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# Differential responses of osteoblasts and macrophages upon *Staphylococcus aureus* infection

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## Abstract

**Background:** *Staphylococcus aureus* (*S. aureus*) is one of the primary causes of bone infections which are often chronic and difficult to eradicate. Bacteria like *S. aureus* may survive upon internalization in cells and may be responsible for chronic and recurrent infections. In this study, we compared the responses of a phagocytic cell (i.e. macrophage) to a non-phagocytic cell (i.e. osteoblast) upon *S. aureus* internalization.

**Results:** We found that upon internalization, *S. aureus* could survive for up to 5 and 7 days within macrophages and osteoblasts, respectively. Significantly more *S. aureus* was internalized in macrophages compared to osteoblasts and a significantly higher (100 fold) level of live intracellular *S. aureus* was detected in macrophages compared to osteoblasts. However, the percentage of *S. aureus* survival after infection was significantly lower in macrophages compared to osteoblasts at post-infection days 1–6. Interestingly, macrophages had relatively lower viability in shorter infection time periods (i.e. 0.5–4 h; significant at 2 h) but higher viability in longer infection time periods (i.e. 6–8 h; significant at 8 h) compared to osteoblasts. In addition, *S. aureus* infection led to significant changes in reactive oxygen species production in both macrophages and osteoblasts. Moreover, infected osteoblasts had significantly lower alkaline phosphatase activity at post-infection day 7 and infected macrophages had higher phagocytosis activity compared to non-infected cells.

**Conclusions:** *S. aureus* was found to internalize and survive within osteoblasts and macrophages and led to differential responses between osteoblasts and macrophages. These findings may assist in evaluation of the pathogenesis of chronic and recurrent infections which may be related to the intracellular persistence of bacteria within host cells.

**Keywords:** Infection, Internalization, Intracellular bacteria, *S. aureus*, Chronic infection, Recurrent infection

## Background

*Staphylococcus aureus* (*S. aureus*) is one of the primary causes of bone infections [1–3]. These infections are often chronic, difficult to eradicate, and have high morbidity rates [4]. *S. aureus* can infiltrate deep into bone and soft tissue as a result of severe trauma or surgical implants [5]. Although *S. aureus* has traditionally been considered an extracellular pathogen, it has been reported by several groups that this bacterium can invade and survive within

a variety of cells such as neutrophils, macrophages, T-lymphocytes, epithelial cells, endothelial cells, fibroblasts, and osteoblasts [6–16]. One hypothesis, not yet proven, about chronic and recurrent infections is that bacteria internalize into host cells and the internalization may lead to the bacteria's evasion of the host's immune responses and provide protection from most conventional antibiotics [17,18].

The primary role of osteoblasts is to synthesize bone components and induce bone matrix mineralization [19]. Osteoblasts are not traditionally considered part of the immune system. However, osteoblasts were recently found to be able to induce inflammatory cytokines and chemokines upon *S. aureus* internalization [20,21]. This finding may suggest an important role for osteoblasts in

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triggering immune responses after *S. aureus* infection. *S. aureus* can be internalized into osteoblasts and its internalization is believed to be mediated by binding of fibronectin-binding proteins on *S. aureus* surfaces and fibronectins on osteoblast surfaces, which are connected to the integrin dimer  $\alpha_5\beta_1$  molecule [6]. Protein-ligand interaction leads to *S. aureus* adhesion and invasion by a “zipper-like” mechanism [15]. Eventually, internalized bacteria escape into the cytoplasm and may lead to host cell death by apoptosis [22]. In addition, live osteoblasts are necessary for *S. aureus* internalization as *S. aureus* could not internalize into formalin-fixed osteoblasts [10,23].

The major role of phagocytes like macrophages, neutrophils, and dendritic cells is to engulf and eliminate a wide variety of invading pathogens. As a result, high influxes of such phagocytes are expected at the infection site upon pathogen invasion. For instance, a high influx of neutrophils was detected at the infection site of *S. aureus* bone infection [24]. Unfortunately, some pathogens can survive within these phagocytes after being phagocytized which may lead to chronic diseases [25,26]. It was reported that *S. aureus* can survive within neutrophils and its survival may have contributed to infection persistence as well as dissemination *in vivo* [7]. Neutrophils are short-lived and are unlikely to carry intracellular pathogens for long [27]. Macrophages, however, are long-lived and may possibly allow surviving pathogens to invade the circulatory system from localized infection sites [28] and thereby may be more likely to contribute to chronic and recurrent infections.

The aims of this study were to compare *S. aureus* internalization in a phagocytic cell (i.e. macrophage) to a non-phagocytic cell (i.e. osteoblast) and to investigate macrophage and osteoblast responses upon *S. aureus* infection. We hypothesized that *S. aureus* can internalize into macrophages and osteoblasts and lead to differential responses.

## Results

### Characterization of *S. aureus* infection of osteoblasts and macrophages

*S. aureus* was incubated with osteoblasts or macrophages for 2 h, with a multiplicity of infection (MOI) from 100:1 to 1000:1; the MOI represents the *S. aureus* to osteoblast or macrophage ratio. Osteoblasts and macrophages were both found to be infected. However, significantly higher (~100 fold) numbers of intracellular *S. aureus* were found within macrophages compared to osteoblasts (Figure 1A); the intracellular colony forming units (CFUs) for infected macrophages and osteoblasts were approximately  $3.5 \times 10^6$  and  $3.1 \times 10^4$  CFU/( $10^5$  cells), respectively. No significant differences were observed in the same cell type at the various MOIs studied (i.e. 100:1, 500:1, and 1000:1). By contrast, significantly lower viability was observed in macrophages compared to osteoblasts at 2 h infection; the viability of macrophages and osteoblasts were 62-78%

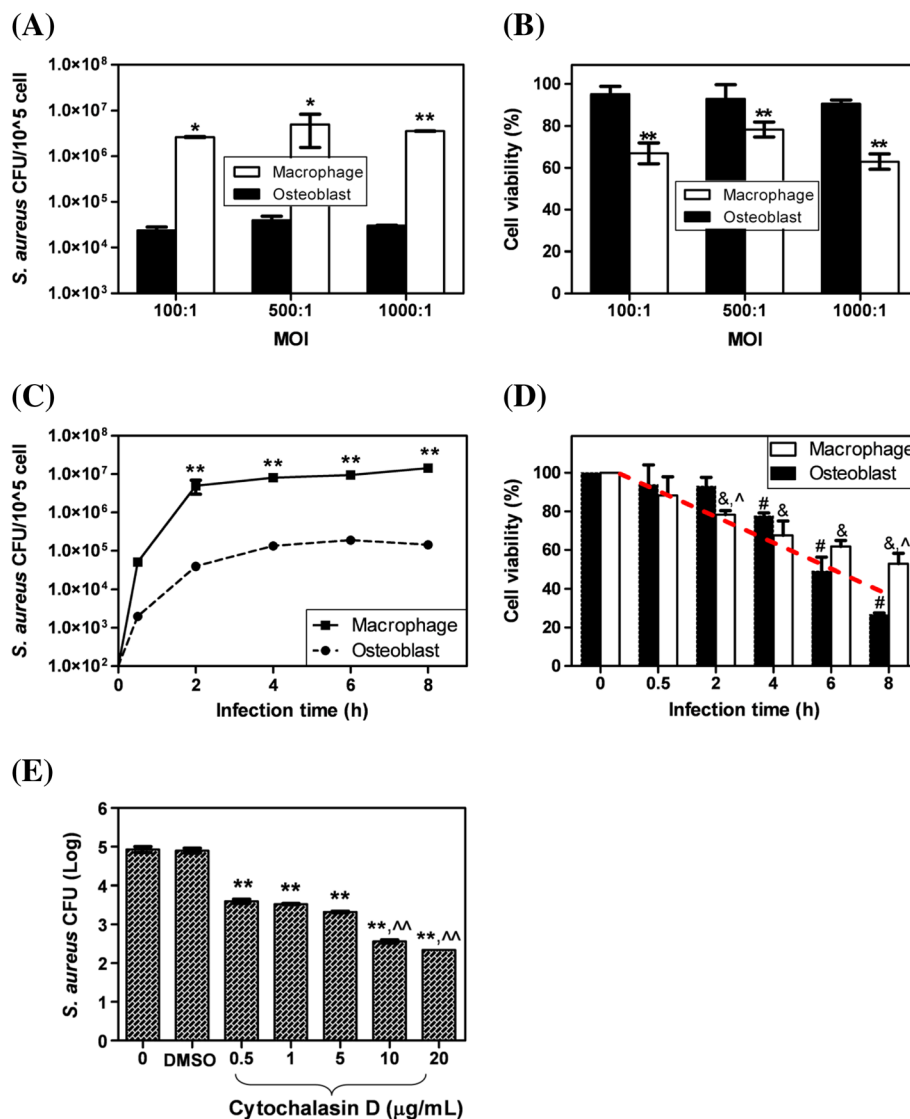
and 90-95%, respectively (Figure 1B). No significant differences in viability for the same cell type at the MOIs investigated (i.e. 100:1, 500:1, and 1000:1) were noted following the 2 h infection.

At an MOI of 500:1, the number of intracellular *S. aureus* for both macrophages and osteoblasts increased with increasing infection time and reached a plateau at 2 h, at which point the intracellular CFUs for macrophages and osteoblasts were  $5.0 \times 10^6$  and  $3.9 \times 10^4$  CFU/( $10^5$  cells), respectively (Figure 1C). At infection times of 2–8 h, the intracellular CFUs for macrophages were significantly higher (about 100 fold) than those of osteoblasts. At an MOI of 500:1, the viability of macrophages and osteoblasts decreased approximately linearly with increasing infection time. The viability of macrophages at infection times of 2, 4, 6, and 8 h was significantly lower than that of both macrophage control and at infection time of 0.5 h. The viability of osteoblasts at infection times of 4, 6, and 8 h was significantly lower than that of both osteoblast control and at infection time of 0.5 h (Figure 1D). In addition, the viability of macrophages was significantly lower at 2 h infection but significantly higher at 8 h infection compared to osteoblasts at corresponding infection time periods (Figure 1D).

The *S. aureus* infection of osteoblasts was also found to be significantly inhibited by the addition of cytochalasin D. The intracellular CFUs of *S. aureus* decreased significantly with increasing cytochalasin D at the dose range studied (0.5-20  $\mu$ g/mL), reaching 50% inhibition at 20  $\mu$ g/mL (Figure 1E). Relatively higher cytochalasin D doses of 10 and 20  $\mu$ g/mL also led to significantly lower intracellular CFUs of *S. aureus* compared to the doses of 0.5, 1, and 5  $\mu$ g/mL (Figure 1E).

*S. aureus* was found to be able to survive within macrophages and osteoblasts for approximately a week; live intracellular *S. aureus* was found in macrophages and osteoblasts for 5 and 7 days, respectively (Figure 2). The percentage of live intracellular *S. aureus* for both macrophages and osteoblasts decreased continuously with increasing culturing time after infection, and significantly reduced survival of *S. aureus* was found in macrophages compared to osteoblasts at the same post-infection time period (Figure 2). In addition, no differences in osteoblast proliferation were observed between infected and non-infected osteoblasts within one week post-infection (data not shown).

Confocal microscopy and transmission electron microscopy (TEM) images confirmed that *S. aureus* was internalized and could survive within macrophages and osteoblasts (Figure 3). Meanwhile, substantially more (likely 100 fold) *S. aureus* was internalized (not necessarily alive) by macrophages compared to osteoblasts (Figures 3A and B). Confocal microscopy imaging also found that all live *S. aureus* was inside the osteoblasts and there was no live *S. aureus*



**Figure 1** *S. aureus* infection of osteoblasts and macrophages. **(A)** Live intracellular *S. aureus* and **(B)** viability of osteoblasts and macrophages at different MOIs (100:1, 500:1, and 1000:1) for 2 h. \**p* < 0.05 and \*\**p* < 0.001 compared to osteoblasts at the same MOI. **(C)** Live intracellular *S. aureus* and **(D)** viability of osteoblasts and macrophages at an MOI of 500:1 for various infection times. \*\**p* < 0.001 compared to osteoblasts at the same infection time, #*p* < 0.01 compared to macrophages at infection times 0 and 0.5 h, ^*p* < 0.01 compared to osteoblasts at the same infection times, and #*p* < 0.05 compared to osteoblasts at infection times 0 and 0.5 h. **(E)** Effect of cytochalasin D on *S. aureus* internalization in osteoblasts. \*\**p* < 0.001 compared to the controls and ^^*p* < 0.001 compared to 0.5, 1, and 5 μg/mL.

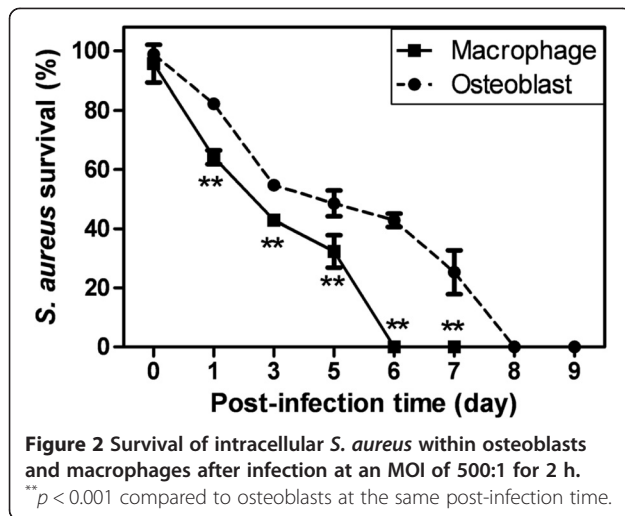
on the surface of the osteoblasts after gentamicin treatment (Figure 3C); this finding was consistent with the bacterial culturing of the washing media after gentamicin treatments – no colonies were found from the washing media. The internalization of *S. aureus* within osteoblasts was found to be non-uniform as some osteoblasts had multiple *S. aureus* bacteria while others had none (Figure 3D).

#### Biological responses of osteoblasts and macrophages upon *S. aureus* infection

Significantly higher hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) levels were observed at 0.5 and 1 h in infected osteoblasts compared

to non-infected osteoblasts, i.e. control (Figure 4A). Significantly higher H<sub>2</sub>O<sub>2</sub> levels were also observed following a 1 h infection in macrophages compared to the non-infected control (Figure 4A). No significant changes in superoxide anion (O<sub>2</sub><sup>-</sup>) production in osteoblasts were observed at the infection time periods studied (i.e. 0.5, 1, and 2 h), while significantly higher O<sub>2</sub><sup>-</sup> levels were found in macrophages at 0.5 and 1 h infection (Figure 4B).

Significantly lower alkaline phosphatase (ALP) enzyme activity was observed at post-infection day 7 in the infected osteoblasts compared to the non-infected cells (i.e. control); no significant changes in ALP enzyme activity were found



between infected and non-infected osteoblasts at days 1 and 4 (Figure 4C). The macrophage phagocytosis activity studies showed that the ability to ingest bacteria was much higher for infected macrophages (83%) compared to non-infected ones (44%) (Figure 4D).

## Discussion

*S. aureus* has been traditionally considered as an extracellular pathogen; however, it has been shown to invade and survive within both non-phagocytic and phagocytic cells. By nature, the internalization and survival of *S. aureus* within non-phagocytic and phagocytic cells would be expected to be different, and may play significantly different roles in related diseases. The main goal of the present study was to compare the internalization behavior and related biological responses of *S. aureus* in a non-phagocytic cell (i.e. osteoblast) and a phagocytic cell (i.e. macrophage); our findings may contribute to the understanding of the pathogenesis of many chronic and recurrent infections.

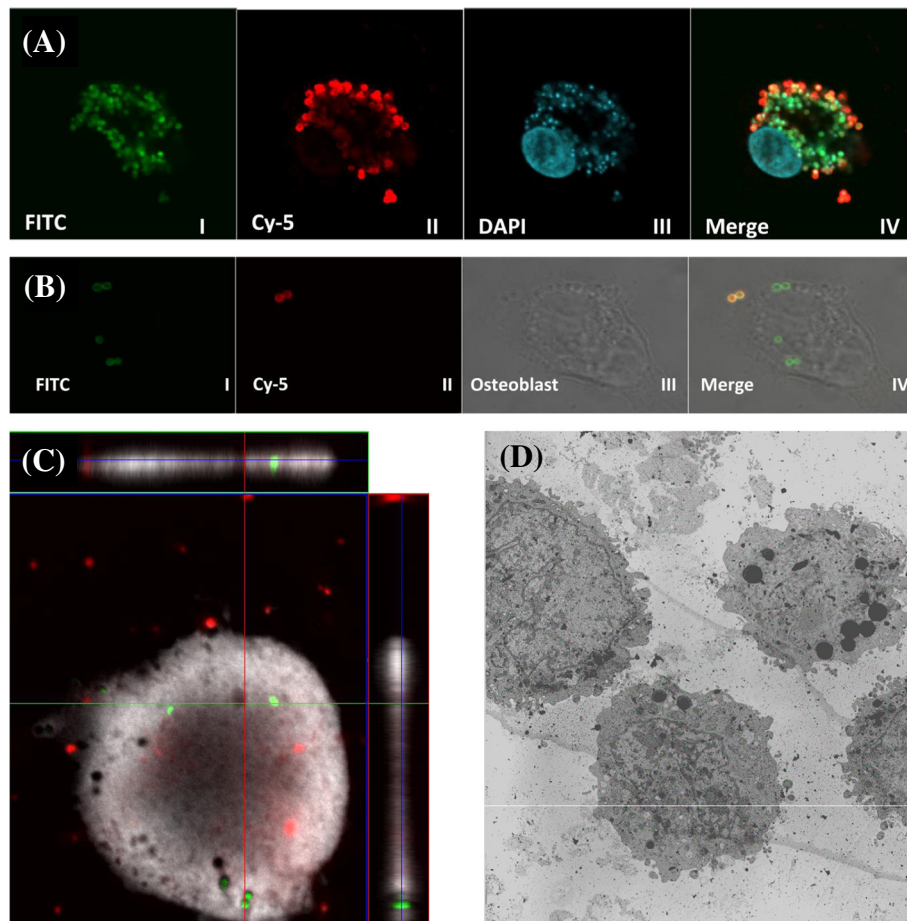
In this study, *S. aureus* was internalized by both osteoblasts and macrophages. The infection of osteoblasts and macrophages was observed as early as 0.5 h at an MOI of 500:1. With increasing infection time, the intracellular CFUs of both osteoblasts and macrophages increased significantly from 0.5 h to 2 h followed by a plateau from 2 h to 8. Our data indicated that an intracellular load of approximately one *S. aureus* per osteoblast (Figure 1C) was sufficient to induce the death of approximately 10% of the osteoblast population within 2 h and 70% within 8 h (Figure 1D). Since macrophages are supposed to engulf and eliminate pathogens on contact, it was not surprising to find that, at the same infection conditions (i.e. MOI of 500:1 for 2 h), significantly more (approximately 100 fold) *S. aureus* (live and dead) was phagocytized by macrophages compared to those

internalized by osteoblasts. Similarly, significantly more live intracellular *S. aureus* was seen in macrophages compared to osteoblasts during infection times of 2–8 h. Macrophages had significantly lower viability at a shorter infection time period (i.e. 2 h) and significantly higher survival at a longer infection time (i.e. 8 h) compared to infected osteoblasts. In addition, it is possible that the accumulation of toxins produced by *S. aureus* [29,30] and the significantly higher levels of H<sub>2</sub>O<sub>2</sub> in infected osteoblasts and macrophages and O<sub>2</sub><sup>-</sup> in infected macrophages affected the viability of macrophages and osteoblasts; both decreased (almost linearly) with increasing infection time. Rasigade *et al.* reported that phenol-soluble modulins, a class of membrane-damaging exoproteins, acted as intracellular toxins and were involved in killing of the host cells like osteoblasts, and they found that the intracellular expression of *psmA* gene resulted in extensive cell damage [31]. It seems that actin cytoskeleton formation played a major role in *S. aureus* internalization since cytochalasin D, an inhibitor that disrupts actin filament polymerization, led to up to 50% inhibition of *S. aureus* internalization. This finding was consistent with other studies [23,32] where actin filament was determined to play a dominant role in *S. aureus* internalization. One limitation of this study was that MOIs lower than 100:1 were not investigated and the higher MOI may only apply to infected tissues where numerous bacteria may exist. Future studies may need to consider lower MOIs.

Note that our data confirmed that gentamicin treatment was effective in eliminating extracellular *S. aureus* and the post-infection CFU was indeed from live intracellular *S. aureus*. Gentamicin treatment is commonly used to eliminate extracellular bacteria [21,32], but such a procedure lacks direct confirmation of live intracellular bacteria. In this study, besides culturing the washing media collected after gentamicin treatment, the dual staining approach combined with confocal microscopy presented direct evidence that no live extracellular *S. aureus* was observed after the gentamicin treatment (Figure 3C).

*S. aureus* has been thought to be a frequent cause for several types of chronic and recurrent infections including osteomyelitis, endovascular diseases, and chronic lung infections [33], and *S. aureus* infections have been reported in clinical cases to persist asymptotically with relapses occurring months or even years after apparent antimicrobial cure of the infections [34,35]. In these cases, *S. aureus* may have protected itself and escaped antibiotics and immune response of the host by “hiding” intracellularly and establishing a latent bacterial reservoir. This was supported by our observation that *S. aureus* could survive intracellularly for up to 5 and 7 days, respectively, within macrophages and osteoblasts (Figure 2). As a phagocytic cell, macrophages were obviously more effective than osteoblasts at not only phagocytizing but also destroying the

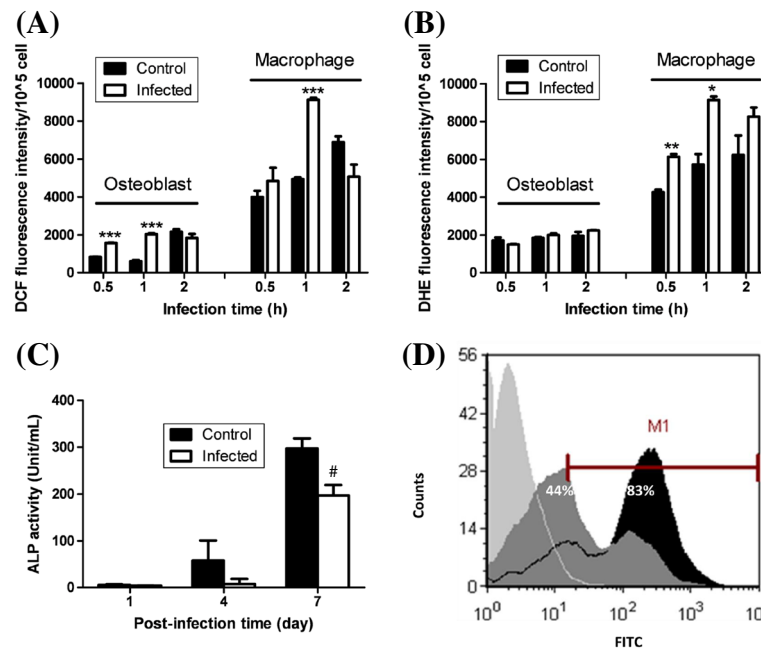




**Figure 3 Visualization of intracellular *S. aureus* within (A) macrophages and (B-D) osteoblasts.** Osteoblasts and macrophages were infected with *S. aureus* at an MOI of 500:1 for 2 h. (A and B) *S. aureus* was stained with FITC before infection. Infected osteoblasts and macrophages were fixed, blocked, stained first with primary antibody to *S. aureus* surface protein A, and then secondary antibody conjugated to Cy-5. The nuclei of the macrophages were additionally stained with DAPI. Visualized at (I) 488 nm, (II) 633 nm, and (III) 405 nm. (IV) Merged images of (I), (II), and (III). As a result, intracellular *S. aureus* is shown in green (FITC) and extracellular *S. aureus* is co-localized with both red (Cy-5) and green (FITC). (C) Z-stack sections were used to confirm that all live *S. aureus* was inside osteoblasts as determined by the X-Y planes. Live *S. aureus* are green (Syto 9) and dead *S. aureus* are red (PI). Osteoblasts were infected with Syto 9-labeled *S. aureus*, then extracellular bacteria were killed with gentamicin and washed. Osteoblasts were detached from the wells and stained with PI. Approximately 20 cells (randomly selected) were examined. (D) Confirmation of intracellular *S. aureus* within osteoblasts using TEM.

intracellular bacteria. This was supported by our data showing that *S. aureus* infection not only significantly increased the  $H_2O_2$  levels in macrophages at 1 h infection but also significantly increased the  $O_2^-$  levels in macrophages at infection times of 0.5 and 1 h. In contrast, *S. aureus* infection induced significantly higher levels of  $H_2O_2$  in osteoblasts at 0.5 and 1 h infection but did not induce significant changes in  $O_2^-$  levels in osteoblasts. As a result, a significantly higher number of intracellular CFUs was found in macrophages immediately after infection while significantly less intracellular *S. aureus* survived in the days following infection. Survival of *S. aureus* within host cells was also reported in tissues and other cell types. For instance, Tuchscher *et al.* reported that *S. aureus* could persist within host cells and/or tissues for several

weeks [36], survive within human lung epithelial cells for up to 2 weeks [37], persist in macrophage vacuoles for 3–4 days before escaping into the cytoplasm and causing macrophage lysis [6], and remain viable for up to 5 days within HT-29 and Caco-2 enterocytes [38]. It was proposed that, once inside osteoblasts, macrophages, or other cells, *S. aureus* may undergo phenotypic switching to small colony variants (SCVs), which are associated with increased anchoring of fibronectin-binding proteins and clumping factors on the bacterial surface [36,39]. These proteins may function as substrates for bacterial enzymes that are needed to evade phagocytic oxidative killing [6,40] thereby contributing to the intracellular survival of *S. aureus*. Moreover, *S. aureus* produces catalase, which catalyzes the decomposition of  $H_2O_2$ , thereby protecting



**Figure 4** Cellular and molecular responses of osteoblasts and macrophages infected with *S. aureus* at an MOI of 500:1 for 2 h. **(A)** DCF fluorescence intensity as an indicator of H<sub>2</sub>O<sub>2</sub> production by non-infected controls and *S. aureus*-infected osteoblasts and macrophages. **(B)** DHE fluorescence intensity as an indicator of O<sub>2</sub><sup>-</sup> production by non-infected controls and *S. aureus*-infected osteoblasts and macrophages. **(C)** Osteoblast ALP activity measured at post-infection days 1, 4, and 7. **(D)** Macrophage phagocytosis activity (ingestion). Light gray area: control macrophages without incubation with *S. aureus*; dark gray area: non-infected macrophages; black area: infected macrophages. \**p* < 0.01, \*\**p* < 0.001, \*\*\**p* < 0.0001, and #*p* < 0.05 compared to control.

itself inside host cells such as macrophages [41]. It was believed that the phenotypic switching of *S. aureus* may make the bacteria more resistant to antibiotics [17,42]. Similarly, *S. epidermidis* was found to persist in macrophages and also in peri-implant tissues in mice despite antibiotic treatments [43,44]. The survival of *S. aureus* within cells like macrophages and osteoblasts and the possible phenotypic switching of *S. aureus* may explain why antibiotics have so often failed to cure *Staphylococcal* infections [2,17,36]. In addition, the presence of antibodies (e.g. anti-tumor necrosis factor-related apoptosis-inducing ligand or TRAIL antibody) may improve the viability of infected host cells and provide better protection for the intra-cellular bacteria [45]. Alexander *et al.* found that in the presence of 1 µg/mL of anti-TRAIL antibody, the percentage of apoptotic cells decreased from the control (in the absence of antibody) of 32% to 28% [45]. Future studies may focus on investigation of the possible changes that occur to *S. aureus* after internalization into osteoblasts and macrophages and the effect of a variety of opsonins potentially present *in vivo*. This study was limited to *in vitro* cell studies which may not reflect what is happening in patients with chronic infections. *In vivo* studies using chronic infection animal models, which may allow monitoring of intracellular presence of *S. aureus* with time, are needed in the future.

*S. aureus* infection also resulted in significantly increased levels of H<sub>2</sub>O<sub>2</sub> in infected osteoblasts at infection times of 0.5 and 1 h and in infected macrophages at infection time of 1 h. The O<sub>2</sub><sup>-</sup> levels in infected macrophages significantly increased at infection times of 0.5 and 1 h. The increase in reactive oxygen species indicates that *S. aureus* internalization induced significant stress in osteoblasts and macrophages which would produce such chemicals to eliminate the invading pathogens. *S. aureus* infection also led to much higher phagocytosis activity of macrophages and significantly lower ALP activity of osteoblasts at day 7 after infection. This effect could be associated with the significant increase in H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> levels. It is noteworthy that, besides the significant changes in reactive oxygen species, *S. aureus* internalization in osteoblasts also led to significantly higher production of IL-6 and IL-12 [21,46], macrophage chemoattractant protein 1, IL-8, IP-10, RANTES [21,46], and RANK-L and prostaglandin E<sub>2</sub> (two important molecules that can promote osteoclastogenesis and bone resorption) [47].

## Conclusions

We compared *S. aureus* internalization in a phagocytic cell (i.e. macrophage) to a non-phagocytic cell (i.e. osteoblast) and investigated the cells' responses upon infection. We found that *S. aureus* could internalize within macrophages

and osteoblasts and, upon infection, a significantly higher number of live intracellular *S. aureus* was observed in macrophages compared to osteoblasts. The viability of macrophages and osteoblasts both decreased with increasing infection time and macrophages had significantly lower viability during 2 h infection and significantly higher viability during 8 h infection compared to osteoblasts. Moreover, intracellular *S. aureus* was found to survive within macrophages and osteoblasts for approximately 5 and 7 days, respectively. The percentage of *S. aureus* survival within macrophages and osteoblasts decreased with increasing post-infection time, and the percentage of *S. aureus* survival within macrophages was significantly lower compared to that within osteoblasts. Moreover, compared to non-infected controls, *S. aureus* infection resulted in (i) significantly increased hydrogen peroxide production in macrophages and osteoblasts, (ii) significantly increased superoxide anion production in macrophages but not in osteoblasts, (iii) significantly lower alkaline phosphatase activity in infected osteoblasts, and (iv) higher phagocytosis activity in infected macrophages.

## Methods

### Reagents

Tryptic soy agar (TSA, w/5% sheep blood) plates, tryptic soy broth (TSB), phosphate buffered saline (PBS), fetal bovine serum (FBS), 0.25% trypsin/2.21 mM ethylenediaminetetraacetic acid (EDTA) solution, 45% glucose solution, 7.5% sodium bicarbonate, sodium pyruvate, and HEPES buffer were all obtained from Fisher Scientific (Pittsburgh, PA). Dulbecco's Modified Eagle Media: Nutrient Mixture F-12 (DMEM/F12) and RPMI-1640 media were purchased from LONZA (Walkersville, MD). DiI fluorescent dye, Syto-9, propidium iodide (PI), and 100 U/mL penicillin/100 mg/mL streptomycin solution were from Invitrogen (Carlsbad, CA). Gentamicin, Triton X-100, cytochalasin D, dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), lysostaphin, fluorescein isothiocyanate (FITC), paraformaldehyde, and glutaraldehyde were obtained from Sigma (St. Louis, MO). Primary antibody Ab20920 and goat polyclonal to rabbit IgG secondary antibody-Cy5 ab6564 were obtained from Abcam (Cambridge, MA). Dichlorofluorescein diacetate (H<sub>2</sub>DCF-DA) and dihydroethidium (DHE) were from Life Technologies (Grand Island, NY).

### Bacteria

*S. aureus* (ATCC 25923) was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Bacteria were prepared as we previously reported [48-52]. Briefly, a fresh inoculum was prepared by suspending 5 colonies of *S. aureus*, grown on a blood agar plate, in 5 mL TSB and incubating at 37°C for 18 h. After incubation, the *S. aureus* inoculum was centrifuged at 3750 rpm for 15 min at 4°C,

washed once with 10 mL PBS, and the bacteria pellet was diluted to (6–8) × 10<sup>8</sup> CFU/mL with sterile PBS. Next, the bacteria were centrifuged again and the bacteria pellet was then re-suspended in either DMEM/F12 for the infection of osteoblasts or in RPMI-1640 medium for the infection of macrophages; both cell culture media were free from streptomycin/penicillin and FBS.

### Infection of osteoblasts with *S. aureus*

Rat osteoblasts (UMR-106) were obtained from ATCC and grown in full-supplemented DMEM/F12 medium containing 10% FBS and 1% penicillin/streptomycin solution. As previously reported [53,54], 3 × 10<sup>5</sup> cells/mL were seeded in 12-well plates (Fisher Scientific) and cultured in full-supplemented DMEM/F12 medium for at least 24 h at 37°C in a 5% CO<sub>2</sub> incubator until they reached ~80% confluence. Osteoblasts were infected and the effects of MOI and infection time on osteoblast infection were investigated: (1) To examine the effect of MOI on osteoblast infection, the osteoblast monolayer was washed 3 times with PBS and then fresh DMEM/F12 medium was added (free from streptomycin/penicillin and FBS). Immediately, *S. aureus* was added at MOIs of 100:1, 500:1, and 1000:1 and incubated for 2 h. (2) To examine the effect of infection time on osteoblast infection, the osteoblast monolayer was washed 3 times with PBS and then fresh DMEM/F12 medium (free from streptomycin/penicillin and FBS) was added. *S. aureus* was added at an MOI of 500:1 and incubated for different times, i.e. infection times, of 0.5, 2, 4, 6, and 8 h. After each treatment, the osteoblast monolayer was washed 3 times with PBS and treated with 100 µg/mL gentamicin (an antibiotic known not to penetrate mammalian cell membranes within a few hours [55,56]) for 2 h at 37°C in a 5% CO<sub>2</sub> incubator. Osteoblasts were then washed 3 times with PBS and immediately lysed with 0.1% Triton X-100 in PBS for 10 min at 37°C; the cell lysates were diluted in PBS and plated on blood agar plates overnight. The washing PBS was collected and plated on blood agar plates overnight as well. To determine viability, osteoblasts were detached by incubating them at 37°C for 3 min in a 0.25% trypsin/2.21 mM EDTA solution; trypsinization was stopped by adding DMEM/F12 medium supplemented with 10% FBS. Viable osteoblasts were counted using a hemocytometer (Bright-Line Hausser Scientific, Horsham, PA) [57] following the trypan-blue exclusion assay which depends on cellular membrane integrity; dead cells allow the stain to penetrate while live cells do not.

### Infection of macrophages with *S. aureus*

A rat alveolar macrophage cell-line (NR 8383) was obtained from ATCC and grown in full-supplemented RPMI-1640 medium containing 10% FBS, 1% streptomycin/penicillin,

45% glucose solution, 7.5% sodium bicarbonate, and sodium pyruvate. The infection of macrophages with *S. aureus* was studied at different MOIs and infection times. The protocols for infecting macrophages were similar to those of infecting osteoblasts as described previously. In brief, to achieve adherence,  $3 \times 10^5$  cells/mL were seeded in 12-well plates and cultured in full-supplemented RPMI-1640 medium for at least 24 h at 37°C in a 5% CO<sub>2</sub> incubator. Cultured macrophages were washed 3 times with PBS and then infected with *S. aureus* at different MOIs (100:1, 500:1, and 1000:1) or infection times (0.5-8 h). Infected macrophages were washed, treated with gentamicin, washed again (the washing media were collected and plated on blood agar plates overnight), and then lysed to determine the number of live intracellular *S. aureus*. To determine the viability of macrophages, adherent macrophages were scraped using a cell scraper (Fisher Scientific) and combined with floating macrophages from the same sample for trypan-blue exclusion assay and hemocytometry.

The viability of osteoblasts and macrophages after infection with *S. aureus* was calculated relevant to their control (non-infected) cells according to the following equation:

$$\text{Viability(\%)} = \frac{\frac{\text{Number of live cell in infected sample}}{\text{Number of live and dead cells in infected sample}}}{\frac{\text{Number of live cells in control sample}}{\text{Number of live and dead cells in control sample}}} \times 100\%$$

Note that the total cell numbers in the infected and control samples were the same at the beginning of the infection (i.e. infection time = 0 h) but were different at later infection time periods (i.e. 0.5-8 h).

#### Inhibition of *S. aureus* internalization in osteoblasts

Cytochalasin D was reconstituted in 1% DMSO.  $3 \times 10^5$  cells/mL were seeded in 12-well plates and cultured in full-supplemented DMEM/F12 medium to reach ~80% confluence. The osteoblast monolayer was washed 3 times with PBS and then fresh DMEM/F12 medium was added (free from streptomycin/penicillin and FBS) together with cytochalasin D (0.5, 1, 5, 10, and 20 µg/mL). After culturing for 30 min, *S. aureus* was added at an MOI of 500:1 and further incubated for 2 h. Following treatment with gentamicin, infected osteoblasts were lysed and plated to determine the number of live intracellular bacteria.

#### Survival of *S. aureus* within osteoblasts or macrophages

Osteoblasts or macrophages were infected with *S. aureus* at an MOI of 500:1 for 2 h, treated with gentamicin, washed, and cultured for up to a week in DMEM/F12 (for osteoblasts) or RPMI-1640 (for macrophages) supplemented with 5% FBS and 5 µg/mL lysostaphin; lysostaphin does not penetrate mammalian cell membranes

for long time periods, e.g. weeks [58-60]. The cell culture medium was changed every 3 days. At post-infection days 0, 1, 3, 5, 6, 7, and/or 8 and 9, independent samples of infected cells were washed with PBS, lysed with 0.1% Triton X-100, and plated on blood agar plates to determine the number of live intracellular *S. aureus*. The percentage of live intracellular CFUs [53] at different times following infection was calculated based on the live intracellular CFUs immediately after infection (i.e. post-infection day 0).

#### Confocal microscopy

A dual staining approach [61,62] was adopted to visualize intracellular *S. aureus*. Osteoblasts or macrophages were cultured on rounded cover-slips for at least 24 h in full-supplemented DMEM/F12 or RPMI-1640, respectively. Fresh *S. aureus* was cultured for 18 h at 37°C in a 5% CO<sub>2</sub> incubator. After washing the bacteria once with PBS, the pellet was stained with 100 µg/mL FITC in PBS for 30 min at room temperature prior to infection. Excess FITC was removed by washing with PBS and centrifuging at 3750 rpm for 15 min at 4°C. After infecting with the stained *S. aureus* for 2 h at an MOI of 500:1, osteoblasts were trypsinized using a 0.25% trypsin/2.21 mM EDTA solution for 30 seconds at room temperature to remove adherent extracellular *S. aureus*; no trypsinization was used in the macrophage samples. Osteoblasts or macrophages were then fixed with 4% paraformaldehyde in distilled water for 30 min at room temperature. Fixed cells were washed 3 times with PBS and blocked with 5% BSA for 1 h at room temperature. To further label the extracellular *S. aureus*, the fixed cells were incubated overnight at 4°C with a primary antibody Ab20920S in 5% BSA, washed 3 times with PBS (to remove excess free primary antibody), and then incubated in the dark with a secondary antibody-conjugated Cy5 fluorescent dye in 2.5% BSA for 45 min at room temperature. After washing the excess secondary antibody with PBS, the cover-slips were flipped onto microscopic glass slides and used for image observation; macrophage samples were mounted in the presence of 4',6-diamidino-2-phenylindole (DAPI) fluorescent dye to visualize the nuclei of macrophages. Slides were visualized using a 159 Plan-Apochromat 63x/1.40 oil objective on an LSM 510 confocal microscope (Zeiss, Jena, Germany).

To confirm the presence of live intracellular *S. aureus* and the efficacy of gentamicin at killing extracellular *S. aureus*, osteoblasts were seeded on a rounded cover-slip overnight. The monolayer was then stained with DiI fluorescent dye for 20 min at 37°C in the dark. The monolayer was washed once with PBS and infected with Syto-9 labeled *S. aureus* as aforementioned. After gentamicin treatment, infected osteoblasts were washed 3 times with HEPES buffer and PI stain was added for 15 min at room temperature in the dark. Immediately after washing off



the excess PI, the slides were examined under the LSM 510 confocal microscope and images of Z-stack sections were taken to confirm the live intracellular *S. aureus*. Z-stack sections were generated and the X-Y planes showed that all live (green) *S. aureus* was inside the osteoblasts.

#### Transmission electron microscopy (TEM)

Osteoblasts were infected with *S. aureus* at an MOI of 500:1 for 2 h, washed once with PBS, and detached using trypsin-EDTA. Osteoblasts were then collected by centrifugation at 1200 rpm at 4°C for 7 min, and the pellet was washed twice with PBS. Slides were then prepared as previously reported [63]. In brief, osteoblasts were fixed with 2% paraformaldehyde and 4% glutaraldehyde mixed with 0.075 M PBS for 30 min at room temperature. The fixed cell mass was collected in 1.5 mL Eppendorf tubes. The cell pellet was washed 3 times with PBS, post-fixed in 1% osmium tetroxide for 2 h at room temperature, washed 3 times with PBS, treated with aqueous 1% tannic acid for 1 h at room temperature, and then dehydrated in a gradient ethanol series. The cells were embedded in pure LR white resin solution and polymerized at 60°C for 24–48 h. Thin (0.1 µm) sections were cut and placed on nickel grids, stained with 2% uranyl acetate and lead citrate, and viewed using TEM (JEOL, Peabody, MA).

#### Reactive oxygen species production

Osteoblasts and macrophages were infected with *S. aureus* at an MOI of 500:1. At pre-determined time points (0.5, 1, and 2 h), samples of infected osteoblasts or macrophages were taken, washed once with PBS, and then incubated with H<sub>2</sub>DCF-DA or DHE at 37°C for 1 h in the dark; separate samples were used for the staining of H<sub>2</sub>DCF-DA and DHE. Non-infected osteoblasts and macrophages were used as controls and were treated the same as the infected cells except no *S. aureus* was added. Viable cells of infected and control samples at the pre-determined time points were obtained using hemocytometry and were used to analyze the final fluorescent data. The fluorescence intensity was measured using a fluorescent microplate reader (BioTek Instrument, Inc., Winooski, VT) at 492 nm/520 nm for 2',7'-dichlorofluorescein (DCF), converted intracellularly from H<sub>2</sub>DCF-DA, and 492 nm/620 nm for DHE. H<sub>2</sub>DCF-DA and DHE are commonly used to stain intracellular H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup>, respectively [64]. The acetate groups of H<sub>2</sub>DCF-DA are cleaved by intracellular esterases and oxidation and convert to highly fluorescent DCF.

#### Osteoblast alkaline phosphatase (ALP) activity

Osteoblasts were cultured in 12-well plates at a density of  $5 \times 10^4$  cells/mL, infected at an MOI of 500:1 for 2 h following the aforementioned infection protocol. Non-infected (control) and infected osteoblasts were cultured

in DMEM/F12 supplemented with 5% FBS and 5 µg/mL lysostaphin solution for a week; medium was changed every 3 days. On post-infection days 1, 4, and 7, osteoblast monolayers were washed with PBS once and the ALP activity was determined using an ALP assay kit (Abcam) and expressed as Unit/mL.

#### Macrophage phagocytosis assay

Macrophage phagocytosis (ingestion) activity was tested by measuring the uptake of FITC-labeled *S. aureus* by non-infected (control) macrophages and macrophages infected with *S. aureus* (unlabeled) at an MOI of 500:1 for 2 h. After incubating  $5 \times 10^5$  cells/mL non-infected (control) and infected macrophages separately with FITC-labeled *S. aureus* at 10:1 MOI for 2 h, macrophages (infected and non-infected) were treated with 100 µg/mL gentamicin for 2 h at 37°C in a 5% CO<sub>2</sub> incubator. Macrophages were then scraped and collected for flow cytometry analysis using BD-FACS Calibur (BD, Franklin Lakes, NJ); 10,000 events were collected. Data were acquired in logarithmic mode for the forward scatter (FSC), side scatter (SSC), and green fluorescence channel FL-1H (i.e. FITC). Control macrophages were subjected to the same experimental protocols as the infected cells but without infection with *S. aureus*. The percentage of macrophages with FITC fluorescent intensity corresponds to the ingestion activity of macrophages.

#### Statistical analysis

Statistical analyses were performed using JMP Statistical Visualization Software (SAS Institute, Cary, NC). Experiments were repeated at least twice on separate days to verify reproducibility. All data were expressed as mean ± SD and analyzed using one-way analysis of variance (ANOVA). Statistical significance was set at  $p < 0.05$ , 0.01, 0.001, or 0.0001.

#### Ethics statement

No human subjects, human material, or human data were involved.

#### Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions

TH participated in the design of the study, carried out the experiments, assisted in the data interpretation, and drafted the manuscript. BL conceived and designed the study, interpreted data, and revised the manuscript. Both authors read and approved the final manuscript.

#### Authors' information

TH is currently a postdoctoral research associate at the Department of Microbiology and Immunology at the University of Maryland in Baltimore. BL is an Associate Professor in the Department of Orthopaedics, West Virginia University and a member of American Society for Microbiology (ASM), Orthopaedic Research Society (ORS), Society for Biomaterials (SFB), and American Chemical Society (ACS).

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