Diabetes Exacerbates Infection via Hyperinflammation by Signaling through TLR4 and RAGE

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ABSTRACT For more than a century, diabetic patients have been considered immunosuppressed due to defects in phagocytosis and microbial killing. We confirmed that diabetic mice were hypersusceptible to bacteremia caused by Gram-negative bacteria (GNB), dying at inocula nonlethal to nondiabetic mice. Contrary to the pervasive paradigm that diabetes impedes phagocytic function, the bacterial burden was no greater in diabetic mice despite excess mortality. However, diabetic mice did exhibit dramatically increased levels of proinflammatory cytokines in response to GNB infections, and immunosuppressing these cytokines with dexamethasone restored their resistance to infection, both of which are consistent with excess inflammation. Furthermore, disruption of the receptor for advanced glycation end products (RAGE), which is stimulated by heightened levels of AGEs in diabetic hosts, protected diabetic but not nondiabetic mice from GNB infection. Thus, rather than immunosuppression, diabetes drives lethal hyperinflammation in response to GNB by signaling through RAGE. As such, interventions to improve the outcomes from GNB infections should seek to suppress the immune response in diabetic hosts.

IMPORTANCE Physicians and scientists have subscribed to the dogma that diabetes predisposes the host to worse outcomes from infections because it suppresses the immune system. This understanding was based largely on *ex vivo* studies of blood from patients and animals with diabetes. However, we have found that the opposite is true and worse outcomes from infection are caused by overstimulation of the immune system in response to bacteria. This overreaction occurs by simultaneous ligation of two host receptors: TLR4 and RAGE. Both signal via a common downstream messenger, MyD88, triggering hyperinflammation. In summary, contrary to hundred-year-old postulations about immune suppression in diabetic hosts, we find that diabetes instead predisposes to more severe infections because of additional inflammatory output through dual activation of MyD88 by not only TLR4 but also RAGE. It is the activation of RAGE during GNB infections in those with diabetes that accounts for their heightened susceptibility to infection compared to nondiabetic hosts.

KEYWORDS diabetes mellitus, Gram-negative bacteria, infection, inflammation, RAGE, TLR4

Twenty-six million people in the United States suffer from diabetes, a disease projected to afflict one-fifth of the population by 2050 (1). People with diabetes are three times more likely to develop bacterial sepsis and suffer far worse outcomes (2–5). In particular, diabetes is a risk factor for significantly increased mortality from infections caused by Gram-negative bacteria, especially *Acinetobacter baumannii* (6–9). Over a century of literature has indicated that defective phagocytes in diabetic hosts are responsible for worse outcomes from bacterial infections, leading to the characterization of diabetes as an immunosuppressive state. This immunosuppression has long been ascribed to deficits in neutrophil phagocytosis and microbial killing found *ex vivo* in cells taken from diabetic hosts (4, 5, 10–22), yet neutropenia is not a substantial risk factor for worse outcomes from *A. baumannii* infections (23, 24). Indeed, even in those with cancer, the majority of patients who develop *A. baumannii* infections are not neutropenic (23), and neutropenia is not an independent predictor of outcome from these infections (24). We therefore suspected that suppression of phagocytic activity was unlikely to explain why diabetic hosts would be more susceptible to infections caused by *A. baumannii* and other Gram-negative bacteria.

While studying *A. baumannii* pathogenesis, we found that the principal cause of lethality in nondiabetic mice was inflammation driven by stimulation of Toll-like receptor 4 (TLR4) by lipopolysaccharide (LPS) (25). It has also been reported that high levels of inflammatory mediators in mice correlated with increased susceptibility to bacterial infection (26). Thus, we investigated a novel hypothesis: rather than immunosuppression, worse outcomes in diabetic hosts with Gram-negative bacterial infections are due to overactivation of the innate immune system. As such, we sought to determine the etiology for increased mortality from infections caused by Gram-negative bacteria and to define the source of excess inflammation in the diabetic host.

RESULTS

We confirmed that C57BL/6 mice with diabetes induced by streptozotocin (STZ) (27) were hypersusceptible to lethal *A. baumannii* bacteremia at an inoculum nonlethal to nondiabetic mice (Fig. 1A). Even mice with diet-induced-obesity (DIO) diabetes, which causes mild hyperglycemia consistent with clinical type 2 diabetes (28–30), had significantly increased mortality compared to that in nondiabetic mice (Fig. 1B). One possible explanation for greater mortality in diabetic mice is that hyperglycemia creates preferable growth conditions for the bacteria. However, we found no difference in bacterial growth in DIO diabetic versus nondiabetic mouse serum, indicating that simple growth enhancement caused by hyperglycemia was not operative (Fig. 1C). Moreover, despite clear survival differences between DIO diabetic and nondiabetic mice, there was no disparity in blood bacterial density (Fig. 1D), indicating no deficiency in phagocytic clearance of bacteria in DIO diabetic mice.

Despite similar bacterial densities, diabetic but not nondiabetic mice experienced hypothermia, severe metabolic acidemia (low blood pH, low serum bicarbonate, and high base deficit), and markedly higher serum levels of interleukin-1β (IL-1β), IL-10, and tumor necrosis factor (TNF) than nondiabetic mice ($P < 0.05$ for all comparisons), plus a strong trend toward higher levels of IL-6 ($P = 0.05$) (Fig. 2A and B). Finally, diabetic mice developed higher ratios of IL-10 to TNF (Fig. 2C), consistent with prior clinical findings that predict worse survival in bacteremic patients (31).

The worse survival rates and increased levels of inflammatory cytokines in diabetic mice with no correlation between bacterial burden and survival suggested that hyperinflammation was driving the increased lethality. To test this hypothesis, we immunosuppressed diabetic mice by treating them with a potent corticosteroid (dexamethasone at 4 mg/kg of body weight). Diabetic mice treated once with dexamethasone 20 min postinfection had markedly improved survival compared to diabetic control mice treated with a placebo (Fig. 3A). Thus, diabetes did not exacerbate lethality by inhibiting clearance of *A. baumannii* but by amplifying the inflammatory response to the organism.

We have previously shown that *A. baumannii* infections result in profound hyper-
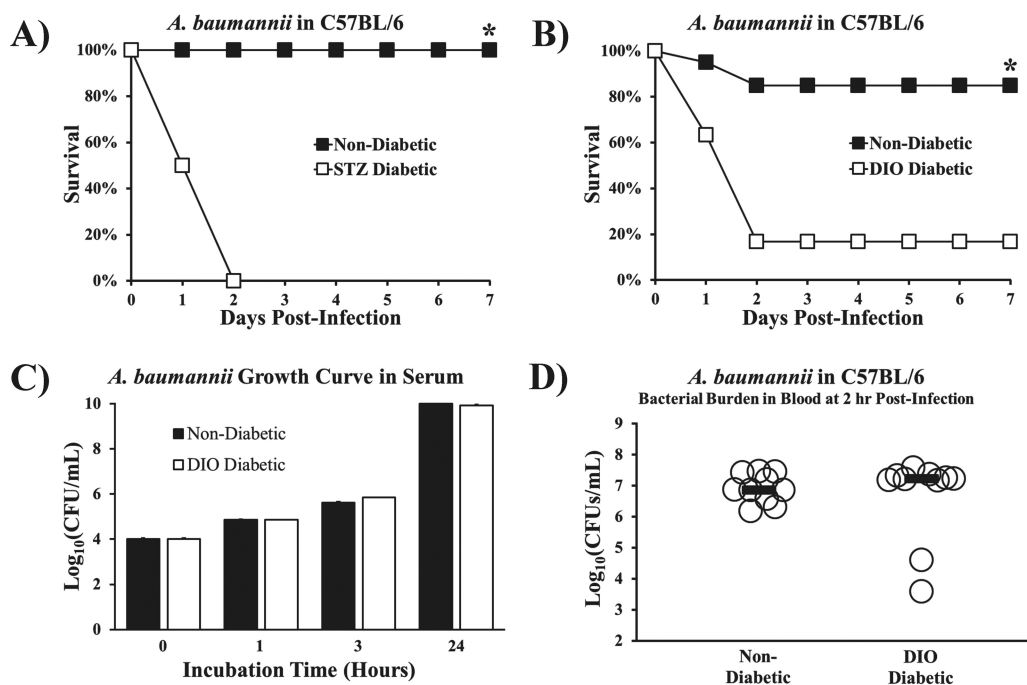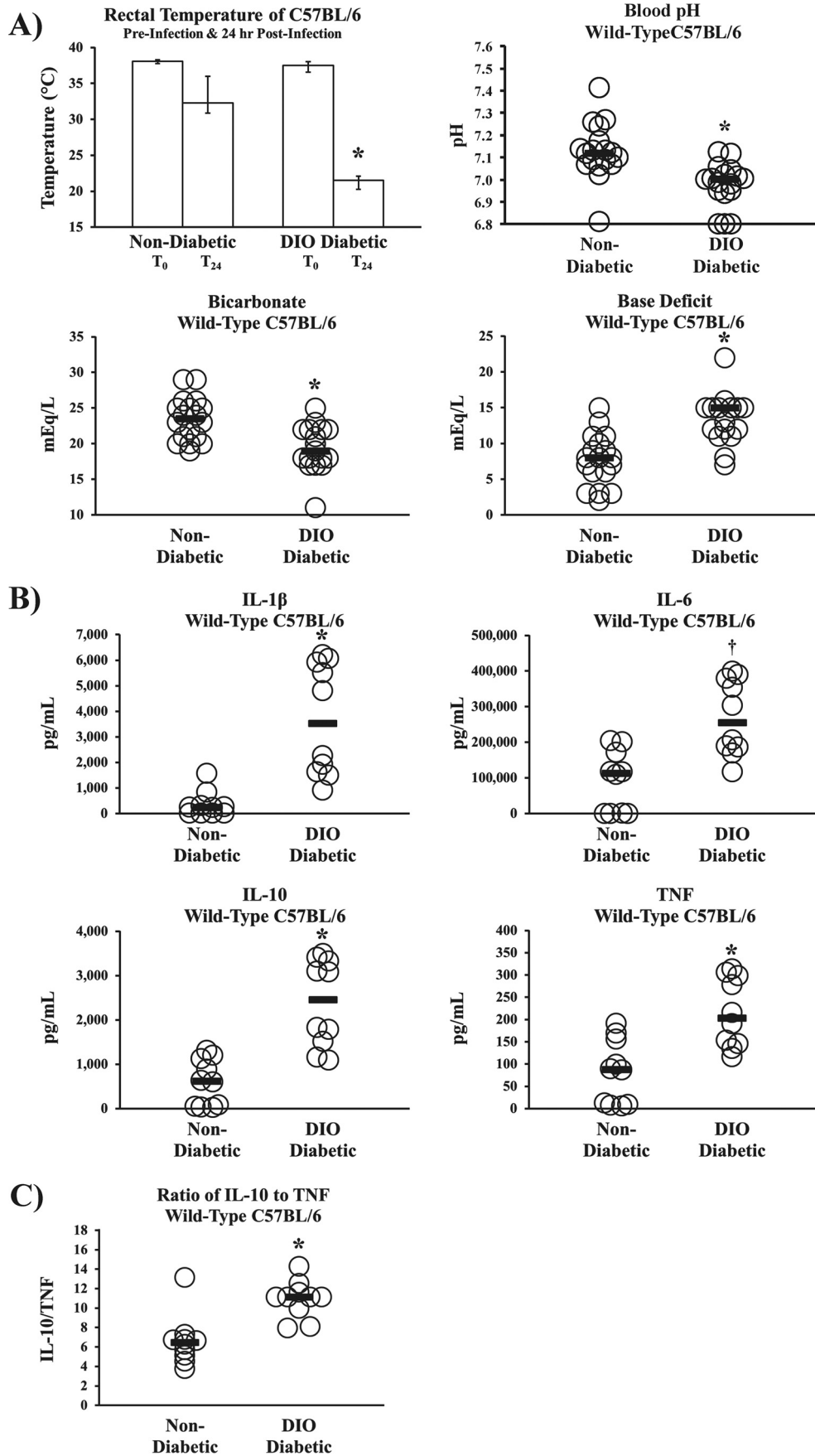
FIG 1 Diabetic mice were hypersusceptible to *A. baumannii* sepsis despite having bacterial densities similar to those in nondiabetic mice. (A, B) Survival curves for diabetic and nondiabetic mice. (A) C57BL/6 mice with streptozotocin-induced (STZ) diabetes (severe, type 1 model) developed lethal bloodstream infections due to the hypervirulent *A. baumannii* strain HUMC1 (7×10^6-CFU inoculum), whereas nondiabetic control mice cleared the infection ($n = 8$ mice per group). *, $P < 0.05$. (B) Mice with diet-induced-obesity (DIO) diabetes (mild, type 2 model) also developed lethal infections due to *A. baumannii* (2×10^7-CFU inoculum), whereas nondiabetic control mice cleared the infection ($n = 30$ mice per group). *, $P < 0.05$. (C) The rates of growth for log-phase *A. baumannii* were identical in DIO diabetic and nondiabetic mice. (D) DIO diabetic mice had blood bacterial burdens nearly identical to those in nondiabetic control mice at 2 h postinfection ($n = 10$ mice per group; bars display median values).

inflammation and sepsis in nondiabetic mice via TLR4 (25). We therefore sought to determine whether TLR4 disruption also mitigated mortality in diabetic mice. While all wild-type mice (C3H/Fe) with streptozotocin-induced diabetes died from *A. baumannii* infection, diabetic TLR4 mutant mice (C3H/He) had markedly improved survival (Fig. 3B). Similarly, a proprietary small-molecule competitive TLR4 antagonist (E5564, 10 mg/kg/day intravenously [i.v.] for 4 days postinfection) completely protected mice from otherwise lethal *A. baumannii* infection (Fig. 3C). Furthermore, knocking out TLR4 in C57BL/6 mice did not affect the hyperglycemia in DIO mice (Fig. 3D) but did completely protect them from lethal infection (Fig. 3E). These data confirm that diabetic mice are protected against *A. baumannii* infection in the absence of TLR4 signaling, as was previously found in nondiabetic mice.

Despite worse survival, DIO diabetic mice did not have greater bacterial burdens or higher serum endotoxin levels than nondiabetic mice, whether wild type or TLR4 knockout (TLR4 KO) (Fig. 4A and B). Tellingly, DIO diabetic TLR4 KO mice had marked improvements in sepsis biomarkers and near-baseline (32–34) levels of inflammatory cytokines compared to DIO diabetic wild-type mice (Fig. 4C and D). These data further underscore the lack of correlation between outcomes of infection and innate immune clearance of bacteria, instead attributing mortality to hyperinflammation in diabetic hosts.

To explore the generalizability of these findings, we established models of infection in DIO diabetic mice caused by a clinical bloodstream isolate of *Escherichia coli* expressing an extended-spectrum β-lactamase (ESBL) (strain type 131/H30 with the bla_{CTX-M} gene) and a strain of pan-drug-resistant *Klebsiella pneumoniae* expressing a *Klebsiella pneumoniae* carbapenemase (KPC). DIO diabetic mice were significantly more susceptible than nondiabetic mice to both Gram-negative *Enterobacteriaceae* species

A)

Rectal Temperature of C57BL/6
Pre-Infection & 24 hr Post-Infection

Blood pH
Wild-Type C57BL/6

Bicarbonate
Wild-Type C57BL/6

Base Deficit
Wild-Type C57BL/6

B)

IL-1β
Wild-Type C57BL/6

IL-6
Wild-Type C57BL/6

IL-10
Wild-Type C57BL/6

TNF
Wild-Type C57BL/6

C)

Ratio of IL-10 to TNF
Wild-Type C57BL/6

(Fig. 5A) despite similar blood bacterial densities (Fig. 5B). Just as we found with *A. baumannii*, DIO diabetic TLR4 KO mice were more resistant to *K. pneumoniae* and *E. coli* infection than DIO diabetic wild-type mice (Fig. 5C).

Since both diabetic and nondiabetic mice express TLR4 but diabetic mice die from inocula nonlethal to nondiabetic mice despite similar bacterial burdens, we hypothesized that a second inflammatory signaling pathway unique to diabetic mice must drive sepsis, in addition to TLR4. One well-studied mechanism by which diabetes induces endovascular inflammation proceeds via stimulation of the receptor for advanced glycation end products (RAGE), due to elevated levels of advanced glycation end products (AGEs) (35, 36). Recently, TLR4 and RAGE were shown to share the downstream proinflammatory signaling transducer MyD88 (37). We therefore tested the ability of two soluble RAGE antagonists to affect the outcome from infection, FPS-ZM1 (small molecule) and RAP (peptide antagonist) (38, 39). Antagonizing RAGE during *A. baumannii* infection was protective in mice with DIO diabetes but not in nondiabetic mice (Fig. 6A), demonstrating that the effect was related to diabetes. Despite improving the survival of DIO diabetic mice, RAGE antagonism did not alter the bacterial burdens (Fig. 6B) but did significantly ameliorate the metabolic acidemia and cytokine storms seen in the DIO diabetic mice (Fig. 6C and D). DIO diabetic mice also experienced significantly higher ratios of IL-10 to TNF than mice treated with the small-molecule RAGE antagonist (FPS-ZM1), similar to the results for the nondiabetic wild-type mice in the experiment whose results are shown in Fig. 2C (Fig. 6E). Additionally, antagonizing RAGE in DIO diabetic mice infected with *E. coli* (Fig. 6F) or *K. pneumoniae* (Fig. 6G) significantly improved survival without affecting bacterial burdens, demonstrating that the protective effect was generalizable across Gram-negative species.

To ensure that off-target effects of the two RAGE antagonists were not responsible for the observed phenotype, we sought to corroborate the RAGE antagonist results in knockout mice. We first demonstrated that DIO wild-type and DIO RAGE KO mice had similarly elevated levels of fasting blood glucose (Fig. 7A). Like the outcome of treatment with soluble RAGE antagonists, knocking out RAGE in diabetic mice also made them highly resistant to lethal infection compared to the susceptibility of diabetic wild-type control mice (Fig. 7B). Indeed, knocking out RAGE in diabetic mice reverted their susceptibility to infection to the level of nondiabetic wild-type mice (compare Fig. 1B and 7B). Severing RAGE signaling also resulted in marked reductions of inflammatory cytokine levels in diabetic mice (Fig. 7C), to levels comparable to those in nondiabetic wild-type mice (compare Fig. 2B and 7C). Diabetic wild-type mice again developed higher ratios of IL-10 to TNF than diabetic RAGE KO mice (Fig. 7D), resembling the ratios in nondiabetic wild-type mice (compare Fig. 2C and 7D).

As previously mentioned, a recent study found a common signaling pathway between RAGE and TLR4, whose unique cytoplasmic effectors both bind to TIRAP and signal through MyD88, which transduces downstream signals that activate the transcription of inflammatory cytokines (37). We therefore sought to determine whether MyD88 signaling was a common pathway driving the increased susceptibility of diabetic mice to infections caused by Gram-negative bacteria. DIO wild-type and DIO MyD88 KO mice had similar levels of fasting blood glucose, which were significantly higher than the levels in nondiabetic wild-type mice (Fig. 8A). Despite intact TLR4 and RAGE, diabetic MyD88 KO mice were completely resistant to lethal infection compared to the susceptibility of diabetic wild-type control mice (Fig. 8B).

FIG 2 Diabetic mice had worse sepsis biomarkers from *A. baumannii* sepsis despite bacterial densities similar to those in nondiabetic mice. (A) Body temperatures ($n = 10$ mice per group) and blood sepsis biomarkers demonstrated severe metabolic acidosis (low blood pH, low serum bicarbonate, and high base deficit) consistent with septic shock at 18 h postinfection with *A. baumannii* (2×10^7-CFU inoculum) in DIO diabetic wild-type mice but not nondiabetic wild-type control mice ($n = 18$ nondiabetic control mice, and $n = 17$ DIO diabetic wild-type mice). (B) Inflammatory cytokine levels were markedly elevated in DIO diabetic wild-type mice compared to the levels in nondiabetic wild-type control mice ($n = 10$ mice per group). *, $P < 0.05$; †, $P = 0.05$. (C) During *A. baumannii* bacteremia, diabetic mice experienced significantly higher ratios of IL-10 to TNF than nondiabetic mice ($n = 10$ mice per group). Bar graphs show medians and interquartile ranges; bars on dot plots display medians.
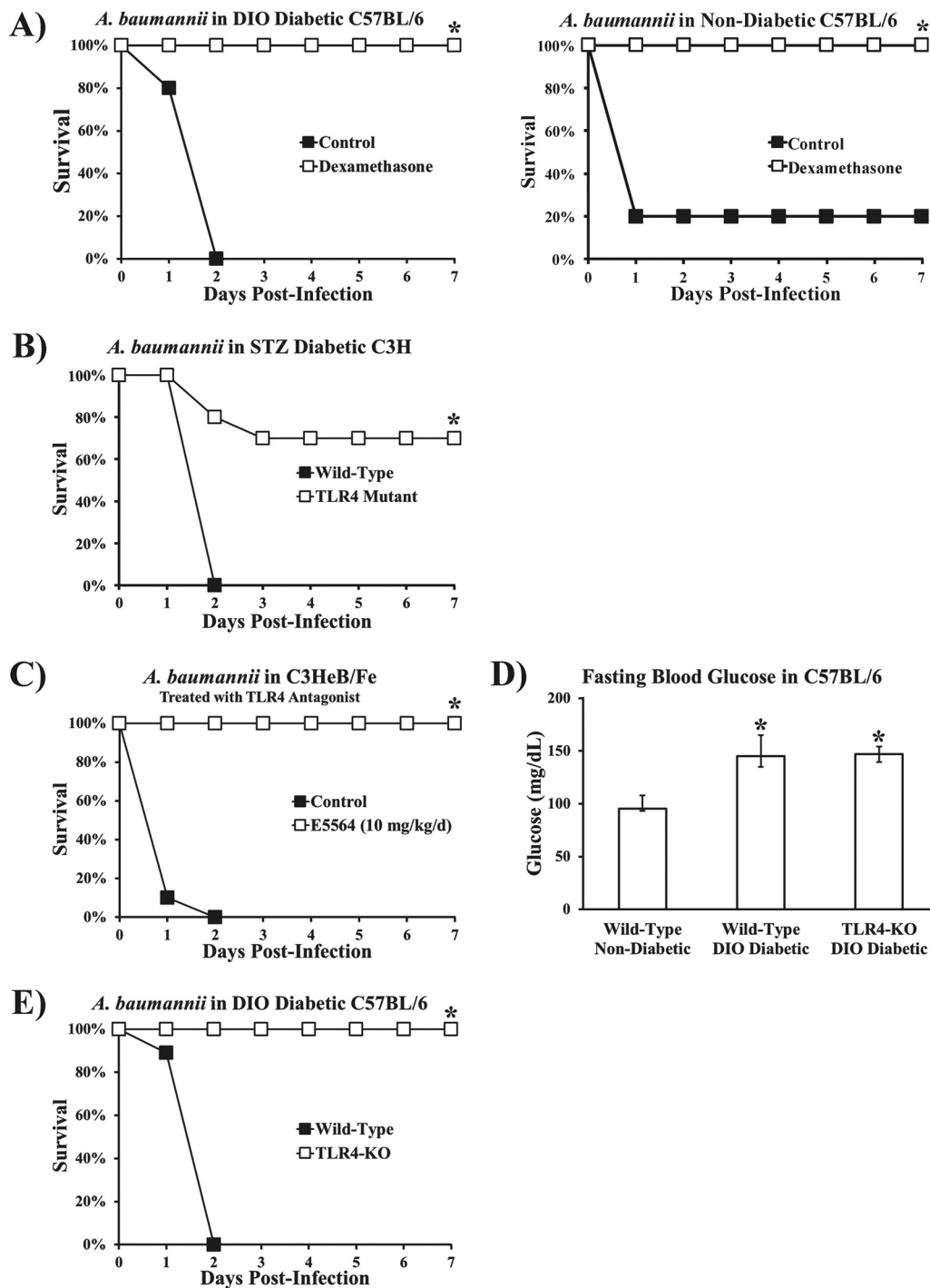
FIG 3 Treatment with the potent immunosuppressive corticosteroid dexamethasone and disruption of TLR4 restored resistance to infection in diabetic mice. (A) DIO diabetic and nondiabetic mice were infected with a normally lethal dose of *A. baumannii* (2×10^7-CFU inoculum). Mice treated 20 min postinfection with 4 mg/kg of dexamethasone, a potent corticosteroid, were resistant to infection, while those treated with saline (control) succumbed to infection within 48 h ($n = 5$ mice per group). *, $P < 0.05$. (B) Streptozotocin-induced diabetic wild-type C3H (C3HeB/Fe) and TLR4 mutant (C3H/He) mice were infected with *A. baumannii* (2×10^7-CFU inoculum). TLR4 mutant mice had significantly improved survival ($n = 10$ mice per group). (C) A TLR4 antagonist (E5564) protected mice from lethal *A. baumannii* bacteremia caused by i.v. infection. E5564 was administered i.v. at 10 mg/kg/day for 4 days ($n = 8$ mice per group). (D) DIO wild-type and DIO TLR4 KO mice had significantly higher levels of fasting blood glucose than nondiabetic wild-type mice ($n = 10$ mice per group; median values and interquartile ranges are shown). *, $P < 0.05$ versus the results for nondiabetic wild-type mice. (E) DIO diabetic TLR4 KO mice were resistant to *A. baumannii* bacteremia, while DIO diabetic wild-type mice all died ($n = 10$ mice per group). *, $P < 0.05$ versus the results for the control.
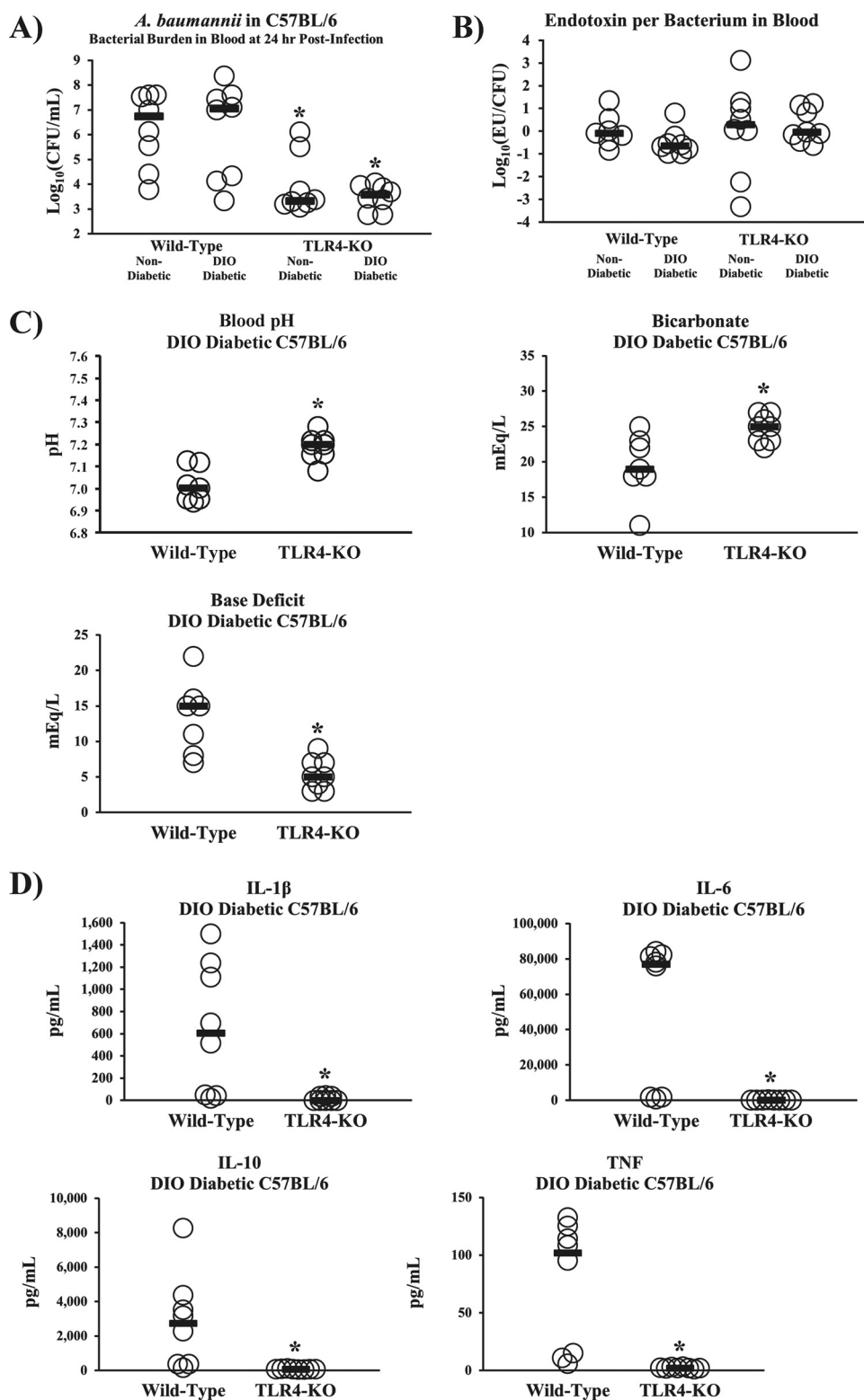
FIG 4 TLR4 disruption abrogated the hypersusceptibility of diabetic mice to sepsis from *A. baumannii* and other Gram-negative bacteria. (A) DIO diabetic mice had bacterial burdens at 24 h postinfection similar to those in nondiabetic mice, whether on a wild-type or TLR4 KO background ($n = 8$ mice per group). *, $P < 0.05$ versus the results for DIO diabetic and nondiabetic wild-type mice. (B) DIO diabetic and nondiabetic wild-type and TLR4 KO mice had nearly identical levels of lipopolysaccharide (LPS), measured as endotoxin units (EU) per bacterial CFU in the blood ($n = 7$ mice for the nondiabetic wild type, and $n = 8$ mice for all other groups). (C, D) DIO diabetic TLR4 KO mice were protected from severe acidosis/acidemia (C) and cytokine storms (D) that occurred in DIO diabetic wild-type mice infected with *A. baumannii* (2×10^7-CFU inoculum) ($n = 7$ mice per group). Bars represent median values. *, $P < 0.05$ versus the results for the control.
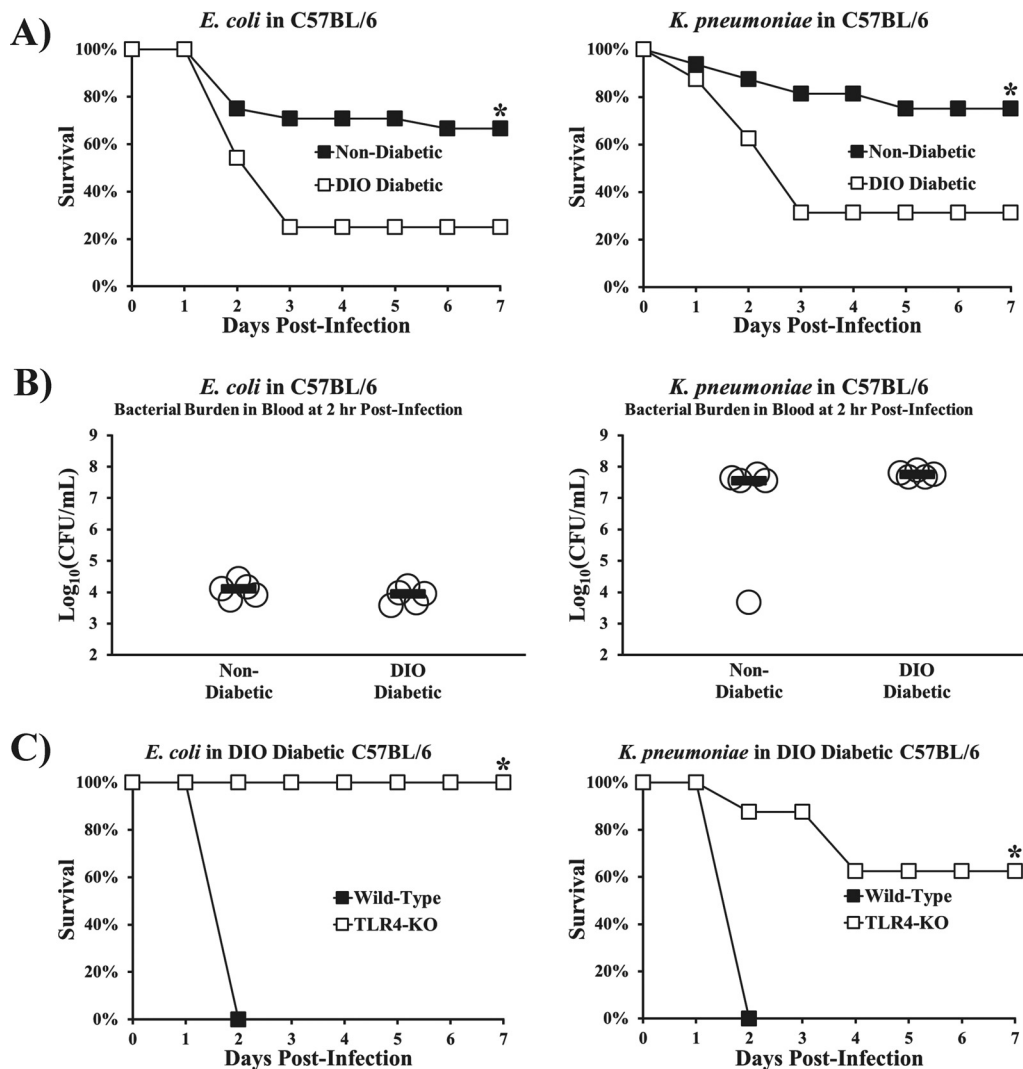
FIG 5 TLR4 disruption abrogated the hypersusceptibility of diabetic mice to sepsis from other Gram-negative bacteria. (A) Survival curves for diabetic and nondiabetic mice. DIO diabetic wild-type mice were more susceptible than nondiabetic wild-type mice to infections with ESBL *E. coli* and pan-drug-resistant, KPC-expressing *K. pneumoniae* ($n = 24$ mice for *E. coli* groups, and $n = 16$ mice for *K. pneumoniae* groups from 2 and 3 experiments, respectively). *, $P < 0.05$ versus the results for the control. (B) DIO diabetic wild-type mice had no significant difference in blood bacterial burdens at 2 h postinfection compared to the blood bacterial burdens in nondiabetic wild-type mice ($n = 5$ mice per group). $P > 0.1$ for both comparisons. (C) DIO diabetic TLR4 KO mice are rescued from the increased susceptibility of DIO diabetic mice to both organisms ($n = 8$ per group). *, $P < 0.05$ versus the results for the control. Bars on dot plots display median values.

DISCUSSION

The increased susceptibility of diabetic patients to acute bacterial infections—particularly those caused by Gram-negative bacteria—was noted in the 19th century (40). As early as 1907, the mechanism driving the increased severity of infections in diabetic patients was attributed to suppressed phagocytic clearance of microbes (15–18, 20–22, 41–43). Notwithstanding these conclusions, which were primarily drawn from *in vitro* and *ex vivo* assays of phagocyte function, we now report that diabetes causes the host to become hypersusceptible to infections caused by Gram-negative bacteria through immune paralysis, independent of bacterial density/clearance. Rather, the hypersusceptibility of diabetic hosts to Gram-negative bacterial infections is primarily due to hyperinflammation—not immunosuppression—inherent to the diabetic host.

TLR4 signaling occurs during infection for both diabetic and nondiabetic mice, but
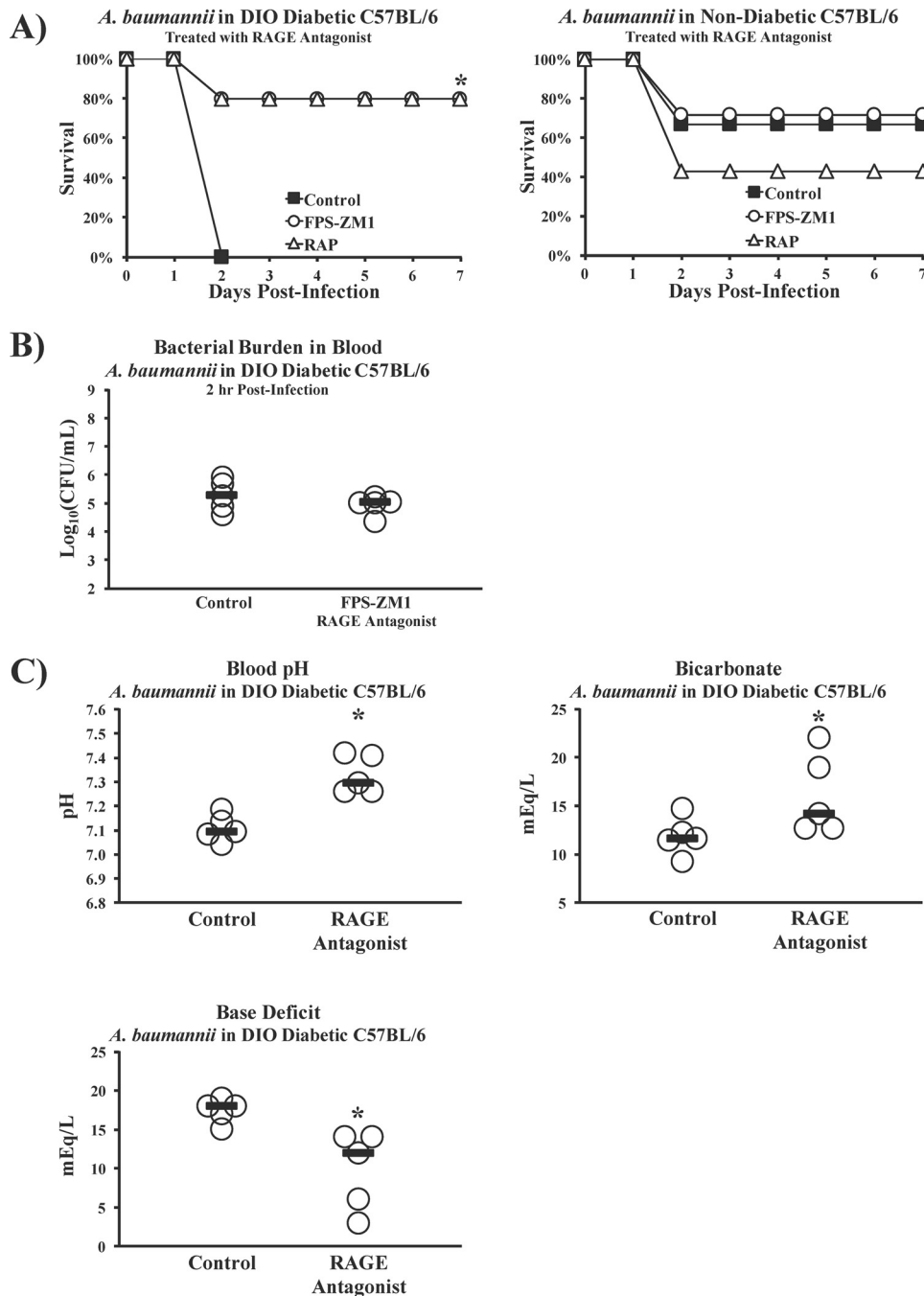
FIG 6 RAGE disruption ameliorates hypersusceptibility of diabetic mice to Gram-negative bacterial sepsis. (A) DIO diabetic and nondiabetic C57BL/6 mice were infected with *A. baumannii* and treated once daily for 4 days with PBS (control), a small-molecule RAGE antagonist (FPS-ZM1 at 75 μg/day), or a peptide RAGE antagonist (RAP at 300 μg/day). The nondiabetic C57BL/6 mice ($n = 6$ mice for control group, and $n = 7$ mice per treated group) were infected with an inoculum of *A. baumannii* (3×10^7 CFU) that kills only some control mice to maximize the chances of detecting some protective effect of RAGE antagonism; the DIO diabetic wild-type mice ($n = 5$ mice per group) were infected with an inoculum (2×10^7 CFU) that is 100% lethal for diabetic mice. RAGE antagonism improved the survival of diabetic but not nondiabetic mice. (B) RAGE antagonism did not alter bacterial burdens in DIO diabetic mice. Bars represent median values. *, $P < 0.05$ versus the results for the control. (C) Sepsis biomarkers improved significantly in DIO diabetic mice infected with *A. baumannii* (2×10^7-CFU inoculum) upon treatment with the small-molecule RAGE antagonist FPS-ZM1 ($n = 5$ mice per group). Bars represent median values. *, $P < 0.05$ versus the results for the control. (D) DIO diabetic mice are protected from cytokine storms after infection with *A. baumannii* (2×10^7-CFU inoculum) upon treatment with the small-molecule RAGE antagonist FPS-ZM1 ($n = 5$ mice per group). Bars represent median values. *, $P < 0.05$ versus the results for the control. (E) During *A. baumannii* bacteremia, DIO diabetic mice experienced higher ratios of IL-10 to TNF than mice treated with the small-molecule RAGE antagonist FPS-ZM1, similar to the results for the nondiabetic wild-type mice in the experiment whose results are shown in Fig. 2C ($n = 10$ mice per group). *, $P < 0.05$. (F) DIO diabetic mice were infected with an inoculum of *E. coli* (2×10^8 CFU) that kills some diabetic mice. They were treated once daily for 4 days with PBS (control) or the small-molecule RAGE antagonist FPS-ZM1 (250 μg/day). RAGE antagonism significantly improved survival in the diabetic mice (left), even though the bacterial burdens were no different between control and RAGE antagonist-treated mice (right). *, $P < 0.05$ versus the results for the control. (G) DIO diabetic mice were infected with inoculum of *K. pneumoniae* (2×10^8 CFU) that kills all diabetic mice. They were treated once daily for 4 days with PBS (control) or the small-molecule RAGE antagonist FPS-ZM1 (250 μg/day). RAGE antagonism significantly improved survival in the diabetic mice (left), even though the bacterial burdens were no different between control and RAGE antagonist-treated mice (right). *, $P < 0.05$ versus the results for the control.
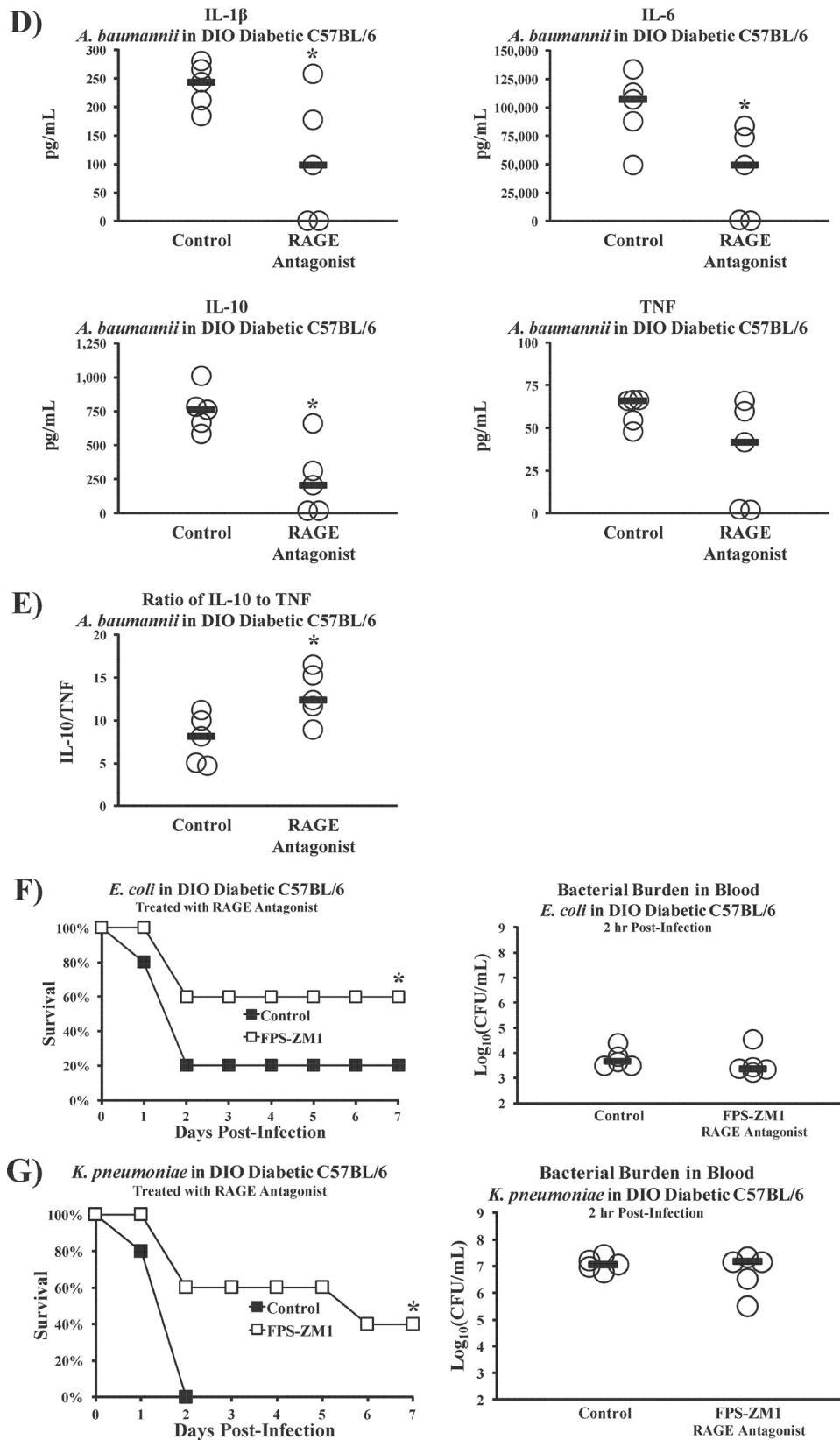
D)

IL-1β
A. baumannii in DIO Diabetic C57BL/6

IL-6
A. baumannii in DIO Diabetic C57BL/6

IL-10
A. baumannii in DIO Diabetic C57BL/6

TNF
A. baumannii in DIO Diabetic C57BL/6

E)

Ratio of IL-10 to TNF
A. baumannii in DIO Diabetic C57BL/6

F)

E. coli in DIO Diabetic C57BL/6
Treated with RAGE Antagonist

Bacterial Burden in Blood
E. coli in DIO Diabetic C57BL/6
2 hr Post-Infection

G)

K. pneumoniae in DIO Diabetic C57BL/6
Treated with RAGE Antagonist

Bacterial Burden in Blood
K. pneumoniae in DIO Diabetic C57BL/6
2 hr Post-Infection
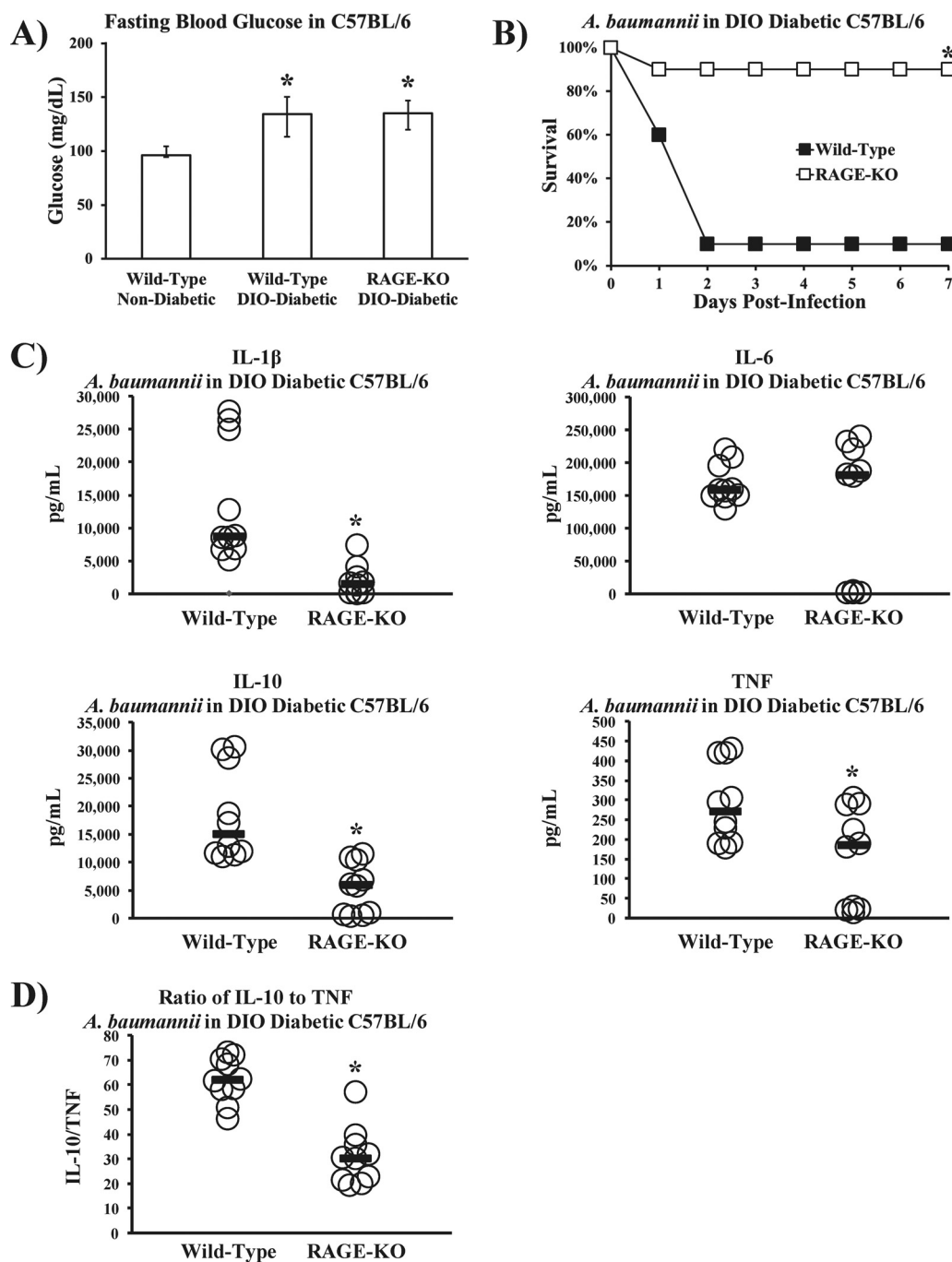

FIG 6 (Continued)

FIG 7 Knocking out RAGE reverts the susceptibility of diabetic mice to *A. baumannii* sepsis to the level of nondiabetic wild-type mice. (A) DIO diabetic wild-type and DIO diabetic RAGE KO mice had significantly higher levels of fasting blood glucose than nondiabetic wild-type control mice ($n = 10$ mice per group). *, $P < 0.05$ versus the results for nondiabetic wild-type mice. (B) DIO diabetic RAGE KO mice were much more resistant to *A. baumannii* bloodstream infection (2×10^7-CFU inoculum) than DIO diabetic wild-type mice ($n = 10$ mice per group). *, $P < 0.05$ versus the results for the control. (C) DIO diabetic RAGE KO mice are protected from cytokine storms that occur in DIO diabetic wild-type mice infected with *A. baumannii* (2×10^7-CFU inoculum) ($n = 10$ mice per group). Bars represent median values. *, $P < 0.05$ versus the results for the control. (D) During *A. baumannii* bacteremia, diabetic wild-type mice experienced significantly higher ratios of IL-10 to TNF than diabetic RAGE KO mice, similar to the nondiabetic wild-type mice in the experiment whose results are shown in Fig. 2C ($n = 10$ mice per group). *, $P < 0.05$.

RAGE signaling increases susceptibility to infection exclusively in diabetic mice. By eliminating RAGE signaling in diabetic mice, their susceptibility to infection was reverted to the level of nondiabetic mice. TLR4 and RAGE signaling converge at MyD88, and hence, diabetic mice experience dual activation of MyD88 from two distinct
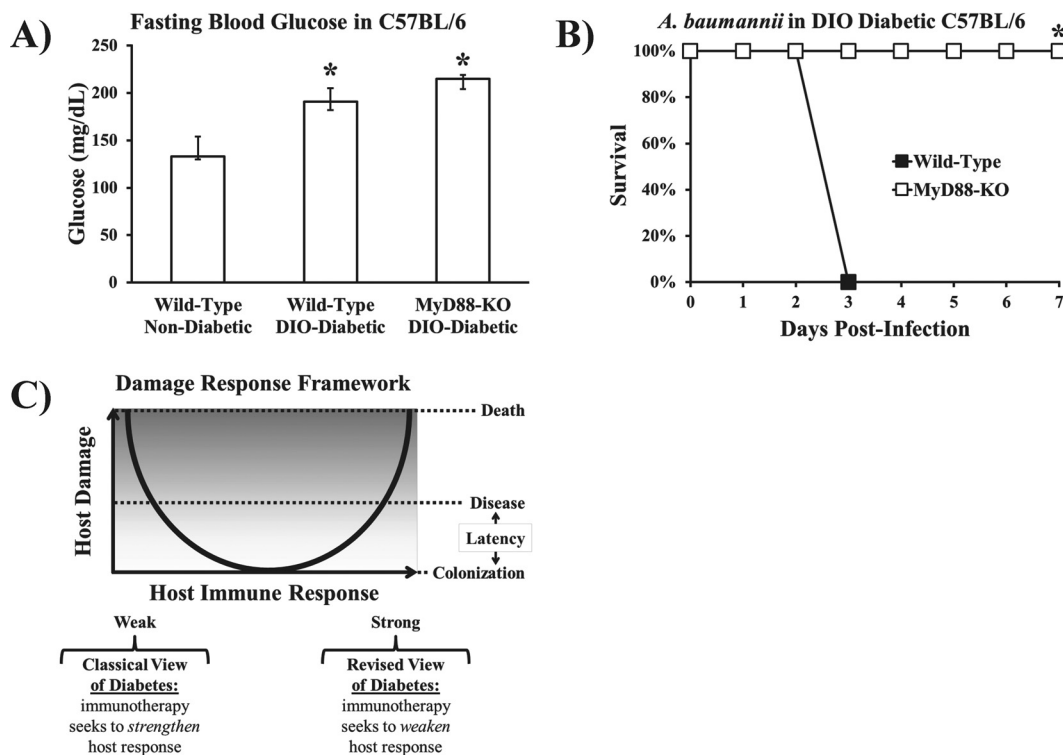
FIG 8 MyD88 disruption completely abrogated the susceptibility of diabetic mice to *A. baumannii* sepsis. (A) DIO diabetic wild-type and DIO diabetic MyD88 KO mice had significantly higher levels of fasting blood glucose than nondiabetic wild-type control mice ($n = 5$ mice per group). *, $P < 0.05$ versus the results for nondiabetic wild-type mice. Bar graph show median values and interquartile ranges. (B) DIO diabetic MyD88 KO mice were completely resistant to *A. baumannii* bloodstream infection (2×10^7-CFU inoculum), whereas all DIO diabetic wild-type mice died ($n = 5$ mice per group). *, $P < 0.05$ versus the results for the control. (C) Damage response framework basic curve, adapted from Casadevall and Pirofski (44) (reprinted from *Nature Reviews Microbiology* with permission of the publisher). The traditional view of diabetes is that it substantially weakens the host's immune response, predisposing the host to worse outcomes from infection. The current results indicate that diabetes predisposes to worse outcomes from infection by amplifying the innate immune/inflammatory response to Gram-negative bacterial infection.

receptors, whereas nondiabetic mice activate MyD88 through TLR4 alone. Our findings thus indicate that diabetic mice had increased mortality from infections caused by Gram-negative bacteria not because of immunosuppression but because of hyperinflammation driven by TLR4 and compounded by RAGE, both signaling through a common pathway via MyD88.

In 2003, Casadevall and Pirofski proposed the damage response framework model (Fig. 8C) (44). In short, the framework underscores that outcomes of infection depend on contributions from both the microbe and the host. Clinical symptoms and outcomes from infection can therefore result from excessive host inflammatory responses to infection. Diabetes has traditionally been viewed as an immunocompromising condition, causing suppression of phagocytic host defense mechanisms that lead to poor outcomes from infection, an interpretation that pins diabetic hosts to the weak-response side of the damage response framework. Instead, our results indicate that diabetes causes excessive host damage through hyperactive innate immune inflammation in response to Gram-negative bacteria, an interpretation that pins diabetic hosts to the (opposite) strong-response side of the damage response framework.

These results imply that efforts to stimulate the immune system will imperil rather than protect diabetic hosts from acute, Gram-negative bacterial infections. On the contrary, dampening the innate immune response to such infections offers a promising therapeutic approach to such infections in diabetic hosts. Specifically, antagonism of TLR4, RAGE, and MyD88 were each highly effective in protecting mice from lethal

infections in our model. Such a strategy has tremendous potential as adjunctive therapy by bolstering antibacterial agents, which alone are often inadequate against Gram-negative bacterial infections in patients with diabetes. Similar pathogenesis and potential therapies may also be relevant to other infections that are more common and more severe in those with diabetes.

MATERIALS AND METHODS

Bacterial and mouse strains. Strain HUMC1 is an *A. baumannii* clinical blood and lung isolate (from a patient with bacteremic ventilator-associated pneumonia) that is resistant to all antibiotics except for colistin and is highly virulent in animal models (25, 45, 46). Strain KPC KP1 is a panresistant (resistant to all antibiotics, including tigecycline and colistin) clinical bloodstream isolate of *K. pneumoniae*. Strain JJ1886 is a sequence type 131, ESBL-expressing, H30 strain-derived clinical isolate of *E. coli* (courtesy of James Johnson).

C57BL/6, C57BL/6 congenic TLR4 KO (*Tlr4$^{1ps-del}$*), C57BL/6 congenic MyD88 KO (*Myd88$^{tm1.1Defr}$*), C3HeB/Fe, and C3H/He (*Tlr4$^{1ps-def}$*) mice were purchased from Jackson Laboratories. DIO diabetic mice were fed a high-fat diet consisting of 60% calories from fat (D12492; Research Diets). Ann Marie Schmidt's laboratory graciously provided us with RAGE KO mice (47).

Infection models. We used our well-established tail vein infection method as the model for bacteremia (25, 45, 46). In this model, a relatively low inoculum (2×10^7 to 4×10^7 CFU) of *A. baumannii* results in lethal infection in susceptible mice, and the model mimics the second most common clinical *A. baumannii* syndrome (bacteremia), typically initiated by a catheter. Streptozotocin was administered as previously described (46). Some mice were treated with a parenteral competitive TLR4 antagonist, E5564 (supplied by Eisai, Inc.), starting on the day of infection. Based on pilot studies, the drug was dosed at 10 mg/kg, administered i.v. once daily for 4 days when treating i.v. infections.

Bacteria were grown overnight in tryptic soy broth (TSB) at 37°C with shaking. The bacteria were passaged to mid-log growth phase in TSB at 37°C with shaking. Cells were washed twice with phosphate-buffered saline (PBS) and resuspended at the appropriate concentration for infection. Bacteria were administered i.v. via the tail vein. All animal experiments were approved by the Institutional Committees on the Use and Care of Animals (IACUC) at the Los Angeles Biomedical Research Institute and the Keck School of Medicine at the University of Southern California, following the National Institutes of Health guidelines for animal housing and care (48).

Growth curve in serum. Mice were sedated with 100 mg/kg ketamine and 10 mg/kg xylazine. Serum was isolated from blood obtained by cardiac puncture. *A. baumannii* HUMC1 was added to 300 μl serum at 1×10^4 CFU/ml and placed on a rotator in a humidified 37°C incubator. Aliquots were taken at 0, 1, 3, and 24 h postinoculation and then diluted in PBS and plated on tryptic soy agar (TSA) to count CFU.

Cytokine and sepsis biomarkers. Serum cytokines were quantified by using the MSD Multi-Spot assay (Meso Scale Diagnostics, LLC) according to the manufacturer's instructions. Blood sepsis biomarkers (pH, base deficit, and bicarbonate) were analyzed using the i-STAT system. For experiments in which i-STAT measurements were made, it was necessary to treat mice with intraperitoneal heparin (25 U; Sigma-Aldrich, St. Louis, MO) with simultaneous sedation by intraperitoneal ketamine (100 mg/kg) and xylazine (10 mg/kg) prior to cardiac puncture to prevent clotting in the i-STAT cartridges. Whole blood was drawn into a 25-gauge syringe and aliquoted into i-STAT cartridges. Values were read on an i-STAT portable clinical analyzer.

Temperatures were measured rectally using a digital thermometer (model BAT-12; Physitemp Instruments, Inc.).

Statistics. Survival was compared by the nonparametric log rank test. Categorical variables were compared with the Wilcoxon rank sum test for unpaired comparisons.

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