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Short Communication

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Silver-nanoparticles increase bactericidal activity and radical oxygen responses against bacterial pathogens in human osteoclasts

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Abstract

Bone infections are difficult to treat and can lead to severe tissue destruction. Acute bone infections are usually caused by *Staphylococcus aureus*. Osteoclasts, which belong to the monocyte/macrophage lineage, are the key cells in bone infections. They are not well equipped for killing bacteria and may serve as a reservoir for bacterial pathogens. Silver has been known for centuries for its bactericidal activity. Here, we investigated the bactericidal effects of nano-silver particles in bacteria infected human osteoclasts. We found that nano-silver in *per se* non-toxic concentration enhanced the bactericidal activity in osteoclasts against intracellular Methicillin-resistant, virulent *Staphylococcus aureus.* The reduced bacterial survival in nano-silver pretreated cells correlated with increased reactive oxygen responses towards the invading pathogens. Overall, these results indicate that nano-silver compounds should be considered as an effective treatment and prevention option for bacterial bone and orthopedic implant infections.

Key words: Monocyte-derived osteoclasts; Bone infection; Antibacterial activity; Silver-nanoparticle; Reactive oxygen species

Bone and orthopedic implant infections are caused by microorganisms and are generally associated with highly inflammatory processes leading to bone destruction and implant loss.[1,2] Acute bone infections are predominantly caused by *Staphylococcus aureus.*[3] Conservative treatments of bone infections with antibiotics offer poor results when not combined with debridement.[1,4]

Silver has been used throughout the ages for its antimicrobial activities.[5] Especially the use of orthopedic implant with nanoparticulate silver coatings was intensively investigated.[6–8] The problem is that silver nanoparticles (Ag-NP) at antibacterial concentrations have been reported to be cytotoxic against bone cells *in vitro.*[9]

The rationale for the present study was to investigate biological effects on human monocyte derived cells mediated by Ag-NP that are independent of directly exerted cytotoxicity. Our study demonstrates for the first time that Ag-NP induces a strong bactericidal activity against problematic pathogens in human osteoclasts that is potentially mediated by increased radical oxygen responses (ROS) independently of direct silver toxicity.

Methods

In vitro generation of human macrophages and osteoclasts

Human osteoclasts and macrophages were generated as described.[10] For the differentiation of monocytes towards

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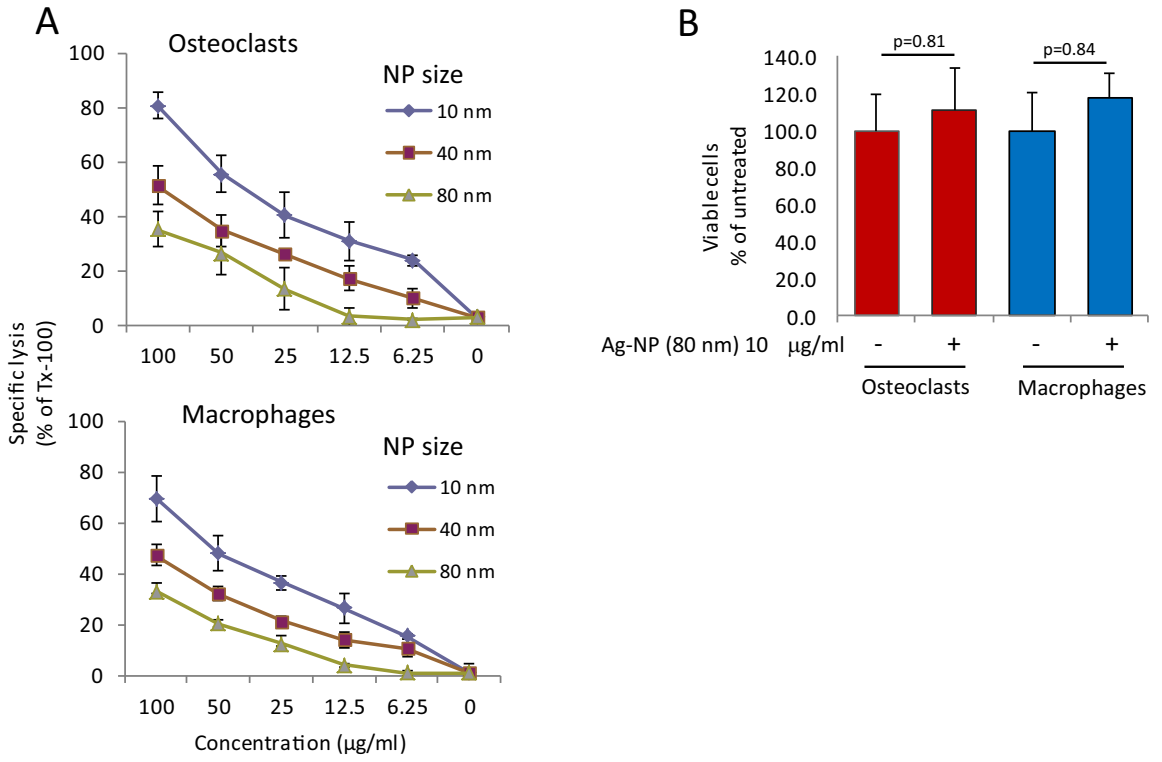

Figure 1. Viability of the host cells after treatment with Ag-NP of various sizes. Osteoclasts and macrophages were treated with Ag-NP of indicated size for 4 hours. Cell viability was assessed by the fluorescence release from BCECF pre-labeled cells (**A**) or by the release of lactate dehydrogenase, LDH (**B**). In A, the average +/− SEM is indicated. In **B**, bars represent average +/− SEM normalized to untreated cells.

osteoclasts RANK-L (10 µg/ml) and M-CSF (10 ng/ml; both Miltenyi) was applied.

Generation of pro-inflammatory primed supernatant

Human macrophages were infected with *Salmonella typhimurium* strain SL1344. The primed supernatant was cleared by centrifugation and filtration.

Ag-Np treatment, bacterial infection and colony forming unit assays (CFU)

Cells were seeded in 24-well plates overnight. For indicated experiments, the medium was supplemented with primed supernatant (at 1:10 dilution) or with 2.5 ng/ml interferon-γ (Miltenyi). Cells were treated with nano-silver particles (80 nm, NanoComposix) at 10 µg/ml for 4 hours and then infected with *E. coli* (BL21) at a multiplicity of infection (MOI) of 10 or with MRSA *S. aureus* (USA300) at a MOI of 1. At indicated times, samples were hypotonically lysed and spread on LB-Agar plates.

Assessing cell viability in BCEFCF fluorescence and LDH release assays

Fluorescence release assays using BCEFCF-AM (Sigma) were performed according to[11] and LDH release was measured according to manufacturer's recommendations (Roche).

Phagocytosis

E. coli (Alexa Fluor® 488 conjugate; Fisher Scientific) as well as CFSE (Sigma) labeled *S. aureus* were used to determine the phagocytosis of bacteria by flow cytometry and confocal microscopy.

ROS measurements

ROS responses to bacteria were measured using Ampliflu™ Red according to manufacturer's recommendations (Sigma).

Results

Consistent with earlier studies,[9,12] we found significant cell damage in fluorescence (Figure 1, *A*) and LDH release (Figure 1, *B*) assays induced by Ag-NP in a size-dependent manner. Smaller particles were more toxic than particles of larger size. 80-nm particles exerted no nanotoxicity at concentrations of 12.5 µg/ml and below. All subsequent experiments were performed with 80-nm particles at a concentration of 10 µg/ml.

Intracellular, non-virulent *E. coli* multiplied in untreated osteoclasts (Figure 2, *A*, left panel). Ag-NP treatment enabled the osteoclasts to significantly reduce viable bacteria. Macrophages, known antibacterial effector cells, were able to efficiently reduce viable *E. coli* load without Ag-NP pretreatment. However, we found an even further reduced bacterial load in Ag-NP

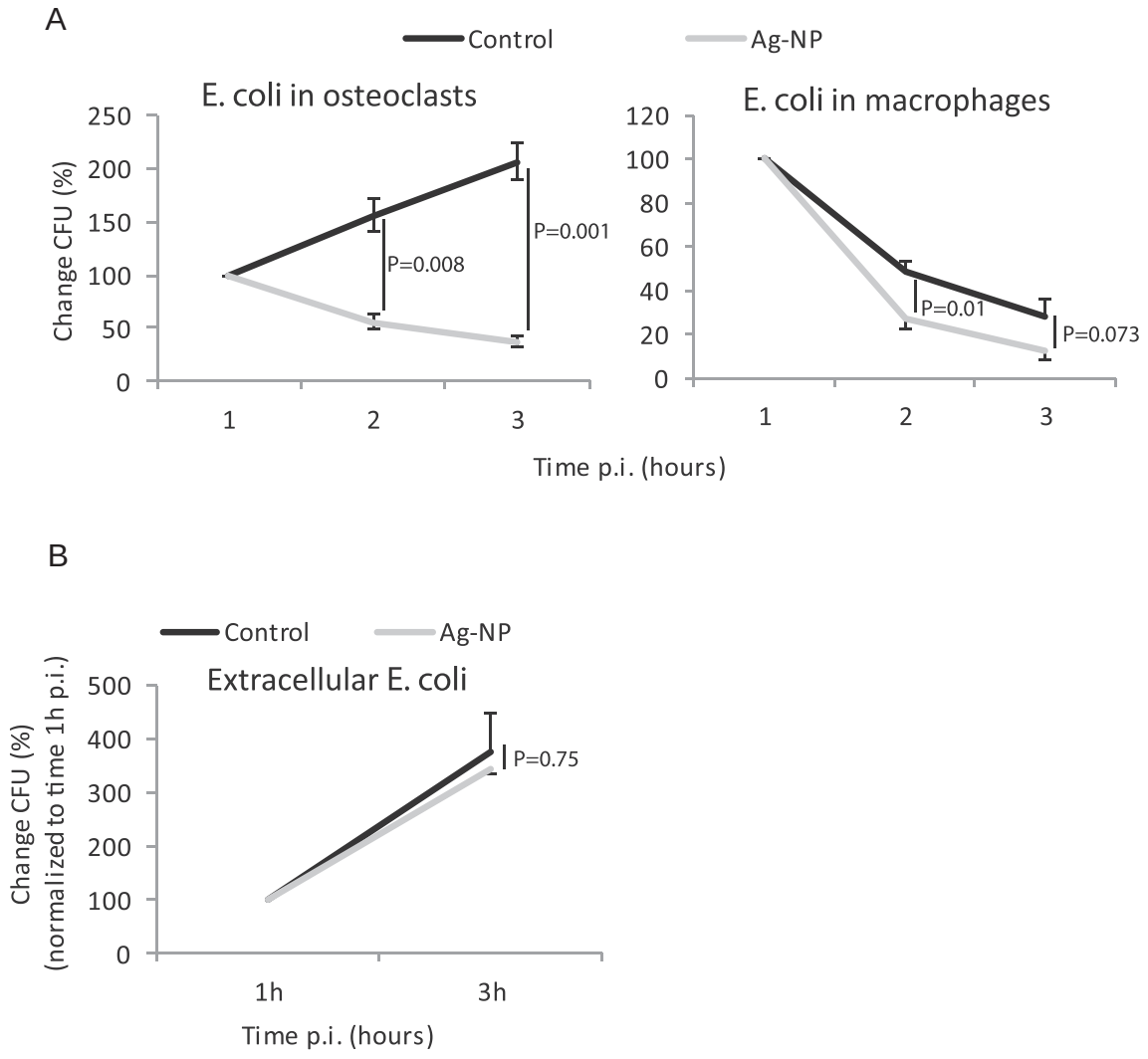

Figure 2. Viability of intracellular and extracellular *E. coli* after Ag-NP treatment. Cells were challenged with 10 MOI *E. coli* Bl21 after Ag-NP treatment. Intracellular bacteria load was enumerated in CFU assays. CFU counts were normalized to the 1-hour postinfection time point to focus on intracellular survival and to diminish interexperimental differences of the raw CFU counts (see also Figure S1). In **B**, extracellular bacteria were grown in presence +/− Ag-NP to determine silver toxicity.

pre-treated macrophages Ag-NP treatment at the earlier postinfection time point suggesting a more rapid response in Ag-pretreated cells as compared to control-treated cells (Figure 2, *B* right panel; compare Figure S1). Extracellular *E. coli* grew unaffected in the presence of Ag-NP at chosen concentration and size (Figure 2, *B*).

Consistent with recent reports,[13] in cells without previous pro-inflammatory activation intracellular virulent, drug-resistant *S. aureus* expanded unimpaired as compared to extracellular growth (Figure 3, *A* and *B*). *S. aureus* also grew in osteoclasts, activated by the addition of pro-inflammatory primed supernatant (Figure 3, *C* and *D*) or interferon-γ (IFN-γ; Figure 3, *E*). However, the pretreatment with Ag-NP enabled activated osteoclasts to efficiently kill intracellular virulent *S. aureus*. Pro-inflammatory activated macrophages were able to eliminate engulfed *S. aureus* (Figure 3, *C* and *E*) that was further reduced by Ag-NP ($p = 0.057$).

Silver pre-incubation neither altered the uptake of *E. coli* (Figs. 4, *A* and *B*; see also Figure S1) nor the intracellular engulfment of *S. aureus* (Figure 4, *C*) in osteoclast or macrophages.

Silver pre-incubation enhanced ROS responses in osteoclasts towards both *S. aureus* and *E. coli* challenge (Figure 5). Generally, we found higher ROS responses in macrophages, consistent with the CFU assays.

Discussion

Osteoclasts have a crucial role in the pathogenesis of bone infections. Being capable of phagocytizing bacteria and not properly armed to kill them, they serve as a bacterial reservoir.[14]

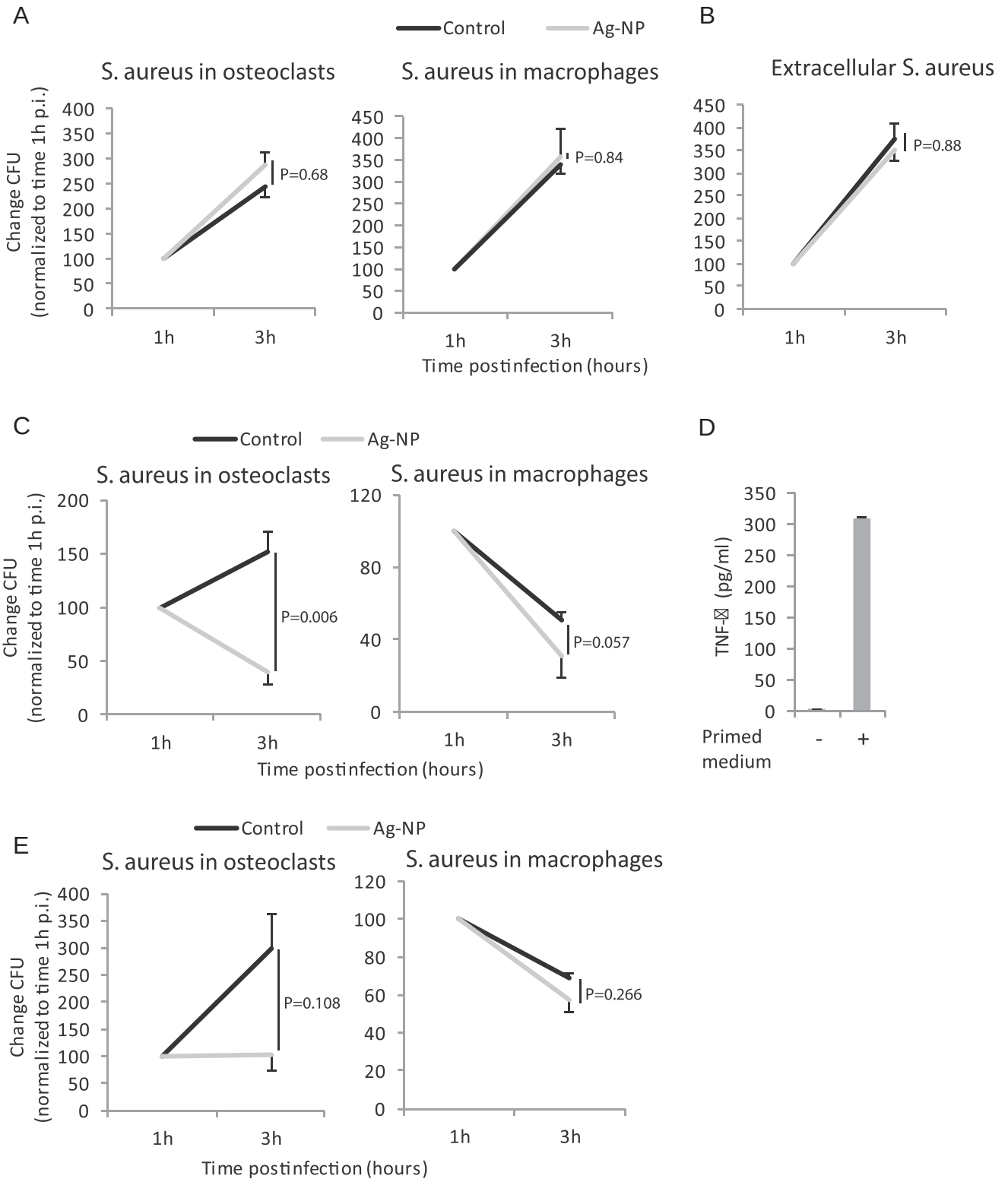

Figure 3. Viability of *S. aureus* in activated cells after Ag-NP incubation. Cells were treated with Ag-NP and were then infected with *S. aureus* MRSA. CFU assays from not previously activated cells are shown in **A**, from cells activated by the addition of primed supernatant are demonstrated in **C**, and from IFN-γ activated cells are indicated in **E**. Extracellular bacteria are shown in **B**. TNF-α levels, measured by ELISA, after addition of primed supernatant to the growth medium is demonstrated in **D**.

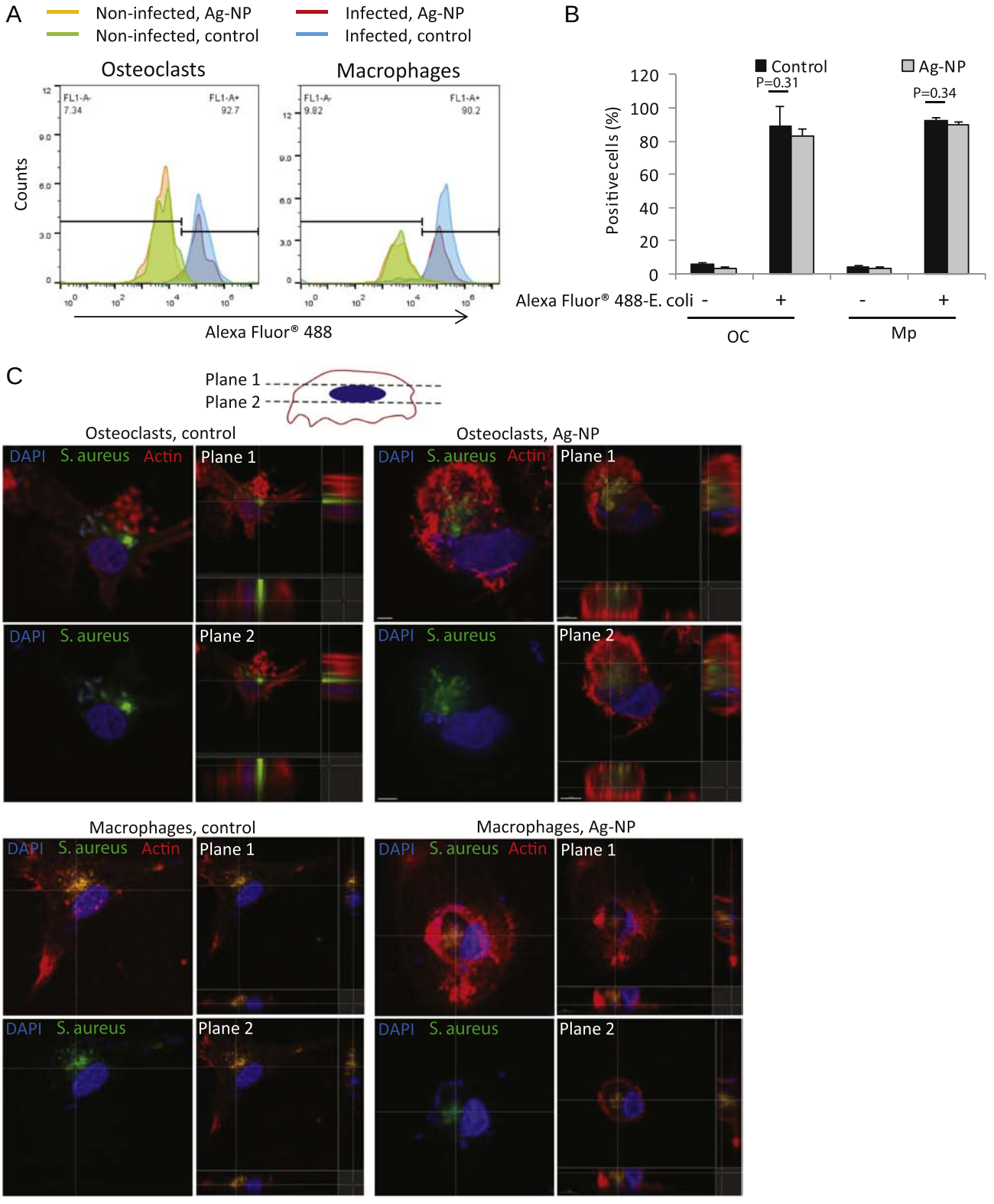

Figure 4. Ag-NP do not interfere with bacterial uptake. Cells were treated with Ag-NP before challenge with green fluorescent *E. coli* or *S. aureus* before fixation and preparation for flow cytometry (**A** and **B**) or confocal microscopy (**C**). In **B**, averages +/− SEM are shown. In **C**, the cells were counterstained with phalloidin-AF594 (actin cytoskeleton in red) and DAPI (blue nuclear stain) to indicate cell dimensions and nucleus. Maximum intensity projections (left images) and 2 distinct focal planes within the infected host cells (see cartoon) are shown.

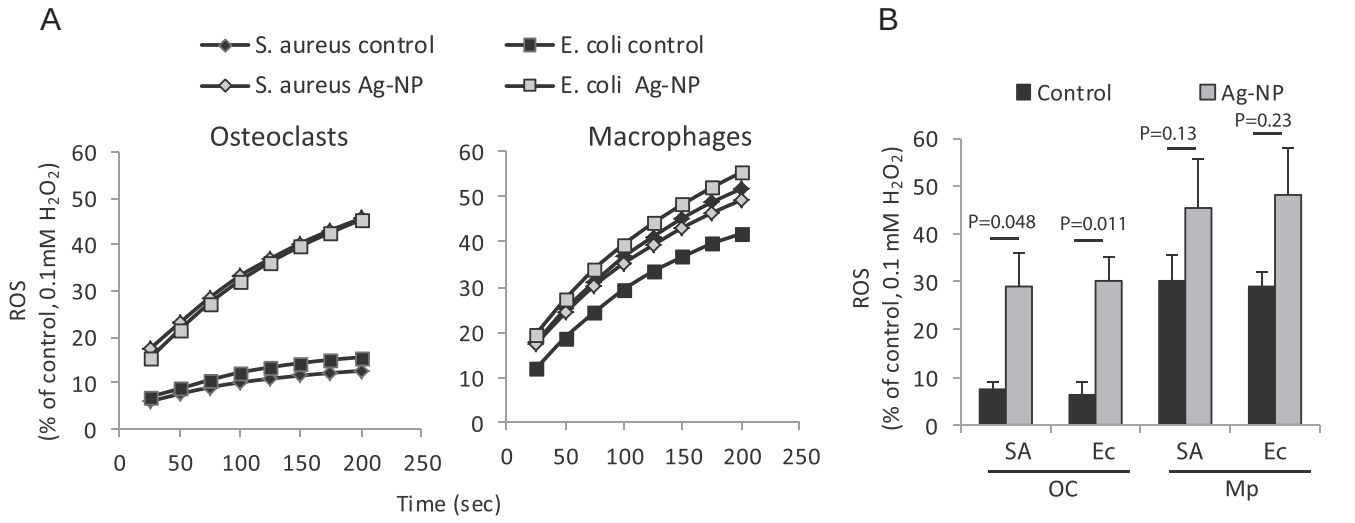

Figure 5. AG-NP increase ROS responses towards bacteria in osteoclasts. Cells were treated with Ag-NP before challenge with live *S. aureus* or *E. coli*. ROS were detected using Ampliflu. Kinetic readings of representative experiments are shown in **A**. In **B**, averages +/− SEM at the 150-second time point are presented.

The major finding of this study was that Ag-NP treatment, at a non-toxic concentration, significantly enhanced bactericidal activity against both non-virulent *E. coli* and virulent, multi-drug resistant *S. aureus*. Previous activation of the cells in a pro-inflammatory environment was necessary for efficient elimination of *S. aureus*. Such a pro-inflammatory environment is found also *in vivo* during the pathogenesis of hematogenous bone infection as bacteremia is often a prerequisite for the disease.

Macrophages, as functional antibacterial effector cells, were used as a control population.[15] However, not previously activated macrophages, were not capable of controlling the growth of virulent *S. aureus*. *S. aureus,* especially community-acquired strains, evolved an impressive tool set to survive in professional phagocytes.[16] However, polarizing the macrophages towards a M1 phenotype enabled the cells to kill engulfed *S. aureus.* Consistent with the previous literature,[17] activation with pro-inflammatory primed supernatant was more efficient than activation with IFN-γ alone.

Importantly, AG-NP treatment did not impair bacteria uptake. Instead, we found elevated ROS responses in Ag-NP treated osteoclasts. A recent study demonstrated that osteoclast precursors potently kill bacteria in a ROS-dependent manner, especially in pro-inflammatory environment *via* the activation of Toll-like receptor 4. Further differentiation into mature osteoclasts diminished this bactericidal activity.[18] Ag-NP treatment, in addition to pro-inflammatory activation, might reactivate the antibacterial function still present in osteoclast precursors.

Conclusion

Research demonstrated toxicity of Ag-NP when used at bactericidal concentrations.[9] As such, they are not widely used in clinic. Here we demonstrate that Ag-NP induce bactericidal activity and ROS generation in osteoclasts independently of direct silver toxicity, which renders silver compounds excellent candidates for a rational therapy design.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nano.2017.11.006.

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