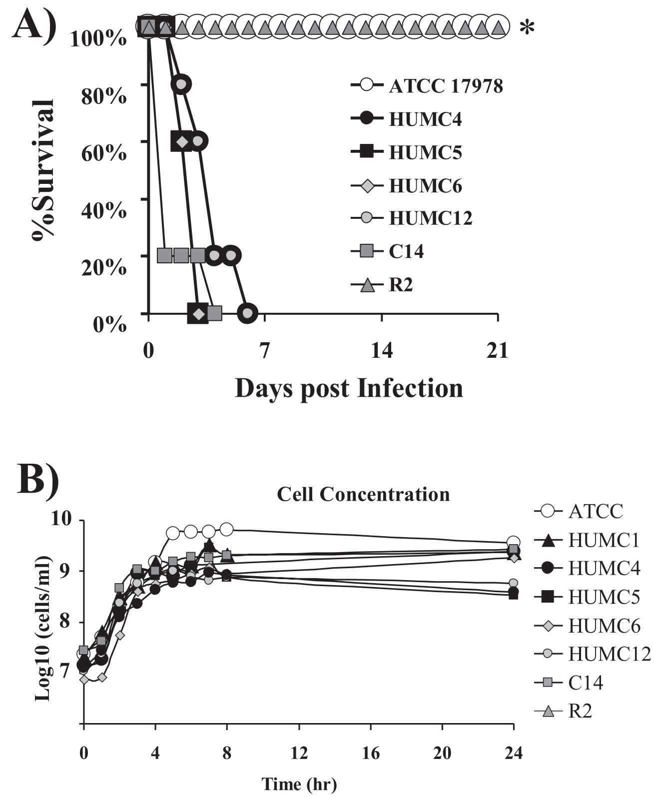

FIG 4 Various virulences of clinical isolates, including colistin-resistant strains. (A) C3H/FeJ mice were infected iv with 5×10^7 HUMC4, -5, -6, and -12 and R2 and C14 bacteria, all of which caused 100% mortality ($n = 5$ to 8 mice per group). ATCC 17978 and R2 were avirulent. (B) *In vitro* growth rates did not differ substantially among strains irrespective of virulence *in vivo*.

changes to lipid A (R2 and C14) (Table 1) (43), were tested as well. The colistin-resistant C14 ($pmrB^{\Delta 160}$) clinical isolate, isolated from the wound of a Brazilian patient, caused rapid, lethal infection. In contrast, the other colistin-resistant strain, R2 ($pmrB^{T235I}$), caused no mortality (Fig. 4A). Growth curves demonstrated similar growth rates for all of these strains (Fig. 4B), so growth rates did not account for differences in virulence.

Total LPS content (ng LPS/bacillus) was found to be similar when comparing strains with high versus low virulence (e.g., HUMC1 versus ATCC 17978 versus R2) (Fig. 5A). Thus, there was no apparent relationship between LPS density and strain virulence *in vivo*. LPS extracted from each strain was tested for intrinsic TLR4-activating potency using HEK-Blue cells transfected with a TLR4-linked colorimetric reporter gene. The highest TLR4 acti-

TABLE 1 Strains used[a]

Strain	Source and comments (reference)
ATCC 17978	Isolated from cerebral spinal fluid in 1951 from a 4-mo-old with fatal meningitis (61)
HUMC1	Blood and sputum clinical isolate
HUMC4	Deep endotracheal aspirate clinical isolate
HUMC5	Bronchoalveolar lavage clinical isolate
HUMC6	Sputum clinical isolate
HUMC12	Wound infection clinical isolate
R2	Laboratory-derived colistin-resistant mutant derived by serial passage of ATCC 1798 in colistin; $pmrB^{T235I}$
C14	Polymyxin-resistant clinical isolate

[a] HUMC strains are described in reference 42; R2 and C14 are described in reference 43.

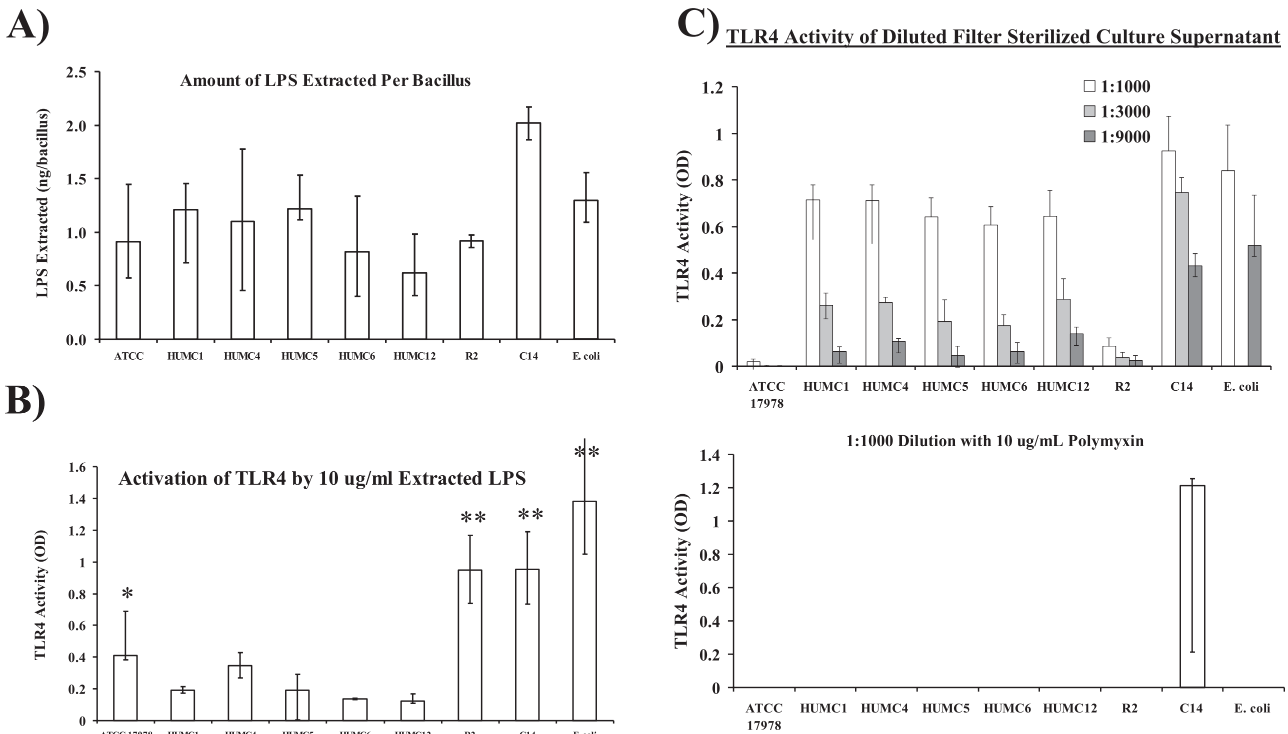

FIG 5 *In vitro* correlate of *in vivo* virulence. (A) LPS content (ng/bacillus) was similar across strains of highly varying virulences (e.g., HUMC1 versus ATCC 17978 versus R2). Results are from at least two separate extractions, each done in duplicate. (B) TLR4-activating potency of extracted LPS was higher for R2 and C14 than all other *A. baumannii* strains (**, $P < 0.05$ versus results for all other strains except *E. coli*). Of the colistin-susceptible *A. baumannii* strains, TLR4-activating potency was highest among *A. baumannii* ATCC 17978, which was avirulent (*, $P < 0.05$ versus HUMC strains). Results are from a minimum of two assays per strain, each done in duplicate. (C) Filter-sterilized culture supernatant induced a much stronger TLR4 signal from strains that caused lethal infections *in vivo* than from avirulent strains. Also, addition of polymyxin blocked the TLR4 activation from all strains except those highly resistant to colistin (C8 and C14), which were not affected by polymyxin. Results are from a minimum of three assays per strain, each done in duplicate. For all panels, median and interquartile ranges are graphed.

vation in extracted LPS was found in the C14 colistin-resistant strain of *A. baumannii*, which had activity comparable to that of LPS extracted from *E. coli* (Fig. 5B). Of the colistin-susceptible strains, the highest TLR4-activating potency was found in LPS extracted from the least-virulent strain, ATCC 17978 (Fig. 5B). Intriguingly, the other hypovirulent strain, R2, which has a single point mutation in the regulatory gene *pmrB* that leads to upregulation of *pmrC* and decoration of lipid A of LPS by phosphoethanolamine (43), led to a nearly 2-fold increase in LPS proinflammatory potency, to a level almost the same as that of C14, which also had upregulated *pmrC*. Thus, intrinsic TLR4-activating potency of LPS extracted from each strain appeared to be related to the dysregulation of *pmrC* that caused polymyxin resistance, but it did not correlate with virulence.

Since LPS density per bacillus and TLR4-activating potency of extracted LPS did not correlate with *in vivo* virulence, shedding of LPS during growth was analyzed. Culture supernatants from cells at the mid-logarithmic phase of growth were filter sterilized (confirmed by no growth in the cultured filtrate) and tested in the TLR4 activation assay. Culture supernatants from the most virulent strains, HUMC1 and C14, were by far the most potent at inducing TLR4 activation and came the closest to the potency of *E. coli* culture supernatant (Fig. 5C). Boiling of the supernatants or exposure to proteinase K did not alter TLR4 activation (data not shown), implicating a heat-stable, nonproteinaceous inflammatory inducer (such as LPS). Furthermore, polymyxin abrogated TLR4 activation by the supernatants of all strains except the colistin-resistant strain, C14 (the LPS of which, it being a polymyxin-resistant isolate, would not bind well to polymyxin), confirming that TLR4 activation was due to LPS. Finally, the limulus amebocyte lysate assay confirmed that despite the substantial intrinsic TLR4-activating potency of their extracted LPS, the LPS activity in culture filtrates from ATCC 17978 and R2 was lower than that from the other strains, consistent with their reduced virulence.

Inhibition of LPS biosynthesis did not kill *A. baumannii* but enhanced opsonophagocytosis and decreased inflammation, resulting in protection of mice from lethal infection. Small-molecule inhibitors of a key enzyme, LpxC, involved in the first committed step in the synthesis of LPS lipid A, are in advanced preclinical development as antibiotics (44, 45). These agents have broad activity against GNB, but most do not have *in vitro* killing activity against *A. baumannii* and therefore have been assumed to be incapable of treating *A. baumannii* infections and have not been studied for this purpose. Nevertheless, based on the correlation between LPS shedding and strain virulence, an investigational LpxC inhibitor that is in advanced preclinical development, LpxC-1, was obtained for testing. LpxC-1 had no detectable MIC when tested against the HUMC *A. baumannii* strains at concentrations up to 512 μg/ml. Nevertheless, overnight growth followed by a 3-h passage of the *A. baumannii* strains in the presence of 4 μg/ml of LpxC-1 resulted in diminished TLR4-activating potency

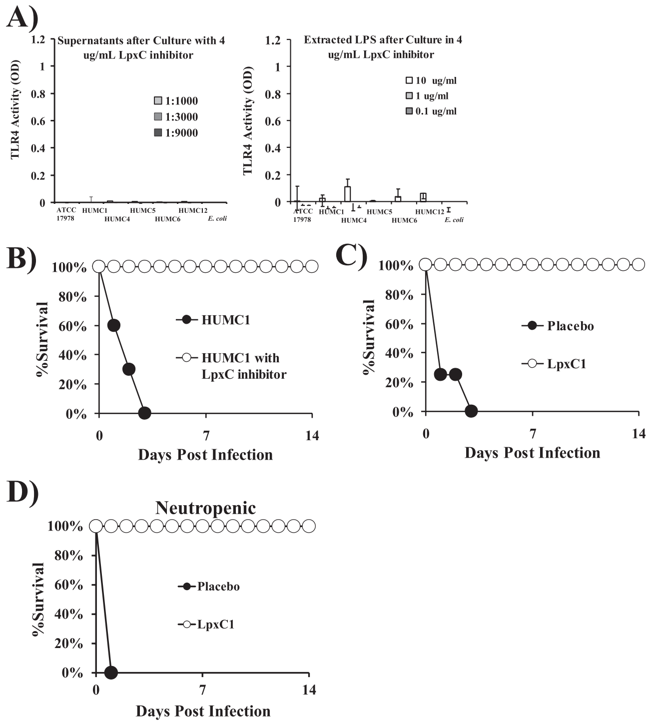

FIG 6 Inhibition of LPS biosynthesis with an inhibitor of LpxC blocked TLR4 activation *in vitro* and abrogated virulence *in vivo*. (A) TLR4-activating potency of filtered culture supernatant and extracted LPS from *A. baumannii* strains passaged to log phase in the presence of 4 μg/ml of LpxC inhibitor (LpxC-1). The results with LpxC-1 were run concurrently with those without LpxC-1 (compare signal with and without LpxC-1 inhibitor in Fig. 5C versus Fig. 6A). (B) Survival of wild-type C3H/FeJ mice ($n = 10$ per group) which were either infected with normal *A. baumannii* HUMC1 and treated with a placebo (40% cyclodextrin in water i.v. once daily) for 3 days starting on the day of infection or infected with *A. baumannii* HUMC1 that was cultured overnight and during log passage in the presence of 4 μg/ml of LpxC inhibitor and treated with LpxC-1 (100 mg/kg in 40% cyclodextrin i.v.) for 3 days postinfection. (C) Survival of wild-type C3H/FeJ mice ($n = 10$ per group) which were infected with *A. baumannii* HUMC1 and treated with a placebo (40% cyclodextrin in water) or LpxC-1 (100 mg/kg in 40% cyclodextrin i.v.) starting 1 h after infection and for 3 days postinfection. (D) Survival of BALB/c mice ($n = 11$ in the placebo group; $n = 10$ in the LpxC1-treated group) made neutropenic with cyclophosphamide, infected with *A. baumannii* HUMC1, and treated with placebo or LpxC-1 starting after infection and for 3 days postinfection.

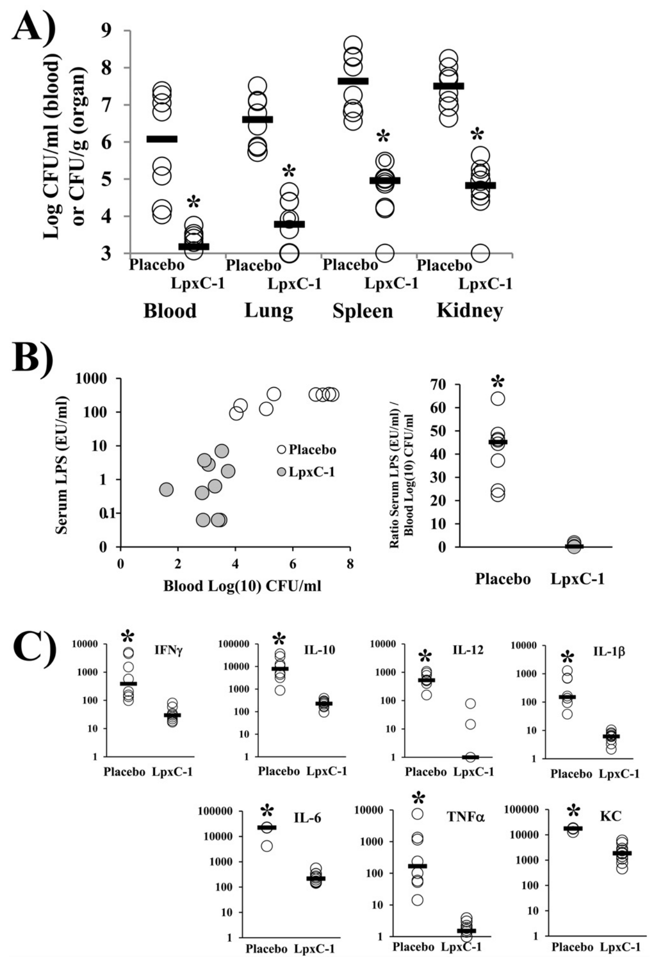

FIG 7 Bacterial densities in blood and tissue and serum LPS and cytokine concentrations for mice treated with LpxC-1 or a placebo. C3H/FeJ mice ($n = 15$ per group) were infected with *A. baumannii* HUMC1. At 1 h and 24 h, infected mice were treated i.v. with LpxC-1 (100 mg/kg). Five control mice died before the 24-h time point; no treated mice died. (A) Bacterial densities in blood and tissue for treated versus control mice. (B) Serum LPS levels for treated versus control mice. (C) Serum cytokine levels for treated versus control mice. *, $P < 0.01$ versus results for the control.

of the culture supernatant as well as that of LPS extracted from the bacilli (Fig. 6A).

Exposure of bacteria to LpxC-1 prior to tail vein infection in mice, followed by treatment of the mice with LpxC-1, completely protected them from lethal infection (Fig. 6B). Subsequently, mice were infected with *A. baumannii* HUMC1 grown overnight and passaged to log phase without exposure to LpxC-1, and treatment with LpxC-1 was initiated after infection. Treatment of established infection was also completely protective (Fig. 6C). To determine the efficacy of LpxC-1 in a compromised host model, neutropenic mice were infected with *A. baumannii* and treated with LpxC-1 or the placebo. LpxC-1 markedly improved survival of neutropenic mice (Fig. 6D).

The impact of LpxC-1 on bacterial density and cytokine production in C3H/FeJ mice was determined. Infected mice were treated with LpxC-1 and euthanized at 24 or 48 h postinfection to harvest blood and organs. The infection was substantially lethal, resulting in deaths of 5 of the 15 control mice before the 24-h time point. No LpxC-1-treated mice died. At 24 h, LpxC-1-treated mice had a 500-fold reduction in median blood and tissue bacterial densities (Fig. 7A), as well as a median 600-fold reduction in serum LPS (Fig. 7B) and 10- to 100-fold reductions in median levels of serum proinflammatory cytokines (e.g., IFN-γ, IL-12, IL-1β, IL-6, TNF-α, and KC) and the anti-inflammatory cytokine (IL-10) (Fig. 7C). By 48 h, only 2 control mice were alive, but similar trends were seen in the differences between bacterial density and serum LPS and cytokine levels (data not shown). Blood bacterial density and serum LPS concentrations correlated for placebo-treated mice ($P = 0.008$) but not for LpxC-1-treated mice (Fig. 7B). Also, placebo-treated mice had a 90-fold-higher median ratio of serum LPS concentration to blood bacterial density than did LpxC-1-treated mice ($P < 0.001$) (Fig. 7B).

Finally, to determine how LpxC-1 could alter *in vivo* bacterial density even though it did not kill the bacteria *in vitro*, we com-

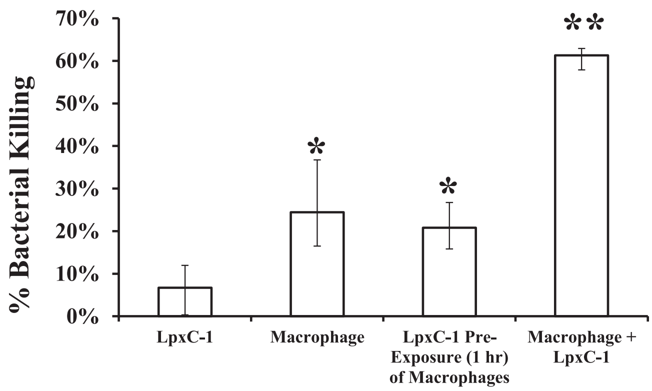

FIG 8 Macrophage killing of *A. baumannii* is enhanced by exposure to LpxC-1. Reduction of CFU at 1 h of *A. baumannii* incubation with LpxC-1 alone, macrophages alone, macrophages that had been preexposed to LpxC-1 for 1 h followed by rinsing away the LpxC-1, and macrophages plus LpxC-1. Median and interquartile ranges of killing are shown. Results from are from eight samples per group. *, $P < 0.05$ versus results for LpxC-1 alone; **, $P < 0.05$ versus results for all other groups.

pared MICs of LpxC-1 with and without serum and found no change in MICs in the presence of serum. Since LpxC-1 did not affect complement susceptibility of the bacteria (i.e., no growth inhibition in the presence of serum or change in LpxC-1 MIC in the presence of serum), we compared macrophage killing of bacteria in the presence or absence of LpxC-1. Exposure of *A. baumannii* HUMC1 to LpxC-1 during the 1-h coincubation with RAW cells resulted in a marked increase in macrophage killing of the bacteria (Fig. 8). Preexposure of the macrophages to LpxC-1 for 1 h, followed by rinsing away the LpxC-1, did not affect macrophage killing of the bacteria.

DISCUSSION

The finding that LPS-TLR4 interactions govern *in vivo* virulence of *A. baumannii* and that an LpxC inhibitor antibiotic with no *in vitro* activity against *A. baumannii* protected mice from lethal infection are of considerable biological and translational importance. The protection despite a similar tissue bacterial burden but with reduced inflammatory cytokines in TLR4-mutant mice demonstrates that protection was driven by immunomodulation rather than by altering the bacterial density of infection. Interestingly, despite the lack of detectable *in vitro* killing of *A. baumannii* by LpxC-1 in standard susceptibility tests, treatment of mice with LpxC-1 markedly reduced tissue bacterial density, serum LPS levels, and serum inflammatory cytokine levels. As a result, LpxC-1 totally protected mice from lethal infection. Exposure of bacteria to the LpxC-1 inhibitor increased their susceptibility to opsonophagocytic killing by macrophages. The LpxC-1 inhibitor also reduced the LPS levels in serum relative to the bacterial density in blood, so the effect on reducing immunopathogenesis was greater than would be expected to be caused by another antibiotic that reduced CFU without reducing LPS density in the bacteria. Since LpxC inhibitors are already in advanced preclinical development, these results indicate that such inhibitors should be tested clinically in patients infected with *A. baumannii* irrespective of *in vitro* susceptibility results. Since there are few if any drugs in development with the potential to treat lethal XDR/PDR *A. baumannii* infections, the discovery that an entirely new class of compounds has therapeutic potential is of great potential clinical importance. Furthermore, the colistin-mediated *in vitro* neutralization of LPS activation of TLR4 suggests that adjunctive colistin therapy, potentially at lower and hence less-toxic doses than are typically used clinically, could reduce *A. baumannii* virulence *in vivo* irrespective of bactericidal activity. Thus, low-dose colistin merits study as an adjunctive, combination therapy even for *A. baumannii* strains that are susceptible to β-lactam antibiotics.

While colistin-resistant strains of *A. baumannii* are reported to have reduced virulence in mice (46), the current findings indicate that colistin resistance does not necessarily intrinsically affect virulence. Indeed, several publications have defined varying strain virulences unrelated to colistin resistance (47, 48). In the current study, two strains with regulatory mutations affecting polymyxin resistance through addition of phosphoethanolamine to LPS (43), C14 and R2, were found to have highly divergent *in vivo* virulences. The clinical isolate C14 was as virulent *in vivo* as carbapenem-resistant, colistin-susceptible HUMC isolates. In contrast, R2 was avirulent. Extracted LPS from both strains led to enhanced TLR4 stimulation relative to that with LPS extracted from other, non-colistin-resistant strains. However, the *pmrC* mutation in R2 did not increase its LPS shedding relative to that of its hypovirulent parent strain, 17978, and thus its virulence was not affected. In contrast, strain C14 had both increased TLR4 activation from extracted LPS and a very high level of LPS shed during growth, resulting in enhanced *in vivo* virulence. The molecular genetics and structure of LPS that result in greater shedding by the more-virulent strains merits investigation, since elucidating these factors should result in novel targets for therapeutic intervention.

These results also provide direct experimental confirmation of the host-pathogen damage model of Casadevall and Pirofski (49). Specifically, the *A. baumannii* bacterial burdens were similar during lethal and nonlethal infection in wild-type versus mutant mice, and evaluation of bacterial burden or clearance did not describe virulence for this pathogen. Rather, virulence was related to induction of host hyperinflammation resulting in lethal sepsis. Thus, investigation of infections caused by *A. baumannii*, whether preclinical or clinical, should focus as much on host response biomarkers as on microbiological eradication. Furthermore, caution must be exercised when evaluating the severity of infection in experimental models based solely on microbial burden. Microbial burden may not accurately reflect "damage" to the host, or actual outcome of infection, particularly in models that do not assess actual physiological consequences of infection (e.g., nonlethal models). For example, in a previous study, TLR4-KO mice on a C57BL/6 background were reported to be susceptible to *A. baumannii* infection, which may appear to be discordant with our results. However, the previous study used a nonlethal model of infection and found slower early clearance of the organism from the lung (41). By 48 h, the organism had been cleared similarly by wild-type and TLR4-KO mice, and there was no apparent clinical or physiological consequence for the mice of this slower initial bacterial clearance. Our data also showed a nonsignificant, modestly lower bacterial burden in tissue of C3H/HeJ TLR4-mutant mice than in wild-type mice and demonstrate that the clinical outcomes were not driven by the tissue bacterial burden but rather by the host response to the bacteria. Thus, our data are not discordant from those of the previous study and must be interpreted in the context of lethal versus nonlethal models.

How the LpxC-1 inhibitor enhances phagocytosis is not clear. The effect was not due to a direct impact of LpxC-1 on macrophages, because pretreatment of macrophages with the LpxC-1 inhibitor, followed by rinsing away the inhibitor, resulted in no substantive change in macrophage killing of the bacteria. Mutation of Lpx is known to result in upregulation of genes responsible for the biosynthesis of poly-β-1,6-N-acetylglucosamine (PNAG), which presumably replaces LPS as a predominant oligosaccharide in the outer membrane, enabling the bacteria to maintain cell viability (50). It has long been known that the macrophage mannose receptor binds to *N*-acetylglucosamine (51), which may account for the enhanced phagocytosis of *A. baumannii* in the setting of LpxC-1 exposure.

Antimicrobial discovery screens and development programs are typically built around lead compounds with low *in vitro* MICs, preferably with microbicidal activity, against target bacteria. However, such screens fail to detect the potential for antimicrobial drugs to modulate pathogenesis aside from microbicidal activity against the organism. Most LpxC inhibitors, including LpxC-1, do not have *in vitro* activity against *A. baumannii* by standard susceptibility testing. However, *A. baumannii* is known to express LpxC (52), and the current study demonstrates that while the LpxC inhibitor tested did not inhibit *A. baumannii* growth, it did markedly modulate the ability of the cells to activate TLR4 and induce septic shock *in vivo*. These data underscore the importance of finding new, physiologically relevant ways to screen for small-molecule and biological agents to treat XDR/PDR GNB and other highly resistant microbes in order to discover novel therapeutic classes.

Bacteremia is one of the most common clinical syndromes caused by *A. baumannii* and is often accompanied by sepsis syndrome (15, 18, 53–55). Such infections typically occur in patients hospitalized in the ICU, most likely via bolus entry from catheters, which is similar to the mode of entry in the model studied. An advantage of the C3H model of infection is that relatively low inocula (e.g., 2×10^7) induce fatal infection even without having to cause overt immunocompromise. This lethal inoculum is similar to that required to cause fatal infections by other virulent bacteria in noncompromised mice, such as *Staphylococcus aureus*, *Enterococcus*, and *Pseudomonas aeruginosa* (56, 57). In contrast, the same inoculum of *A. baumannii* in other mouse models, such as BALB/c mice, is nonfatal unless accompanied by induction of diabetes mellitus or neutropenia (42, 58, 59).

In summary, LPS-mediated activation of TLR4 was a primary pathogenic factor during systemic *A. baumannii* infection, and TLR4 was antiprotective against lethal infection. Of great translational importance is that inhibition of LpxC resulted in diminished LPS-mediated TLR4 activation and protected mice from lethal infection despite a lack of *in vitro* susceptibility of the bacteria to the inhibitor by traditional testing. These results underscore the urgent and pressing need to find *in vitro* screens that predict *in vivo* efficacy in a physiological way and the potential for small-molecule and biological therapies to be effective antibacterial agents even if they do not directly kill the target pathogen.

MATERIALS AND METHODS

Strains and mouse model of infection. Nine clinical isolates of *A. baumannii* were used (Table 1). Wild-type (C3H/FeJ) and TLR4-mutant (C3H/HeJ) mice and congenic C57BL/6 and congenic TLR4-knockout (KO) mice were used (Jackson Laboratories). In some experiments, BALB/c mice were made neutropenic using cyclophosphamide (200 mg/kg of body weight given intraperitoneally [i.p.] on day −2 relative to infection, with a repeat dose of 150 mg/kg 5 days later), as we have previously described (58). *A. baumannii* strains were grown overnight at 37°C with shaking in tryptic soy broth (TSB). The bacteria were passaged to mid-log-phase growth at 37°C with shaking. Cells were washed twice with phosphate-buffered saline (PBS) and resuspended at the appropriate concentration for infection. Infections with 2×10^7 or 5×10^7 bacteria were administered intravenously (i.v.) via the tail vein. The final concentration was confirmed by quantitative culturing of the inocula. The LpxC inhibitor LpxC-1 (Pfizer Inc.) was dissolved in 40% cyclodextrin in sterile water. Mice were treated subcutaneously with 100 mg/kg/day for 3 days starting on the day of infection, based on previously published pharmacokinetic information for related compounds (45). Control mice were treated with placebo (40% cyclodextrin in sterile water) alone. All animal experiments were approved by the Institutional Committee on the Use and Care of Animals at the Los Angeles Biomedical Research Institute, following the National Institutes of Health guidelines for animal housing and care.

Organ histopathology and immunofluorescence. Organs were fixed in zinc-buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) or stained by immunofluorescence. For immunofluorescence, the slides were deparaffinized and stained with immune sera from mice surviving previous sublethal infection with HUMC1 (convalescent-phase serum obtained 1 month after infection) and counterstained with goat anti-mouse, fluorescein isothiocyanate (FITC)-conjugated IgG.

Cytokine and sepsis biomarkers. Mice were sedated with ketamine, and blood was obtained by cardiac puncture. Serum cytokines were quantified by the MSD Multi-Spot assay (Mesoscale) per the manufacturer's instructions. Whole-blood pH was analyzed using the i-STAT system. For experiments in which i-STAT measurements were made, it was necessary to anticoagulate the mice with intraperitoneal heparin (100 U given i.p.; Sigma-Aldrich, St. Louis, MO) simultaneously with sedation by intraperitoneal ketamine (100 mg/kg) 5 to 15 min prior to cardiac puncture to prevent clotting in the i-STAT cartridges. Whole blood was aspirated into a 25-gauge syringe and aliquoted into i-STAT cartridges. Values were read on an i-STAT portable clinical analyzer. To measure serum LPS levels, the limulus amebocyte assay (LAL) was used (Associates of Cape Cod, East Falmouth, MA).

Temperature was measured using a digital thermometer, Physitemp model Bat-12 (Physitemp Instruments Inc., Clifton, NJ). The probe was inserted rectally to its hilt and maintained in this position until the temperature reading stabilized. Temperature and weights were recorded between 8 and 9 AM each day.

TLR4 assay. *A. baumannii* strains were grown overnight at 37°C with shaking in TSB. The bacteria were passaged at 37°C with shaking. Cells were washed three times with PBS and resuspended to an optical density of 1.0. LPS was isolated from *A. baumannii* strains using an LPS extraction kit (iNtRON Biotechnology, Inc.).

Each passaged strain was plated in TSB agar to determine the amount of LPS per bacillus. Isolated LPS was stored in polystyrene tubes at 4°C, and these were assayed for LPS activity within 1 month. For collection of culture supernatants, the bacteria were passaged to an optical density of 1.5 at 37°C with shaking. The cultures were spun down at 4,000 rpm for 10 min, and supernatants were sterile filtered using 0.22-μm syringe filters (Millipore Corp.). To verify that there were no live cells in the filtered supernatants, the supernatants were plated in TSB agar.

Dilutions of filter-sterilized culture supernatant and isolated LPS were made in sterile glass tubes. The filtered supernatants and isolated LPS were then assayed for TLR4 activity using the HEK-Blue LPS detection kit (InvivoGen). HEK-Blue-4 cells were passaged in HEK-Blue selection medium until they were 60 to 80% confluent. Right before an assay, the cells were washed with PBS to remove the selection medium and then diluted to 2×10^5 cells/ml in HEK-Blue detection medium. In each well of a

96-well plate, 20 μl of sample, 100 μl of cells, and 100 μl of HEK-Blue detection medium were added. The plate was incubated for 18 h at 37°C in 5% CO_2 and read using a spectrophotometer (BioTek Instruments, Inc.) at 630 nm. For some experiments, bacteria were grown overnight and passaged to log phase in the presence of 4 μg/ml of LpxC-1 before extraction of LPS or harvesting of supernatant. For other experiments, polymyxin B (Sigma-Aldrich) was added to TLR4 assay test wells to block LPS effects or supernatants were boiled at 100°C for 20 min to determine the impact of this on TLR4 activation.

Growth curves. *A. baumannii* strains were cultured overnight in TSB at 37°C, passaged by placing 100 μl of overnight culture in 10 ml of TSB, and serially sampled to determine optical density and bacterial density by quantitative culturing. Optical density was measured at an absorbance of 600 nm (Implen OD600 DiluPhotometer).

Killing assay. Bacterial killing by macrophages was assessed using our previously published method (60). In brief, RAW 264.7 macrophage cells (both from American Type Culture Collection, Rockville, MD) were cultured at37°C in 5% CO_2 in RPMI 1640 (Irvine Scientific, Santa Ana, CA) with 10% fetal bovine serum (FBS), 1% penicillin, streptomycin, and glutamine (Gemini Bioproducts), and 50 μM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). RAW cells were activated by 3 days of exposure to 100 nM phorbol myristate acetate (PMA) (Sigma-Aldrich). Activated RAW 264.7 macrophages were harvested after scraping with BD Falcon cell scrapers (Fischer Scientific) and cocultured in polystyrene snap cap tubes in a rotating drum at 37°C at a ratio of 20 bacteria to 1 macrophage. After a 1-h incubation, the tubes were sonicated and quantitatively plated in tryptic soy agar (TSA). Colony-forming units (CFU) of the cocultured tubes were compared to CFU of growth control tubes containing only microbes with no macrophages. Percent killing was calculated as 1 − (CFU from coculture tubes/CFU from growth control tubes).

Statistics. Survival was compared by using the nonparametric log rank test. Categorical variables were compared using the Wilcoxon rank-sum test for unpaired comparisons or the Wilcoxon signed-rank test for paired comparisons.

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