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Dexamethasone exhibits its anti-inflammatory effects in *S. aureus* induced microglial inflammation via modulating TLR-2 and glucocorticoid receptor expression

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ABSTRACT

Microglial inflammation plays crucial role in the pathogenesis of CNS infections including brain abscesses. *Staphylococcus aureus* (*S. aureus*) is considered as one of the major causative agents of brain abscesses. Due to the emergence of multidrug resistant bacteria the available treatment options including conventional antibiotics and steroid therapy become ineffective in terms of inflammation regulation which warrants further investigation to resolve this health issue. Microglial TLR-2 plays important roles in the bacterial recognition as well as induction of inflammation whereas glucocorticoid receptor (GR) triggers anti-inflammatory pathways in presence of glucocorticoids (GCs). The main objective of this study was to figure out the interdependency between TLR-2 and GR in presence of exogenous dexamethasone during microglial inflammation as an alternative therapeutic approach. Experiments were done either in TLR-2 neutralized condition or GR blocked condition in presence of dexamethasone. Free radicals production, arginase, superoxide dismutase (SOD), catalase enzyme activities and corticosterone concentration were measured along with Western blot analysis of TLR-2, GR and other inflammatory molecules. The results suggested that dexamethasone pre-treatment in TLR-2 neutralized condition efficiently reduces the inflammatory consequences of *S. aureus* induced microglial inflammation through up regulating GR expression. During TLR-2 blocking dexamethasone exerted its potent anti-inflammatory activities via suppressing reactive oxygen species (ROS), NO production and up regulating arginase, SOD and catalase activities at the time point of 90 min. Further in-vivo experiments are needed to conclude that dexamethasone could resolve brain inflammation possibly through microglial phenotypic switching from pro-inflammatory M1 to anti-inflammatory M2.

1. Introduction

Microglial inflammation is considered as the hallmark phenomenon of brain abscesses caused by different pathogenic bacterial strains like *Staphylococcus aureus* [1]. Microglia provides an initial line of defence against invading microorganism to the CNS but exposure of microglia to *S. aureus* leads to microglial over activation in terms of pro-inflammatory mediator release as well as cellular stress [2]. So, regulation of microglial activation should be the ultimate way out for controlling bacterial brain abscesses, a major health issue in developing countries [3]. Beside antibiotics the application of steroids in acute CNS infections is not only controversial [4] but becomes limited due to the prevalence of steroid resistance [5].

The enhanced expression of cell surface TLR-2 in activated microglia plays pivotal role in bacterial recognition. Previous studies indicated that targeting of TLR-2 signalling components might have promising role in the development of drugs to treat infectious diseases and inflammatory disorders [6]. Attenuation of microglial activation in the brain may result less damage to normal parenchyma and improvement in cognitive and neurological functions, associated with the pathological changes in brain abscesses [7]. It was reported that some prostaglandins may regulate TLR-2 mediated inflammatory events in brain immune cells [8]. So, the understanding of mechanisms that govern infection induced TLR-2 signalling in glial cells, with respect to inflammatory markers like COX-2 expression, will undoubtedly contribute to the effective management of bacterial brain abscesses.

It was demonstrated that all classes of glial cells including microglia express glucocorticoid receptor (GR) that can translocate to the nucleus in presence of hormone. These observations suggested that microglial cells are major targets for GR directed control of gene transcription in

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the brain [9]. The activation of glucocorticoid receptors in CNS is known to exert strong immunomodulatory effects through neuro-endocrine crosstalk [10]. Moreover, literature reveals that GR is a susceptible target for bacterial infectious agents. The GR number and its ligand affinity could be reduced by live microorganism infection or stimulation with their degraded products like LPS [11]. Evidences suggest that exogenous glucocorticoid (GC) abolishes pro-inflammatory cytokine expression in microglia whereas GR antagonist prolongs the inflammatory consequences [12].

The physiological action of glucocorticoids is mediated by glucocorticoid receptor (GR, NR3C1), a member of nuclear receptor super family [13]. Upon binding of glucocorticoids, cytosolic GR becomes active by dissociating from hsp90 followed by importin mediated nuclear translocation [14]. GR can modulate other inflammatory gene expression primarily by physical association with activator protein-1 (AP-1) and nuclear factor-κB (NF-κB). Glucocorticoids probably show its immunosuppressive effects through inhibiting those transcription factors [15]. Literature showed that TLR-2 ligand (Pam3Cys) pretreatment diminishes *S. aureus* induced retinal microglial inflammation but surprisingly increases its phagocytic activity. Moreover, pro-inflammatory mediator expression was also attenuated by either functional blocking of TLR-2 with anti-TLR-2 antibody or by siRNA knockdown of TLR-2 [16]. Other reports indicated that TLR-2 agonist could oppose the dexamethasone induced reduction in neutrophil extracellular trap formation against *S. aureus* [17]. However, the depletion of endogenous glucocorticoids or application of GR antagonist RU486 could increase the mortality from endotoxin and even non-lethal doses of staphylococcal super antigen [18]. Therefore, TLR-2 and GR might have some distinctive roles during onset of *S. aureus* induced microglial activation as well as in its resolution. The interrelationship between these two receptors in infected microglia could have some therapeutic importance to find out an alternative way to treat microglial inflammation. Moreover, an understanding of mechanisms that govern TLR-2 signalling in microglia, with respect to glucocorticoid receptor (GR) expression, will undoubtedly facilitate the design of therapeutic regimens for CNS infections.

Immediately after bacterial recognition via TLR-2, it triggers pro-inflammatory cytokines and ROS production by activating NF-κB and MAPKs whereas, Dexamethasone via upregulating GR had been shown to reduce ROS production, NO release and other inflammatory reactions of activated microglia [19]. However, several studies have reported that the number of cytosolic GR was diminished following treatment with IL-1, IL-2, IL-6 and TNF-α in contrast to very few reports showing up regulation of GR with same group of cytokines [20].

There are two different states of microglial polarization coexist depending upon the variable microenvironment and cellular reactivity [21]. Pro-inflammatory M1 subtype is known to be induced by LPS stimulation or Th1 cytokines (i.e. IFN-γ, TNF-α). This activated M1 state confers potent microbicidal activities during inflammation [22]. However, alternative M2 microglia can be driven by Th2 cytokines. Literatures suggest that higher levels of IL-10, TGF-β, arginase activity and glucocorticoids are responsible for the anti-inflammatory M2 polarization [23]. As glucocorticoid receptor (GR) activation has some modulatory effects on M1/M2 switching during live *S. aureus* infection [24] further investigations should be needed to delineate the neuro-endocrine-immune mechanism of GR signalling in the resolution of microglial inflammation through polarization switching.

From the previous reports it was found that TLR-2 facilitates the innate immune response to pathogen along with induction of inflammation whereas GC could show the immunosuppressive effects during infection through GR mediated pathway but no study has been conducted to define the crosstalk between TLR-2 and GR in terms of controlling microglial inflammation. In this study, an attempt has been taken to establish the inter-relationship between TLR-2 and glucocorticoid receptor (GR) in *S. aureus* infected microglial cells as an alternative way to regulate microglial activation. However, this experimental setup was also attempted to figure out the underlying mechanisms by which both of these receptors work during exogenous glucocorticoid administration or to modulate endogenous corticosterone release in murine microglia. Microglial TLR-2/GR dependency was investigated by blocking cell surface TLR-2 with anti TLR-2 antibody or cytosolic GR with RU486 in terms of ROS, NO and anti-oxidant enzyme activities and other inflammatory markers. Moreover, dexamethasone was used as a synthetic ligand for GR in presence of endogenous corticosterone during acute inflammation. This study will also be helpful to delineate the contribution of TLR-2 and GC induced GR activation on microglial M1/M2 polarization switching expecting better resolution of brain inflammation.

2. Materials and methods

2.1. Maintenance of animals

Adult male Swiss albino mice (6–8 weeks of age with body weight 20 ± 4 g) were used throughout this study. Upon arrival animals were kept under controlled environment (temperature 21–24 °C and 40–60% humidity) and fed a normal rodent diet. All the animal experiments were performed according to the protocols that had been approved by the Institutional Animal Ethics Committee (IAEC), Department of Physiology, University of Calcutta, under the guidance of CPCSEA [Proposal number: IAEC/IV/Proposal/BB-01/2017, approved on 20.03.2017], Ministry of Environment and Forest, Govt. of India. During brain tissue collection mice were anesthetized with ketamine hydrochloride (Sigma, Life Sciences) at a dose of 1 mg/kg body weight through the tail vein followed by cervical dislocation.

2.2. Isolation of murine primary microglial cells

Brains from adult male Swiss albino mice (4 mice/group) were isolated in HBSS and dispersed in 0.3% collagenase D and 10 mM HEPES buffer dissolved in HBSS followed by 30 min of incubation at 37 °C. After incubation brain tissues were homogenized and filtered in 70 μm pore cell strainers (Becton Dickinson), centrifuged at 1500 rpm for 7 min, washed, and resuspended in 70% isotonic Percoll (Sigma-Aldrich). Then the cell suspension was transferred to centrifuge tubes containing 25% isotonic Percoll, which were sequentially layered on top with PBS. Now, after centrifugation (30 min, 800 ×g, 4 °C), the 70%:25% Percoll interphase layers were collected, and the cells were washed to remove Percoll. Finally, the adherent cells, which contained CD11b+ cells (determined by FACS), were cultured in DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml sodium pyruvate, and 10 mM HEPES buffer. Microglial cells were washed with PBS and resuspended in medium containing 1% heat-inactivated FBS, and then cultured for the required times at 37 °C [25].

2.3. Preparation of bacteria

The *Staphylococcus aureus* strain AG-789 was used in this study. It was obtained from Apollo Gleneagles Hospital, Kolkata, West Bengal, India and maintained in our laboratory. *S. aureus* (AG-789) grown overnight in Muller Hinton broth (MHB) (High media, Bombay, India) was diluted with fresh broth and cultured until mid-logarithmic phase of growth. Bacteria were harvested, washed twice with sterile PBS and adjusted to the desired inoculum spectrophotometrically before infection by adjusting O.D. of 0.2 at 620 nm which corresponds to 5.0×10^7 cells/ml for *S. aureus* and the colony forming unit (CFU) count of the desired inoculums was confirmed by serial dilution and cultured on blood agar [26]. From the biochemical analysis and bio typing for characterization of the *S. aureus* (AG-789) isolate, it was found that *S. aureus* (AG-789) is catalase and coagulase positive and resistant to methicillin.

2.4. Anti TLR-2 antibody, GR antagonist (RU486) and dexamethasone pre-treatment in primary microglial cells followed by viable *S. aureus* infection

After isolation primary microglial cells were divided into following groups, i.e. C (control microglia), C + IsoAb (control microglia treated with isotype antibody), C + TLR2Ab (control microglia treated with anti TLR-2 antibody), C + RU (control microglia treated with RU486), C + Dex (control microglia treated with dexamethasone), C + TLR2Ab + Dex (control microglia treated with anti TLR-2 antibody and dexamethasone), C + RU + Dex (control microglia treated with RU486 and dexamethasone), SA (viable *S. aureus* infected microglia), IsoAb + SA (isotype antibody pre-treated and *S. aureus* infected), TLR2Ab + SA (anti TLR-2 antibody pre-treated and *S. aureus* infected), RU + SA (RU486 pre-treated and *S. aureus* infected), Dex + SA (dexamethasone pre-treated and *S. aureus* infected), TLR2Ab + Dex + SA (anti TLR-2 antibody and dexamethasone pre-treated and *S. aureus* infected) and RU + Dex + SA (RU486 and dexamethasone pre-treated and *S. aureus* infected). Both of mouse isotype-matched control antibody (Biorbyt, cat no. orb22987) and rabbit anti-mouse TLR-2 antibody (Biorbyt, cat no. orb11487) were used at a concentration of 5 μg/ml [27]. A GR antagonist mifepristone (RU486) (Sigma, M8046) was applied to the cultured primary microglia at a concentration of 10 μM to inhibit the function of GR [28]. 150 nM concentration of dexamethasone (Sigma, D1756) was applied to the microglia as an exogenous source of corticosterone [29].

After pre-incubation with such antibodies, antagonist and dexamethasone respective groups of microglial cells were infected with live *S. aureus* (AG-789) at a ratio of 1: 2 (5×10^6 microglial cells were infected with 10^7 *S. aureus*) followed by 30, 60 and 90 min of incubation at 37 °C [30]. Post infection supernatants were collected and stored at −20 °C until further analysis.

2.5. Determination of microglial motility by agarose spot assay

The agarose spot assay was performed to determine the microglial chemotaxis in the presence or absence of isotype antibody, anti TLR-2 antibody, RU486 and dexamethasone with respect to live *S. aureus* infection. A 0.5% low-melting point agarose was prepared in sterile PBS. The solution was heated in microwave and mixed well until dissolved completely. Then the mixture was cooled to 40 °C. From that 90 μl of agarose solution was pipetted into a 1.5 ml microcentrifuge tube containing 10 μl of PBS with or without live *S. aureus* and mixed well. The 10 μl spots of agarose were pipetted onto a 35 mm diameter glass-bottomed cell culture dish and allowed to cool for 10 min at 4 °C. Spots containing only sterile PBS were considered as PBS control. Microglial cells pre-treated with isotype antibody, anti TLR-2 antibody, RU486 and dexamethasone were plated separately into those dishes (5×10^6 cells in 1 ml DMEM) and then incubated at 37 °C in 5% CO_2 to allow the cells to adhere and migrate. After 6 h, microglial cells under the spot were observed under phase contrast microscope. The expressed values were the mean obtained from three separate dishes [31].

2.6. Assay for quantification of hydrogen peroxide (H_2O_2) production

After time-dependent incubation, cell culture supernatants were collected and H_2O_2 assay was performed according to the method as described earlier with slight modification [32]. Briefly 70 μl of supernatant, 20 μl of Horse Radish peroxidase (HRP) (500 μg/ml), 70 μl of Phenol red (500 μg/ml) and 40 μl of medium were added 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 was measured at 620 nm. Control set received 70 μl of HBSS in place of supernatant. A standard H_2O_2 curve was plotted and H_2O_2 release in supernatants was evaluated and expressed in $\mu M/10^6$ cells.

2.7. Assay for quantification of superoxide anion (O_2^-) production

Superoxide anion release assay measures the change in color of cytochrome C (cyt C), when reduced by O_2^- released from the stimulated cells infected with *S. aureus* pre-treated with or without exogenous SOD (catalogue no SC-11407) (2.78 μg/ml). The difference between the amounts of cyt C reduced in the presence and absence of SOD represented the amount of superoxide anion generated during the incubation. The instrument was blanked on the samples containing cyt C plus SOD and instructed sequentially to read the absorbance of all the samples at 550 nm in reference to the blank. The absorbance read in this manner is closest to the true SOD inhibitable cyt C reduction. Cell supernatant obtained after the time-dependent phagocytosis by the microglial cells, infected with *S. aureus* were incubated in presence of cyt C (100 μl at 2 mg/ml). The production of superoxide anion was monitored spectrophotometrically at 550 nm with reference to the blank. The amount of superoxide anion production was calculated by the following formula: nanomoles of superoxide anion = (mean absorbance at 550 nm × 15.87) [33].

2.8. Assay for quantification of nitric oxide (NO) production

The production of nitric oxide was assayed by measuring the nitrite levels of the stable nitric oxide metabolite in culture medium which was prepared as described previously [34]. Briefly, accumulation of nitrite in the medium was determined by colorimetric Griess assay. Microglial cells (5×10^6 cells per well) in 24-well plates in 500 μl culture medium were stimulated with viable *S. aureus* for 30, 60 and 90 min. 100 μl of culture supernatant reacted with an equal volume of Griess reagent (one part 0.1% naphthylethylenediamine and other part 1% sulfanilamide in 5% H_3PO_4) in culture plates for 10 min at room temperature in the dark. The absorbance at 550 nm was determined using a microplate reader (Bio Rad). The total NO production was determined by comparing with a standard $NaNO_3$ curve.

2.9. Assay for quantification of arginase enzyme activity

Arginase enzyme activity of microglial cells was measured in all different conditions. After time dependent incubation with *S. aureus* microglial cells were lysed with 50 μl of PBS containing 0.1% Triton-X 100 and incubated for 30 min at room temperature. Then 50 μl of 10 mM $MnCl_2$ and 50 mM Tris-HCl, pH 7.5 were added and the enzyme was activated by heating the mixture at 55 °C for 10 min. The hydrolysis of arginine was initiated by the addition of 25 μl of 0.5 M arginine, pH 9.7 to a 25 μl aliquot of the previously activated lysate. Then they were incubated for 60 min at 37 °C and the reaction was stopped by the addition of 400 μl of an acid mixture containing H_2SO_4, H_3PO_4 and H_2O at a ratio of 1:3:7. The formation of urea was measured colorimetrically at 540 nm after adding 25 μl of 9% α-isonitrosopropiophenone and heating the mixture at 100 °C for 45 min. The arginase activity was determined by comparing with the standard curve of urea [35].

2.10. Assay for superoxide dismutase (SOD) enzyme activity

The levels of antioxidant enzymes display the intracellular host defence mechanisms which have the ability to scavenge free radicals. So, determination of SOD activity became relevant to this study. A 100 μl of cell-free lysate was mixed separately with 1.5 ml of Tris-EDTA-HCl buffer (pH 8.5), followed by addition of 100 μl of 7.2 mM/L pyrogallol 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 absorbance was measured at 420 nm. One unit was determined as the amount of enzyme that inhibited the oxidation of pyrogallol by 50%. The SOD enzyme activity was expressed as U/mg protein [36].

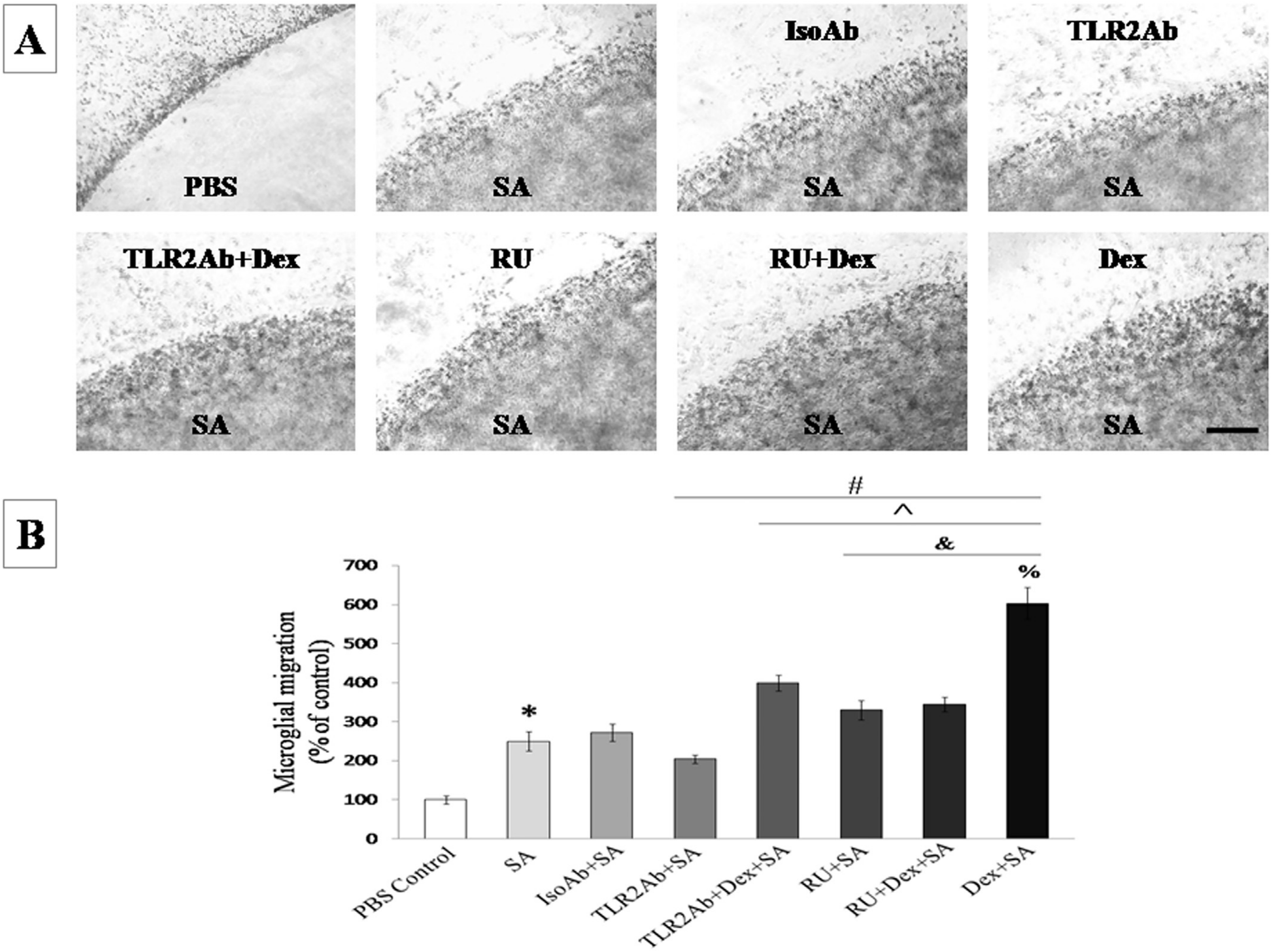

Fig. 1. Effects of anti TLR-2 antibody, GR antagonist RU486 and dexamethasone treatment on microglial motility in response to live *S. aureus* infection.
(A) Phase contrast microscopic images of agarose spot assay. Microglial cells with or without pre-treatment were placed on 35 mm cover slips with spots containing either PBS or *S. aureus* (SA). Scale bar: 200 μm. (B) Cells migrated into the spots were counted and graphically represented. Results were shown as mean ± SD from three independent experiments. The differences were statistically significant at $p < 0.05$ level. '*' indicates significant difference in comparison to PBS control, '#' indicates significant difference in comparison to SA, '^' indicates significant difference in comparison to TLR2Ab + SA, '%' indicates significant difference in comparison to RU + SA and '&' indicates significant difference compared to TLR2Ab + Dex + SA.

2.11. Assay for catalase enzyme activity

The catalase enzyme activity was estimated in the cell-free lysate of microglia both before and after infection in the presence or absence of isotype antibody, anti TLR-2 antibody, RU-486 and dexamethasone by measuring the decrease in H_2O_2 concentration at 240 nm. At time zero, 100 μl of the lysate was added separately to 2.89 ml of potassium phosphate buffer (pH 7.4) taken in a quartz cuvette. To it 0.1 ml of 300 mM H_2O_2 was added and absorbance was measured at 240 nm for 5 min at 1 min intervals. Catalase enzyme activity was expressed in terms of nM/min mg protein [37].

2.12. Assay for quantification of corticosterone release

The amount of corticosterone released from live *S. aureus* infected primary microglial cells in presence or absence of anti TLR-2 antibody, GR antagonist and dexamethasone was measured by 2, 4 DNPH method with slight modifications. After time dependent incubation cell-free lysates were dissolved in 500 μl of methanol. An equal volume of DNPH reagent was added to it and mixed well. Now, the test tubes were placed in a water bath maintaining 60 °C temperature for 90 min to proceed the reaction by protecting them from direct light. Then they were allowed to cool for 2 min and 500 μl of 4 N NaOH solution was added to it with gentle shaking. After 20 min absorbance was measured at 470 nm using spectrophotometer against the reagent blank. The quantity of corticosterone was determined using standard corticosterone curve [38].

2.13. Protein extraction and Western blot analysis for the detection of TLR-2, GR, iNOS, COX-2, NF-κB, SAPK/JNK expression in primary microglial cells

Microglial cell pellets from different groups were resuspended in ice-cold RIPA-NP40 buffer containing 0.5 mM PMSF, 1 mM sodium orthovanadate, and 1 μl/ml protease inhibitor cocktail (1 mg/ml leupeptin, 1 mg/ml aprotinin, 10 mg/ml soybean trypsin inhibitor, 1 mg/ml pepstatin). After 30 min incubation on ice lysates were subjected to sonication (2 × 5 s at level 40) and followed by centrifugation at 12,500 ×g for 10 min at 4 °C. Supernatants from the cell extracts were collected [39] and total protein contents were measured using Lowry method. Samples containing equal amounts of protein (120 μg/lane) were separated by SDS-PAGE and transferred to a 0.1 μm pore nitrocellulose membrane. Non-specific binding sites were blocked with Tris-buffered saline and Tween-20 (TBST) (20 mM Tris–HCl, 150 mM

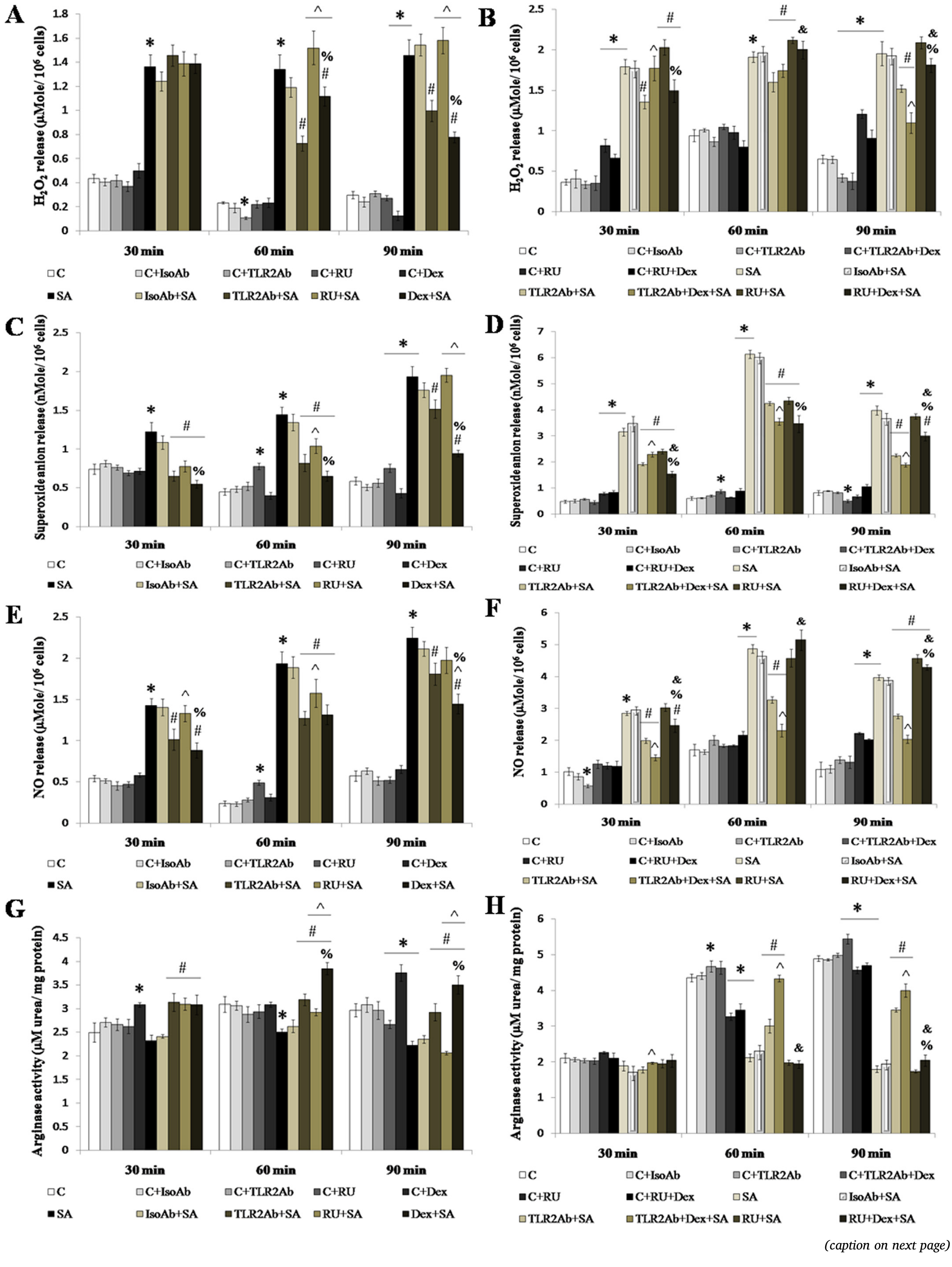

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Fig. 2. Effects of dexamethasone pre-treatment on the production of H_2O_2 (A–B), superoxide anion (C–D), Nitric oxide (E–F) and Arginase activity (G–H) from both TLR-2 and GR opened or blocked conditions.
Murine microglia (5×10^6 cells/ml) were allowed to interact with *S. aureus* (10^7 bacteria/ml) in 1:2 ratio and incubated at 37 °C for 30, 60 and 90 min in the presence or absence of anti-TLR-2 antibody, RU486 and dexamethasone pre-treatment. Results were represented as mean ± SD from three independent experiments. The differences were statistically significant at $p < 0.05$ level. '*' indicates significant difference with respect to control, '#' indicates significant difference in comparison to SA, '^' indicates significant difference in comparison to TLR2Ab + SA, '%' indicates significant difference in comparison to RU + SA and '&' indicates significant difference compared to TLR2Ab + Dex + SA group.

NaCl, 0.1% Tween 20) containing 5% non-fat milk, membranes were washed three times in TBST and probed overnight at 4 °C with rabbit anti-mouse TLR2 antibody (Biorbyt, cat no. orb11487), Glucocorticoid receptor (GR) antibody (Biorbyt, cat no. orb127396), COX-2 antibody (Biorbyt, cat no. orb106537), iNOS antibody (Biorbyt, cat no. orb13614), NF-κB p65 antibody (Biorbyt, cat no. orb11118) and SAPK/JNK antibody (Biorbyt, cat no. orb14628) separately in TBS followed by washing and 2 hour incubation with appropriate horseradish peroxidase-conjugated secondary antibody, developed with the super-signal chemiluminescent substrate (Thermo Scientific, USA) and exposed to X-Omat BT films (Kodak). Beta-tubulin had been used as a loading control for Western blot by using rabbit anti beta-tubulin polyclonal antibody (Biorbyt, cat no. orb11537) to ensure equal loading throughout the gel [40].

2.14. Statistical analysis

Isolated microglial cells from mice were pooled together to obtain the requisite amount of cells (5×10^6 cells/ml) and the different parameters were measured. This was repeated for three times for each parameter (for e.g., H_2O_2 production), then the mean values of these triplicate experiments were taken for final calculation. Data was expressed as mean ± S.D. One-way model 1 ANOVA (Analysis of Variance) was done between the groups. $p < 0.05$ was considered to be the significant level. All statistical analyses were done using Origin Pro-8 software (Origin Lab Corporation, Northampton, MA).

3. Results

3.1. Dexamethasone increases but TLR-2 neutralization or GR blocking decreases microglial chemotaxis in response to viable S. aureus stimulation

As microglial chemotaxis is indirectly related to the microglial reactivity as well as functionality it was an obvious measurable parameter for the present study. Agarose spot assay had been performed by putting PBS or viable *S. aureus* within the spot and microglia in the culture plate were pre-treated with anti-TLR-2 antibody, GR antagonist RU486 both in presence or absence of dexamethasone. Observations were made under phase contrast microscope (Fig. 1A).

When TLR-2 and GR both remains open: From Fig. 1B it was found that microglial migration was significantly increased in SA group compared to the PBS control ($p < 0.05$). Dexamethasone pre-treatment (Dex + SA) sharply increased microglial chemotaxis compared to the SA group as well as TLR-2 or GR blocking conditions (TLR2Ab + SA and RU + SA). Moreover, the motility in Dex + SA group was found to be significantly higher ($p < 0.05$) than TLR-2 neutralized and dexamethasone pre-treated group (TLR2Ab + Dex + SA).
When TLR-2 is blocked but GR remains open: TLR-2 neutralization (TLR2Ab + SA) significantly reduced microglial motility compared to the SA group ($p < 0.05$) but the pre-treatment with dexamethasone (TLR2Ab + Dex + SA) reversed the situation by increasing the motility with respect to both SA and TLR2Ab + SA group.
When GR is blocked but TLR-2 remains open: Microglial GR blocking by pre-treatment with RU486 (RU + SA) significantly enhanced microglial motility compared to SA and TLR2Ab + SA group ($p < 0.05$). Fig. 1B indicated that microglial chemotaxis in RU + SA group was found to be lower than TLR2Ab + Dex + SA group but no alteration was observed during dexamethasone pre-treated condition (RU + Dex + SA).

3.2. TLR-2 neutralization in presence of exogenous dexamethasone diminishes H_2O_2, superoxide anion, NO release from infected microglia but increases during GR blocked condition

Free radical production is considered as a part of cellular immune response and it also causes pathogen induced cellular stress when produced in higher amounts within the cell. Therefore, we were interested to measure those free radicals production in our experimental set up. At first we have isolated microglial cells from a group of animals and investigated the effects of TLR-2 neutralization by anti TLR-2 antibody, GR blocking with RU486 and dexamethasone treatment when both of TLR-2 and GR remain open in *S. aureus* infected microglial cells. Then to extrapolate our findings separate experimental set up was prepared and microglia were isolated from different groups of animals for finding out the effects of dexamethasone pre-treatment over two different conditions i.e., TLR-2 neutralized condition or GR blocking condition in infected microglia. For inter group comparison and statistical analysis few groups of the first set (i.e., C, SA, IsoAb + SA, TLR2Ab + SA, RU + SA etc.) were repeated again in second set. Hence, the two sets of different data were presented side by side in Fig. 2A–B, C–D, E–F and G–H respectively.

When TLR-2 and GR both remains open: From Fig. 2 we have found that significant rises in H_2O_2, superoxide anion, NO production were observed in *S. aureus* infected microglia (SA) with respect to the control cells (C) irrespective of their post infection time ($p < 0.05$). There was no significant difference found when microglial cells were treated with isotype antibody prior to the infection (IsoAb + SA). However dexamethasone pre-treatment (Dex + SA) significantly ($p < 0.05$) reduced H_2O_2 production compared to the SA group as well as TLR-2 neutralized (TLR2Ab + SA) and GR blocked infected microglia (RU + SA) particularly at 60 and 90 min (Fig. 2A).
In case of superoxide anion release (Fig. 2C) Dex + SA group showed marked reduction compared to the SA group at 30, 60 and 90 min but with respect to the GR blocked infected microglia (RU + SA) particularly at 60 min and both to the TLR2Ab + SA and RU + SA groups at 90 min ($p < 0.05$).
NO release assay (Fig. 2E) showed that Dex + SA group significantly diminishes the value than the SA group at all three time points. It was also found to be lower than RU + SA group at 30 min and lower than both of TLR2Ab + SA and RU + SA groups at 90 min ($p < 0.05$).
When TLR-2 is blocked but GR remains open: TLR-2 neutralization prior to *S. aureus* infection (TLR2Ab + SA) significantly lowered H_2O_2, superoxide anion, NO production compared to the SA group ($p < 0.05$) at 30, 60 and 90 min (Fig. 2B, D, E). When dexamethasone pre-treatment was applied to this TLR-2 neutralized condition (TLR2Ab + Dex + SA) the overall free radicals production was significantly ($p < 0.05$) suppressed compared to the only TLR-2 neutralized group (TLR2Ab + SA) but variations were

observed in H_2O_2, superoxide anion, NO production at different time points. In case of H_2O_2 and superoxide release assay this reduction was significant at only 90 min and at both of 60 and 90 min respectively (Fig. 2B, D), whereas NO production (Fig. 2F) was significantly lowered in TLR2Ab + Dex + SA group at 30, 60 and 90 min with respect to the TLR2Ab + SA group ($p < 0.05$).

When GR is blocked but TLR-2 remains open: Surprisingly, GR blocking with RU486 (RU + SA) significantly raised H_2O_2 production compared to the SA group at 30 and 60 min but in case of NO production it was found to be significantly higher only at 90 min ($p < 0.05$). No observable differences were found in case of superoxide anion release assay. Dexamethasone pre-treatment in GR blocking condition (RU + Dex + SA) significantly reduced free radicals production with respect to the RU + SA group but it was still significantly higher than TLR2Ab + Dex + SA group at 60, 90 min for H_2O_2 (Fig. 2B), at only 90 min for superoxide anion release (Fig. 2D) and in case of NO assay it was valid for all three time points (i.e., 30, 60 and 90 min) (Fig. 2F).

3.3. Exogenous dexamethasone recovers *S. aureus* induced reduction of arginase activity in microglia during TLR-2 neutralized condition

When TLR-2 and GR both remains open: From Fig. 2G it was found that arginase enzyme activity was markedly increased even when the control microglia was treated with dexamethasone (C + Dex) with respect to the control (C) particularly at 30 and 90 min. After live *S. aureus* infection (SA) arginase activity was significantly reduced ($p < 0.05$) compared to the control cells at 60 and 90 min (Fig. 2G, H) but dexamethasone administration prior to the infection (Dex + SA) could reversed the situation by augmenting the activity with respect to the SA, TLR2Ab + SA and RU + SA groups at the time point of 60 and 90 min.

When TLR-2 is blocked but GR remains open: Anti TLR-2 antibody pretreatment in *S. aureus* infected group (TLR2Ab + SA) markedly enhanced arginase activity compared to SA at 60 and 90 min. Further increment was observed when the cells were pre-treated with dexamethasone in the same condition (TLR2Ab + Dex + SA) with respect to the TLR2Ab + SA group irrespective of the post infection times (Fig. 2H).

When GR is blocked but TLR-2 remains open: In contrast to the TLR-2 blocking pre-treatment with GR antagonist RU486 showed no observable changes in arginase activity in comparison to the SA group. When dexamethasone was applied in this condition (RU + Dex + SA) the higher value was found at 90 min compared to the RU + SA group. On the other hand the activity in RU + Dex + SA group was found to be significantly ($p < 0.05$) lower than TLR2Ab + Dex + SA group particularly at 60 and 90 min (Fig. 2H).

3.4. Superoxide dismutase (SOD) and catalase activity is augmented during TLR-2 neutralized condition but diminished by RU486 application both in presence or absence of dexamethasone

Anti-oxidant enzyme activities were measured from different groups of microglia in a same experimental set up but for the better understanding of the results and statistical analysis all the experimental groups were not presented in the same figure. Some data sets were repeated in two column diagrams on the basis of comparison in between the groups (Fig. 3A, B and C, D).

When TLR-2 and GR both remains open: SOD and catalase enzyme activities were significantly increased in SA group compared to the control microglia ($p < 0.05$). Dexamethasone administration prior to infection (Dex + SA) markedly raised SOD activity compared to the TLR2Ab + SA and RU + SA groups at 60 and 90 min (Fig. 3A). In case of catalase activity this observation was valid for 90 min only (Fig. 3C).

When TLR-2 is blocked but GR remains open: TLR-2 blocking prior to infection significantly reduced both SOD and catalase activities with respect to the SA group at all three time points. Surprisingly, dexamethasone pre-treatment (TLR2Ab + Dex + SA) up regulated both of these enzyme activities at 60 and 90 min when comparing with only TLR2Ab + SA group ($p < 0.05$) (Fig. 3B, D).

When GR is blocked but TLR-2 remains open: From Fig. 3A, B and C, D it was found that RU + SA group showed marked decline in SOD and catalase activities compared to the SA group at 60, 90 min. Dexamethasone pre-treatment in GR blocking condition at 90 min showed recovery of both of their activities from GR blocking condition (RU + SA). A significant diminution ($p < 0.05$) of both SOD and catalase activities were observed in RU + Dex + SA group compared to the TLR2Ab + Dex + SA group at 60 and 90 min of post infection time (Fig. 3B, D).

3.5. Alterations of corticosterone concentration either in presence or absence of exogenous dexamethasone during TLR-2 neutralized and GR blocked condition

In our experimental set up the effects of exogenous dexamethasone was observed in three different conditions. Therefore, the microglial corticosterone concentration was a logical parameter to measure the cumulative effects of endogenous corticosterone and exogenously applied dexamethasone over receptor blocking conditions.

When TLR-2 and GR both remains open: Fig. 3E indicated that dexamethasone treatment in control cells (C + Dex) showed higher corticosterone concentration than C group. The *S. aureus* infection (SA) induced corticosterone release was found to be significant than control group at all three time points. Dexamethasone treatment before infection (Dex + SA) further raised the value with respect to the SA, TLR2Ab + SA and RU + SA groups at 60 and 90 min ($p < 0.05$).

When TLR-2 is blocked but GR remains open: TLR-2 neutralization prior to *S. aureus* infection (TLR2Ab + SA) reduced the corticosterone concentration compared to the SA group irrespective of the post infection times. Dexamethasone pre-treatment (TLR2Ab + Dex + SA) totally reversed the situation by significantly increasing ($p < 0.05$) the value compared to the TLR2Ab + SA group at 30, 60 and 90 min (Fig. 3F). Moreover the optimum concentration of corticosterone was found at 90 min of post infection time.

When GR is blocked but TLR-2 remains open: GR blocking with RU486 (RU + SA) significantly diminished *S. aureus* induced corticosterone concentration particularly at 30 and 90 min. Dexamethasone administration (RU + Dex + SA) slightly enhanced the value compared to the only GR blocking condition at 30 and 90 min but the same value in RU + Dex + SA group was found to be significantly lower ($p < 0.05$) than TLR2Ab + Dex + SA group at 60 and 90 min (Fig. 3F).

3.6. Microglial GR expression inversely modulates TLR-2 expression and down regulates other inflammatory markers like COX-2, iNOS, NF-κB and SAPK/JNK in presence of dexamethasone

From the above results it was found that dexamethasone induced optimum effects were found at the time point of 90 min in both cases of TLR-2 neutralization and GR blocking. Therefore, we have performed Western blot analysis of the major two receptors like TLR-2, GR along with some inflammatory markers at the time point of 90 min to find out their functional relationship during *S. aureus* infection.

When TLR-2 and GR both remains open: From Fig. 4 it was found that TLR-2 expression was significantly increased in SA group with

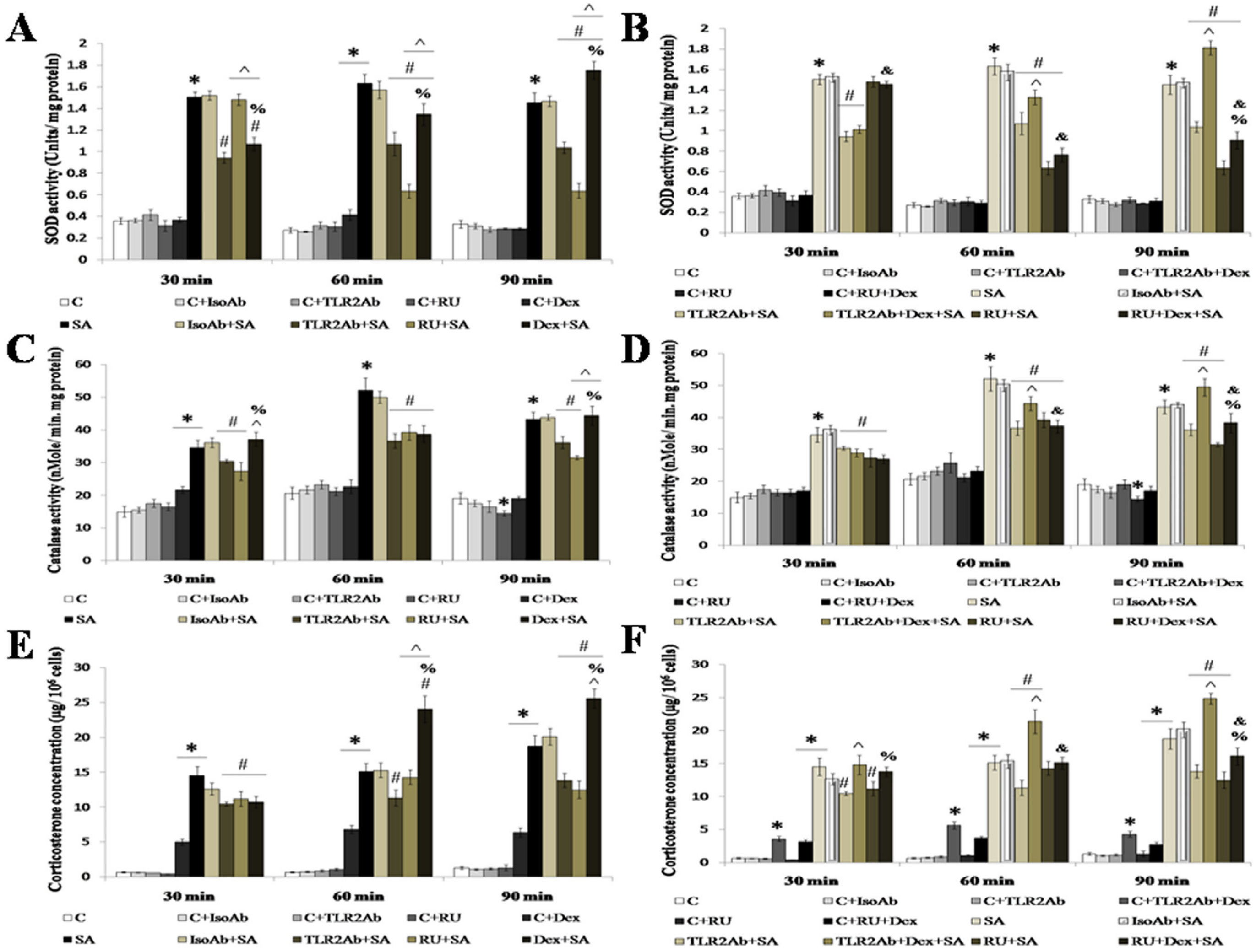

Fig. 3. Effects of dexamethasone administration in presence or absence of GR antagonist RU486 and anti TLR-2 antibody on *S. aureus* induced alterations of SOD and catalase activities and corticosterone concentration.

SOD enzyme activity (A–B) was expressed as U/mg protein, catalase enzyme activity (C–D) was measured in terms of nM/min.mg protein and corticosterone concentration (E–F) was expressed as µg/10^6 cells. Results were shown as mean ± SD from three independent experiments. The differences were statistically significant at $p < 0.05$ level. '*' indicates significant difference with respect to control, '#' indicates significant difference in comparison to SA, '^' indicates significant difference in comparison to TLR2Ab + SA, '%' indicates significant difference in comparison to RU + SA, '&' indicates significant difference with respect to TLR2Ab + Dex + SA group.

respect to control (C). No marked variation was observed when infected microglia was pre-treated with isotype antibody (IsoAb + SA). Dexamethasone pre-treated and *S. aureus* infected cells (Dex + SA) showed significant reduction in TLR-2 expression compared to SA and RU + SA groups but it was found to be higher when comparing with TLR2Ab + SA group ($p < 0.05$).

In case of GR blot dexamethasone treatment to control microglia (C + Dex) and *S. aureus* infection (SA) significantly increased GR expression with respect to control microglia ($p < 0.05$). Further significant elevation was observed in Dex + SA group compared to the SA, TLR2Ab + SA and RU + SA groups (Fig. 4).

Other inflammatory markers and signalling molecules like COX-2, iNOS, NF-κB, JNK showed almost identical patterns of expression in Western blot (Fig. 5). Their expressions were significantly increased in SA group when comparing with the control ($p < 0.05$). After dexamethasone pre-treatment COX-2, iNOS, NF-κB expression were significantly reduced with respect to the SA, TLR2Ab + SA and RU + SA groups but in case of SAPK/JNK it was significantly lower in comparison to the SA and RU + SA groups only.

When TLR-2 is blocked but GR remains open: Cell surface TLR-2 blocking with anti TLR-2 antibody before *S. aureus* infection (TLR2Ab + SA) significantly reduced both of TLR-2 and GR expressions with respect to SA ($p < 0.05$). After dexamethasone pretreatment (TLR2Ab + Dex + SA group) marked elevation of GR expression was observed compared to the only TLR-2 neutralized *S. aureus* infected group (TLR2Ab + SA) in contrast to the sharply reduced TLR-2 expression in the same group (TLR2Ab + Dex + SA) (Fig. 6).

From Fig. 6 it was found that COX-2, iNOS, NF-κB, JNK expression follows almost similar patterns like TLR-2 expression (Fig. 6). In all the cases their expressions were seemed to be lowest when TLR-2 neutralization was accompanied with dexamethasone pre-treatment (TLR2Ab + Dex + SA) compared to the SA and TLR2Ab + SA groups ($p < 0.05$).

When GR is blocked but TLR-2 remains open: During GR blocking with RU486 the opposite scenario of TLR-2 neutralization was observed. RU + SA group showed significant diminution of GR expression compared to both of SA and TLR2Ab + SA groups ($p < 0.05$). In case of TLR-2 expression it was slightly reduced compared to the *S. aureus* infected microglia (SA) but significantly higher than

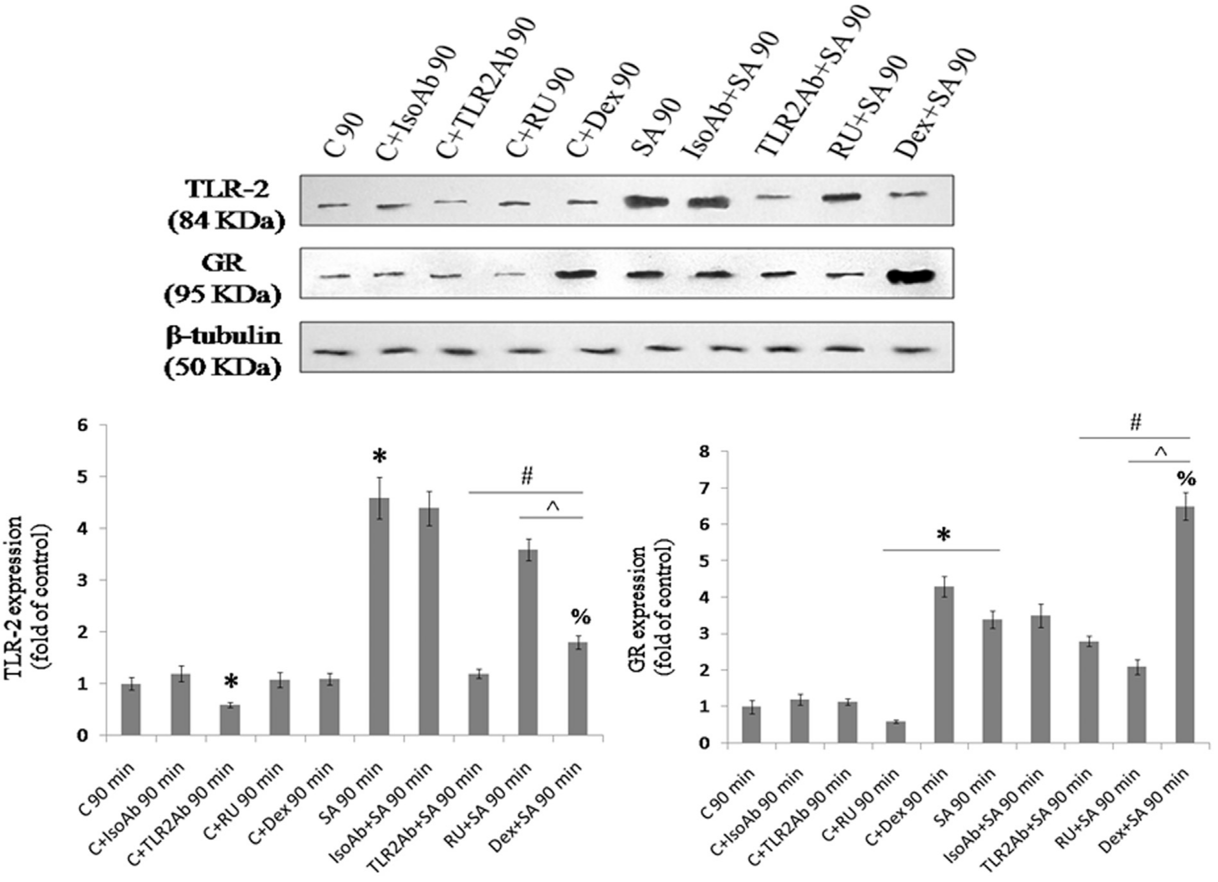

Fig. 4. Western blot analysis of microglial TLR-2 and glucocorticoid receptor (GR) expression during TLR-2 neutralization, GR blocking or dexamethasone application.

Whole cell lysates were prepared for analysis of TLR-2 and GR expression at 90 min. All the samples were probed with β-tubulin to show equal protein loading. '*' indicates significant difference with respect to control, '#' indicates significant difference in comparison to SA, '^' indicates significant difference in comparison to TLR2Ab + SA, '%' indicates significant difference in comparison to RU + SA group at $p < 0.05$ level.

TLR2Ab + SA group (Fig. 4). From Fig. 6 it could be said that RU486 mediated suppression of GR expression and augmentation of TLR-2 expression was reduced slightly by the application of dexamethasone in the GR blocking condition.

Other markers of inflammation were found to be elevated (COX-2, NF-κB) or remain unaltered (iNOS, JNK) in GR blocking condition with respect to the SA group ($p < 0.05$) but slightly recover in dexamethasone pre-treated condition (RU + Dex + SA). The only exception was that SAPK/JNK expression is not altered even after dexamethasone application. The similarity between all of them was that RU + Dex + SA group in all the cases showed significantly higher value ($p < 0.05$) compared to the TLR2Ab + Dex + SA group (Fig. 6).

3.7. Dexamethasone pre-treatment induces percent inhibition of TLR-2 but reduces percent inhibition of GR expression

From Western blot analysis percent inhibition of TLR-2 and GR expression were measured with respect to the *S. aureus* infection. The fold increase values of these two receptors during TLR-2 neutralization and GR blocking were taken into account for percent inhibition calculation.

When TLR-2 is blocked but GR remains open: From Table 1 it was found that in absence of exogenous dexamethasone the percent inhibition of TLR-2 expression is 75.6 which is increased to 83.7 after dexamethasone application. Percent of GR inhibition was 41.9 without dexamethasone whereas it was sharply reduced to −24.4% after dexamethasone treatment. It implied that 24.4% enhancement was observed in case of GR expression.

When GR is blocked but TLR-2 remains open: In this condition the percent inhibition of TLR-2 expression was found to be 23.3 without dexamethasone but after dexamethasone application TLR-2 expression showed an increased inhibition of 45.3%. The percent inhibition of GR expression in absence of dexamethasone was 62.8 which were slightly reduced to 50% in presence of exogenous dexamethasone.

4. Discussion

Regulation of microglial activation caused by bacterial brain abscesses is still a challenging and debatable health issue especially in developing countries [41]. Brain abscesses represent a significant mortality rates ranging from around 30 to 80%. A large proportion of brain abscess patients experiences long-term complications, such as seizures, loss of mental acuity, and focal neurological defects [42]. The opportunistic bacteria *S. aureus* can initiate microglial inflammation in the infected brain tissues followed by profuse neuroinflammation in the surrounding areas [43]. The conventional antibiotic therapy sometimes able to kill the pathogen but could not reach to their optimum therapeutic level due to emergence of multidrug resistant strain [44]. Though killing of bacteria directly reduces bacterial load in the CNS they are not able to manage the infection induced inflammatory consequences which are more damaging to the host [45]. Early studies using a mouse experimental brain abscess model revealed that Toll-like receptors (TLRs) act as a complex mediator for disease pathogenesis

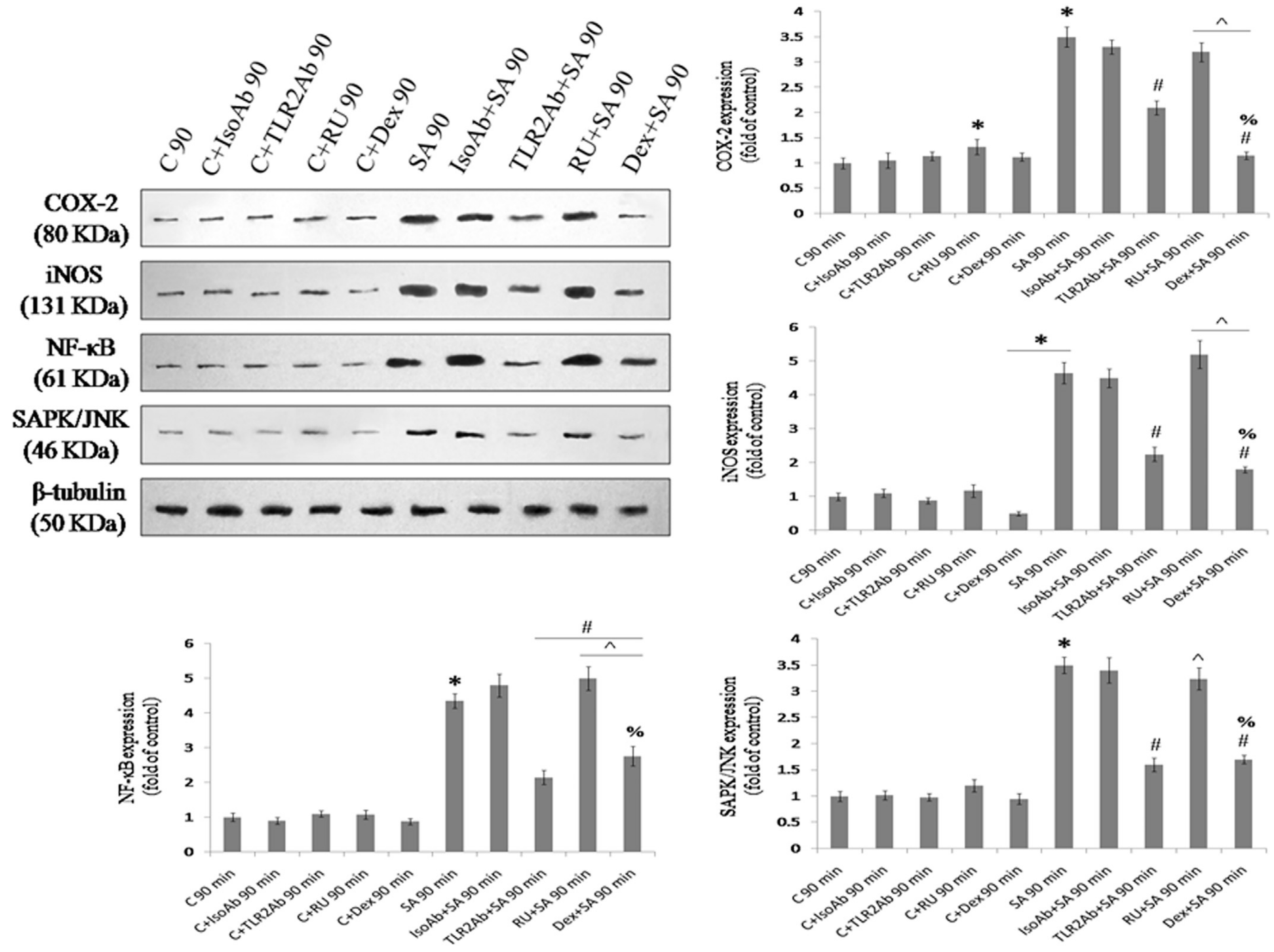

Fig. 5. Effects of TLR-2 and GR blocking or dexamethasone pre-treatment on the infection induced COX-2, iNOS, NF-κB and SAPK/JNK expression in primary microglial cells.

Cell free lysates were prepared for analysis of inflammatory molecules like COX-2, iNOS, NF-κB and SAPK/JNK after 90 min of *S. aureus* infection. All the samples were probed using β-tubulin to show equal protein loading. '*' indicates significant difference with respect to control, '#' indicates significant difference in comparison to SA, '^' indicates significant difference in comparison to TLR2Ab + SA, '%' indicates significant difference in comparison to RU + SA group at $p < 0.05$ level.

and progression [46]. Previously steroids were used as adjunctive therapeutic agents but their excessive use causes steroid resistance [47]. It was reported that microglial surface TLR-2 and intracellular GR play crucial roles in induction as well as suppression of inflammation. Therefore, we have targeted TLR-2 and GR to figure out their interdependency during acute *S. aureus* infection as an alternative approach for controlling microglial over activation. Moreover, the effects of dexamethasone administration were investigated over TLR-2 neutralized and GR blocked condition separately in terms of free radicals production, antioxidant enzyme activities and other inflammatory marker expression which ultimately lead to microglial M1/M2 polarization switching.

Microglia is considered as the principal phagocytic cells of the CNS and its motility directly contributes to the phagocytosis as well as microglial reactivity [48]. From agarose spot assay it was evident that microglial motility with respect to the *S. aureus* infection was highest when cells were pre-treated with dexamethasone in presence of both TLR-2 and GR. The reduced motility due to the functional blocking of GR proves that presence of GR is a crucial factor for microglial reactivity [49]. Our finding was supported by a previous study showing that minute concentration of corticosterone could modulate macrophage function [29]. Moreover, TLR-2 acts as a pattern recognition receptor which can contribute to the pathogen induced microglial activation [50]. The diminution of microglial motility followed by TLR-2 neutralization was probably due to the incomplete recognition of its natural ligand *S. aureus* peptidoglycan. Literature suggested that experiments with TLR-2 knock out microglia shows no alterations in cellular motility in response to the TLR-2 agonist Pam3CSK4 [31].

During *S. aureus* infection the overall free radicals (H_2O_2, superoxide anion and NO) production was increased as a component of respiratory burst in microglia [51]. Being an anti-inflammatory agent dexamethasone was reported to be responsible for enhanced intracellular survival of bacteria [52]. This is probably due to the lowering of free radical production following dexamethasone pre-treatment to the *S. aureus* infected microglia as depicted from our results. The pretreatment with GR antagonist RU486 raised free radical production probably by down regulating GR expression as opposed to the immunosuppressive effect of dexamethasone [53]. This increased production of reactive oxygen species (ROS) and NO could lead to the increased cellular stress in infected microglia [54].

As TLR-2 is responsible for initiating pro-inflammatory cascade of events the suppression of oxidative burst was observed when TLR-2 is blocked with anti TLR-2 antibody [55]. This reduction in free radical production was further attenuated in presence of exogenous

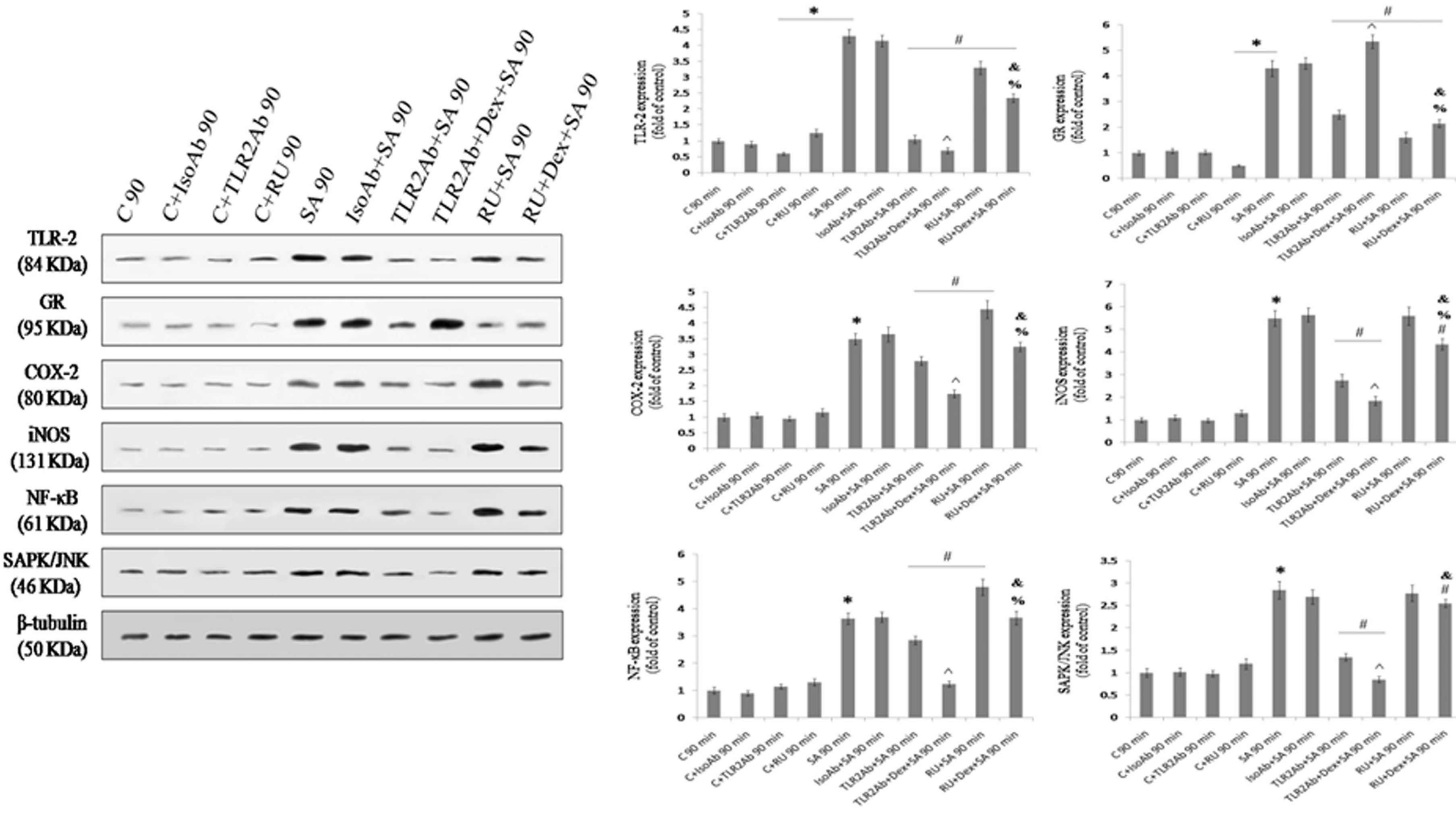

Fig. 6. Effects of dexamethasone administration on the *S. aureus* induced expressions of TLR-2, GR, COX-2, iNOS, NF-κB and SAPK/JNK either in TLR-2 neutralized or in GR blocked condition.
Western blot analysis was done using primary microglial cells infected with *S. aureus* after incubation with TLR-2 antibody or RU486 at the time point of 90 min. Cell free lysates were prepared for the analysis of TLR-2, GR, COX-2, iNOS and NF-κB and SAPK/JNK expression. To ensure the equal protein loading in different lanes samples were probed with β-tubulin. '*' indicates significant difference with respect to control, '#' indicates significant difference in comparison to SA, '^' indicates significant difference in comparison to TLR2Ab + SA, '%' indicates significant difference in comparison to RU + SA and '&' indicates significant difference with respect to TLR2Ab + Dex + SA group at $p < 0.05$ level.

Table 1
Percent inhibition of TLR-2 and GR expression with respect to *S. aureus* infection in different experimental conditions with or without dexamethasone.

Receptor	Percent inhibition of receptor expression with respect to infected group (SA) from Western blot analysis		Exogenous dexamethasone administration
	TLR-2 opened GR blocked + SA	GR opened TLR-2 blocked + SA	
TLR-2	23.3	75.6	(−)
GR	62.8	41.9	
TLR-2	45.3	83.7	(+)
GR	50	−24.4	

Percent inhibition of receptor expression was calculated by the following formula: [(a − b) / a] × 100, where receptor expression in SA group was taken as 'a' and expressions in different receptor blocked groups were taken as 'b'. The fold increase values in arbitrary unit were taken for this calculation.

dexamethasone. Western blot analysis indicated that administration of dexamethasone could able to induce GR expression that led to the down regulation of TLR-2 [56] as well as COX-2, iNOS expression [57,58] at 90 min and ultimately exerts potent anti-inflammatory effects by attenuating H_2O_2, superoxide anion and NO release in TLR-2 blocking condition.

In contrast to the free radical production, arginase enzyme activity was found to be reduced following bacterial infection [59]. Actually, two enzymes iNOS and arginase 1 act upon the same substrate L-arginine depending upon their expression levels and decides whether microglia would be shifted towards M1 or M2 polarization. Higher arginase activity triggered microglial M2 switching [60]. Our observation clearly indicated that dexamethasone in presence of anti TLR-2 antibody promotes M1 to M2 switching [61] particularly around 60 to 90 min of infection but GR blocking with RU486 could reverse the situation by triggering M1 polarization probably via augmenting iNOS expression [62]. This is to be further added that dexamethasone in presence of GR antagonist could slightly increase the arginase activity after 90 min.

Superoxide dismutase (SOD) and catalase, two antioxidant enzymes raised their activity after TLR-2 neutralization as well as dexamethasone pre-treatment in TLR-2 neutralized condition. The higher activity of SOD and catalase might be responsible for sharp decline in superoxide anion and H_2O_2 release from infected microglia [63]. So, we

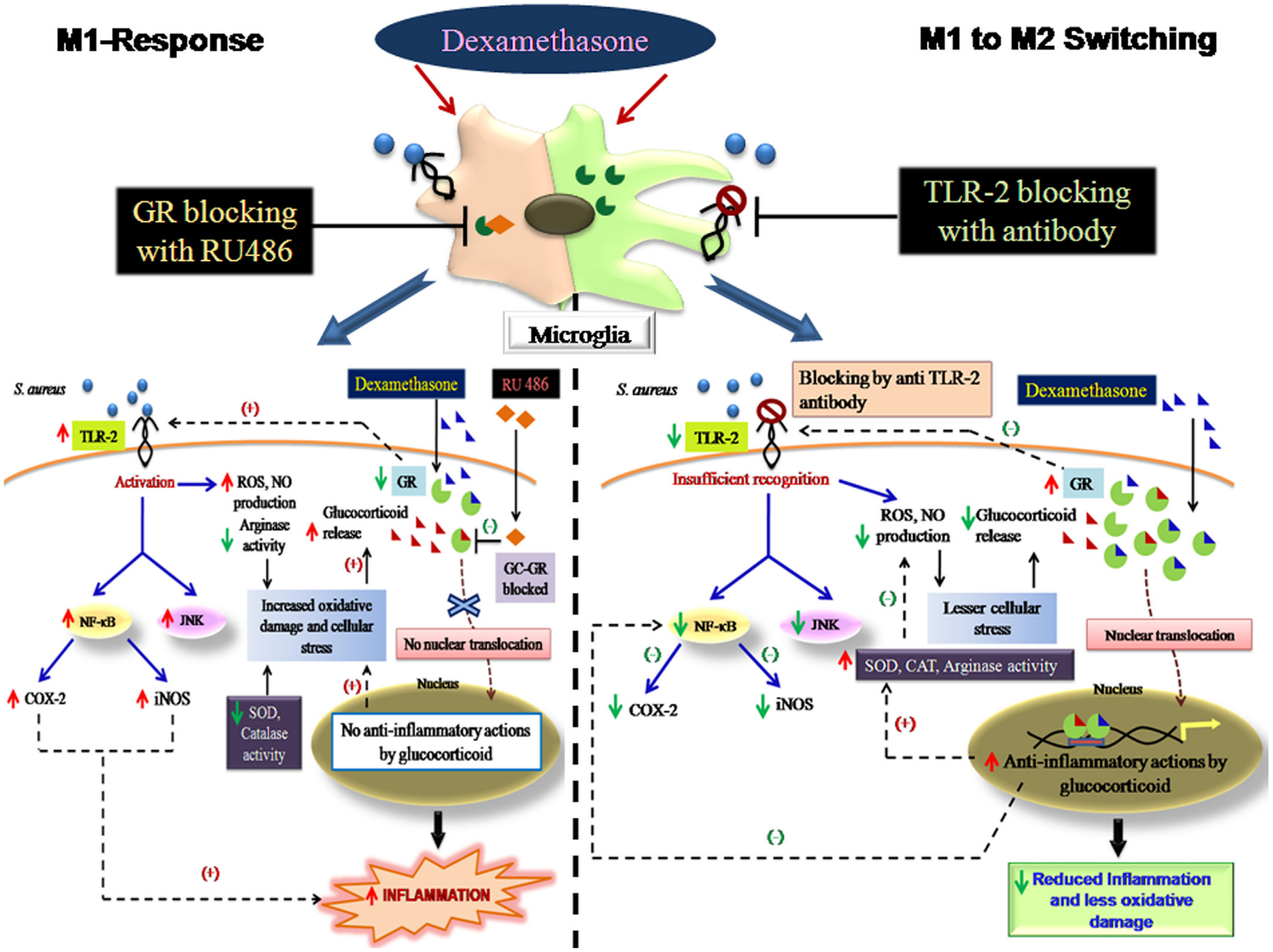

Fig. 7. Schematic representation of *S. aureus* induced microglial inflammation regulation by dexamethasone pre-treatment in TLR-2 blocking condition.
Left panel: Showing the effect of dexamethasone treatment during GR blocking with RU486 in *S. aureus* infected microglia. Live *S. aureus* infection causes increased oxidative damage, stress and enhanced inflammation which triggers pro-inflammatory M1 polarization of microglia via down regulating GR and up regulating TLR-2 expression. Right panel: Intracellular mechanism of microglia after dexamethasone treatment in presence of TLR-2 neutralizing antibody. The simultaneous inhibition of TLR-2 induced pro-inflammatory events along with higher GR expression promoted anti-inflammatory effects of GC through M1 to M2 polarization switching. [Red arrows represent up-regulation or increment; green arrows indicate down-regulation or decrement and (+) indicates facilitation; (−) indicates inhibition.] (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

can say that dexamethasone may exert better anti-inflammatory, protective effects during TLR-2 neutralization via up regulating antioxidant enzyme activities [64]. As the modulation of GR in terms of infection was one of our major focuses we have studied the effects of a synthetic corticosterone, dexamethasone over TLR-2 or GR blocking conditions. Actually, the modulation of GR expression occurred by the cumulative effects of exogenous dexamethasone and endogenous corticosterone release [65]. From corticosterone assay it could be assumed that *S. aureus* infection increases endogenous corticosterone concentration possibly by stress induced mechanism [66]. In TLR-2 blocking condition dexamethasone pre-treatment enhanced the corticosterone release gradually and reached to the peak value at 90 min as supported by the higher expression of GR at the same time point. Western blot analysis proved that GR antagonist RU486 successfully blocked infection induced corticosterone release [67] via down regulating GR. Application of dexamethasone in GR blocked condition could not recover the situation due to the competitive binding of RU486 with GR [68].

Western blot data revealed that TLR-2 and GR might play antagonistic role in *S. aureus* induced microglial inflammation. *S. aureus* infected microglia could induce both of TLR-2 and GR expression and after treatment with anti TLR-2 antibody TLR-2 expression was drastically reduced, this also proves the successful blocking of TLR-2. Due to the lack of sufficient TLR-2 expression other pro-inflammatory markers like COX-2, iNOS expression was also diminished [69] and possibly regulates infection induced cellular stress. Moreover, dexamethasone pre-treatment increased GR expression via down regulating TLR-2 or other inflammatory markers. Hence, it can be said that GR expression can negatively modulates TLR-2 expression and it could be beneficial for the host in terms of inflammation regulation [70]. The consequences of GR blocking with RU486 showed just opposite effects via augmenting TLR-2 induced microglial inflammation [71]. The dependency between TLR-2 and GR worked via NF-κB and JNK mediated pathway [72]. Therefore, it can be speculated that dexamethasone induced up regulation of GR provides protection mostly in TLR-2 neutralized condition by switching the microglial polarization towards M2.

From the overall study (Fig. 7) it can be concluded that dexamethasone pre-treatment during TLR-2 neutralized condition effectively reduced *S. aureus* induced microglial inflammation via modulating TLR-2/GR mediated pathway. When functional blocking of GR was done using RU486 but TLR-2 remained open (Fig. 7: left panel) live *S. aureus* could initiate inflammatory responses to the cell via utilizing cell surface TLR-2. As a result ROS and NO production were increased by suppressing arginase activity. The decreased anti-oxidant enzyme activities induce cellular stress which could trigger endogenous GC release. Application of dexamethasone increased the numbers of cytosolic ligands of GR but they could not exert their anti-inflammatory effects due to blocking of GR and interrupted GC-GR translocation to nucleus. Moreover, TLR-2 activation induced NF-κB and JNK mediated pathways and enhanced iNOS and COX-2 expression [73] which ultimately leading to excess microglial inflammation. The resulting reduced expression of GR ultimately indicates the pro-inflammatory M1 response of microglia which was supported by enhanced TLR-2, iNOS, COX-2, NF-κB and JNK expression in contrast to the lower arginase activity and corticosterone concentration. In case of TLR-2 blocking (Fig. 7: right panel) cytosolic GR remained open and facilitates the GC-GR mediated nuclear translocation. Firstly, the incomplete recognition of *S. aureus* was observed due to the presence of anti TLR-2 antibody. This insufficient recognition reduced the overall free radicals production, iNOS, COX-2, NF-κB and JNK expression accompanied with enhanced SOD, catalase and arginase activities [52]. Though due to the reduced cellular stress endogenous corticosterone release was low it was replenished with the dexamethasone pre-treatment. This increased availability of GR ligands up regulated GR expression in contrast to the sharp decline in TLR-2 expression. Evidences suggested that reduction of overall inflammatory markers along with increased arginase activity triggered M1 to M2 switching [74]. Moreover, it is important to be noted that uninterrupted GC-GR mediated anti-inflammatory pathway was further strengthen by cell surface TLR-2 blocking and ultimately confers protection from microglial inflammation possibly through M1 to M2 switching. Though we have focused only on the modulation of total GR expression cytosolic or nuclear discriminations should be done to understand the detailed GR signalling. As the microglial M1/M2 switching can be speculated from the imbalance of pro-inflammatory and anti-inflammatory mediators which warrants further studies on M1/M2 signature markers of microglia.

From the above discussion it can be recommended that TLR-2/GR dependency might be targeted as an alternative way to control microglial activation and the complications related to the steroid resistance should be avoided via TLR-2 neutralization before steroid administration ensuring better resolution of inflammation. Further studies should be done with some bactericidal agents along with TLR-2 blocking to validate our findings in terms of bacterial killing as well as microglial polarization switching. Actually, our work is based upon the in-vitro experiments that were attempted to resolve *S. aureus* induced microglial inflammation by an alternative way. The major pathophysiological concern is the management of bacterial brain abscesses. As microglia was considered as the key player for initiating neuroinflammation in the brain we were interested to find out the ligand induced crosstalk of TLR-2 and GR within the microglia in response to dexamethasone treatment. However, dexamethasone administration should be done in TLR-2 neutralized condition at the very initial stages of brain abscess formation as depicted from our proposed study that GR expression reached its optimum level within 90 min of live *S. aureus* infection. It could be expected that neutralization of TLR-2 facilitates the potent anti-inflammatory activities of dexamethasone in the experimental brain abscess model and prevents further progression of brain inflammation possibly via microglial polarization switching.

This is to further add that *S. aureus* induced in-vivo brain abscess model might be required to validate our current in-vitro findings. Though experimental models of this disease are limited Kielian et al. had developed an experimental murine brain abscess model to accurately reflect the disease progression in humans, providing pathophysiological and immunomodulatory basis of therapeutic agents [75]. It was also reported that intravenously injected *S. aureus* via tail vein could able to penetrate the blood-brain barrier and persisted in mouse brain [76]. However, TLR-2 knockout mice could be used for brain abscess induction to evaluate the efficacy of this alternative therapeutic strategy.

Declaration of competing interest

All authors declare that there are no conflicts of interest.

Acknowledgments

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