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# ■ INFECTION

# Is intracellular *Staphylococcus aureus* associated with recurrent infection in a rat model of open fracture?

T. Gao,
J. Lin,
C. Zhang,
H. Zhu,
X. Zheng

*Department of Orthopaedic Surgery, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, China*

## Aims

The purpose of this study was to determine whether intracellular *Staphylococcus aureus* is associated with recurrent infection in a rat model of open fracture.

## Methods

After stabilizing with Kirschner wire, we created a midshaft femur fracture in Sprague-Dawley rats and infected the wound with green fluorescent protein (GFP)-tagged *S. aureus*. After repeated debridement and negative swab culture was achieved, the isolation of GFP-containing cells from skin, bone marrow, and muscle was then performed. The composition and viability of intracellular *S. aureus* in isolated GFP-positive cells was assessed. We suppressed the host immune system and observed whether recurrent infection would occur. Finally, rats were assigned to one of six treatment groups (a combination of antibiotic treatment and implant removal/retention). The proportion of successful eradication was determined.

## Results

Green fluorescent protein-containing cells were successfully isolated after the swab culture was negative from skin (n = 0, 0%), muscle (n = 10, 100%), and bone marrow (n = 10, 100%) of a total of ten rats. The phagocytes were predominant in GFP-positive cells from muscle (73%) and bone marrow (81%) with a significantly higher viability of intracellular *S. aureus* (all p-values < 0.001). The recurrent infection occurred in up to 75% of rats after the immunosuppression. The proportion of successful eradication was not associated with implant retention or removal, and the efficacy of linezolid in eradicating intracellular *S. aureus* is significantly higher than that of vancomycin.

## Conclusion

Intracellular *S. aureus* is associated with recurrent infection in the rat model of open fracture. Usage of linezolid, a membrane-permeable antibiotic, is an effective strategy against intracellular *S. aureus*.

### Article focus
- Is intracellular *Staphylococcus aureus* associated with recurrent infection in a rat model of open fracture?
- Can the host immune system eradicate *S. aureus* before or after implant removal in this rat model?
- Can systemic administration of membrane-permeable antibiotics increase the probability of eradicating intracellular *S. aureus*?

### Key messages
- The phagocytes were responsible for the major bacterial burden of intracellular *S. aureus*. After host immunosuppression, the intracellular reservoir of *S. aureus* can cause recurrence of infection, and the higher the dose of mycophenolate sodium (MPS), the higher the recurrence rate of infection.
- Implant removal did not increase the probability of eradicating *S. aureus* in our

Correspondence should be sent to X. Zheng; email: zhengxianyou@126.com

model, possibly because no biofilm was formed on the implant.

■ Usage of linezolid, a membrane-permeable antibiotic, is an effective strategy against intracellular *S. aureus*.

## Strengths and limitations

■ This is the first study to evaluate the relationship between intracellular *S. aureus* and the recurrent infection in a rat model of open fracture.

■ Large animal and clinical studies are needed to further validate our findings based on a rat model.

## Introduction

The prevalence of infection after open fractures is a frequently encountered complication at less than 1% for Gustilo-Anderson grade I and up to 30% for Gustilo-Anderson grade III fractures.[1–3] Infections after open fractures are occasionally difficult to eradicate and finally lead to chronic and recurrent infections.[4–6]

*Staphylococcus aureus* is the pathogen most frequently isolated from the open fracture wound[7,8] and is often surprisingly successful in avoiding host immune attacks, antimicrobial therapy, and surgical debridement.[9–11] Current microbiological studies have revealed an important mechanism for recurrent infection of *S. aureus* that bacteria may hide in host cells, replicate intracellularly, and break out when the host immune system is compromised.[12–14] To date, *S. aureus* has been proven to be able to invade epithelial and endothelial cells, keratinocytes, fibroblasts, osteoblasts, and phagocytes (neutrophils and macrophages).[15,16] In this study, we first established an infection model after open fracture. After elimination of extracellular infection by surgical debridement, we investigated several questions: 1) which type of tissue and cell is responsible for the major bacterial burden of intracellular *S. aureus* in the rat model of open fracture?; 2) can the intracellular reservoir of *S. aureus* cause recurrence of infection when the host immune system is compromised?; 3) can the host immune system eradicate intracellular *S. aureus* before and after implant removal?; and 4) can systemic administration of membrane-permeable antibiotics increase the probability of eradicating intracellular *S. aureus*?

## Methods

All animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of our hospital.

**Bacterial inoculum preparation.** To generate green fluorescent protein (GFP)-tagged *S. aureus* (ATCC BAA-1556 is a clone of the multiresistant pathogen *S. aureus* USA300), pMV158GFP[17], a plasmid that carries the gene encoding the GFP, was introduced into competent *S. aureus* by electroporation. Then these bacteria were maintained on tryptic soy agar with 5% sheep blood (BD,

Franklin Lakes, New Jersey, USA) and selected by 10 μg/ml chloramphenicol (Sigma-Aldrich, St. Louis, Missouri, USA). Fresh culture was prepared for inoculation by harvesting several colonies and suspending in 5 ml of tryptic soy broth supplemented with 10 μg/ml chloramphenicol. The broth was then incubated overnight on a shaker at 37˚C. After washing the bacteria with phosphate buffered saline (PBS) three times, we adjusted the cell concentration to $1 \times 10^8$ colony-forming units (CFUs) per millilitre based on a standard curve of optical density.

**Antibiotic susceptibility tests.** The vancomycin and linezolid minimum inhibitory concentrations (MICs) of these *S. aureus* strains were determined with an Etest using Etest strips (bioMérieux, Marcy lÉtoile, France) and Mueller-Hinton agar plates (Eiken Chemical, Tokyo, Japan) according to the manufacturer's instructions.

**Surgical technique for stabilization and fracture creation.** The femur was first stabilized with intramedullary Kirschner wire (K-wire) before fracture creation. Briefly, after satisfied anaesthetization, thorough sterile draping of the surgical site was performed before incision. The skin over the knee was sharply incised for approximately 3 mm in a longitudinal fashion. The knee joint was then opened in a suprapatellar approach to expose the intercondylar notch of distal femur. We first created an entrance hole with a 22-gauge needle at the notch. We then reamed a femoral canal in a retrograde fashion with 20- and 18-gauge needles and finally inserted a K-wire (1 mm in diameter) through the reamed tract. We left a 1 mm protruding segment in the knee joint for possible removal. The joint and skin incision was closed with interrupted 4-0 sutures. A midshaft femoral fracture and injury to soft tissue was then created using the drop-weight impactor (800 g, 10 cm). The fracture creation was then confirmed by radiograph (Figure 1). The fracture site was opened to mimic the open fracture by stripping the surrounding periosteum and inoculating with 100 μl inoculation solution with $1 \times 10^7$ CFUs of *S. aureus*. The skin
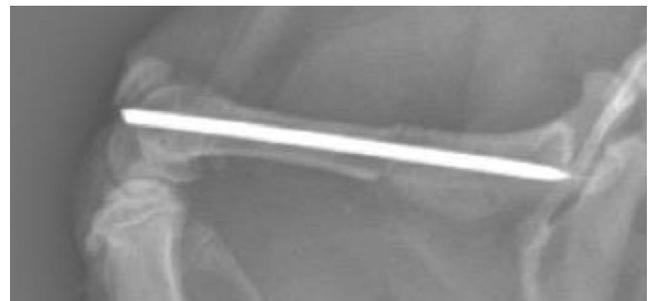


**Fig. 1**

A representative lateral radiograph after fracture creation and fixation. The femur was first stabilized with intramedullary Kirschner wire, and 1 mm protruding of the Kirschner wire was left in the knee joint for possible removal. Then, the joint and skin incision was closed with interrupted 4-0 sutures. A midshaft femoral fracture and injury to soft tissue was then created using the drop-weight impactor (800 g, 10 cm).

incision was closed with interrupted 4-0 sutures and left for 48 hours to establish surgical site infection. After 48 hours, these rats repeatedly received surgical debridement and irrigation with normal saline every 48 hours until the multipoint swab culture around the wound was negative. Negative swab culture was achieved in all rats within four times of debridement.

**GFP-positive cell isolation and composition analysis by flow cytometry.** After negative swab culture was achieved in all rats, the skin, muscle, and bone marrow of ten rats were harvested to isolate GFP-positive cells. After thorough sterile draping, skin and muscle around the wound was harvested with sterile scissors. Bone marrow was harvested by curettage of femoral canal and flushing with 2 ml alpha minimum essential medium ($\alpha$MEM) three times. The harvested tissues were first minced with a sterile blade and scissors in a sterile cell culture dish to facilitate further tissue digestion. The harvested tissue was then digested with collagenase IV 200 U/ml and collagenase I 200 U/ml (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and supplemented with 3 mm calcium chloride ($CaCl_2$) in $\alpha$MEM for 60 minutes at 37°C. Dispersed cells were filtered out through a sterile nylon mesh and washed several times with PBS.

The GFP-positive cells were isolated with a flow cytometer (BD). The composition of GFP-positive cells was further analyzed with allophycocyanin (APC)-conjugated CD11b and PE-Cy7-conjugated CD45 antibodies (BD). CD45 is widely expressed by all immune cells and thus used to distinguish immune and non-immune cells.[18] CD11b is a common antigen expressed by all phagocytes including neutrophils, macrophages, and dendritic cells.[19]

**Assessing viability of intracellular *S. aureus*.** In total, 10,000 bacteria-containing phagocytes (GFP+, CD45+, CD11b+), non-phagocytic immune cells (GFP+, CD45+, CD11b-), and non-immune cells (GFP+, CD45-, CD11b-) obtained from the above isolation experiment were incubated with lysostaphin (Sigma-Aldrich) to remove any remaining extracellular *S. aureus* at 4 $\mu$g/ml for 30 minutes and then lysed with 100 µl of autoclaved water. We then inoculated 30 µl cell lysate on tryptic soy agar with 5% sheep blood (BD) and 10 µg/ml chloramphenicol for bacterial selection (Sigma-Aldrich). Bacterial colonies were quantified using ImageJ analysis (National Institutes of Health and LOCI, Madison, Wisconsin, USA).

**Tissue culture and bacterial identification.** The skin from knee to hip was prepared and draped to maintain sterility. Samples including skeletal muscles, the femur, and the K-wire (if applicable) were harvested. All tissues and K-wires were transferred immediately into sterile tubes after harvesting. We then added 3 ml normal saline solution into each tube. The bone and soft tissues were homogenized with a sterile tissue grinder at 4°C. K-wires were first sonicated to dissolve the biofilm and release bacteria from the biofilm. The supernatant was then inoculated

and incubated on tryptic soy agar with 5% sheep blood (BD) and selected by 10 µg/ml chloramphenicol (Sigma-Aldrich) for 24 hours. The positive results were defined as the formation of at least one colony. Bacterial colonies were quantified using ImageJ analysis. Bacterial identification was conducted with 16S ribosomal DNA sequencing by using the MicroSeq 500 microbial identification system (Thermo Fisher Scientific). The expression of GFP in *S. aureus* was assessed via real-time polymerase chain reaction (PCR) (Applied Biosystems (ABI); Thermo Fisher Scientific) to confirm that the isolated *S. aureus* was the inoculated strain.

**Immunosuppression and detection of recurrent infection.** Before the immunosuppressive treatment, we performed biopsy of muscle and cultured the obtained muscle tissue to confirm the carriage of intracellular *S. aureus*. We then gave mycophenolate sodium (MPS) (2 mg/kg, 10 mg/kg, and 20 mg/kg; 20 rats per group) intramuscular injections on a daily basis for one week to suppress the host immune system. The fracture site was then opened, and the swab culture was performed. The recurrent infection was defined as positive culture of specimens obtained from swab.

**Intracellular carriage of *S. aureus* eradicated by host immune system.** After negative swab culture was achieved, 120 rats were assigned randomly to one of six treatment groups: implant retention and no antibiotics; implant removal and no antibiotics; implant retention and linezolid (membrane-permeable); implant removal and linezolid; implant retention and vancomycin (non-membrane-permeable); implant removal and vancomycin. Implant removal was performed one month after this group assignment. Vancomycin (40 mg/kg) and linezolid (10 mg/kg) were given intravenously on three consecutive days one month after assignment. After three months, the proportion of successful eradication was determined using tissue culture of bone, muscle, and implant if applicable.

**Statistical analysis.** Differences in proportion between groups were compared using Fisher's exact test. Significance was evaluated using the non-parametric Mann-Whitney U test for the comparison of colony counts between different groups. The Statistical Package for the Social Sciences (SPSS, Chicago, Illinois, USA) was used for statistical analysis.

## Results

All bacteria grown on culture in this study were identified as the identical strain of inoculated *S. aureus* by using the MicroSeq 500 (Thermo Fisher Scientific) microbial identification system and real-time PCR, without colonization of any other bacteria indicating that there was no unexpected microbial contamination.

**Antibiotic susceptibility tests.** The strain used in this study is susceptible to both vancomycin and linezolid,
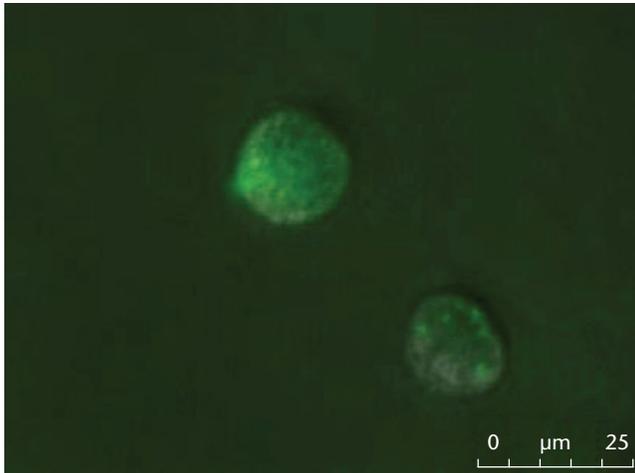
**Fig. 2**

A representative fluorescence microscopy image of the green fluorescent protein (GFP)-positive infected leucocytes. After being isolated with a flow cytometer, the GFP-positive leucocytes were detected under a fluorescence microscope. The green points indicate where GFP-tagged *S. aureus* were found within leucocytes.



**Fig. 3a**



**Fig. 3b**

The composition of green fluorescent protein (GFP)-positive cells in a) muscle and b) bone marrow. After negative swab culture was achieved, no GFP-positive cells were successfully isolated from the skin, however they were successfully isolated from the muscle and bone marrow of all rats. The phagocytes (CD45$^+$, CD11b$^+$) were predominant in GFP-positive cells from muscle (73%) and bone marrow (81%).

and the MICs of vancomycin and linezolid against this *S. aureus* strain are 2 μg/ml and 1 μg/ml, respectively.

**Composition of GFP-positive cells.** We then attempted to isolate GFP-containing cells from skin, bone marrow, and muscle using flow cytometry in a total of ten rats after the last debridement. No positive result was observed after culture of the implant. GFP-positive cells were successfully isolated from the muscle and bone marrow of all rats. Figure 2 shows the GFP-positive infected cells under a fluorescence microscope. No GFP-positive cell was successfully isolated from skin.

We hypothesized that the intracellular *S. aureus* might mainly reside in immune cells, especially phagocytes because of their defensive nature. Thus, the composition of GFP-positive cells was first analyzed with CD45 and CD11b antibodies. The phagocytes (CD45$^+$, CD11b$^+$) were predominant in GFP-positive cells from muscle (73%) and bone marrow (81%). The composition of GFP-positive cells is shown in Figure 3.

The bacterial burden of *S. aureus* in different subpopulations of GFP-positive cells (after pooling cells from muscle and bone marrow together) was then evaluated with bacterial culture after lysis of 10,000 cells. The colony number per thousand cells of each subpopulation is shown in Figure 4. The mean colony number of phagocytes was significantly higher than that of any other subpopulation (phagocytes: 135.7 (SD 55.4); non-phagocytic immune cells: 26.4 (SD 11.3); non-immune cells: 21.4 (SD 8.8); both p-values < 0.001, Mann-Whitney U test). Together, these data indicate that phagocytes were responsible for the major bacterial burden of intracellular *S. aureus*.

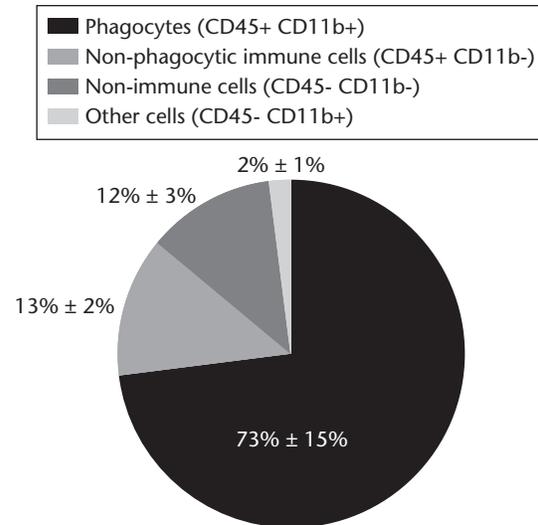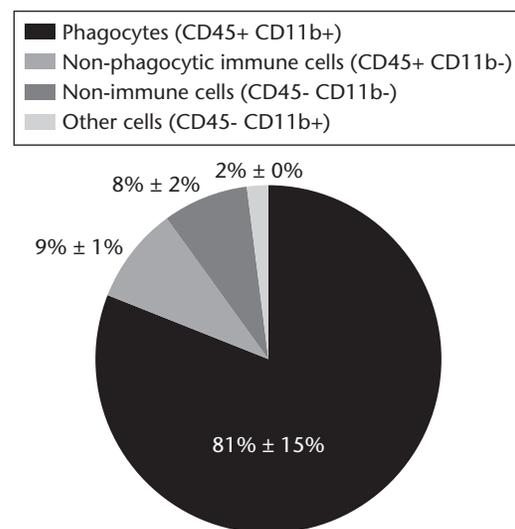**Immunosuppression induces recurrent infection of *S. aureus*.** After immunosuppression was induced with 2 mg/kg MPS, the recurrent infection occurred in one out of 20 rats (5%) with intracellular *S. aureus* carriage. The incidence of recurrent infection significantly increased after a higher dose of MPS (10 mg/kg: ten out of 20, 50%; 20 mg/kg: 15 out of 20, 75%). Visible abscesses were found in zero, eight, and 13 out of 20 rats in each group (2 mg/kg, 10 mg/kg, and 20 mg/kg), respectively. The incidence of recurrent infection is summarized in Table I.

**Eradication of intracellular *S. aureus* by host immune system, implant removal, and antibiotics.** The proportion of successful eradication was not associated with implant retention or removal (no antibiotics: 5% retention
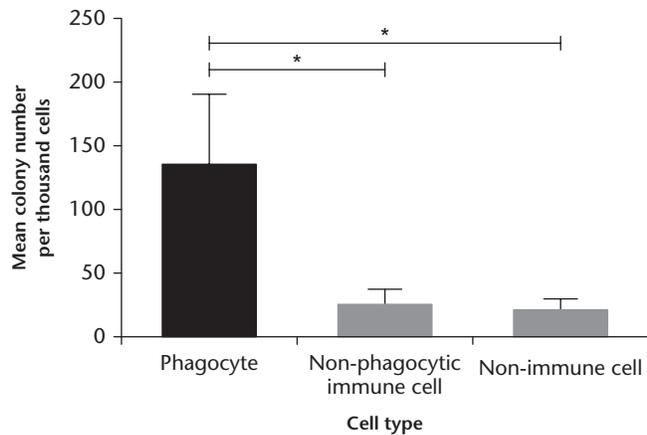
**Fig. 4**

The colony number per thousand cells in each subpopulation of green fluorescent protein (GFP)-positive cells. After pooling cells from muscle and bone marrow together, isolated GFP-positive cells were divided into different subpopulations by allophycocyanin (APC)-conjugated CD11b and PE-Cy7-conjugated CD45 antibodies. The bacterial burden of *Staphylococcus aureus* in different subpopulations was then evaluated with bacterial culture after lysis of 10,000 cells. The mean colony number of phagocytes was significantly higher than that of any other subpopulation (phagocytes: 135.7 (SD 55.4); non-phagocytic immune cells: 26.4 (SD 11.3); non-immune cells: 21.4 (SD 8.8); both p-values < 0.001, Mann-Whitney U test). *p < 0.005.

**Table I.** Proportions of recurrent infection of *Staphylococcus aureus* by mycophenolate sodium dose

| Variable | MPS dose | | |
|---|---|---|---|
| | **2 mg/kg** | **10 mg/kg** | **20 mg/kg** |
| Positive culture, n (%) | 1 (5) | 10 (50)* | 15 (75)† |
| Visible abscesses, n (%) | 0 (0) | 8 (40)* | 13 (65)† |

*p = 0.003 versus 2 mg/kg, Fisher's exact test.
†p < 0.001 versus 2 mg/kg, Fisher's exact test.
MPS, mycophenolate sodium.

compared with 10% removal, p = 0.553; linezolid: 90% retention compared with 85% removal, p = 0.632; vancomycin: 15% retention compared with 20% removal, p = 0.677; all p-values calculated using Fisher's exact test). The efficacy of linezolid in eradicating intracellular *S. aureus* is significantly higher than that of vancomycin despite implant retention or removal (retention: 90% compared with 15%, p < 0.001; removal: 85% compared with 20%, p < 0.001; both Fisher's exact test). The associated data are summarized in Table II.

**Table II.** Proportions of successful eradication of intracellular *Staphylococcus aureus* by different treatment

| Treatment | No antibiotics | Linezolid | Vancomycin | p-value* |
|---|---|---|---|---|
| Implant retention, n (%) | 1 (5) | 18 (90) | 3 (15) | < 0.001 |
| Implant removal, n (%) | 2 (10) | 17 (85) | 4 (20) | < 0.001 |
| p-value† | 0.553 | 0.632 | 0.677 | N/A |

*Linezolid versus vancomycin; Fisher's exact test.
†Implant retention versus removal; Fisher's exact test.
N/A, not applicable.

## Discussion

The role of intracellular *S. aureus* in recurrent infection including skin infection, tonsillitis, rhinosinusitis, and sepsis has been well established by multiple studies.[20–23] The finding of intracellular *S. aureus* in bone and joint infection could be traced back to the case report published more than a decade ago in a patient with chronic osteomyelitis.[24] *S. aureus* can invade various types of cells including epithelial and endothelial cells, keratinocytes, fibroblasts, osteoblasts, and phagocytes (neutrophils and macrophages).[15,16] In this study, as expected, eradication of extracellular *S. aureus* after open fracture was insufficient to eliminate all pathogens from host. The host cells carrying *S. aureus* intracellularly resided in muscle and bone marrow in the rat model. We also demonstrated that the viability of intraphagocytic *S. aureus* was significantly higher than those in other cells. Taken together, we showed that the phagocytes were responsible for the major bacterial burden of intracellular *S. aureus* in this rat model of open fracture.

Biofilm is produced by bacteria after binding to host proteins that adhere to the implant.[25] The biofilm effectively shields the included bacteria from immune cells and antibiotics.[26] Thus, the biofilm on the implant is associated with recurrence of infection. However, there was no biofilm formed on the implants in our study. As a result, implant removal did not increase the probability of eradicating *S. aureus* in our model.

Multiple strategies including antibody-antibiotic conjugate, intracellular drug delivery, and synthetic antimicrobial peptides have been established to kill intracellular *S. aureus*.[27–30] The rationales for these studies were mainly based on the difficulty in killing intracellular *S. aureus* with antibiotics in vitro, especially for those non-membrane-permeable antibiotics.[31,32] The minimum bactericidal concentration (MBC) for intracellular *S. aureus* is much higher than the maximal dose that host could tolerate.[27,32] However, it is clearly hard to kill all bacteria with antibiotics alone in vivo. Another important finding of this study was that regular doses of membrane-permeable antibiotics could eliminate intracellular *S. aureus* efficiently in vivo.

There are some limitations in our study. Firstly, chloramphenicol was used for bacterial selection in order to exclude the bacterial contamination. It is possible that a few bacteria became chloramphenicol-sensitive due to lack of selection pressure in the animal. This subpopulation of *S. aureus* was clearly neglected in the CFU determination. Thus, the bacterial burden of intracellular *S. aureus* was underestimated. Notably, because the GFP-positive cell subpopulation data were obtained under identical conditions (all possibly underestimated), we believe that our conclusions that phagocytes were responsible for the major bacterial burden of intracellular *S. aureus* can still be reliable. Secondly, when assessing the bacterial burden of *S. aureus* in different subpopulations of GFP-positive cells,

the influence of the size of the cells in intracellular bacterial numbers was not considered. It may be a potential mechanism for phagocytes' higher intracellular bacterial burden. However, our conclusions that the phagocytes were responsible for the major bacterial burden of intracellular *S. aureus* in this rat model of open fracture are still accurate.

In conclusion, intracellular *S. aureus* is associated with recurrent infection in the rat model of open fracture. Usage of linezolid, a membrane-permeable antibiotic, is an effective strategy against intracellular *S. aureus*.

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**Author information**
- T. Gao, MBBS, PhD Candidate
- J. Lin, MBBS, PhD Candidate
- C. Zhang, MD, PhD, Department Director
- H. Zhu, MD, Orthopaedic Specialist
- X. Zheng, MD, PhD, Orthopaedic Specialist Department of Orthopaedic Surgery, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, China

**Author contributions**
- T. Gao: Prepared the draft manuscript, Conducted the animal experiments.
- J. Lin: Co-authored the study, Conducted the animal experiments.
- C. Zhang: Designed and conducted the statistical calculations.
- H. Zhu: Designed and co-authored the study.
- X. Zheng: Designed the study, Revised the manuscript.