Published in final edited form as:

Nanomedicine. 2020 February ; 24: 102158. doi:10.1016/j.nano.2020.102158.

Harnessing Iron-oxide Nanoparticles towards the Improved Bactericidal Activity of Macrophage against *Staphylococcus aureus*

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Abstract

Iron oxide nanoparticles (IONPs) have been increasingly used in various biomedical applications in preclinical and clinical settings. Although the interactions of IONPs with macrophages have been well-reported in the context of nanoparticle toxicity, harnessing the capacity of IONPs in reprogramming macrophages towards bactericidal activity has not been explored. Here, using an *in vitro* culture model of macrophages and an *in vivo* mouse model of skin wound infection by *Staphylococcus aureus* (*S. aureus*), we demonstrated that IONPs in combination with a strategy to trigger the Fenton reaction could significantly enhance bactericidal effects of macrophages against intracellular *S. aureus* by inducing a M1 macrophage polarization that stimulates the production of reactive oxygen species. Our study supports that harnessing the characteristic of IONPs to tune macrophage polarization to exhibit a bactericidal activity may provide a new strategy for treating infectious diseases.

Graphical Abstract

The current study demonstrates that the capacity of IONPs to trigger a Fenton reaction can be harnessed to promote the bactericidal activity of macrophages. The uptake of IONPs by macrophages enhance a killing of intracellular *S. aureus* by promoting the generation of reactive oxygen species (ROS). The capacity of ascorbic acid (VC) in catalyzing the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) can further enhance a IONPs-mediated killing of intracellular *S. aureus* by increasing the generation of Fe^{2+} and hydroxyl radicals ($\text{OH}^{\bullet}$).

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Data availability

The data sets generated during the current study are available from the corresponding author (M.K) upon reasonable request.

Conflicts of interest

Authors declare no conflict of interest for this study.

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Keywords

iron oxide nanoparticles; macrophages; Fenton reaction; reactive oxygen species; *S. aureus*

Background

Staphylococcus aureus (*S. aureus*) is a major human pathogen that causes persistent infections in skin and soft tissues as well as in implanted medical devices [1]. *S. aureus* can survive within macrophages, which often leads to the persistence of bacteria that leads to chronic infection in the host [2]. Macrophages are key mediators of the immune response due to their characteristics to exhibit phenotypic plasticity depending on environmental cues associated with various physiological and pathological conditions [3]. In particular, a tightly regulated macrophage polarization toward a M1-like phenotype, characterized by the production of pro-inflammatory mediators and reactive oxygen species (ROS), is critical for innate immune defense against bacterial pathogens [4]. As such, insufficient ROS production in macrophages has been associated with a failure to kill the pathogen [5]. Therefore, a strategy to promote the generation of sufficient quantities of ROS in macrophages can be a potential therapeutic strategy to prevent dissemination of bacterial pathogens and resolve infections.

Iron oxide nanoparticles (IONPs) have been increasingly used in a wide range of biomedical applications including contrast agents, drug carriers, hyperthermia therapy, and iron supplement in preclinical and clinical settings [6, 7]. However, the use of IONPs has been limited due to the redox activity of iron to catalyze several deleterious reactions including the Fenton reaction that triggers the generation of ROS [8-10]. In view of this, there have been numerous studies to examine the capacity of IONPs to augment the expression of pro-inflammatory cytokines and generation of ROS in macrophages, in the context of nanoparticle toxicity [11, 12]. This can be detrimental to the host tissue if not properly controlled. For example, excessive accumulation of irons in macrophages were associated with prolonged inflammation in chronic venous ulcers, which led to impaired wound healing [13]. Conversely, a strategy to fine-tune the capacity of IONPs to convert macrophage polarization toward a pro-inflammatory M1-like phenotype can be beneficial for certain pathologic conditions that require robust innate immune responses such as cancer and infectious diseases. Indeed, it has been shown that the potential toxicities of nanoparticles could be harnessed to exhibit an anticancer activity through the mechanism that generates ROS and pro-inflammatory responses in macrophage [14]. However, harnessing the capacity of IONPs in reprogramming macrophage towards bactericidal activity has not been explored.

The objective of this study is to examine the capacity of IONPs in macrophage polarization and its impact on macrophage-mediated bactericidal activity. We have reasoned that the internalization of IONPs by macrophages would promote the killing of intracellular bacteria by stimulating macrophage polarization towards the enhanced generation of ROS, when they are properly tuned to trigger a Fenton reaction. To address this, using *in vitro* culture models of macrophage-like RAW 264.7 cells and *in vivo* mouse models of skin wound infections by *S. aureus*, we have examined if IONPs, alone or in combination with a strategy to trigger the

Fenton reaction, can be beneficial for macrophage-mediated bactericidal and pro-inflammatory immune responses against *S. aureus*.

Methods

Iron oxide nanoparticles and reagents

Suspensions of 100-nm dextran coated iron oxide particles (100 nm, NanoMAG-D-SPIO) were purchased from Micromod Partikeltechnologie, GmbH (Rostock, Germany). The Vitamin C, 2,2'-bipyridine (bipyridyl, BIP), and thiourea (THI) were purchased from Sigma-Aldrich (St. Louis, MO). Fluorescein (FITC)-conjugated *Escherichia coli* (*E. coli*) Bioparticles (opsonized), 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA) and hydroxyphenyl fluorescein (HPF) were purchased from Thermo Fisher Scientific (Waltham, MA).

Preparation of RAW 264.7 cells

RAW 264.7 cells (ATCC, Manassas, VA) were seeded at a density of 40,000 cells per well (100 μ L total volume per well) in a 96-well plate. The cells were cultured in DMEM medium supplemented with heat-inactivated FBS (10%), glutamine (2 mM), penicillin (100 U/mL), and streptomycin sulfate (100 mg/mL) for 24 h and then subjected to various treatments for subsequent assays.

Cytotoxicity of IONPs to RAW 264.7 cells

The cytotoxicity of IONPs was assessed using an MTT assay. For this, RAW 264.7 cells prepared in a 96-well plate were treated with IONPs at varying concentrations (0-5 mg/mL) and incubated for 24 h. The MTT assay was performed by following the manufacture protocol (Trevigen, Gaithersburg, MD).

Culture of *S. aureus*

A single colony of *S. aureus* (ATCC 6538 strain, ATCC, Manassas, VA) was transferred to the tryptic soy broth (TSB) medium and cultured for 18 h at 37°C on a shaking incubator at 180 rpm. The colony forming unit (CFU) number of viable *S. aureus* was counted by measuring the optical density at 600 nm and the bacteria were diluted to 1×10^6 CFU/mL for subsequent experiments.

Collection of planktonic and biofilm conditioned medium from *S. aureus*

For the collection of planktonic conditioned medium (PLK-CM), *S. aureus* bacteria cultured on a shaking incubator were pelleted by centrifugation at 3,500g and 4 °C for 15 min and supernatant of the pellet was collected. For the collection of biofilm conditioned medium (BIO-CM), the *S. aureus* bacteria were seeded into the wells of 24-well plate and incubated at 37°C in a static culture condition for 6 days as described [15]. The supernatant of the biofilm culture of *S. aureus* was collected, filtered using a 0.2- μ m filter and stored at -80°C for further experimental use.

Measurement of the levels of intracellular total ROS and hydroxyl radicals in RAW 264.7 cells

The level of intracellular total ROS and intracellular hydroxyl radicals was quantified using carboxy-H₂DCFDA and HPF, respectively. Following treatments, RAW 264.7 cells were subsequently incubated with either carboxy-H₂DCFDA or HPF, and fluorescence intensity was measured using a spectrophotometer (SpectraMax® M4 Multi-Mode Microplate Reader, Molecular Devices, Sunnyvale, CA).

Real time quantitative PCR (qPCR) analysis

Total RNA was isolated from RAW 264.7 cells or primary macrophages isolated from wounds of mice using a RNA extraction kit (OMEGA BIO-TEK, Norcross, GA). The qPCR analysis was performed with a Real-Time PCR System (Realplex² -Master cycler, Eppendorf, Hauppauge, NY) as described [16]. Results were normalized with respect to the level of housekeeping gene *Gapdh* and expressed as a fold-change in mRNA. The primer sequences of genes used in this study were listed in Supplementary Table 1, which include *Gapdh*, inducible nitric oxide synthase (*iNos*), interleukin-1 beta (*IL-1β*), tumor necrosis factor-α (*Tnf-α*), arginase-1 (*Arg-1*), and *Cd206*.

ELISA assay

RAW 264.7 cells were seeded into a 24-well plate (2.5×10^5 cells per well) and the supernatant of cell culture was collected following treatments and protein levels of pro-inflammatory cytokines including IL-1β, IL-12, and TNF-α were determined using ELISA kits (BioLegend, San Diego, CA).

Measurement of intracellular iron concentration

The intracellular level of iron in RAW 264.7 cells was measured using an iron assay kit (Abcam, Cambridge, MA) after homogenizing the cells in iron assay lysis buffer. The intracellular concentration of iron was normalized to the protein content in the cells. The relative levels of ferrous iron (Fe²⁺) were normalized to the untreated control group and expressed as a fold change.

Antibiotic protection assay for quantifying bactericidal activity of macrophages

The effect of IONPs on the bactericidal activity of macrophages against intracellular bacteria was assessed using an antibiotic protection assay as described [16]. In brief, RAW 264.7 cells seeded into a 24-well plate (2.5×10^5 cells per well) were co-cultured with planktonic *S. aureus* at a ratio of 1:10 for 1 h and washed with PBS to remove any non-adherent bacteria. Then, the cells were treated with gentamicin (25 μg/mL, Sigma-Aldrich, St. Louis, MO) for 18 h to eradicate extracellular bacteria and the lysates of RAW 264.7 cells were serially diluted and plated on TSA plates for CFU counting.

Mouse model of cutaneous wound *S. aureus* infection

C57BL/6 mice (male mice, 8-12 weeks old) were obtained from Jackson laboratory (Bar Harbor, ME). Mice were anesthetized with 1.5% isoflurane gas inhalation and one full-thickness, circular wound was made on the dorsal surface of a mouse using a 6 mm sterile

biopsy punch (Acuderm Inc., Fort Lauderdale, FL). The wound was covered with a transparent, semipermeable Tegaderm dressing (3M, Maplewood, MN) and 1×10^6 CFU of *S. aureus* was injected under the dressing to the wounded skin. At day 1 post infection, 40 μ L of sterile saline, IONPs (3 mg/mL in 40 μ L of saline), or IONPs (3 mg/mL) with VC (500 μ M) were locally injected to the area of the wound infection. At day 2 post-infection, the wounded skin was excised and homogenized for bacterial CFU counting. For the phenotyping of wound macrophages, cells were isolated from the excisional wounds of mice by enzymatic digestion method with collagenase I, collagenase XI and hyaluronidase (Sigma-Aldrich, St. Louis, MO). Then, macrophages were sorted from the wound-isolated cells using a magnetic activated cell sorting (MACS) method by positively selecting F4/80+ cells with MACS magnetic column (MACS® Columns, Miltenyi Biotec) as described in our recent study [17]. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Kent State University.

Statistical analysis

All statistical analysis were performed using an Origin 2015 software (Origin Lab, Northampton, MA). A two-tailed unpaired t-test was used to determine statistical significance between two groups. Statistical significance among multiple groups was determined using one-way ANOVA followed by Turkey's posttest for secondary analysis for comparison. All the data were given as mean $\pm$ standard error (SE) and *p*-value of less than 0.05 were considered statistically significant.

Results

IONPs promoted M1-like pro-inflammatory responses in RAW 264.7 macrophages

We first sought to determine the range of IONPs concentrations that can exhibit a pro-inflammatory activation in macrophages, while maintaining minimal toxicity to the cells. This was determined by quantifying the viability of RAW 264.7 macrophages at varying concentrations of IONPs (0-5 mg/mL) using a MTT cell viability assay. The treatment of IONPs to RAW 264.7 cells did not alter the viability of the cells at concentration up to 3 mg/mL compared to untreated control group, while the cell viability decreased by 10 % at 5 mg/mL concentration ($p < 0.05$) (Fig. 1 A). This result is in line with the result of recent study that shows minimal cytotoxicity of IONPs at 3 mg Fe/mL [14]. We next assessed the capacity of IONPs to trigger pro-inflammatory responses in macrophages by quantifying the expression of pro-inflammatory genes. For this, RAW 264.7 macrophages were pre-treated with IONPs at 3 mg/mL concentration in the absence and presence of *S. aureus*, and the expression of *iNos*, *Il-1 β* and *TNF- α* mRNAs were quantified by qPCR (Fig. 1B). Our results show that IONPs-treated RAW 264.7 macrophages exhibited an increased expression of *iNos* mRNA by 2-fold, with albeit lesser increase for *Il-1 β* and *Tnf- α* mRNAs, compared to an untreated control group. Interestingly, the expression of *iNos*, *Il-1 β* , and *Tnf- α* mRNAs were significantly augmented in IONPs-treated RAW 264.7 cells in the presence of *S. aureus*. The expression of pro-inflammatory cytokines (IL-1 β , TNF- α , and IL-12) in the protein levels was in line with qPCR results (Fig. 1C). These results suggest the capacity of IONPs that can amplify M1-like pro-inflammatory responses in the presence of an infectious challenge.

IONPs promoted a bactericidal activity of RAW 264.7 macrophages against intracellular *S. aureus*, associated with the generation of ROS

The capacity of macrophage to produce a sufficient amount of ROS is essential for conferring innate immune defense against intracellular bacteria [18]. Once internalized by cells, IONPs are degraded in endocytic organelles, resulting in the release of iron ions in the cytoplasm [19]. The newly formed iron ions can considerably affect the intracellular redox signaling that leads to the generation of ROS inside cells via a Fenton reaction [20]. Thus, we sought to investigate whether a IONPs-triggered Fenton reaction to generate ROS was sufficient to exhibit a bactericidal activity against *S. aureus* survived within macrophages. To ascertain this, we have assessed the capacity of IONPs to produce ROS in RAW 264.7 cells by treating the cells with varying concentrations of IONPs (0-3 mg/mL) and then quantifying the extent of total ROS generation using carboxy-H₂DCFDA, fluorogenic dye that can detect hydroxyl, peroxy and other ROS activity within the cell. The levels of intracellular ROS in RAW 264.7 cells in response to IONPs were increased in a dose dependent manner (Fig. 2A). Additionally, the extent of ROS generation in IONPs-treated RAW 264.7 cells was further increased in the presence of *S. aureus*, compared with cells treated with either IONPs or *S. aureus* alone (Fig. 2B). We subsequently engaged in the study to examine whether IONP-triggered generation of ROS was sufficient enough to elicit a bactericidal activity against intracellular *S. aureus* by quantifying the number of *S. aureus* survived within macrophages (Fig. 2C). The CFU number of viable intracellular *S. aureus* was decreased with increasing concentrations of IONPs (Fig. 2D). However, the improved bactericidal activity was not associated with the alteration in the phagocytic capacity of RAW 264.7 cells to IONPs (Fig. S1). Taken together, our results support that IONPs are capable of eliciting a bactericidal function of macrophages against intracellular *S. aureus* and this is associated with the capacity of IONPs to trigger the generation of ROS in macrophages.

IONPs restored an impaired bactericidal activity of macrophages against *S. aureus* biofilm

It has been shown that *S. aureus* infections involving the formation of biofilm are linked with macrophage polarization towards a M2-like phenotype [21, 22]. We have recently reported that secreted factors from *S. aureus* biofilm could significantly impair the bactericidal activity of macrophages [16]. By observing the capacity of IONPs to elicit pro-inflammatory and bactericidal activities in RAW 264.7 macrophages, we next examined whether priming with IONPs can restore the bactericidal activity of macrophages against *S. aureus* biofilms. For this, RAW 264.7 cells were pre-treated with IONPs (3 mg/mL) for 24 h, followed by incubation with BIO-CM (Fig. 3A). Then, the bactericidal activity of RAW 264.7 cells was examined and the result was compared with cells exposed to PLK-CM. The priming of RAW 264.7 cells with IONPs resulted in significant reduction in the number of surviving bacteria by ~50% in the presence of BIO-CM, compared to an untreated control group ($P<0.05$), albeit to a lesser extent than the one treated with PLK-CM (Fig. 3B). This result supports that the treatment of IONPs could convert macrophages polarization towards an anti-biofilm phenotype, which might contribute to the improved bactericidal activity against *S. aureus* biofilm.

Harnessing the capacity of IONPs to generate hydroxyl radicals via a Fenton reaction enhanced a bactericidal activity of macrophages

Our results demonstrate that the capacity of IONPs to promote bactericidal and pro-inflammatory responses in macrophages was associated with the generation of ROS. It is conceivable that the uptake of IONPs by macrophages might result in the release of ferric iron (Fe^{3+}) in the cytoplasm, resulting in increased ROS formation via the Fenton reaction [23, 24]. In particular, since the ability to increase the availability of ferrous iron (Fe^{2+}) in the cytoplasm is critical for ROS formation, we hypothesized that the treatment of a reducing agent would enhance a bactericidal activity of macrophages via catalyzing a Fenton reaction. To test this hypothesis, we first examined if the treatment of ascorbic acid, vitamin C (VC), a known reductant to drive the Fenton reaction by reducing Fe^{3+} to Fe^{2+} , can exhibit an enhanced bactericidal activity against intracellular *S. aureus*. The ROS generation and bactericidal activity in RAW 264.7 macrophages were not altered by the treatment of VC alone (500 μM) (Fig. 4A and 4B). However, a combined treatment of IONPs (3 mg/mL) with VC (500 μM) to RAW 264.7 cells could significantly augment the generation of total ROS compared to IONPs alone (Fig. 4A), which was associated with an increased bactericidal activity (Fig. 4B). To further determine if the improved ROS generation and bactericidal activity were associated with a Fenton reaction due to increased release of Fe^{2+} , the levels of Fe^{2+} were compared between RAW 264.7 cells treated with IONPs alone and IONPs with VC. The treatment of IONPs alone could significantly increase the level of Fe^{2+} in RAW 264.7 cells by 3-fold compared to untreated cells, and its level was further augmented by 2-fold in the presence of VC compared to IONPs only (Fig. 4C).

Among various forms of ROS, hydroxyl radical ($\text{OH}^{\bullet}$) is highly cytotoxic by causing oxidative damage to DNA and cell membrane [25]. In the Fenton reaction-mediated generation of ROS, the oxidation of Fe^{2+} by hydrogen peroxide (H_2O_2) produces highly reactive hydroxyl radical [26]. To test if enhanced bactericidal activity of RAW264.7 cells with IONPs in combination with VC could be a consequence of Fe^{2+} release and the generation of hydroxyl radicals, the extent of hydroxyl radical generation and bactericidal activity in RAW 264.7 cells with a combined treatment of IONP and VC were quantified using the chelator of Fe^{2+} (BIP) [27], or scavenger of hydroxyl radicals (THI) [28]. The treatment of BIP (500 μM) to RAW 264.7 cells significantly decreased the IONPs/VC-induced generation of hydroxyl radicals up to the level of IONPs treatment only (Fig. 4D), which was associated with decrease in bactericidal activity of RAW 264.7 cells (Fig. 4E). Additionally, the inhibition of hydroxyl radical formation by BIP was sufficient enough to reverse IONPs/VC-induced bactericidal activity of RAW 264.7 cells, which was comparable to the level induced by THI treatment. Taken together, our results suggest that the treatment VC can synergistically enhance IONPs-mediated bactericidal activity of macrophages by triggering the Fenton reaction involving the release of Fe^{2+} and the generation of hydroxyl radical (Fig. 4F).

The treatment of IONPs and VC synergistically reduced the number of bacteria in wounds of mice infected with *S. aureus*

By observing the capacity of IONPs, in combination with VC, in promoting the bactericidal activity of RAW 264.7 cells *in vitro*, we next sought to validate its efficacy *in vivo* using a

murine model of wound infection by *S. aureus*. For this, C57BL/6 mice were inoculated with 1×10^6 CFU in the wounds at day 0 and then a defined amount of IONPs (3 mg/mL in 40 μ L sterile saline per wound) were topically applied to the wounds of mice at day 1 post-infection. The CFU number of *S. aureus* was quantified from the wounded skin harvested at day 2 post-infection (Fig. 5A). The treatment of IONPs to the wound could reduce a bacterial burden by 25% compared to the control group ($p < 0.05$) (Fig. 5B). In consistence with our results from the *in vitro* study, the combined treatment of VC (500 μ M) with IONPs significantly reduced *S. aureus* numbers in the wound by 75% compared to the control group ($p < 0.05$). We next examined whether the reduced bacterial burden was associated with the polarization of macrophages. For this, F4/80⁺ macrophages were isolated from the wound at day 2 post-infection and the expression of pro-inflammatory M1 (*iNos* and *Il-1 β*) and anti-inflammatory M2 markers (*Arg-1* and *Cd206*) were determined. The macrophages from wounds of mice treated with IONPs and VC displayed a significantly increased expression of *iNos* and *Il-1 β* compared to either IONPs alone or untreated control group, which was associated with an attenuated expression of M2 markers including *Arg-1* and *Cd206* (Fig. 5C). It has been reported that IONPs or VC alone could inhibit the growth of *S. aureus* [29, 30]. However, the direct application of IONPs (up to 3 mg/mL) or VC (up to 2 mM) to the culture of *S. aureus* did not alter the viability of the bacteria, at least in our experimental system here (Fig. S2). Thus, we hypothesize that the improved clearance of *S. aureus* in the wounds of mice treated with IONPs and VC is, in part, attributed to the polarization of macrophages towards the M1-like pro-inflammatory phenotype.

Discussion

Our study supports that harnessing the characteristic of IONPs to tune macrophage polarization to exhibit a bactericidal activity can provide a new strategy for treating bacterial infections. This was supported by our data from both *in vitro* and *in vivo* studies, which demonstrated that IONPs could exhibit an enhanced bactericidal activity of macrophages against intracellular *S. aureus* by inducing a M1 macrophage polarization that stimulates the production of ROS. Importantly, a strategy for facilitating a Fenton reaction by the addition of a reducing agent could significantly enhance the bactericidal activity of macrophages.

The capacity for *S. aureus* to survive within macrophages is a critical factor for facilitating the dissemination of the pathogens in the host [2]. Upon phagocytosis of the bacterial pathogens, the production of sufficient quantities of ROS, in large part by mitochondrial respiratory chain, is critical for killing the pathogens [31]. However, *S. aureus* has developed several strategies to escape ROS-mediated killing by eliciting a transcriptional regulation of ROS detoxifying enzymes. For example, *S. aureus* is capable of encoding super oxide dismutase by transcribing genes such as *sodA* and *sodM* and encoding catalase by transcribing *kata*, which result in the enzymatic degradation of superoxide and hydrogen peroxide, respectively [32, 33]. However, no cellular detoxification mechanism for hydroxyl radicals has been found in *S. aureus*. Thus, we speculated that a strategy to generate hydroxyl radicals by IONPs would render intracellular *S. aureus* to be more susceptible to bactericidal attacks by macrophages. We hypothesized that the liberation of Fe³⁺ ions from IONPs can drive the generation of hydroxyl radicals via a Fenton reaction. To ascertain this, we sought to utilize the capacity of ascorbic acid (VC) in catalyzing the reduction of ferric

iron to ferrous iron, which in turn can react with H₂O₂ to generate hydroxyl radicals [34]. Our results validated the ability of VC to synergistically enhance an IONP-mediated bactericidal activity of macrophages, which was associated with the increased generation of Fe²⁺ ions and hydroxyl radicals. The functional role of hydroxyl radicals in the IONPs and VC-mediated killing of intracellular *S. aureus* was supported by the finding that a scavenger of hydroxyl radicals could diminish a bactericidal activity of RAW 264.7 macrophages.

Our data from the *in vitro* study using RAW 264.7 cells demonstrated that the capacity of IONPs to generate ROS was associated with the polarization of macrophages towards a M1-like pro-inflammatory phenotype. In line with the findings from the *in vitro* study, the treatment of IONPs to the wounds of mice could significantly increase the expression of M1 markers (*iNos* and *Il-1β*), whereas the expression of M2 markers (*Arg-1*, *Cd206*) were significantly attenuated. Importantly, the co-treatment of IONPs with VC could further amplify the expression of *iNos* and *Il-1β* in wound macrophages, along with a concomitant decrease in *Arg-1* and *Cd206*. It may be possible that the increased level of intracellular iron or subsequent generation of ROS might activate NF-κB [35, 36], resulting in the polarization of macrophages towards the M1-like activation. An appropriate activation of macrophages to produce pro-inflammatory cytokines and chemokines at the site of infection is critical for innate immune defense by promoting the recruitment of neutrophils into the infected skin [37]. Thus, apart from the direct role of IONPs in promoting a ROS-mediated bactericidal activity of macrophages against intracellular *S. aureus*, we do not rule out the possibility that the capacity of IONPs to promote the pro-inflammatory response of macrophages in the wounds of *S. aureus* infection might also be beneficial to the host to combat infection through indirect effects on wound microenvironment.

Our study implicates the translational potential of IONPs as an immune adjuvant to treat bacterial infections by means of promoting anti-bacterial and pro-inflammatory responses in macrophages. The delivery of free iron ions to the macrophages is limited due to the tightly regulated mechanism of iron transport and homeostasis for balancing iron within safe limits, in order to avoid cellular damage associated with iron overload [38]. It has been shown that macrophages acquire iron via transferrin receptor-dependent endocytosis of transferrin, and the iron is then transported into the cytoplasm by the divalent metal transporter 1 [39, 40]. However, the increase in total body iron triggers the production of hepcidin that inhibits the entry of iron to the cell [41]. In view of this, the delivery of iron in the form of nanoparticles can be advantageous to target macrophages and may hold promise for treating infectious diseases in that macrophages are highly phagocytic cells that are capable of phagocytosing nanoparticles efficiently. Additionally, similar types of IONP drug formulations have received FDA approvals, which include Feraheme, Feridex, and Gastromark [42]. However, it also should be noted that, despite the beneficial effect of IONPs on reprogramming macrophages towards an anti-bacterial phenotype, either excessive activation of macrophages or non-specific targeting effects may result in uncontrolled inflammation [13]. The future study should be directed towards optimizing the dosing and frequency of IONPs treatment as well as achieving a targeted delivery of IONPs to the target tissue and cells, which may ensure sufficient bacterial killing while minimizing toxicity due to overdose of IONPs.

In summary, our study demonstrates that harnessing the characteristic of IONPs to skew macrophage polarization to exhibit a bactericidal activity by means of triggering a Fenton reaction can provide a new strategy for treating infectious diseases as an immune adjuvant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding support: This study was funded by a grant from the National Institute of Health (NIH R01 NR015674).

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Figure 1. Effects of IONPs on the viability and expression of pro-inflammatory genes in RAW 264.7 macrophages.

A. The effect of IONPs on the viability of RAW 264.7 cells. RAW 264.7 cells were treated with varying concentrations of IONPs (0-5 mg/mL) for 24 h and cell viability were quantified using an MTT assay. B. The qPCR analysis of pro-inflammatory genes in RAW 264.7 cells. RAW 264.7 cells were pre-treated with IONPs (3 mg/mL) for 18 h and then exposed to *S. aureus* (1×10^6 CFU/mL) for 1 h. C. The ELISA analysis of pro-inflammatory proteins (IL-1 β , TNF- α , and IL-12) in RAW 264.7 cells. RAW 264.7 cells were pre-treated with IONPs (3 mg/mL) for 18 h and then exposed to *S. aureus* (1×10^6 CFU/mL) for 4 h. N=3-5 independent experiments for each experimental group. *: $p < 0.05$. **: $p < 0.01$. N.S: Not Significant ($p > 0.05$).

Figure 2. Effects of IONPs on the generation of ROS and bactericidal activity in RAW 264.7 macrophages against intracellular *S. aureus*.

A. The quantification of ROS generation in the RAW 264.7 cells treated with varying concentration of IONPs (0-3 mg/mL) for 18 h. B. The quantification of ROS generation in the RAW 264.7 cells treated with IONPs in the presence or absence of *S. aureus*. The cells were pre-treated with IONPs (3 mg/mL) for 18 h and then exposed to *S. aureus* (1×10^6 CFU/mL) for 1 h. C. A schematic diagram of the experimental protocol for phagocytosis and killing of intracellular bacteria by antibiotic protection assay. D. The effect of varying concentrations of IONPs (0-3 mg/mL) on the bactericidal activity of RAW 264.7 cells assessed by antibiotic protection assay. N=3-5 independent experiments for each experimental group. *: $p < 0.05$. **: $p < 0.01$. N.S: Not Significant ($p > 0.05$).

Figure 3. Effects of IONPs on the bactericidal activity in RAW 264.7 macrophages in response to *S. aureus* biofilm.

A. A schematic diagram of the experimental protocol for the collection and treatment of conditioned medium from the planktonic and biofilm phase of *S. aureus* to the RAW 264.7 cells. RAW 264.7 cells were pre-treated with IONPs for 18 h and then exposed to PLK-CM or BIO-CM for 1 h. B. The quantification of the bactericidal activity of IONPs-primed RAW 264.7 cells in the presence of conditioned medium collected from planktonic or biofilm phase of *S. aureus*. Data are from three (N=3) independent experiments for each experimental group. *: $p < 0.05$.

Figure 4. The effects of VC on IONPs-mediated generation of ROS and bactericidal activity in RAW 264.7 macrophages against intracellular *S. aureus*.

(A-C). The intracellular level of total ROS (A), bactericidal activity (B), and intracellular level of ferrous iron (Fe^{2+}) (C) in RAW 264.7 cells treated with IONPs (3 mg/mL) alone, VC (500 μ M) alone, or IONPs with VC. The cells were incubated with or without IONPs for 18 h and then subsequently treated with VC for 30 min. D. The intracellular level of hydroxyl radical concentration in RAW 264.7 cells treated with IONPs (3 mg/mL) alone, IONPs with VC (500 μ M), or IONPs with VC and BIP (500 μ M). The cells were incubated with or without IONPs for 18 h and then subsequently treated with VC or VC mixed with BIP for 30 min. E. The bactericidal activity of RAW 264.7 cells treated with IONPs (3 mg/mL) with VC (500 μ M), IONPs with VC and BIP (500 μ M), or IONPs with VC and THI (200 mM), assessed by antibiotic protection assay. F. A schematic on the proposed mechanism by which IONPs and VC can promote the killing of intracellular bacteria via triggering a Fenton reaction that generates intracellular ROS. N=3-5 independent experiments for each experimental group. *: $p < 0.05$. N.S: Not Significant ($p > 0.05$).

Figure 5. The in vivo validation of the efficacy of IONPs against *S. aureus* using a mouse model of wound infection.

A. A schematic on the experimental protocol for cutaneous wound infection by *S. aureus* and topical treatment of IONPs or IONPs/VC to the site of infection. *S. aureus* (1×10^6 CFU/ wound) was inoculated to 6-mm skin wounds at 0 day followed by topical application of IONPs (3 mg/mL in 40 μ L saline) or IONPs/VC (500 μ M) on each wound at 1 day. The skin wound tissues were dissected at 2 day for bacteria CFU counting and qPCR analysis. B. The quantification of bacterial CFU number from wounds of mice. * $p < 0.05$, N=6 per group. C. The expression of M1 marker (*iNos* and *Il-1β*) and M2 marker (*Arg-1* and *Cd206*) in the F4/80+ macrophages isolated from wounds mice treated with IONPs or IONPs with VC. N=4 independent experiments for each experimental group. * $p < 0.05$.