Acinetobacter baumannii phenylacetic acid metabolism influences infection outcome through a direct effect on neutrophil chemotaxis

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Edited by Ralph R. Isberg, Howard Hughes Medical Institute, Tufts University School of Medicine, Boston, MA, and approved June 27, 2016 (received for review November 23, 2015)

Innate cellular immune responses are a critical first-line defense against invading bacterial pathogens. Leukocyte migration from the bloodstream to a site of infection is mediated by chemotactic factors that are often host-derived. More recently, there has been a greater appreciation of the importance of bacterial factors driving neutrophil movement during infection. Here, we describe the development of a zebrafish infection model to study *Acinetobacter baumannii* pathogenesis. By using isogenic *A. baumannii* mutants lacking expression of virulence effector proteins, we demonstrated that bacterial drivers of disease severity are conserved between zebrafish and mammals. By using transgenic zebrafish with fluorescent phagocytes, we showed that a mutation of an established *A. baumannii* global virulence regulator led to marked changes in neutrophil behavior involving rapid neutrophil influx to a localized site of infection, followed by prolonged neutrophil dwelling. This neutrophilic response augmented bacterial clearance and was secondary to an impaired *A. baumannii* phenylacetic acid catabolism pathway, which led to accumulation of phenylacetate. Purified phenylacetate was confirmed to be a neutrophil chemoattractant. These data identify a previously unknown mechanism of bacterial-guided neutrophil chemotaxis in vivo, providing insight into the role of bacterial metabolism in host innate immune evasion. Furthermore, the work provides a potentially new therapeutic paradigm of targeting a bacterial metabolic pathway to augment host innate immune responses and attenuate disease.

Acinetobacter baumannii | zebrafish | neutrophils | chemotaxis | phenylacetate

The opportunistic Gram-negative bacterium, *Acinetobacter baumannii*, is now threatening our current antimicrobial armamentarium. This bacterium has a particular predilection for infecting patients with compromised innate immune defenses such as those in intensive care units, where it is responsible for a diverse range of infections including ventilator-associated pneumonia, bacteremia, and urinary tract, skin, and wound infection (1). Despite an increase in *A. baumannii* infections and outbreaks in health care facilities (1), relatively little is known about its pathogenesis. As with other Gram-negative bacteria, *A. baumannii* lipopolysaccharide plays a critical role in immune stimulation, particularly through Toll-like receptor 4 (TLR4) and CD14, leading to the production of the neutrophil chemotactic factor IL-8 (2) and the proinflammatory cytokine TNF-α (3). Mice lacking TLR4 and CD14 are more susceptible to *A. baumannii* pulmonary infection (3). Neutrophil recruitment is also critical, with neutrophil depletion being associated with more severe disease and greater bacterial burdens and dissemination (4, 5). The specific drivers of neutrophil trafficking during *A. baumannii* infection have not yet been defined.

Fundamental advances in the understanding of host–pathogen interactions have recently emerged through the use of the vertebrate model system *Danio rerio* (zebrafish) (6). The zebrafish is a tropical freshwater fish that has a remarkably similar immune system to humans, with elaborate innate and adaptive immune response pathways (7). As embryos, zebrafish rely solely on innate immune defenses, including soluble components such as opsonins, cytokines, chemokines, and complement, as well as the innate cellular repertoire of antigen presenting and phagocytic cells (7, 8). The establishment of a functional adaptive immune system is delayed to approximately 3 wk postfertilization (7). This distinction in immune development makes zebrafish embryos ideally suited to study host innate immune responses to bacterial pathogens (9, 10). High-resolution, real-time imaging enables the mechanistic dissection of how host innate immune cells migrate and respond to a bacterial pathogen in vivo (6). For example, work in zebrafish has led to a paradigm change in the understanding of the importance of macrophages harboring and disseminating mycobacterial infection (11, 12), and the conserved role for nerve growth factor β and its receptor tyrosine kinase TrkA signaling in pathogen-specific host immunity against *Staphylococcus aureus* (13).

In the present study, we exploited the advantages of zebrafish to better understand *A. baumannii* pathogenesis and interrogated

Significance

***Acinetobacter baumannii* is one of the most significant hospital-acquired bacterial pathogens, able to cause life-threatening infections and develop resistance to all currently available antibiotic agents. Here, we established zebrafish as a model to study real-time interactions between innate immune cells and *A. baumannii* during infection. We identified a bacterial metabolic pathway that, when inhibited, leads to enhanced immune responses toward the bacteria, improving bacterial clearance and reducing severity of disease. The enhanced immune response was secondary to accumulation of a metabolic by-product, which acted as a direct, bacterial-mediated attractant of neutrophils, the key immune cell important in response to bacterial infections. These results pave the way for novel therapeutic targeting of bacterial metabolism to stimulate immune responses to fight off infection.**

Author contributions: M.S.B., G.J.L. and A.Y.P. designed research; M.S.B., F.E., X.K., G.M.C., and M.H.M.M. performed research; F.E., G.L.M., G.M.C., K.E.S., M.H.M.M., J.L., D.J.C., G.J.L., and A.Y.P. contributed new reagents/analytic tools; M.S.B., F.E., G.L.M., X.K., D.J.C., G.J.L., and A.Y.P. analyzed data; and M.S.B., G.L.M., and A.Y.P. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1523116113/-/DCSupplemental.

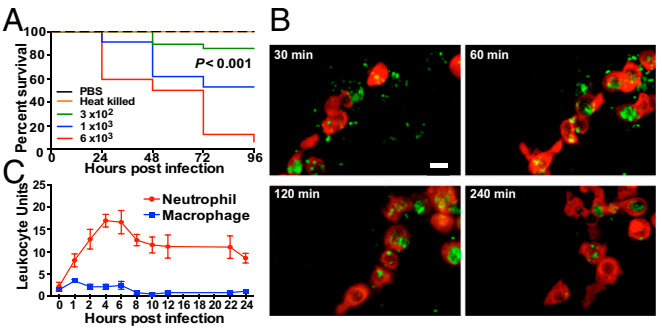

Fig. 1. *A. baumannii* infection in zebrafish. (*A*) Survival after bloodstream infection with live *A. baumannii* ($n = 30$ embryos, three biological replicates; *P* value is a comparison of 3×10^2 and 1×10^3 cfu per embryo by log-rank test). (*B*) Time-lapse confocal scanning laser microscopy showing red neutrophils [Tg(*lyz*:DsRed)^{nz50}] phagocytosing and clearing *A. baumannii*–GFP. (Scale bar: 20 μm.) (*C*) LUs after infection into the somatic muscle of zebrafish after adjustment for trauma (PBS solution injection; mean ± SEM, $n = 5$ per experiment, three biological replicates).

bacterial factors that determine neutrophil behavior in vivo. We showed that *A. baumannii* causes a lethal systemic infection in zebrafish, and that bacterial drivers of disease are conserved between zebrafish and mammals. Neutrophils were the dominant phagocyte responders to *A. baumannii* infection, and we identified that the *A. baumannii* phenylacetic acid catabolism pathway is an important mechanism used by the bacteria to assist in immune evasion. Loss of function of this metabolic pathway led to by-product accumulation dominated by phenylacetate (PA), which acted as a potent bacterial chemoattractant. These data identify a previously unknown mechanism of bacterial-guided neutrophil behavior in vivo and provide an example of the interaction between bacterial metabolism and host immunology.

Results

Systemic Infection with Live *A. baumannii* Causes Lethal Disease in Zebrafish. One of the most life-threatening forms of *A. baumannii* disease is bloodstream infection (1). As *A. baumannii* is not known to be a natural zebrafish pathogen, we assessed its capacity to cause disease in zebrafish embryos. A reference hospital-acquired *A. baumannii* strain [American Type Culture Collection (ATCC) 17978] was inoculated into the circulation of embryos 48 h postfertilization (hpf). As few as 300 cfu of bacteria caused lethal disease, with mortality dependent on the infecting inoculum (Fig. 1*A*). Similar findings were observed for other clinical *A. baumannii* strains (AB307, A9844, AB0059, ATCC 19606) (Fig. S1 *A*–*D*). Neither injection of washed, heat-killed bacteria (Fig. 1*A*) nor culture filtrates from exponential or stationary-phase growth caused disease. These data show that live bacterial cells are required for *A. baumannii* pathogenesis in zebrafish.

To determine the ability of zebrafish to mount an effective response to *A. baumannii* bloodstream infection, we performed in vivo bacterial growth kinetic studies after infection with 1×10^3 cfu of *A. baumannii* per embryo (Fig. S1*E*). The bacterial burden increased to 20 h postinfection (hpi) but then subsided by 44 h to undetectable levels (Fig. S1*E*), suggesting that zebrafish can mount an innate immune response that clears an initially expanding bacterial population. Conversely, after inoculation of *A. baumannii* into the zebrafish yolk sac, a site devoid of innate immune cells (14), zebrafish were hypersusceptible to lethal disease (Fig. S1*F*), further supporting a role of innate immune responses in the defense against *A. baumannii*.

Protective Phagocyte Responses to *A. baumannii* Infection in Zebrafish. In mammals, neutrophils play an important role in response and clearance of *A. baumannii* infection (4, 5). To examine phagocyte–*A. baumannii* interactions in zebrafish, we used transgenic embryos with fluorescently labeled leukocytes. Localized infection with GFP-expressing bacteria (*A. baumannii*) was established within somatic muscle. Within minutes of inoculation, neutrophils migrated to the site of *A. baumannii* infection, where they accumulated, phagocytosed bacteria, and then migrated away, clearing the bulk of infection by 4 h (Fig. 1*B* and Movie S1). Histopathology of the infection site confirmed neutrophilic phagocytosis of bacteria (Fig. S1*G*). Macrophages, which have also been shown to be important first responders to *A. baumannii* infection in mammals (15), were also recruited to the infection site but appeared to have less phagocytic activity (Movie S2), and similar numbers were seen with a tissue trauma control (PBS solution injection) (Fig. 1*C*). These data highlight the specificity of neutrophils in responding to pathogen-associated factors and macrophages in responding to tissue damage.

Within neutrophils, pathogen degradation typically occurs by hydrolytic enzymes within acidic phagolysosomes, whereas the remainder of the cytoplasm remains at neutral pH (16). To characterize the intracellular handling of *A. baumannii* following phagocytosis by neutrophils in vivo, we labeled *A. baumannii* with pHrodo-dextran, which emits red fluorescence only under low pH conditions. In Tg(*mpx*:GFP) zebrafish with green fluorescent neutrophils, red pHrodo-dependent fluorescence emerged within cytoplasmic vacuoles at 50 min postinfection (Fig. 2*A*, Fig. S2*A*, and Movie S3), confirming that intracellular handling and degradation of *A. baumannii* following neutrophil phagocytosis is within acidic phagolysosomes.

To further characterize the functional role of leukocytes in *A. baumannii* infection, we depleted zebrafish of neutrophils by knocking down the gene encoding the zebrafish ortholog of the granulocyte colony stimulating factor receptor [colony stimulating factor 3 receptor (*csf3r*)] (17). This led to greater susceptibility of zebrafish embryos to lethal *A. baumannii* infection (Fig. 2*B*). In contrast, *irf8* knockdown, which depletes macrophages but expands the neutrophil population (18), did not impact on *A. baumannii* infection survival (Fig. 2*C* and Fig. S2 *B* and *C*). These data suggest a greater functional requirement for neutrophils than macrophages in controlling *A. baumannii* infection.

Bacterial Determinants of Virulence in Mammals Are Conserved in Zebrafish. Although a number of bacterial virulence mechanisms are host-specific (19), some are likely conserved across a range of higher-order species, including insects, fish, and mammals (20). To assess the predictive potential of zebrafish for *A. baumannii* virulence, we first assessed *A. baumannii* mutants with deletions in genes that encode a sensor histidine kinase (Δ*gacS*) and a response regulator (Δ*gacA*), both of which have displayed marked attenuation for virulence in a murine septicemia model (21). *A. baumannii* Δ*gacS* and Δ*gacA* were significantly attenuated for virulence compared with wild-type *A. baumannii* in a zebrafish bloodstream infection model (Fig. 3*A*).

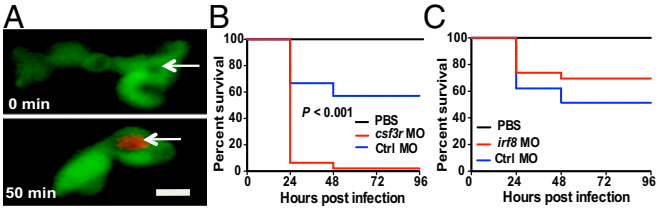

Fig. 2. Neutrophils are indispensible for zebrafish survival from *A. baumannii* infection. (*A*) Phagocytosed *A. baumannii* (pHrodo-dextran stain, white arrow) is handled within acidic phagolysosomes within a neutrophilic vacuole at 50-min postinfection [Tg(*mpx*:GFP)^{i114} embryos with green fluorescent neutrophils were used]. (Scale bar: 20 μm.) Survival of neutrophil-depleted embryos (*csf3r* MO) (*B*) or macrophage-depleted embryos (*irf8* MO) (*C*) after bloodstream infection with *A. baumannii*. Comparison is made vs. *A. baumannii*-infected embryos injected with control morpholino (Ctrl MO; $P < 0.001$; $n = 30$ embryos, three biological replicates).

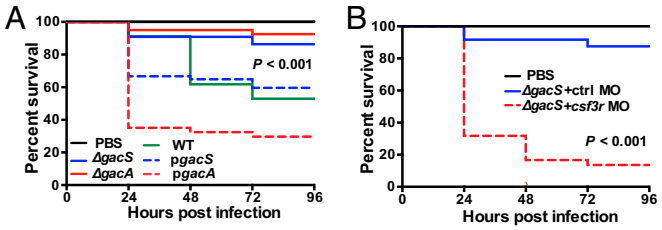

Fig. 3. *A. baumannii* virulence is conserved between zebrafish and mammals. (*A*) Zebrafish survival after bloodstream infection with *A. baumannii* mutants defective in virulence regulation (Δ*gacS* and Δ*gacA*) and corresponding complemented strains (p*gacS* and p*gacA*; *P* value is comparison between Δ*gacS* and p*gacS*; $n = 30$ embryos, three biological replicates). (*B*) Neutrophil depletion using a *csf3r* morpholino (MO) restored the virulence of *A. baumannii* Δ*gacS* compared with zebrafish injected with a control morpholino (Ctrl MO; $P < 0.001$).

Complementation by expressing the full-length copies of the deleted genes restored virulence to wild-type *A. baumannii* levels (Fig. 3*A*). Moreover, neutrophil depletion using the *csf3r* morpholino restored the virulence capabilities of *A. baumannii* Δ*gacS* (Fig. 3*B*), suggesting that the virulence attenuation of this mutant was neutrophil-dependent.

Quorum-sensing is an evolutionary conserved signaling mechanism between cells within a population that facilitates the control of growth, nutrient acquisition, virulence, and other biological processes in bacteria (22, 23). Quorum sensing is yet to be established as a virulence factor in *Acinetobacter*. We tested an *Acinetobacter* M2 mutant with a deletion of the autoinducer synthase gene (*abaI*), which is necessary for 3-hydroxy-C_{12}-homoserine lactone production (24). Compared with wild-type *Acinetobacter* M2 infection, zebrafish infected with *Acinetobacter* Δ*abaI* had significantly greater survival (Fig. S2*D*). Virulence was partly restored with complementation. These findings were then corroborated in a mammalian septicemia model (Fig. S2*E*). Together, these data show that diverse intrinsic *Acinetobacter* virulence determinants are shared for infections in zebrafish and mammals, and support the use of zebrafish to study established, as well as new, mechanisms of *Acinetobacter* disease.

An *A. baumannii* Global Virulence Regulator Drives Extravascular Neutrophil Migration Patterns During Infection. We have shown that neutrophils orchestrate a coordinated response to a localized *A. baumannii* infection including rapid migration to the site of infection, phagocytosis, and bacterial processing within phagolysosomes, followed by migration away. Thus far, infection model studies have concentrated on host-derived factors that determine neutrophil chemotaxis such as IL-8, leukotriene B4, C5a, and hydrogen peroxide (2, 10, 25, 26). Less is known about bacterial factors that directly drive neutrophil migratory patterns and trafficking. During localized tissue infections into the zebrafish somatic muscle, we observed a difference in neutrophil behavior with the *A. baumannii* Δ*gacS* mutant. Although the neutrophils migrated to the infection site and phagocytosed bacteria, they failed to migrate away as occurred in wild-type *A. baumannii* infection (Fig. 4*A* and Movie S4). This resulted in persisting clusters of neutrophils at the infection site until 48 hpi (Fig. 4*B* and Fig. S3*A*). Importantly, complementation of Δ*gacS* restored neutrophil migratory patterns to those of wild-type (Fig. 4*B*). As a control, other virulence-attenuating *A. baumannii* deletion mutants (Δ*abaI* and Δ*argH*) were tested but showed wild-type neutrophil migratory patterns (Fig. 4*B*), highlighting the specificity of this response to *A. baumannii* Δ*gacS*. To control for site of infection, we also infected zebrafish in the otic vesicle, which, under normal conditions, is devoid of neutrophils, but is another established site for studying a localized infection. Similar findings were observed (Fig. 4*C* and Fig. S3*B*).

To characterize neutrophil dynamics in more detail, we used time-lapse confocal microscopy and 3D cell tracking software to quantify neutrophil migratory parameters (Fig. 4*D* and Fig. S3*C*). Following infection with wild-type *A. baumannii*, neutrophils migrated to and from the site of infection consistently during the 5-h observation period (Fig. 4*D*). However, following infection with *A. baumannii* Δ*gacS*, there was intense neutrophil migration to the site of infection during the first 75 min, followed by minimal further neutrophil ingress or egress for the remainder of the observation period (Fig. 4*D*). To determine if this neutrophil response coincided with increased host-derived chemotactic stimuli, we quantified at 1 h and 12 h after tail muscle infection the expression of mRNA encoding the chemokine IL-8, which is an established host-derived chemotaxin in tissue infection (2), and leukotriene A4 (LTA4) hydrolase that converts LTA4 to LTB4 (27). The expression of both mRNAs increased with infection, but no difference was seen between wild-type and *A. baumannii* Δ*gacS* (Fig. S4*A*). Collectively, these data indicate the presence of a strong and persistent chemoattractant in *A. baumannii* Δ*gacS* infection driving vigorous neutrophil influx followed by migratory arrest, and opens the possibility that this is bacterially derived.

Neutrophil Dwelling in *A. baumannii* Infected Tissue Is Mediated by an Impaired Phenylacetic Acid Catabolic Pathway. *A. baumannii* GacS is a global virulence regulator, controlling 674 genes across diverse functions (21). Interestingly, after deletion of *A. baumannii gacS*, the genes with greatest repression (as much as 200-fold) belonged to a single operon made up of 15 coding sequences known as the *paa* operon (21). The function of this operon is to degrade aromatic compounds such as PA, which is derived from phenylalanine, to form acetyl- and succinyl-CoA used in the TCA cycle (28). We therefore hypothesized that *A. baumannii* Δ*gacS* is altering neutrophil responses through repression of the *paa* operon, leading to accumulation of aromatic compounds and subsequent bacterial-driven chemoattraction.

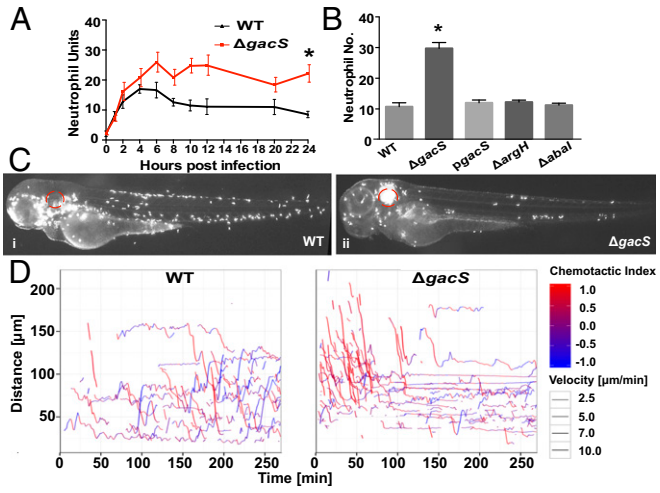

Fig. 4. Altered in vivo neutrophil migratory patterns during *A. baumannii* infection. (*A*) Localized somatic muscle infection with *A. baumannii* Δ*gacS* led to greater neutrophil units at the site of infection and a loss of migration away compared with wild-type *A. baumannii* infection (mean ± SEM from three biological replicates). (*B*) Neutrophil units at the site of a localized somatic muscle infection 48 hpi showed restoration of neutrophil numbers with *gacS* complementation (p*gacS*) and no abnormalities with other *A. baumannii* virulence-associated mutants (Δ*argH*, Δ*abaI*; mean ± SEM, three biological replicates). (*C*) Infection of the otic vesicle (red circle) with wild-type *A. baumannii* (*i*) and Δ*gacS* (*ii*) imaged at 48 hpi. (Magnification: 10×.) (*D*) Cell tracking analysis of neutrophil movement after infection with WT and Δ*gacS* strains. A chemotactic index (red, movement toward infection; blue, movement away) and velocity (thickness of the line) are indicated (representative experiment from five biological replicates). Asterisks in *A* and *B* denote comparison between wild-type *A. baumannii* and Δ*gacS* (*$P \leq 0.05$).

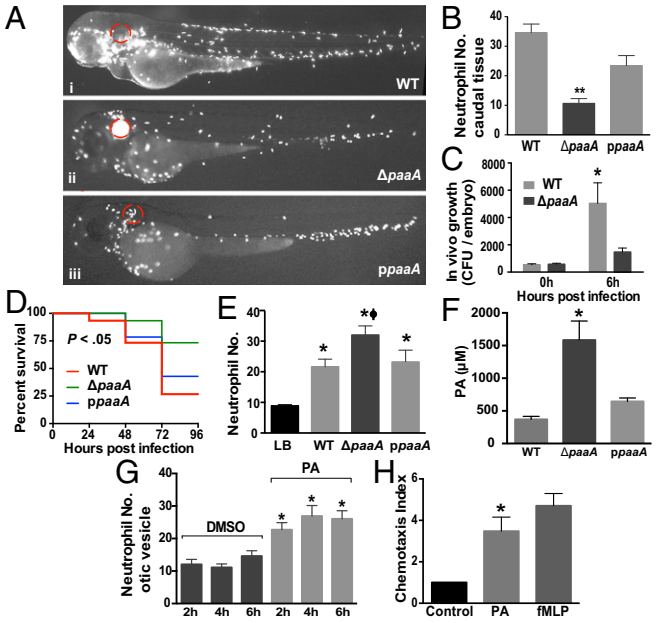

Fig. 5. PA is a bacterial-driven chemoattractant. (*A*) Neutrophil accumulation imaged 48 hpi of the otic vesicle (red circle). (*B*) The observed neutrophil clustering was associated with a reduction in neutrophils normally resident in the caudal hematopoietic tissue assessed at 48 h. The functional effect of this neutrophilic response to *A. baumannii* Δ*paaA* infection was a reduced bacterial burden (*C*) and increased zebrafish survival (*D*) compared with wild-type *A. baumannii* and the complemented strain (p*paaA*; *P* value is a comparison of Δ*paaA* and p*paaA* by log-rank test). (*E*) Attraction of neutrophils to the otic vesicle 6 hpi of culture filtrate. (*F*) Concentration of PA in culture filtrates. (*G*) Injection of purified PA into the otic vesicle was sufficient to attract a greater number of neutrophils within 2 h of injection compared with PBS solution. (*H*) Neutrophil chemotaxis toward PA (0.05 M) in an ex vivo neutrophil transwell migration assay using mouse-derived neutrophils. fMLP was used as a positive control. For all experiments, column bars represent the mean ± SEM, performed at least in triplicate, and the asterisks denote comparison between wild-type *A. baumannii* and Δ*paaA* for *B*, *C*, and *F*, between LB and wild-type, Δ*paaA*, and p*paaA* for *E*, and between PA and PBS control for *G* and *H*; letter ϕ denotes comparison between wild-type and Δ*paaA* for *E* by one-way ANOVA (Kruskal–Wallis test, $*P \leq 0.05$, $**P < 0.01$, and $^{\Phi}P \leq 0.05$). (Magnification: 10×.)

To test whether the Δ*gacS*-mediated neutrophil response is caused by loss of the PA catabolic pathway, we induced a localized infection in the zebrafish otic vesicle with an early PA pathway *A. baumannii* mutant (Δ*paaA*). As shown in Fig. 5*A*, neutrophil responses were the same as that seen for *A. baumannii* Δ*gacS* (Fig. 4*C*), with prolonged neutrophil dwelling at the site of infection compared with wild-type *A. baumannii*. The phenotype was restored to wild-type levels with complementation (p*paaA*) (Fig. 5*A*). Consistent with there being neutrophil relocation and clustering in the otic vesicle and not local neutrophil proliferation, we documented that neutrophil aggregation in the otic vesicle during *A. baumannii* Δ*paaA* infection was concurrent with reduction of neutrophils normally resident in the caudal hematopoietic tissue (Fig. 5*B*). Highlighting the specificity of this neutrophilic response to *A. baumannii* Δ*paaA*, we infected zebrafish with another isogenic *A. baumannii* mutant with a deletion in a *GacS*-regulated gene *csuD* (21). No difference in neutrophil trafficking compared with wild-type was seen (Fig. S4*B*).

To determine the functional impact of this neutrophil dwelling response on bacterial survival, we assessed bacterial densities at 6 hpi for wild-type *A. baumannii*, Δ*paaA*, and complement strains (all grow similarly in vitro). Significantly fewer bacteria were found for Δ*paaA* (Fig. 5*C*), and this was associated with attenuated virulence for the Δ*paaA* mutant compared with wild-type and complement strains (Fig. 5*D*). Together, these data indicate that the *A. baumannii* PA catabolic pathway is important in mediating neutrophil chemotaxis and tissue responses to acute infection, and may be a mechanism by which *A. baumannii* uses for immune evasion and disease progression.

PA Mediates Neutrophil Chemotaxis. To confirm whether a metabolic by-product was accumulating in the tissues after infection with *A. baumannii* Δ*paaA*, we first injected the otic vesicle of zebrafish with *A. baumannii* culture filtrate taken from stationary-phase in vitro growth (Fig. 5*E*). Neutrophil chemotaxis and dwelling occurred with supernatant taken from *A. baumannii* Δ*paaA*, whereas neutrophil chemotaxis was less with supernatant from wild-type and complemented *A. baumannii* strains (Fig. 5*E*). Interestingly, injection of washed, heat-killed *A. baumannii* Δ*paaA* caused neutrophil chemotaxis, but neutrophil dwelling was not observed (Fig. S4*C*), indicating that metabolism from live bacteria is required for the production of the causative factor.

To identify the molecule responsible for the observed neutrophil responses, we performed untargeted global metabolomics by MS on culture filtrate taken from wild-type *A. baumannii*, Δ*paaA* and the complemented strain. In total, more than 1,600 metabolite features were detected, and the only metabolite that showed a significant accumulation (more than twofold; $P < 0.05$) in *A. baumannii* Δ*paaA* compared with the wild-type and complemented strains was putatively identified as PA (Fig. S5 and Dataset S1). Subsequent identification and accurate quantification of PA by using targeted liquid chromatography (LC)/MS confirmed fourfold accumulation of PA in Δ*paaA* supernatant compared with wild-type (Fig. 5*F*). PA is an intermediate product of the bacterial phenylalanine metabolic pathway, and a direct substrate of *paaA*. To confirm that PA was the causative driver of the observed neutrophil tissue responses, we injected purified PA (Sigma) into the otic vesicle of zebrafish at varying concentrations. This showed a bell-curve dose–response relationship characteristic of chemoattractants (29), with a concentration as low as 3.4 ng per embryo having an effect, and peaking at 37 ng per embryo (Fig. S6*A*). Neutrophil migration was seen within 2 hpi (Fig. 5*G*). When purified PA was added to wild-type culture filtrate and injected into the otic vesicle of zebrafish, we observed the same neutrophil trafficking characteristics as observed for infection with *A. baumannii* Δ*paaA* (Fig. S6*B*). Furthermore, the addition of purified PA to wild-type *A. baumannii* cells before infection led to greater embryo survival compared with infection with wild-type *A. baumannii* alone (Fig. S6*C*). Finally, to determine if PA was acting directly on neutrophils rather than through stimulation of endogenous cytokines, and to assess whether this effect was observed with mammalian neutrophils, we performed ex vivo neutrophil migration assays by using murine bone marrow-derived neutrophils. Purified PA caused significantly greater neutrophil transmigration compared with the control (Fig. 5*H*). Together, these data confirm that, in the absence of a functional PA catabolic pathway, PA accumulates with live bacterial cells and then acts as a bacterial-driven neutrophil chemoattractant.

Discussion

A. baumannii continues to be a problematic Gram-negative bacterial pathogen within health care institutions, with reports of pandrug resistance threatening our modern-day antibiotic armamentarium. As a consequence, we urgently require an improved understanding of the interactions between *A. baumannii* and the host during acute infection to provide the foundations for future therapeutic strategies. We developed a zebrafish model to study the molecular mechanisms of *A. baumannii*–host interactions. Through use of real-time fluorescent microscopy and cell tracking, we demonstrated an interaction between bacterial metabolism and host innate immune response. Inhibition of the *A. baumannii* phenylacetic acid catabolism pathway led to the accumulation of PA as a metabolic by-product, which was shown to be a direct bacterial-driven chemoattractant, causing

neutrophils to avidly migrate to the site of infection and dwell. The functional impact of this pronounced neutrophilic response was a reduction in bacterial burden and attenuated disease. Targeting a metabolic pathway with the intention of augmenting host innate immune response provides a potentially new paradigm for the treatment of challenging superbugs such as *A. baumannii*.

Zebrafish have now been used as a model to study the pathogenesis of diverse infectious diseases (9, 30–33). Bacteria such as *Mycobacterium marinum*, *Salmonella enterica* serovar Typhimurium, *S. aureus*, and *Shigella flexneri* have all displayed inoculum-dependent acute lethality in zebrafish, with survival correlating with bacterial growth in the host. By testing defined *A. baumannii* mutants, we were able to show that, for some virulence factors, virulence in zebrafish correlated with that observed in a mammalian model. *A. baumannii* GacS is a two-component signal transduction system that is essential for disease in a mouse septicemia model (21); likewise, mutants in this system were highly attenuated for disease in zebrafish. We also showed that quorum sensing is required for *A. baumannii* virulence in zebrafish and a mammalian host. These data support the utility of zebrafish as a facile, in vivo model for the study of new, as well as established, bacterial virulence mechanisms, and strengthen the idea that conservation of bacterial virulence may exist across a range of multicellular eukaryotic species.

Zebrafish and humans share very similar immune systems in terms of cellular anatomy and genetic regulation (6). Young zebrafish embryos (< 3 wk post fertilization) rely solely on innate immunity, which makes them ideal for the study of initial host responses to bacterial pathogens. By using several lines of evidence, we showed that innate immune cells, particularly neutrophils, were indispensible for zebrafish survival from *A. baumannii* infection. A lower inoculum of *A. baumannii* administered i.v. was cleared by zebrafish after ∼44 h, whereas administration of the same dose into the yolk sac (devoid of phagocytic cells) was lethal. In a localized somatic muscle infection, neutrophils predominated as infection-specific first responders and showed greater phagocytic function than macrophages. Finally, selective depletion of zebrafish neutrophils increased susceptibility to acute lethal infection, whereas depletion of macrophages had no significant effect. Neutrophil depletion also restored the virulence of bacterial mutants that were attenuated for disease. These data support findings observed in mammalian hosts whereby depletion of neutrophils exacerbated *A. baumannii* infection (5, 34), whereas macrophage depletion had a limited impact on disease (15).

An impressive feature of zebrafish is the ability to visualize real-time infection dynamics at the cellular level, made possible by their optical transparency and the availability of well-characterized transgenic reporter fish lines that selectively fluoresce host innate immune cells. By using fluorophore-marked bacteria, we used high-resolution, time-course imaging experiments to delineate individual leukocyte responses to *A. baumannii* invasion in vivo, and, more specifically, study the drivers of neutrophil migration during an acute infection. For one of our bacterial mutants (Δ*gacS*), we observed that neutrophils avidly migrated to the site of infection and then failed to migrate away as seen with wild-type bacterial infection. By using a series of isogenic *A. baumannii* mutants, we showed that a mutant in the phenylacetic acid catabolism pathway mirrored the phenotype observed with Δ*gacS*. Δ*paaA* culture filtrate alone was sufficient to replicate the phenotype, but this was abolished with infection of washed, heat-killed Δ*paaA* cells, both suggesting that a bacterial-driven metabolic by-product was responsible for the altered neutrophil behavior.

Bacterial-guided leukocyte chemotaxis has thus far been well described for molecules such as LPS, lipopeptides, peptidoglycan, flagellin, and nucleic acids (35). However, these microorganism-associated molecular patterns lead to leukocyte recruitment indirectly via Toll-like receptors and activation of chemokines (e.g., IL-8). Very little is known about directly acting bacterial factors that drive neutrophil recruitment and behavior. By using global metabolomic analyses of culture supernatants, we showed that PA was significantly and specifically increased for the Δ*paaA* mutant compared with supernatants taken from wild-type and complemented bacteria. To confirm the independent effect of this metabolite on neutrophil migration, purified PA was injected into the otic vesicle of zebrafish. Neutrophil chemotaxis and dwelling was observed. Purified PA was also sufficient to recreate the phenotype when added to culture supernatant from wild-type bacteria. These observations were then extended using ex vivo mammalian neutrophils, which showed that purified PA was independently capable of inducing neutrophil recruitment. Together, these data confirmed the role of PA as a direct bacterial-driven chemoattractant for zebrafish and mammalian neutrophils.

The role of PA in bacterial metabolism has not been fully elucidated. In *Burkholderia cenocepacia*, a Gram-negative pathogen related to *A. baumannii*, deletion of genes in the phenylalanine degradation pathway (*paaABCDE*) led to accumulation of phenylacetic acid and inhibited bacterial quorum sensing (36). Virulence attenuation has also been shown with disruption of the *paa* catabolic pathway; however, the mechanism of this remains unclear (37). Here we propose a mechanism that is driven by augmentation of host innate immune response. By deleting the *A. baumannii* phenylacetic acid catabolism pathway, an accumulation of PA ensues that enhances neutrophil migration and clustering at the site of infection, leading to greater bacterial killing and improved host survival. The significance of these data are that inhibition of this bacterial metabolic pathway could form a novel adjunctive therapy. The global emergence of antimicrobial resistance has led to a desperate need in identifying antimicrobials with new mechanisms of action. Augmentation of host immune responses to a bacterial pathogen could provide an alternative treatment strategy in the face of extreme drug-resistant and pandrug-resistant Gram-negative bacteria such as *A. baumannii*.

Methods and Materials

Bacterial Strains and Growth Conditions. *A. baumannii* strains (Table S1) were grown in LB liquid or solid media at 37 °C supplemented with carbenicillin (150 μg/mL) or kanamycin (50 μg/mL) as appropriate. GFP expression and complementation of *Acinetobacter* M2 Δ*abaI* and ATCC 17978Δ*paaA* were performed by using pWH1266 as described elsewhere (38). Primers used for this study are shown in Table S2.

Zebrafish Strains and Maintenance. Wild-type Tübingen, Tg(*lyz*:DsRed)^{nz50} (39), Tg(*mpeg1*:mCherry)^{gl23} (40), Tg(*mpx*:GFP)^{i114} (41), and Tg(*mpeg1*:Gal4FF)^{gl25} × Tg(UAS-E1b:Eco.*NfsB*-mCherry)^{c264} zebrafish embryos were maintained in the Monash University FishCore facility according to standard protocols (*SI Materials and Methods*).

Microinjection of *A. baumannii* into Zebrafish Embryos. Overnight bacterial cultures were adjusted to OD_{600} of 1, washed, and resuspended in 2% (wt/vol) polyvinylpyrrolidone in PBS solution. Microinjections were performed at 48 hpf, and all inocula were confirmed by colony counts (42, 43). Embryos were monitored daily for survival to 96 hpi, and survival analyses were performed by using the Kaplan–Meier method. The Mantel–Cox test was used for statistical significance ($P < 0.05$). A range of 500–800 cfu of bacteria were injected into the muscle or otic vesicle for neutrophil and macrophage migration assays. Methods for in vivo *A. baumannii* growth kinetics and quantitative real-time PCR are shown in *SI Materials and Methods*.

Morpholino Depletion of *cfs3r* and *irf8*. Antisense morpholino oligomers (MOs) directed at *csf3r* (30) and *irf8* (18) were purchased from Gene Tools. Volumes of 1 nL were microinjected into the yolk of one-cell embryos, delivering MOs at the following final concentration in distilled water: 25 μM of *csf3r*^{ATG} MO and 25 μM of *irf8*^{ATG} MO. A standard control MO was used as a negative control.

Leukocyte Enumeration. Leukocyte units (LUs), a surrogate parameter proportional to leukocyte numbers determined by analysis of digital images, were computed as previously described and validated (43). When appropriate, LUs are referred to as "neutrophil units" or "macrophage units." Leukocyte numbers were referred to when the total number of neutrophils was counted. A Mann–Whitney test was used for statistical significance ($P < 0.05$).

MICROBIOLOGY

Microscopy and Image Processing. For microscopy, zebrafish were immobilized in 1% low melting temperature agar. Routine bright-field and fluorescence imaging was performed by using a Zeiss Lumar V12 stereo dissecting microscope with an AxioCam MRm camera running AxioVision 4.8 software. Confocal microscopy was performed with a Zeiss LSM 710 device with a Plan-Apochromat 20×, 0.8 N.A. objective, and Zen software (v4.0) (*SI Materials and Methods*). Images were processed in Imaris (Bitplane) for cell-tracking mode, and data were analyzed in the R program using ggplot2 (26). Vibratome sectioning, histopathology, and pHrodo staining are described in *SI Materials and Methods*.

Murine Disseminated Infection Model. The model was performed as described previously (21) using female BALB/c mice aged 6–8 wk (n = 15 per group) infected via intraperitoneal injection (*SI Materials and Methods*). Animal protocols were approved by the Monash University Animal Ethics Committee.

Metabolomics. Metabolite samples were prepared from cell culture supernatants by solvent extraction and analyzed by LC/MS with hydrophilic interaction liquid chromotography (HILIC) and high-resolution accurate MS (44) (*SI Materials and Methods*). Metabolomics data were analyzed with the IDEOM software (45), and PA abundance confirmed with Tracefinder (Thermo). Quantification of PA was confirmed by using a complementary analytical method, reversed-phase chromatography coupled to high-resolution MS (Q-Exactive; Thermo; *SI Materials and Methods*).

Ex Vivo Neutrophil Chemotaxis Assay. Neutrophils were isolated from the bone marrow of C57BL/6 mice by Histopaque density gradient centrifugation, and chemotaxis was performed as described previously (46) (*SI Materials and Methods*).

ACKNOWLEDGMENTS. We thank Dr. Connie Wong and Alyce Nicholls for supporting the chemotaxis assay; Monash Micro Imaging for use of the imaging facilities; Monash FishCore facilities for the care and maintenance of zebrafish; and Max Cryle and Matthew Belousoff for assistance with the metabolomics assays. This work was supported by an Australian National Health and Medical Research Council (NHMRC) Career Development fellowship (to A.Y.P. and D.J.C.), and an Australian Leadership Award by the Australian government (to M.S.B.). G.J.L. is an NHMRC Senior Research Fellow. The Australian Regenerative Medicine Institute is supported by funds from the State Government of Victoria and the Australian Federal Government.

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