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ORIGINAL ARTICLE

Glucomannan enhanced the macrophage activity in exposure to methicillin-resistant *Staphylococcus aureus* (MRSA): *in-vitro* study

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ABSTRACT

Background and Objectives: The increasing number of methicillin-resistant *Staphylococcus aureus* persuade the need for preventive measures. Glucomannan is a polysaccharide choice for developing immunological strategies. This study aimed to investigate changes in gene expression and phagocytic activity of macrophage cells in the presence of glucomannan.
Materials and Methods: The effect of different concentrations of glucomannan (25, 50, and 100 µg/mL) on the phagocytic activity of macrophage cells was measured using the colony count method. The expression of Tumor Necrosis Factor-alpha (TNF-α) and Inducible Nitric Oxide Synthase (iNOS) genes was evaluated by Real-Time PCR.
Results: The concentrations of glucomannan significantly reduced the bacterial Colony-Forming Unit (CFU) and increased the phagocytic activity of macrophage cells. The maximum effect of glucomannan on iNOS and TNF-A genes expression was 100 µg/mL.
Conclusion: Glucomannan should be considered an adjuvant that stimulates the immune system. It may increase the expression of TNF-α and iNOS genes and the phagocytic activity of macrophage cells against methicillin-resistant *Staphylococcus aureus*.

Keywords: Glucomannan; Macrophage; Methicillin-resistant *Staphylococcus aureus*; Inducible nitric oxide synthase; Tumor necrosis factor-alpha

INTRODUCTION

Staphylococcus aureus is responsible for causing an extended range of infections worldwide, causing skin or lung infections or life-threatening infections such as necrotizing pneumonia, skin, soft tissue, and bloodstream infections in the human body (1). Over the past few decades, the incidence of *S. aureus* infection has increased significantly, causing many medical problems due to its resistance to antibiotics (2, 3).

Methicillin-resistant *S. aureus* has been considered a major nosocomial pathogen associated with hospitals and healthcare facilities, even though community-associated MRSA infection has also become an emerging problem recently (4, 5). Macrophages are among the most significant immune cells to fight against *S. aureus.* After exposure to bacteria, macrophages combat *S. aureus* infections by producing cytokines, regulating immune function, phagocytosis,

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and intracellular killing (6, 7).

TNF-α is the principal regulator of the proinflammatory cytokine cascade that can regulate macrophage function. TNF-α is released immediately after antigen exposure and plays a significant role in activating proinflammatory cells. Macrophages are usually activated through the toll-like receptor signaling pathways and produce TNF-α, which cooperates with other factors such as IFN-γ (8). Once activated, macrophages migrate to the site of inflammation. Therefore, the induction of inducible nitric oxide synthase (iNOS), production of nitric oxide (NO), and increased production of toxic oxygen species help macrophages to remove pathogens (8).

One of the main ways activated macrophages fight *S. aureus* is to produce nitric oxide (NO) induced by inducible NO synthase (iNOS). Cytokines such as tumor necrosis factor-alpha (TNF-α) can regulate iNOS levels. Increasing iNOS increases NO production and causes the loss of vital components of *S. aureus* (9, 10). However, *S. aureus,* particularly the MRSA strain, can resist the immune system and antibiotic therapies due to multiple virulence factors (11).

Glucomannan is a polysaccharide that can alter the expression of cytokines by binding to TLR of immune cells, increasing the translocation of NF-kB, and regulating the immune function cells such as macrophages against pathogens due to its immunomodulatory properties (12).

In the present study, the variations of macrophage's phagocytosis activity against MRSA in the glucomannan proximity were examined. Furthermore, the expression of iNOS and TNF-α genes associated with the immune, survival, and apoptotic functions of NF-kB was analyzed using a quantitative Real-Time PCR method in the presence of glucomannan.

MATERIALS AND METHODS

***S. aureus* growth conditions.** MRSA COL strain was used in this study. The bacteria were inoculated into the nutrient broth medium (Merck, Germany) and incubated overnight at 37°C. The optical density (OD) was measured using a spectrophotometer, and the number of bacteria was measured using the following formula: OD600 of $1.0=8\times10^8$. Due to the toxicity of the bacterial culture medium to macrophage cells, the medium was centrifuged at 4000 RPMI for 10 minutes, and the supernatant was removed. The bacterium was ready to be inoculated into the cell culture plate after adding RPMI 1640 + fetal bovine serum (FBS).

Isolation of mouse peritoneal macrophage cells. Ten female BALB/c mice aged six weeks and weighing 25 g were received from Pasteur Institute of Iran (Karaj, Iran). Mice were kept in cages in an animal house facility according to the instructions defined by Tehran University of Medical Sciences. Then, 5 ml of sterile thioglycollate was injected into the peritoneum of each mouse to induce peritoneal ascites for macrophage accumulation. After five days, 10 ml of sterile saline was gently injected into the peritoneal cavity, aspirated again, and transferred to a sterile falcon tube. The number of live macrophages were collected and counted using the trypan blue staining method.

Cell culture and treatment. Macrophage cells were suspended in a 10 ml medium (improved RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin) after washing with cold and sterile PBS. In the next step, the falcon tubes were centrifuged, and after removing the supernatant, 5 ml of lysis buffer was added to each tube, and after 5 minutes, 5 ml of saline + FBS 10% solution was added to each falcon to neutralize the lysis buffer and centrifuged twice for 10 minutes. The cells were then slowly pipetted into cell culture plate wells (700000 cells each well), and after 2 hours of incubation at 37°C in a CO_2 incubator, the supernatant was removed, and 500 μl of the new medium was added to each well. Finally, concentrations of glucomannan were added. Macrophages were adjacent for 24 hours at 25, 50, and 100 μg/mL glucomannan concentrations. The cells and bacteria were then exposed to each other in a one-to-one ratio in cell culture plate wells. Group E containing macrophages and 1 μl lipopolysaccharide (LPS) was considered the positive control, and group F containing only macrophages was considered the negative control.

Survival of bacteria and CFUs count assay. The effect of diverse concentrations of glucomannan added to the media on bacterial survival was assayed by counting bacterial colony-forming units (CFUs). Assays were made on 4-well plates containing either a mixture of macrophages and *S. aureus* cells with or without the different concentrations of glucomannan. After 90 minutes of incubation of the cell culture plate at 37°C in a CO_2 incubator, the supernatant of

the wells containing the bacteria was picked up and, after dilution, was cultured for colony count on a blood agar medium. Bacterial colonies were counted after overnight incubation.

Real-time PCR. Total RNA extraction from the macrophage cells was performed by TRIzol following the manufacturer's instructions by phase separation method. After isolation, total RNA was exposed to DNase I.

cDNA synthesis. cDNA was produced using the ThermoScript RT-PCR System Revert Aid™ First Strand cDNA Synthesis Kit protocol (Fermentas) according to the manufacturer's recommendations for oligo (dT) 20 primed cDNA-synthesis. cDNA synthesis was performed on 1-3 µg RNA, at 60°C. Finally, cDNA was diluted 1:5 prior use in QPCR. RT (reverse transcription) consisted of 20 µl of the reaction solution stated above, 1 µl of RevertAid M-MuLV RT (200 U/µL), 4 µl of 5× PrimeScript Buffer, and 1 µl of 10 mM dNTP Mix complemented with PCR water to a total volume of 20 µl. The reverse transcription reactions were carried out at 42°C for 60 min and 70°C for 5 min. Target mRNA was quantified by Real-Time RT-PCR (TaqMan®) using a Applied Biosystems StepOne™ Real-Time PCR system. Real-Time RT-PCR was carried out in a 9-well plate (in duplicate) using SYBR Green Real-Time PCR Master Mix according to the manufacturer's instructions. TaqMan threshold cycle number (Ct) was normalized using the $2^{\Delta\Delta Ct}$ method:

$$\Delta\Delta Ct = (Ct_{Target} - Ct_{Actin})\ \text{treatment} - (Ct_{Target} - Ct_{Actin})\ \text{control}$$

The primers used in this study are listed in Table 1. After the PCR, the amplicon melting curve was checked for PCR specificity (Fig. 1).

Statistical analysis. GraphPad Prism 6 software (GraphPad, San Diego, CA, USA) was used to analyze the results. One-way Analysis of Variance (ANOVA) was used to find the comparison among all groups. Data were expressed as mean ± standard error (SEM). $p < 0.05$ were considered as statistically significant.

Ethics approval and consent to participate. All methods and animal experiments implicated in this study were carried out by the Tehran University of Medical Sciences (Ethics Code: IR.TUMS.SPH.REC.1398.271). All procedures were performed according to international guidelines and guidelines for ethical behavior in the care and use of animals. This study was based on ARRIVE guidelines.

Table 1. The primers of TNF-α, iNOS and standard b-actin used in Real-Time PCR.

Primer Name	Sequence 5'→ 3' (10-50 bp)*
TNF-α	Forward: ATGTTGTAGCAAACCCTGAAGCT
	Reverse: ATTGGCCAGGAGGGCATT
iNOS	Forward: CTGTCCTTGGAAATTTCTGTT
	Reverse: TGGCCAGATGTTCCTCTATT
β-Actin	Forward: TCCTCCTGAGCGCAAGTAC
	Reverse: CCTGCTTGCTGATCCACATCT

RESULTS

Effects of glucomannan on TNF-α expression. In this study, the Real-Time PCR method was used to investigate the effect of glucomannan on TNF-α gene expression (Fig. 2). The results showed that the expression of this gene in the groups containing glucomannan, bacteria, and macrophages (A, B, C) increased significantly compared to other groups. On the other hand, the group treated with 100 concentrations of glucomannan compared to 25 and 50 concentrations showed a significant increase in the expression of this gene. In groups containing macrophages and glucomannan (G, H, I), due to the absence of bacteria, TNF-α gene expression was lower than in similar groups containing bacteria. The highest TNF-α gene expression was related to the positive control group (E). The fewest expression was related to the negative control group (F).

Effects of glucomannan on iNOS expression. In the present study, the expression of the iNOS gene in murine macrophage cells was investigated using a Real-Time PCR method in the presence and absence of *S. aureus* and doses of 0, 25, 50, and 100 glucomannan. The results showed that the expression of this gene in the groups containing glucomannan, bacteria, and macrophages increased significantly compared to other groups (Fig. 3). On the other hand, the group treated with 100 concentrations of glucomannan compared to 25 and 50 concentrations showed a significant increase in the expression of this gene.

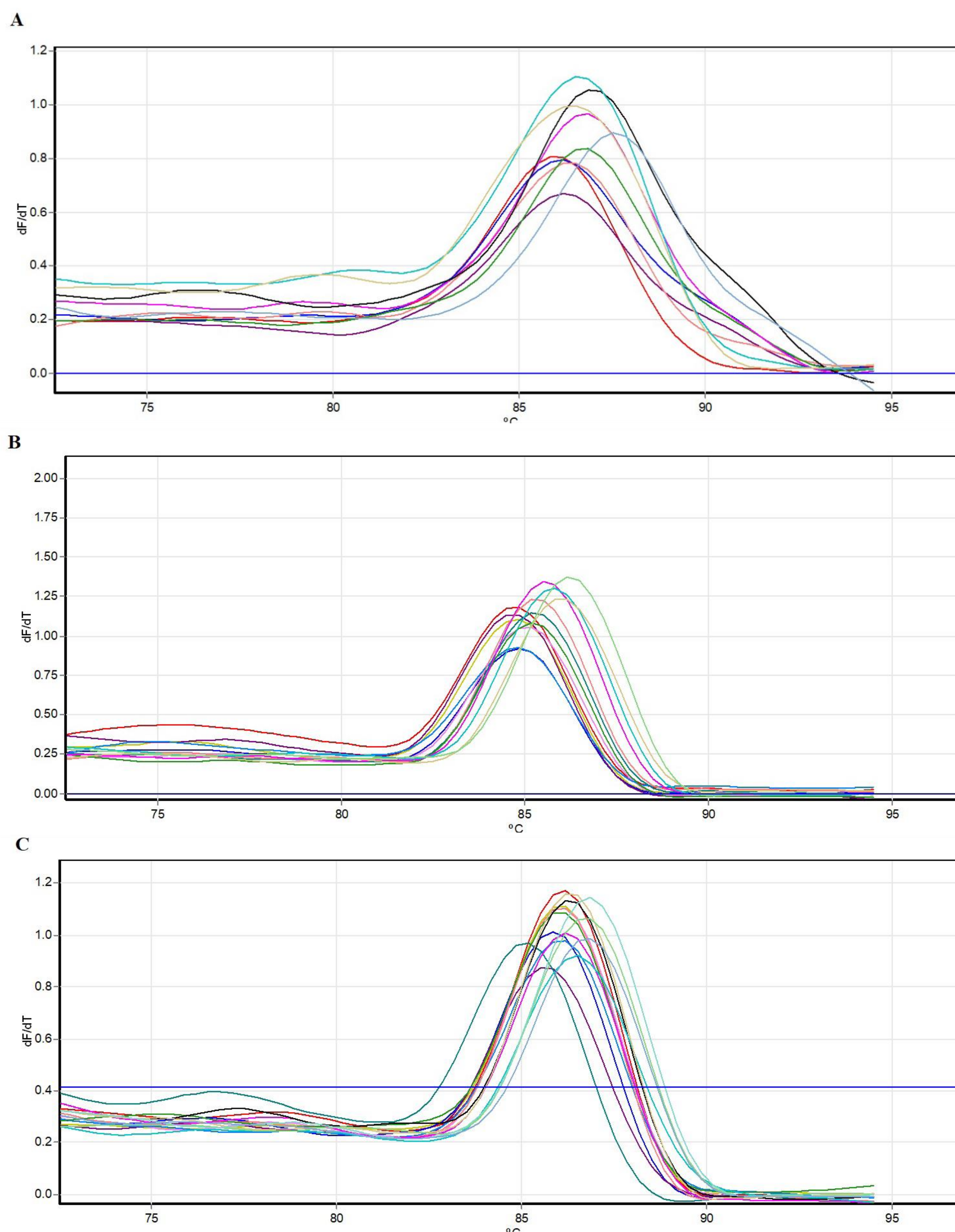

Fig. 1. Melting curve of Real-time PCR reaction of β-Actin gene (A), iNOS gene (B), TNF-α gene (C)

Effects of glucomannan on macrophage cell phagocytic activity and CFU. The results of this study showed that the proximity of macrophages with different doses of glucomannan affects their phagocytic ability (Fig. 4). After macrophage and *S. aureus* exposure with doses of 0, 25, 50, and 100 µg/mL glucomannan, the supernatant was removed and cultured in a blood agar medium. The results showed that the number of colonies decreased with increasing glucomannan dose (Table 2).

DISCUSSION

S. aureus is one of the most important pathogenic bacteria causing many infections (13, 14). Despite

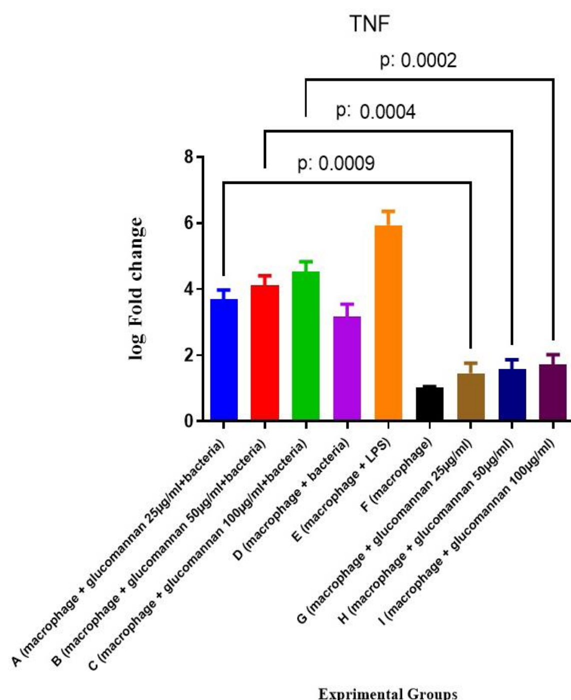

Fig. 2. The expression rate of the TNF-α gene. Group A contains: Macrophage + *S. aureus* + 25 µg/ml glucomannan, Group B contains: Macrophage + *S. aureus* + 50 µg/ml glucomannan, Group C contains: Macrophage + *S. aureus* + 100 µg/ml glucomannan, Group D contains: Macrophage + *S. aureus,* Group E contains: macrophage + LPS, group F contains macrophage, group G contains macrophage + 25 µg/ml glucomannan, group H contains: macrophage + 50 µg/ml glucomannan, group I contains: macrophage + 100 µg/ml glucomannan. ($p < 0.05$ were considered as statistically significant).

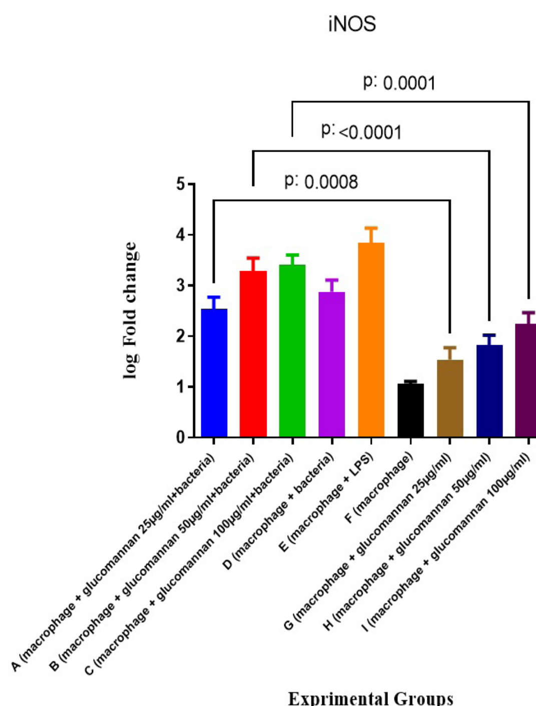

Fig. 3. The expression rate of the iNOS gene. Group A contains: Macrophage + *S. aureus* + 25 µg/ml glucomannan, Group B contains: Macrophage + *S. aureus* + 50 µg/ml glucomannan, Group C contains: Macrophage + *S. aureus* + 100 µg/ml glucomannan, Group D contains: Macrophage + *S. aureus*, Group E contains: macrophage + LPS, group F contains: macrophage, group G contains macrophage + 25 µg/ml glucomannan, group H contains: macrophage + 50 µg/ml glucomannan, group I contains: macrophage + 100 µg/ml glucomannan. ($p < 0.05$ were considered as statistically significant).

antibiotic resistance, the use of second-line antibiotics is still one of the treatments for MRSA infections; nevertheless, using these antibiotics, including vancomycin, linezolid, and daptomycin, is expensive and can be associated with side effects (15). Consequently, the increasing number of resistant strains of *S. aureus* persuade the need for a preventative approach.

TNF-α, and iNOS are the most critical factors in regulating the function of immune system cells. Therefore, the variations in the expression of these genes in macrophages exposed to glucomannan were examined. The results showed that the expression of these genes was dose-dependently associated with glucomannan.

Glucomannan is a biologically active carbohydrate and mainly contains mannose and glucan (12). Glucomannan has been used in diets and therapies for centuries and has anti-inflammatory properties (16). The results of several studies have shown that the use of polysaccharides such as glucomannan, because of its effect on the regulation of immune functions, can be an effective way to treat and prevent bacterial infections (17, 18). In the present study, the effect of glucomannan on the phagocytic function of macrophages and changes in the expression of iNOS and TNF-α genes in these cells was investigated. The results showed that the expression of these genes and phagocytosis were dose-dependently associated with glucomannan. The TNF-α evaluation results showed, in comparison with the control group, in glucomannan-treated macrophages, the expression of this gene increased in a dose-dependent manner which can help the macrophages to Phagocyte more pathogens.

The binding of signaling molecules and cytokines

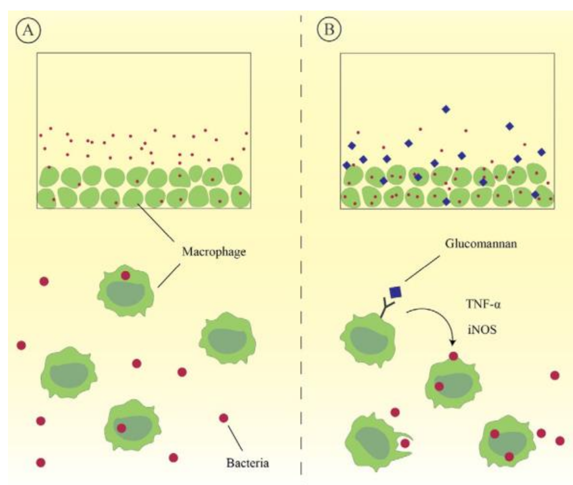

Fig. 4. Glucomannan on macrophage cell phagocytic activity. A: Macrophages are activated after exposure to the pathogen and begin phagocytosis. B: By binding to TLRs, glucomannan increases the expression of proinflammatory cytokines such as TNF-α and iNOS, thereby increasing macrophage phagocytic activity.

Table 2. Number of colonies grown on blood agar after escaping from phagocytosis

Group name	Contents	Number of colonies counted
A	Macrophage + *S. aureus* + 25 µg/ mL glucomannan Macrophage +	3
B	*S. aureus* + 50 µg/ mL glucomannan	2
C	Macrophage + *S. aureus* + 100 µg/ mL glucomannan	1
D	Macrophage + *S. aureus*	3

such as TNF-α to the corresponding macrophage receptors activates a signal transduction cascade, inducing a proinflammatory response in these cells. Macrophage activation increases the expression of proinflammatory genes such as iNOS and the concomitant effector proteins that produce reactive oxygen species (ROS) and reactive nitrogen species, which help eliminate pathogens (19). In the present research, results of iNOS evaluation showed that macrophage treatment with glucomannan increases the expression of this gene in a dose-dependent manner, while in untreated macrophages, the expression of the iNOS gene was lower.

The results of previous studies have shown that the induction of iNOS expression is dependent on TNF receptor-1 signaling in macrophages (20).

NO is one of the inflammatory mediators and plays an essential role in regulating the function of the immune system (21, 22). In addition, due to its antimicrobial effects, this molecule can help the immune system eliminate pathogens (22, 23).

Cytokines such as IFN gamma and TLR ligands induce iNOS production in many cells, including macrophages (24). Macrophages are one of the primary NO-producing cells. The effects of NO on macrophages cause their cytotoxic and antimicrobial function. iNOS can affect the function and differentiation of immune cells (25). In this study, results of the phagocytosis assay showed that the addition of glucomannan to the co-culture of macrophages and bacteria caused more effectiveness in this process.

In a study on the regulatory role of NO in cell dendritic function and macrophages, Xiong et al. (26) showed that NO could inhibit NF-κB transcription and interleukin-12 production in these cells. Additionally, iNOS can play a role in balancing macrophages M1 and M2, and its deficiency in animal models makes them more susceptible to inflammatory diseases (25).

The results of previous studies have shown that the presence of glucomannan with macrophage cells can increase the production of inflammatory cytokines from these cells. Cai et al. (27), in an in-vitro study, showed that glucomannan-containing compounds could increase the expression of iNOS, NO, and other cytokines from macrophages due to their immunomodulatory properties.

Onishi et al. (28) investigated the effect of glucomannan oral powder on inflammation and skin immune response in animal models. For this purpose, they used histopathological tests and measured levels of proinflammatory cytokines. The results showed that the glucomannan diet significantly repressed skin lesions in animal models. In addition, the overproduction of cytokines such as TNF-α in the glucomannan-fed group was suppressed, indicating a role in regulating glucomannan immunity. HE et al. (29) examined the effect of glucomannan on THP-1 cells and its immunomodulatory properties. The results showed that in the positive control group (treated with LPS), the amount of TNF-α secretion increased significantly after two hours of exposure to LPS. However, this increase occurred gradually in the glucomannan-treated groups. In their study, a

comparison between glucomannan and LPS showed that the cells reacted to both substances despite the difference in mechanism of action; nevertheless, glucomannan also played an anti-inflammatory and immune-regulating role.

Cai et al. (27) measured the effect of glucomannan on macrophage activity using the neutral red uptake method. They showed that the phagocytic activity increased up to 63% in the proximity of glucomannan. Although glucomannan overdose reduced phagocytosis, macrophages still performed better phagocytosis than the control group. Gurusmatika et al. (30) investigated the effects of glucomannan on the activity of rat peritoneal macrophage cells. Results showed that usage of glucomannan increases macrophage phagocytic activity due to its immunomodulatory properties. Exposure to glucomannan for 6 hours resulted in a 50% increase in phagocytosis compared to the control group.

By binding to TLRs, glucomannan initiates proinflammatory cascades, which in turn increase TNF-α, thereby increasing macrophage activity. However, TNF-α can also play a role in inhibiting and regulating immune function. In a study, Gan et al. (31) examined the effects of glucomannan on stimulating MR clustering on macrophages and thereby stimulated the cells into an M2 phenotype. The results showed that the use of higher doses of glucomannan improved the symptoms of colitis and inflammatory bowel disease in an animal model, and increase the ability of the immune system to fight pathogens. In addition, this regulatory role at higher doses can prevent an overactive immune system and inflammation (28). The present study results did not well illustrate the role of glucomannan in preventing inflammation due to the smaller dose range of glucomannan. In addition, the use of only one strain of *S. aureus* could further limit the results. Researchers may consider these two issues in future studies.

CONCLUSION

iNOS as a cytoplasmic enzyme is not present in resting macrophages, and its expression is induced in response to microbial antigens. TNF-α is an inflammatory cytokine that is increased by *S. aureus* infection and activation of macrophage cells. These two play essential roles in inhibiting *S. aureus* infection. In this study, the immunomodulatory effects of glucomannan on macrophages were investigated in the presence of MRSA. The results showed that glucomannan may increase the expression of TNF-α and iNOS genes and the phagocytic activity of macrophage cells against MRSA and aid the immune system in the combat against pathogens. More studies are needed in order to candidate glucomannan as an adjuvant in vaccine development.

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