View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Dartmouth Digital Commons (Dartmouth College)

Dartmouth College
Dartmouth Digital Commons

Open Dartmouth: Faculty Open Access Articles

11-2014

Acidosis Potentiates the Host Proinflammatory Interleukin-1β Response to Pseudomonas Aeruginosa Infection

I. M. Torres
Dartmouth College

Y. R. Patankar
Dartmouth College

Tamer B. Shabaneh
Dartmouth College

E. Dolben
Dartmouth College

Deborah Hogan
Dartmouth College

See next page for additional authors

Follow this and additional works at: https://digitalcommons.dartmouth.edu/facoa

Part of the Infectious Disease Commons, and the Medical Microbiology Commons

Recommended Citation

Torres, I. M.; Patankar, Y. R.; Shabaneh, Tamer B.; Dolben, E.; Hogan, Deborah; Leib, David; and Berwin, Brent L., "Acidosis Potentiates the Host Proinflammatory Interleukin-1β Response to Pseudomonas Aeruginosa Infection" (2014). *Open Dartmouth: Faculty Open Access Articles*. 919.
https://digitalcommons.dartmouth.edu/facoa/919

This Article is brought to you for free and open access by Dartmouth Digital Commons. It has been accepted for inclusion in Open Dartmouth: Faculty Open Access Articles by an authorized administrator of Dartmouth Digital Commons. For more information, please contact dartmouthdigitalcommons@groups.dartmouth.edu.

Authors
I. M. Torres, Y. R. Patankar, Tamer B. Shabaneh, E. Dolben, Deborah Hogan, David Leib, and Brent L. Berwin

This article is available at Dartmouth Digital Commons: https://digitalcommons.dartmouth.edu/facoa/919

IAI Journals.ASM.org

Acidosis Potentiates the Host Proinflammatory Interleukin-1β Response to *Pseudomonas aeruginosa* Infection

Iviana M. Torres, Yash R. Patankar, Tamer B. Shabaneh, Emily Dolben, Deborah A. Hogan, David A. Leib, Brent L. Berwin

Department of Microbiology and Immunology, Geisel School of Medicine at Dartmouth, Lebanon, New Hampshire, USA

Infection by *Pseudomonas aeruginosa*, and bacteria in general, frequently promotes acidification of the local microenvironment, and this is reinforced by pulmonary exertion and exacerbation. However, the consequence of an acidic environment on the host inflammatory response to *P. aeruginosa* infection is poorly understood. Here we report that the pivotal cellular and host proinflammatory interleukin-1β (IL-1β) response, which enables host clearance of the infection but can produce collateral inflammatory damage, is increased in response to *P. aeruginosa* infection within an acidic environment. Synergistic mechanisms that promote increased IL-1β release in response to *P. aeruginosa* infection in an acidic environment are increased pro-IL-1β induction and increased caspase-1 activity, the latter being dependent upon a functional type III secretion system of the bacteria and the NLRC4 inflammasome of the host. Using an *in vivo* peritonitis model, we have validated that the IL-1β inflammatory response is increased in mice in response to *P. aeruginosa* infection within an acidic microenvironment. These data reveal novel insights into the regulation and exacerbation of inflammatory responses to *P. aeruginosa*.

Pseudomonas aeruginosa is a Gram-negative opportunistic bacterium that causes a variety of acute and chronic infections. These infections are highly prevalent in patients with cystic fibrosis (CF) and in immunocompromised tissues, can be acquired iatrogenically, and are a cause of ocular keratitis (1–4). Recent evidence supports that a key proinflammatory cytokine elicited in response to *P. aeruginosa* infection is interleukin-1β (IL-1β) (5, 6). IL-1β is produced predominantly by macrophages and plays a pivotal role in the recruitment of neutrophils and subsequent bacterial clearance (1, 5, 6). Correspondingly, IL-1β pathway impairment or neutropenia results in susceptibility to *P. aeruginosa* infection and pathogenesis (7). However, excessive IL-1β levels or chronic IL-1β production, exemplified during CF disease, can result in organ damage, dysfunction, and even lethality (8).

The molecular basis for the cellular IL-1β response to *P. aeruginosa* is beginning to be elucidated. The pro-IL-1β precursor (p31) is upregulated upon Toll-like receptor (TLR) engagement. A second signal is required for the processing and release of the active form of IL-1β (p17). In the case of *P. aeruginosa*, the second signal is the type III secretion system (T3SS)-dependent injection of stimulatory ligands such as the PscL rod proteins or flagellin into the host cell cytoplasm, which activates the NLRC4 (NOD-like receptor, CARD domain-containing protein 4) inflammasome complex (9–13). This complex contains NLRC4, caspase-1, and the adaptor molecule ASC (apoptosis associated speck-like protein containing a CARD domain). Caspase-1, activated through autoproteolysis, cleaves pro-IL-1β into the active form of IL-1β and enables its release (14, 15). Infection with *P. aeruginosa* strains deficient in the T3SS, including the *popB* mutant used here, results in attenuated IL-1β-dependent responses (5, 16). The T3SS is also required for transport of cytotoxic effector proteins (ExoS, ExoT, ExoU, and ExoY) into the eukaryotic host cell cytoplasm, and increased mortality has correlations with *P. aeruginosa* T3SS function (17); however, the Exo proteins are not required for inflammasome activation (13, 16, 18, 19). Despite these mechanistic insights into IL-1β release in response to *P. aeruginosa* infection, how the extracellular microenvironment influences the inflammatory response during infection is less understood.

The microenvironments surrounding inflammatory sites feature acidosis of tissues and fluids to pH levels well below the physiological norm of ~7.4 (20, 21). Relevant to our studies, this is observed during bacterial infection, where anaerobic glycolysis, lactic acid accumulation, hypoxia, bacterial fatty acids, and hypochlorous acid (HOCl) production by activated neutrophils contribute to local acidosis with measured pH values ranging from 5.9 to 7.0, depending upon the disease process and method of measurement (22–27). In CF, the loss of CF transmembrane conductance regulator (CFTR)-mediated bicarbonate transport has been proposed to contribute to pulmonary acidosis, which is supported by pH measurements of airway surface liquid, submucosal gland fluid, and mucopurulent airway secretions that range from ~6.1 to 6.9 (28–33). While the importance of homeostatic maintenance of pH has been well studied, reports on how low pH affects inflammation are just now emerging (23, 24). In particular, recent reports have demonstrated that *in vitro* acidosis can enhance IL-1β release from lipopolysaccharide (LPS)-primed mouse glial cells and human monocytes (34–37). In a study by Rajamaki et al., the IL-1β response was found to be dependent on the activation of the NLRP3 inflammasome by the acidic environment, and it was proposed that acidosis serves as a novel danger signal (37). Therefore, based on the clinical observations of acidosis during bacterial infection and the pivotal role of IL-1β in disease pathogenesis, we investigated how physiologically relevant changes in pH alter the inflammatory response to *P. aeruginosa*.

Received 7 May 2014 **Returned for modification** 18 June 2014
Accepted 13 August 2014
Published ahead of print 25 August 2014
Editor: A. Camilli
Address correspondence to Brent L. Berwin, berwin@dartmouth.edu.
Copyright © 2014, American Society for Microbiology. All Rights Reserved.
doi:10.1128/IAI.02024-14

In this report we demonstrate that a low-pH microenvironment enhances IL-1β production *in vitro* and *in vivo* in response to infection with *P. aeruginosa*. We demonstrate the specificity of this response and identify two intracellular mechanisms that generate the amplified response observed during *P. aeruginosa* infection under acidic conditions: there is increased production of pro-IL-1β and increased caspase-1-dependent cleavage to its active form. Additionally, in contrast to previous *in vitro* studies (34–37), *in vivo* analyses revealed that bacterial T3SS function is required to trigger acidosis-enhanced IL-1β responses; acidosis, even in the presence of bacterial LPS, is not sufficient to enable robust *in vivo* IL-1β responses. These studies are the first to show that extracellular pH governs the host response against *P. aeruginosa* through modulation of cytokine release, and they provide new insights into the role of acidic pH in the regulation of the innate immune response during infections.

MATERIALS AND METHODS

Mice. C57BL/6 wild-type (WT) mice were obtained from the National Cancer Institute (Bethesda, MD). C57BL/6 NLRC4$^{-/-}$, NLRP3$^{-/-}$, and ASC$^{-/-}$ mice (10, 38) were obtained from V. Dixit (Genentech, CA). Caspase-1$^{-/-}$ mice were purchased from the Jackson Laboratory (Bar Harbor, ME); these mice were shown to also be deficient in caspase-11 (15, 39). Studies were compliant with the *Guide for the Care and Use of Laboratory Animals* of the National Research Council and were approved by the Dartmouth Institutional Animal Care and Use Committee.

Reagents. Hanks balanced salt solution (HBSS) was purchased from Corning Cellgro (Manassas, VA), LPS, *o*-nitrophenyl-β-D-galactopyranoside (ONPG), EGTA, and HEPES from Sigma-Aldrich (St. Louis, MO), the DuoSet enzyme-linked immunosorbent assay (ELISA) kits for mouse and human IL-1β and the polyclonal goat anti-mouse IL-1β (AF-401-NA) from R&D Systems (Minneapolis, MN), pan-caspase inhibitor Z-VAD-FMK from InvivoGen (San Diego, CA), FAM-YVAD-FMK FLICA caspase-1 stain from ImmunoChemistry Technologies (Bloomington, MN), horseradish peroxidase (HRP)-conjugated bovine anti-goat antibody (sc-2350) from Santa Cruz Biotechnology (Santa Cruz, CA), anti-mouse CD45-allophycocyanin (APC) monoclonal antibody (clone 30-F11) and anti-mouse IL-1β Pro-form phycoerythrin-(PE) monoclonal antibody (clone NJTEN3) from eBioscience (San Diego, CA), APC-conjugated anti-mouse Ly6G antibody (clone 1A8) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse F4/80 antibody (clone BM8) from BioLegend (San Diego, CA), fluorescein sodium salt (46960) from Fluka (Buchs, Switzerland), and 1 M HEPES at pH 7.3 (BP299-100), used for infecting mice *in vivo*, from Fisher BioReagents (Pittsburgh, PA).

Cell culture. Bone marrow-derived dendritic cells (BMDC) were cultured using a modification of the protocol of Inaba et al. (40) as previously described (41). Briefly, BMDC in culture medium (RPMI 1640 medium, 10% heat-inactivated fetal bovine serum [FBS], 100 units/ml penicillin-streptomycin, and 50 mM β-mercaptoethanol [β-ME], supplemented with granulocyte-macrophage colony-stimulating factor [GM-CSF]) were plated in six-well plates. The cells were washed and refed on days 2 and 4, and semiadherent cells were harvested for use at day six or seven.

THP-1 monocytic cells were differentiated into macrophages using 50 ng/ml of phorbol myristate acetate (PMA) for 24 h and then washed with HBSS before use.

The culture medium pH was adjusted by buffering HBSS with a final concentration of 25 mM HEPES of various pH values such that, following equilibration with a preincubation in 12- or 24-well plates for 1 h at 37°C and 5% CO_2, the pH of the culture medium was 6.7, 7.0, or neutral (7.3).

Bacteria. *Pseudomonas aeruginosa* PA14 strains were obtained from G. O'Toole and D. Hogan (Geisel School of Medicine at Dartmouth, NH). All strains have been previously used and published (42–44). Bacteria were cultured overnight at 37°C in Luria broth (LB), and subsequently subcultured for 3 h in LB.

β-Galactosidase assay. T3SS gene expression was assessed using the $P_{exsD\text{-}lacZ}$ construct in WT PA14 (44). Subcultured bacteria (optical density at 600 nm [OD_{600}], ~0.5) were resuspended in preequilibrated HBSS buffered to the indicated pH with 25 mM HEPES, in the presence or absence of 2 mM EGTA. Subsequently, the cultures were incubated in a 12-well plate for 2.5 h at 37°C and 5% CO_2. Following incubation, the cultures were resuspended in 1 ml of Z buffer, and the Miller assay was performed as previously described (45). Expression of *lacZ* was determined by the Miller unit equation (46).

NanoString. NanoString nCounter (NanoString Technologies) analyses were used to quantify *P. aeruginosa* gene expression from WT PA14 bacteria incubated in preequilibrated HBSS and 2 mM EGTA at 37°C and 5% CO_2 (47). Briefly, each reaction mixture contained 80 ng of bacterial RNA, hybridization buffer, reporter probes, and capture probes, and 6 positive and 8 negative controls were included. Overnight hybridization of RNA with reporter and capture probes at 65°C was followed by sample preparation using the NanoString prep station. Finally, targets were counted on the nCounter using 255 fields of view per sample. Data were analyzed using nSolver Analysis software v1.1. Raw counts for T3SS-related transcripts (*popB*, *exoU*, and *exoT*) were normalized to the arithmetic mean for six positive controls and to the geometric mean for three *P. aeruginosa* housekeeping genes (*fbp*, *ppiD*, and *rpoD*).

***In vitro* bacterial infection.** A total of 2.5×10^5 cells per well (24-well plate) were infected with subcultured bacteria at a multiplicity of infection (MOI) of 1 in preequilibrated HBSS, 1% FBS, and 25 mM HEPES. Following coincubation for 3 h at 37°C and 5% CO_2, cell-free supernatants were collected and analyzed by ELISA. Where indicated, BMDC were preincubated with 20 μM Z-VAD-FMK for 1 h before infection and throughout the course of the assay. FAM-YVAD-FMK-FLICA and propidium iodide (PI) stainings were done as previously described (42).

Intracellular staining for pro-IL-1β. A total of 2.5×10^5 cells per well in a 24-well plate were coincubated with subcultured bacteria at an MOI of 0.2 in the presence of preequilibrated HBSS containing 1% FBS with 25 mM HEPES. As a control, BMDC were stimulated with LPS at 50 ng/ml for 3 h. At 3 h postinfection (hpi), cells were harvested, washed, blocked with monoclonal antibody 2.4G2, stained with anti-CD45 antibody, fixed, permeabilized, and stained for intracellular pro-IL-1β.

Immunoblotting. A total of 10^6 cells per well in a 12-well plate containing preequilibrated HBSS with 25 mM HEPES were infected at an MOI of 1. BMDC and bacteria were coincubated for 3 h as described above. Cell-free supernatants and cell lysates were processed for Western analyses as previously described (42). IL-1β was detected with polyclonal antibody (R&D Systems AF-401-NA or GeneTex GTX74034).

***In vivo* bacterial infection.** WT mice were injected intraperitoneally (i.p.) with 1 ml of 4% thioglycolate solution and then 4 to 5 days later with 10^6 CFU of the indicated genotype of PA14. For infection, 100 μl of bacteria suspended in phosphate-buffered saline (PBS) was mixed with 400 μl of sterile filtered 1 M HEPES buffer (500 μl total at a final pH of 5.4, 7.6, or 8.7). For IL-1β and cellular recruitment analyses, mice were sacrificed at 2 hpi, and i.p. lavage was performed using 1 ml PBS. Cellularity was determined by fluorescence-activated cell sorter (FACS) analyses following staining with APC-conjugated anti-mouse Ly6G antibody and FITC-conjugated anti-mouse F4/80 antibody, with gating to exclude lymphocytes, bacteria, and debris. The intraperitoneal pH was measured at either 30 min postinfection or 2 hpi, using a fluorescein-based fluorescence assay with a standard curve obtained using 100 mM phosphate buffer at pH increments of 0.5 from pH 5 to pH 7.5.

Statistics. Means ± standard deviations (SD) obtained from independent experiments with technical duplicates are shown. Two-way analysis of variance (ANOVA) with Dunnett's *post hoc* analysis (denoted by hash symbols) and, as appropriate, the unpaired Student *t* test with Welch's correction or one-way ANOVA with Dunnett's *post hoc* analysis (denoted by asterisks) were performed using Prism 4.0a to analyze statistical significance.

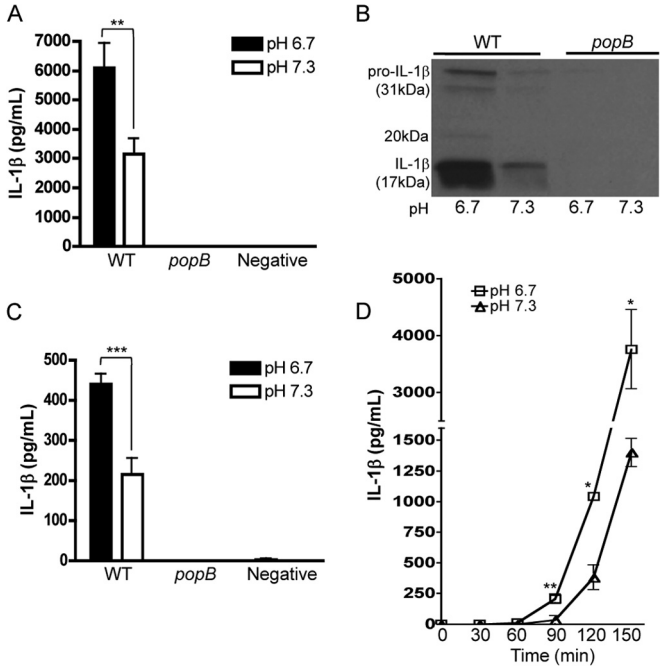

FIG 1 Acidic pH enhances IL-1β production by murine BMDC and human THP-1 macrophages infected with *P. aeruginosa*. BMDC from C57BL/6 mice (A, B, and D) or differentiated THP-1 cells (C) were uninfected (negative) or infected with *P. aeruginosa* strain PA14 (WT) or the PA14 *popB* isogenic mutant (deficient in T3SS) at an MOI of 1 at the indicated pH values. (A and C) Culture media were analyzed by ELISA for IL-1β production at 3 hpi. (B) Relative pro-IL-1β and cleaved IL-1β protein present in the supernatants at 3 hpi were analyzed by Western blotting. (D) The kinetics of IL-1β release were analyzed by ELISA at the indicated time points (open squares, pH 6.7; open triangles, pH 7.3). The data in panels A, B, and D are derived from at least two independent experiments ($n \geq 4$). ***, $P \leq 0.0005$; **, $P \leq 0.005$; *, $P \leq 0.01$.

RESULTS

Acidosis enhances cellular IL-1β responses to *P. aeruginosa*. Low pH is present at sites of inflammation and may contribute to the inflammatory response. To test the effect that pH has on the cellular IL-1β response to *P. aeruginosa*, we infected murine BMDC with the PA14 strain of *P. aeruginosa* at neutral and acidic pHs and subsequently analyzed the media for IL-1β content. An acidic pH value of 6.7 was chosen since it is within the range of measured physiological acidosis found at inflammatory sites and within the CF lung (26, 28, 29, 32, 48). BMDC infected at this acidic pH exhibited a greater IL-1β response than those infected at neutral pH (Fig. 1A). The *popB* mutant of PA14, which lacks a functional T3SS and is thereby attenuated in inflammasome activation (5, 42), was used as a control for specificity of the IL-1β response. BMDC infected with *popB* bacteria did not elicit a measurable IL-1β response at acidic pH, and the response was comparable to that of the uninfected control, which indicates that acidic pH alone does not drive the release of IL-1β even in the presence of bacterial LPS (Fig. 1A). Western blotting confirmed that secretion of the cleaved, active form of IL-1β (17 kDa) was greater at acidic pH when infection was with WT PA14 (Fig. 1B). Under acidic conditions, low levels of the 20-kDa IL-1β fragment, previously described to have biological activity (36), were also observed (Fig. 1B). To corroborate our findings with human cells, we utilized cultured human THP-1 macrophages. THP-1 macrophages infected with *P. aeruginosa* at acidic pH also responded with increased IL-1β production (Fig. 1C), validating the results observed with murine BMDC. To analyze the kinetics of IL-1β release in response to *P. aeruginosa* infection at acidic pH, media were collected every 30 min and analyzed by ELISA. Significantly enhanced secretion of IL-1β at low pH was observed as early as 90 min postinfection. Moreover, the kinetic analysis revealed an increasingly differential accumulation of IL-1β at low versus neutral pH over time (Fig. 1D). These results indicate that the cellular response to *P. aeruginosa* is pH dependent, is exacerbated at low pH, and requires a functional bacterial T3SS even in the presence of bacterial LPS.

The pH-dependent IL-1β response to *P. aeruginosa* requires NLRC4 and is independent of NLRP3. *P. aeruginosa* activates the NLRC4 inflammasome at physiological pH (12, 13, 19). However, extracellular acidosis has been shown to activate the NLRP3 inflammasome in LPS-stimulated macrophages, leading to the release of IL-1β (37). Therefore, we tested whether the enhanced response observed in Fig. 1 was due to a pH-dependent triggering of the NLRP3 inflammasome or an enhanced NLRC4-dependent response. Loss of NLRC4, but not NLRP3, led to a marked decrease of mature IL-1β secretion in response to WT PA14 infection (Fig. 2A and B), demonstrating that the pH-dependent IL-1β production in response to *P. aeruginosa* requires NLRC4. Use of the bacterial *popB* mutant again resulted in loss of IL-1β secretion at all pH values tested (Fig. 2A and B).

To assess the specificity of the NLRC4 inflammasome activity, we next tested whether ASC and caspase-1, components of the NLRC4 inflammasome (49, 50), were required for IL-1β secretion at acidic pH. Indeed, the pH-dependent IL-1β response was significantly and substantially decreased in ASC$^{-/-}$ BMDC (Fig. 2C and D). We used two complementary approaches to test the contribution of caspase-1 to the pH-dependent IL-1β response (Fig. 3). First, pharmacological caspase inhibition using Z-VAD-FMK abrogated the IL-1β response both at neutral and acidic pHs (Fig. 3A and B). Second, the use of caspase-1$^{-/-}$ BMDC subsequently revealed that the genetic loss of caspase-1 phenocopies the use of Z-VAD-FMK (Fig. 3C and D), indicating that caspase-1 is the major enzyme contributing to the IL-1β response. These results establish that NLRC4, ASC, and caspase-1 are predominantly required for the enhanced IL-1β secretion in response to *P. aeruginosa* under acidic conditions, rather than the NLRP3-dependent inflammasome response to bacterial LPS.

***P. aeruginosa* T3SS expression is independent of acidosis.** Since some species of bacteria alter their secretory pathways in response to pH changes (51, 52), we tested the hypothesis that acidic pH may increase the expression of components of the *P. aeruginosa* T3SS and thereby contribute to the observed increase in IL-1β response. To test this hypothesis, we employed bacteria that express a reporter construct of the *lacZ* gene under the control of the *exsD* promoter ($P_{exsD\text{-}lacZ}$) (44). *exsD* is a T3SS-regulatory gene that, akin to many of the bacterial T3SS genes, is induced under calcium-limited conditions or through contact with the host cell (16, 53). WT PA14 carrying the $P_{exsD\text{-}lacZ}$ reporter incubated at acidic and neutral pHs under T3SS-inducing (with EGTA) or noninducing (lacking EGTA) conditions was analyzed for β-galactosidase activity. Consistent with previous reports, T3SS expression was enhanced under inducing conditions (44, 54, 55). Importantly, induction was similar at both acidic and neutral pHs (Fig. 4A). To confirm and extend this analysis, we quantita-

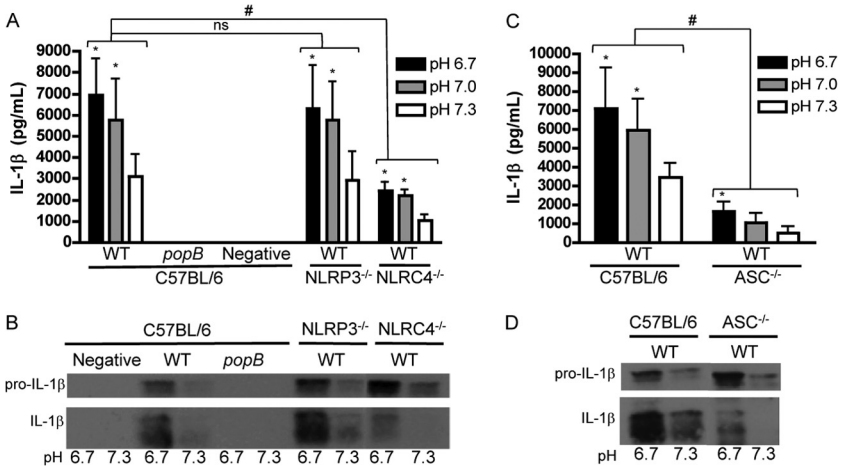

FIG 2 IL-1β production enhanced by acidosis is dependent on the NLRC4 inflammasome. BMDC from C57BL/6 mice and, where indicated, NLRP3$^{-/-}$, NLRC4$^{-/-}$, and ASC$^{-/-}$ mice were infected with WT or *popB* PA14 at the indicated pH values (MOI = 1). (A and C) Culture supernatants analyzed by ELISA for IL-1β production at 3 hpi. (B and D) Western blot analyses of relative pro-IL-1β and cleaved IL-1β protein in the media at 3 hpi. Data are representative of at least two independent experiments ($n \geq 4$). *, $P \leq 0.05$, compared with neutral control within the same mouse genotype; ns, not significant; #, $P \leq 0.0001$, compared with C57BL/6 group.

tively assayed the expression of T3SS effector genes of WT PA14 cultured at acidic or neutral pH under T3SS-inducing conditions. Gene expression analyses revealed no difference in the effector genes *exoU* and *exoT* or the T3SS translocon gene *popB* at neutral versus acidic pH (Fig. 4B). Thus, the enhanced IL-1β production observed under acidic conditions is likely not due to a differential upregulation of the bacterial T3SS or its effectors, suggesting that the observed increased response is due to a host-dependent mechanism in response to *P. aeruginosa* infection at low pH.

Cellular mechanisms responsible for enhanced IL-1β responses to *P. aeruginosa* in an acidic microenvironment. We next endeavored to determine the host mechanisms that underlie the increased production of IL-1β in response to *P. aeruginosa* at acidic pH. Previous data support that pro-IL-1β is regulated by NF-κB (56) and that low environmental pH can increase NF-κB activation (57). To test whether acidic pH elicits increased pro-IL-1β synthesis upon infection with *P. aeruginosa*, we analyzed pro-IL-1β protein levels in cell lysates by Western blotting (Fig. 5A) and in intact cells by flow cytometry (Fig. 5B and C). Infection with WT PA14 at acidic pH elicited higher expression of pro-IL-1β than that at neutral pH as assessed by Western (Fig. 5A) and FACS (Fig. 5B and C) analyses. Consistent with pro-IL-1β induction at neutral pH via TLR-dependent signaling and independent of the core inflammasome components, the pro-IL-1β levels ex-

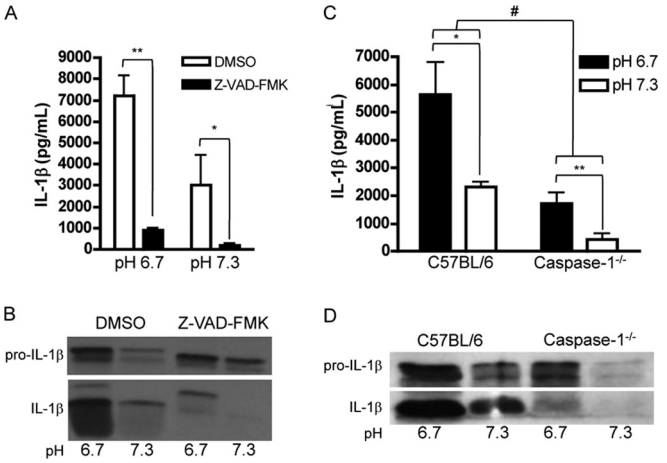

FIG 3 Caspase-1 is required for the pH-dependent IL-1β response. (A and B) BMDC from C57BL/6 mice were pretreated with pan-caspase inhibitor Z-VAD-FMK before infection with WT PA14 at the indicated pH values. (C and D) BMDC from C57BL/6 mice and, where indicated, caspase-1$^{-/-}$ mice were infected with WT PA14 at the indicated pH values. Media were analyzed by ELISA (A and C) and by Western blotting analyses (B and D) for IL-1β production at 3 hpi ($n = 4$). All experiments utilized an MOI of 1. **, $P \leq 0.005$; *, $P \leq 0.05$ (compared with control). #, $P \leq 0.0001$ (compared with C57BL/6 group).

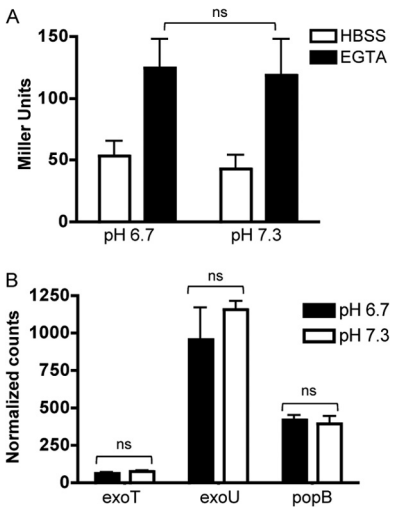

FIG 4 *P. aeruginosa* T3SS expression is not increased under acidic conditions. (A) WT PA14 harboring the $P_{exsD\text{-}lacZ}$ construct was incubated in the presence or absence of 2 mM EGTA in HBSS medium at the indicated pH values for 2.5 h, and β-galactosidase activity (Miller units) was subsequently measured ($n \geq 6$). (B) Normalized transcript counts of T3SS-related genes, analyzed by NanoString nCounter, from WT PA14 cultured at the indicated pH values in the presence of EGTA ($n = 2$). ns, not significant.

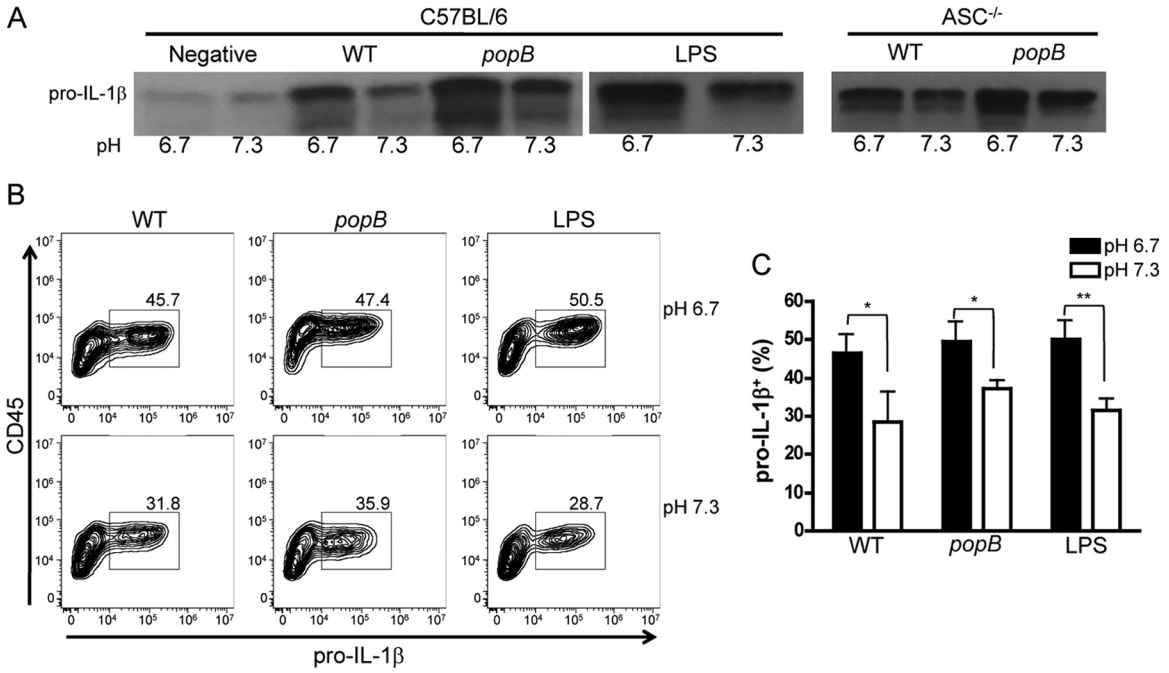

FIG 5 Acidic pH induces enhanced pro-IL-1β expression. BMDC from C57BL/6 and, where indicated, $ASC^{-/-}$ mice were either treated with LPS (50 ng/ml) or infected with WT or *popB* PA14 at an MOI of 1 (A) or 0.2 (B and C) for 3 h. (A) Cell lysates were analyzed by Western blotting for pro-IL-1β protein expression. (B and C) Gating was used to exclude the bacteria, and the gated BMDC were analyzed for expression of CD45 and intracellular pro-IL-1β by flow cytometry. Representative contour plots (B) and cumulative quantitative analyses (C) of the percentage of pro-IL-$1\beta^+$ cells from the $CD45^+$ population are shown ($n = 4$). **, $P \leq 0.005$; *, $P \leq 0.05$.

hibited similar levels of pH-mediated enhancement when stimulated with the *popB* mutant and when $ASC^{-/-}$ cells were used (Fig. 5A to C). The pH-dependent pro-IL-1β responses could be recapitulated with purified LPS, which obviated the requirement for live bacteria (Fig. 5A to C). These results demonstrate that there is a pH-dependent increase of pro-IL-1β that contributes to the enhanced IL-1β response during *P. aeruginosa* infection in an acidic environment. Additionally, pro-IL-1β induction by purified LPS demonstrates that pH-induced bacterial responses, such as modifications to LPS or other TLR ligands, are not required for the enhanced IL-1β response.

Although the pH-dependent pro-IL-1β induction provides one explanation for the enhanced response, we noted that pro-IL-1β induction at pH 6.7 was 2-fold greater than that at neutral pH (Fig. 5), while at the same pH values the secreted IL-1β response was >3-fold (Fig. 1D). These observations led us to hypothesize that an additional basis for enhanced IL-1β production under acidic conditions is an increase in the rate of pro-IL-1β cleavage. This hypothesis was also supported by studies of apoptosis showing increased catalytic efficiency of some mammalian and yeast caspases at lower pH (58, 59). To test whether caspase-1 activity was higher when BMDC were infected at an acidic pH compared to neutral pH, we used a caspase-1-specific fluorescent probe, FAM-YVAD-FLICA, to measure caspase-1 activity. Acidic pH induced higher caspase-1 activation in BMDC upon infection with WT PA14. However, infection with the *popB* mutant led to similar caspase-1 activity regardless of pH (Fig. 6A and B). As a complementary assay, we measured *P. aeruginosa*-induced cytotoxicity. Cell death, as assessed by propidium iodide (PI)-positive cells (Fig. 6C), was consistent with the IL-1β results observed in Fig. 1A and D. Notably, cell death trends began to be observable at approximately 1 h and were robustly observable by 3 h (Fig. 6C). Bacteria deficient in T3SS (*popB*) induced less cytotoxicity in host cells (Fig. 6C), and, consistent with previous reports (16, 19, 42), analyses of the cytotoxicity revealed contributions of both NLRC4-dependent (pyroptotic) and -independent mechanisms (data not shown). Thus, acidic pH increases caspase-1 activity, which leads to higher IL-1β release and cell death.

Acidosis exacerbates the *in vivo* IL-1β response to *P. aeruginosa*. In order to test whether acidic pH within a microenvironment leads to higher IL-1β production *in vivo*, we used a modification of an established *P. aeruginosa* peritonitis model that mimics iatrogenic infections such as those acquired during continuous ambulatory peritoneal dialysis (13, 60). Thioglycolate-stimulated C57BL/6 mice were injected intraperitoneally with PA14 in the presence of buffered medium in order to temporarily shift the peritoneal microenvironment to a desired pH. To induce acidic conditions, we used medium that was initially buffered to a pH of 5.4, which, due to *in vivo* pH equilibration, was empirically determined to maintain a pH of ~6.3 throughout the experiment. At 2 hours postinfection, peritoneal lavage samples were collected for IL-1β analysis and for determining cellularity by flow cytometry. Mice infected with WT PA14 under acidic conditions elicited robust and significantly higher IL-1β responses than mice infected with WT PA14 under neutral or basic conditions (Fig. 7A). As a control both for specificity of the response to *P. aeruginosa* and for the acidic conditions, we employed the PA14 *popB* mutant. Mice infected with *popB* bacteria under acidic conditions failed to elicit an IL-1β response (Fig. 7A), demonstrating the requirement of a functional T3SS and that acidic conditions are not singularly suf-

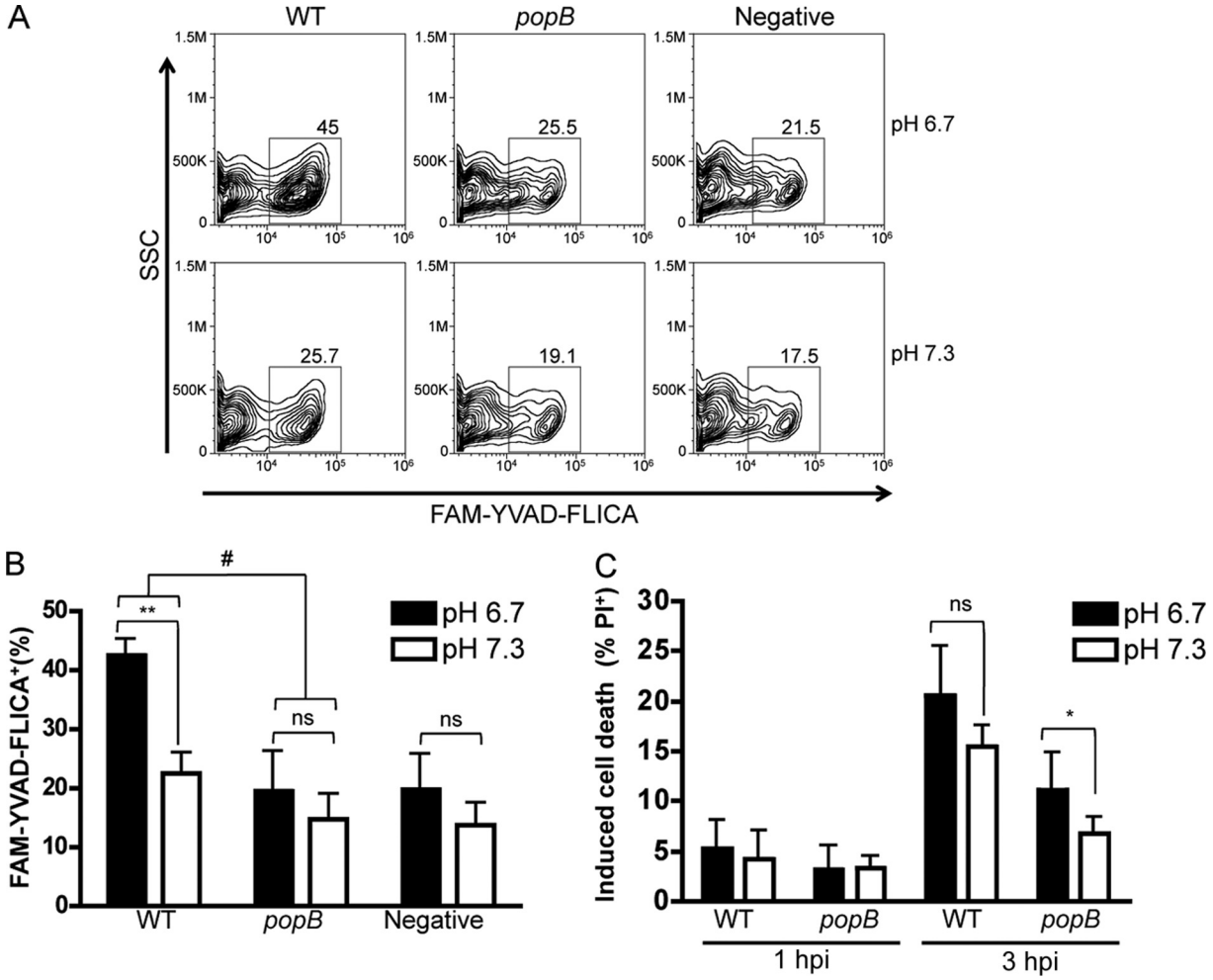

FIG 6 Decreased pH during *P. aeruginosa* infection results in enhanced caspase-1 activity. (A and B) BMDC from C57BL/6 mice were infected with WT or *popB* PA14 (MOI = 1), followed by incubation with FAM-YVAD-FLICA. Bacteria were excluded, and the BMDC were analyzed for caspase-1 activity as assessed by FLICA fluorescence with flow cytometry. Representative contour plots (A) and cumulative quantitative analyses of the percentage of FLICA$^+$ cells (B) are shown ($n = 4$). (C) Percentage of propidium iodide (PI)-positive cells due to infection with WT or *popB* PA14 (MOI = 1) at the indicated pH ($n = 4$). ns, not significant; **, $P \leq 0.005$; *, $P \leq 0.05$ (compared with neutral control). #, $P \leq 0.0001$ (compared with WT PA14).

ficient to elicit IL-1β, even in the presence of bacterial LPS. FACS analyses revealed comparable number of F4/80$^+$ macrophages and Ly6G$^+$ neutrophils in the peritoneal lavage fluid under all conditions at 2 hpi (Fig. 7B). Hence, the increase in IL-1β is not due to an increase in the recruitment of IL-1β-producing cells but instead is due to an amplification of the response to *P. aeruginosa* infection within an acidic environment.

DISCUSSION

The effect of acidic microenvironments on inflammatory responses during infection is underappreciated. This is directly relevant to bacterial infections in which acidosis is frequently observed during acute septic shock with increased lactate levels and during chronic infections (28–30, 61–66). However, how acidosis directly alters the host inflammatory response to *P. aeruginosa* infection has not previously been defined.

Based on low pH acting as a danger signal for inflammasome activation and the development of local acidic microenvironments postinfection, we hypothesized that infection with *P. aeruginosa* in an acidic environment will lead to enhanced IL-1β release. Here we provide the first report of pH-dependent changes of IL-1β release in the context of a *P. aeruginosa* infection. The IL-1β response significantly increased, dose dependently, as the pH of the microenvironment dropped from neutral (7.3) to 6.7. The enhanced release of IL-1β in response to *P. aeruginosa* at low pH was dependent on NLRC4, ASC, and caspase-1; this is the first report of pH modulation of responses by the NLRC4 inflammasome. This led to several important mechanistic insights. First, it revealed that the canonical NLRC4-dependent inflammasome is the predominant mechanism responsible for the observed increase in IL-1β responses to *P. aeruginosa* under acidic conditions. Second, the enhanced IL-1β response was surprisingly independent of NLRP3. Since acidosis is reported to act as a trigger for NLRP3-dependent inflammasome responses to purified LPS (37), we initially hypothesized that NLRP3 would contribute to the release of IL-1β. However, our data indicate that during an active infection by *P. aeruginosa*, the NLRC4 inflammasome contributions are dominant to those of the NLRP3 inflammasome. Concomitantly, a requisite for IL-1β release was a functional bacterial T3SS rather than solely the presence of LPS. However, under con-

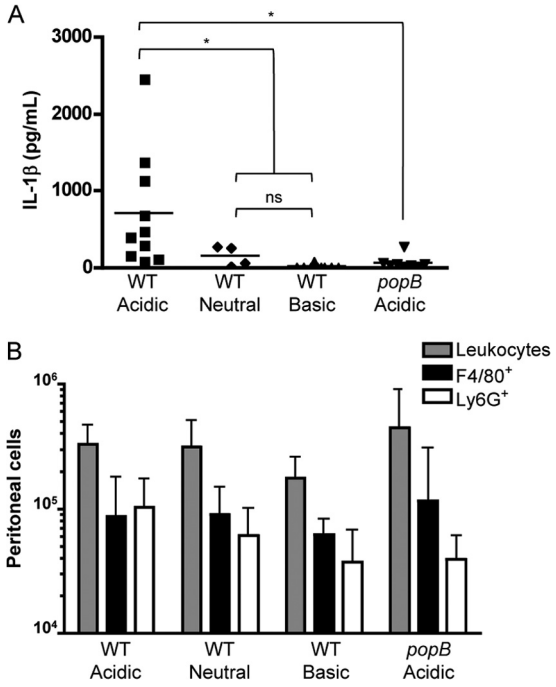

FIG 7 Peritoneal acidosis promotes *P. aeruginosa*-induced IL-1β production *in vivo*. Thioglycolate-induced C57BL/6 mice were injected i.p. with 10^6 CFU of WT or *popB* PA14 in an acidic (5.4), neutral (7.6), or basic pH (8.7) buffer (WT acidic, $n = 10$; WT neutral, $n = 4$; WT basic, $n = 9$; *popB* acidic, $n = 8$). Mice were sacrificed at 2 hpi, and peritoneal lavage fluid was collected. (A) Cell-free supernatants were analyzed by ELISA for IL-1β production. (B) The total number of leukocytes and the numbers of $F4/80^+$ and $Ly6G^+$ cells in the peritoneal lavage fluid were quantified by FACS analyses and were not significantly different (WT acidic, $n = 5$; WT neutral, $n = 4$; WT basic, $n = 5$; *popB* acidic, $n = 4$). ns, not significant; *, $P \leq 0.05$.

ditions in which the *P. aeruginosa* T3SS was induced by low extracellular Ca^{2+} concentrations (44, 55), the T3SS expression was not dependent on pH as assessed by two complementary assays. From this we infer that the pH dependency of the IL-1β release was regulated predominantly by the host cell and was not due to an upregulation of the bacterial T3SS at low pH.

Importantly, we have identified two complementary mechanisms that underlie the enhanced cellular IL-1β response to *P. aeruginosa* under acidic conditions. Pro-IL-1β priming increased under acidic conditions following infection with either WT PA14 or the T3SS-deficient *popB* mutant or in the presence of purified LPS. This supports that TLR ligands are sufficient to prime increased pro-IL-1β induction in response to acidic microenvironments and is consistent with previous observations based on LPS treatment in the context of acidic conditions (34, 37). However, the kinetics and magnitude of IL-1β release under acidic conditions were not entirely accounted for by pro-IL-1β induction, and in the search for a complementary mechanism we were guided by previous studies of apoptosis that had identified that many caspases display greater activity upon acidification of the cytosol (58, 59). This corollary between the apoptosome and the inflammasome was upheld, as we showed that caspase-1 activity increased at lower pH and contributed to more rapid processing of IL-1β to its active form; unlike pro-IL-1β induction, this activity was absolutely dependent upon stimulation derived from the bacterial T3SS. *Pseudomonas*-induced cytotoxicity followed a parallel outcome, as assessed both by kinetics and by pH and T3SS dependence. These mechanisms likely have broad physiological relevance, since *P. aeruginosa* infections frequently happen in, or induce, acidic extracellular microenvironments that can directly influence the intracellular pH (37). As a formal test of potential *in vivo* relevance, we infected mice with *P. aeruginosa* in buffered solutions with various pHs. Mice infected with bacteria at acidic pH triggered the release of significantly larger amounts of IL-1β than mice infected with bacteria at relatively neutral or alkaline pH. This experiment demonstrated the necessity for bacterial T3SS activity rather than the mere presence of LPS to elicit IL-1β release and demonstrated for the first time that *in vivo* shifts in pH can dramatically alter the inflammatory response to *P. aeruginosa*. Additionally, we observed a drop in the peritoneal pH of mice originally infected with bacteria at neutral pH (data not shown), which may indicate a temporary drop in pH at the site of infection due to increased metabolic activity and inflammation. Such drops in the pH in the microenvironment of infection could have broad effects on the amplification of the IL-1β response, as demonstrated by the release of significantly larger amounts of IL-1β *in vivo* under acidic conditions in our study. Based on the broad underlying requirements identified, i.e., a TLR agonist in combination with an inflammasome stimulus in the presence of physiologically low pH, we speculate that our findings would apply to host inflammatory responses to many genera of bacteria.

In a broader context, our data support that pH neutralization may provide therapeutic benefit in situations where deleterious IL-1β responses contribute to the pathology. Notably, excessive IL-1β production during chronic infection in CF patients is proposed to lead to increased pulmonary damage in the host and to subsequent mortality. This is supported by murine studies in which IL-1 receptor (IL-1R) signaling contributes to bacterial clearance (5, 7, 67) but excessive IL-1R signaling in response to *P. aeruginosa* is deleterious to the host (6, 8). Intriguingly, the pulmonary airway surface liquid pH is acidified both in clinical CF patients (30, 62) and in the porcine CF model (29). Data from the latter demonstrated that the low pH reduced bacterial killing in the CFTR-deficient porcine lung and, in combination with our data, suggest that even incremental increases of the pulmonary fluid pH within localized regions of infection may provide a therapeutic effect by reducing both bacterial burden and IL-1β-mediated lung pathology.

In conclusion, our principal finding is that the proinflammatory IL-1β response is activated at higher levels within physiologically acidic environments in response to *P. aeruginosa* infection. We have described the molecular underpinnings for this exacerbated response and have validated our conclusion with the use of an *in vivo* peritonitis model. These data provide novel insights into the regulation of inflammasome responses in acidic microenvironments and how neutralization of the acidic microenvironment may provide a therapeutic benefit to the host.

ACKNOWLEDGMENTS

We thank Rustin Lovewell, George O'Toole, Bruce Stanton, and Jeffrey Hollomon (Geisel School of Medicine at Dartmouth, NH), Matthew Poynter and Matthew Wargo (University of Vermont, VT), and Jeanne Hardy and Barbara Osborne (University of Massachusetts—Amherst, MA) for assistance and advice.

This work was supported by National Institutes of Health (NIH) COBRE grants (P30 RR032136-01 and P30 GM106394), Cystic Fibrosis

Foundation Research Development Program grants (STANTO11R0 to B.L.B. and STANTO07R0 to D.A.H.), an NIH R01 grant (EY009083-21S2 to D.A.L.), NIH support through grants P42ES007373 and UL1TR001086, the Copenhaver and Thomas Fellowship (Y.R.P.), and NIH grant T32AI007363 (to I.M.T.).

We have no conflicts to report.

REFERENCES

1. **Lavoie EG, Wangdi T, Kazmierczak BI.** 2011. Innate immune responses to Pseudomonas aeruginosa infection. Microbes Infect. **13:**1133–1145. http://dx.doi.org/10.1016/j.micinf.2011.07.011.
2. **Kurahashi K, Kajikawa O, Sawa T, Ohara M, Gropper MA, Frank DW, Martin TR, Wiener-Kronish JP.** 1999. Pathogenesis of septic shock in Pseudomonas aeruginosa pneumonia. J. Clin. Invest. **104:**743–750. http://dx.doi.org/10.1172/JCI7124.
3. **Fagon JY, Chastre J, Hance AJ, Montravers P, Novara A, Gibert C.** 1993. Nosocomial pneumonia in ventilated patients: a cohort study evaluating attributable mortality and hospital stay. Am. J. Med. **94:**281–288. http://dx.doi.org/10.1016/0002-9343(93)90060-3.
4. **Pearlman E, Sun Y, Roy S, Karmakar M, Hise AG, Szczotka-Flynn L, Ghannoum M, Chinnery HR, McMenamin PG, Rietsch A.** 2013. Host defense at the ocular surface. Int. Rev. Immunol. **32:**4–18. http://dx.doi.org/10.3109/08830185.2012.749400.
5. **Wangdi T, Mijares LA, Kazmierczak BI.** 2010. In vivo discrimination of type 3 secretion system-positive and -negative Pseudomonas aeruginosa via a caspase-1-dependent pathway. Infect. Immun. **78:**4744–4753. http://dx.doi.org/10.1128/IAI.00744-10.
6. **Schultz MJ, Rijneveld AW, Florquin S, Edwards CK, Dinarello CA, van der Poll T.** 2002. Role of interleukin-1 in the pulmonary immune response during Pseudomonas aeruginosa pneumonia. Am. J. Physiol. Lung Cell. Mol. Physiol. **282:**L285–L290.
7. **Konstan MW, Davis PB.** 2002. Pharmacological approaches for the discovery and development of new anti-inflammatory agents for the treatment of cystic fibrosis. Adv. Drug Deliv. Rev. **54:**1409–1423. http://dx.doi.org/10.1016/S0169-409X(02)00146-1.
8. **Cohen TS, Prince AS.** 2013. Activation of inflammasome signaling mediates pathology of acute P. aeruginosa pneumonia. J. Clin. Invest. **123:**1630–1637. http://dx.doi.org/10.1172/JCI66142.
9. **Miao EA, Mao DP, Yudkovsky N, Bonneau R, Lorang CG, Warren SE, Leaf IA, Aderem A.** 2010. Innate immune detection of the type III secretion apparatus through the NLRC4 inflammasome. Proc. Natl. Acad. Sci. U. S. A. **107:**3076–3080. http://dx.doi.org/10.1073/pnas.0913087107.
10. **Franchi L, Amer A, Body-Malapel M, Kanneganti TD, Ozoren N, Jagirdar R, Inohara N, Vandenabeele P, Bertin J, Coyle A, Grant EP, Nunez G.** 2006. Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages. Nat. Immunol. **7:**576–582. http://dx.doi.org/10.1038/ni1346.
11. **Miao EA, Alpuche-Aranda CM, Dors M, Clark AE, Bader MW, Miller SI, Aderem A.** 2006. Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. Nat. Immunol. **7:**569–575. http://dx.doi.org/10.1038/ni1344.
12. **Miao EA, Ernst RK, Dors M, Mao DP, Aderem A.** 2008. Pseudomonas aeruginosa activates caspase 1 through Ipaf. Proc. Natl. Acad. Sci. U. S. A. **105:**2562–2567. http://dx.doi.org/10.1073/pnas.0712183105.
13. **Sutterwala FS, Mijares LA, Li L, Ogura Y, Kazmierczak BI, Flavell RA.** 2007. Immune recognition of Pseudomonas aeruginosa mediated by the IPAF/NLRC4 inflammasome. J. Exp. Med. **204:**3235–3245. http://dx.doi.org/10.1084/jem.20071239.
14. **Li P, Allen H, Banerjee S, Franklin S, Herzog L, Johnston C, McDowell J, Paskind M, Rodman L, Salfeld J, Towne E, Tracey D, Wardwell S, Wei FY, Wong W, Kamen R, Seshadri T.** 1995. Mice deficient in IL-1 beta-converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock. Cell **80:**401–411. http://dx.doi.org/10.1016/0092-8674(95)90490-5.
15. **Kuida K, Lippke JA, Ku G, Harding MW, Livingston DJ, Su MS, Flavell RA.** 1995. Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. Science **267:**2000–2003. http://dx.doi.org/10.1126/science.7535475.
16. **Hauser AR.** 2009. The type III secretion system of Pseudomonas aeruginosa: infection by injection. Nat. Rev. Microbiol. **7:**654–665. http://dx.doi.org/10.1038/nrmicro2199.
17. **Hauser AR, Cobb E, Bodi M, Mariscal D, Valles J, Engel JN, Rello J.** 2002. Type III protein secretion is associated with poor clinical outcomes in patients with ventilator-associated pneumonia caused by Pseudomonas aeruginosa. Crit. Care Med. **30:**521–528. http://dx.doi.org/10.1097/00003246-200203000-00005.
18. **Frithz-Lindsten E, Du Y, Rosqvist R, Forsberg A.** 1997. Intracellular targeting of exoenzyme S of Pseudomonas aeruginosa via type III-dependent translocation induces phagocytosis resistance, cytotoxicity and disruption of actin microfilaments. Mol. Microbiol. **25:**1125–1139. http://dx.doi.org/10.1046/j.1365-2958.1997.5411905.x.
19. **Franchi L, Stoolman J, Kanneganti TD, Verma A, Ramphal R, Nunez G.** 2007. Critical role for Ipaf in Pseudomonas aeruginosa-induced caspase-1 activation. Eur. J. Immunol. **37:**3030–3039. http://dx.doi.org/10.1002/eji.200737532.
20. **Neri D, Supuran CT.** 2011. Interfering with pH regulation in tumours as a therapeutic strategy. Nat. Rev. Drug Discov. **10:**767–777. http://dx.doi.org/10.1038/nrd3554.
21. **Mansson B, Geborek P, Saxne T, Bjornsson S.** 1990. Cytidine deaminase activity in synovial fluid of patients with rheumatoid arthritis: relation to lactoferrin, acidosis, and cartilage proteoglycan release. Ann. Rheum. Dis. **49:**594–597. http://dx.doi.org/10.1136/ard.49.8.594.
22. **Ng AW, Bidani A, Heming TA.** 2004. Innate host defense of the lung: effects of lung-lining fluid pH. Lung **182:**297–317. http://dx.doi.org/10.1007/s00408-004-2511-6.
23. **Lardner A.** 2001. The effects of extracellular pH on immune function. J. Leukoc. Biol. **69:**522–530.
24. **Kellum JA, Song M, Li J.** 2004. Science review: extracellular acidosis and the immune response: clinical and physiologic implications. Crit. Care **8:**331–336. http://dx.doi.org/10.1186/cc2900.
25. **Rotstein OD, Nasmith PE, Grinstein S.** 1987. The Bacteroides by-product succinic acid inhibits neutrophil respiratory burst by reducing intracellular pH. Infect. Immun. **55:**864–870.
26. **Simmen HP, Blaser J.** 1993. Analysis of pH and pO2 in abscesses, peritoneal fluid, and drainage fluid in the presence or absence of bacterial infection during and after abdominal surgery. Am. J. Surg. **166:**24–27. http://dx.doi.org/10.1016/S0002-9610(05)80576-8.
27. **Bryant RE, Rashad AL, Mazza JA, Hammond D.** 1980. β-Lactamase activity in human pus. J. Infect. Dis. **142:**594–601. http://dx.doi.org/10.1093/infdis/142.4.594.
28. **Song Y, Salinas D, Nielson DW, Verkman AS.** 2006. Hyperacidity of secreted fluid from submucosal glands in early cystic fibrosis. Am. J. Physiol. Cell Physiol. **290:**C741–C749. http://dx.doi.org/10.1152/ajpcell.00379.2005.
29. **Pezzulo AA, Tang XX, Hoegger MJ, Alaiwa MH, Ramachandran S, Moninger TO, Karp PH, Wohlford-Lenane CL, Haagsman HP, van Eijk M, Banfi B, Horswill AR, Stoltz DA, McCray PB, Jr, Welsh MJ, Zabner J.** 2012. Reduced airway surface pH impairs bacterial killing in the porcine cystic fibrosis lung. Nature **487:**109–113. http://dx.doi.org/10.1038/nature11130.
30. **Coakley RD, Grubb BR, Paradiso AM, Gatzy JT, Johnson LG, Kreda SM, O'Neal WK, Boucher RC.** 2003. Abnormal surface liquid pH regulation by cultured cystic fibrosis bronchial epithelium. Proc. Natl. Acad. Sci. U. S. A. **100:**16083–16088. http://dx.doi.org/10.1073/pnas.2634339100.
31. **Poulsen JH, Fischer H, Illek B, Machen TE.** 1994. Bicarbonate conductance and pH regulatory capability of cystic fibrosis transmembrane conductance regulator. Proc. Natl. Acad. Sci. U. S. A. **91:**5340–5344. http://dx.doi.org/10.1073/pnas.91.12.5340.
32. **Yoon SS, Coakley R, Lau GW, Lymar SV, Gaston B, Karabulut AC, Hennigan RF, Hwang SH, Buettner G, Schurr MJ, Mortensen JE, Burns JL, Speert D, Boucher RC, Hassett DJ.** 2006. Anaerobic killing of mucoid Pseudomonas aeruginosa by acidified nitrite derivatives under cystic fibrosis airway conditions. J. Clin. Invest. **116:**436–446. http://dx.doi.org/10.1172/JCI24684.
33. **Garland AL, Walton WG, Coakley RD, Tan CD, Gilmore RC, Hobbs CA, Tripathy A, Clunes LA, Bencharit S, Stutts MJ, Betts L, Redinbo MR, Tarran R.** 2013. Molecular basis for pH-dependent mucosal dehydration in cystic fibrosis airways. Proc. Natl. Acad. Sci. U. S. A. **110:**15973–15978. http://dx.doi.org/10.1073/pnas.1311999110.
34. **Edye ME, Lopez-Castejon G, Allan SM, Brough D.** 2013. Acidosis drives damage-associated molecular pattern (DAMP)-induced interleukin-1 secretion via a caspase-1-independent pathway. J. Biol. Chem. **288:**30485–30494. http://dx.doi.org/10.1074/jbc.M113.478941.
35. **Jancic CC, Cabrini M, Gabelloni ML, Rodriguez Rodrigues C, Salamone G, Trevani AS, Geffner J.** 2011. Low extracellular pH stimulates the

production of IL-1beta by human monocytes. Cytokine **57:**258–268. http://dx.doi.org/10.1016/j.cyto.2011.11.013.
36. **Takenouchi T, Iwamaru Y, Sugama S, Tsukimoto M, Fujita M, Sekigawa A, Sekiyama K, Sato M, Kojima S, Conti B, Hashimoto M, Kitani H.** 2011. The activation of P2X7 receptor induces cathepsin D-dependent production of a 20-kDa form of IL-1beta under acidic extracellular pH in LPS-primed microglial cells. J. Neurochem. **117:**712–723. http://dx.doi.org/10.1111/j.1471-4159.2011.07240.x.
37. **Rajamaki K, Nordstrom T, Nurmi K, Akerman KE, Kovanen PT, Oorni K, Eklund KK.** 2013. Extracellular acidosis is a novel danger signal alerting innate immunity via the NLRP3 inflammasome. J. Biol. Chem. **288:**13410–13419. http://dx.doi.org/10.1074/jbc.M112.426254.
38. **Sutterwala FS, Ogura Y, Szczepanik M, Lara-Tejero M, Lichtenberger GS, Grant EP, Bertin J, Coyle AJ, Galan JE, Askenase PW, Flavell RA.** 2006. Critical role for NALP3/CIAS1/cryopyrin in innate and adaptive immunity through its regulation of caspase-1. Immunity **24:**317–327. http://dx.doi.org/10.1016/j.immuni.2006.02.004.
39. **Kayagaki N, Warming S, Lamkanfi M, Vande Walle L, Louie S, Dong J, Newton K, Qu Y, Liu J, Heldens S, Zhang J, Lee WP, Roose-Girma M, Dixit VM.** 2011. Non-canonical inflammasome activation targets caspase-11. Nature **479:**117–121. http://dx.doi.org/10.1038/nature10558.
40. **Inaba K, Inaba M, Deguchi M, Hagi K, Yasumizu R, Ikehara S, Muramatsu S, Steinman RM.** 1993. Granulocytes, macrophages, and dendritic cells arise from a common major histocompatibility complex class II-negative progenitor in mouse bone marrow. Proc. Natl. Acad. Sci. U. S. A. **90:**3038–3042. http://dx.doi.org/10.1073/pnas.90.7.3038.
41. **Amiel E, Alonso A, Uematsu S, Akira S, Poynter ME, Berwin B.** 2009. Pivotal advance: Toll-like receptor regulation of scavenger receptor-A-mediated phagocytosis. J. Leukoc. Biol. **85:**595–605. http://dx.doi.org/10.1189/jlb.1008631.
42. **Patankar YR, Lovewell RR, Poynter ME, Jyot J, Kazmierczak BI, Berwin B.** 2013. Flagellar motility is a key determinant of the magnitude of the inflammasome response to Pseudomonas aeruginosa. Infect. Immun. **81:**2043–2052. http://dx.doi.org/10.1128/IAI.00054-13.
43. **Cisz M, Lee PC, Rietsch A.** 2008. ExoS controls the cell contact-mediated switch to effector secretion in Pseudomonas aeruginosa. J. Bacteriol. **190:**2726–2738. http://dx.doi.org/10.1128/JB.01553-07.
44. **Anderson GG, Yahr TL, Lovewell RR, O'Toole GA.** 2010. The Pseudomonas aeruginosa magnesium transporter MgtE inhibits transcription of the type III secretion system. Infect. Immun. **78:**1239–1249. http://dx.doi.org/10.1128/IAI.00865-09.
45. **Redelman CV, Chakravarty S, Anderson GG.** 2014. Antibiotic treatment of Pseudomonas aeruginosa biofilms stimulates expression of the magnesium transporter gene mgtE. Microbiology **160:**165–178. http://dx.doi.org/10.1099/mic.0.070144-0.
46. **Miller JH.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
47. **Geiss GK, Bumgarner RE, Birditt B, Dahl T, Dowidar N, Dunaway DL, Fell HP, Ferree S, George RD, Grogan T, James JJ, Maysuria M, Mitton JD, Oliveri P, Osborn JL, Peng T, Ratcliffe AL, Webster PJ, Davidson EH, Hood L, Dimitrov K.** 2008. Direct multiplexed measurement of gene expression with color-coded probe pairs. Nat. Biotechnol. **26:**317–325. http://dx.doi.org/10.1038/nbt1385.
48. **Martinez D, Vermeulen M, Trevani A, Ceballos A, Sabatte J, Gamberale R, Alvarez ME, Salamone G, Tanos T, Coso OA, Geffner J.** 2006. Extracellular acidosis induces neutrophil activation by a mechanism dependent on activation of phosphatidylinositol 3-kinase/Akt and ERK pathways. J. Immunol. **176:**1163–1171. http://dx.doi.org/10.4049/jimmunol.176.2.1163.
49. **Mariathasan S, Newton K, Monack DM, Vucic D, French DM, Lee WP, Roose-Girma M, Erickson S, Dixit VM.** 2004. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. Nature **430:**213–218. http://dx.doi.org/10.1038/nature02664.
50. **Stehlik C, Lee SH, Dorfleutner A, Stassinopoulos A, Sagara J, Reed JC.** 2003. Apoptosis-associated speck-like protein containing a caspase recruitment domain is a regulator of procaspase-1 activation. J. Immunol. **171:**6154–6163. http://dx.doi.org/10.4049/jimmunol.171.11.6154.
51. **Nakayama S, Watanabe H.** 1995. Involvement of cpxA, a sensor of a two-component regulatory system, in the pH-dependent regulation of expression of Shigella sonnei virF gene. J. Bacteriol. **177:**5062–5069.
52. **Beuzon CR, Banks G, Deiwick J, Hensel M, Holden DW.** 1999. pH-dependent secretion of SseB, a product of the SPI-2 type III secretion system of Salmonella typhimurium. Mol. Microbiol. **33:**806–816. http://dx.doi.org/10.1046/j.1365-2958.1999.01527.x.
53. **Frank DW.** 1997. The exoenzyme S regulon of Pseudomonas aeruginosa. Mol. Microbiol. **26:**621–629. http://dx.doi.org/10.1046/j.1365-2958.1997.6251991.x.
54. **Vakulskas CA, Brady KM, Yahr TL.** 2009. Mechanism of transcriptional activation by Pseudomonas aeruginosa ExsA. J. Bacteriol. **191:**6654–6664. http://dx.doi.org/10.1128/JB.00902-09.
55. **Dasgupta N, Ashare A, Hunninghake GW, Yahr TL.** 2006. Transcriptional induction of the Pseudomonas aeruginosa type III secretion system by low Ca^{2+} and host cell contact proceeds through two distinct signaling pathways. Infect. Immun. **74:**3334–3341. http://dx.doi.org/10.1128/IAI.00090-06.
56. **Cogswell JP, Godlevski MM, Wisely GB, Clay WC, Leesnitzer LM, Ways JP, Gray JG.** 1994. NF-kappa B regulates IL-1 beta transcription through a consensus NF-kappa B binding site and a nonconsensus CRE-like site. J. Immunol. **153:**712–723.
57. **Bellocq A, Suberville S, Philippe C, Bertrand F, Perez J, Fouqueray B, Cherqui G, Baud L.** 1998. Low environmental pH is responsible for the induction of nitric-oxide synthase in macrophages. Evidence for involvement of nuclear factor-kappaB activation. J. Biol. Chem. **273:**5086–5092.
58. **Garcia-Calvo M, Peterson EP, Rasper DM, Vaillancourt JP, Zamboni R, Nicholson DW, Thornberry NA.** 1999. Purification and catalytic properties of human caspase family members. Cell Death Differ. **6:**362–369. http://dx.doi.org/10.1038/sj.cdd.4400497.
59. **Matsuyama S, Llopis J, Deveraux QL, Tsien RY, Reed JC.** 2000. Changes in intramitochondrial and cytosolic pH: early events that modulate caspase activation during apoptosis. Nat. Cell Biol. **2:**318–325. http://dx.doi.org/10.1038/35014006.
60. **Szeto CC, Chow KM, Leung CB, Wong TY, Wu AK, Wang AY, Lui SF, Li PK.** 2001. Clinical course of peritonitis due to Pseudomonas species complicating peritoneal dialysis: a review of 104 cases. Kidney Int. **59:**2309–2315. http://dx.doi.org/10.1046/j.1523-1755.2001.00748.x.
61. **Ricciardolo FL, Gaston B, Hunt J.** 2004. Acid stress in the pathology of asthma. J. Allergy Clin. Immunol. **113:**610–619. http://dx.doi.org/10.1016/j.jaci.2003.12.034.
62. **Tate S, MacGregor G, Davis M, Innes JA, Greening AP.** 2002. Airways in cystic fibrosis are acidified: detection by exhaled breath condensate. Thorax **57:**926–929. http://dx.doi.org/10.1136/thorax.57.11.926.
63. **Douidar SM.** 1997. Nebulized sodium bicarbonate in acute chlorine inhalation. Pediatr. Emerg. Care **13:**406–407. http://dx.doi.org/10.1097/00006565-199712000-00014.
64. **Kostikas K, Papatheodorou G, Ganas K, Psathakis K, Panagou P, Loukides S.** 2002. pH in expired breath condensate of patients with inflammatory airway diseases. Am. J. Respir. Crit. Care Med. **165:**1364–1370. http://dx.doi.org/10.1164/rccm.200111-068OC.
65. **Bakker J, Coffernils M, Leon M, Gris P, Vincent JL.** 1991. Blood lactate levels are superior to oxygen-derived variables in predicting outcome in human septic shock. Chest **99:**956–962. http://dx.doi.org/10.1378/chest.99.4.956.
66. **Deshpande GG, Heidemann SM, Sarnaik AP.** 2000. Heat stress is associated with decreased lactic acidemia in rat sepsis. Crit. Care **4:**45–49. http://dx.doi.org/10.1186/cc649.
67. **Mijares LA, Wangdi T, Sokol C, Homer R, Medzhitov R, Kazmierczak BI.** 2011. Airway epithelial MyD88 restores control of Pseudomonas aeruginosa murine infection via an IL-1-dependent pathway. J. Immunol. **186:**7080–7088. http://dx.doi.org/10.4049/jimmunol.1003687.