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Possible Role of Toll-like Receptor-2 in the Intracellular Survival of *Staphylococcus aureus* in Murine Peritoneal Macrophages: Involvement of Cytokines and Anti-Oxidant Enzymes

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Abstract

Effects of blocking toll-like receptor-2 (TLR-2) on the survival of *Staphylococcus aureus* (*S. aureus*) and cytokine production in peritoneal macrophages of Swiss albino mice were analysed. Macrophages were infected with *S. aureus* in the presence and absence of anti-TLR-2 antibody. Tumour necrosis factor-α (TNF-α) interleukin-6 (IL-6), interferon-gamma (IFN-γ), interleukin-1β (IL-1β), interleukin-12 (IL-12) and interleukin-10 (IL-10) concentrations were measured. Expressions of TLR-2, NF-κB, MyD 88 were analysed by Western Blot. Expression of TLR-2 was increased in *S. aureus*-infected macrophages with respect to control and was MyD 88 independent. TLR2 blocking significantly reduced TNF-α, IL-6, IL-1β and IL-10 and increased IFN-γ and IL-12 production. Decreased catalase activity and increased superoxide dismutase (SOD) by *S. aureus* with concomitant increase in H_2O_2 and nitric oxide (NO) were observed in the case of prior TLR-2 blocking. To understand whether catalase contributing in the intracellular survival, was of bacterial origin or not, 3-amino, 1, 2, 4-triazole (ATZ) was used to inhibit specifically macrophage-derived catalase. Catalase enzyme activity from the whole staphylococcal cells in the presence of ATZ suggested that the released catalase were of extracellular origin. From the intracellular survival assay, it was evident that pretreatment of macrophages with ATZ reduces the bacterial burden in macrophages when infected with the recovered bacteria only from the anti-TLR-2 antibody-treated macrophages after phagocytosis. Catalase protein expression from the whole staphylococcal cells recovered after phagocytosis also indicated the catalase release from *S. aureus*. Capturing of *S. aureus* via TLR-2 induces inflammatory reactions through activation of NF-κB-signalling pathways which was MyD88-independent.

Introduction

In the innate immune response, toll-like receptors (TLRs) that are predominantly expressed in cells involved in immuno-inflammatory responses play pivotal roles in the host defence against microbial pathogens by recognizing pathogen-associated molecular pattern (PAMP)s and activating intracellular signalling pathways [1]. Among the known TLRs, TLR-2 is a key sensor for detecting *S. aureus* infection [2, 3]. Furthermore, TLR-2 induces the synthesis of pro-inflammatory mediators to rapidly activate the innate immune system [4, 5]. The absence of TLR-2 has been shown to provide protective effects to the host organisms in certain models suggesting that TLR-2 has not only beneficial [6] but also detrimental roles in host innate response against *S. aureus* infections [7]. Results of either single or dual TLR blockade before or upon acute bacterial infection have been shown to play a central role of TLR-2 in sensing bacterial challenge *in vivo* and the capacity to protect from shock upon subsequent or synchronous antibiotic therapy [8]. Another interesting field worthy of study in susceptibility to infection is the ability developed by many virulent strains of pathogens to evade immunity through TLRs. Such is the case with the bacteria *Mycobacterium tuberculosis*, *Yersinia enterocolitica*, *Yersenia pestis*, *Yerseinia pseudotuberculosis* and fungi such as *Candida albicans* and *Aspergillus fumigates* which activate a TLR-2-mediated mechanism to induce anti-inflammatory pattern

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that down modulates the microbicidal function of leucocytes [9, 10]. Certain pathogens used TLR-based strategies to evade host defence [11]. This regulation by TLR-2 seemed to be beneficial to bacteria and dependent on the strain of bacteria, as TLR-2-deficient mouse macrophages were reported to have a greater capacity to kill *S. aureus* organisms compared with wild-type macrophages [12–14]. The activation of TLR signalling with these infections promotes sustained efforts to develop novel strategy to block these pathways for a variety of disease infections [15]. Taken together, understanding negative regulation of TLR signalling may be helpful to develop methods of artificial manipulation of TLR signalling to restore inflammatory diseases, overcome uncontrolled inflammation and make counter measures against infection [16]. It was suggested that targeting immunosuppressive microenvironment using anti-TLR-2 antibody is a novel therapeutic strategy for combating inflammation [17]. Addition of anti-TLR2 antibodies to *Mycobacterium avium subsp. paratuberculosis* (MAP)-infected monocytes promoted phagosome maturation and organism killing [18]. Anti-TLR-2 antibody significantly decreased spontaneous cytokine production from rheumatoid arthritis synovial tissue explants cultures [19]. Studies also indicated that blocking TLR-2 activity is a novel therapeutic strategy for anti-metastasis that combats the immunosuppressive environment [20, 21]. Immunoneutralization of TLR-2 with antibody directed against it has been reported to cause modulatory response to *S. aureus* [22, 23]. Thus, it appears that even though *S. aureus* is recognized by macrophages through TLR-2, *S. aureus* may utilize TLR-2 as part of its survival mechanism. This makes TLR-2 a very intriguing molecule to study when trying to understand the survival mechanism of *S. aureus*. Expression of distinct set of TLR-2 and differences in reactivity to microbial molecules in BALB/C and C57BL6 mice have been discussed earlier [24]. Currently, there is no study on the expression of TLR-2 and role of TLR-2 in the intracellular survival of *S. aureus* in macrophages in wild-type Swiss albino mice. We have recently reported on cellular events and intracellular survival of *S. aureus* during infection of murine macrophages of Swiss albino mice [25]. The role of cell surface TLR-2 in the survival of *S. aureus* using Swiss albino mice has not been studied. The roles of TLRs in the sensing of oxidants by cells and tissues *in vitro* have been investigated [26]. TLR2 was required for oxidant-induced inflammation *in vivo*. Therefore, it seems that as in higher vertebrates, *S. aureus* has evolved a diversified array of antioxidant tools, both enzymatic and non-enzymatic, to resist immune-mediated oxidative attack [27]. Catalase has been proposed to be a potential virulence factor in many bacterial pathogens, because its activity might protect them from the reactive oxygen species (ROS) generated by eukaryotic cells [28]. Thus, this enzyme has been demonstrated to be an essential factor for the intracellular survival of bacteria [29].The present study was performed to investigate the involvement of macrophage cell surface TLR-2 in the intracellular survival of *S. aureus* in peritoneal macrophages of Swiss albino mice.

Materials and methods

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. Wild-type male Swiss albino mice were used throughout the study. Macrophages were prepared from peritoneal fluids of thioglycolate-administered mice. The *Staphylococcus aureus* (*S. aureus*) strain AG-789 was obtained from Apollo Gleneagles Hospital, Calcutta, West Bengal, India.

Preparation of bacteria. *Staphylococcus aureus* strain (AG-789) grown overnight in Luria Bertini broth were 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 inoculum 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 [30].

Isolation and stimulation of peritoneal macrophages. The mice, used at 6–8 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 exudate was harvested by lavage of the peritoneal cavities of mice with endotoxin-free Hanks' solution 4–5 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 RPMI 1640 medium supplemented with 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin and then were allowed for plastic adherence non-adherent 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 [31]. Murine peritoneal macrophages (5×10^6 cells/ml) were infected with *S. aureus* (1×10^6, 5×10^6 and 1×10^7 CFU/ml) for 48 h at 37 °C and expression of TLR-2 on macrophages were performed.

In a separate set of experiment, murine peritoneal macrophages (5×10^6 cells/ml) were infected with *S. aureus* (5×10^6 CFU/ml) for 24, 48 and 72 h at 37 °C and expression of TLR-2 on macrophages were performed.

Blocking antibody reagents and culture conditions. For TLR-2 blocking assays, polyclonal antibodies against TLR-2 (Biorbyt Limited, Cambridge, UK) were added at 10 μg/ml, and the macrophages were incubated for 1 h at 37 °C

in 5% CO_2 with *S. aureus* or medium alone were then added, and the cells were incubated for an additional 24, 48 or 72 h at 37 °C in 5% CO_2. Cell-free culture supernatants were collected at 24, 48 and 72 h [32, 33]. Saturation studies were performed beforehand and were found useful at a concentration of 10 μg/ml as suggested earlier [34].

Assays for colony-forming ability of engulfed bacteria. 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 [35] in RPMI-FBS (5%) and incubated at 37 °C cell culture incubator for different times in the presence and absence of anti-TLR-2 antibody (10 μg/mL). 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, note that we were unable to use antibiotics to kill bacteria present outside of macrophages because engulfed bacteria died quickly during the period necessary for the action of antibiotics. 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.

Determination of catalase activity of whole staphylococcal cells recovered after time-dependent phagocytosis in the presence or absence of anti-TLR-2 antibody. The bacteria that survived after time-dependent phagocytosis, as obtained from the plates of respective phagocytic time were used to estimate the whole staphylococcal cell catalase activity as described previously [25]. The bacterial cells were grown overnight and washed twice in sterile distilled water and once in sterile 50 mM potassium phosphate buffer (pH 7.0). The washed bacterial cells were dispersed in phosphate buffer (pH 7.0) by shaking glass beads and diluting to give an absorbance of 0.62 at 600 nm. A 1 ml amount of the suspension of culture with an absorbance of 0.62 contained approximately 1.1×10^8–1.5×10^9 CFU/ml by standard plate counts. Based on preliminary experiments, 5×10^6–1.5×10^8 CFU/ml was used to determine catalase activity of cells in the presence of 15 μmoles of H_2O_2/ml phosphate buffer. After 10 min of incubation, bacterial cells were centrifuged and supernatants were collected and stored at −20 °C. Catalase activity in the supernatant was estimated in a process as mentioned in Materials and methods section.

Culture of intracellular viable S. aureus *and preparation of whole-cell extract (cell lysate) for determination catalase expression by immunoblot*. The bacteria that were survived after time-dependent phagocytosis as obtained from the plates of respective phagocytic time were further cultured in nutrient broth. Cells were washed twice in phosphate-buffered saline (PBS), vortexed, passed through a 5-μm pore size filter, diluted to an optical density at 650 nm of 0.4 (10^6 CFU/ml) and resuspended in lysis buffer. Bacterial suspensions (10 ml) were washed twice with lysis buffer containing 1 mM disodium EDTA, pH 7.2, 0.5 mM PMSF and finally resuspended in 2 ml buffer. Suspensions were sonicated on ice at 8 μm for 90 s (six 15 s bursts with 15 s cooling periods) and then centrifuged at 16,000 *g* for 45 min at 4 °C. Supernatants (lysates) were filtered and stored at −20 °C. Protein content of this crude bacterial lysate was determined using Bradford method.

Immunoblot analysis for catalase. Bacterial cells lysates samples were resuspended in SDS-PAGE sample loading buffer, subjected to SDS-PAGE, and transferred onto BioTrace, PVDF membrane, (PALL; Gelman Laboratory, Port Washington, NY, USA) as described previously [36]. Membranes were probed at room temperature with rabbit antiserum to catalase (Primary antibody to catalase; Biorbyt Limited) at a 1:3000 dilution and immunoreactivity was detected with a goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated antibody (Biorbyt Limited). Blots were developed with a chemiluminescent substrate (SuperSignal West Pico Chemiluminescent Substrate; Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Use of catalase inhibitor 3-amino 1, 2, 4-triazole and assay of catalase activity. In another set of experiments, macrophages were treated with 1 mM 3-amino, 1, 2, 4-triazole (ATZ) solution, an intracellular catalase inhibitor at 37 °C for 30 min [37]. After proper washing, the ATZ pretreated or non-treated macrophages were allowed to interact with equal numbers of *S. aureus* and incubated at 37 °C for 48 h in the presence or absence of anti-TLR-2 antibody. Supernatants and cell-free lysates were prepared from this set. Catalase activity in the supernatant and cell-free lysate was determined spectrophotometrically by measuring the decrease in H_2O_2 concentration at 240 nm as described below.

Effect of ATZ on the whole-cell staphylococcal catalase activity of the bacteria recovered after 48 h of phagocytosis. The bacteria recovered after 48 h of phagocytosis in the presence or absence of anti-TLR-2 antibody were used to determine catalase activity in the presence of 15 μmoles of H_2O_2/ml of phosphate buffer [25]. Bacterial cells (1.5×10^8 CFU/ml) were further allowed to interact with ATZ for 15, 30, 45 and 60 min at 37 °C followed by centrifugation and collection of supernatant. Catalase contents of the supernatant were determined spectrophotometrically as described in the Materials and methods section.

Intracellular survival of recovered bacteria by macrophages in the presence of ATZ. To address whether the intracellular survival of recovered *S. aureus* by macrophages in the presence of ATZ was altered, the bacteria recovered after 48 h of phagocytosis in the presence or absence of anti-TLR-2 antibody were further cultured and used for intracellular survival assay. Freshly isolated peritoneal

macrophages were treated with 1 mM ATZ solution, an intracellular catalase inhibitor at 37 °C for 30 min [37]. After proper washing, the ATZ pretreated or non-treated macrophages were allowed to interact with equal numbers of *S. aureus* and incubated at 37 °C for 24, 48 and 72 h. Number of surviving bacteria was represented as CFU/ml of macrophages.

Assay of catalase activity. Catalase activity in the medium or cell-free lysate was determined spectrophotometrically by measuring the decrease in H_2O_2 concentration at 240 nm. At time zero, 100 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 this, 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 m. mole/min mg protein [38].

Assay of superoxide dismutase activity. About 100 μl of the medium (supernatant) or cell-free lysate was mixed separately with 1.5 ml of a Tris-EDTA-HCl buffer (pH 8.5), then 100 μl of 7.2 mM 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 U/mg protein [39].

Hydrogen peroxide (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 [40]. Briefly 70 μl of peritoneal macrophages, 20 μl Horse Raddish peroxidase (HRP; 500 μg/ml), 70 μl of Phenol red (500 μg/ml) and 40 μl of *S. aureus* 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 bacteria. A standard H_2O_2 curve was plotted and H_2O_2 release in supernatants and lysate was evaluated and expressed in μM/10^6 cells.

Assay for the determination of nitric oxide. Nitric oxide (NO) release was determined by the Griess assay. Thioglycolate-elicited mouse peritoneal macrophages in serum-free RPMI 1640 medium were mixed with *S. aureus* and the mixture was incubated in the presence or absence of anti-TLR-2 antibody for different times at 37 °C with 5% CO_2 and centrifuged. About 50 μl of supernatant or lysate was incubated separately in 40 μM Tris (pH 7.9) containing 40 μM of the reduced form of β-nicotinamide adenine dinucleotide phosphate, 40 μM flavine adenine dinucleotide and 0.05 U/ml nitrate reductase at 37 °C for 15 min. Reduced samples were incubated with an equal volume of Griess reagent consisting of sulphanilamide [0.25% (w/v)] and *N*-1-naphthylethylenediamine [0.025% (w/v)], and the mixture was incubated for 10 min and the absorbance at 550 nm was measured. The total nitrate/nitrite concentration was determined by comparison to a reduced $NaNO_3$ standard curve [41].

Tumour necrosis factor α, interferon-gamma, interleukin-6, interleukin-1beta, interleukin-10 and interleukin 12 p40 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 [35] in RPMI-FBS (5%) and incubated at 37 °C cell culture incubator for different times in the presence and absence of anti-TLR-2 antibody. 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 tumour necrosis factor-α (TNF-α), interferon-gamma (IFN-γ), IL-6, IL-10, IL-1β and IL-12 p40 production was assayed using ELISA kits according to the instructions provided by the manufacturer (RayBiotech, Inc., Norcross, GA, USA) in a Bio-Rad ELISA Reader.

Western blot analysis to study the expression of TLR-2, MyD-88 and NF-κB. The effect of anti-TLR-2 antibody on the expression of TLR-2 cytoplasmic proteins were prepared from macrophages and analysed by Western blot. The following buffers were used for sample preparation: Buffer A (5 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA pH 8.0, 10 mM EGTA pH 8.0, 1 mM dithiothreitol (DTT), protease inhibitor cocktail. Cells from each experimental group were washed three times with ice-cold PBS and suspended in 200 μl buffer A. Cells were lysed by ultrasonic disruption using a Microtip Probe for 10 pulses of 5 s each. Lysates were centrifuged at 8,385 *g* for 20 min at 4 °C. The supernatants containing cytoplasmic proteins were collected and stored at −80 °C. Protein concentrations were normalized to the protein content by Bradford method before the assay. Proteins were denatured at 100 °C for 5 min in loading buffer (60 mM Tris, 2.5% sodium dodecyl sulphate, 10% glycerol, 5% mercaptoethanol, 0.01% bromophenol blue). Aliquots containing an equal amount of total proteins from each sample were separated by SDS-PAGE (10% gel) and transferred onto BioTrace, PVDF, transfer membranes (PALL; Gelman Laboratory; 100 V, 1.0 h, 4 °C). After blocking for 2 h at 4 °C in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20) containing 5% non-fat milk, membranes were washed three times in TBST and probed overnight at 4 °C with appropriate primary antibodies [42] (anti-TLR2; Biorbyt, Cambridgeshire, UK anti-MyD88 and anti-NF-κB [p65] from Abcam, Cambridge, UK) in TBST containing 1% bovine serum albumin. Blots were washed three times in TBST, incubated for 2 h with appropriate HRP-conjugated secondary antibodies, developed with the Super Signal chemiluminescent substrate (SuperSignal West Pico Chemiluminescent Substrate; Thermo Scientific) and

exposed to X-Omat BT films (Kodak, Windsor, CO, USA). Bands were quantified using QUANTITY ONE software (Bio-Rad, Inc., Hercules, CA, USA).

Statistical analysis. Data were represented as the mean ± SD. Parameters between groups were compared by one-way ANOVA using the statistical software Origin, version 8. Post hoc comparison of means was done by Scheffe's test. Significance for all tests was set at $P < 0.05$.

Results

Effect of viable S. aureus infection on the expression of TLR-2 in murine peritoneal macrophages of Swiss albino mice

Since live *S. aureus* is known to activate macrophages and induce release of cytokines, the question arises whether *S. aureus* itself has any role in the expression of TLR-2 on the surface of murine peritoneal macrophages of Swiss albino mice concomitant with the release of cytokines from the same source. The data presented in Fig. 1 showed that stimulation of peritoneal macrophages with viable *S. aureus* at different doses also induced expression of TLR-2 in macrophages after *in vitro* infection (Fig. 1).

Time kinetic study showed that the *S. aureus* infection-induced expression of TLR-2 was maximum at 48 h post infection which was reduced at 72 h after infection. Preincubation of peritoneal macrophages with anti-TLR-2 antibody strongly inhibited the *S. aureus* infection-induced TLR-2 expression in macrophages at 48-h post-infection (Fig. 2).

Phagocytosis of S. aureus by peritoneal macrophages of Swiss albino mice was increased due to surface TLR-2 blocking

Increased phagocytic activity has been observed for murine peritoneal macrophages with surface TLR-2 blocking prior to viable *S. aureus* infection. Our results showed that the intracellularly viable bacterial count (CFU) was least when the murine peritoneal macrophages were preincubated with anti-TLR-2 antibody for 1 h prior to *S. aureus* infection. The decrease in the mean bacterial CFU counts for 24 h (250 ± 14.2), 48 h (420 ± 28.28) and 72 h (340 ± 14.42) of phagocytic time in the presence of antibody were found to be significant ($P < 0.05$) when compared to the mean CFU count after time–dependent phagocytosis with no antibody (Table 1).

Alteration in antioxidant enzyme activity after TLR-2 blocking followed by S. aureus infection

As we have already reported that bacterial catalase and superoxide dismutase (SOD) played an important role in the intracellular survival of *S. aureus* in murine peritoneal macrophages, we became interested to find out whether there occur any changes in these antioxidant enzyme activities after TLR-2 blocking followed by *S. aureus* infection. A marked decrease in the catalase enzyme activity has been observed both in the culture supernatant (media) and in the cell-free lysate when macrophages were incubated with anti-TLR-2 antibody prior to *S. aureus* infection than that of macrophages infected with *S. aureus*

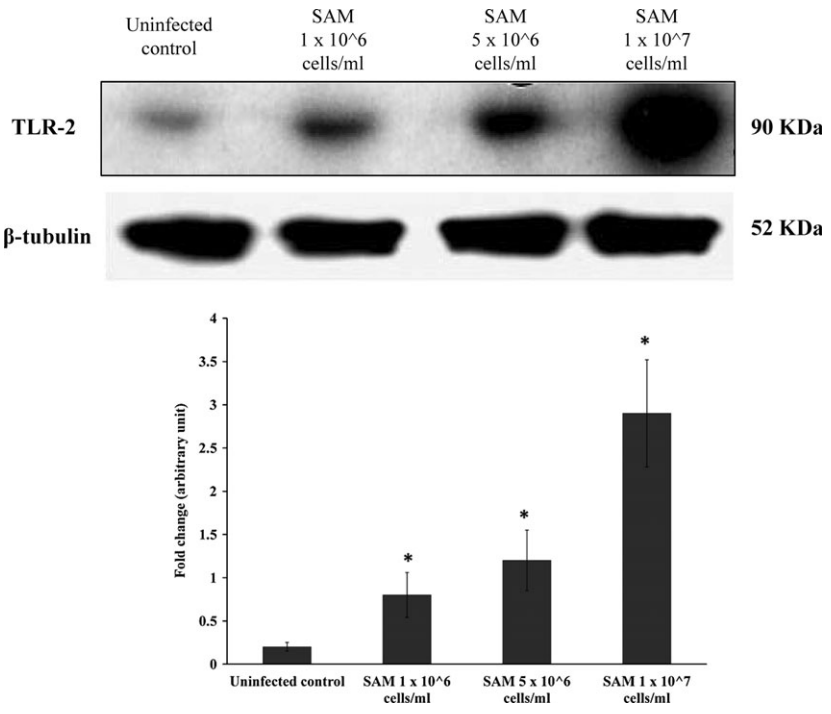


Figure 1 Live *Staphylococcus aureus* infection induces toll-like receptor 2 (TLR-2) expression. Murine peritoneal macrophages (5×10^6 cells/ml) were infected with *S. aureus* (1×10^6, 5×10^6 and 1×10^7 CFU/ml) for 48 h at 37 °C and expression of TLR-2 on macrophages were performed from the whole-cell lysate. All the samples were probed with β-tubulin to show equal protein loading. Results shown are representative of three independent experiments. *Significant difference with respect to uninfected control macrophages ($P < 0.05$). SAM-*S. aureus*-infected macrophages.

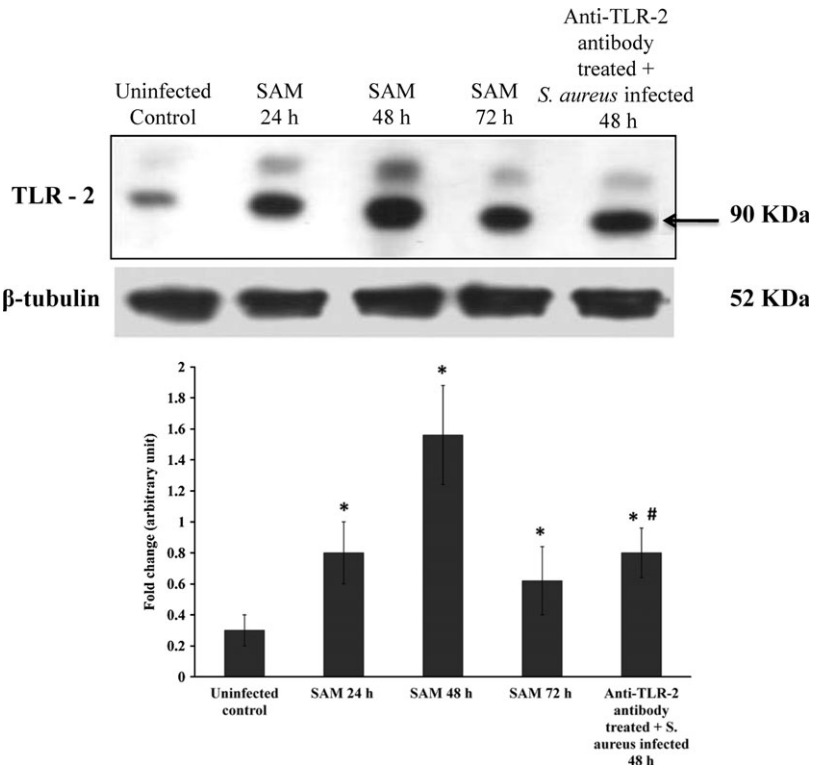


Figure 2 Up-regulated expression of toll-like receptor 2 (TLR-2) by live *Staphylococcus aureus* in murine peritoneal macrophages. The peritoneal macrophages of Swiss albino mice were isolated. Murine peritoneal macrophages (5×10^6 cells/ml) were infected with *S. aureus* (5×10^6 CFU/ml) for 24, 48 and 72 h at 37 °C. Whole-cell lysates were prepared for the analysis of TLR-2 by western blot. All the samples were probed with β-tubulin to show equal protein loading. Results shown are representative of three independent experiments. *Significant difference with respect to uninfected control macrophages. #Significant difference with respect to SAM 48 h ($P < 0.05$). SAM-*S. aureus*-infected macrophages.

Table 1 Colony-forming unit of the engulfed *Staphylococcus aureus* recovered after time-dependent phagocytosis with murine peritoneal macrophages in the presence or absence of anti-toll-like receptor-2 (TLR-2) antibody.

Groups	CFU count/ml of cell-free lysate (Mean ± SD)
S. aureus-infected macrophage for 24 h	360 ± 28.28
S. aureus-infected macrophage for 48 h	560 ± 42.42
S. aureus-infected macrophage for 72 h	720 ± 56.56
Anti-TLR-2 antibody-treated Macrophage + *S. aureus* infection for 24 h	250 ± 14.20*
Anti-TLR-2 antibody-treated Macrophage + *S. aureus* infection for 48 h	420 ± 28.28*
Anti-TLR-2 antibody-treated Macrophage + *S. aureus* infection for 72 h	340 ± 14.42*

Staphylococcus aureus (5×10^6 CFU/ml) were allowed to interact with murine peritoneal macrophages (5×10^6 CFU/ml) in the presence or absence of anti-TLR-2 antibody and incubated for different times at 37 °C. Macrophages were lysed, plated and incubated overnight to obtain the CFU of the intracellularly survived bacteria next day. Results were shown as mean ± SD of three independent experiments.
*Significant difference compared with *S. aureus* infected (antibody untreated) group ($P < 0.05$).

alone (Table 2). However, the SOD activity has been found to be increased due to TLR-2 blocking before *S. aureus* infection than that of macrophages infected only with *S. aureus* (Table 3).

Alteration in antioxidant enzyme activity from the recovered S. aureus after time-dependent phagocytosis in the presence or absence of antibody

Crude bacterial lysates of the recovered bacteria after time-dependent phagocytosis in presence and absence of anti-TLR-2-mediated receptor blocking were prepared as mentioned previously. A marked decrease in the bacterial catalase enzyme activity has been observed in the cell-free lysate of recovered bacteria when macrophages were incubated with anti-TLR-2 antibody prior to *S. aureus* infection than that of macrophages infected with *S. aureus* alone (Table 4). However, the SOD activity from the recovered bacteria has been found to be increased due to TLR-2 blocking before *S. aureus* infection than that of macrophages infected only with *S. aureus* (Table 5).

Alteration in catalase expression from the recovered S. aureus after time-dependent phagocytosis

Crude bacterial lysates from the recovered bacteria after time-dependent phagocytosis in the presence and absence of anti-TLR-2-mediated receptor blocking were prepared as described earlier. A marked decrease in the bacterial catalase protein has been observed in the cell-free lysate of recovered bacteria when macrophages were incubated with anti-TLR-2 antibody prior to *S. aureus* infection than that of macrophages infected with *S. aureus* alone

Table 2 Alteration in catalase enzyme activity after toll-like receptor-2 (TLR-2) blocking followed by *Staphylococcus aureus* infection.

Groups	Unit of superoxide dismutase (SOD)/mg of protein in the medium (culture supernatant)	Unit of SOD/mg of protein in the lysate (cell-free extract)
S. aureus-infected macrophage for 24 h	3.8 ± 0.3	3.77 ± 0.26
S. aureus-infected macrophage for 48 h	4.8 ± 0.2	6.56 ± 0.82
S. aureus-infected macrophage for 72 h	6.2 ± 0.34	5.32 ± 0.84
Anti-TLR-2 antibody-treated macrophage + *S. aureus* infection for 24 h	9.85 ± 1.85*	11.82 ± 2.80*
Anti-TLR-2 antibody-treated macrophage + *S. aureus* infection for 48 h	11.66 ± 0.16*	26.80 ± 0.12*
Anti-TLR-2 antibody-treated macrophage + *S. aureus* infection for 72 h	11.72 ± 0.62*	14.24 ± 0.24*

Catalase activity was expressed in terms of nmole/min mg of protein in the supernatant or lysate. Results are shown as mean ± SD of three independent experiments.
*Significant difference at $P < 0.05$ level compared to *S. aureus*-infected (antibody untreated) group.

Table 3 Alteration in superoxide dismutase (SOD) enzyme activity after toll-like receptor-2 (TLR-2) blocking followed by *Staphylococcus aureus* infection.

Groups	n mole/min mg protein in the medium (culture supernatant)	n mole/min mg protein in the lysate (cell-free extract)
S. aureus-infected macrophage for 24 h	33.73 ± 3.68	37.33 ± 3.26
S. aureus-infected macrophage for 48 h	43.52 ± 0.76	46.56 ± 0.82
S. aureus-infected macrophage for 72 h	56.92 ± 0.29	65.32 ± 1.84
Anti-TLR-2 antibody-treated macrophage + *S. aureus* infection for 24 h	19.85 ± 1.85*	21.82 ± 2.80*
Anti-TLR-2 antibody-treated macrophage + *S. aureus* infection for 48 h	15.66 ± 0.16*	16.80 ± 0.12*
Anti-TLR-2 antibody-treated macrophage + *S. aureus* infection for 72 h	11.72 ± 0.62*	14.24 ± 0.24*

Superoxide dismutase activity was expressed in terms of unit of SOD/mg of protein in the supernatant or lysate. Results are shown as mean ± SD of three independent experiments.
*Significant difference at $P < 0.05$ level compared with *S. aureus*-infected (antibody untreated) group.

Table 4 Alteration in catalase enzyme activity of whole staphylococcal cells from the recovered *Staphylococcus aureus* after time-dependent phagocytosis in the presence or absence of antibody.

Groups	Recovered bacteria after phagocytosis (hours)	Bacterial Catalase activity (n mole/min mg bacterial protein)
S. aureus-infected macrophage	24	35.63 ± 2.36
S. aureus-infected macrophage	48	48.68 ± 2.82
S. aureus-infected macrophage	72	46.65 ± 2.08
Anti-TLR-2 antibody-treated macrophage + *S. aureus* infection	24	18.60 ± 0.14*
Anti-TLR-2 antibody-treated macrophage + *S. aureus* infection	48	17.24 ± 0.26*
Anti-TLR-2 antibody-treated macrophage + *S. aureus* infection	72	14.26 ± 0.12*

The recovered *S. aureus* after time-dependent phagocytosis in the presence or absence of anti-toll-like receptor-2 (TLR-2) antibody were used to determine catalase activity in the presence of 15 μmoles of H_2O_2/ml of phosphate buffer. Catalase activity was expressed in terms of m. mole/min mg protein. Results were shown as mean ± SD of three independent experiments.
*Significant difference at $P < 0.05$ level compared with *S. aureus*-infected (antibody untreated) group.

Table 5 Bacterial superoxide dismutase (SOD) activity from the recovered *Staphylococcus aureus* after time-dependent phagocytosis.

Groups	Recovered bacteria after phagocytosis (hours)	Bacterial SOD activity (Units of SOD/mg bacterial protein)
S. aureus-infected macrophage	24	13.77 ± 1.36
S. aureus-infected macrophage	48	18.68 ± 1.82
S. aureus-infected macrophage	72	26.65 ± 2.08
Anti-TLR-2 antibody-treated macrophage + *S. aureus* infection	24	18.60 ± 1.14*
Anti-TLR-2 antibody-treated macrophage + *S. aureus* infection	48	37.24 ± 2.26*
Anti-TLR-2 antibody-treated macrophage + *S. aureus* infection	72	44.26 ± 4.12*

The recovered *S. aureus* after time-dependent phagocytosis in the presence or absence of anti-toll-like receptor-2 (TLR-2) antibody were used to determine SOD activity and was expressed in terms of unit of SOD/mg of bacterial protein. Results were presented as mean ± SD of three independent experiments.
*Significant difference at $P < 0.05$ level compared with *S. aureus*-infected (antibody untreated) group.

(Fig. 3). However, we did not perform the protein expression for SOD from the recovered bacteria in this system.

Catalase assay of the cell-free lysates of murine peritoneal macrophages after 48 h of infection with S. aureus in the presence or absence of ATZ or anti-TLR-2 antibody

Table 6 shows a marked increase in the catalase release in the supernatant or lysate of peritoneal macrophages infected with *S. aureus* after 48 h of phagocytosis compared with the non-infected control group. A marked decrease in the catalase release was observed in the lysates of ATZ-treated murine peritoneal macrophages after 48 h of infection compared with the ATZ non-treated infected group. This decrease in the catalase release was even more prominent when macrophages were pretreated with ATZ and anti-TLR-2 antibody before *S. aureus* infection.

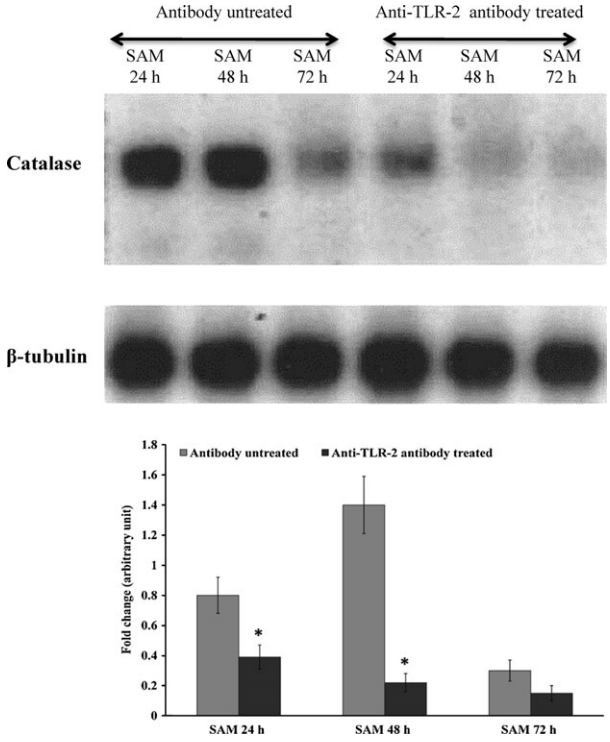


Figure 3 Expression of catalase from recovered bacteria. Protein samples from the recovered bacteria after time-dependent phagoytosis in the presence or absence of antitoll-like receptor-2 antibody were subjected to SDS-PAGE and transferred onto PVDF membranes. Membranes were probed with rabbit anti-serum to catalase and immunoreactivity was detected with a goat anti-rabbit IgG horseradish peroxidase HRP)-conjugated antibody (Abcam). Blots were developed with a chemiluminescent substrate according to the manufacturer's instructions. *Significant difference with respect to antibody untreated SAM ($P < 0.05$). SAM- *Staphylococcus aureus*-infected macrophages.

Intracellular survival of S. aureus in macrophages

Table 7 shows intracellular survival of *S. aureus* in macrophages when ATZ pretreated macrophages were infected either with recovered *S. aureus* from anti-TLR-2 antibody treated or anti-TLR-2 antibody non-treated macrophages. We found that pretreatment of macrophages with ATZ significantly reduces the bacterial burden in macrophages when they were infected only with the recovered *S. aureus* from the anti-TLR-2 antibody-treated macrophages (Table 7).

Effect of ATZ on the catalase activity of S. aureus recovered after 48 h of infection

We have found that incubation of H_2O_2-stimulated whole staphylococcal cells (recovered from anti-TLR-2 antibody untreated macrophages) with ATZ for 30 min inhibited the catalase release maximally, after which the inhibitory effect of ATZ on catalase release seemed to have declined when compared to with ATZ non-treated bacteria. Identical results were also obtained for the *S. aureus* recovered from anti-TLR-2 antibody treated macrophages, when stimulated with H_2O_2 in the presence or absence of ATZ (Fig. 4).

Tumour necrosis factor α, Interferon-gamma, interleukin-6, interleukin-1beta, interleukin-10 and interleukin 12p40 released in the media after phagocytosis

Figure 5 showed a gradual decrease in the TNF-α, IL-6, IL-10, IL-1β level in the media (culture supernatant) with increase in phagocytic time in the presence of anti-TLR-2 antibody significantly ($P < 0.05$) when compared to the cytokine production after time–dependent phagocytosis with no antibody. However, the IFN-γ and IL-12p40 level has been found to be significantly ($P < 0.05$) increased due to TLR-2 blocking before *S. aureus* infection than that of macrophages infected only with *S. aureus* (Fig. 5A–F).

Determination of H_2O_2 release by the murine peritoneal macrophages after S. aureus infection

The amount of H_2O_2 (μM/10^6 cells) released in the supernatant and lysates were estimated. The increase in the H_2O_2 released in supernatant for 24 h (13.0 ± 0.3), 48 h (15.7 ± 0.21) and 72 h (15.8 ± 0.4) of phagocytic time in the presence of antibody were found to be significant ($P < 0.05$) when compared to the H_2O_2 release after time-dependent phagocytosis with no antibody (Fig. 6A) The amount of H_2O_2 (μM/10^6 cells) released in the lysates (Fig. 6B) only at 72 h of phagocytosis in the presence of antibody were found significantly higher than that of released at 72 h of phagocytosis with no anti-TLR-2 antibody.

Table 6 Catalase release in the lystaes of 3-amino, 1, 2, 4-triazole (ATZ) pretreated or non-treated peritoneal macrophages infected with *Staphylococcus aureus* in the presence or absence of anti-toll-like receptor-2 (TLR-2) antibody.

Groups	Catalase activity from the culture supernatant (m. mole/min. mg tissue protein)	Catalase activity from the cell lysate (m. mole/min. mg tissue protein)
Without aminotriazole (ATZ) pretreatment		
Control macrophage	10.54 ± 0.93	10.89 ± 0.85
Macrophage + anti-TLR-2 antibody	8.83 ± 0.71	8.03 ± 0.79
Macrophage + *S. aureus*	44.65 ± 2.36*	46.07 ± 2.84*
Macrophage + anti-TLR-2 antibody + *S. aureus*	15.66 ± 1.27#,$	16.98 ± 1.41#,$
With pretreatment of aminotriazole (ATZ)		
Macrophage + ATZ	8.79 ± 0.89	7.41 ± 0.67
Macrophage + ATZ + anti-TLR-2 antibody	6.13 ± 0.45	7.11 ± 0.52
Macrophage + ATZ + *S. aureus*	24.57 ± 1.89*	21.57 ± 1.61*
Macrophage + ATZ + anti-TLR-2 antibody + *S. aureus*	12.24 ± 1.18#,$	11.32 ± 1.04#,$

In ATZ pretreated or non-treated sets, macrophages were treated with 1 mM 3 amino 1, 2, 4 triazole (ATZ) solution at 37 °C for 15 min and were allowed to interact with equal numbers of *S. aureus* and incubated at 37 °C for 48 h in presence or absence of anti-TLR-2 antibody. Cell-free lystates from both ATZ treated and non-treated sets were prepared. Catalase activity in the supernatant and lystaes was determined spectrophotometrically at 240 nm. Catalase activity was expressed in terms of m mole/min mg protein. Results were shown as mean ± SD of three independent experiments.
*Significant difference in comparison with control macrophage ($P < 0.05$), in both ATZ pretreated and non-treated group.
#Significant difference in comparison to macrophage treated with *S. aureus* ($P < 0.05$), in both ATZ pretreated and untreated group.
$Significant difference in comparison to macrophage treated with anti-TLR-2 antibody ($P < 0.05$), in both ATZ pretreated and untreated group.

Table 7 Intracellular survival assay.

Experimental groups	CFU/ml macrophage (mean ± SD)
Intracellular survival assay of macrophages infected with the recovered bacteria from the anti-TLR-2 antibody non-treated macrophages	
Staphylococcus aureus-infected macrophage 24 h	356 ± 28.51
S. aureus-infected macrophage 48 h	498 ± 31.47
S. aureus-infected macrophage 72 h	624 ± 42.31
ATZ-pretreated macrophage + *S. aureus*-infected 24 h	405 ± 27.54
ATZ-pretreated macrophage + *S. aureus*-infected 48 h	514 ± 46.39
ATZ-pretreated macrophage + *S. aureus*-infected 72 h	708 ± 51.28
Intracellular survival assay of macrophages infected with the recovered bacteria from the anti-TLR-2 antibody-treated macrophages	
S. aureus-infected macrophage 24 h	355 ± 21.56
S. aureus-infected macrophage 48 h	465 ± 43.12
S. aureus-infected macrophage 72 h	560 ± 43.69
ATZ-pretreated macrophage + *S. aureus*-infected 24 h	210 ± 19.06*
ATZ-pretreated macrophage + *S. aureus*-infected 48 h	345 ± 23.15*
ATZ-pretreated macrophage + *S. aureus*-infected 72 h	407 ± 31.27*

The bacteria recovered after 48 h of phagocytosis in the presence or absence of anti-toll-like receptor-2 (TLR-2) antibody were further cultured and used for intracellular survival assay. Freshly isolated peritoneal macrophages were treated with 1 mM 3-amino, 1, 2, 4-triazole (ATZ) solution, an intracellular catalase inhibitor at 37 °C for 30 min. After proper washing, the ATZ pretreated or non-treated macrophages were allowed to interact with equal numbers of *S. aureus* and incubated at 37 °C for 24, 48 and 72 h. Number of surviving bacteria was represented as CFU/ml of macrophages. Results were shown as mean ± SD of three independent experiments.
*Significant difference in ATZ pretreated macrophage + *S aureus* infection in comparison with macrophages infected with *S. aureus* alone group.

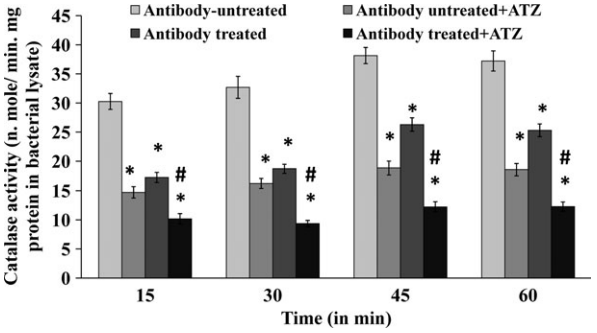


Figure 4 Effect of 3-amino, 1, 2, 4-triazole (ATZ) on the intracellularly survived whole-cell staphylococcal Catalase activity after 48 h of phagocytosis. The intracellularly survived staphylococcal cells recovered after 48 h of phagocytosis in the presence or absence of anti-toll-like receptor-2 antibody or ATZ were used to determine catalase activity in the presence of 15 μmoles of H_2O_2/ml of phosphate buffer. Recovered *Staphylococcus aureus* (1.5×10^8 CFU/ml) were further allowed to interact with 1 mM ATZ for 15, 30, 45 and 60 min, respectively, at 37 °C followed by centrifugation and collection of supernatant. Catalase contents of the supernatants were estimated spectrophotometrically. Catalase activity was expressed in terms of m mole/min mg protein. Results were shown as mean ± SD of three independent experiments. *indicates significant difference with respect to antibody untreated macrophages; #indicates significant difference with respect to antibody treated macrophages at $P < 0.05$.

Determination of nitric oxide production by the murine peritoneal macrophages after S. aureus infection

The amount of NO ($\mu M/10^6$ cells) released in the supernatant and lysates were measured. The increase in the NO released in supernatant (Fig. 7A) for 24 h (13.21 ± 0.62), 48 h (17.46 ± 1.64) and 72 h (19.83 ± 1.24) of phagocytic time in the presence of antibody were found to be significant ($P < 0.05$) when compared to the NO release after time–dependent phagocytosis with no anti-TLR-2 antibody (Fig. 7A). The amount of NO ($\mu M/10^6$ cells) released in the lysates (Fig. 7B) at 24 h (9.12 ± 0.21), 48 h (9.8 ± 0.23) and 72 h (9.7 ± 0.7) h of phagocytosis in the presence of antibody were found significantly higher

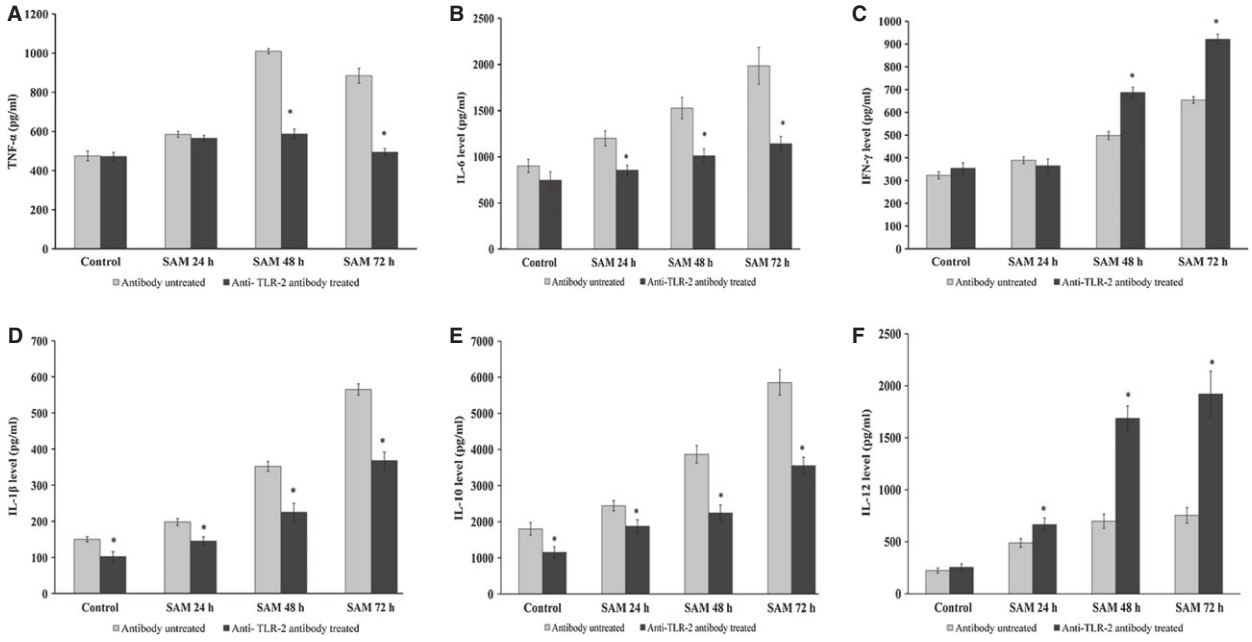


Figure 5 (A–F) Cytokine release in the supernatant. Levels of tumour necrosis factor-α (TNF-α) (A), IL-6 (B), interferon-gamma (IFN-γ) (C) IL-1β (D), IL-10 (E) and IL-12p40 (F) in the supernatants collected after 24, 48, 72 h *Staphylococcus aureus* –infected macrophages in the presence or absence of anti-toll-like receptor-2 antibody were determined by utilizing ELISA according to the manufacturer's recommendations and were expressed from triplicate experiments. Without infection control versus *S. aureus* AG-789 alone, *, indicates significant difference in TNF-α, IFN-γ, IL-6, IL-1β, IL-10 and IL-12 levels with respect to antibody untreated SAM at each time point ($P < 0.05$); SAM- *S. aureus*-infected macrophages.

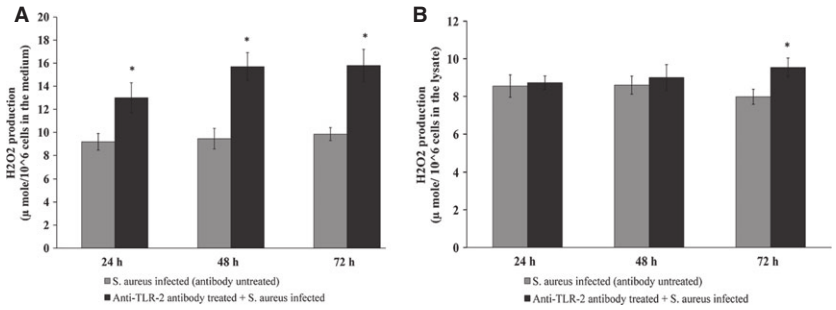


Figure 6 Evaluation of hydrogen peroxide (H_2O_2) release by the murine peritoneal macrophages after *Staphylococcus aureus* infection in the presence or absence of anti-toll-like receptor-2 (TLR-2) antibody. Murine peritoneal macrophages (5×10^6 cells/ml) were allowed to interact with *S. aureus* (5×10^6 CFU/ml) in a 1:1 cell/bacterium ratio incubated at 37 °C cell culture incubator for different times in the presence and absence of anti TLR-2 antibody. Supernatants (A) and lysates (B) were prepared as mentioned earlier. H_2O_2 content was expressed in terms of μmole/10^6 cells. Results are shown as mean ± SD of three independent experiments. * Significant difference compared with *S. aureus*-infected (antibody untreated) group ($P < 0.05$).

than that of released in the lysates with no anti-TLR-2 antibody.

MyD88 and NF-KB expression by murine peritoneal macrophages was affected due to surface TLR-2 blocking

Toll-like receptor-2 serves as the primary receptor for *S. aureus*. Myeloid differentiation factor 88 (MyD88) is the central adapter protein for signal transduction of all TLRs, with the exception of TLR-3. In this investigation, no increase in Swiss albino peritoneal macrophage expression of MyD88 was detected in response to *S. aureus* stimulation. Neither were the levels of MyD88 molecules reduced by blockade of TLR-2 with anti-TLR-2 antibody (Fig. 8). The intensity of the signals derived from MyD88 does not seem to differ in the samples obtained with and without anti-TLR-2 antibody treatment. Western blot analysis of nuclear proteins showed that viable *S. aureus* infection-induced NF-κB activation in murine peritoneal macrophages of Swiss albino mice. However, preincubation with anti-TLR-2 antibody before *S. aureus* infection at 48 h increased the expression of the NF-κB (Fig. 8) when compared to the *S. aureus* infection alone at 48 h.

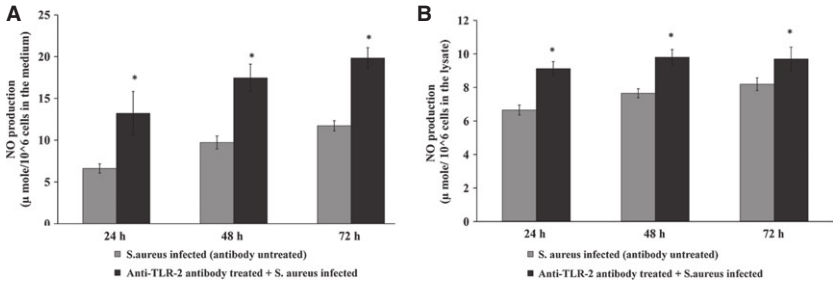


Figure 7 Evaluation of nitric oxide (NO) release by the murine peritoneal macrophages after *Staphylococcus aureus* infection in the presence or absence of anti-toll-like receptor-2 (TLR2) antibody. Murine peritoneal macrophages (5×10^6 cells/ml) were allowed to interact with *S. aureus* (5×10^6 CFU/ml) in a 1:1 cell/bacterium ratio incubated at 37 °C cell culture incubator for different times in the presence and absence of anti-TLR-2 antibody. Results in this figure represent mean NO content (μM) in the medium (A) or from cell-free lysate (B) of murine peritoneal macrophages from different groups of triplicate experiments. *Significant difference compared with *S. aureus*-infected (antibody untreated) group ($P < 0.05$).

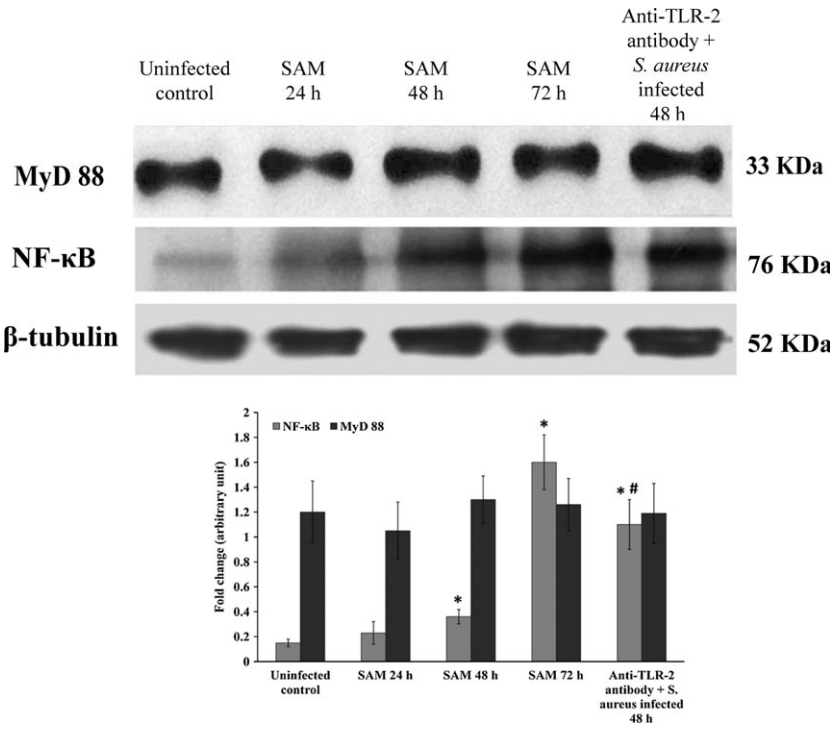
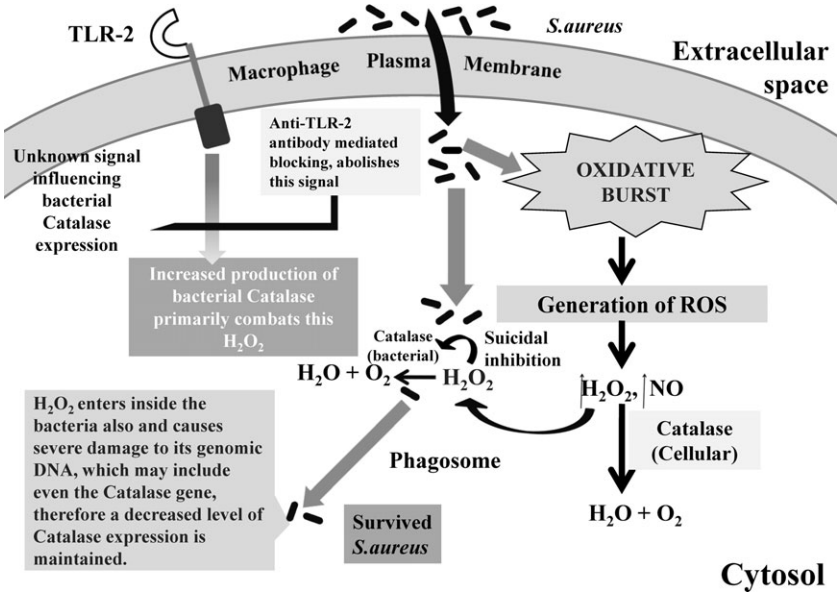


Figure 8 Effect of anti-toll-like receptor-2 (TLR2) antibody on MyD88 and NF-κB expression in *Staphylococcus aureus*-stimulated murine peritoneal macrophages: Murine peritoneal macrophages (5×10^6 cells/ml) were preincubated with anti-TLR-2 antibody. Cells were then infected with *S. aureus* (5×10^6 CFU/ml) for different times as indicated. Whole-cell lysates were then prepared for the analysis of MyD88 and NF-kB by Western blot as described previously. All the samples were probed with β-tubulin to show equal protein loading. Results shown are representative of three independent experiments. *Significant difference with respect to uninfected control macrophages. #Significant difference with respect to antibody untreated SAM 48 h. SAM-*S. aureus*-infected macrophages.

Discussion

The presence of specific TLR-2 receptor on the surface of murine peritoneal macrophages of Swiss albino mice has been reported in this study. Blocking of cell surface TLR-2 by antibody enhances the peritoneal macrophage's ability to clear staphylococcal infection, attenuates pro-inflammatory cytokine production and increases secretion of IL-12, IFN-γ and production of H_2O_2 and NO with an elevated NF-KB expression. We have also established that peritoneal macrophage respond to stimulation with *S. aureus*, that is, TLR-2 ligand, according to previous reports [43], and this effect is partially, but significantly, decreased by anti-TLR-2 antibodies. Previous studies indicated that 10 μg/ml of a polyclonal receptor–neutralizing antibody to TLR-2 was sufficient to saturate TLR-2 receptors in 10^6 macrophages [34] indicating saturation of cell surface TLR-2 in Swiss mice macrophages using this amount of anti-TLR-2 antibody.

It has long been known that professional phagocytes may serve as intracellular reservoirs of *S. aureus*. In keeping with this idea, recent *in vitro* studies have confirmed high-level resistance by *S. aureus* to macrophage-mediated killing [44, 45]. It may be suggested that macrophage cell surface TLR-2 of Swiss mice were involved in the recognition of viable *S. aureus*. It can also be suggested that prior incubation of macrophages with anti-TLR-2 antibody might lead to reduced recognition of viable bacteria;



Scheme 1. Schematic representation of the study.

however, the number of bacteria that have already been internalized were killed by the macrophages. As there was no further recognition as well as internalization due to receptor blocking, less intracellular survival inside the macrophages might be suggested. However, our data indicate that blocking of TLR-2 acts to accelerate the elimination of bacteria. This is in accordance with the previous observation that TLR-2 responds to pathogenic microbes and activates signalling pathways that lead to the production of the immunosuppressive environment [16]. It is therefore possible that TLR-2 is an immunosuppressive PRR, in both humoral and cellular innate immune responses, at least for certain types of microbes. The absence of TLR-2 has also been shown to provide protective effects to the host organism in certain models [7].

Reactive oxygen species, such as hydrogen peroxide and superoxide, are generated during the macrophage respiratory burst in response to microbial infection. Bacterial pathogens must overcome the toxic effects of ROS to establish infections. Production of SOD and catalase enzymes, which decompose superoxide and peroxide radicals, respectively, have been reported to contribute to the virulence of a number of pathogens. Thus, the ability of catalase to decompose peroxide radicals increases survival of bacteria in the presence of peroxide [46, 47].

Catalase is an enzyme that is involved in oxidative stress resistance and converts H_2O_2 generated during cellular metabolism to water and molecular oxygen. As a result, catalase has been proposed as a potential virulence factor in many bacterial pathogens [48, 49], because its activity might protect them from the ROS generated by eukaryotic cells, mainly by the inflammatory cells during phagocytosis. In *S. aureus*, catalase has been implicated as a virulence-determinant [50]. However, recent studies with *S. aureus* katA mutants, deficient in catalase enzyme, have revealed no differences in virulence with the corresponding wild-type strains in different murine models of infection [36]. As we obtained reduced catalase activity after TLR-2 blocking, degradation of macrophage-derived H_2O_2 by catalase is prevented, leading to enhanced killing of *S. aureus* by H_2O_2 and less intracellular survival is suggested.

Contribution of catalase was further verified by the assay of catalase in the presence of ATZ. Previous studies using ATZ, a specific catalase inhibitor, have marked their role in endogenous catalase inhibition and thereby leading to a moderate rise in H_2O_2 production [51] and subsequently a greater host microbicidal activity. Pretreatment of macrophages with ATZ specifically inhibited the macrophage-derived catalase; therefore, the enzyme activity obtained in the supernatant or lysate indicated that the released catalase was of bacterial origin. This decrease was even more prominent when macrophages were pretreated with ATZ as well as anti-TLR-2 antibody before infection.

In support of this argument, we established the whole staphylococcal catalase activity after stimulation with H_2O_2. The amount of catalase released by the bacteria in ATZ non-treated set was quite higher compared with the ATZ treated set and established our speculation that the released catalase was of bacterial origin.

Moreover, the bacteria recovered after time-dependent phagocytosis in the presence of anti-TLR-2 antibody also shows reduced catalase activity. This might indicate that reduced bacterial catalase enzyme activity is the limiting factor for less intracellular survival of *S. aureus* inside the macrophages due to TLR-2 blocking. This phenomenon of

decreased catalase activity of the recovered bacteria after phagocytosis in the presence of anti-TLR-2 antibody may suggest that following phagocytosis, a signal might have been transmitted in macrophages, the bacterium that remain viable inside the cell may express some factors which promote growth, leading to expression of some genes which can downregulate or repress the catalase gene or mutate the bacteria. However, the mutation directly associated with loss of catalase activity has not been identified [52].

We have demonstrated the marked decrease in the catalase activity of the bacteria recovered after phagocytosis in the presence of anti-TLR-2 antibody. This also suggests an increase in released H_2O_2 levels after phagocytosis. These changes in recovered bacterial catalase activity after phagocytosis were not due to loss of catalase during the washing procedure or culture in medium. It has been reported that catalase cannot be saturated by its substrate, to a certain limit, this enzymes works faster when more H_2O_2 is available [53]. This may support that decreased level of catalase in bacteria was maintained after they were recovered and cultured in medium. The reason for low catalase activity in the bacteria isolated after engulfment by macrophages may be given (Scheme 1) as follows: (1) The reduced activity of catalase in the *S. aureus* after phagocytosis by macrophages may lie in excess H_2O_2 in macrophages diffusing in to the bacterial cell where a slight increase in the level of H_2O_2 may cause 'suicidal inactivation' of bacterial catalase by its own substrate [54]. Suicidal inactivation of catalase and peroxidases by their substrate such as H_2O_2 has been reported, (2) diffusion of H_2O_2 in to bacterial and subsequent diffusion of some H_2O_2 in to the macrophages from the host cell, in fact does not cause much change in the level of H_2O_2, or (3) blockage by TLR 2 antibody may trigger H_2O_2 generation from some non-mitochondrial pathway, and as catalase remains inhibited, this H_2O_2 addition does not get metabolized quickly and that may be the reason for H_2O_2 level not getting altered in macrophages even after block by TLR 2 antibody [55].

It has been reported that long-term cultures (72-h post-infection) of infected phagocytes permitted recover of viable wild-type *Campylobacter jejuni,* in contrast no viable KatA mutant bacteria have been recovered. This observation indicates that catalase is essential for intramacrophage persistence and growth and suggests a novel mechanism for intracellular survival [49]. Moreover, bacteria recovered 72-h post-infection remained culturable, survived and multiplied significantly higher numbers. In a recent study it has been shown that *S. aureus* phagocytosed by human monocyte derived macrophages can survive intracellularly for 4–7 days without affecting cell viability. This proceeds until the cells are suddenly lysed by escaping bacteria, which then go on to proliferate to high numbers [2]. These findings also supported the maintenance of virulence factors (e.g. catalase) even after recovery from long-term culture or phagocytosis. It seems that some intracellular bacteria are capable of resisting several hours or even for days the microbicidal assault by neutrophils, which in time may release live bacteria on sites [56] and maintain its existence over there.

SODs are responsible for converting superoxides to hydrogen peroxide and oxygen. Thereafter, catalase converts hydrogen peroxide to water and oxygen. In comparison with catalase, SOD activity is increased after phagocytosis in the presence of anti-TLR-2 antibody. Enhanced SOD activity might indicate a greater accumulation of H_2O_2 inside the cell-mediating enhanced bacterial killing.

We have measured the catalase and SOD activity from the recovered bacteria after time-dependent phagocytosis in the presence or absence of anti-TLR-2 antibody to find out the origin of catalase enzyme in the recovered bacteria. The decreased catalase and increased SOD in the bacterial lysate of the recovered bacteria might indicate their role in scavenging murine macrophage–derived H_2O_2. Our findings raise the possibility that decreased catalase may enhance H_2O_2 accumulation and ultimately limit H_2O_2 decomposition. This data confirmed the role catalase and SOD in the bacterial intracellular survival and that the released catalase and SOD during phagocytosis were predominantly of bacterial origin. This was further verified by the Western blot analysis of the crude bacterial lysate of the recovered bacteria after phagocytosis. Therefore, here, we report that reduced staphylococcal catalase expressions due to surface TLR-2 blocking might protect the host macrophage-derived H_2O_2 leading to bacterial killing by murine peritoneal macrophages of Swiss albino mice. It has also been reported that absence of catalase reduces long-term survival of *Helicobacter pylori* in macrophage phagosomes [48]. As a result, strains that do not produce catalase have reduced virulence [57].

It has to be elucidated whether parts of the ROS in phagolysosomes may be a trigger for the stringent response in *S. aureus* [58]. Little is known about the intracellular behaviour of *S. aureus*. Growing evidence suggests that *S. aureus* can survive after phagocytosis and persists in neutrophils or macrophages to hide from the immune system, as well as to travel to and infect distant sites in the host [59, 60]. However, the importance of single virulence factors, as of catalase in this case, contributing to intracellular survival seems to be dependent on the type of host cell and bacterial strains analysed. Further studies using Swiss albino peritoneal macrophages will delineate this hypothesis in future.

Additionally, we performed the intracellular survival of *S. aureus* in macrophages, where ATZ pretreated macrophages were infected either with recovered *S. aureus* from anti-TLR-2 antibody treated or anti-TLR-2 antibody non-treated macrophages, we found that pretreatment of

macrophages with ATZ significantly reduces the bacterial burden in macrophages when they were infected with the recovered *S. aureus* from the anti-TLR-2 antibody-treated macrophages (Table 7). As the recovered bacteria from anti-TLR-2 antibody-treated macrophages have decreased catalase expression, after infecting the freshly isolated and ATZ pretreated macrophages with them the degradation of H_2O_2 either by macrophage derived catalase or by the bacterial catalase is not possible; therefore, the H_2O_2-mediated killing of bacteria is favoured and we obtained reduced intracellular survival as evident from Table 7. This was also identical with our previous findings where we have reported that pretreatment of mice with ATZ reduces the bacterial burden in the blood and spleen after *in vivo* infection with the recovered bacteria [35]. However, infection of freshly isolated and ATZ pretreated macrophages with the recovered bacteria from anti-TLR2 antibody non-treated macrophages shown non-significant increased intracellular survival. It may be hypothesized that since the bacteria that were recovered from anti-TLR-2 antibody non-treated macrophages, the bacteria might have increased or normal catalase expression, which can decompose the macrophage derived H_2O_2, as a result the bacteria survived inside the macrophage and shows increased CFU (Table 7), although it is insignificant.

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 analysed the response of murine peritoneal macrophages to viable bacteria. Heat killing might denature the cell wall in a way that buries TLR-2 ligands such that they are harder for the receptor to see without proteolytic processing of the cell wall or without concentrating TLR-2 in phagosomal membranes. As a high ratio of IL-10 to TNF-α was associated with fatal outcome in patients with infection [61], we focused our study on the production of pro-inflammatory and an anti-inflammatory cytokines. Measurement of TNF-α, IL-1β and IL-6 from the mononuclear cells after incubation with TLR ligand for TLR-2 is suggested as a clinical assay to evaluate toll-like-receptor function and to find out possible molecular defect in the innate immune system related to TLR function [62]. This data demonstrate activation of Swiss albino peritoneal macrophage cells in response to bacteria resulting in production of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6. Furthermore, TNF-α, IL-1β and IL-6 production by *S. aureus* stimulated Swiss albino peritoneal macrophage was significantly reduced by incubation with the blocking anti-TLR-2 antibody. This has also corroborated with previous study where TLR-2-deficient macrophages demonstrated marked impairment in the *S. aureus*-induced TNF-α.

The data obtained in this study also demonstrates that production of inflammatory cytokines by Swiss albino peritoneal macrophage was attenuated by anti-TLR-2 antibody administered before *S. aureus* -stimulation. These results implicate antibody-mediated TLR-2 blockage on immune cells as a promising strategy for attenuation of potentially fatal host response amplification in the course of acute infection. Moreover, we also observed that the production of TNF-α, IL-1β and IL-6 from *S. aureus*-stimulated peritoneal macrophages occurred via a TLR-2-dependent-signalling mechanism. This is also supported by previous study where *S. aureus* induced IL-6 production was inhibited in the presence of neutralizing anti-TLR-2 antibody [14, 63]. It was reported that TLR-4, but not TLR-2, blockade by antibody on human PBMCs inhibited rapid IFN- γ release but not IL-10 reduction upon *Escherichia coli* infection [64]. Furthermore, IFN- γ challenge enhanced TLR2-specific human PBMC activation if applied 3 h before TLR-2 challenge implicating IFN-γ as a MyD88 – driven inducer of regulation of surface TLR-2 expression and infection-related TLR-2 sensitivity [8]. Therefore, elevated IFN-γ and reduced IL-10 level after TLR-2 blockade might lead to activation of macrophages as the immunosuppressive effects of *S. aureus* induced IL-10 is withdrawn. It was reported that in the presence of IFN-γ, IL-12 is induced by *S. aureus* in monocytes/macrophages while IL10 is suppressed, leading to expression of high levels of MHC class II and costimulatory molecules. However, when macrophages are targeted by microbes (especially bacteria and some IL-10-producing/inducing viruses), such as *S. aureus*, which cause recurrent infections, high levels of IL-10 as well as Th17 promoting IL-6 and IL-23, but not IL-2 may be produced. High levels of IL-10 might inhibit the innate immune response by decreasing the release of inflammatory cytokines and simultaneously inhibiting antigen presentation by down modulating MHC class II molecules and costimulatory molecules [48]. Therefore, In the presence of increased IFN-γ due to TLR-2 blockade the elevated IL-12, while suppression of IL-10 is not surprising as evident from our study. Moreover, it is also reported that TLR-2 antibody treatment markedly reduced the level of IL-10 and restored the release of IFN-γ [17]. Therefore, increased IFN-γ might regulate macrophage activation and also determines the outcome of *S. aureus* infection.

Recent evidence also suggested that ligation of TLR-2 by pathogenic microorganisms such as fungi or mycobacteria induce a Th-2 anti-inflammatory bias either through the release of IL-10 or through inhibition of IFN-signalling. This leads to down modulation of the microbicidal function of leucocytes and evasion of host defence [65]. The increased levels of IFN-γ and IL-12 due to TLR-2 blockade before *S. aureus* infection appears to regulate the expression of TLR-2 and might be increased with the concentration and duration of treatment with bacterial pathogens, and the increase was amplified by several cytokines [66].

In the TLR2-mediated signal transduction pathway, the NF-κB pathway is closely related to the inflammatory response. Nuclear factor-κB is the generic name for a

family of dimers formed by a several proteins: NF-κB1 (also known as p50/p105), NF-κB2 (also known as p52/p100) and REL, RELA (also known as p65/NF-κB3) and RELB. The different heterodimers bind to specific promoters to initiate transcription of a wide range of genes that influence the inflammatory response as well as cell death and survival and tissue repair. The most common NF-κB dimer comprises a p65 and p50 heterodimer. IκBα binds NF-κB in the cytoplasm to block its nuclear translocation. A variety of stimuli lead to phosphorylation of critical serine residues on IκBα, targeting it for ubiquitination and degradation by the proteasome, thus allowing NF-κB to enter the nucleus and mediate transcription. This study indicates that nuclear expression of NF-κB in Swiss albino peritoneal macrophage was increased in response to *S. aureus* infection and was not inhibited by administration of anti-TLR-2 antibody before stimulation. These data support the hypothesis that TLR-2 induces inflammatory reactions through activation of NF-κB-signalling pathways. In this model signalling via pattern recognition receptors which activate NF-KB transcriptional factors may explain the partial activation of macrophages, during anti-TLR-2 blocking. We observed activation of NF-KB shortly after phagocytosis during TLR-2 blocking. Furthermore, it can be suggested that the increased production of IFN-γ and IL-12 by *S. aureus* – stimulated Swiss albino peritoneal macrophage associated with TLR-2 blockade is mediated by induction of NF-κB nuclear transfer at latter stages of infection. In this study, no increase in Swiss albino peritoneal macrophage expression of MyD88 was detected in response to *S. aureus* stimulation, as evident from the intensity of signals derived from MyD88 obtained from the samples with and without anti-TLR-2 antibody treatment. It has been reported that MyD88-dependent [6] and MyD88-independent pathways exist as part of TLR-mediated signalling pathways after bacterial infection. Thus, MyD88-independent pathways may also play a major role in TLR2-mediated signalling pathway in these Swiss albino mice. Previous results acquired after treatment with anti-IFN-γ suggest that NO release after LPS challenge is mediated through IFN-γ [67]. In our case, due to availability of the inducer cytokine like IFN-γ for iNOS, the NO level gets increased. Our results also indicated that elevated IL-12, which is one of the main inducers of IFN-γ in cells, leading to enhanced IFN-γ is involved in the regulation of NO production and to some extent gives protection also supported by earlier study [68, 69].

Conclusion

Blocking of cell surface TLR-2 by antibody enhances the peritoneal macrophage's ability to clear staphylococcal infection, attenuates pro-inflammatory cytokine production, and increases secretion of IL-12, IFN-γ, and production of H_2O_2 and NO with concomitant signalling of NF-κB. To understand whether catalase contributing in the intracellular survival, was of bacterial origin or not, 3-amino 1, 2, 4-triazole (ATZ) was used to inhibit specifically macrophage derived catalase. Catalase enzyme activity from the whole staphylococcal cells in presence of ATZ suggested that the released catalase were of extracellular origin. Catalase protein expression from the whole staphylococcal cells recovered after phagocytosis also indicated the catalase release from *S. aureus*. From the intracellular survival assay, it was evident that pretreatment of macrophages with ATZ reduces the bacterial burden in macrophages when infected with the recovered bacteria only from the anti-TLR-2 antibody treated macrophages after phagocytosis. Our findings raise the possibility that decreased catalase may help to enhance H_2O_2 accumulation and ultimately limit H_2O_2 decomposition. Therefore, here, we report that reduced staphylococcal catalase expressions due to surface TLR-2 blocking might protect the host macrophage derived H_2O_2 leading to bacterial killing by murine peritoneal macrophages of Swiss albino mice. The increased levels of IFN-γ and IL-12 and enhanced NF-κB expression due to TLR-2 blockade before *S. aureus* infection appear to regulate the killing of bacteria by inducing H_2O_2 and NO promotes bacterial killing.

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