In Vitro Anti-inflammatory and Immunomodulatory Effects of Ciprofloxacin or Azithromycin in *Staphylococcus aureus*-Stimulated Murine Macrophages are Beneficial in the Presence of Cytochalasin D

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ABSTRACT—We hypothesized that if internalization of *Staphylococcus aureus* could be blocked by using cytochalasin D (an inhibitor of phagocytosis and phagolysosome fusion), then the intracellular entry and survival of the pathogen in host's phagocytic cells recruited to the inflammatory site can be restricted. At the same time, if we use antimicrobial agents (*e.g.*, ciprofloxacin and azithromycin) having potent intracellular and extracellular microbicidal activity against the bacterium that have not entered into the phagosome and remains adhered to the phagocytic cell membrane, then they can be eradicated from the site of infection without compromising the host cell. To validate this, role of ciprofloxacin (CIP) and azithromycin (AZM) in eliminating *S. aureus* by suppressing the phagocytic activity of macrophages with cytochalasin D before infection was investigated. CIP and AZM were used either alone or in combination with cytochalasin D. Supernatant and lysate obtained from the culture of macrophages were used for quantification of reactive oxygen species, lysozymes, antioxidant enzymes, and cytokines produced. Azithromycin was better than ciprofloxacin in combination with cytochalasin D for eradicating *S. aureus* and regulating cytokine release. Further studies are required for ensuring proper delivery of this combination at the site of infection.

KEY WORDS: azithromycin; ciprofloxacin; cytochalasin D; intracellular survival.

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INTRODUCTION

Staphylococcus aureus is a major human pathogen causing significant morbidity and mortality in both community- and hospital-acquired infections [1]. *S. aureus* which often causes chronic or relapsing diseases has been reported to persist as an opportunistic intracellular organism both *in vitro* and *in vivo* [2]. Localized *S. aureus* infections have been followed by bacterial invasion of the vascular system, leading to bacteremia and sepsis. Whether an infection is contained or spreads depends on a complex interplay between *S. aureus* virulence determinants and host defense mechanisms [3]. *S. aureus* has been reported to express a wide array of secreted and cell surface-associated virulence factors to help evade immune responses [4]. The treatment of *S. aureus* infections has been increasingly problematic due to the high prevalence of multi-antibiotic-resistant strains, such as methicillin-resistant *S. aureus* (MRSA) [5], and the emergence of and vancomycin-resistant *S. aureus* strains (VRSA) [6].

S. aureus have been reported to survive within phagocytic cells both in polymorphonuclear neutrophil (PMN) and monocytes [7]. However, recent experiments assessing invasion and the intracellular survival of *S. aureus* in endothelial, epithelial cells, macrophages, and osteoblasts [8–14] suggested that such events may contribute to the persistence of *S. aureus* during infections such as septic arthritis [15–17]. Moreover, it has long been known that professional phagocytes may serve as intracellular reservoirs of *S. aureus* [18]. In order to survive and induce infection, pathogenic bacteria have to cope with their changing environment, as well as continuous attacks of the host antimicrobial defense system. In keeping with this idea, recent *in vitro* studies have confirmed high-level resistance by *S. aureus* to neutrophil- [19] and macrophage- [20] mediated killing *via* several virulence factors such as surface proteins that support colonization on host tissues, like coagulase, staphylokinase, leukocidins, etc., that promote bacterial spreading, protein A that inhibits phagocytic engulfment, and toxins, like TSST, hemolysins, leukotoxins, exotoxins, etc., that damage host cell membranes.

It has been suggested by an expert panel of the Infectious Diseases Society of America (IDSA) that vancomycin dosing should be monitored for treatment of infections due to MRSA strains with reduced susceptibility to vancomycin, but the question that still remains to be addressed is what would be the optimal therapy in case of vancomycin resistance. It may be suggested that alternative antibiotic regimen might be used or to find out the role of suitable combination therapy to treat such infections [21]. Thus, there is a need of designing some alternative strategy to treat infections due to MRSA that can eradicate these pathogens completely and ameliorates the progression of the disease processes.

S. aureus may occasionally become intracellular, at least within monocytes, macrophages, and PMN when host defense mechanisms are activated [22]. The facultative intracellular persistence of staphylococci may play an important role in the pathogenesis, because this localization protects them from both humoral and cell-mediated immune responses. The intracellular habitat of *S. aureus* calls for antibiotics with intracellular activity toward *S. aureus*. However, antibiotics have routinely been tested only for their *in vitro* activity on extracellular bacteria. Obviously, the intracellular activity of antibiotics can significantly differ from that exerted extracellularly. In general, it seems impossible to deduce the intracellular antibacterial activity of antibiotics from standard susceptibility tests [23]. Moreover, it has been reported that approximately one third of clinical *S. aureus* isolates not only survived in but also killed their eukaryotic host cells, which was accompanied by increased *in vivo* virulence [24]. Therefore, a successful anti-staphylococcal therapy should include the elimination of intracellular bacteria and the rescue of host cells from staphylococci-induced cell death.

The invading bacterium initially adheres to the cell membrane and following transient actin polymerization at sites proximal to the site of entry is thought to trigger ingestion by the formation of a phagocytic vesicle enclosing bacterium [25]. It has been reported that cytochalasin D, a fungal metabolite and specific actin polymerization inhibitor, which binds to actin and modifies its polymerization reversibly has been used as an inhibitor of bacterial uptake and an inducer of cell–cell contact disruption by a variety of cell types [26]. Cytochalasin D permeates cell membrane and causes cells to stop ruffling, stop translocating, and stop rounding up. Functionally, cytochalasin D inhibits microfilament function and polymerization by blocking actin monomer addition at the rapidly growing end of the actin filament [27]. Azithromycin (AZM) belongs to macrolide group of antibiotics that interfere with function of neutrophils and macrophages, disrupting the process of chemotaxis, migration, and cellular activity [28, 29]. This disruption affects cell survival as they induce apoptosis of lymphocytes and neutrophils [30–33]. These antibiotics are also involved in the processes of adherence and diminish the expression of the molecules needed for adherence [34, 35]. Pathogenic bacteria and other infectious agents can stimulate monocytes or macrophages directly, initiating a release of pro-inflammatory cytokines to sustain inflammation and the immunological response. Tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1, IL-6, and IL-8 are biologically active peptides produced by phagocytic macrophages, PMN, eosinophils, and monocytes induced by the pathogen organisms, endotoxin, and other stimuli [36, 37]. Antibiotics are also capable of reducing the production of pro-inflammatory cytokines like IL-1, IL-6, IL-8, TNF-α, and interferon-gamma (IFN-γ) during acute phase inflammatory processes [38, 39]. Furthermore, various oxidizing species that take part in the innate immunity of the host phagocytic cells, such as superoxide anion and nitric oxide, are also diminished by the action of these groups of antibiotics. Their activity is not only restricted within the cytosol of the cell, and they can cause pronounced diminishing effects on several nuclear transcription factors such as NF-κB and activator protein-1 (AP-1), and it is speculated that glutathione deficiency could activate this mechanism [40–43]. Ciprofloxacin (CIP) which belongs to group of fluoroquinolones is a

potent synthetic agent active against a variety of bacterial species *in vitro*. These drugs appear to antagonize the A subunit of the bacterial enzyme DNA gyrase [44–46] and thus block DNA replication. Additional incompletely understood processes typically result in the rapid killing of bacteria [47].

Recent investigation conducted to figure out the role of intracellularly active antibiotics in elimination of intracellular *S. aureus* reveals that withdrawal of antibiotics leads to cytotoxic activity of the surviving population of *S. aureus* inside the phagocytic cells which eventually leads to killing of host cells. Thus, uses of antibiotics that are active against intracellular pathogen do not confer full protection from them. This situation demands some alternative strategy which may help to eliminate intracellular pathogen load and also concurrently aid in survival of the phagocytic cells. We, therefore, investigated the role of antibiotics either alone or in combination in eliminating intracellular or extracellular *S. aureus* by suppressing the phagocytic activity of murine peritoneal macrophages with cytochalasin D before infection to address whether internalization of *S. aureus* is required or not for antibiotic-mediated killing. This novel strategy might be helpful in reducing intracellular persistence of *S. aureus* in pathogenesis of several disease processes. In order to address this problem, we hypothesized that if internalization of *S. aureus* could be blocked by using cytochalasin D (an inhibitor of phagocytosis and phagolysosome fusion), then the intracellular entry and survival of the pathogen in host's phagocytic cells recruited to the inflammatory site can be restricted. At the same time, if we use antimicrobial agents (*e.g.*, CIP and AZM) having potent intracellular and extracellular microbicidal activity against the bacterium that has not entered into the phagosome and remains adhered to the phagocytic cell membrane, then they can be eradicated from the site of infection without compromising the host cell. Moreover, it has been reported that combination therapy is beneficial over monotherapy. Since no synergistic interaction has been obtained in the *in vitro* killing of *S. aureus*, we have omitted the CIP + AZM combination in the cytochalasin D-treated macrophages during *S. aureus* infection. Thus, we choose combination therapy with an antibiotic (CIP or AZM) and cytochalasin D combination in treatment of *S. aureus* infection *in vitro* in isolated murine peritoneal macrophages. Azithromycin was found to be more beneficial in action compared to ciprofloxacin in combination with cytochalasin D in eradicating *S. aureus* from the infected macrophages. Azithromycin alone was also good enough to decrease the pathogen burden within the macrophages, but owing

to intracellular persistence after azithromycin therapy, several diseases posed difficulties in treatment using only antimicrobial therapy. Taken together, it could be suggested that actions of CIP and AZM are enhanced in the presence of cytochalasin D indicating that internalization of *S. aureus* by macrophages is not essential for antibiotic-mediated killing at least in this *in vitro* study. Although both the tested antibiotics got access inside the cell as evident from effective pathogen killing, further *in vitro* studies are required for ensuring proper delivery of this combination at the site of infection or within the phagocytic cells for considering this mode of therapy in cases of intracellular persistence of *S. aureus*.

MATERIALS AND METHODS

Biochemical Analysis and Biotyping for Characterization of the *S. aureus* (AG-789) Isolate

Catalase Test

Catalase test was performed according to the protocol given by Cowan [48]. Single colonies of the *S. aureus* (AG-789) maintained on tryptic soya agar plates were used for the test.

Slide Test

Single colony from the solid medium was transferred to a drop of 30 % hydrogen peroxide (H_2O_2)

Tube Test

About 3 % aqueous H_2O_2 solution was poured on growth containing slant or broth and observed for bubbling.

Coagulase Test

The procedure for coagulase testing was followed such as single colony of *S. aureus* (AG-789) was directly placed in diluted plasma [49] and was placed in water bath and monitored at 2, 4, 6, 8, and 24 h, respectively, for clot formation [50] and examined according to the recommendation of American Public Health Association.

Growth on Glucose-Containing Media

Twenty-hour-old culture of *S. aureus* (AG-789) in tryptic soy broth was transferred to semisolid Brewer's fluid thioglycolate media which was steamed and cooled

to 50 °C. After solidification at room temperature, tubes were incubated at 37 °C for 72 h [51]. Growth was observed in the anaerobic zone by looking at the tubes through a light source.

Bovine Plasma Coagulation

Eighteen to twenty four hour broth culture of *S. aureus* (AG-789) was prepared in which 0.5 ml of bovine plasma (diluted) was added in an aluminum-capped test tube. The contents of the tube were mixed and placed in water bath at 37 °C in a water bath. Readings were made at an interval of 30 min to determine the exact time of clotting up to 8 h. Controls containing no *S. aureus* were prepared to determine the clot formation [52].

Growth on Crystal Violet Agar

Crystal violet agar was prepared by adding crystal violet in different dilutions to nutrient agar. Single colonies of *S. aureus* were taken and transferred to crystal violet agar plates and incubated overnight at 37 °C, and the resulting growth was observed and examined as purple or white colonies [53].

Antimicrobial Agents and Chemicals

The study drugs which included AZM, CIP, methicillin (MET), and chloramphenicol (CLO) (HiMedia, Bombay, India) were used for all *in vitro* testing. Ciprofloxacin was dissolved in sterile double-distilled water, whereas azithromycin was dissolved in absolute alcohol to prepare a stock solution of 100 μg/ml, which was further diluted in sterile water before use for *in vitro* assays. Cytochalasin D was obtained from Enzo Life Sciences, USA. Cytochalasin D was prepared to the desired dose by dissolving the chemical in 2 mM dimethyl sulfoxide (DMSO) Merck Ltd, Mumbai, India. The solution was stored at −20 °C until used.

In Vitro Susceptibility Tests

In vitro susceptibilities of the isolate were performed per National Committee for Clinical Laboratory Standards (NCCLS) guidelines. Minimum inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) were determined by the microtitre broth dilution method in MHB (HiMedia, Bombay, India), and disk agar diffusion (DAD) test was performed using Mueller-Hinton agar supplemented with 5 % sheep blood following NCCLS guidelines [54].

Test for Synergism and Antagonism

Checkerboard assay was performed according to the method described earlier [55]. For each combination, a synergy test was performed in a 96-well microtitre plate containing two antimicrobial agents in twofold dilutions (4×MIC to 1/32×MIC) dispensed in a checkerboard fashion on the day of the assay. $\sum$ Fractional inhibitory concentrations (FICs) were calculated and were used to classify the effect of combination of antimicrobial agents as the following: synergistic, for FIC indexes ≤0.5; no interaction, for FIC indexes >0.5–4; and antagonistic, for FIC indexes >4.

Maintenance of Animals and Cells

All experiments involving animals were conducted according to the protocols that had been approved by the Institutional Animal Ethics Committee (IAEC), Department of Physiology, University of Calcutta, under the guidance of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) [Approval Number 820/04/ac/CPCSEA dated August 5, 2013], Ministry of Environment and Forest, Government of India. Wild-type male Swiss albino mice were used throughout the study. To minimize the feeling of hypoxia or discomfort before and during mouse dissection and tissue collection, mice were anesthetized with inhaling anesthetics (ether) before terminal surgery. Euthanasia was performed by general anesthesia followed by vital tissue removal using 2–3 % ether for induction and 1 % for maintenance. Macrophages were prepared from peritoneal fluids of thioglycolate-administered mice. The *S. aureus* strain AG-789 was obtained from Apollo Gleneagles Hospital, Calcutta, West Bengal, India. The strain AG-789 was found to be methicillin-resistant and catalase-positive.

Preparation of Bacteria for In Vitro Infection to Peritoneal Macrophages

The *S. aureus* strain AG-789 was obtained from Apollo Gleneagles Hospital, Calcutta, West Bengal, India. *S. aureus* strain (AG-789) grown overnight in Luria-Bertani broth (HiMedia, Bombay, India) was diluted with fresh broth and cultured until mid-logarithmic phase of growth. Bacteria were harvested, washed twice with sterile saline, and adjusted to the

desired inoculums spectrophotometrically before infection ($OD_{620} = 0.2$ for 5.0×10^7 cells/ml for *S. aureus*), and the colony-forming unit (CFU) count of the desired inoculum was confirmed by serial dilution and culture on blood agar [15–17].

Isolation and Stimulation of Peritoneal Macrophages

The mice, used at 6 to 8 weeks of age and fed standard laboratory chow and water, were injected intraperitoneally with 2 ml of 4 % sterile thioglycolate broth, and the resulting peritoneal exudates were harvested by lavage of the peritoneal cavities of mice with endotoxin-free Hanks' solution 4 to 5 days later. Peritoneal macrophages were suspended in 0.83 % ammonium chloride solution containing 10 % (*v/v*) Tris buffer (pH 7.65) to lyse erythrocytes. The cells were resuspended in Roswell Park Memorial Institute (RPMI) medium supplemented with 10 % fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin and then were allowed for plastic adherence. Nonadherent cells were removed by aspiration and washing with RPMI 1640 medium before the addition of *S. aureus*. The adherent macrophages, more than 95 % of which appeared to be typical macrophages by light microscopy, were used for each experiment [11–13]. The peritoneal macrophages collected were suspended in Hank's balanced salt solution (HBSS) solution. Murine peritoneal macrophages (5×10^6 cells/ml) were infected with *S. aureus* (5×10^6 CFU/ml) for 30, 60, and 90 min at 37 °C, in humidified 5 % CO_2 incubator (Heal Force, Model HF-151, China) in the presence or absence of antibiotics and cytochalasin D at a dose of 5 μg/ml [28] either alone or in combination with azithromycin or ciprofloxacin at a dose of four times the MIC for the *S. aureus* strain (AG-789).

Assay for Intracellular Killing

Murine peritoneal macrophages (5×10^6 cells/ml) were mixed with *S. aureus* (5×10^6 CFU/ml) in a 1:1 cell/bacterium ratio [11–13] in RPMI-FBS (5 %) and incubated at 37 °C cell culture incubator for different times in the presence and absence of antibiotics and cytochalasin D either alone or in combination. After centrifugation, cell culture supernatants were collected and stored for further assay. Phagocytosis was stopped by adding cold (4 °C) RPMI-1640, and extracellular *S. aureus* were removed by washing the suspension in RPMI. The pellets were disrupted in sterile water containing 0.01 % bovine serum albumin (BSA) by vigorously vortexing to release intracellular bacteria in the lysate. The lysates containing bacteria were plated at serial dilutions on mannitol agar plates. The plates were incubated at 37 °C for a day or two, and the number of colonies was determined.

H_2O_2 Production

After time-dependent phagocytosis, supernatants were collected, and cell lysates were prepared from the pellet. H_2O_2 assay of the supernatant and lysate was performed according to the method as described earlier with slight modification [56]. Briefly 70 μl of supernatant or lysate, 20 μl horseradish peroxidase (HRP) (500 μg/ml), 70 μl of phenol red (500 μg/ml), and 40 μl medium were added in each of the microtiter plate and was allowed for incubation for 2 h at 37 °C. The reaction was stopped by adding 25 μl of 2 (N) NaOH, and the absorbance reading was taken at 620 nm. Control set received 40 μl of HBSS in place of supernatant/lysate. A standard H_2O_2 curve was plotted, and H_2O_2 release in supernatants and lysate was evaluated and expressed in micromolar per 10^6 cells.

Assay for Determination of Superoxide Anion

Superoxide anion release assay measures the change in color of cytochrome C (cytC), when reduced by O^{2-} released from the stimulated macrophages. Cell-free supernatants recovered from *S. aureus*-stimulated macrophages in the presence or absence of antibiotics and cytochalasin D either alone or in combination were incubated in the presence of cytochrome C (100 μl at 2 mg/ml in HBSS). The reaction was terminated by placing the tubes in ice for 5 min. The production of superoxide anion was monitored spectrophotometrically (UV-1800 UV–VIS spectrophotometer, Shimadzu, Japan) at 550 nm in reference to the blank. The amount of superoxide production was calculated by the following formula: nanomoles of superoxide anion = (mean absorbance at 550 nm × 15.87) [57].

Determination of Antioxidant Enzyme Activity in Supernatant and Lysate After Time-Dependent Phagocytosis of *S. aureus* in the Presence or Absence of Antibiotics and Cytochalasin D Either Alone or in Combination

Assay of Catalase Enzyme Activity

Catalase enzyme activity in the supernatant or cell-free lysate was determined spectrophotometrically by measuring the decrease in H_2O_2 concentration at 240 nm. At time 0, 10 μl of the supernatant or cell-free lysate was added separately to 2.89 ml of potassium phosphate buffer (pH 7.4) taken in a quartz cuvette. To it, 0.1 ml of 300 mM

H_2O_2 was added and absorbance was taken at 240 nm for 5 min at 1-min intervals. Catalase activity was expressed in terms of millimole per minute per milligram protein [15–17].

Assay of Superoxide Dismutase (SOD) Enzyme Activity

One hundred microliters of the supernatant or cell-free lysate was mixed separately with 1.5 ml of a Tris-ethylenediamine hydrochloric acid (Tris–EDTA–HCl) buffer (pH 8.5), then 100 μl of 7.2 mmol/L pyrogallol was added and the reaction mixture was incubated at 25 °C for 10 min. The reaction was terminated by the addition of 50 μl of 1 M HCl and measured at 420 nm. One unit was determined as the amount of enzyme that inhibited the oxidation of pyrogallol by 50 %. The activity was expressed as units per milligram protein [15–17].

Assay of Reduced Glutathione (GSH) Content

Reduced glutathione content (as acid-soluble sulfhy-dryl) was estimated by its reaction with DTNB (Ellman's reagent) following the method of Sedlac and Lindsey with some modifications [58]. Of the sample, 0.3 ml was mixed with 0.3 ml 10 % TCA followed by vortexing. Then, the mixture was centrifuged at 5,000 rpm for 10 min at 4 °C. Two hundred fifty microliters of the resultant supernatant was added with 500 μl 0.8 M Tris–HCl followed by the addition of 25 μl of DTNB. The absorbance was measured at 412 nm using a UV–VIS spectrophotometer to determine GSH content. The values were expressed as nanomoles of GSH per milligram protein

Assay of Bactericidal Lysozyme Enzyme Release

The supernatants and cell-free lysates obtained in the phagocytosis assay were used to estimate the lysozyme content after varying phagocytic times. Lysozyme content was estimated according to the method described by Colowick *et al.* [59]. A suspension of *Micrococcus lysodeikticus* in 0.15 M potassium phosphate buffer, pH 6.2, was prepared and its optical density was fixed at 0.6–0.7 at 450 nm. Of this suspension, 900 μl was mixed with 50 μl of 1 % Triton X-100, and the reaction was started by adding 50 μl of the supernatants or cell-free lysates. The decrease in optical density was recorded at 450 nm as a function of time. The change in absorbance for the first minute only was used to determine the rate of the reaction. One unit of enzyme is defined as the amount of enzyme that produces a decrease in absorbance of 0.001/min at 450 nm.

Assay for Protein Estimation of the Sample

Protein content of the respective supernatant and lysate was measured by the method [60] as described earlier.

Tumor Necrosis Factor-α, Interferon-Gamma, Interleukin-6, and Interleukin-10 ELISA Assays

Murine peritoneal macrophages (5×10^6 cells/ml) were mixed with *S. aureus* (5×10^6 CFU/ml) in a 1:1 cell/bacterium ratio [11–13] in RPMI-FBS (5 %) and incubated at 37 °C cell culture incubator for different times in the presence and absence of antibiotics and cytochalasin D either alone or in combination. After incubation, cell culture supernatants were collected and stored at −70 °C prior to analysis. Supernatants from different groups were normalized to the protein content by Bradford method before the assay, and levels of TNF-α, IFN-γ, IL-6, and IL-10 production were assayed using enzyme-inked immunosorbent assay (ELISA) kits according to the instructions provided by the manufacturer (RayBiotech, Inc. USA) in a BioRad ELISA reader.

Statistical Analysis

Isolated peritoneal macrophages from mice were pooled together to obtain the requisite amount of macrophages (5×10^6 cells/ml), and the different parameters were measured. This was repeated for three times for each parameter (*e.g.*, H_2O_2 production), then the mean value of these triplicate experiments was taken for calculation. Data was expressed as mean±SD. Means were compared between groups by using analysis of variance (ANOVA). $P < 0.05$ was considered significant.

RESULTS

Biochemical Analysis and Biotyping for Characterization of the *S. aureus* (AG-789) Isolate

Catalase Test. Bubbling was observed in case of both slide and tube test upon addition of H_2O_2 which confirms *S. aureus* (AG-789) to be catalase-positive.

Coagulase Test. In the second hour, slight coagulation was seen. In the fourth hour, coagulation was seen in one fourth of the volume of the tube. In 24th hour, a slight increase in coagulation than the fourth hour was observed. These observations hold *S. aureus* (AG-789) to be coagulase-positive. The clot can be described as 2+.

Anaerobic Growth in Glucose-Containing Media. Dense uniform anaerobic growth denoted by ++ was seen in the anaerobic zone of the tubes.

Bovine Plasma Coagulation. Coagulation was observed after incubation for 2 h, and subsequently, the size of clot got increased till the eighth hour after which no increase in the size of the clot was visualized. Diluted plasma coagulated less as compared to plasma without dilution. Thus, it can be said that *S. aureus* (AG-789) can coagulate bovine plasma.

Growth on Crystal Violet Agar

Positive growth of *S. aureus* (AG-789) was seen in all dilutions of crystal violet agar except the plate containing lowest dilution of crystal violet (*i.e.*, 100 μg/ml). Maximum growth was visualized in the plate containing highest dilution of crystal violet (1 μg/ml). Thus, it can be said that *S. aureus* (AG-789) can grow on crystal violet containing agar, but crystal violet inhibits growth at higher concentration.

In Vitro Susceptibility Test

The tested strain AG-789 was found to be resistant to methicillin. This MRSA was found to be sensitive to ciprofloxacin, chloramphenicol, and azithromycin. MBC and zone diameter obtained from DAD test were found to be in compliance with the MIC value for the tested strain. Results of MIC, MBC, and DAD were represented in Table 1.

Evaluation of Synergy by Checkerboard Assay

Table 2 illustrates the interaction between the study drugs in different combination that might be of therapeutic use. The results of this assay showed no synergistic interaction between the antibiotics, ammonium chloride, or cytochalasin D. Antagonism was observed with methicillin interacting with ciprofloxacin or azithromycin. Interaction of these antibiotics with cytochalasin D showed indifference in action. Thus, combination of two antibiotics could not be recommended for use in this experimental setup.

Effect of Cytochalasin D and Antibiotic Treatment Either Alone or in Combination on Phagocytosis of *S. aureus* by Peritoneal Macrophages

Control macrophages were readily able to phagocytose *S. aureus*. After 90 min of incubation, the number of viable bacteria within macrophages declined. However, the number bacteria phagocytosed by cytochalasin D-treated macrophages appeared to be lower than that observed for cytochalasin D-untreated macrophages at the same incubation times. Concentration of DMSO used for reconstitution of cytochalasin D did not show any significant alteration of phagocytosis of *S. aureus* by macrophages (Table 3). Overall, the experimental results indicated that cytochalasin D treatment leads to a reduction in the uptake of *S. aureus* cells by macrophages presumably by interfering with actin-mediated phagocytosis. CIP and AZM do not affect the viability of macrophages (data not shown) but almost immediately after application to infected macrophages inhibit the *S. aureus* growth and replication. There were statistically significant differences in the counts of viable bacteria recovered from cytochalasin plus antibiotic-pretreated macrophages before *S. aureus* infection when compared with cytochalasin D-pretreated plus *S. aureus*-infected macrophages (Table 3). This result clearly showed that cytochalasin D in the presence of either CIP or AZM reduced intra-macrophage killing of *S. aureus*.

Table 1. *In Vitro* Susceptibility Tests

Antibiotic	MIC (μg/ml)		MBC (μg/ml)		Zone diameter (mm)	
	ATCC 25923	AG-789	ATCC 25923	AG-789	ATCC 25923	AG-789
CIP	0.5[b]	0.06[b]	1	0.12	28	24
MET	2[b]	>32[c]	0.5	_[d]	17	_[a]
CHLO	1[b]	0.25[b]	0.12	2	23	21
AZM	1[b]	2[b]	0.12	1	24	22

CIP ciprofloxacin, *MET* methicillin, *CLO* chloramphenicol, *AZM* azithromycin
[a] No zone of inhibition detected
[b] Sensitive
[c] Resistant
[d] Resistant up to 32 μg/ml

Table 2. Evaluation of Synergy by Checkerboard Assay

Combination (A + B)		FIC A	FIC B	∑ FIC	Remarks
Antibiotic A	Antibiotic B				
Methicillin	Ciprofloxacin	4.0	1.0	5	Antagonism
Methicillin	Chloramphenicol	1	0.031	1.031	Indifference
Methicillin	Azithromycin	1.0	4.0	5.0	Antagonism
Ciprofloxacin	Chloramphenicol	0.5	0.0624	0.5624	Indifference
Ciprofloxacin	Azithromycin	4	4	8	Antagonism
NH$_4$Cl	Azithromycin	1	4	5	Antagonism
NH$_4$Cl	Ciprofloxacin	_[a]	_[a]	_[a]	_[a]
Cytochalasin D	Ciprofloxacin	_[a]	_[a]	_[a]	_[a]
Cytochalasin D	Azithromycin	1	1	2	Indifference

∑ FIC (FIC A + FIC B) value <0.5, synergy; 0.5–4, indifference; >4, antagonism
FIC fractional inhibitory concentration
[a] Resistant to the combination

Effect of Cytochalasin D and Antibiotic Treatment on *S. aureus* Infection-Induced Hydrogen Peroxide Production in the Supernatant and Lysate by Murine Peritoneal Macrophages

Amount of H$_2$O$_2$ released in the supernatant or lysate was significantly higher ($P<0.05$) in *S. aureus*-infected macrophage than that of control macrophages only at 60 and 90 min after *S. aureus* infection. Statistically significant increment in the amount of H$_2$O$_2$ production only in the supernatant but not in lysate was observed ($P<0.05$) in cytochalasin D-pretreated macrophages before infection at 60 and 90 min post-incubation, when compared with the only *S. aureus*-infected macrophages (Table 4).

Macrophages pretreated either with CIP or AZM alone before *S. aureus* infection also showed reduced production of H$_2$O$_2$ in the supernatant or lysate in comparison to the amount released by the only *S. aureus*-infected macrophages. However, administration of either CIP or AZM to the cytochalasin D-pretreated macrophages before *S. aureus* infection showed reduced H$_2$O$_2$ release in the supernatant when compared with cytochalasin D plus *S. aureus*-infected macrophages (Table 4).

Effect of Cytochalasin D and Antibiotic Treatment on *S. aureus* Infection-Induced Superoxide Anion Release in the Supernatant and Lysate by Murine Peritoneal Macrophages

The amount of superoxide anion (O$_2^{\cdot-}$) (nanomoles/10^6 cells) released in the supernatant and lysates was also estimated. The increase in the O$_2^{\cdot-}$ released in supernatant and lysate after different times of phagocytosis in the presence of *S. aureus* infection was found to be significant ($P<0.05$) when compared to the O$_2^{\cdot-}$ release of noninfected control macrophages (Table 5). Statistically significant increase in the amount of O$_2^{\cdot-}$ released both in the supernatant and lysate was observed ($P<0.05$) in cytochalasin D-pretreated macrophages before infection, when compared only with the *S. aureus*-infected macrophages at 30 and 60 min post-incubation. Treatment of macrophages with CIP alone before *S. aureus* infection showed increased amount of O$_2^{\cdot-}$ released in the supernatant but decreased in the lysate at 60 and 90 min post-incubation when compared with *S. aureus* alone-infected macrophages. Treatment of macrophages with AZM alone before *S. aureus* infection also showed increased amount of superoxide anion released in the supernatant in comparison to the only *S. aureus*-infected macrophages. However, the administration of either CIP or AZM to the cytochalasin D-pretreated macrophages before *S. aureus* infection showed significantly reduced amount of O$_2^{\cdot-}$ release in the supernatant as well as in the lysate when compared with cytochalasin D plus *S. aureus*-infected macrophages after phagocytosis (Table 5).

Effect of Cytochalasin D and Antibiotic Treatment on *S. aureus* Infection-Induced Alteration in the Catalase Enzyme Activity in the Supernatant and Lysate by Murine Peritoneal Macrophages

Figure 1 shows a marked increase in the catalase enzyme activity in the supernatant or lysate of peritoneal macrophages infected with *S. aureus* after 30, 60, and 90 min of phagocytosis compared with the noninfected control group. A significant decrease in the catalase

Table 3. Effect of Cytochalasin D and Antibiotic Treatment Either Alone or in Combination on Phagocytosis of *S. aureus* by Peritoneal Macrophages

Groups	Number of CFU/ml lysate at 30 min post-infection (mean±SD)	Number of CFU/ml lysate at 60 min post-infection (mean±SD)	Number of CFU/ml lysate at 90 min post-infection (mean±SD)
Control macrophage (no DMSO in medium and without infection)	ND	ND	ND
S. aureus-infected macrophage (no DMSO in medium)	91±6.67[a]	75±5.33[a]	52.33±4.93[a]
Cytochalasin D (in DMSO) -pretreated macrophages	ND	ND	ND
Macrophages + DMSO in medium +*S. aureus*	87.66±9.3	73±3.6	52.33±3.51
Cytochalasin D-pretreated + *S. aureus*-infected macrophage	55±6.29[b]	47.33±7.52[b]	40±5.89[b]
S. aureus-infected macrophages + azithromycin-treated	42±4.87	38.07±3.57	35.42±5.37
S. aureus-infected macrophages + ciprofloxacin-treated	39±8.56	32±6.27	27.35±5.65
Cytochalasin D-pretreated +*S. aureus*-infected macrophages + azithromycin-treated	23. 28±4.07	18.33±4.08[c]	13. 39±4.87[c]
Cytochalasin D-pretreated + *S. aureus*-infected macrophages + ciprofloxacin-treated	20±6.47[d]	16.33±3.21[d]	14.08±3.64[d]

[a] Significant difference with respect to control macrophages at *P*<0.05
[b] Significant difference with respect to *S. aureus*-infected macrophage at *P*<0.05
[c] Significant difference with respect to azithromycin-treated *S. aureus*-infected macrophages at *P*<0.05
[d] Significant difference with respect to ciprofloxacin-treated *S. aureus*-infected macrophages at *P*<0.05

enzyme activity was observed in the supernatants of cytochalasin D-treated murine peritoneal macrophages before infection compared with the cytochalasin D-nontreated infected group. Treatment of macrophages with either

Table 4. Effect of Cytochalasin D and Antibiotic Treatment on *S. aureus* Infection-Induced Hydrogen Peroxide Production in the Supernatant and Lysate by Murine Peritoneal Macrophages

Groups	H_2O_2 production in supernatant (mM/10^6 cells)			H_2O_2 production in lysate (mM/10^6 cells)		
	30 min	60 min	90 min	30 min	60 min	90 min
CM	0.032±0.01	0.053±0.01	0.077±0.01	0.85±0.05	0.17±0.03	0.24±0.03
SAM	3.56±0.15[a]	4.08±0.16[a]	4.91±0.10[a]	1.90±0.08[a]	2.76±0.04[a]	3.03±0.09[a]
Cyto D + SAM	3.23±0.19[b]	4.56±0.18[b]	6.72±0.43[b]	1.15±0.09[b]	1.95±0.04[b]	2.81±0.06[b]
SAM + CIP	3.05±0.13[b]	2.71±0.21[b]	3.27±0.11[b]	1.54±0.20[b]	2.17±0.12[b]	3.46±0.14[b]
SAM + AZM	4.56±0.34[b]	2.96±0.18[b]	2.28±0.09[b]	1.16±0.12[b]	2.51±0.08[b]	2.41±0.05[b]
Cyto D + SAM + CIP	2.83±0.08[cd]	3.85±0.13[cd]	3.20±0.39[cd]	1.02±0.12[c]	1.61±0.15[cd]	1.78±0.13[cd]
Cyto D + SAM + AZM	2.39±0.08[c]	3.90±0.18[c]	1.03±0.29[cd]	1.81±0.06[c]	1.55±0.13[c]	1.03±0.07[cd]

CM control macrophage, *SAM S. aureus*-infected macrophages, *Cyto D + SAM* cytochalasin D-pretreated + *S. aureus*-infected macrophages, *SAM + CIP S. aureus*-infected macrophages treated with ciprofloxacin, *SAM + AZM S. aureus*-infected macrophages treated with azithromycin, *Cyto D + SAM + CIP* cytochalasin D-pretreated macrophages infected with *S. aureus* and then treated with ciprofloxacin, *Cyto D + SAM + AZM* cytochalasin D-pretreated macrophages infected with *S. aureus* and then treated with azithromycin
[a] Significant difference with respect to CM
[b] Significant difference with respect to SAM
[c] Significant difference with respect to either ciprofloxacin (CIP) or azithromycin (AZM) plus *S. aureus*-infected macrophages
[d] Significant difference with respect to cytochalasin D (Cyto D) plus *S. aureus*-infected macrophages at *P*<0.05 level of significance

Table 5. Effect of Cytochalasin D and Antibiotic Treatment on *S. aureus* Infection-Induced Superoxide Anion Release in the Supernatant and Lysate by Murine Peritoneal Macrophages

Groups	Superoxide anion release in the supernatant (nM)			Superoxide anion release in the lysate (nM)		
	30 min	60 min	90 min	30 min	60 min	90 min
CM	0.20±0.11	0.19±0.12	0.21±0.11	0.15±0.01	0.16±0.04	0.18±0.03
SAM	2.39±0.10[a]	1.94±0.11[a]	1.87±0.12[a]	1.08±0.09[a]	1.38±0.14[a]	1.84±0.07[a]
Cyto D + SAM	3.26±0.13[b]	2.46±0.11[b]	1.83±0.05	1.89±0.08[b]	1.38±0.08	0.83±0.03[b]
SAM + CIP	2.53±0.07[b]	3.23±0.06[b]	4.26±0.05[b]	1.60±0.06[b]	0.99±0.01[b]	1.55±0.02[b]
SAM + AZM	2.73±0.14[b]	2.15±0.11	1.35±0.10[b]	1.47±0.04[b]	1.32±0.07[b]	0.71±0.07[b]
Cyto D + SAM + CIP	3.52±0.11[d]	2.31±0.07[d]	1.56±0.17[d]	0.76±0.08[c]	0.24±0.09[cd]	0.34±0.02[cd]
Cyto D + SAM + AZM	2.48±0.16[cd]	1.58±0.12[cd]	0.38±0.07[cd]	1.65±0.09[c]	1.04±0.04[cd]	0.45±0.09[cd]

CM control macrophage, *SAM S. aureus*-infected macrophages, *Cyto D + SAM* cytochalasin D-pretreated + *S. aureus*-infected macrophages, *SAM + CIP S. aureus*-infected macrophages treated with ciprofloxacin, *SAM + AZM S. aureus*-infected macrophages treated with azithromycin, *Cyto D + SAM + CIP* cytochalasin D-pretreated macrophages infected with *S. aureus* and then treated with ciprofloxacin, *Cyto D + SAM + AZM* cytochalasin D-pretreated macrophages infected with *S. aureus* and then treated with azithromycin
[a] Significant difference with respect to CM
[b] Significant difference with respect to SAM
[c] Significant difference with respect to either ciprofloxacin (CIP) or azithromycin (AZM) plus *S. aureus*-infected macrophages
[d] Significant difference with respect to cytochalasin D (Cyto D) plus *S. aureus*-infected macrophages at $P<0.05$ level of significance

CIP or AZM alone before *S. aureus* infection showed decreased amount of catalase enzyme activity in the supernatant when compared with *S. aureus* alone-infected macrophages. This decrease in the catalase enzyme activity was even more prominent when cytochalasin D-pretreated macrophages were incubated with either CIP or AZM
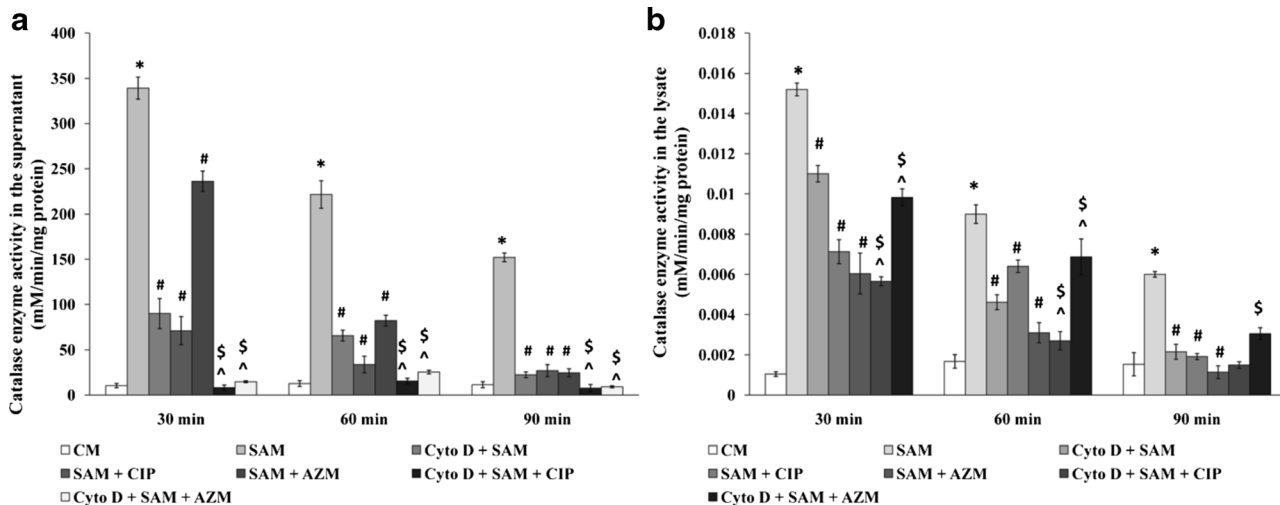

Fig. 1. Effect of cytochalasin D and antibiotic treatment on *S. aureus* infection-induced alteration in the catalase enzyme activity in the supernatant and lysate by murine peritoneal macrophages. The supernatant and lysate recovered after time-dependent phagocytosis in the presence or absence of cytochalasin D or antibiotic treatment as described in the "MATERIALS AND METHODS" section were used to determine catalase activity in the presence of 15 μmol of H_2O_2/ml of phosphate buffer. Catalase activity was expressed in terms of millimoles per minute milligram protein. Results were shown as mean±SD of three independent experiments. *CM*, control macrophage; *SAM*, *S. aureus*-infected macrophages; *Cyto D + SAM*, cytochalasin D-pretreated + *S. aureus*-infected macrophages; *SAM + CIP*, *S. aureus*-infected macrophages treated with ciprofloxacin; *SAM + AZM*, *S. aureus*-infected macrophages treated with azithromycin; *Cyto D + SAM + CIP*, cytochalasin D-pretreated macrophages infected with *S. aureus* and then treated with ciprofloxacin; *Cyto D + SAM + AZM*, cytochalasin D-pretreated macrophages infected with *S. aureus* and then treated with azithromycin. *Asterisk:* Significant difference with respect to CM. *Number sign:* Significant difference with respect to SAM. *Dollar sign*: Significant difference with respect to SAM plus CIP *versus* SAM plus AZM-treated macrophages. *Circumflex accent:* Significant difference with respect to either ciprofloxacin (CIP) or azithromycin (AZM) plus *S. aureus*-infected macrophages *versus* cytochalasin D plus SAM, at $P<0.05$ level of significance.

before *S. aureus* infection in comparison to cytochalasin D plus *S. aureus*-infected macrophages (Fig. 1).

Effect of Cytochalasin D and Antibiotic Treatment on *S. aureus* Infection-Induced Alteration in the SOD Enzyme Activity in the Supernatant and Lysate by Murine Peritoneal Macrophages

Figure 2 shows a significant increase in the superoxide dismutase enzyme activity in the supernatant or lysate of peritoneal macrophages infected with *S. aureus* after 30, 60, and 90 min of phagocytosis compared with the noninfected control group ($P<0.05$). A significant decrease in the SOD enzyme activity was observed in the supernatants and lysates of cytochalasin D-treated murine peritoneal macrophages before infection compared with the cytochalasin D-nontreated infected group. Treatment of macrophages with either CIP or AZM alone before *S. aureus* infection showed decreased amount of SOD enzyme activity in the supernatant and lysate when compared with *S. aureus* alone-infected macrophages. This decrease in the SOD enzyme activity was even more prominent in the supernatant at 60 and 90 min when cytochalasin D-pretreated macrophages were incubated with either CIP or AZM before *S. aureus* infection in comparison to cytochalasin D plus *S. aureus*-infected macrophages (Fig. 2).

Effect of Cytochalasin D and Antibiotic Treatment on *S. aureus* Infection-Induced Alteration in the GSH Content in the Supernatant and Lysate by Murine Peritoneal Macrophages

Content of GSH in the supernatant or lysate was significantly higher ($P<0.05$) in *S. aureus*-infected macrophage than that of control macrophages at 30, 60, and 90 min after *S. aureus* infection. Statistically significant increase in the amount of GSH only in the supernatant but not in lysate was observed ($P<0.05$) in cytochalasin D-pretreated macrophages before infection at 30, 60, and 90 min post-incubation, when compared with the only *S. aureus*-infected macrophages (Fig. 3).

Macrophages pretreated with CIP alone before *S. aureus* infection also showed increased level of GSH in the supernatant at 30 and 60 min post-incubation but decreased at 90 min in comparison to the amount released by the only *S. aureus*-infected macrophages. Macrophages pretreated with AZM alone before *S. aureus* infection showed decreased amount of GSH content in the supernatant at 60 and 90 min of post-incubation when compared to the amount of superoxide anion released by the only *S. aureus*-infected macrophages. However, administration of CIP to the cytochalasin D-pretreated macrophages before *S. aureus* infection showed increased GSH content in the supernatant but decreased in the lysate when compared
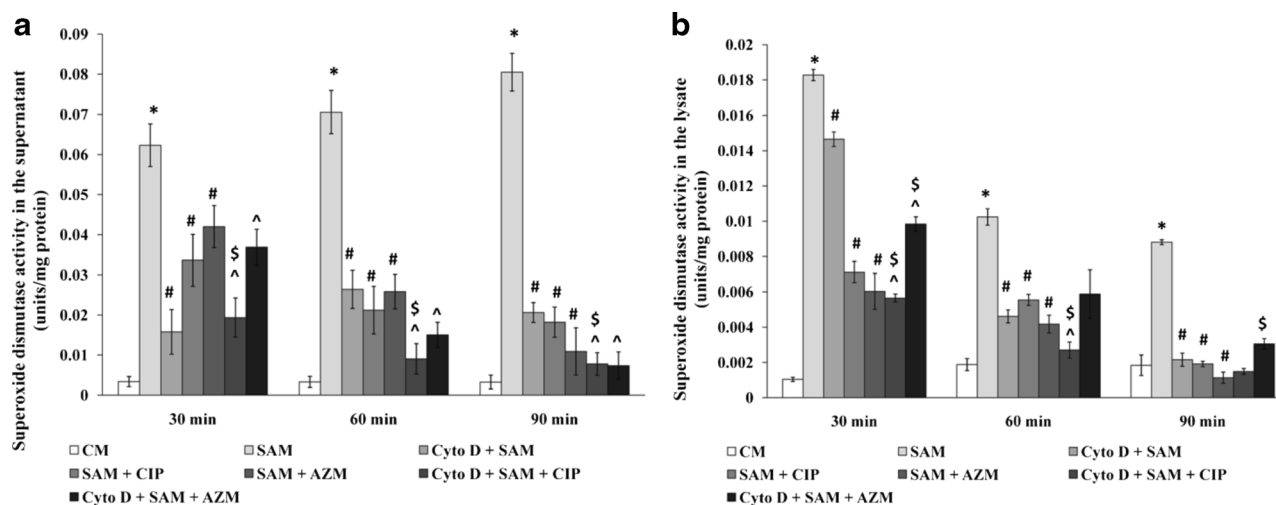

Fig. 2. Effect of cytochalasin D and antibiotic treatment on *S. aureus* infection-induced alteration in the superoxide dismutase (*SOD*) enzyme activity in the supernatant and lysate by murine peritoneal macrophages. The supernatant and lysate which recovered after time-dependent phagocytosis in the presence or absence of cytochalasin D or antibiotics were used to determine SOD activity and were expressed in terms of the unit of SOD per milligram protein. Results were presented as mean±SD of three independent experiments. *Asterisk*: Significant difference with respect to CM. *Number sign:* Significant difference with respect to SAM. *Dollar sign*: Significant difference with respect to SAM plus CIP *versus* SAM plus AZM-treated macrophages. *Circumflex accent:* Significant difference with respect to either ciprofloxacin (*CIP*) or azithromycin (*AZM*) plus *S. aureus*-infected macrophages *versus* cytochalasin D plus SAM, at $P<0.05$ level of significance.
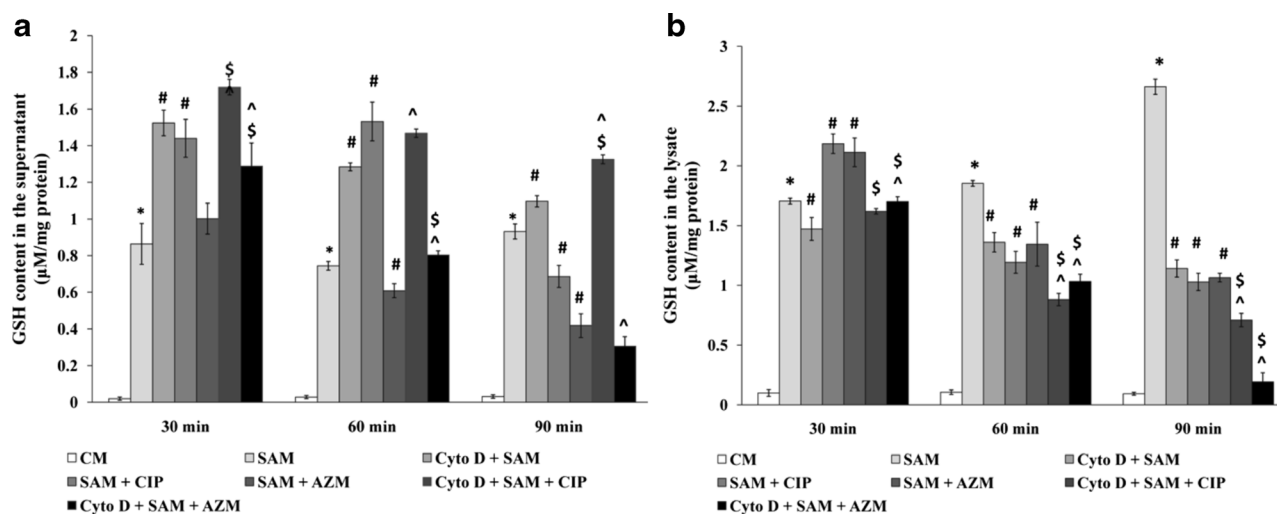
Fig. 3. Effect of cytochalasin D and antibiotic treatment on *S. aureus* infection-induced alteration in the GSH content in the supernatant and lysate by murine peritoneal macrophages. The supernatant and lysate which recovered after time-dependent phagocytosis in the presence or absence of cytochalasin D or antibiotics were used to determine the GSH content and were expressed in terms of micromolar per milligram protein. Results were presented as mean±SD of three independent experiments. *Asterisk:* Significant difference with respect to CM. *Number sign:* Significant difference with respect to SAM. *Dollar sign:* Significant difference with respect to SAM plus CIP *versus* SAM plus AZM-treated macrophages. *Circumflex accent:* Significant difference with respect to either ciprofloxacin (*CIP*) or azithromycin (*AZM*) plus *S. aureus*-infected macrophages *versus* cytochalasin D plus SAM, at *P*<0.05 level of significance.

with cytochalasin D plus *S. aureus*-infected macrophages at 60 and 90 min post-incubation (Fig. 3). Administration of AZM to the cytochalasin D-pretreated macrophages before *S. aureus* infection showed reduced GSH level both in the supernatant and in the lysate in comparison to the GSH level of cytochalasin D plus *S. aureus*-infected macrophages at 60 and 90 min post-incubation (Fig. 3).

Effect of Cytochalasin D and Antibiotic Treatment on *S. aureus* Infection-Induced Alteration in the Lysozyme Enzyme Activity in the Supernatant and Lysate by Murine Peritoneal Macrophages

Figure 4 shows a significant increase in the lysozyme enzyme activity in the supernatant or lysate of peritoneal macrophages infected with *S. aureus* after 30, 60, and 90 min of phagocytosis compared with the noninfected control group (*P*<0.05). A significant decrease in the lysozyme enzyme activity was observed in the supernatants and lysates of cytochalasin D-treated murine peritoneal macrophages before infection when compared with the cytochalasin D-nontreated infected group. Treatment of macrophages with either CIP or AZM alone before *S. aureus* infection also showed decreased amount of lysozyme enzyme activity in the supernatant and lysate when compared with *S. aureus* alone-infected macrophages. However, the lysozyme enzyme activity was restored significantly in the lysate at 30 and 90 min when cytochalasin

D-pretreated macrophages were incubated with either CIP or AZM before *S. aureus* infection in comparison to cytochalasin D plus *S. aureus*-infected macrophages (Fig. 4).

Effect of Cytochalasin D and Antibiotic Treatment on *S. aureus* Infection-Induced TNF-α, IFN-γ, IL-6, and IL-10 Production by Murine Peritoneal Macrophages

Figure 5 showed a gradual decrease in the IL-6 (Fig. 5c), IL-10 (Fig. 5d), and IFN-γ (Fig. 5b) level in the media (culture supernatant) with an increase in phagocytic time in the presence of cytochalasin D significantly (*P*<0.05) when compared to the cytokine production after time-dependent phagocytosis with cytochalasin D-nontreated infected macrophage. Macrophages pretreated either with CIP or AZM alone before *S. aureus* infection also showed reduced level of IL-6, IL-10, and IFN-γ in the supernatant in comparison to the amount released by the only *S. aureus*-infected macrophages. However, the level of IL-6, IL-10, and IFN-γ was decreased significantly in the medium at 60 and 90 min when cytochalasin D-pretreated macrophages were incubated with either CIP or AZM before *S. aureus* infection in comparison to cytochalasin D plus *S. aureus*-infected macrophages. Level of TNF-α was decreased significantly in the medium at 60 and 90 min when cytochalasin D-pretreated macrophages were incubated with either CIP or AZM before *S. aureus*

infection in comparison to cytochalasin D plus *S. aureus*-infected macrophages (Fig. 5a).

DISCUSSION

In view of the fact that staphylococcal infections have a tendency to become chronic, the fate of *S. aureus* within macrophages is an important consideration. Intracellular persistence of *S. aureus* within the macrophages, especially the antibiotic resistant types, has posed serious problems and difficulties in treatment of several diseases. Moreover, antibiotics with known intracellular bactericidal activity when used have been shown to be uptaken within the host cell, but recent evidences have suggested that they no longer confer protection *in vitro*. The possible reasons for that discrepancy are numerous: (i) the degree of plasma membrane permeability may determine concentration gradients of the antibiotic inside and outside of the cell, (ii) the subcellular localization of antibiotics and bacteria may segregate in diverse intracellular compartments, (iii) antibiotics may be inactivated by the intracellular milieu, and (iv) differences in the physiological state of the internalized pathogen (resting *versus* dividing) can modulate the susceptibility to antibiotics [61]. Another complicating factor is the extracellular multiplication of the surface-associated

bacteria or those released from lysed infected cells, since these organisms would be subject to repeated endocytic events.

Efforts to obviate this difficulty by adding appropriate antibiotics and by restricting the entry of viable staphylococci associated with the cells surface *in vitro* require a novel combination of drugs involving an efficacious antibiotic with intracellular bactericidal activity and an inhibitor of phagocytosis, cell adherence, and phagolysosome fusion so that the situation can be controlled by killing the pathogens in the extracellular environment and also ameliorating their intracellular multiplication within phagocytic cells. Thus, the aim of our study was to formulate an efficacious combination using *in vitro* susceptibility studies and to observe the effect of such combination on uptake of staphylococcal cells by isolated murine peritoneal macrophages and their subsequent lysis to reduce the pathogen load within the phagocytic cells of the host. We chose mouse peritoneal macrophages as we could investigate phagocytosis within 2 h of isolation, compared with 1 week, which is required for differentiation of human monocyte-derived macrophages [62, 63]. Moreover, peritoneal macrophages used in the study were elicited with thioglycolate broth but did not potentially affect intracellular killing as evident from our earlier study [11–13]. Therefore, inclusion of data set by using untreated or
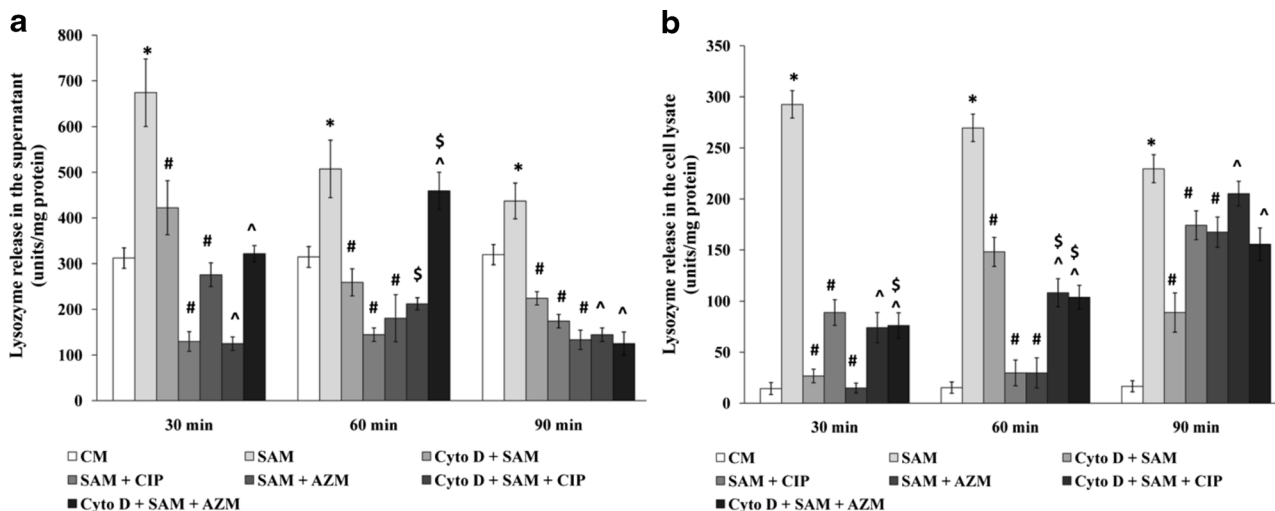

Fig. 4. Effect of cytochalasin D and antibiotic treatment on *S. aureus* infection-induced alteration in the lysozyme enzyme activity in the supernatant and lysate by murine peritoneal macrophages. The supernatants and cell-free lysates obtained in the phagocytosis assay were allowed to interact with a suspension of *Micrococcus lysodeikticus* in 0.15 M potassium phosphate buffer, pH 6.2, and optical density fixed at 0.6–0.7 at 450 nm. One unit of lysozyme was defined as the amount of enzyme that produces a decrease in absorbance of 0.001/min at 450 nm, and the lysozyme enzyme activity was expressed in terms of units per milligram protein. Results were presented as mean±SD of three independent experiments. *Asterisk*: Significant difference with respect to CM. *Number sign:* Significant difference with respect to SAM. *Dollar sign:* Significant difference with respect to SAM plus CIP *versus* SAM plus AZM-treated macrophages. *Circumflex accent:* Significant difference with respect to either ciprofloxacin (*CIP*) or azithromycin (*AZM*) plus *S. aureus*-infected macrophages *versus* cytochalasin D plus SAM, at *P*<0.05 level of significance.
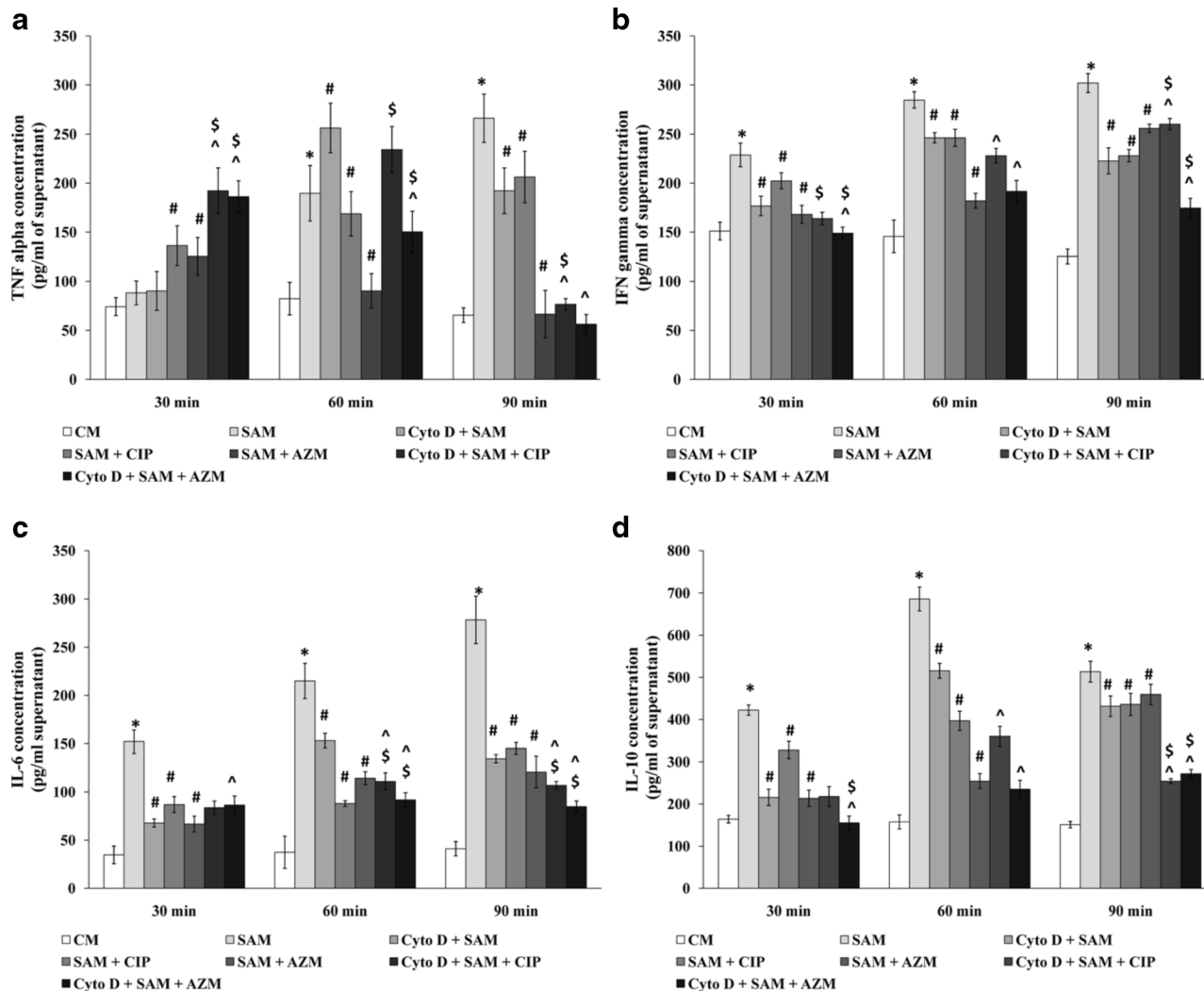
Fig. 5. a–d Cytokine release in the supernatant. Levels of tumor necrosis factor-alpha (*TNF-α*) (**a**), interferon-gamma (*IFN-γ*) (**b**), interleukin-6 (*IL-6*) (**c**), and interleukin-10 (*IL-10*) (**d**) in the supernatants collected after 30, 60, and 90 min of *Staphylococcus aureus*-infected macrophages in the presence or absence of cytochalasin D or antibiotics were determined by utilizing ELISA according to the manufacturer's recommendations and were expressed from triplicate experiments. *Asterisk*: Significant difference with respect to CM. *Number sign*: Significant difference with respect to SAM. *Dollar sign:* Significant difference with respect to SAM plus CIP *versus* SAM plus AZM-treated macrophages. *Circumflex accent:* Significant difference with respect to either ciprofloxacin (*CIP*) or azithromycin (*AZM*) plus *S. aureus*-infected macrophages *versus* cytochalasin D plus SAM, at *P*<0.05 level of significance.

nonelicited macrophages, such as alveolar macrophages or splenic macrophages, is not essential in our study. When testing the cytochalasin D as an augmenter of the antibiotics, several assays like intracellular killing, production of reactive oxygen species, antioxidant enzymes, lysozyme release, and finally measurement of cytokines released by host cells in response to all these treatments before *S. aureus* infection and the rationale for performing these above assays have already been mentioned in the "MATERIALS AND METHODS" section. Therefore, the above assays are very much linked and do not stand individually.

In this study, we tested the *in vitro* susceptibility of the clinical isolate *S. aureus* (AG-789). This *S. aureus* strain was found to be methicillin-resistant and was thus termed MRSA. Results of MIC, MBC, and DAD revealed that this strain was sensitive to AZM and CIP both of which have potent intracellular bactericidal activities. We used checkerboard technique to investigate synergism of the combinations studied against the isolated MRSA (AG-789). Since various concentrations of antibiotic combinations can be studied by twofold broth dilutions, the synergistic effect can be detected by determining the summation

fractional inhibitory concentration (Σ FIC) from the respective individual FIC value of each antibiotic in combination. Synergy is defined as a decrease in the viable organism as a result of the combination when compared with the most effective antibiotic when tested alone. Our results show that none of the tested combinations showed synergy. Azithromycin showed indifference, whereas ciprofloxacin was resistant to the pathogen in combination with cytochalasin D, respectively.

Another aim of this study was to determine the intracellular anti-staphylococcal activities of antibiotics in the presence of cytochalasin D, an established inhibitor of phagocytosis and phagolysosome fusion. The role of microfilament and cytoskeleton rearrangement in phagocytosis by mammalian cells and other has been documented in a number of published reports [64, 65]. The cellular processes are normally dependent on actin polymerization and are sensitive to actin polymerization inhibitor cytochalasin D. Cytochalasin D is a specific actin polymerization inhibitor [66] that has been extensively used to study actin polymerization-dependent processes in mammalian cells [67]. It is well established that *S. aureus* invade phagocytic cells like macrophages, and here, we have also observed that in the absence of cytochalasin D phagocytosis process was normal but in presence of cytochalasin D phagocytosis was impaired in the macrophages. It was reported that viability of macrophages over 48 h was not affected by treatment with cytochalasin D [68]; therefore, alteration of macrophage viability after 90 min of incubation with cytochalasin D in our study could be avoided. DMSO (0.1 %) which was used to dissolve cytochalasin D did not affect the growth rate of bacteria. Macrophages that had received no treatment or had been pretreated with DMSO were included as control cultures. These control co-cultures were used to rule out any possible impact of this solvent on phagocytosis of macrophages. Since counts of bacteria recovered from these control co-cultures were similar to those obtained for co-cultures that used untreated macrophages, the concentration of DMSO used was assumed to not have an adverse effect on phagocytosis of *S. aureus* by macrophages (Table 3). In fact, phagocytosis of macrophages containing DMSO in medium did not show any significant alteration when compared with the phagocytosis of *S. aureus* by macrophages containing no DMSO in the cell culture medium (Table 3). Moreover, addition of either azithromycin or ciprofloxacin to the medium of culture was able to completely eradicate *S. aureus.* The distinct subcellular localization of *S. aureus* might impair, at least in part, the accessibility for antibiotics. A high intracellular concentration of a given antibiotic does not guarantee effective intracellular antimicrobial activity. What counts is the concentration of free active antibiotic where the bacteria are. Thus, the concentrations of azithromycin and ciprofloxacin acting on *S. aureus* in the cytoplasm may be low in relation to the total amount of drug present in the cell. The rapid lysis of staphylococci, streptococci, and other pyogenic bacteria exposed to cell wall active antibiotics such as beta-lactams and vancomycin results in exaggerated release of bacterial products and an augmented and potentially harmful host inflammatory response. Therefore, optimal treatment of sepsis and other severe bacterial infections and or other mediators that blunt the host inflammatory response and dampen the cytokine cascade could be a choice [32, 69]. In our study, CIP and AZM do not affect the growth of the macrophage but almost immediately after application to infected macrophages inhibit *S. aureus* growth and replication. This was supported by a recent report which stated that the macrolide antibiotic AZM at low doses significantly improved the phagocytosis of apoptotic bronchial epithelial cells by bronchoalveolar lavage (BAL)-derived alveolar macrophages from normal and chronic obstructive pulmonary disease (COPD) subjects [70]. The current findings of improvement of peritoneal macrophage's phagocytic ability for *S. aureus* in the presence of AZM may thus provide a novel approach to supplement existing therapies in septic shock. Importantly, there have been many reports of the anti-inflammatory properties of AZM [71, 72]. As a result, low-dose AZM has already been used for clinical trials in various disease states including sepsis. The current findings suggest that the effects of AZM may be mediated by improved phagocytic ability of macrophages for *S. aureus* in addition to other anti-inflammatory properties of the antibiotic.

Cells are provided with very strong biological antioxidant enzymatic and nonenzymatic molecules with enormous capabilities to mitigate the deleterious and potentially harmful effects of reactive oxygen species (ROS) and other free radicals. One of the primary antioxidant defense mechanisms is the GSH redox system [73]. Several studies have suggested that catalase serves as a virulence factor due to its ability to cleave hydrogen peroxide, a reactive oxygen intermediate (ROI) responsible for bactericidal activities of phagocytes. Catalases of pathogenic bacteria are important for optimal detoxification of H_2O_2 and survival in macrophages [11–13].

It has been reported that cytochalasin activates superoxide anion release and exocytosis of beta-*N*-acetylglucosaminidase and lysozyme from polymorphonuclear leukocytes (neutrophils). Moreover, they can also

activate both nicotinamide adenine dinucleotide phosphatase (NADPH) oxidase and selective degranulation of neutrophils during interaction with the cell [74]. Thus, cytochalasin D also aids in the bactericidal function of the leukocytes [75]. Therefore, enhanced bactericidal action of cytochalasin D-treated macrophages is by releasing hydrogen peroxide, superoxide is not surprising as evident from our study.

Ciprofloxacin, apart from being a bactericidal fluoroquinolone, also increases the production of reactive oxygen species in the bacteria. The involvement of superoxide anion ($O_2^{\cdot-}$) and H_2O_2 in the antibacterial action of ciprofloxacin has been well established using superoxide dismutase, catalase, and alkyl hydroperoxide reductase knockout strains of *Escherichia coli*. Our findings are consistent with those reported earlier that glutathione gives protection against fluoroquinolones and not against nonfluoroquinolone antibiotics [76].

Macrolides like AZM are able to inhibit the production of ROS from neutrophils, and it has been reported that this effect is incubation time-dependent [77]. It has been suggested that this is due in part to cell membrane stabilization. Macrolides attenuate the membrane-destabilizing effect of bioactive phospholipids, such as lysophosphatidylcholine, platelet-activating factor (PAF), and lyso-PAF, and this is accompanied by a dose-related inhibition of superoxide production. Interference with phospholipase/phosphatidic acid phosphohydrolase may also decrease superoxide generation by phagocytes [78].

The kinetics study of $O_2^{\cdot-}$ production in the supernatant of macrophage cultures has revealed higher concentration of this anion in infected cytochalasin D-pretreated and CIP-treated infected macrophages but not in the AZM-treated infected macrophages after 60 min of incubation. The phagocytosis of microorganisms activates oxidase dependence on adenine dinucleotide phosphatase (NADPH), which induces the production of high levels of superoxide, a process commonly denominated as respiratory burst. Herein, the generation of superoxide anion in supernatant of peritoneal macrophages increased in the 60 to 90 min of the incubation period compared to the control setup as well as the *S. aureus*-infected macrophages [79].

Antioxidant enzymes are considered to be a primary defense that prevents biological macromolecules from oxidative damage. SOD rapidly dismutates superoxide anion to less dangerous H_2O_2, which is further degraded by catalase to water and oxygen. The results of the present study showed a significant fall in SOD and catalase enzyme activity which may be due to dispose of the free radicals, produced due to *S. aureus* infection. Incubation of cytochalasin D-treated macrophages with AZM before *S. aureus* infection shows increased SOD activity both in the supernatant and lysate at 30 min suggesting that azithromycin was better than ciprofloxacin in mediating its bactericidal action in combination with cytochalasin D.

Glutathione system is an important antioxidant system in cell and is involved in protection against free radicals, peroxides, and toxic compounds, and their reactive intermediates formed intracellularly either spontaneously or enzymatically in cellular systems. The depletion of antioxidant enzyme activity was may be due to the inactivation of the enzyme proteins by MRSA infection-induced depletion of the enzyme substrates and/or downregulation of transcription and translational processes [80]. Increased lysozyme released in the supernatant following incubation with azithromycin to cytochalasin D-pretreated macrophages before *S. aureus* infection may be helpful for increased killing activity of the macrophages with time.

When an infection occurs, the innate immune system is the first line to address the whole bacteria. As infection also implies a contact with whole bacteria and not with purified microbial products, we analyzed the response of murine peritoneal macrophages to viable bacteria. It has been reported that a high ratio of IL-10 to TNF-α was associated with fatal outcome in patients with infection [81], and thus, we focused our study on the production of pro-inflammatory and an anti-inflammatory cytokines. Increased TNF-α from the cytochalasin D plus CIP or cytochalasin D plus AZM-treated macrophages at early infection (at 30 min) indicated bacterial killing and better efficacy of both these antibiotics in the presence of cytochalasin D during early infection. However, suppression of TNF-α level at late stage of infection by both antibiotics in the presence of cytochalasin D indicated that the combination was able to downregulate inflammation with increasing time which was beneficial to the host cell. Suppression of IFN-γ as well as IL-6 by the cytochalasin D- and AZM-treated macrophages at late stage of infection indicated downregulation of inflammation by this combination. Macrolide antibiotics reportedly repress the production of pro-inflammatory cytokines in macrophages [71]. The present study examined the hypothesis that cytochalasin D in combination with AZM could suppress macrophage TNF-α production in response to whole bacteria, in this case clinical isolates of MRSA. Given the important role of TNF-α in sepsis, such suppression by combination therapy could have a therapeutic effect [82]. Invading pathogens are recognized *via* pathogen recognition receptors such as toll-like receptor (TLR)-2 and TLR-4, scavenger receptors, and mannose receptors and are

phagocytosed. It has been reported that *S. aureus* is able to "attack" or form pores in macrophages, and TNF-α secretion occurs even in the absence of TLR-2 and TLR-4 sensors. Therefore, another mechanism independent of TLR must exist for cytochalasin D- and AZM-mediated anti-inflammatory actions, at least in *S. aureus* infections. Consistent with other studies, the current authors noted a significant decrease in TNF-α in the presence of AZM [83, 84].

IL-10, an anti-inflammatory cytokine level, remained decreased at all times following combination treatment compared to only *S. aureus*-infected and antibiotic-treated macrophages. Thus taken together, decreased pro-inflammatory cytokine levels reveal that combination therapy has eradicated not only the bacterial burden within the macrophages but have also led to the inhibition of signalling pathways that lead to the production of pro-inflammatory cytokines, though we have not observed an increase in anti-inflammatory cytokine level (IL-10) following the treatment.

As an alternative to traditional antibiotics in an era of increasing bacterial resistance, new attention has focused upon development of agents that target bacterial virulence factors, effectively disarming the pathogen burden and allowing clearance by the host [85, 86]. Our observation suggests that cytochalasin D in combination with AZM deserves consideration as a therapeutic target because inhibition of phagocytosis not only reduce bacterial uptake but may in specific cases have the dual benefit of increasing susceptibility to commonly used antibiotics including CIP or AZM. It can be concluded from this *in vitro* study that the action of AZM in mediating bacterial killing is enhanced in the presence of cytochalasin D indicating internalization of *S. aureus* by macrophages is not essential for killing of bacteria by AZM. Reduction of the amount of pro-inflammatory/toxic pathogen-derived products, avoids overstimulation of resident migrating immune cells including macrophages, and thereby protects the peripheral tissue. It appears desirable to keep the concentration of pro-inflammatory products of pathogens in the tissue low during the whole course of an infection including its treatment. In clinical practice, a favorable outcome depends on the antibiotic treatment after hospital admission. Therefore, the reduction of potentially deleterious pathogen-derived compounds by rapid initiation of an effective antibiotic therapy or by choosing compounds which do not release large amounts of pathogen products is a promising strategy to overstimulation of phagocytic cells and decrease tissue injury. In arthritis, while a diversity of organisms are present in deep infection, the dominant bacteria are Gram-positive pathogens with *S. aureus*. Established treatments rely on the controlled release of antibiotics. Therefore,

nano-conjugation of antibacterial agents (*e.g.*, AZM) along with the inhibitor of phagocytosis (*e.g.*, cytochalasin D) might be validated by *in vivo* study in the amelioration of arthritis. Moreover, liposome-mediated targeted delivery of cytochalasin D plus AZM to the site of infection can be extrapolated in some *in vivo* animal models of arthritis for control of overstimulation of phagocytic cells at the site of tissue injury as well as controlled release of AZM to kill the infiltrated *S. aureus* into the synovial tissue in septic arthritis. Reduction of oxidative stress burden on the *S. aureus*-infected macrophages may pave the way for better killing of internalized *S. aureus* by cytochalasin D plus CIP or cytochalasin D plus AZM. However, cytochalasin D plus AZM combination seems to possess better efficacy, at least, in our *in vitro* incubations.

CONFLICT OF INTEREST. For the manuscript entitled "*In vitro* anti-inflammatory and immunomodulatory effects of ciprofloxacin or azithromycin in *Staphylococcus aureus* stimulated murine macrophages are beneficial in the presence of cytochalasin D" by Somrita Dey *et al.*, the authors declared that they have no conflict of interest for this manuscript toward submission in *Inflammation*. The authors also state that they do not have a direct financial relation with the commercial identities mentioned in this manuscript that might lead to a conflict of interest for any of the authors.

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