

The Capsule of *Acinetobacter baumannii* Protects against the Innate Immune Response

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Keywords

Acinetobacter baumannii · Pneumonia · Lung · Host-pathogen interactions · Type I interferon · Type III interferon · Capsule · Innate response

Abstract

Acinetobacter baumannii is an opportunistic pathogen that has recently emerged as a global threat associated with high morbidity, mortality, and antibiotic resistance. We determined the role of type I interferon (IFN) signaling in *A. baumannii* infection. We report that *A. baumannii* can induce a type I IFN response that is dependent upon TLR4-TRIF-IRF3 and phagocytosis of the bacterium. Phase variants of *A. baumannii* that have a reduced capsule, lead to enhanced TLR4-dependent type I IFN induction. This was also observed in a capsule-deficient strain. However, we did not observe a role for this pathway in vivo. The enhanced signaling could be accounted for by increased phagocytosis in capsule-deficient strains that also lead to enhanced host cell-mediated killing. The increased cytokine response in the absence of the capsule was not exclusive to type I IFN signaling. Several cytokines, including the proinflammatory IL-6, were increased in cells stimulated with the capsule-deficient strain, also observed in vivo. After 4 h in our acute pneumonia mod-

el, the burden of a capsule-null strain was significantly reduced, yet we observed increases in innate immune cells and inflammatory markers compared to wild-type *A. baumannii*. This study underscores the role of phase variation in the modulation of host immune responses and indicates that the capsule of *A. baumannii* plays an important role in protection against host cell killing and evasion from activation of the innate immune response.

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Introduction

Acinetobacter baumannii is an important Gram-negative pathogen responsible for significant morbidity and mortality associated with pneumonia, bacteremia, and wound infections [1]. It is a prominent nosocomial as well as a community-acquired pathogen and has a very high mortality rate [2–5]. It has been the only Gram-negative organism to have been associated with an increase in ICU-related pneumonias, both in the USA and globally [6, 7]. Reasons for its surge in prominence are its evolu-

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tion of enhanced virulence [3, 8] and its high capacity for the acquisition of new genetic determinants such as those encoding antibiotic resistance [9]. Up to two-thirds of isolates are multidrug resistant, with antibiotic pan-resistance associated with carbapenemases of increasing prevalence [10–13]. In part due to this resistance, the WHO has it listed as “priority critical” and the number 1 ranked organism recommended for new research and development [14].

The capsule of *A. baumannii* has been shown to play significant roles in protection against several host processes. The capsule aids in resistance to desiccation, disinfectants, antimicrobials, and antibiotics, consequently mutations in genes responsible for capsule production severely affect virulence in vivo [15–19]. Similarly, alteration in capsule structure can impact virulence [20]. A recently described property of *A. baumannii* is its ability to undergo phase variation. Phase variation in *A. baumannii* manifests as a conversion between more translucent colony morphology and typically opaque colonies [21]. These translucent variants have been shown to have reduced capsule production and subsequently attenuated resistance to antimicrobial compounds and the ability to persist in vivo [22].

Type I interferon (IFN) signaling is a pathway that often involves the activation of innate immune sensors (surface, endosomal, and cytosolic) that initiate the activation of IFN- α /IFN- β via several different IFN regulator factor (IRF) transcriptional activators. Although initially identified for their roles in curtailing viral infection, it is now clear type I IFNs play multifaceted roles, including modulating bacterial infections [23]. Many extracellular and intracellular bacteria have been shown to activate this pathway. The outcome of bacterial infection related to activation of type I IFNs has been shown to be both beneficial and detrimental depending upon the pathogen [23]. We have a long-standing interest in the ability of bacterial pathogens to activate type I and the related type III IFN signaling pathways [24–29]. In this study, we sought to determine the capacity of *A. baumannii* to activate the type I IFN response, its role in infection, as well as the role of the capsule and other factors that initiate this process and the influence they play on the innate immune response.

Materials and Methods

Bacterial Culture

A. baumannii 5075 and the capsule mutant (*wzc*) were grown in LB broth at 37°C to exponential phase (OD₆₀₀ nm of 1.0) before use in the experiments. Growth curves were conducted using cul-

tures diluted 1:100 from exponential phase (OD₆₀₀ nm of 1.0), in LB at 37°C, sampled every 15 min using a SpectraMax i3x plate reader at OD₆₀₀ nm (Molecular Devices). Heat-killed *A. baumannii* was prepared by incubating cultures at 65°C for 1 h.

Cell Culture

Immortalized WT and knockout murine macrophages were cultured as described previously [24, 25, 28]. Inhibitors were used at the following concentrations 30 min prior to bacterial stimulation: cytochalasin D 20 μ M, chloroquine (CQ) 10 μ M, and *Rhodobacter sphaeroides* LPS 10 μ g/mL. Bone marrow-derived dendritic cells (BMDCs) were generated from the bone marrow of WT C57BL/6J and *Tlr4*^{-/-} (Jackson Laboratories) mice and differentiated in RPMI medium containing 10% heat-inactivated fetal bovine serum, penicillin/streptomycin, and GM-CSF (20 ng/mL) at 37°C with 5% CO₂ in sterile petri dishes for 7 days. BMDCs were stimulated at an MOI of 10 for 24 h for cytokine quantification by ELISA and 2 h (MOI of 100) for RNA extraction in qRT-PCR experiments. Neutrophils were isolated from the bone marrow of the femurs and tibias of naive female and male mice by density gradient centrifugation as previously described [30]. Briefly, bone marrow cells were overlaid on a Histopaque 1119 and Histopaque 1077 gradient (Sigma) and centrifuged for 30 min at 720 g at room temperature without brakes. Thereafter, neutrophils were collected at the interface between the two layers. After assessment of cell numbers and viability, neutrophils were resuspended in HBSS containing Ca²⁺, Mg²⁺, and 0.1% gelatin. Neutrophils were used for experiments on the day of purification. Peritoneal macrophages were used immediately after isolation from peritoneal lavage washes (3 mL of PBS) of mice and cultured in RPMI media with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin.

Killing Assays

To evaluate bacterial killing capacity, neutrophils were incubated with *A. baumannii* (MOI of 10) for 60 min with slow rotation at 37°C, after which the reaction was stopped at 4°C, and cells were lysed with 1% saponin. Bacterial numbers were determined by serial dilutions. Killing in BMDCs and peritoneal macrophages was assessed after 2 h of incubation at 37°C and an MOI of 10.

Phagocytosis Assays

A. baumannii cultures were grown to an OD₆₀₀ nm of 1 and harvested by centrifugation at 10,000 g for 5 min before resuspending the pellet in 1 mL of PBS. FITC (10 μ g/mL FITC) was added to the suspension and mixed by vortexing. The mixture was then incubated in the dark for 30 min at room temperature. The bacterial suspension was then washed three times with PBS to remove unbound dye and resuspended in 1 mL of PBS. The labeled bacteria were then used to infect a monolayer of BMDCs at an MOI of 10 and incubated up to 30 min. The infected cells were then washed by PBS to remove extracellular bacteria and detached using TrypLE Express (Thermo Fisher). Phagocytosis was quantified using data acquired with a LSRFortessa X-20 cytometer (Becton Dickinson), and results were analyzed using FlowJo v.10.

Capsule Extraction and Staining

Capsule extraction was carried out as described by Tipton and Rather [31]. Briefly, bacteria were streaked on LB agar plates and cultured overnight, after which they were harvested by scraping

and the collected pellets (adjusted to equivalent OD600 nm density and bacterial cell numbers) resuspended in lysis buffer (60 mM Tris, pH 8; 10 mM MgCl₂; 50 μM CaCl₂; 20 μL/mL DNase and RNase; and 3 mg/mL lysozyme). This was followed by incubation at 37°C for 1 h and three liquid nitrogen/37°C freeze-thaw cycles. Additional DNase and RNase were added, and the samples were incubated at 37°C for 30 min. SDS was added to 0.5%, followed by incubation at 37°C for an additional 30 min. The samples were then processed with boiling and 2 mg/mL of proteinase K treatment. Polysaccharides in culture supernatants were precipitated in 75% ice-cold ethanol overnight, followed by pelleting, air-drying, resuspending with SDS sample buffer, and boiling for 5 min. Equivalent amounts of polysaccharide cell lysates and supernatant precipitants were loaded in SDS-PAGE and stained with Alcian blue.

Cytokine Quantification

Cytokine levels were quantified by ELISA for IFN-β (PBL IFN), IFN-λ (R&D Systems), and TNF-α (Biolegend). Multiplex cytokine analysis was performed by Eve Technologies (Calgary, AB, Canada).

RNA Analysis

RNA was isolated using the E.Z.N.A. total RNA kit (Omega Biotek), followed by DNase digestion using DNasefree (Life Technologies). cDNA was synthesized using the high-capacity cDNA reverse transcription kit (Applied Biosystems). qRT-PCR was performed using Power SYBR green PCR master mix (Applied Biosystems) on a Quantstudio 6 thermal cycler (Applied Biosystems) using the ΔΔCt method. Samples were normalized to β-actin. Data were calculated using the formula $2^{-(\Delta\Delta Ct)}$ where the ΔCt is the gene of interest Ct minus the β-actin control Ct. The ΔΔCt equals the test ΔCt minus the unstimulated control (PBS) ΔCt.

Animal Studies

Female and male C57BL/6J WT, *Ifnar1*^{-/-}, *Ifnlr1*^{-/-}, or *Ifnar1*^{-/-}/*Ifnlr1*^{-/-} double knockout (DKO) mice [32], 6–8 weeks old, were infected intranasally while under ketamine/xylazine anesthesia with 1×10^7 CFU of *A. baumannii* for either 4 h or 24 h [30]. Bronchoalveolar lavage fluid (BALF) was obtained from 3 successive washes with 1 mL of PBS. BALF was used to enumerate bacterial counts and quantify cytokine levels (mouse 32-plex; Eve Technologies) and to stain cells for flow cytometry. Lung and spleen tissue were homogenized in 400 μL of PBS and used to enumerate bacterial counts. Sepsis experiments were performed following intraperitoneal injection of 1×10^5 CFU of *A. baumannii* for 24 h.

Flow Cytometry

Cells in BALF were stained with fluorescently labeled antibodies: CD45-Alexa Fluor 700 (30-F11), CD11b-PE-Cy7 (M1/70), Ly6G-PerCP-Cy5.5 (1A8), CD11c-BV605 (N418), CD45-Alexa Fluor 700 (30-F11), BV510-CD103 (2E7), SiglecF-Alexa Fluor 647 (E50-2440; BD Biosciences), MHCII-APC-Cy7 (M5/114.15.2), Ly6C-PE-Texas red (AL-2; BD Biosciences), and DAPI to assess cell viability. Uniform dyed microspheres, Dragon Green (Bangs Laboratories, Inc.), were used for cell counting. Antibodies were purchased from BioLegend. Data were collected by flow cytometry using an LSRFortessa X-20 cytometer (Becton Dickinson), and results were analyzed using FlowJo v.10.

Statistics

Animal data were assessed using a nonparametric Mann-Whitney test. All experiments were conducted on at least two separate occasions. In vitro experiments were assessed using parametric Student's *t* test. Normal distribution is verified using the Shapiro-Wilk test. Multiple comparisons were conducted using one-way analysis of variance with Dunnett's multiple comparison test. A *p* value less than 0.05 was considered significant. Statistics were performed with Prism software (GraphPad, La Jolla, CA, USA).

Results

A. baumannii Activates a Robust Type I IFN Response

We sought to determine if *A. baumannii* activates the type I IFN pathway. In our previous study investigating differences in *A. baumannii* infection based on biological sex, we undertook RNA-seq on mouse lungs [26]. We mined this data for evidence of type I IFN signaling. Interrogation of the RNA-seq data revealed a robust IFN signature (Fig. 1a). We observed induction of *Ifnb1* and the receptor *Ifnar1* as well as genes encoding the IRFs 1, 5, 7, and 9. We also observed the induction of known IFN-regulated genes, such as *Isg15*, *Usp18*, *Oasl2*, and *Mx1* (Fig. 1a). To confirm this transcriptional data at the protein level, we utilized BMDCs, which induce significant levels of IFN. Stimulation of BMDCs with *A. baumannii* induced levels of IFN-β averaging 4 ng/mL (*p* < 0.0001; Fig. 1b). We also saw a significant increase (*p* < 0.05) in the related cytokine, IFN-λ, which is part of type III IFN signaling (Fig. 1c).

Type I IFN Signaling by *A. baumannii* Is Dependent upon TLR4

To define how *A. baumannii* activates type I IFN signaling, we utilized a series of inhibitors and knockout cell lines. Stimulation of macrophages with heat-killed preparations of *A. baumannii* did not alter the response to live cells, indicating that an active bacterial process was not required to induce this pathway (Fig. 1d). Uptake of bacterial cells was required, as indicated by the significant reduction in *Ifnb* in the presence of the actin polymerization inhibitor cytochalasin D (Fig. 1d).

The endosomal acidification inhibitor, CQ, had no effect, indicating that endosomal TLRs were not involved in the induction of *Ifnb* (Fig. 1d). Given the Gram-negative status of *A. baumannii*, a logical ligand for activation is its LPS and consistent with this observation with CQ, we observed that *Ifnb* induction occurs through the TLR4-TRIF pathway. Utilizing knockout cell lines inactivated for both the receptor and adapter we saw zero *Ifnb*

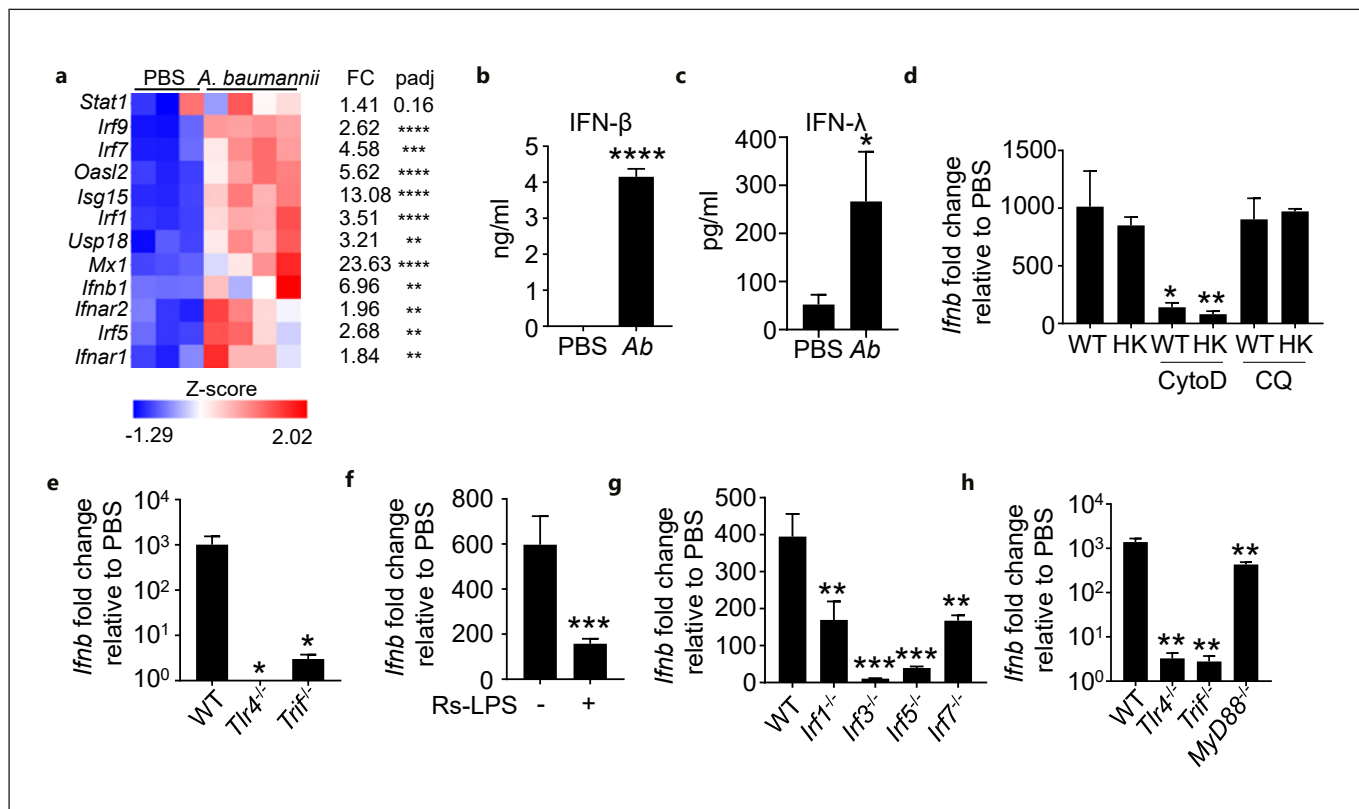


Fig. 1. *A. baumannii* activates type I IFN signaling via TLR4 and IRF3/5. **a** Heat map of type I IFN-related genes from RNA-seq of murine lungs infected with *A. baumannii* and uninfected controls. FC-fold change in *A. baumannii* infected samples compared to uninfected controls. **b, c** Bone marrow-derived macrophages were stimulated overnight with PBS or *A. baumannii* for ELISA quantitation. **d–g** Bone marrow macrophages were stimulated with *A. baumannii* for 2 h before qRT-PCR analysis. **d** WT and HK *A. baumannii* were added to macrophages in the presence/absence of

cytoD and CQ. **e** Role of TLR receptors using knockout macrophages. **f** Inhibition of IFN signaling using *R. sphaeroides* LPS (Rs-LPS). **g** Role of IRF was determined using knockout macrophages. **h** BMDCs from WT and knockout mice were quantified for *Ifnb*. $N \geq 3$ from at least two independent experiments. **** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$ relative to untreated or WT controls using a Student's *t* test (**b, c, e, f**) or ANOVA (**d, g, h**). Ab, *A. baumannii*; ANOVA, one-way analysis of variance; HK, heat-killed; cytoD, cytochalasin D.

induction in the absence of TLR4 ($p < 0.01$) (Fig. 1e). We confirmed the involvement of TLR4 by stimulating cells with *A. baumannii* in the presence of LPS from *R. sphaeroides*. *R. sphaeroides* LPS acts as a TLR4 antagonist due to its decreased level of acylation [33]. *A. baumannii* stimulated cells antagonized with *R. sphaeroides* LPS had 76% ($p < 0.001$) less *Ifnb* induction (Fig. 1f). After activation of the innate receptor, IRFs are activated before inducing *Ifnb* transcription. Utilizing immortalized knockout macrophage cell lines, we investigated the IRF requirements for *A. baumannii* *Ifnb* induction. We observed reductions in both *Irf1*^{-/-} and *Irf7*^{-/-} cells. A 90% reduction was observed in the absence of IRF5 ($p < 0.001$), while a 97% reduction was observed in the absence of IRF3 ($p < 0.001$; Fig. 1g). This indicates while IRF3 is the most im-

portant IRF, there are contributions from other IRFs. We further confirmed the role of TLR4 in activating *Ifnb* by stimulating primary BMDCs from WT, *Tlr4*^{-/-}, *Trif*^{-/-}, and *MyD88*^{-/-} mice. While we did observe a decrease in *Ifnb*, in the absence of MyD88, we further confirmed that the TLR4-TRIF pathway was the major requirement for *Ifnb* reduction, as in their absence there was more than a 99% reduction (Fig. 1h).

Decreased Capsule Expression Leads to an Enhanced Type I IFN Response

During our investigation on how *A. baumannii* activates type I IFN, we documented the appearance of colony variants that exhibited a translucent colony morphology (designated as WT-T). It is now published that these

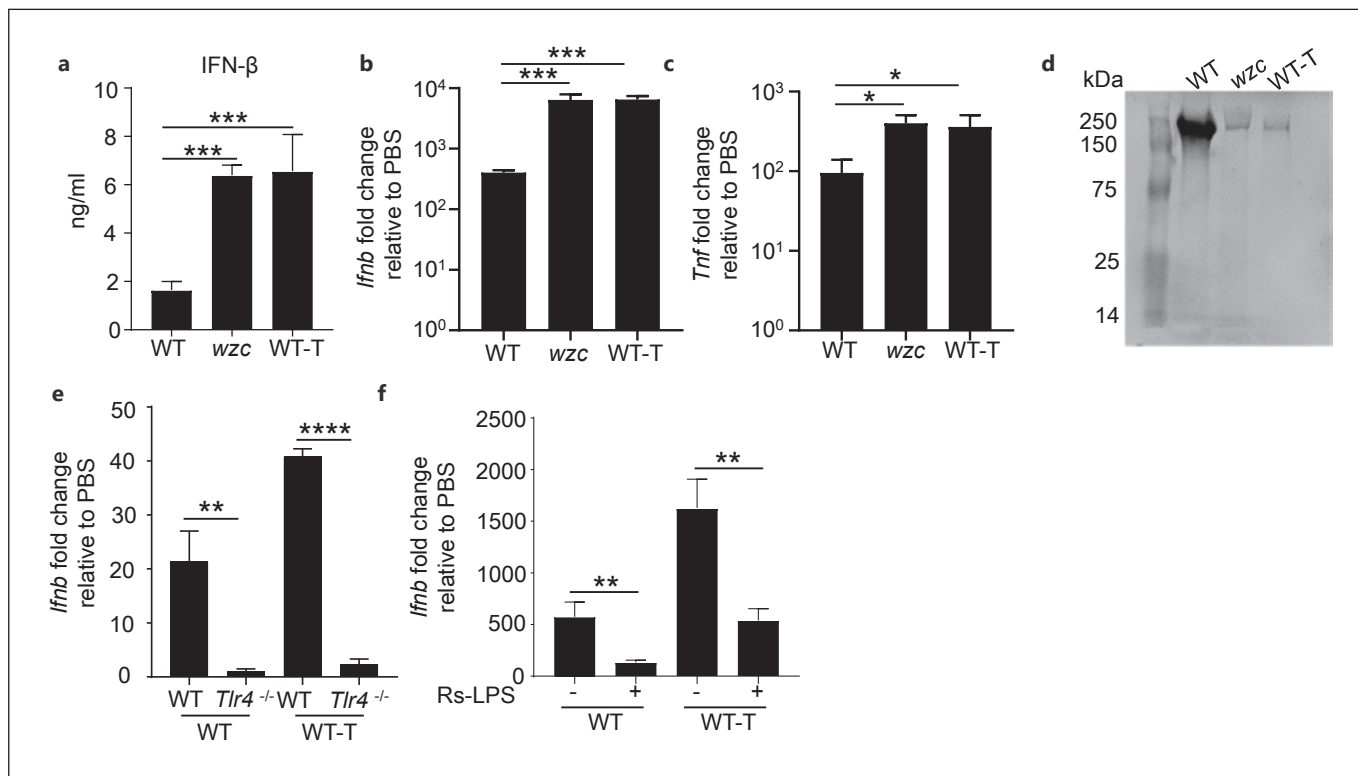


Fig. 2. Type I IFN expression is increased in the absence of the capsule in a TLR4-dependent manner. **a** BMDCs were stimulated for 24 h with *A. baumannii* and supernatants quantified for IFN- β . **b, c** BMDCs were stimulated for 2 h before *Ifnb* and *Tnf* were quantified by qRT-PCR. **d** Alcian blue-stained SDS-PAGE gel of capsule extraction from strains. **e** WT and *Tlr4*^{-/-} BMDCs were infected with WT or translucent variants (WT-T) of *A. baumannii* for 2 h and *Ifnb* expression was assessed by qRT-PCR. Values were

normalized to PBS controls. **f** BMDCs were stimulated with *A. baumannii* WT or translucent (WT-T) strains for 2 h with or without *R. sphaeroides* LPS (Rs-LPS), before RNA extraction and qRT-PCR analysis. $N \geq 6$ from at least two independent experiments. **** $p < 0.0001$, *** $p < 0.001$, and ** $p < 0.01$ compared to WT controls using an ANOVA with Dunnett's post test. ANOVA, one-way analysis of variance.

variants are the result of changes in capsule levels [16, 22]. We investigated the capacity of these variants to also induce the type I IFN pathway. We observed that the variants induced significantly higher levels of type I IFN at both the protein and RNA levels and that this was comparable to the enhanced levels observed in a capsule mutant, *wzc*, strain (Fig. 2a, b). Additional *Tnf* was also observed in vitro (Fig. 2c). To confirm with published reports that the translucent variant had a decreased capsule, we extracted the capsule from the WT, capsule-mutant, and variant strains. We observed a comparable level of polysaccharide in the variant strain to the capsule-null strain (Fig. 2d). We were able to demonstrate that the enhanced type I IFN induction was TLR4-dependent like what we observed with the WT strain. In the absence of TLR4, *Ifnb* was essentially not induced upon stimulation with the variant strain (Fig. 2e). Likewise, when cells were

stimulated with the variant in the presence of the *R. sphaeroides* LPS that acts as a TLR4 antagonist, we also observed a significant drop in *Ifnb* induction (Fig. 2f).

Capsule Is Important for Protection against Innate Cell Killing

Given the many roles of capsule in bacterial pathogenesis, we investigated the mechanism behind the ability of strains with altered capsule levels to induce increased type I IFN. We did not detect any difference in the ability of LPS from WT and capsule-deficient strains to activate cells, nor could we detect any capacity for capsule to activate type I IFN (data not shown). The capsule is known to protect pathogens from phagocytosis by immune cells and we tested if this was true for *A. baumannii*. WT (opaque) and translucent strains, along with the *wzc* strain were fluorescently labeled and phagocytosis quan-

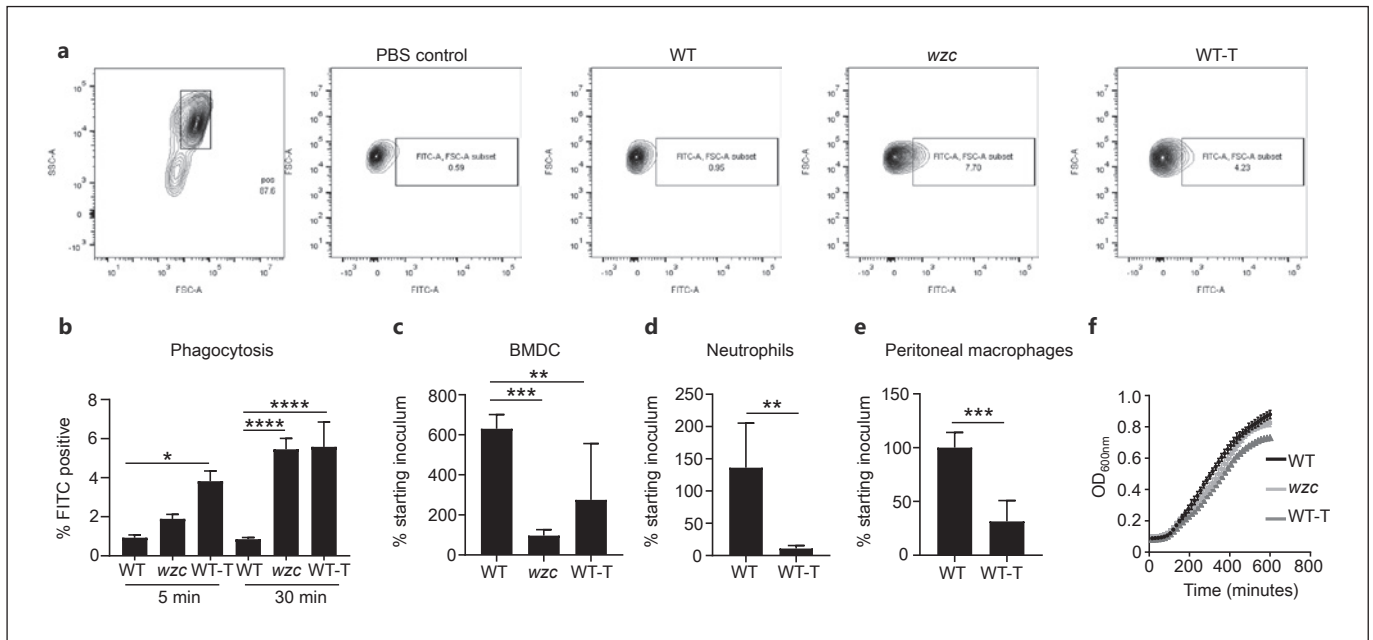


Fig. 3. *A. baumannii* capsule-mutant and translucent strains have increased susceptibility to host cell phagocytosis and killing. BMDCs were infected with FITC labeled *A. baumannii* WT, capsule-mutant (*wzc*), or translucent variants (WT-T) for 5 or 30 min. Cells were harvested and subjected to flow cytometry. **a** Representative flow cytometry plots of gated cells and positive populations at 30 min. **b** Quantification of phagocytosis. BMDCs (**c**), neutro-

phils (**d**), or peritoneal macrophages (**e**) were infected with *A. baumannii* strains for 2 h before bacteria were quantified. **f** Growth of WT, *wzc*, and WT-T strains. Graphs display means with standard deviation. $N \geq 6$ for all experiments. $***p < 0.001$, $**p < 0.01$, and $*p < 0.05$ using a Student's *t* test (**d, e**) or ANOVA (**b, c**). ANOVA, one-way analysis of variance.

phils by flow cytometry. While 5 min after incubation with BMDCs we did not observe any differences in cellular uptake, by 30 min we observed a significant increase in BMDCs containing the capsule-mutant and translucent variant (Fig. 3a, b), indicating capsule normally aids in protection from phagocytosis.

To determine the cellular consequence of the increased phagocytosis susceptibility, we then investigated the ability of *A. baumannii* to survive against host cell killing. It is known that the capsule can protect *A. baumannii* from antimicrobial products and disinfectants as well as enable survival in the environment [16]. We assessed the capacity of WT opaque and translucent variants to defend against innate immune cell killing. In many of the cell types we assessed in vitro, we observed that the WT strain was able to avoid killing and replicated in the presence of the immune cells (Fig. 3c–e). This was not the case with reductions in capsule expression. In BMDCs, the *wzc* strain was reduced by 85% ($p < 0.001$) and we also saw a 54% decrease in the translucent variant ($p < 0.001$). Likewise, the translucent variant was also reduced with neutrophils (Fig. 3d). Lastly, we assessed the survival of strain

against peritoneal macrophages and again observed a significant decrease in the ability of the translucent variant strain to survive against killing (Fig. 3e). These phenotypes were not growth related as lack of capsule does not inhibit growth to stationary phase in complete media (Fig. 3f), unlike growth in minimal media [22]. We did observe some reduced growth with the WT-T strain; however, there was no difference in growth between the strains in the first 2 h when most of the assays were performed. These data show that capsule expression is important in protection against phagocytosis and killing by the innate immune system.

Type I IFN Signaling Is Not Involved in the Pathogenesis of Acute A. baumannii Infection

To determine the role of type I IFN signaling in *A. baumannii* pneumonia, we utilized mice lacking the type I IFN receptor, IFNAR. We recently published that mice exhibit differences in susceptibility to *A. baumannii* pulmonary infection based on sex [30], so we, therefore, infected both male and female mice and cumulated the data separately. As expected, we observed female mice to have

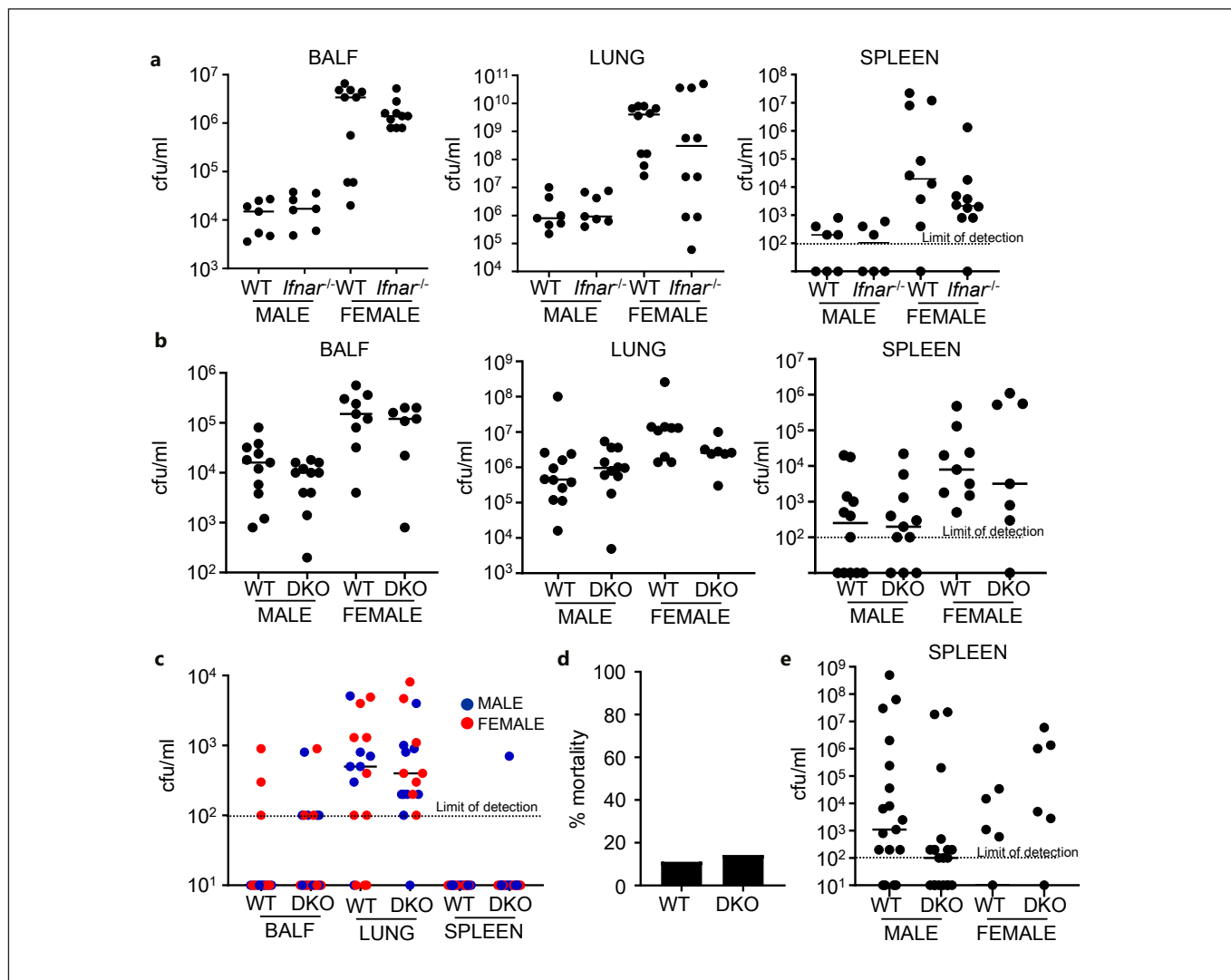


Fig. 4. Role of type I and III IFN signaling in *A. baumannii* infection. **a** Mice were intranasally infected with 10^7 *A. baumannii* 5075 for 24 h before BALF, lung homogenates, and spleen homogenates were enumerated for bacteria. **b** WT and DKO mice were intranasally infected with 10^7 *A. baumannii* 5075 for 24 h before BALF, lung homogenates and spleen homogenates were quantified for bacteria. WT and DKO mice were infected as before and samples

were collected 48 h later for bacterial burdens (**c**) and survival (**d**). **e** Mice were infected intraperitoneally with 10^5 *A. baumannii* 5075 and bacterial counts enumerated 24 h later. Each point represents a mouse. Line display median. Data were assessed using a non-parametric Mann-Whitney test. DKO, double knockout *Ifnar^{-/-}/Ifnlr^{-/-}*.

increased bacterial burdens to males but did not observe any difference in either sex in *Ifnar1^{-/-}* mice compared to WT (Fig. 4a). We did observe a 38% reduction in bacterial burden in the BALF of females, but this was not statistically significant (Fig. 4a). As noted, before (Fig. 1c), the type III IFN pathway induces a similar set of genes upon its induction. As type III IFN is also being induced, our lack of phenotype might have been due to compensatory changes. We therefore also infected mice that were

DKO for type I and III IFN pathways, *Ifnar1^{-/-}/Ifnlr1^{-/-}*. In the DKO animals, we were also unable to observe any differences in bacterial burden (Fig. 4b). We examined bacterial burdens in WT and DKO mice 48 h after infection to determine if there were differences at a later time point. We observed no differences also at this time point nor did we observe differences between the sexes (Fig. 4c). This was also evidence pathologically, with no differences in the infected groups at this time (online suppl. Fig. 1;

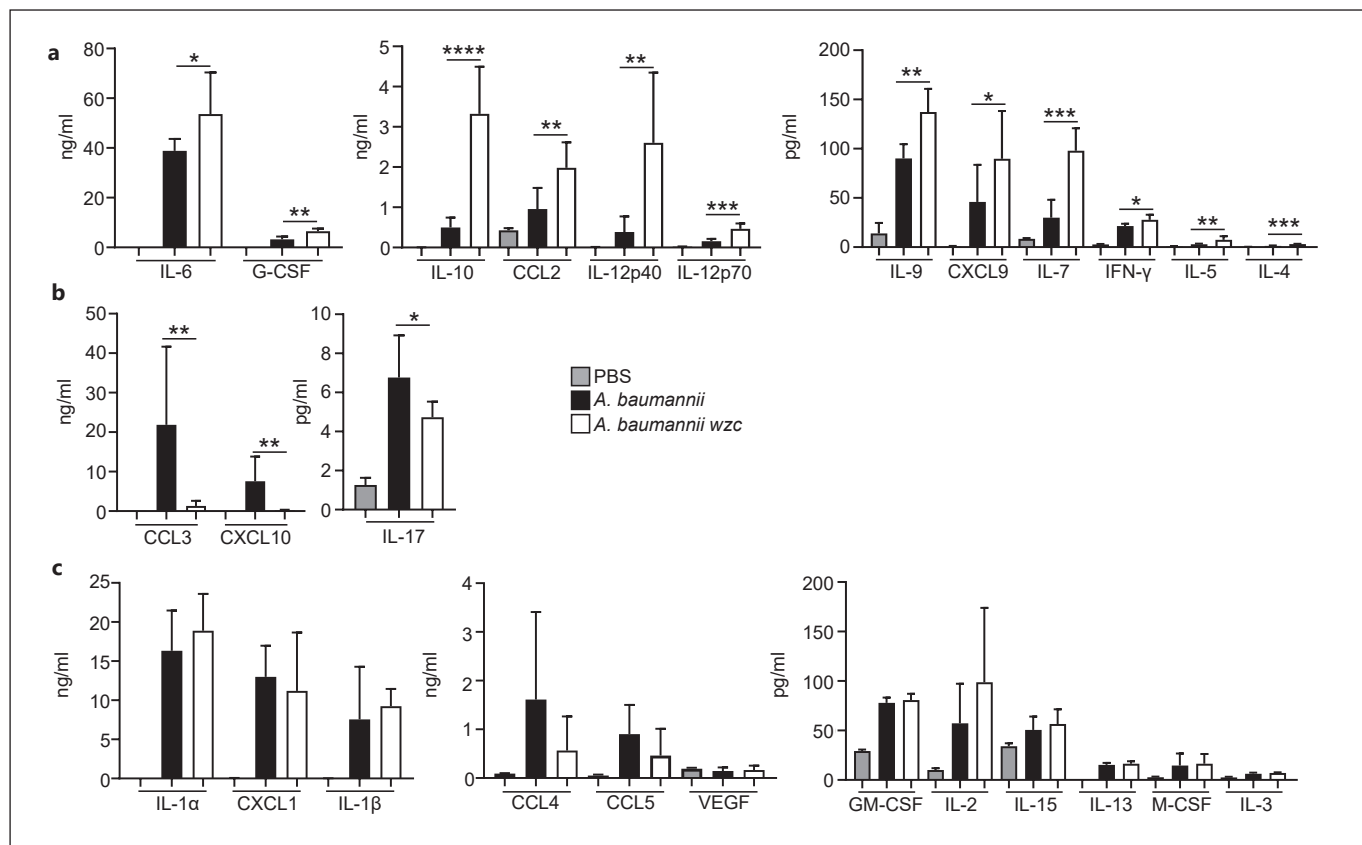


Fig. 5. Absence of the capsule enhances cytokine production. BMDCs were stimulated with WT *A. baumannii* 5075 or a *wzc* mutant for 24 h. Supernatants were collected for cytokine quantification. **a** Cytokines with increased expression in the absence of the capsule. Decreased cytokines in the absence of the capsule

(b) and unaltered cytokines **(c)**. Gray, PBS control; black, WT *A. baumannii*; white, *wzc* mutant. Graphs display means with standard deviation. $N = 9$ for infected samples and 2 for PBS controls. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$ using a Student's *t* test.

see www.karger.com/doi/10.1159/000522232 for all online suppl. material). At the 48-h time point, some mice had died; however, we did not observe differences between the genotypes (Fig. 4d). To determine if this lack of difference was evident across all sites or specific to the lung, we repeated the infection in DKO mice in a model of septicemia and also did not observe a difference in bacteria clearance (Fig. 4e).

Capsule Limits Cytokine Expression

We sought to define if the absence of the capsule led to alterations in cytokine expression outside of the type I IFN pathway. We exposed BMDCs to either WT *A. baumannii* 5075 or the *wzc* strain for 24 h, before collecting cell-free supernatant and quantifying cytokine expression with multiplex ELISA. We observed that several cytokines were significantly increased in the absence of the capsule. Major cytokines such as IL-6, G-CSF, CCL2

(MIP2), and IL-12 were all significantly increased (Fig. 5a). Potentially counteracting these changes, we also observed a greater than 6-fold increase ($p < 0.0001$) in the anti-inflammatory IL-10 (Fig. 5a). We also noted 3 analytes that were decreased in the absence of the capsule (Fig. 5b). CCL3 (MIP-1 α) and CXCL10 (IP-10) were reduced by 95% and 98%, respectively ($p < 0.01$). IL-17 was also reduced. Several cytokines also showed no significant changes. These data, along with our prior results, would suggest that the capsule protects the *A. baumannii* cell wall from activating host innate receptors.

Capsule Aids in *A. baumannii* Evasion from the Innate Immune Response

Our data demonstrate that in the absence of the capsule *A. baumannii* activates enhanced levels of type I IFN, in addition to several other cytokines (Fig. 1, 2, 5). We also know that the capsule helps in its capacity to resist

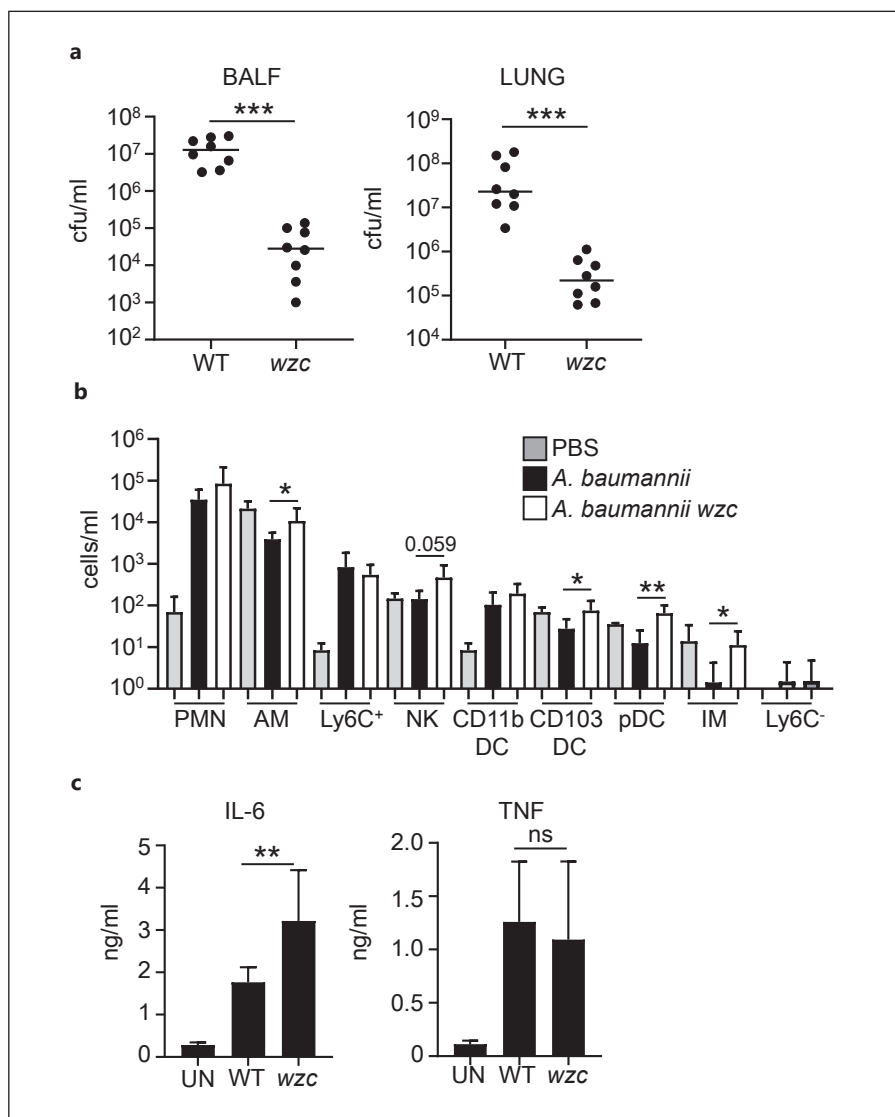


Fig. 6. Capsule of *A. baumannii* protects against the innate immune response during infection. C57BL/6J mice were intranasally infected with 10^7 CFU of *A. baumannii* 5075 for 4 h. **a** Bacterial counts in BALF and lung homogenates. Each point represents a mouse. Lines display median. **b** Flow cytometric analysis of innate immune cells in BALF. **c** Quantification of cytokines in BALF using ELISA. $N = UN-2, WT-8, wzc-7$. Graphs show means with standard deviation. UN, uninfected; ns, not significant. *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$ using a nonparametric Mann-Whitney test (**a, b**) and Student's *t* test (**c**).

immune cells' killing (Fig. 4). It has been previously demonstrated that capsule-deficient strains are attenuated in vivo [16]. Given our data, we wanted to test the hypothesis that capsule mutants are cleared faster in vivo, in part due to their capacity to better activate the innate immune response. To answer this question, we utilized our acute pneumonia model and chose an early time point, 4 h, after inoculating mice. We chose this time point as by 24 h, significant clearance of the capsule-deficient strains has occurred [16] and the innate response might be reduced due to the diminished bacterial burden. We observed that after 4 h of infection, the capsule-deficient strain was already 300-fold less than the WT strain in BALF ($p < 0.001$; Fig. 6a) and 165-fold decreased in lung tissue ($p < 0.001$; Fig. 6a). While the capsule-deficient strain was more rap-

idly cleared, we did observe increased innate immune cell infiltrates in the airway. After infection with the WT strain, several cell types were decreased compared to the uninfected control that were present at significantly higher levels in the *wzc* mutant (Fig. 6b). This included the most abundant resident immune cell in the airway, the alveolar macrophage (2.7-fold increase, $p < 0.05$), in addition to $CD103^+$ dendritic cells, plasmacytoid dendritic cells, and interstitial macrophages (Fig. 6b). Infection with the WT strain led to a reduction in alveolar macrophages that was not evident with the capsule mutant. Furthermore, consistent with our in vitro cytokine data, we also observed increased proinflammatory cytokines with the capsule mutant. While TNF was unchanged, we did observe a 182% ($p < 0.01$) increase in IL-6 levels (Fig. 6c).

Cytokines IL-17, CCL2, and IL-1 β were not induced at this time point. These data suggest that in the absence of the capsule, an increased innate immune response (increased alveolar macrophages and proinflammatory cytokines) further precipitates the rapid removal of *A. baumannii* lacking the capsule, which normally acts to protect itself from the innate immune system.

Discussion

In this study, we have further expanded upon the multiple roles the capsule plays in *A. baumannii* infection. We have identified that *A. baumannii* can activate a robust type I IFN response that is dependent upon the TLR4 signaling pathway. This signaling is increased in the absence of the capsule, as seen in both capsule-null strains and phase variants that transcriptionally decrease capsule production. Absence of capsule facilitates efficient phagocytosis by immune cells, which ultimately leads to killing by eukaryotic cells. We provide further evidence that the capsule also prevents *A. baumannii* from activating proinflammatory cytokines, likely through preventing surface interactions and phagocytosis that further precipitate clearance of *A. baumannii* in vivo through innate immune cell recruitment.

We have shown that the capsule is an important evasion mechanism from host cell killing. Strains lacking a capsule were attenuated in their ability to propagate in the presence of several different immune cell types. The attenuation in survival is in part due to their decreased ability to avoid phagocytosis by these immune cells. It has also been shown by multiple groups that the capsule is important for protection against a variety of agents and virulence traits, such as desiccation, biofilm formation, antibiotics, and disinfectants [15, 16, 18, 22, 34]. These data in sum substantiate the rapid clearance of mutants and strains that have reduced capsule in vivo [16, 17, 22], which we also observed even after 4 h in our pneumonia model. Thus, the decreased ability to avoid phagocytosis and the enhanced susceptibility to host products significantly attenuates strains lacking the capsule.

In addition to the capsule acting as a direct mechanism to avoid host cell killing, it also limits the innate immune response. We observed this both in our characterization of type I IFN activation as well as in other cytokines, and the response to WT and capsule-deficient strains in vivo. In vivo, we observed that there was an increase in the number of innate cells in mice infected with capsule-deficient versus WT strains. Alveolar macrophages were de-

creased after infection with the WT strain, which was not evident with the capsule-deficient strain. Possibly as a result of the capsule mutant being rapidly cleared, the alveolar macrophage population was not overwhelmed. The capsule in other species is known to potentially mask the pathogen [35], and we also observed that with *A. baumannii*. Given all our data, it is most likely that the enhanced susceptibility to killing and phagocytosis by capsule deficiency leads to more rapid digestion and release of TLR ligands that leads to this increased innate immune response. There is also some evidence that TLR ligation and activation can stimulate phagocytosis, thus creating a cycle of activation, phagocytosis, and digestion that self-propagates, particularly in the absence of the capsule [36–39]. This further demonstrates that the presence of the capsule not only offers protection against immune cell uptake and killing, but also the associated consequences of these processes, which is inflammatory cytokine production and innate cell recruitment to further precipitate clearance of the bacterium.

We observed that *A. baumannii* was able to activate type I IFN signaling via TLR4. Our delineation of the TLR4-TRIF-IRF3 pathway concurs with an earlier observation that suggested TRIF was important for activation [40]. We were also able to demonstrate that this signaling was enhanced in the absence of the capsule. Based on our data we can assume that this increase in induction is a result of the release of pathogen-associated molecular patterns, namely, lipopolysaccharide, which is released during digestion of the bacterial cell, and this is subject to further investigation. Given that the strains without the capsule were more readily phagocytosed and susceptible to host cell killing, they are likely facilitating enhanced activation of innate signaling pathways. We did not see a role for type I or III IFN signaling in the pathogenesis of *A. baumannii* airway infection. This contrasts to prior work that showed clearance was impeded in mice lacking IFNAR [40]. The difference in phenotype may have been a result of differences in the *A. baumannii* strain used, which would suggest that type I IFN signaling is not a universally important pathway for *A. baumannii*. This conclusion would be consistent with what we and others have observed in other species of bacteria, with varying degrees of importance that vary even within a given species [23].

We demonstrate in this study that the capsule is an important component of *A. baumannii* to protect itself against the host innate immune response. Due to the enhanced response evoked by the immune system in the absence of the capsule, the rate at which *A. baumannii* switches to a translucent variant could impact the overall

survival of the population and thus be potentially advantageous for the bacterium to tightly regulate this switching process to avoid enhanced activation of the innate response to facilitate its persistence.

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Statement of Ethics

Animal work in this study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (NIH), the Animal Welfare Act, and U.S. federal law. Protocol 201800040 was approved by the Institutional Animal Care and Use Committee of Rutgers New Jersey Medical School of Newark New Jersey.

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

L.A. conducted experiments, analyzed data, and wrote and edited the manuscript. S.P. and J.K. conducted experiments, analyzed data, and edited the manuscript. D.P. conducted experiments, analyzed data, wrote and edited the manuscript, and provided resources and funding.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material. Further inquiries can be directed to the corresponding author.

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