RESEARCH ARTICLE

Simultaneous blockade of TLR4 and TNFR1 attenuates TLR2 sensitivity in LPS-stimulated macrophages through TNFR2-mediated pathway

Biswadev Bishayi[2*], Rajen Dey[1], Rituparna Ghosh[2], Mehak Kanwar[2]

ABSTRACT

Background: Recent studies have found TLR2 to be a significant player in initiating immune responses in the host during bacterial infection. Macrophage polarization is one of the vital factors in the amelioration of sepsis. It is well established that recognition and binding of LPS with cell surface TLR4 could induce the production of a wide array of pro-inflammatory cytokines that initiate an organism's inflammatory responses. Recent studies claimed that augmented expression of TLR2 shows better responsiveness to LPS, thus increasing its affinity to the ligand. *Objectives:* Our study attempts to demonstrate the underlying mechanisms of how TLR2 sensitivity is altered during the simultaneous blocking of TLR4 and TNFR1 and how TLR2 contributes towards the phenotypic switching of macrophages. We were also interested to see whether blocking TLR4, in any way, affects the LPS/TLR2 interactions and influences some major cytokine receptors. *Materials and Methods:* Murine peritoneal macrophages (5×10^6 cells/mL) were pre-treated with TLR4 and TNFR1 antibody (alone or in combination) and then stimulated with LPS for 60 minutes. FACS analyses were performed to determine M1 and M2 polarized cell populations. Assays from the cell-free supernatant determined ROS generation, and the activities of antioxidant enzymes were determined from the cell-free lysate. Western blot analysis was used to determine receptor expressions. *Results:* Our results indicated that blocking both receptors markedly reduced ROS levels due to its scavenging by the elevated antioxidant enzymes. Western blot data confirmed that combinatorial blockade of TLR4 and TNFR1 augmented TLR2 and TNFR2 expression in contrast to the attenuation of IL-1R. *Conclusion:* Therefore, the regulation of TLR2 expression was found to be TLR4-dependent, and it can show reduced NF-κB activation in response to LPS in TLR4 and TNFR1 blocked macrophages. Moreover, dual blocking can promote M2 polarization by up-regulating TNFR2. This approach could be taken as an alternative therapeutic strategy to treat LPS-sepsis.

Keywords: Inflammation, LPS stimulation, Macrophage, TLR2, TLR4, TNFR1.

Indian Journal of Physiology and Allied Sciences (2024); DOI: 10.55184/ijpas.v76i03.247 **ISSN:** 0367-8350 (Print)

INTRODUCTION

With its potential to trigger downstream inflammatory responses during bacterial infection, Toll-like receptor 2 (TLR2) plays a significant role in the early response of the innate immunity system.[1] TLR2 recognizes a wide variety of pathogens and the formation of TLR2 heterodimers with either other TLR members or non-TLR cellular molecules serves as a prerequisite in cellular activation initiation.[2] While earlier research has shown that TLR2 signaling inhibits nuclear factor-κB (NF-κB) p65[3] to control macrophage polarization, the underlying mechanisms remain unclear. Macrophages play a pivotal role in the pathophysiology of inflammatory responses.[4] Thus, their dysregulations become a major cause of immune alterations in lipopolysaccharide (LPS) sepsis. The primary constituent of the outer wall of the gram-negative bacteria, LPS, is one of the main causative agents of infection-induced septic shock.[5] Located on the cell surface, the TLR4 and TLR2 recognize microbial components like endotoxins (LPS) and superantigens, respectively.[6] Endotoxin-induced septic shock is a systemic toxic inflammatory response caused by excessive secretion of pro-inflammatory mediators, such as tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), and reactive oxygen species (ROS), mainly resulting in cytotoxicity.[7] NADPH oxidase, or NOX, expressed in phagocytes, also contributes to ROS generation. NOX has also been shown to have a role in immune cell polarization and differentiation.[8] The ROS generated has been further found to influence macrophage M1/M2 polarization, ultimately leading to NF-κB activation and pro-inflammatory gene transcription.[9]

LPS, on binding to TLR4, induces the production and release of critical pro-inflammatory cytokines, which activate inflammatory responses in an organism.[10] Interestingly, studies revealed that over-expression of TLR2 confers responsiveness to LPS stimulation in pleural mesothelial cells.[11] Thus, when TLR4 binds to its ligand, i.e. LPS, it results in an increased affinity of TLR2 towards its ligand.[12] Researchers suggested TLR-2 could activate NF-κB in response to LPS

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How to cite this article: Bishayi B, Dey R, Ghosh R, Kanwar M. Simultaneous blockade of TLR4 and TNFR1 attenuates TLR2 sensitivity in LPS-stimulated macrophages through TNFR2-mediated pathway. *Indian J Physiol Allied Sci* 2024;76(3):42-55.

Conflict of interest: None

Submitted: 13/05/2024 **Accepted:** 31/07/2024 **Published:** 30/09/2024

stimulation in mice.[13] Thus, studies on TLR-2-mediated LPS signaling might provide some clues on regulating sepsis.[14,15] Macrophages are specifically involved in the production of cytokines; indeed, TNF-α, IL-1β, IL-6, IL-10, and IL-12 are released by activated macrophages following the activation of TLR2 and TLR4.[16] On recognizing pathogen-associated molecular patterns (PAMPs) and disease-associated molecular patterns (DAMPs), TIR domain-containing adaptor proteins such as Myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) are recruited by the TLRs.[17] These initiate signal transduction pathways, which activate NF-κB, IRFs, or mitogen-activated protein kinase (MAP kinase). Since ROS have been found to play critical roles in sepsis,[18] their counteraction by the various anti-oxidants also requires significant attention. Superoxide dismutase (SOD) is one of the main anti-oxidants involved in regulating the redox balance in the event of the onset of sepsis,[19] alongside catalase (CAT). SOD acts as a first line of defense in biological systems by converting the superoxide radicals into hydrogen peroxide and molecular oxygen. Following its participation in Fenton's reaction, this H_2O_2 produces OH; H_2O is produced by GSH/GPx, and H_2O/O_2 is produced through CAT.[20] The adequate clearance of hydrogen peroxide thus requires sufficient concentration of these antioxidant enzymes. Excessive generation of these ROS during sepsis alongside suppressed levels of these anti-oxidants creates an imbalance leading to cellular damage.

Like many other molecules, a paradoxical role of TNF-α can be attributed to triggering a TNF-α-dependent cascade leading to either an inflammatory or anti-inflammatory process.[21] Since soluble TNF/TNFR1 may play a role in promoting inflammation and mTNF/TNFR2 might result in immune modulation and tissue regeneration,[22] new therapeutics that selectively target sTNF/TNFR1 have evolved. TNFR1-selective antagonists and sTNF-special antagonists have been suggested to leave the mTNF/TNFR2 signaling pathway undisturbed, which may diminish the detrimental effects caused by TNF-α.[23] This mechanism thus provides protective TNF-mediated responses by immune modulation without promoting inflammation. This means that TNFR2 can be the main receptor for controlling inflammation and suggests that mTNF and TNFR2 control the inflammatory process, although TNFR1 also plays a significant role in regulating the expression level of TNFR2 on immune cells.[24] Furthermore, identifying the cytoplasmic domain of the IL-1 receptor type I (IL-1R1) in the Toll protein[25] further demonstrated the significance of members of the IL-1 family to the innate response. The functional domain of the cytosolic component of IL-1R1/2 is termed the Toll interleukin-1 receptor (TIR) domain. The TIR domain is significantly homologous to the TIR domains of all the Toll-like receptors (TLR), including TLR4 and TLR2.[26]

A previously published study from our group demonstrated that the simultaneous blocking of TLR4 and TNFR1 facilitates the polarization of the M1 macrophages toward the M2 phenotype.[27] This phenotypic shift might be beneficial in designing an alternate therapeutic approach to treat sepsis. Therefore, in this study, an attempt has been made to investigate the contribution of single or dual blockade of the TLR4 and TNFR1 receptors over the sensitivity of TLR2 to ensure better protection against the inflammatory consequences of bacterial LPS-induced macrophage toxic responses, which have not been investigated so far. We are interested in finding out whether the blockade of TLR4 has any effect on LPS/TLR2 interaction that can influence the TNF/TNFR mediated pathway. Additionally, the contributions of IL-1R and TNFR2 would be extensively studied to develop an alternative approach to control LPS-induced inflammatory responses in terms of polarization switching (M1–M2) in peritoneal macrophages.

MATERIALS AND METHODS

Experimental Animals and Isolation of Murine Peritoneal Macrophages

All experimental procedures involving animals were carried out adhering to the protocols approved by the Institutional Animal Ethics Committee (IAEC), Department of Physiology, University of Calcutta, under the guidance of the Committee for the Control and Supervision of Experiments on Animals (CCSEA) [Approval number- IAEC-V/P/BB-1/2019 dated 07.08.2019], Ministry of Environment and Forest, Government of India. For this *in-vitro* study, peritoneal macrophages were isolated and pooled together from 5 male Swiss albino mice (6–8 weeks of age with a body weight of 20 ± 4 g) and divided into the following groups: Control macrophages (without any pretreatment), control macrophages treated with TLR4Ab (C+TLR4Ab), control macrophages treated with TNFR1Ab (C+TNFR1Ab), control macrophages treated with both TLR4Ab and TNFR1Ab (C+TLR4Ab+TNFR1Ab), LPS- treated macrophages (LPS), macrophages pre-treated with TLR4Ab followed by LPS stimulation (TLR4Ab+LPS), macrophages pre-treated with TNFR1Ab followed by LPS stimulation (TNFR1Ab+LPS) and macrophages pre-treated with both TLR4Ab and TNFR1Ab followed by LPS stimulation (TLR4Ab+TNFR1Ab+LPS). A total of 40 animals were used to perform each set of experiments, and the final results were obtained from three independent experiments.

All these experimental animals were subjected to intraperitoneal injections of 4% sterile thioglycolate broth. The resulting peritoneal exudates were harvested with endotoxin-free Hanks' solution 4 to 5 days later. The cells, thus obtained, were suspended in 0.83% ammonium chloride solution containing 10% (v/v) tris buffer (pH 7.65). This was done to lyse the erythrocytes. After that cells were washed in PBS to remove ammonium chloride. The cells were then resuspended in RPMI 1640 medium supplemented with 10% FBS along with 100 IU/mL penicillin and 100 µg/mL streptomycin and allowed to adhere for 2 hours at 37°C.[28] The non-adherent cells were removed, and adherent cells were collected by frequent aspiration with a Pasteur pipette.

After that, the cells were washed and resuspended in RPMI containing 10% FBS at a density of 5×10^6cells/mL. Finally, the trypan blue dye exclusion technique was performed to examine the adherent cell viability. On achieving 95% of cell viability, the cells were used for the experiments. These adherent cells were pooled from at least 5 mice to obtain the requisite number of peritoneal macrophages.[29]

Antibody Blocking and LPS Stimulation

A dose-dependent study was conducted, and a dose-response curve was plotted with various concentrations of LPS before starting the experiments. Results demonstrated that a 100 ng/mL LPS dose was optimum to elicit inflammatory responses in the peritoneal macrophages. The peritoneal macrophages (5×10^6 cells/mL) obtained from the mice were subjected to pre-treatment with primary antibodies for TLR4 (Abcam, cat no. ab13867)[30] and TNFR1[31] at a dose of 10 µg/mL (Biorbyt, cat no. orb27627) either alone or in combination 30 minutes before LPS stimulation. This was followed by washing off the excess antibody by changing the cell culture media. After this, cells were stimulated with LPS (*In-vivo* Gen, cat no. tlrl-eklps) at a dose of 100 ng/mL for 60 minutes. Another round of washing was conducted before FACS analysis so that no excess LPS from the media were left.[32] The schematic representation of our experimental design has been shown in Figure 1. After treatment with the antibodies, the cells were divided into two groups- one was kept as a control while the other group was subjected to LPS stimulation at a dose of 100 ng/mL. These cells were then analyzed by flow cytometer and further experiments were carried out. For the flow cytometric analysis, the pure macrophage population was made to undergo analysis by gating $CD11b^+$ cells using anti CD11b antibody conjugated with PerCP-Cy5.5 (Thermo Fisher Scientific, cat no. 45-0112-82) and next by using M1 specific marker anti CD86 antibody conjugated with PE (Thermo Fisher Scientific, cat no. 12-0862-81) and M2 specific marker anti CD206 antibody conjugated with FITC (Thermo Fisher Scientific, cat no. MA5-16870). Differential analysis was performed for the cell populations. Isotype controls used for FACS analysis yielded results similar to those of the unstained cell population.

MTT Assay for Determining the Viability of the Murine Peritoneal Macrophages

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to examine the macrophage cell viability. Absorbance readings were determined by a Bioradmicroplate reader as described earlier.[33] The percentage viability was calculated using the formula- [(OD of test solution- OD of Blank)/ (OD of Control-OD of Blank) x 100].

Assay for Quantification of Hydrogen Peroxide (H_2O_2) Production

The cell-free supernatants were collected after time-dependent LPS stimulation. About 70 µL of supernatant, 20 µL of Horse Radish peroxidase (HRP) (500 µg/mL), 70 µL of Phenol red (500 µg/mL) along with 40 µL of the medium was added and incubated for 2 hours at 37°C. The reaction was brought to a stop by the addition of 25 µL 2 N NaOH. The absorbance reading was recorded at 620 nm. The experiments were repeated thrice, and the final results were expressed in mean ± SD. The final results were represented as $\mu M/10^6$ cells.[34]

Assay for Quantification of Nitric Oxide (NO) Production

Griess assay was performed to assess the amount of NO release. About 50 µL of the cell-free supernatants were subjected to incubation in 40 µM Tris (pH 7.9) that contained 40 µM b-Nicotinamide adenine dinucleotide phosphate in the reduced form, 40 µM flavin adenine dinucleotide, and 0.05 U/mL nitrate reductase at 37°C for a while of 15 minutes. Then, the reduced samples were again incubated with an equal volume of Griess reagent containing sulphanilamide (0.25% w/v) and N-1-naphthyl ethylenediamine (0.025% w/v). The mixture was incubated for 10 minutes, and the absorbance was measured at a wavelength of 550 nm. A reduced $NaNO_3$ standard curve was used to compare the total NO production.[35]

Assay for Quantification of Superoxide Anion (O_2^-) Production

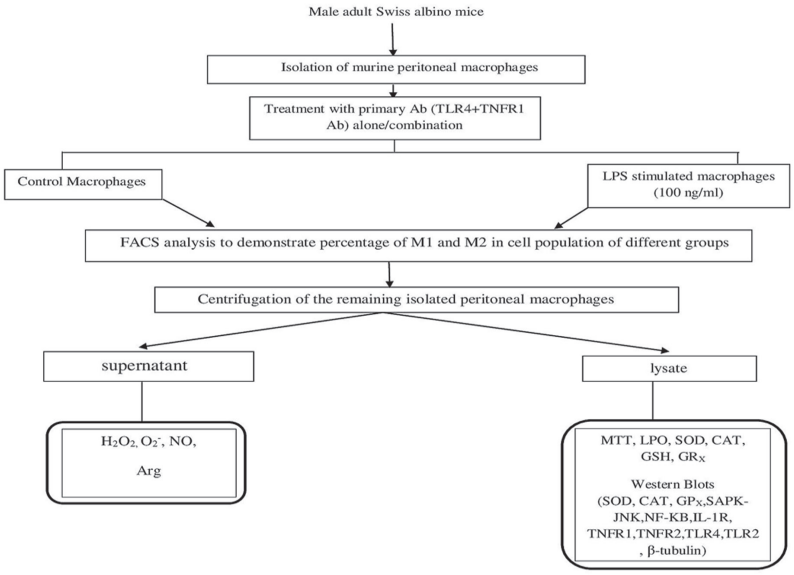
The color change in cytochrome C (cyt C) is evaluated by the superoxide anion release assay when reduced by the O_2^-which is released from the macrophages pre-treated with or without exogenous SOD (2.78 µg/mL). The superoxide anion production was measured by a spectrophotometer at 550 nm, as described earlier. To determine the amount of superoxide anion production, the following formula was used: nanomoles of superoxide anion = (mean absorbance at 550 nm × 15.87).[36] Results were expressed in $nmol/10^6$ cells.

Lipid Peroxidation (LPO) Assay

After LPS stimulation from the different sets, macrophages in the pellet were lysed by adding 100 µL PBS containing 0.1% (vol/vol) Triton X-100 and cell-free lysates were collected separately and used for further experiments. LPO levels were determined as Thiobarbituric acid reactive substances (TBARS). Trichloro acetic acid thiobarbituric acid-hydrochloric acid (TBA-TCA-HCl) reagent was mixed with the cell-free lysates and heated for 20 minutes at 80°C. The absorbance of the pink chromogen present in the clear supernatant was measured in a UV-vis spectrophotometer at 532 nm. Tetraethoxypropane was considered as standard, and the obtained values were expressed in nmoles of TBARS per mg protein.[37]

GSH Assay

A modified Sedlak and Lindsay method was adopted to estimate the reduced glutathione content in the form of acid-soluble sulfhydryl when it reacts with DTNB (Ellman's reagent). A mixture containing 0.3 ml of sample and 0.3 mL of 10% TCA was vortexed. The mixture was centrifuged



Peritoneal macrophages collected from male Swiss Albino mice were subjected to treatment with TLR4 and TNFR1 antibodies. One group of cells was then subjected to LPS stimulation while another group was kept in control. Cells were analyzed by flow cytometry (FACS). The collected cells were then centrifuged to obtain the supernatant. Then macrophages in the pellet were lysed by adding 100 µl PBS containing 0.1% (vol/vol) Triton X-100, and cell-free lysate from different sets was collected separately and then used for further experiments. The assays for H_2O_2, NO, and O_2 were done from the supernatant. Quantification of antioxidant enzyme activities like SOD, CAT, and GSH was performed with cell-free lysates. For the Western blot analyses, the respective cell pellets were lysed separately with RIPA buffer, and these cell-free lysates were used for SDS PAGE followed by Western blot.

Figure 1: Study design

at 40°C for 10 min at 5000 rpm. Following that, we added 500 µL of 0.8M tris–HCl to 250 µL cell-free lysates, 25 µL 5, 5 - dithiobis-2-nitrobenzoic acid (DTNB) was then added to the mixture, after which the absorbance was measured in a UV–vis spectrophotometer at 412 nm. The results were expressed in nmoles of GSH per mg protein.[38]

Assay of Superoxide Dismutase (SOD) Enzyme Activity

To determine the SOD activity, 100 µl of cell-free lysate was mixed with 1.5 mL Tris-EDTA-HCl buffer having a pH of 8.5. Next, 100 µL of 7.2 mmol/L pyrogallol was added to it and incubated at 25°C for 10 minutes. 50 µL of 1M HCl was added to stop the reaction. Absorbance was measured at 420 nm. The SOD enzyme activity was expressed in terms of U/mg protein, where one unit was taken such that that amount of enzyme could inhibit the oxidation of pyrogallol by 50%.[39]

Assay of Catalase Enzyme Activity

We measured the catalase activity in the cell-free lysate. After LPS stimulation from the different sets, macrophages in the pellet were lysed by adding 100 µL PBS containing 0.1% (vol/vol) Triton X-100, and cell-free lysates were collected separately and used for further experiments. 100 µL of cell supernatant was added separately into 2.89 ml of potassium phosphate buffer (pH 7.4) that was taken in a quartz cuvette at time zero. To it, 0.1 mL of 300 mM H_2O_2 was mixed, and absorbance was recorded at 240 nm for 5 minutes at intervals of 1 minute.[40] The mean of the observations from three individual experiments was expressed in terms of mmole/min mg protein.

Assay of Glutathione Reductase Enzyme Activity

Glutathione reductase activity was determined by the oxidation of NADPH to $NADP^+$ during oxidized glutathione reduction. A 1.5 mL reaction mixture was prepared in a quartz cuvette containing 0.3 mM of NADPH and 3 mM oxidized glutathione in 0.2 M K_2HPO_4 buffer (pH = 7.5). The cell-free lysate was added to this mixture. The decrease in the absorbance was recorded at 340 nm for 3 minutes in the spectrophotometer. The enzyme activity was calculated using the millimolar extinction coefficient for NADPH at 340 nm, 6.22.[41] The results were expressed in terms of nmol NADPH/min/mg of protein.

Arginase Enzyme Activity Assay

The supernatants were collected, and macrophages were lysed by adding 100 µL PBS containing 0.1% (vol/vol) Triton X-100. Then, 100 µL of 25 Mm Tris along with 1 mM $MnCl_2$ and the collected supernatant was heated to 55°C for 10 minutes. On cooling, 200 µL 0.5 M arginine in PBS (Sigma-Aldrich) was mixed with the sample solution. This was then incubated at 37°C for 1-hour. The reaction was stopped by adding 900 µL 44.6N H_2PO_4 and 36 N H_2SO_4. The whole mixture was incubated for 30 min at 100°C after 40 µL of 9% (vol/vol) isonitrosopropiophenone in ethanol was added to it. The absorbance was recorded at 550 nm. A standard curve was then plotted using 2-fold serial dilutions of 200 mM urea

(200–3.12 mM), which was used to quantify the arginase enzyme activity assay.[42]

Western Blot Analysis of TLR4, TNFR1, TLR2, TNFR2, IL-1R, SOD, CAT, GP_X, NF-κB, SAPK/JNK Expressions

The cells were centrifuged, the supernatants were decanted, and the cell pellets were lysed using the radioimmunoprecipitation assay (RIPA-NP40) buffer. The protein content of the cell-free lysate was normalized by the Lowry method. Next, the separation of samples was performed, which contained equal amounts of protein in equal volumes of the sample buffer in a denaturing 10% polyacrylamide gel. Then, the sample was finally transferred to a 0.1 mm pore nitrocellulose membrane. As 5% non-fat dry milk was used to block non-specific binding. The nitrocellulose membrane was then incubated with primary antibodies to TLR4 (Abcam, cat no. ab13867), TNFR1 (Biorbyt, cat no. orb27627), TLR2 (Biorbyt, cat no. orb11487) TNFR2 (Biorbyt, cat no. orb224647), IL-1R (Biorbyt, cat no. orb6227), SOD (Biorbyt, cat no. orb67514), CAT (Santacruz cat no SC 50508), GP_X (Biorbyt, cat no. orb6344), SAPK/JNK (Biorbyt, cat no. orb14628), NF-κB (Biorbyt, cat no. orb10182224) in TBS with 0.1% Tween 20 (TBST). The obtained blots were washed thrice in TBST and incubated for 2 hours after adding horseradish peroxidase-conjugated secondary antibodies. The blots were developed with the help of a Super Signal chemiluminescent substrate (Thermo Scientific, USA) and exposed to X-Omat BT films (Kodak). To ensure equal loading of the samples throughout the gel, we used beta-tubulin as the loading control. The quantitative analyses were done using the ImageJ software.

Statistical Analysis

Peritoneal macrophages were isolated from 5 mice and were pooled together to get the requisite number of cells ($5x10^6$ cells/mL). After that, they were cultured in RPMI 1640 medium, which was supplemented with 10% FBS. Now, from these harvested cells, the following groups like control, LPS, and treatments, were segregated *in-vitro*. These were then used to measure the various parameters under study. The experiments were repeated thrice (biological replicate), and the mean value was considered for the final calculations. The number of cells was equal for each of the experiments. Obtained data were expressed in terms of mean ± SD. We performed one-way model 1 ANOVA (Analysis of Variance) between the groups. A Scheffe's F-test for multiple comparisons of the different groups was done when significant p-values were obtained. $p < 0.05$ was found to be the significance level. All analyses were done using Origin Pro 8 software.

RESULTS

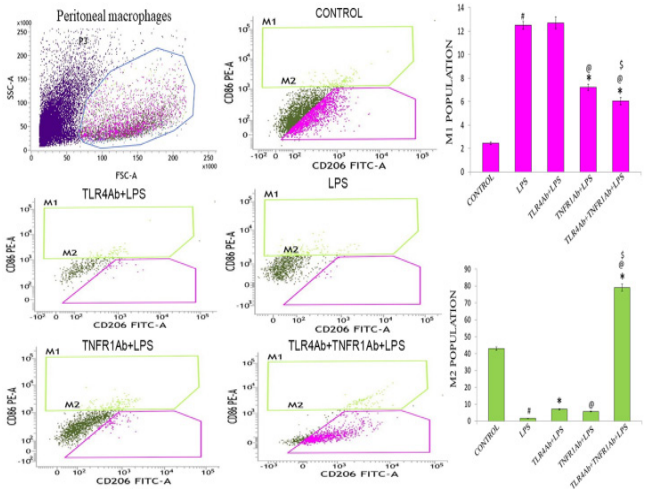
Effect of the TLR4 and TNFR1 Antibodies on the Cell Viability of the Peritoneal Macrophages

On subjecting the murine peritoneal macrophages to treatment with the antibodies and the LPS challenge, the cell viability was determined for all the experimental groups by MTT assay. Results were expressed as the percentage of the control group. We observed that the isolated peritoneal macrophages were >90% viable in dual blocking of the TLR4 and TNFR1 receptors with respect to the control group of cells. LPS stimulation of the cells resulted in >75% viability, while single antibody blocking demonstrated cell viability of >80% (Table 1).

Table 1: Determination of cell viability of various experimental groups of the peritoneal macrophages by MTT assay

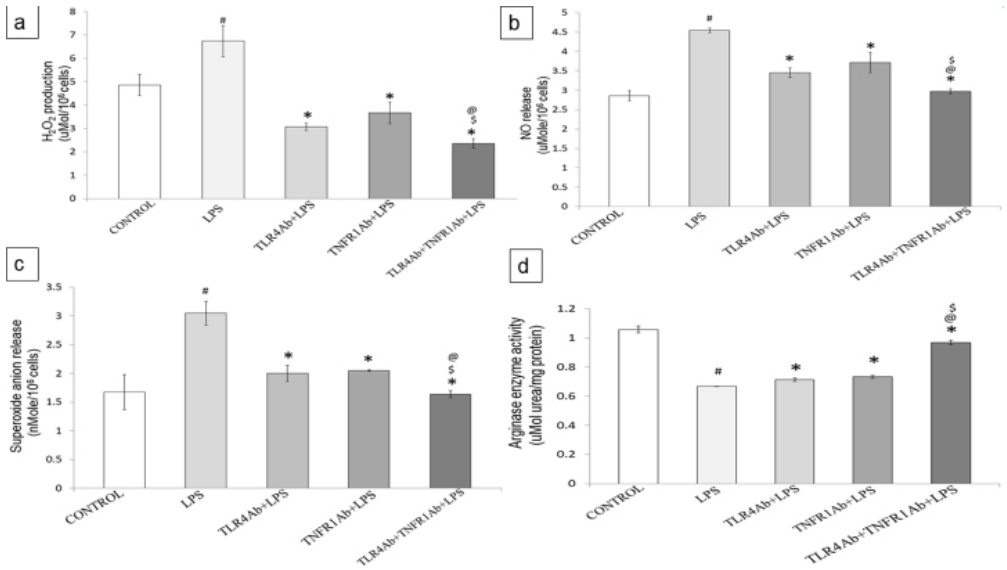
Groups	*Cell Survival (% of control) (Mean ± SD)*
Control	100 ± 0.63
LPS-treated	79.682 ± 0.63
LPS+TLR4 Ab	83.809 ± 1.27
LPS+TNFR1 Ab	85.079 ± 0.63
LPS+TLR4 Ab+TNFR1 Ab	93.492 ± 1.42

Results were shown as Mean±SD from three independent experiments for all five groups and expressed as the % of control viable cells. Peritoneal macrophages were pooled from 5 mice of identical body weight. >75% macrophages were found to be viable in the LPS challenged group while the cells subjected to single antibody treatment (TLR4 or TNFR1) recorded >80% cell viability. Combinatorial blockade of both TLR4 and TNFR1 receptors exhibited >90% cell viability.



Dot plot images of M1/M2 macrophage population analysis. Graphical representation of $CD86^+$ M1 population and graphical representation of $CD206^+$ M2 population in five different groups. Data were represented as mean ± SD from three independent analyses. All the differences were statistically significant at a $p<0.05$ level. '*' indicates a significant difference in comparison to control, '#' indicates a significant difference in comparison to LPS, '@' indicates a significant difference with respect to TLR4Ab+LPS, '$' indicates a significant difference in comparison to TNFR1Ab+LPS.

Figure 2: Flow cytometric analysis of peritoneal macrophages using CD11b-PerCPCy5.5, CD86-PE, and CD206-FITC antibodies after single or dual blocking of TLR4 and TNFR1



Macrophages (5×10^6 cells/ml) were allowed to interact with LPS at 37°C for 60 min in the presence or absence of anti-TLR4 and anti-TNFR1 antibodies. Cell-free supernatant was used for this assay. Results were represented as mean ± SD from three independent experiments. The differences were statistically significant at the p<0.05 level. '*' indicates a significant difference in comparison to control, '#' indicates a significant difference in comparison to LPS, '@' indicates a significant difference with respect to TLR4Ab+LPS, '$' indicates a significant difference in comparison to TNFR1Ab+LPS at p<0.05 level.

Figure 3: Effect of dual blocking of TLR4 and TNFR1 on the production of H_2O_2 (a), NO (b), superoxide anion (c), and arginase enzyme activity(d) from peritoneal macrophages

LPS Challenge Favors Macrophage Polarization Towards the M1 Type While Dual Blocking of TLR4 and TNFR1 Shifts the Phenotype Towards M2

On analysis of the peritoneal macrophages by flow cytometry (Figure 2), it was seen that LPS stimulation significantly increased the M1 population (12.51%) when compared to the control group ($p < 0.05$). Single pre-treatment with TLR4 and TNFR1 antibodies in the LPS stimulated macrophages demonstrated reduced M1 phenotypic population (12.71 and 7.23% respectively), but on simultaneous blocking of both TLR4 and TNFR1 in the macrophages, the most potent reduction in the M1 population was noted (6.04%) in comparison to single antibody treatment ($p < 0.05$).

In the case of the M2 phenotype, LPS stimulation resulted in a reduced macrophage population (1.55%) compared to the control macrophages. On the other hand, individual blocking of the TLR4 and TNFR1 receptors markedly increased the M2 cell population (6.99% and 5.64%, respectively). Furthermore, simultaneous blocking of both the TLR4 and TNFR1 receptors exhibited a significant rise in the M2 macrophage population (79.07%) when compared to single receptor blocking (p<0.05).

Free Radical Production Decreases While Arginase Enzyme Activity Increases on TLR4 and TNFR1 Receptor-Blocking

Cells being stimulated by LPS show higher production of hydrogen peroxide ($p < 0.05$) (Figure 3a)**.** However, on blocking by TLR4 and TNFR1 antibodies, hydrogen peroxide production was diminished ($p < 0.05$). The H_2O_2 production was further decreased when both the TLR4 and TNFR1 receptors were blocked ($p < 0.05$)**.**

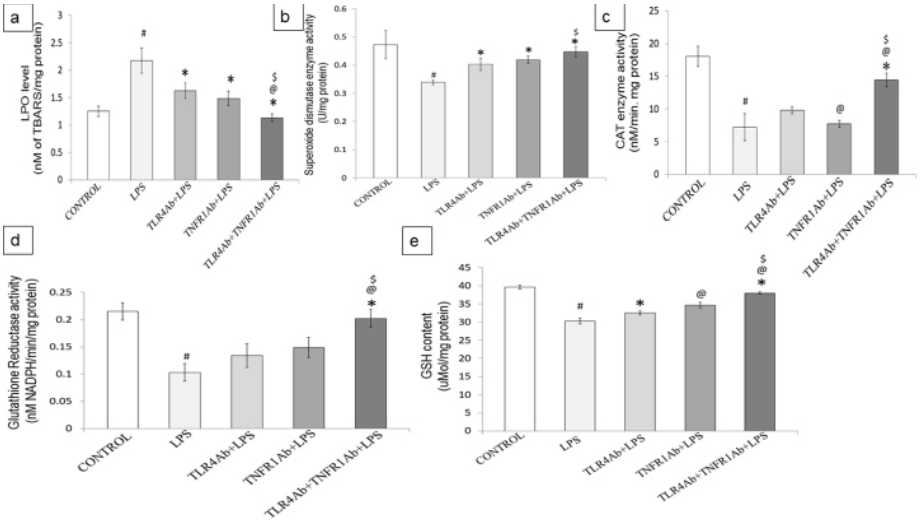
The NO production (Figure 3b) and superoxide anion release were found to be quite high in the LPS-stimulated macrophages when compared to the control group ($p < 0.05$) (Figure 3c). On being subjected to single receptor blocking (TLR4Ab+LPS) and (TNFR1Ab+LPS), the NO production diminished compared to the LPS stimulated group ($p < 0.05$). Simultaneous blocking of the receptors (TLR4Ab+TNFR1Ab+LPS) significantly suppressed the production of both nitric oxide and superoxide anions, which might be due to M2 polarization ($p < 0.05$)**.**

Figure 3d demonstrates that when stimulated with LPS, arginase enzyme activity is significantly reduced ($p < 0.05$) which indicates macrophage polarization towards M1. But on receptor blocking (TLR4Ab+TNFR1Ab+LPS), a rise in the enzyme activity was observed ($p < 0.05$).

Cellular SOD, CAT Enzyme Activity, and GSH Content Increase When Macrophages Shift Towards the M2 Phenotype, Along With a Reduction in the LPO Levels

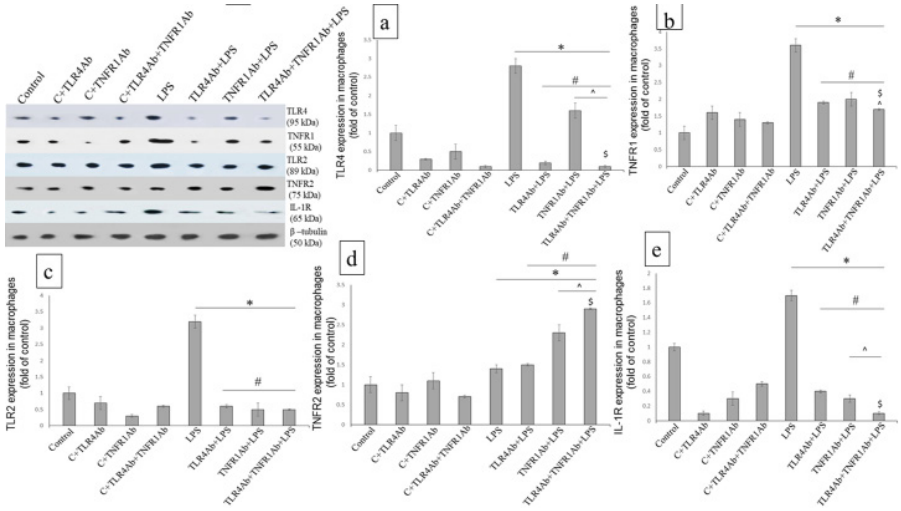
From Figure 4a it can be seen that the lipid peroxidation levels in the LPS-stimulated cells were significantly higher than in the control group ($p < 0.05$). However, the simultaneous blocking of both receptors (TLR4Ab+TNFR1Ab+LPS) significantly decreased ($p < 0.05$) the LPO levels, which indicates the shift towards the M2 phenotype.

From Figure 4b, it was found that when stimulated by LPS, the SOD enzyme activity was markedly reduced ($p < 0.05$) in comparison to control cells. Blocking the cell surface TLR4 and TNFR1 facilitated increased superoxide dismutase enzyme activity in the macrophages, which might indicate a phenotypic shift from the M1 towards the M2 phenotype.



Macrophages (5×10^6 cells/ml) were allowed to interact with LPS at 37°C for 60 min in the presence or absence of anti-TLR4 and anti-TNFR1 antibodies. The cell-free lysate was used for this assay. Results were represented as mean ± SD from three independent experiments. The differences were statistically significant at the $p<0.05$ level. '*' indicates a significant difference in comparison to control, '#' indicates a significant difference in comparison to LPS, '@' indicates a significant difference with respect to TLR4Ab+LPS, '$' indicates a significant difference in comparison to TNFR1Ab+LPS at $p<0.05$ level.

Figure 4: Effects of dual blocking of TLR4 and TNFR1 on the level LPO (a), activities of SOD (b) and CAT (c), GR_X (d) enzymes, and GSH content (e) from peritoneal macrophages



Receptor expression patterns in the LPS stimulated peritoneal macrophages. Whole-cell lysates were prepared for the analysis of TLR4 (a), TNFR1 (b), TLR2 (c), TNFR2 (d), and IL-1R (e) expression after 60 min of LPS stimulation. The densitometric analyses were performed after three repeated experiments.'*' indicates a significant difference in comparison to control, '#' indicates a significant difference in comparison to LPS, '^' indicates a significant difference with respect to TLR4Ab+LPS, '$' indicates a significant difference in comparison to TNFR1Ab+LPS at $p<0.05$ level. All the samples were probed using β-tubulin to ensure equal protein loading.

Figure 5: Western blot analysis of TLR4 (a), TNFR1 (b), TLR2 (c), TNFR2 (d), IL-1R (e) expressions in macrophages during single or dual receptor blockade

Catalase activity dwindled when the macrophages were treated with LPS compared to the control group (Figure 4c). However, a significant improvement was seen in the CAT enzyme activity on single and dual blocking of the TLR4 and TNFR1 receptors.

On measuring the glutathione reductase activity (Figure 4d), we saw that the enzyme activity was significantly reduced on LPS stimulation. However, when TLR4 and TNFR1 were blocked individually (TLR4Ab+LPS or TNFR1Ab+LPS), glutathione reductase enzyme activity was upregulated. Simultaneous blocking of the TLR4 and TNFR1 receptors demonstrated further improvement in the enzyme activity. A significant increase in the cellular GSH content was observed when the macrophages were subjected to single

receptor blocking (TLR4Ab+LPS and TNFR1Ab+LPS) in comparison to the control macrophages ($p < 0.05$) (Figure 4e). When both TLR4 and TNFR1 were blocked at the same time (TLR4Ab+TNFR1Ab+LPS), GSH levels markedly improved, which might be an indication of the phenotypic switch towards the immunoregulatory M2 phenotype.

Simultaneous Blocking of TLR4 and TNFR1 Mitigates LPS-induced Up-regulation of their Expressions but Augmented TNFR2 Expression in the Macrophages

Figure 5 demonstrates that the expressions of both TLR4 and TNFR1 were significantly up-regulated in the macrophages when the cells were subjected to LPS stimulation. However, when the TLR4 and TNFR1 receptors were blocked, their expressions were reduced compared to the LPS-stimulated group. Dual blocking (TLR4Ab+TNFR1Ab+LPS) resulted in the down-regulation of both these receptors in the macrophage in comparison to the LPS stimulated, TLR4Ab+LPS and TNFR1Ab+LPS groups.

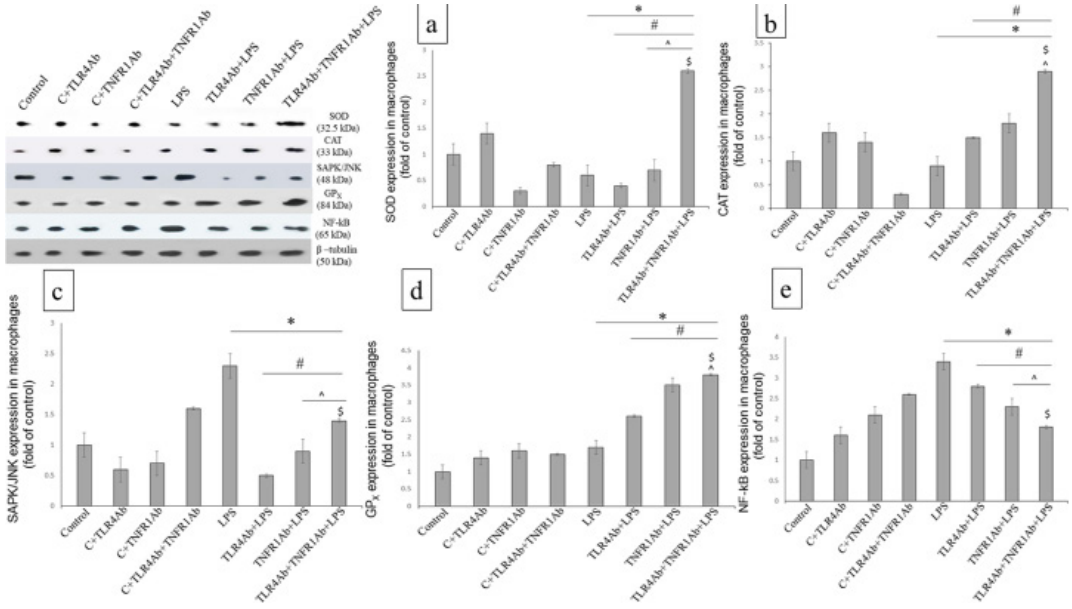
Also, we found that the TLR2 expression was elevated in the macrophages on LPS stimulation but was reduced on single and dual blocking of the TLR4 and TNFR1 receptors. Interestingly, during the dual blockade, the TLR2 expression was found to be greater than that of the TLR4 expression. The TNFR2 expression was also increased in the event of TLR4 and TNFR1 blockade compared to the LPS-stimulated macrophages ($p < 0.05$). The TLR2 expression was significantly up-regulated in the LPS-stimulated macrophages, but the expression plummeted in the event of receptor blocking ($p < 0.05$). The TNFR2 expression was diminished in the LPS-stimulated macrophages compared to the control cells ($p < 0.05$). TNFR2 expression in the macrophages was up-regulated when both the TLR4 and TNFR1 receptors were blocked individually or simultaneously. We also found a significant increase in the IL-1R expression in the LPS-stimulated macrophages compared to the control. On blocking the TLR4 and TNFR1 receptors, single or dual IL-1R expression was significantly reduced ($p < 0.05$) compared to the LPS-stimulated macrophages.

Figure 6 demonstrates that the expressions of antioxidant enzymes such as SOD and CAT were diminished markedly in the LPS-stimulated macrophages in comparison to the control ($p < 0.05$). The expressions were significantly up-regulated on the individual as well as dual blocking of the receptors (TLR4Ab+LPS/TNFR1Ab+LPS and TLR4Ab+TNFR1Ab+LPS) compared to the LPS stimulated macrophages ($p < 0.05$). The SAPK/JNK and NF-κB expression was also found to be significantly diminished ($p < 0.05$) on blocking of the TLR4 and TNFR1 receptors, individually or in combination, when compared to the LPS stimulated macrophages. On the other hand, GP_X showed increased expression in macrophages on single receptor blocking (TLR4Ab+LPS/TNFR1Ab+LPS) ($p < 0.05$). Their expression was further increased in the macrophages when both receptors were simultaneously blocked (TLR4Ab+TNFR1Ab+LPS) ($p < 0.05$).

DISCUSSION

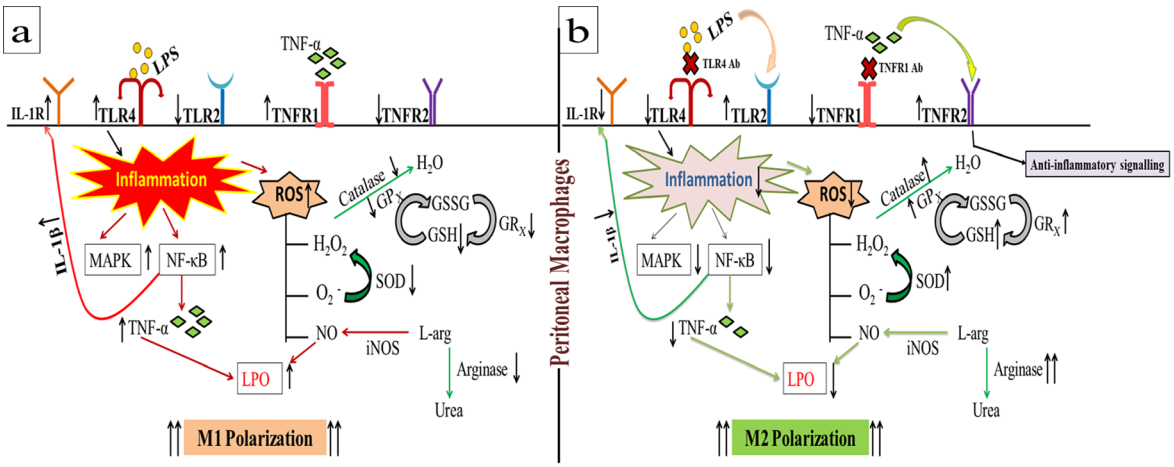
The development of an alternate therapeutic option for LPS sepsis has become imperative due to the approximately 20–30 million patients that are affected by the condition each year worldwide.[43] Septic shock results from systemic inflammatory responses that can be brought on by cytokine storm,[44] among other things. Pro-inflammatory and anti-inflammatory pathways are not balanced, which results in redox dysregulation and cytotoxic reactions.[45]

Macrophage cell surface TLR4 is the major sensory receptor for bacterial LPS, not only TLR4 but also TLR2, which might have gained significant importance in the current research scenario in developing an alternative treatment strategy for sepsis.[46] Previous studies have shown that when splenic macrophages of naive BALB/c mice were cultured in-vitro and treated with LPS, the TLR2 mRNA weakly expressed in un-stimulated macrophages was significantly increased by LPS treatment.[47] Moreover, treatment of a mouse macrophage cell line with LPS, IL-1β, IFN-γ, or TNF-α also significantly increased the TLR2 mRNA expression, whereas TLR4 mRNA expression remained constant. These findings suggest that TLR4 is the dominant receptor for at least some types of LPS, whereas TLR2 is dispensable.[48] The current study also reveals that there is some correlation between TLR2 and TLR4 expressions, similar to other disease models.[49] LPS may increase TLR2 expression indirectly by inducing cytokine secretion (such as TNF-α and IL-1β) in macrophages.[50] Also, the interaction between TLR2 and IL-1R might have a role to play in shifting the macrophage phenotype towards M2. Previous studies proved that simultaneous activation of TLR4 and TLR2 induces IL-1R antagonist gene expression in RAW264.7 macrophage cell lines.[51] Faure and colleagues found that in HMECs, TLR2 mRNA expression, TLR2 protein expression, and TLR2 cell-surface expression were all increased by EcLPS stimulation. Therefore, single (TLR4) or dual (TLR4 + TNFR1) receptor blocking before LPS challenge in murine peritoneal macrophages leads to elevated TLR2 expression as LPS is accessible to more TLR2 on the surface since, on one hand, most of the surface TLR4 are occupied by TLR4Ab and in the other LPS by itself could up-regulate TLR2 in this case. As a result, we found enough TLR2 to mediate macrophage signaling. This TLR4 pathway,[52] coupled with JNK signaling, causes TNF-α expression in response to the LPS challenge.[53] Thus, our strategy of targeting TLR4 is valid and does not hinder TLR2-mediated signaling. This study's primary goals are to determine the fundamental function of TLR2 in macrophage polarization and, as yet unexplored, to determine whether TLR2 sensitivity is affected in any way by concurrently blocking TLR4 and TNFR1 receptors. Additionally, we tried to investigate the function of IL-1R-mediated signaling, which we believe contributes to the development of harmful reactions linked with LPS-induced inflammation. We were also interested in seeing if inhibiting the TLR4 and TNFR1 receptors jointly changed the expression



The alterations of antioxidants and signaling molecules expression patterns were analyzed in peritoneal macrophages. Whole-cell lysates were prepared for the analysis of SOD (a), CAT (b), SAPK/JNK (c), GP_X (d), and NF-κB (e) expression after 60 min of LPS stimulation. The densitometric analyses were performed after triplicate experiments.'*' indicates a significant difference in comparison to control, '#' indicates a significant difference in comparison to LPS, '^' indicates a significant difference with respect to TLR4Ab+LPS, '$' indicates a significant difference in comparison to TNFR1Ab+LPS at $p<0.05$ level. All the samples were probed using β-tubulin to ensure equal protein loading.

Figure 6: Western blot analysis of SOD (a), CAT (b), SAPK/JNK (c), GP_X (d), and NF-κB (e) expression in macrophages during single or dual receptor blockade



From the study, it is observed that the binding of LPS to TLR4 initiates inflammatory responses and promotes NF-κB expression. This results in excessive ROS generation, which leads to oxidative stress. Suppression of anti-oxidant enzyme activities further leads to elevation of the LPO level, which might indicate the polarization of the macrophages towards the M1 phenotype. Also, increased NO production and down-regulation of arginase activity further contribute to the situation that ultimately results in cellular damage (Figure 7a)

However, our experimental studies successfully demonstrate that the simultaneous blocking of the TLR4 and TNFR1 receptors causes an inhibition in the inflammatory responses. A fall in ROS production is seen in the cellular environment, which is scavenged by the increased levels of antioxidants like SOD, CAT, and GSH. The LPO levels decrease due to reduced oxidative stress. With the increase in arginase enzyme activity in blocking the TLR4 and TNFR1 receptors, the NO production decreased, shifting the macrophage phenotype towards the immunoregulatory M2 phenotype (Figure 7b).

Figure 7: Schematic representation of the mechanism of action

of TNFR2, which might lessen the cytotoxic effects of LPS, as TNFR2 is known to have anti-inflammatory properties.

Although TLR4/TNFR1 reliance was demonstrated in our most recent study,[54] both receptors are essential for LPS-induced inflammation. With several studies establishing the LPS-TLR4 pathway as a primary cause for the occurrence of sepsis along with the polarization of the M1/M2 macrophages,[55] our strategy of blocking the TLR4 along with the neutralization of the TNFR1 might be crucial for understanding the potential influence of TLR2 on TNF-TNFR1/TNFR2 response which was not investigated so far in the context of macrophage polarization. Moreover, we wanted to assess the impact on other cytokine receptors, inflammatory mediators, and anti-oxidant enzyme activities over macrophage inflammatory responses. TNF exerts its activities by stimulation of two different types of receptors, TNFR1 and TNFR2, which are both expressed by macrophages. These two TNF receptor types trigger distinct and common signaling pathways that can work in an interconnected manner.[56] A potential trigger for TNFR1-induced inflammation is provided by the exogenous and/or autocrine TNF-α generated in response to TNFR1 and TNFR2 activation. LPS-induced TLR4 signaling not only modifies the necroptotic sensitivity of macrophages for TNF-α in a complex way, but it also has the capacity to initiate an inflammatory-associated toxic response.[57]

Analyzing the flow cytometric data, the LPS challenge results in oxidative damage to the peritoneal macrophages that results from excessive ROS generation and suppression of anti-oxidants. This drives the macrophages toward the M1 phenotype that leads to inflammatory responses.[58] The cumulative effect increases the LPO levels, a clear indicator of the cellular stress responses to LPS in M1 polarized macrophages.[59] However, when TLR4 and TNFR1 receptors were blocked individually or in combination, we saw a reduction in oxidative stress because the formation of free radicals decreased and the activities of antioxidant enzymes increased.[60,61] In this instance, combinatorial receptor blockage was observed to increase the M2 population in the cells.

Upon LPS stimulation to the macrophages, TLR4 primarily recognizes it but during the blockade of both TLR4 and TNFR1, TLR2 sensitivity was increased. Whatever the case, both TLR4 and TLR2 initiate the innate immune responses via promoting H_2O_2 and superoxide anion release.[62] The experimental data shows that dual blockade of TLR4 and TNFR1 could attenuate LPS-induced ROS production. Moreover, the LPS-induced NO production was sharply reduced, possibly due to the competitive activity of iNOS and arginase enzyme in the macrophages[63] subjected to the dual blocking of the TLR4 and TNFR1 receptors. Arginase enzyme activity, which is considered a key player in the polarization of the macrophages,[64] also demonstrated a higher activity level during $CD206^+$ M2 macrophage polarization compared to the $CD86^+$ M1 polarization. This might indicate the higher ornithine levels produced in the M2 macrophages from L-arginine, the common physiological substrate for iNOS.

Western blot demonstrates that the expressions of TLR4 and TNFR1 were diminished in macrophages during the dual blockade. TLR2 expression was also mildly reduced when TLR4 and TNFR1 receptors were blocked, which might be due to decreased affinity of TLR2.[65] Expressions of TNFR2 were seen to be up-regulated in the macrophages on the combinatorial blockade of the receptors (i.e., TLR4 and TNFR1), as TNFR2 is known to initiate anti-inflammatory functions of M2 polarized macrophages.[66] The IL-1R expression was found to be diminished when the macrophages were subjected to simultaneous TLR4 and TNFR1 blocking. The lower expression of IL-1R is possibly due to the reduced IL-1β level when macrophages were subjected to the dual Ab pre-treatment, as justified by our very recent in-vitro study.[54] We have found elevated TLR2 expression with respect to the TLR4 on the surface of peritoneal macrophages when both TLR4 and TNFR1 were neutralized with antibodies. Therefore, during the simultaneous neutralization of TLR4 and TNFR1, its impact on LPS-induced reverse expression/modulation of TLR4 as opposed to TLR2 may be mediated via MD2, which needs to be addressed in future studies. Since TLR4 and TLR2 are both important in the recognition of a variety of pathogens, the binding of LPS to TLR4 causes an increase in the affinity of TLR2 towards its ligand.[67] Therefore, it can be speculated that the attenuation of oxidative stress might also be an outcome of TLR2/TNFR2 cross-talking, which is known to promote anti-inflammatory activities.[68]

We have observed the expressions of SOD, CAT, SAPK/JNK, GPx as well as NF-κB in the macrophages. The combinatorial treatment with the TLR4 and TNFR1 antibodies resulted in the up-regulation of SOD and CAT expressions in comparison to the LPS-challenged macrophages. This augmentation might contribute to higher antioxidant enzyme activities, which will facilitate the shift toward the M2 phenotype. An increase in the anti-oxidant activities facilitated the scavenging of the ROS,[69] as evidenced by our study. This causes the reduction of oxidative stress, which leads to reduced cellular damage. The SAPK/JNK expression was down-regulated in the macrophages on treatment with the antibodies although the expression was significantly higher in the LPS-stimulated macrophages. With a decrease in inflammation, there was also a decrease in the NF-κB expression, the master regulator of inflammation.[70] The higher GP_x expression strongly supported and promoted anti-inflammatory functions in the macrophages. Higher expressions of SOD and CAT, as well as GPx, provide evidence of the fact that neutralization of the receptors facilitated the restoration of redox balance providing a remedy to combat free radicals. Additionally, higher glutathione reductase (GR_X) activity and GSH content after dual blockade markedly suppressed the lipid peroxidation level,[71] as depicted from our data. It was observed that expression of GP_X is increased when the cells were subjected to receptor blockade which provides evidence for the immunoregulatory mechanisms that take

place inside macrophages. Higher levels of GP_X promote the breakdown of H_2O_2 into oxygen and water, which lowers the oxidative stress in the cellular environment. Also, GP_X facilitates the oxidation of GSH to GSSG, which further promotes anti-inflammatory functions in the macrophages.[72] In summary, the binding of LPS to TLR4 initiates inflammation-associated toxic responses and promotes NF-κB expression. This results in excessive ROS generation, which leads to cellular stress. In our study, the up-regulation of TLR2 was found to be TLR4-dependent. It is most likely that this up-regulation of TLR2 by LPS occurs through the activation of NF-κB since the TLR2 promoter contains binding sites for NF-κB in TLR4-blocked macrophages, which do not up-regulate TLR2, show reduced NF-κB activation in response to LPS.[73] The increased cytokine levels in LPS-challenged macrophages might affect the LPS-induced TLR2 expression. In support of these results, the expression of TLR2 in macrophages is up-regulated by LPS, TNF-α, and IL-1β in an NF-κB-dependent manner, whereas TLR4 expression in macrophages is not up-regulated by pro-inflammatory mediators. We have yet to determine whether LPS/TLR2 or LPS/TLR4 interactions also require collaboration with other TLRs, such as TLR1 or TLR6, as shown for some other agonists. Antioxidant enzyme activity suppression also raises the LPO level, which may signify that macrophages are becoming more polarized toward the M1 phenotype.[74] In addition, down-regulating arginase activity and elevated NO production exacerbate the condition and eventually lead to a toxic response in cells.

From the overall study, it can be speculated that dual blocking of TLR4 and TNFR1 receptors on peritoneal macrophages significantly attenuated TLR2 sensitivity to LPS. Moreover, LPS-induced inflammation triggers the production of pro-inflammatory cytokines, i.e., IL-1β and TNF-α. The consequences of cytokine production are directly influenced by the type of receptors. Our data suggests that the higher expression of TNFR2 facilitated anti-inflammatory activities, possibly by triggering arginase enzyme activity or by down-regulating IL-1R, NF-κB, a master regulator of inflammation. Therefore, it could switch M1 polarization towards M2. This study also proved the strong influence on the cellular antioxidant mechanism, which was depicted from the higher expressions of SOD, catalase, and GPx in response to dual treatment. It is to be further added that a recent molecular study from our laboratory confirms that simultaneous TLR4/TNFR1 blockade could suppress STAT1 and STAT3 expression and increase SOCS3 expression in LPS-stimulated macrophages.[75]

Our experimental studies successfully demonstrate that on simultaneous blocking of the TLR4 and TNFR1 receptors, there arises an inhibition in the inflammatory responses through NF-κB. The expression level of NF-κB can be up-regulated by TLR2 by activating the MyD88-dependent signaling pathway, which will eventually induce the production of pro-inflammatory factors such as IL-6, IL-1β, and TNF-α.[76] Blocking of TLR4 and TNFR1 possibly resulted in a significant reduction in the levels of these cytokines, resulting in the amelioration of sepsis through M2 polarization. Our future studies will be aimed at quantifying the levels of the other related cytokines, such as IL-10 as well as IL-6, which are believed to play crucial roles during the onset and progression of the disease. A fall in ROS production is observed in the cellular micro-environment, which might be scavenged through the increased activities of antioxidant enzymes like SOD, CAT, and GR_x. The lipid peroxidation is decreased due to reduced oxidative stress. With the increase in arginase enzyme activity during the dual blockade of TLR4 and TNFR1 receptors, the NO production decreased, which shifts the macrophage phenotype to the anti-inflammatory M2 phenotype (Figure 7a, 7b). As LPS is a major pathogenic factor in sepsis, the observation that the pathway leading to NF-κB activation and cytokine production in response to LPS via TLR4 or TLR2 might be different in macrophages and may have important consequences when the development of therapeutics is being considered not by targeting TLR4 alone but both TLR4 and TLR2 with special reference to the macrophage polarization mediated through TNFR2. The dual blocking of TLR4/TNFR1 could attenuate oxidative damage or cytokine responses, possibly by promoting antioxidant expression. Being an antigen-presenting cell, phagocytosis is one of the crucial cellular mechanisms that could help neutralize bacteria. The proposed treatment strategy might interfere with phagocytosis or antigen presentation to T and B lymphocytes, but TLR2 might be employed as PRR during the blockade of TLR4. So, ultimately, there is an effective reduction of LPS-induced inflammation in response to the dual neutralization of TLR4 and TNFR1.

ACKNOWLEDGMENTS

The authors are indebted to, the Department of Science and Technology, Government of India for providing us with the instruments procured under the DST-PURSE program to the Department of Physiology, University of Calcutta. The author is indebted to CRNN (BD Bioscience Room), Kolkata, for allowing us to use the FACS facilities. During this experiment, research grants for teachers under BI (92)-7 and laboratory working expenses under BI (92)-5, respectively, were received from the Departmental Budget 2023–2024.

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PEER-REVIEWED CERTIFICATION

During the review of this manuscript, a double-blind peer-review policy has been followed. The author(s) of this manuscript received review comments from a minimum of two peer-reviewers. Author(s) submitted revised manuscript as per the comments of the assigned reviewers. On the basis of revision(s) done by the author(s) and compliance to the Reviewers' comments on the manuscript, Editor(s) has approved the revised manuscript for final publication.