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ACINETOBACTER QUORUM SENSING CONTRIBUTES TO INFLAMMATION-INDUCED INHIBITION OF ORTHOPAEDIC IMPLANT OSSEOINTEGRATION

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Abstract

Implant infection impairs osseointegration of orthopaedic implants by inducing inflammation. *Acinetobacter* spp. are increasingly prevalent multi-drug resistant bacteria that can cause osteomyelitis. *Acinetobacter* spp. can also cause inflammation and thereby inhibit osseointegration in mice. The purpose of the present study was to investigate the role of quorum sensing in this context. Therefore, wild-type bacteria were compared with an isogenic *abaI* mutant defective in quorum sensing in a murine osseointegration model. The *abaI* quorum-sensing mutant affected significantly less osseointegration and interleukin (IL) 1β levels, without detectably altering other pro-inflammatory cytokines. Wild-type bacteria had fewer effects on IL1 receptor (IL1R)$^{-/-}$ mice. These results indicated that quorum sensing in *Acinetobacter* spp. contributed to IL1β induction and the resultant inhibition of osseointegration in mice. Moreover, targeting the Gram-negative acyl-homoserine lactone quorum sensing may be particularly effective for patients with *Acinetobacter* spp. infections.

Keywords: *Acinetobacter*, implant infection, osseointegration, osteolysis, quorum sensing.

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List of Abbreviations

A. baumannii — *Acinetobacter baumannii*
A. nosocomialis — *Acinetobacter nosocomialis*
abaI — acyl-homoserine-lactone synthase AbaI
ANOVA — analysis of variance
ASTM — American Society for Testing and Materials
c-fms — colony-stimulating factor-1 receptor
CCL2 — C-C motif chemokine ligand 2

CFU	colony-forming unit
ELISA	enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
FLI	fluorescence imaging
IL	interleukin
IL1R	IL1 receptor
MHB	Muller-Hinton broth
PBS	phosphate-buffered saline
RANKL	receptor activator of nuclear factor kappa-B ligand
ROI	region of interest
TNF	tumour necrosis factor

Introduction

The Gram-negative *Acinetobacter calcoaceticus-A. baumannii* complex is a relatively common cause of osteomyelitis and delayed healing of orthopaedic injuries (Davis *et al.*, 2005; Johnson *et al.*, 2007; Yun *et al.*, 2008). For example, *Acinetobacter* spp. were identified in 50-70 % of osteomyelitis cases in American soldiers wounded in Afghanistan or Iraq (Davis *et al.*, 2005; Johnson *et al.*, 2007; Yun *et al.*, 2008). Those osteomyelitis cases required multiple surgical debridements of necrotic bone and led to delayed fracture healing. Extended courses of antibiotics were administered and were likely responsible for the low frequency of recurrent infection with *Acinetobacter* spp. (Davis *et al.*, 2005; Johnson *et al.*, 2007; Yun *et al.*, 2008). *Acinetobacter* spp. can persist in healthcare environments (Weber *et al.*, 2010) and also frequently acquires multi-drug resistance, further complicating clinical outcomes (Davis *et al.*, 2005; Fily *et al.*, 2019; Munoz-Price and Weinstein, 2008; Perez *et al.*, 2011; Tan and Moenster, 2019; Weber *et al.*, 2010). Consistent with inflammatory responses induced by *Acinetobacter* spp. in soft tissues and the bloodstream (Doi *et al.*, 2015; Dou *et al.*, 2017; Feng *et al.*, 2014; Lin *et al.*, 2012; Mortensen and Skaar, 2012), *Acinetobacter* spp. also cause inflammation and thereby inhibit osteointegration in mice (Choe *et al.*, 2022).

Bacterial implant infection is a devastating complication for orthopaedic patients that induces inflammation and osteolysis and thereby inhibits osteogenesis and osseointegration, causing loosening of previously well-fixed implants (Campoccia *et al.*, 2006). A difficulty in treatment of implant infection is associated with formation of a biofilm. Bacterial biofilms reduce clearance of implant infections by antibiotics and the host immune system (Arnold *et al.*, 2014; Costerton *et al.*, 2007; Lazar *et al.*, 2021). Bacterial density within biofilms is controlled by quorum sensing mediated by autoinducers that regulate bacterial gene expression (Bhargava *et al.*, 2010). Quorum sensing in *Acinetobacter* spp. also regulates virulence, motility, antimicrobial tolerance and modulation of the host immune system (Bhuiyan *et al.*, 2016; Clemmer *et al.*, 2011; Dou *et al.*, 2017; Glucksam-Galnoy *et al.*, 2013; Sun *et al.*, 2021; Tang *et al.*, 2020). Similar to most other Gram-negative bacteria, the primary quorum sensing mediators in *Acinetobacter* spp. are acyl-homoserine lactones, produced by an autoinducer synthase encoded by the *abaI* gene (Anbazhagan *et al.*, 2012; Bhargava *et al.*, 2010; Gonzalez *et al.*, 2009; Niu *et al.*, 2008). The receptor for the acyl-homoserine lactones in *Acinetobacter* spp. is encoded by *abaR* (Bhargava *et al.*, 2010). Recent RNA-sequencing analysis showed that the abaI/abaR quorum-sensing system can regulate expression of numerous genes in *Acinetobacter* spp., including genes that are important for virulence, biofilm formation, antibiotic resistance, energy metabolism, degradation of branched-chain amino acids and lipid metabolism (Sun *et al.*, 2021).

The present study used a previously described murine model to assess effects of implant infection on osseointegration (Choe *et al.*, 2015; 2022) in wild-type mice or mice null for IL1R. *A. nosocomialis* strain M2 was used, which was previously known as *A. baumannii* strain M2 and was originally isolated from a hip infection (Carruthers *et al.*, 2013). To address the role of quorum sensing, a strain M2 mutant was used that lacks quorum sensing and has modestly reduced biofilm formation due to a transposon insertion (abaI::EZTn5<kan>) into *abaI* (Niu *et al.*, 2008). Findings revealed that *Acinetobacter* spp. quorum sensing contributes to inflammation and impaired osseointegration in mice.

Material and Methods

Preparation of implants with adherent bacteria

Titanium alloy screw-shaped implants (Ti-6Al-4V, 3.2 mm length, 1.0 mm diameter, Antrin Miniature Specialties Inc, Fallbrook, CA, USA) were rigorously cleaned following five cycles of alternating treatments in alkali ethanol (0.1 mol/L NaOH and 95 % ethanol at 32 °C) and 25 % nitric acid (Bonsignore *et al.*, 2011). Wild-type *A. nosocomialis* strain M2 was compared with *abaI* isogenic mutant (Niu *et al.*, 2008) to determine effects of quorum sensing. 1 d before each implant surgery, a single colony of wild-type or *abaI* mutant *A. nosocomialis* strain M2 was inoculated into 5 mL of MHB medium (Fisher Scientific) and incubated at 37 °C overnight in a bacterial shaker. Overnight suspensions were diluted 100-fold in MHB medium and incubated at 37 °C until early log phase was reached (A600/0.1 cm light path = 0.05; Nanodrop 1000; Fisher Scientific). Those low-concentration bacterial suspensions (1-3 × 10^9 CFUs/mL) were centrifuged (1,500 ×*g*, 5 min) and resuspended in 1/30 volume of MHB broth to obtain high concentration suspensions (3-9 × 10^{10} CFUs/mL). Rigorously cleaned implants were incubated with high concentration bacterial suspensions for 24 h at 37 °C with gentle shaking to obtain the *A. nosocomialis* strain M2 dose previously found to routinely provide chronic localised implant infections without any signs of systemic sepsis (Choe *et al.*, 2022). Implants with adherent bacteria were rinsed 3-times in PBS (Pro200H, Pro Scientific, Oxford, CT, USA) (pH 7.4) and immediately implanted into mice as described below. Additional implants were simultaneously prepared to measure the adherent CFUs following sonication in PBS with 0.3 % Tween-80 for 10 min (50 W, 43,000 Hz) and vortexing for 5 min (Bernthal *et al.*, 2010; Pribaz *et al.*, 2011). Adherent wild-type and *abaI* (Niu *et al.*, 2008) isogenic mutant *A. nosocomialis* CFUs were 0.3-1 × 10^7 CFUs/implant, without any statistical difference among different bacterial groups.

Animal surgery

Wild-type C57BL/6J and IL1R$^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and MAFIA mice (Burnett *et al.*, 2004; Chinnery *et al.*, 2009) were a gift from Dr Eric Pearlman (CWRU Department of Ophthalmology, Cleveland, OH, USA). All experiments with IL1R$^{-/-}$ mice included wild-type control mice matched for genetic background (C57BL/6J), age (6-8 weeks old) and sex. Mice were maintained in the CWRU Animal Resource Center and all procedures were approved by the CWRU Institutional Animal Care and Use Committee. Mice were randomised among groups, anaesthetised and treated with analgesics (0.5 mg/kg local marcaine and 1.0 mg/kg systemic slow-release buprenorphine) according to a previously established protocol (Choe *et al.*, 2015; 2022). Briefly, a unicortical pilot hole was made manually (0.75 mm pilot hole drill, KLS Martin, Jacksonville, FL, USA) at the anterior medial aspect of the femoral diaphysis and the implants were manually screwed into the pilot hole. The femur fractured during implantation in one of the 169 mice used for the study. That mouse was euthanised immediately and excluded from the analysis. The remaining 168 mice tolerated the surgery well, could ambulate immediately and were included in the study.

FLI

In MAFIA mice, a monocyte/macrophage-specific c-fms promoter drives expression of both enhanced green fluorescent protein and a modified version of fas that can induce apoptosis in response to the small molecule inducer AP20187 (Burnett *et al.*, 2004; Chinnery *et al.*, 2009). Since FLI signals are severely attenuated by overlying tissues, the femora, implants and surrounding soft tissues were exposed for *ex vivo* imaging by dissection and opening the soft tissue. FLI signals were defined by automatic spectral segmentation and quantified in automatically selected ROIs encompassing femora and surrounding soft tissues using a Maestro Imaging System (Perkin Elmer) in the CWRU Center for Imaging Research.

Histomorphometry

Dissected femora were prepared for histomorphology assessment as described previously (Bonsignore *et al.*, 2011). Briefly, femora were fixed in formalin for

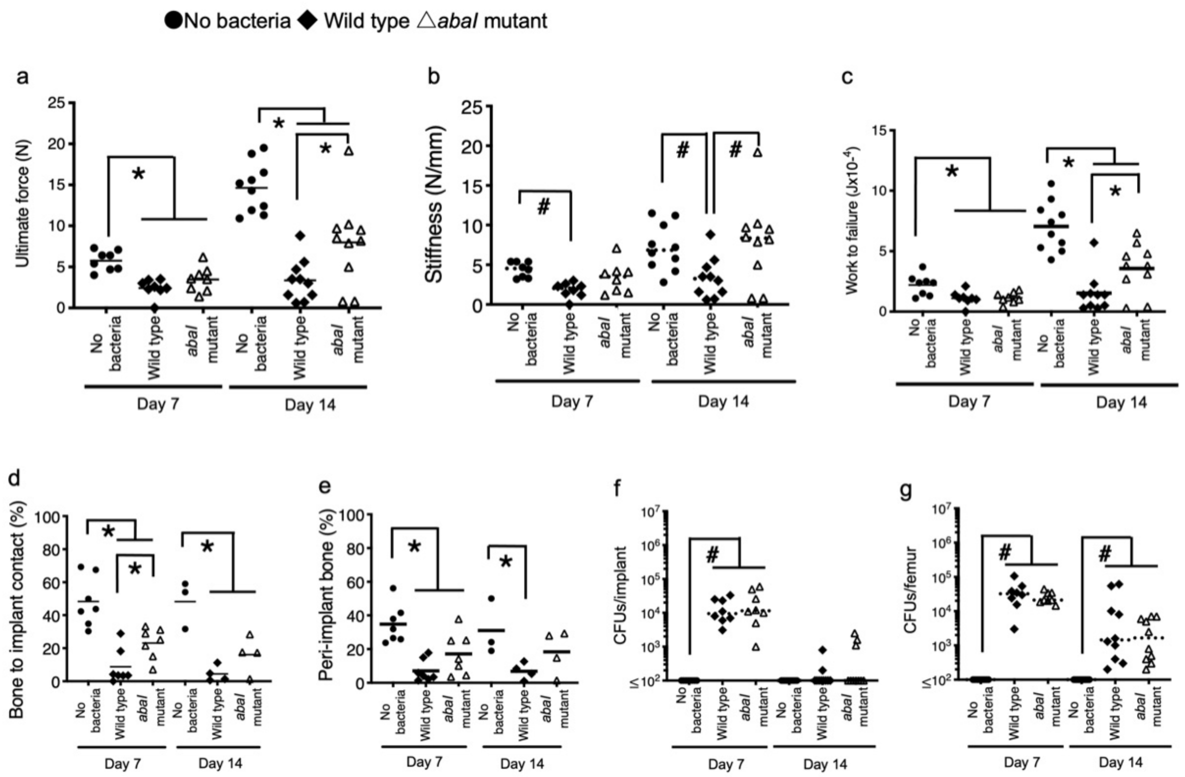

Fig. 1. *abaI* mutation reduced the effect of *Acinetobacter* on osseointegration. (**a**-**c**) Biomechanical and (**d**-**e**) histomorphometric measures of osseointegration and CFUs on (**f**) implants and (**g**) in surrounding femora of control groups without bacteria and of isogenic *A. nosocomialis* strain M2 wild-type and *abaI* mutant groups were measured in C57BL/6J mice. Solid horizontal bars show means for parametric analysis (* $p < 0.05$). Dashed bars show medians for non-parametric analysis ($^{\#}$ $p < 0.05$).

24 h and dehydrated in 70 % ethanol. Undecalcified ground cross-sections (100 μm) were stained with Sanderson's Rapid Bone Stain (Surgipath Medical Industries, Richmond, IL, USA). Bone-to-implant contact and peri-implant bone were measured using ImageJ analysis software (National Institutes of Health). The percentage of direct bone-to-implant contact was calculated in a ROI extending from the periosteal surface of the cortex to the tip of the last implant thread. The percentage of peri-implant bone was calculated in a ROI between the implant threads. The bottom edge of the implant was excluded from all calculations (Bonsignore *et al.*, 2011).

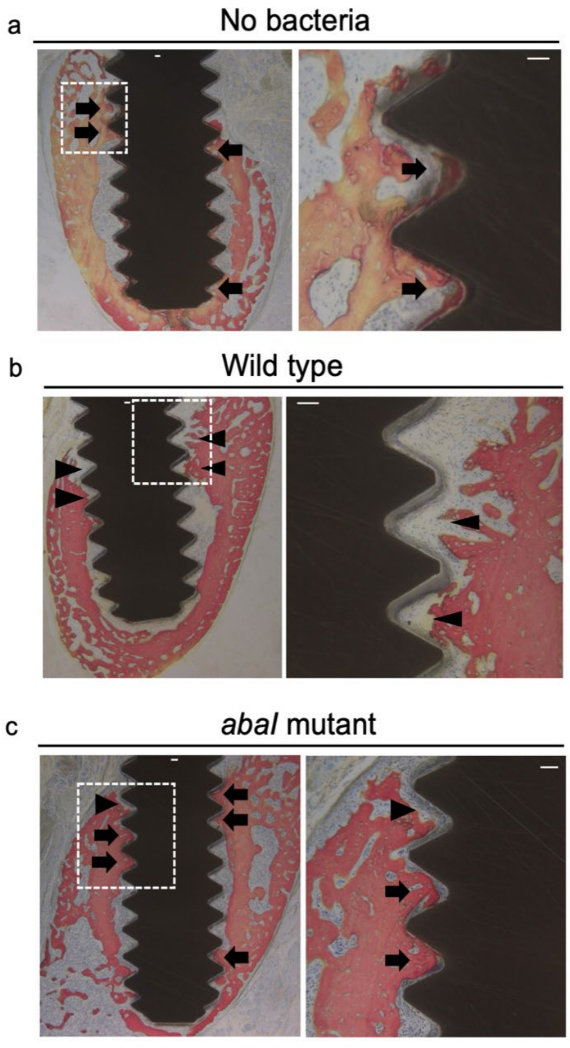

Fig. 2. Histology of osseointegration. (**a-c**) Representative histological images of mice with median histomorphometry results in control group without bacteria and in isogenic *A. nosocomialis* strain M2 wild-type and *abaI* mutant groups at day 14 after implantation. Osseointegration is indicated by contact between bone (stained pink) and implants (dark brown or black) without interposing cells (blue) and by bone formation (stained pink) between threads of the implants (dark brown or black). Osteolysis is indicated by arrow heads and new bone formation is indicated by arrows. All scale bars: 100 μm.

Biomechanical testing
Pull-out testing was performed immediately after euthanasia as previously described (Bonsignore *et al.*, 2011), except a Test Resources 100R Series Single Column Frame (Test Resources, Shakopee, MN, USA) with a 100R Controller was used. Briefly, femora were placed under wire loops embedded in poly-methyl methacrylate and the implant was gripped by a custom-designed jig, which was then attached to the Test Resources Frame. Force was measured through a 4.5 kg capacity load cell and testing was performed at a displacement rate of 1 mm/min. Ultimate force, average stiffness and work to failure were determined from load *versus* displacement curves according to ASTM standards, F543-07. To reduce pre-loading variability, calculations of work began when force equalled 0.3 N.

CFU counting
CFUs on implants and in surrounding femora were quantified after pull-out testing (Choe *et al.*, 2015; 2022). Implants were sonicated for 10 min (50 W, 43,000 Hz) in PBS with 0.3 % Tween-80 followed by vortexing for 5 min (Bernthal *et al.*, 2010; Pribaz *et al.*, 2011). Femora were homogenised in PBS (Bernthal *et al.*, 2010). CFUs in sonicates and homogenates were counted on MHB broth agar plates.

Evaluation of pro-inflammatory cytokines and chemokine
Femur homogenates were centrifuged (9,000 ×g, 10 min) and supernatants were stored at − 20 °C. The concentrations of TNFα, IL1α, IL1β, IL6, RANKL and CCL2 were measured using ELISA DuoSet mini-kits (catalogue numbers DY410, DY400, DY401, DY406, DY462 and DY479, R&D Systems). Biomechanical testing, CFU counting and cytokine measurements were all done on the same mice.

Statistical analysis
All statistical analyses were performed using Prism 7 software (GraphPad Software). Statistical significance was determined by Student's t-test or one-way ANOVA followed by Bonferroni *post-hoc* test in experiments with multiple groups. Non-parametric Mann-Whitney tests or Kruskal-Wallis analysis of variance followed by Student-Newman-Keuls *post-hoc* tests were applied to data sets that were not normally distributed or were not of equal variance. Tests were reported as significant for $p < 0.05$.

Results

Effect of *Acinetobacter* on osseointegration
No signs of systemic infection were observed in any mice. Osseointegration increased in groups without bacteria between 7 and 14 d post-implantation (circles in Fig. 1**a-c**, Fig. 2**a**). In contrast, all three biomechanical (diamonds in Fig. 1**a-c**) and both histomorphometric (diamonds in Fig. 1**d**,**e**, Fig. 2**b**)

measures of osseointegration were reduced by wild-type *A. nosocomialis* strain M2 at both time points.

Effect of *abaI*-deficiency on osseointegration in *Acinetobacter* infection

To determine mechanisms responsible for effects of *A. nosocomialis* strain M2, an *abaI*-deficient isogenic mutant was used that lacks quorum sensing (Niu *et al.*, 2008). The *abaI* quorum-sensing mutant (upward triangles in Fig. 1**a**-**e**, Fig. 2**c**) had less effect than the wild-type strain. For example, the wild-type and *abaI* mutant strains were significantly different regarding all three biomechanical measures of osseointegration at day 14 (Fig. 1**a**-**c**) and with regard to both histomorphometric measures at day 7 (Fig. 1**d**,**e**). Importantly, effects of the *abaI* mutation were not due to different bacterial growth since neither mutant altered the number of bacteria on implants or in surrounding bones (Fig. 1**f**,**g**).

Difference in induction of cytokines between wild-type *Acinetobacter* and mutants

Although *A. nosocomialis* strain M2 infection led to increased macrophage recruitment to the site of infection, the *abaI* mutation did not alter macrophage recruitment (Fig. 3**a**). CCL2, TNFα, IL6, IL1α and IL1β were measured in femora surrounding implants as examples of local inflammatory cytokines. CCL2 and TNFα were not induced at either day 7 or day 14 following implantation (Fig. 3**b**,**c**). IL6 and IL1α were increased equivalently by wild-type *A. nosocomialis* strain M2 and the *abaI* mutant strain (Fig. 3**d**-**f**). In contrast, the IL1β level closely tracked with impaired osseointegration as the *abaI* mutant (upward triangles) had significantly less effect on IL1β at day 7 (Fig. 3**f**), on bone to implant contact at day 7 (Fig. 1**d**) and on all three biomechanical measures of osseointegration at day 14 (Fig. 1**a**-**c**).

Effect of IL1R on cytokine expression and impaired osseointegration in implant infection with *Acinetobacter*

To test the functional role of IL1β, effects of *A. nosocomialis* strain M2 in wild-type and IL1R$^{-/-}$ mice were compared (Glaccum *et al.*, 1997). Consistent with an important role for IL1β, wild-type *A. nosocomialis* strain M2 had less effect on ultimate force in IL1R$^{-/-}$ mice (diamonds in Fig. 4**a**) and there was a trend towards less effect on work to failure (p = 0.07, diamonds in Fig. 4**b**), while stiffness was not affected by IL1R deletion (diamonds Fig. 4**c**). Similarly, levels of CCL2, IL6 and RANKL were reduced in IL1R$^{-/-}$ mice at day 14 (diamonds in Fig. 4**d**-**f**). Importantly, the IL1R deletion did not alter osseointegration or

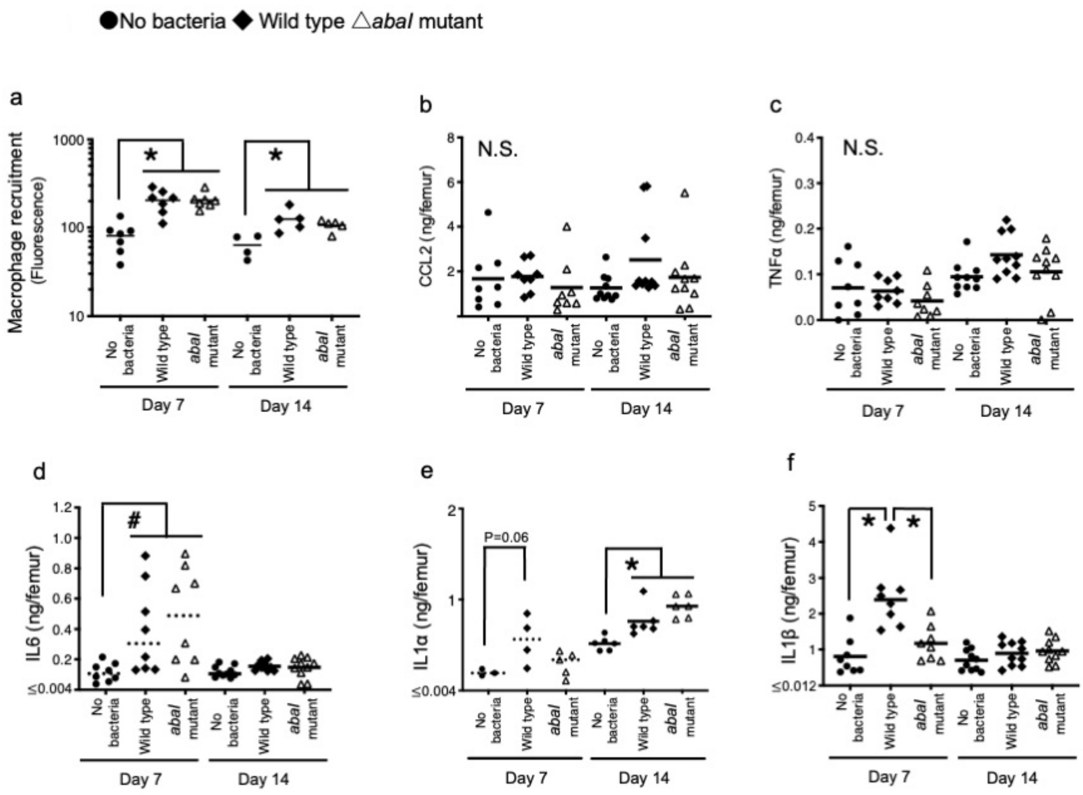

Fig. 3. *abaI* mutation reduced the effects of Acinetobacter on IL1β production.(**a**) Macrophage recruitment was measured in MAFIA mice. Levels of (**b**) CCL2, (**c**) TNFα, (**d**) IL6, (**e**) IL1α and (**f**) IL1β in control groups without bacteria as well as in isogenic *A. nosocomialis* strain M2 wild-type and *abaI* mutant groups were measured in C57BL/6J mice. Assay ranges were (**b**) 58.6 pg-7.5 ng/femur for CCL2, (**c**) 15.6 pg-2 ng/femur for TNFα, (**d**) 15.6 pg-2 ng/femur for IL6, (**e**) 16.4 pg-2.1 ng/femur for IL1α, and (**f**) 78 pg-10 ng/femur for IL1β. Solid horizontal bars show means for parametric analysis (* $p < 0.05$). Dashed bars show medians for non-parametric analysis (# $p < 0.05$). N:S: no significant difference.

cytokine production in the absence of bacteria or in the presence of the *abaI* mutant strain (circles and upward triangles in Fig. 4**a**-**f**). The effects of ILR1 deletion were not due to differential bacterial growth since deletion did not affect the number of wild-type bacteria or the *abaI* mutant on implants or in surrounding bones (Fig. 4**g**,**h**).

Discussion

The present study showed that the quorum-sensing system of *Acinetobacter* spp. infections on implants contributed to macrophage recruitment, production of inflammatory cytokines, osteolysis and impaired osseointegration. These effects are likely due, in part, to regulation by quorum sensing of genes that are important for virulence (Sun *et al.*, 2021; Tang *et al.*, 2020). Also, the effects of *Acinetobacter* spp. quorum sensing were due, in part, to increased induction of IL1β. Those results were consistent with findings that IL1β contributes to inflammatory osteolysis induced by *Staphylococcus aureus* in mice (Bernthal *et al.*, 2011; Putnam *et al.*, 2019; Wang *et al.*, 2020) and that single nucleotide polymorphisms in IL1β and IL1R associate with human osteomyelitis (Alves De Souza *et al.*, 2017; Osman *et al.*, 2016). A limitation of the study was that it was not determined whether the impaired osseointegration was due to reduced osteogenesis, increased osteolysis or both (Choe *et al.*, 2022). The study was also restricted to 14 d following bacterial inoculation and it is, therefore, unknown whether the infection, inflammation or impaired osseointegration would resolve at later time points. Also, it was not determined which virulence factors acted downstream of the quorum-sensing system to regulate IL1β production and osseointegration or which mammalian cells were the direct targets of those virulence factors. It is likely that multiple virulence factors contribute as hundreds of gene are regulated by the quorum-sensing system in *Acinetobacter* spp. (Sun *et al.*, 2021). Virulence factors

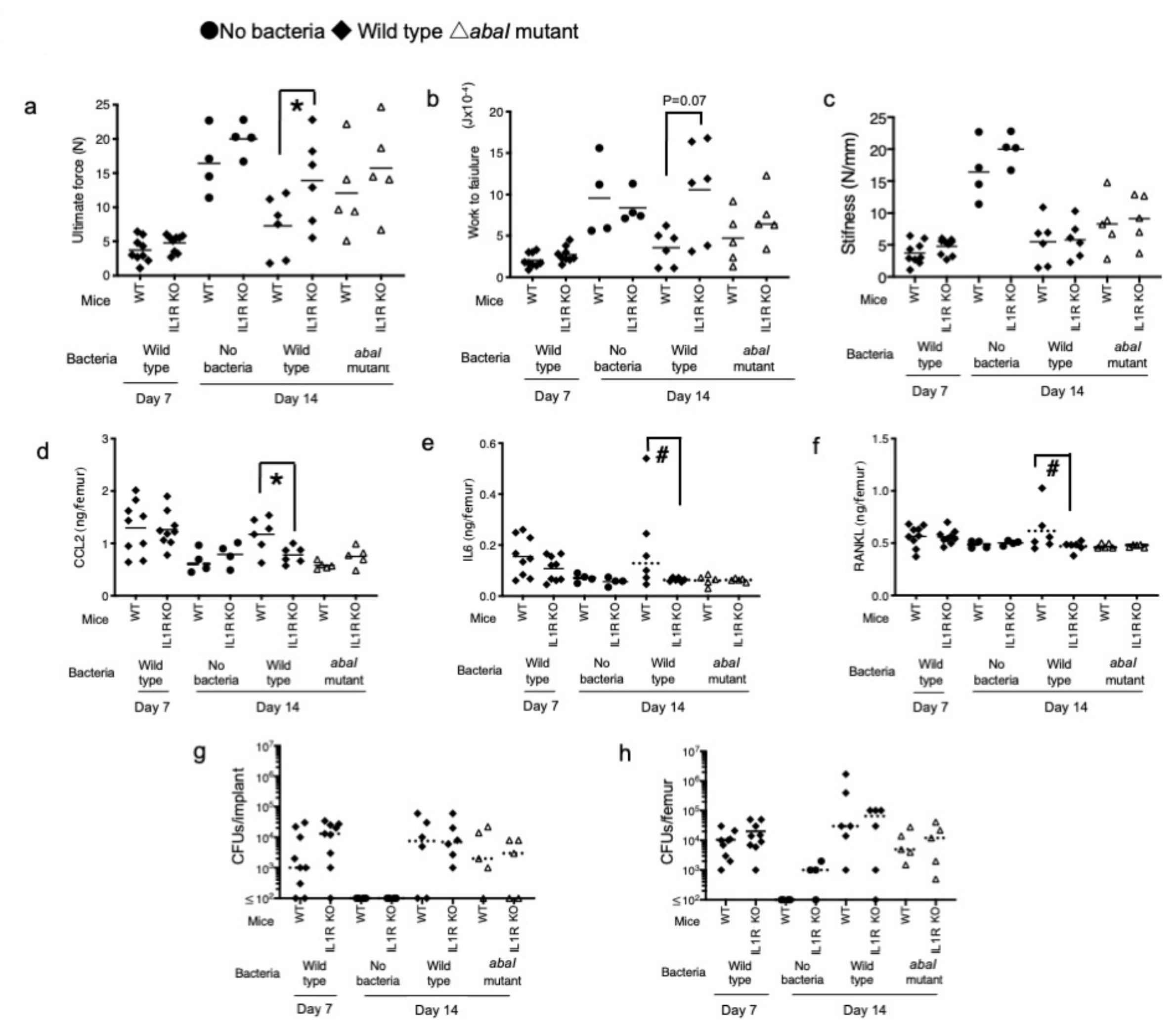

Fig.4. IL-1R mediated the effects of *Acinetobacter* on osseointegration. (**a**-**c**) Biomechanical measures of osseointegration, (**d**-**f**) levels of CCL2, IL6 and RANKL, (**g**,**h**) bacterial burden in control groups without bacteria as well as in isogenic *A. nosocomialis* strain M2 wild-type and *abaI* mutant groups were compared in IL1R1$^{-/-}$ and their wild-type control mice. Assay ranges were (**d**) 19.5 pg-2,5 ng/femur for CCL2, (**e**) 15.6 pg-2 ng/femur for IL6 and (**f**) 31 pg-4 ng/femur for RANKL. Solid horizontal bars show means for parametric analysis (* $p < 0.05$). Dashed bars show medians for non-parametric analysis ($^{\#}$ $p < 0.05$).

that might be involved include the acyl-homoserine lactones themselves acting through mammalian T2R receptors, pathogen-associated molecular patterns that activate mammalian pattern recognition receptors and multiple others (Carey and Lee, 2019; Glucksam-Galnoy *et al.*, 2013; Lin *et al.*, 2012; Bhuiyan *et al.*, 2016; Kale *et al.*, 2017; Morris *et al.*, 2019).

Acinetobacter spp. quorum sensing, similarly to most other Gram-negative bacteria, is mediated by acyl-homoserine lactones (Anbazhagan *et al.*, 2012; Bhargava *et al.*, 2010; Niu *et al.*, 2008). Novel approaches targeting the Gram-negative quorum-sensing system or the T2R mammalian receptors for acyl-homoserine lactones (Carey and Lee, 2019) may, therefore, be particularly effective for *Acinetobacter* spp. infections (Bhargava *et al.*, 2010; Bjarnsholt and Givskov, 2007; Costerton *et al.*, 2007; Lazar *et al.*, 2021). The potential utility of these quorum-quenching approaches is further enhanced by recent reports that acyl-homoserine lactones induce antibiotic resistance in *Acinetobacter* spp. (Dou *et al.*, 2017). Examples of these approaches include developing antagonistic acyl-homoserine lactones (Stacy *et al.*, 2012), engineering thermostable lactonases that can degrade a broad range of acyl-homoserine lactones (Chow *et al.*, 2014) and repurposing drugs that are FDA-approved for other indications (Seleem *et al.*, 2020). The results regarding quorum sensing in the Gram-negative *Acinetobacter* spp. were reminiscent of the extensive literature reviewed by Urish and Cassat (2020) showing that the peptide-based quorum-sensing systems of Gram-positive bacteria also contribute to inflammatory osteolysis.

Importantly, the observed effects of the gene deletions, either in the *A. nosocomialis* strain M2 or in the mice, were not due to differential bacterial growth since none of them altered the number of bacteria on retrieved implants or in surrounding bone. A limitation of the study was use of a transposon mutant without determining whether the effects were reversed in a complemented strain of bacteria. However, it is unlikely that the *abaI* transposon has a polar effect on expression of downstream genes as *abaI* is the last gene in an operon and there are no genes downstream in the same orientation.

In the present study, *A. nosocomialis* strain M2 caused inflammatory osteolysis around implants in addition to impaired osseointegration. This finding would not have been predicted based on the report that *Acinetobacter* spp. increases osteogenesis in mice without detectably inducing osteolysis (Crane *et al.*, 2009). This discrepancy could be due to testing different amounts (Vidlak and Kielian, 2016) or different strains of *Acinetobacter* spp. For example, it is unknown whether the strain used in the previous report (Crane *et al.*, 2009) possesses a quorum-sensing system.

In conclusion, results showed that novel approaches targeting the quorum-sensing system of Gram-negative bacteria may be particularly effective for *Acinetobacter* spp. infections. The murine model will also be useful for future studies to clarify the mechanism of implant failure due to *Acinetobacter* spp. and to assess novel diagnostic tools or therapeutic agents.

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Contributions are as follow. HC, OA, PR, ZL, RB, EG designed the experiments. HC, BH, KH, EG conducted the experiments. HC, OA, PR, ZL, RB, EG wrote the manuscript.

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References

Alves De Souza C, Queiroz Alves De Souza A, Queiroz Alves De Souza MDS, Dias Leite JA, Silva De Morais M, Barem Rabenhorst SH (2017) A link between osteomyelitis and IL1RN and IL1B polymorphisms-a study in patients from Northeast Brazil. Acta Orthop **88**: 556-561.

Anbazhagan D, Mansor M, Yan GO, Md Yusof MY, Hassan H, Sekaran SD (2012) Detection of quorum sensing signal molecules and identification of an autoinducer synthase gene among biofilm forming clinical isolates of Acinetobacter spp. PLoS One **7**: e36696. DOI: 10.1371/journal.pone.0036696.

Arnold WV, Shirtliff ME, Stoodley P (2014) Bacterial biofilms and periprosthetic infections. Instr Course Lect **63**: 385-391.

Bernthal NM, Pribaz JR, Stavrakis AI, Billi F, Cho JS, Ramos RI, Francis KP, Iwakura Y, Miller LS (2011) Protective role of IL-1beta against post-arthroplasty *Staphylococcus aureus* infection. J Orthop Res **29**: 1621-1626.

Bernthal NM, Stavrakis AI, Billi F, Cho JS, Kremen TJ, Simon SI, Cheung AL, Finerman GA, Lieberman JR, Adams JS, Miller LS (2010) A mouse model of post-arthroplasty *Staphylococcus aureus* joint infection to evaluate *in vivo* the efficacy of antimicrobial implant coatings. PLoS One **5**: e12580. DOI: 10.1371/journal.pone.0012580.

Bhargava N, Sharma P, Capalash N (2010) Quorum sensing in *Acinetobacter:* an emerging pathogen. Crit Rev Microbiol **36**: 349-360.

Bhuiyan MS, Ellett F, Murray GL, Kostoulias X, Cerqueira GM, Schulze KE, Mahamad Maifiah MH, Li J, Creek DJ, Lieschke GJ, Peleg AY (2016) *Acinetobacter baumannii* phenylacetic acid metabolism influences infection outcome through a direct effect on neutrophil chemotaxis. Proc Natl Acad Sci U S A **113**: 9599-9604.

Bjarnsholt T, Givskov M (2007) Quorum-sensing blockade as a strategy for enhancing host defences against bacterial pathogens. Philos Trans R Soc Lond B Biol Sci **362**: 1213-1222.

Bonsignore LA, Colbrunn RW, Tatro JM, Messerschmitt PJ, Hernandez CJ, Goldberg VM, Stewart MC, Greenfield EM (2011) Surface contaminants inhibit osseointegration in a novel murine model. Bone **49**: 923-930.

Burnett SH, Kershen EJ, Zhang J, Zeng L, Straley SC, Kaplan AM, Cohen DA (2004) Conditional macrophage ablation in transgenic mice expressing a Fas-based suicide gene. J Leukoc Biol **75**: 612-623.

Campoccia D, Montanaro L, Arciola CR (2006) The significance of infection related to orthopedic devices and issues of antibiotic resistance. Biomaterials **27**: 2331-2339.

Carey RM, Lee RJ (2019) Taste receptors in upper airway innate immunity. Nutrients **11**: 2017. DOI: 10.3390/nu11092017.

Carruthers MD, Harding CM, Baker BD, Bonomo RA, Hujer KM, Rather PN, Munson RS Jr (2013) Draft genome sequence of the clinical isolate *Acinetobacter nosocomialis* strain M2. Genome Announc **1**: e00906-13. DOI: 10.1128/genomeA.00906-13.

Chinnery HR, Carlson EC, Sun Y, Lin M, Burnett SH, Perez VL, McMenamin PG, Pearlman E (2009) Bone marrow chimeras and c-fms conditional ablation (Mafia) mice reveal an essential role for resident myeloid cells in lipopolysaccharide/TLR4-induced corneal inflammation. J Immunol **182**: 2738-2744.

Choe H, Narayanan AS, Gandhi DA, Weinberg A, Marcus RE, Lee Z, Bonomo RA, Greenfield EM (2015) Immunomodulatory peptide IDR-1018 decreases implant infection and preserves osseointegration. Clin Orthop Relat Res **473**: 2898-2907.

Choe H, Tatro JM, Hausman BS, Hujer KM, Marshall SH, Akkus O, Rather PN, Lee Z, Bonomo RA, Greenfield EM (2022) *Staphylococcus aureus* and *Acinetobacter* sp. inhibit osseointegration of orthopaedic implants. Infect Immun **90**: e0066921. DOI: 10.1128/iai.00669-21.

Chow JY, Yang Y, Tay SB, Chua KL, Yew WS (2014) Disruption of biofilm formation by the human pathogen *Acinetobacter baumannii* using engineered quorum-quenching lactonases. Antimicrob Agents Chemother **58**: 1802-1805.

Clemmer KM, Bonomo RA, Rather PN (2011) Genetic analysis of surface motility in *Acinetobacter baumannii*. Microbiology (Reading) **157**: 2534-2544.

Costerton JW, Montanaro L, Arciola CR (2007) Bacterial communications in implant infections: a target for an intelligence war. Int J Artif Organs **30**: 757-763.

Crane DP, Gromov K, Li D, Soballe K, Wahnes C, Buchner H, Hilton MJ, O'Keefe RJ, Murray CK, Schwarz EM (2009) Efficacy of colistin-impregnated beads to prevent multidrug-resistant *A. baumannii* implant-associated osteomyelitis. J Orthop Res **27**: 1008-1015.

Davis KA, Moran KA, McAllister CK, Gray PJ (2005) Multidrug-resistant *Acinetobacter* extremity infections in soldiers. Emerg Infect Dis **11**: 1218-1224.

Doi Y, Murray GL, Peleg AY (2015) *Acinetobacter baumannii*: evolution of antimicrobial resistance-treatment options. Semin Respir Crit Care Med **36**: 85-98.

Dou Y, Song F, Guo F, Zhou Z, Zhu C, Xiang J, Huan J (2017) Acinetobacter baumannii quorum-sensing signalling molecule induces the expression of drug-resistance genes. Mol Med Rep **15**: 4061-4068.

Feng Z, Jia X, Adams MD, Ghosh SK, Bonomo RA, Weinberg A (2014) Epithelial innate immune response to *Acinetobacter baumannii* challenge. Infect Immun **82**: 4458-4465.

Fily F, Ronat JB, Malou N, Kanapathipillai R, Seguin C, Hussein N, Fakhri RM, Langendorf C (2019) Post-traumatic osteomyelitis in Middle East war-wounded civilians: resistance to first-line antibiotics in selected bacteria over the decade 2006-2016. BMC Infect Dis **19**: 103. DOI: 10.1186/s12879-019-3741-9.

Glaccum MB, Stocking KL, Charrier K, Smith JL, Willis CR, Maliszewski C, Livingston DJ, Peschon JJ, Morrissey PJ (1997) Phenotypic and functional characterization of mice that lack the type I receptor for IL-1. J Immunol **159**: 3364-3371.

Glucksam-Galnoy Y, Sananes R, Silberstein N, Krief P, Kravchenko VV, Meijler MM, Zor T (2013) The bacterial quorum-sensing signal molecule N-3-oxo-dodecanoyl-L-homoserine lactone reciprocally modulates pro- and anti-inflammatory cytokines in activated macrophages. J Immunol **191**: 337-344.

Gonzalez RH, Dijkshoorn L, Van den Barselaar M, Nudel C (2009) Quorum sensing signal profile of *Acinetobacter* strains from nosocomial and environmental sources. Rev Argent Microbiol **41**: 73-78.

Johnson EN, Burns TC, Hayda RA, Hospenthal DR, Murray CK (2007) Infectious complications of open type III tibial fractures among combat casualties. Clin Infect Dis **45**: 409-415.

Kale SD, Dikshit N, Kumar P, Balamuralidhar V, Khameneh HJ, Bin Abdul Malik N, Koh TH, Tan GGY, Tan TT, Mortellaro A, Sukumaran B (2017) Nod2 is required for the early innate immune clearance of *Acinetobacter baumannii* from the lungs. Sci Rep **7**: 17429. DOI: 10.1038/s41598-017-17653-y.

Lazar V, Holban AM, Curutiu C, Chifiriuc MC (2021) Modulation of quorum sensing and biofilms in less investigated gram-negative ESKAPE pathogens. Front Microbiol **12**: 676510. DOI: 10.3389/fmicb.2021.676510.

Lin L, Tan B, Pantapalangkoor P, Ho T, Baquir B, Tomaras A, Montgomery JI, Reilly U, Barbacci EG, Hujer K, Bonomo RA, Fernandez L, Hancock RE, Adams MD, French SW, Buslon VS, Spellberg B (2012) Inhibition of LpxC protects mice from resistant Acinetobacter baumannii by modulating inflammation and enhancing phagocytosis. mBio **3**. DOI: 10.1128/mBio.00312-12.

Morris FC, Dexter C, Kostoulias X, Uddin MI, Peleg AY (2019) The mechanisms of disease caused by *Acinetobacter baumannii*. Front Microbiol **10**: 1601. DOI: 10.3389/fmicb.2019.01601.

Mortensen BL, Skaar EP (2012) Host-microbe interactions that shape the pathogenesis of *Acinetobacter baumannii* infection. Cell Microbiol **14**: 1336-1344.

Munoz-Price LS, Weinstein RA (2008) Acinetobacter infection. N Engl J Med **358**: 1271-1281.

Niu C, Clemmer KM, Bonomo RA, Rather PN (2008) Isolation and characterization of an autoinducer synthase from *Acinetobacter baumannii*. J Bacteriol **190**: 3386-3392.

Osman AE, Mubasher M, ElSheikh NE, AlHarthi H, AlZahrani MS, Ahmed N, ElGhazali G, Bradley BA, Fadil AS (2016) Association of single nucleotide polymorphisms in pro-inflammatory cytokine and toll-like receptor genes with pediatric hematogenous osteomyelitis. Genet Mol Res **15**. DOI: 10.4238/gmr.15027718.

Perez F, Ponce-Terashima R, Adams MD, Bonomo RA (2011) Are we closing in on an “elusive enemy”? The current status of our battle with *Acinetobacter baumannii*. Virulence **2**: 86-90.

Pribaz JR, Bernthal NM, Billi F, Cho JS, Ramos RI, Guo Y, Cheung AL, Francis KP, Miller LS (2011) Mouse model of chronic post-arthroplasty infection: noninvasive *in vivo* bioluminescence imaging to monitor bacterial burden for long-term study. J Orthop Res **30**: 335-340.

Putnam NE, Fulbright LE, Curry JM, Ford CA, Petronglo JR, Hendrix AS, Cassat JE (2019) MyD88 and IL-1R signaling drive antibacterial immunity and osteoclast-driven bone loss during *Staphylococcus aureus* osteomyelitis. PLoS Pathog **15**: e1007744. DOI: 10.1371/journal.ppat.1007744.

Seleem NM, Abd El Latif HK, Shaldam MA, El-Ganiny A (2020) Drugs with new lease of life as quorum sensing inhibitors: for combating MDR *Acinetobacter baumannii* infections. Eur J Clin Microbiol Infect Dis **39**: 1687-1702.

Stacy DM, Welsh MA, Rather PN, Blackwell HE (2012) Attenuation of quorum sensing in the pathogen *Acinetobacter baumannii* using non-native N-Acyl homoserine lactones. ACS Chem Biol **7**: 1719-1728.

Sun X, Ni Z, Tang J, Ding Y, Wang X, Li F (2021) The abaI/abaR quorum sensing system effects on pathogenicity in *Acinetobacter baumannii*. Front Microbiol **12**: 679241. DOI: 10.3389/fmicb.2021.679241.

Tan X, Moenster RP (2019) Ceftolozane-tazobactam for the treatment of osteomyelitis caused by multidrug-resistant pathogens: a case series. Ther Adv Drug Saf **11**: 2042098619862083. DOI: 10.1177/2042098619862083.

Tang J, Chen Y, Wang X, Ding Y, Sun X, Ni Z (2020) Contribution of the abaI/abaR quorum sensing system to resistance and virulence of *Acinetobacter baumannii* clinical strains. Infect Drug Resist **13**: 4273-4281.

Urish KL, Cassat JE (2020) *Staphylococcus aureus* osteomyelitis: bone, bugs, and surgery. Infect Immun **88**: e00932-00919.

Vidlak D, Kielian T (2016) Infectious dose dictates the host response during *Staphylococcus aureus* orthopedic-implant biofilm infection. Infect Immun **84**: 1957-1965.

Wang Y, Ashbaugh AG, Dikeman DA, Zhang J, Ackerman NE, Kim SE, Falgons C, Ortines RV, Liu H, Joyce DP, Alphonse MP, Dillen CA, Thompson JM, Archer NK, Miller LS (2020) Interleukin-1beta and tumor necrosis factor are essential in controlling an experimental orthopedic implant-associated infection. J Orthop Res **38**: 1800-1809.

Weber DJ, Rutala WA, Miller MB, Huslage K, Sickbert-Bennett E (2010) Role of hospital surfaces in the transmission of emerging health care-associated pathogens: norovirus, *Clostridium difficile*, and *Acinetobacter* species. Am J Infect Control **38**: S25-33.

Yun HC, Branstetter JG, Murray CK (2008) Osteomyelitis in military personnel wounded in Iraq and Afghanistan. J Trauma **64**: S163-168.

Discussion with Reviewer

Reviewer: In this model, the inoculated bacteria do not establish a long-term infection as evidenced by low or zero CFU at day 14 on the implant. Therefore, any effects of infection are only in the early post-operative phase. Does this suggest that *Acinetobacter* is not a true bone pathogen but rather induces inflammation in contaminated wounds that may extend to bone, without actually inducing osteomyelitis?

Authors: We agree that the CFUs are lower on day 14 than on day 7, especially on the implants (Fig. 1**f**). However, Fig. 1**g** shows substantial CFUs on day 14 in the femora of the groups with either wild-type or *abaI* mutant bacteria. The study was restricted to

14 d after bacterial inoculation and it is, therefore, unknown whether the infection, inflammation or impaired osseointegration would resolve at later time points. *Acinetobacter* is frequently considered to cause osteomyelitis in human patients (Davis *et al.*, 2005; Fily *et al.*, 2019; Johnson *et al.*, 2007; Tan *et al.*, 2019; Yun *et al.*, 2008).

Editor's note: The Scientific Editor responsible for this paper was Fintan Moriarty.