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ORIGINAL ARTICLE

Regulation of *Staphylococcus aureus*-induced CXCR1 expression via inhibition of receptor mobilization and receptor shedding during dual receptor (TNFR1 and IL-1R) neutralization

Puja Dutta[1] · Sahin Sultana[1] · Rajen Dey[1] · Biswadev Bishayi[1]

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Abstract
Our earlier studies proposed a radically new idea suggesting interdependency between TNF-α/TNFR1 and IL-1β/IL-1R pathways in modulation of *Staphylococcus aureus*-induced CXCL8/CXCR1 axis. However, the effects of inhibition of cytokine receptor mobilization at intracellular level and surface TNFR1 and IL-1R shedding on *S. aureus*-induced CXCR1 expression have not been studied so far in peritoneal macrophages. This study aimed to investigate the role of inhibition of receptor mobilization from the intracellular pool (using brefeldin A) and surface receptor shedding (using TAPI-1) on CXCR1 expression during dual receptor (TNFR1 plus IL-1R) neutralization in peritoneal macrophages isolated from wild-type Swiss Albino mice. Release of superoxide anion, nitric oxide, and hydrogen peroxide was measured and cytokine production was done by ELISA. Expression of surface receptors (TNFR1, IL-1R, and CXCR1) and inflammatory mediators was studied by Western blot. It was observed that *S. aureus*-infected macrophages showed elevated ROS production, secretion of TNF-α, IL-1β, and CXCL8, along with increased expression of surface receptors (TNFR1, IL-1R, and CXCR1), and inflammatory markers (iNOS and COX-2) compared with control or treated groups ($p < 0.05$). However, prior treatment of macrophages with BFA or TAPI-1 in the presence of anti-TNFR1 antibody and IRAP during *S. aureus* infection showed significant reduction of all these parameters ($p < 0.05$). We can conclude that targeting of TNFR1 and IL-1R (with major focus on surface expression study) either through blockage of intracellular receptor trafficking pathway or via surface receptor shedding diminishes TNFR1/IL-1R interaction and consequently downregulates CXCR1 expression along with inflammatory signalling pathways during bacterial infections.

Keywords CXCR1 · Inflammation · Interleukin-1 receptor · Peritoneal macrophage · *Staphylococcus aureus* · Tumour necrosis factor receptor 1

Introduction

The peritoneal macrophages, a major subset of macrophage cell line, represent a functionally significant population that play a salient role in modulating immune responses during any pathogenic infection or tissue damage [1]. One such known contagious, opportunistic human pathogen is *Staphylococcus aureus* that can cause a wide range of infections in normal as well as immunocompromised hosts through its various virulence components [2]. The study of cytokine-receptor interaction in response to any pathogen and the underlying network of intercellular signalling mechanisms are essential for regulation of inflammation and its associated biological processes [3]. Since the last few years, various researches have been conducted to target the cytokine receptors on the immune cell surface level to block the inflammatory signalling pathways but how "receptor mobilization" and "receptor shedding" could regulate inflammatory signalling have not yet been explored. In the present study, utmost importance have been given to the inhibition of receptor mobilization and receptor shedding of TNFR1 and IL-1R and its implications on *S. aureus*-induced CXCR1 expression in peritoneal macrophages.

The virulence factors of *S. aureus*-like protein A, adhesion molecules bind to the Toll-like receptor protein (TLR-2) expressed on surface of various immune cells [4] and have been

✉ Biswadev Bishayi
biswadevbishayi4@gmail.com; biswa_dev2@yahoo.com

[1] Department of Physiology, Immunology Laboratory, University of Calcutta, University Colleges of Science and Technology, 92 APC Road, Calcutta, West Bengal 700009, India

shown to activate the pro-inflammatory signalling pathways-NF-κB and MAPK pathways, inducing the secretion of major cytokines like TNF-α, IL-1β, and chemokines CXCL8 which leads to recruitment of macrophages [5]. CXCL8 (or IL-8), a most widely studied chemokine was initially identified as a major neutrophil chemotactic factor [6]. It can bind to both of its functional receptors—CXCR1 and CXCR2 effectively, but the major signalling required for chemotaxis is primarily mediated via CXCR1 [7]. According to previous reports, murine models lack CXCR1 as functional receptor, but fewer studies pointed out the involvement of CXCR1 during infections [8]. Although, we demonstrated previously that live *S. aureus* infection of the murine peritoneal macrophages has been shown to increase the expression of CXCR1 [9, 10]. TNF-α, a pleiotropic cytokine, mediates its function by binding to two types of transmembrane receptors—p55-kDa TNFR1 and the p75-kDa TNFR2. Though TNF-α binds to both these receptors with high affinity, major cellular responses for soluble TNF-α (sTNF-α) seem to be dominated by the binding to TNFR1 and membrane TNF-α (mTNF-α) seemed to be the major physiological stimulator of TNFR2 [3]. Blockage of TNFR1 by specific anti-TNFR1 antibody (as it competes with TNF-α by binding to TNFR1 receptor) inhibits TNF-α-mediated inflammatory response [11]. Interleukin-1β (IL-1β), another major pro-inflammatory cytokine, released from the activated macrophages potentiates CXCL8 production and lead to recruitment of macrophages during bacterial infection [12]. Hence, both TNF-α and IL-1β induce CXCL8 secretion through their respective signalling pathways. IL-1β also regulates TNFR1 expression in infected macrophages [13]. IL-1β is known to bind with the two receptors on target cell surface—interleukin-1 receptors 1 (IL-1R1) and 2 (IL-1R2) with equal affinities. However, only IL-1R1, not IL-1R2, is functional because IL-1R2 lacks a cytoplasmic domain and is thus unable to transmit signals to downstream steps [14]. *S. aureus* mediated CXCL8 chemokine expression is also dependent upon IL-1R1 for its function [15], signifying the role of IL-1β/ IL-1R signalling pathway in regulating CXCR1 expression through activation of NF-κB signalling pathways [16–18]. In our previous studies we demonstrated that administration of anti-TNFR1 antibody (to neutralize TNFR1) or Interleukin 1 receptor antagonist protein (IRAP, to neutralize IL-1R) or both inhibited TNF-α, IL-1β, CXCL8 production and also reduced CXCR1 expression during *S. aureus* infection, suggesting a clear crosstalk among the receptors TNFR1-IL-1R-CXCR1 [10]. However, whether blockage of TNFR1 and IL-1R trafficking from the intracellular pool or receptor shedding could have any influential impact on CXCR1 expression during dual receptor neutralization (TNFR1 plus IL-1R) have not been studied so far in macrophages.

Any disruption or blockage in the intracellular pathway of protein secretion prevents the secreted protein to reach the cell surface. Brefeldin A (a well-known antibiotic with a macrocyclic lactone-ring structure) is widely used for inhibition of protein secretion and intracellular protein trafficking and is preferentially selected over other protein inhibitors like momensin as it neither affects protein synthesis nor involved in receptor-mediated endocytosis [19]. This drug has been used to block albumin secretion in cultured hepatocytes isolated from male Wister rats [19]. According to previous reports, BFA inhibited intracellular collagen degradation at Trans-Golgi Network (TGN) region in lung fibroblasts [20], suggesting its site of action at the Endoplasmic reticulum (ER)-Golgi interface. BFA also have considerable role in insulin-regulated responses through redistribution of Glucose transporter type 4 (GLUT4) from intracellular regions to cell membrane in rat adipocytes [21, 22]. The cytokine receptor TNFR1 is basically situated in the Golgi apparatus in absence of any stimulus or pathogen. Various localization studies suggest that BFA disrupt the structure of trans-Golgi network (TGN) into cluster of vesicles, affecting the redistribution of TNFR1 in the TGN region [23]. During bacterial infection, the TNF-α-TNFR1 complex undergoes internalization within the subcellular organelles, leading to recycling of receptors through the endocytic pathway and maintains a continuous pool of receptors on cell surface. Hence, blockage of this receptor mobilization pathway becomes essential to diminish the receptor count on cell surface resulting in diminution of inflammatory effects. However, the probable role of BFA on other cytokine and chemokine receptors during bacterial infection has not yet been explored. Hence, significance of this inhibitory antibiotic in both TNFR1 and IL-1R trafficking and its effect on CXCR1 expression needs to be studied.

The peritoneal macrophages have its own counterbalancing mechanism to halt the tremendous inflammatory responses by a process called "ectodomain shedding" mediated by some membrane-localized proteolytic enzymes. It involves the cleavage of the extracellular domain of the receptor to prevent cytokine-receptor negotiation at the cell surface. TNF-α-converting enzyme (TACE), a known metalloprotease enzyme of the A disintegrin and metalloproteinase (ADAM) family, has a potential importance in TNFR1 shedding and terminating TNF-α/ TNFR1 signalling pathway [24]. Blockage of TNFR1 signalling pathway by either targeting TNF-α or its receptor with selective antagonists has been recognized in various clinical trials for treatment of various inflammatory and autoimmune diseases [25, 26]. Various studies suggest the role of iNOS/ Nitric oxide induced cGMP in regulating TNFR1 shedding by activation of TACE during sepsis [27]. IL-1β also have an influential role in modulating TNF-α receptor shedding in various in vitro models [28]. The abundance of cell surface TNFR1 is not only governed by mobilization from intracellular stores but also through cleavage from the cell surface [29–31]. However, no reports are available on the effects of inhibition of TNFR1 receptor shedding on *S. aureus* infection-induced CXCR1 expression in murine macrophages. To assess the role of TACE as receptor

sheddase, a TACE inhibitor, TNF-α protease inhibitor (TAPI-1) is commonly used in various experimental studies. Peptidoglycan induced TNFR1 shedding in corneal epithelium and increased release of sTNFR1 into the circulation was inhibited by TAPI-1 [32], hence, suggesting the role of TAPI-1 in inhibiting TACE bioactivity. *S. aureus* and its virulence factor, protein A is also involved in IL-1R2 shedding [33], but no reports on TACE-induced IL-1R1 shedding and its implications on IL-1β/IL-1R signalling during *S. aureus* infection have been identified. Furthermore, the effects of inhibition of TNFR1 and IL-1R shedding in combination with surface receptor neutralization and its impact on *S. aureus* infection-induced CXCR1 expression in murine macrophages needs to be highlighted. The CXCL8/CXCR1 pro-inflammatory signalling cascades also involves increased expression of nitric oxide synthase (iNOS), 5-lipooxygenase (5-LOX), and cyclooxygenase-2 (COX-2), the critical mediators of inflammation [34]. These two enzymatic mediators—iNOS and COX-2 produce nitric oxide and prostaglandin respectively that is responsible for pain, edema, or tissue damage [35]. Hence, effects of BFA and TAPI-1 on iNOS and COX-2 expression under surface receptor-neutralized condition have also been described.

In the current study, we demonstrated that BFA blocked the cytokine receptors TNFR1 and IL-1R to reach the cell surface and TAPI-1 inhibited surface receptor shedding when the surface receptors were neutralized. This down regulation of TNFR1 and IL-1R signalling pathways consequently leads to reduced CXCR1 expression in macrophage. The results obtained also suggested the usefulness of BFA and TAPI-1 in regulation of ROS, cytokine production, and CXCL8/CXCR1 axis at 60 min post-*S. aureus* infection along with other inflammatory responses in macrophages.

Materials and methods

Maintenance of animals

Wild-type male Swiss albino mice had been selected as the main animal model throughout the study. All the experiments involving animals were conducted according to the protocols that had been approved by the Institutional Animal Ethics Committee (IAEC), Department of Physiology, University of Calcutta, under the guidance of CPCSEA (Approval No. IAEC/IV/Proposal/BB-01/2016 dated 18 Jul 2016), Ministry of Environment and Forest, Govt. of India. Upon arrival, mice of 6–8 weeks of age with body weight of 20 ± 4 g were randomized into plastic cages with filter bonnets and saw dust bedding, followed by a 1-week quarantine period. Five mice were housed per cage with food and water ad libitum. Animal holding rooms was maintained at 21–24 °C and 40–60% humidity with a 12-h light dark cycle.

Isolation of murine peritoneal macrophages

Four to five days before killing, 4% sterile thioglycolate broth was injected intraperitoneally to male Swiss albino mice. On the day of killing, the collected peritoneal exudate was harvested by lavage from the peritoneal cavities of mice with endotoxin free Hanks' Balanced Salt Solution (HBSS). The isolated peritoneal macrophages were suspended in 0.83% ammonium chloride solution containing 10% (*v*/v) Tris buffer (pH 7.65) to lyse the erythrocytes. Then, the cells were resuspended in RPMI-1640 medium supplemented with 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin and were allowed to adhere on the plastic surface and the non-adherent cells were removed [36]. More than 95% of peritoneal macrophages were found to be viable for doing experiments as determined by Trypan Blue dye exclusion technique and by flow cytometry cell sorting using macrophage-specific marker (cd11b) [37]. For in vitro study, a minimum of five mice were used to obtain the required amount of peritoneal macrophage cells (5×10^6/ml), counted using haemocytometer and were used for in vitro *S. aureus* infection.

Preparation of bacteria

The bacterial strain *Staphylococcus aureus* (*S. aureus*) (AG-789) was maintained in our laboratory and grown overnight in Luria-Bertani broth, diluted with fresh broth, and cultured until mid-logarithmic phase of growth. Live *S. aureus* infection for in vitro study was prepared by adjusting the desired inoculum before infection at optical density ($(OD)_{620} = 0.2$ for 5.0×10^6 cells/ml for *S. aureus*) suspended in phosphate buffer (PBS) and the OD was measured using a spectrophotometer. By serial dilution technique and culture on blood agar medium, the colony forming unit (CFU) count of the desired inoculum was confirmed [38].

Blocking antibody, reagents, and culture conditions

The isolated peritoneal macrophages were cultured in vitro and divided into various experimental groups as shown in the given scheme (Fig. 1). The macrophages without any treatment or infection were designated as control cells. The macrophages were pre-treated with following antibodies or inhibitors: (a) 5 μg/ml of control immunoglobulin G (IgG) isotype antibody (Cat No. orb22987) [39], (b) 2.5 μg/ml of BFA (Cat No. ab120299) [19], (c) 250 μg/ml of TAPI-1(Cat No. ab142218) [32], (d) 10 μg/ml of polyclonal anti-TNFR1 antibody (Cat No. orb27627), (e) 240 ng/ml of IRAP (from MyBiosource, Cat No. MBS203091) [40, 41]. BFA or TAPI-1-treated macrophages were further incubated with polyclonal anti-TNFR1 antibody or IRAP or both anti-TNFR1 antibody+IRAP for 30 min at 37 °C in 5% CO_2

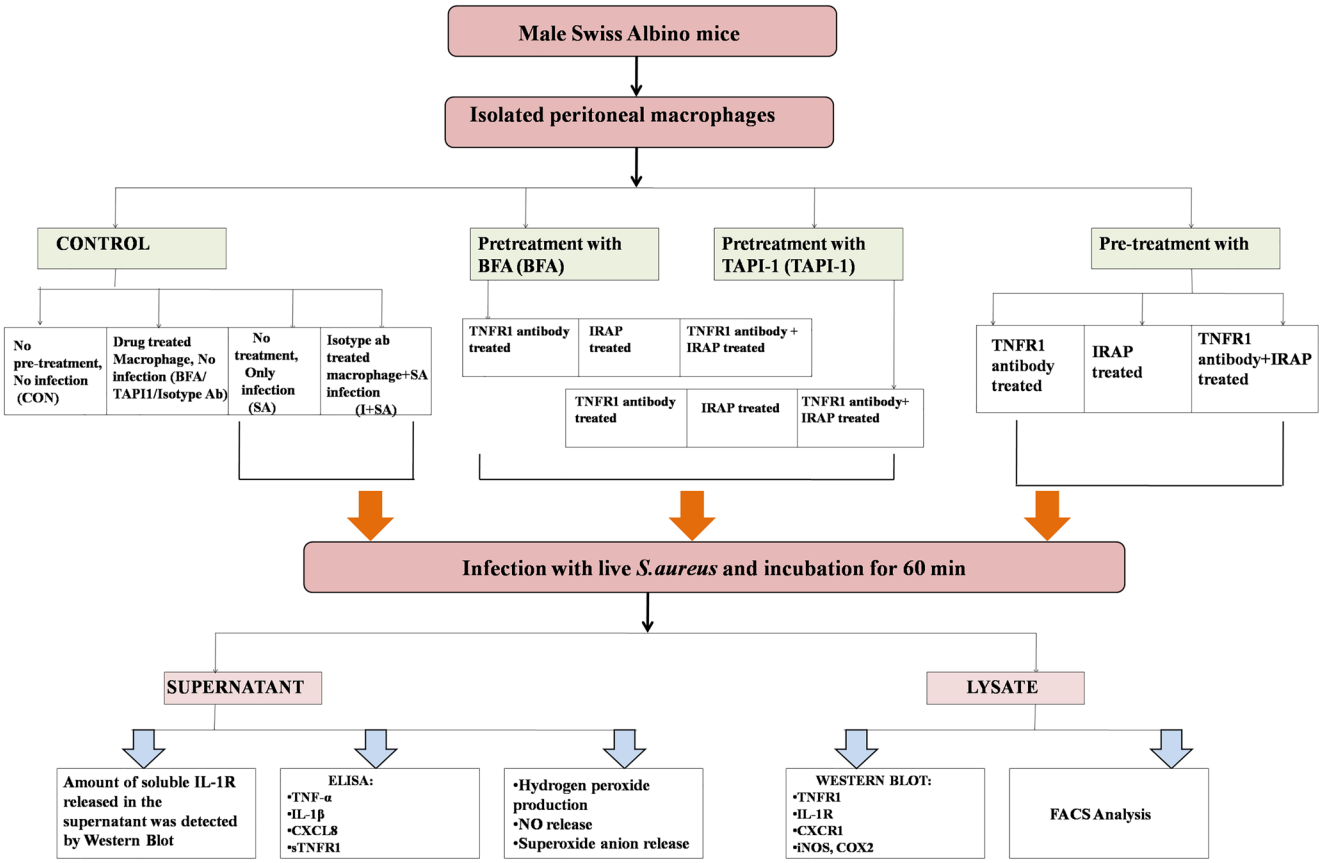


Fig. 1 Experimental design. The peritoneal macrophages were cultured in vitro and subjected to various pre-treatments followed by infection with *S. aureus*. Following the incubation period, the treated or infected cells were centrifuged and supernatant and lysate were separated for further experiments

incubator. The selected doses of BFA and TAPI-1 are non-toxic to the cell (observed in trial studies). Even higher doses of BFA (20 μM) does not have any influence on phagocytosis [42]. However, pre-treatment of macrophages with BFA and TAPI-1 simultaneously was not conducted as excessive drug treatments damaged the normal functioning of the macrophages (obtained from trial studies). The selected anti-TNFR1 antibody and IRAP does not show cross-reactivity with other proteins. Following the incubation period, the treated macrophages were then infected with live *S. aureus* (5×10^6 CFU/ml) as per the experimental groups and further incubated for 60 min at 37 °C in 5% CO_2 incubator. After centrifugation, cell free culture supernatant and lysate were prepared and stored at − 20 °C to carry out further experiments.

Protein estimation

The cell free culture supernatant and lysates were prepared from different sets of macrophages as per the experimental group and stored at − 20 °C for further use. Their protein contents were estimated using the Lowry method [43].

Concentration of supernatants

The supernatant obtained from the stimulated peritoneal macrophages were concentrated to perform western blot analysis for soluble IL-1R (sIL-1R) receptor to study whether it was released in the culture medium. The final-to-original concentration ratio was maintained at 15:1 [44].

Western blot analysis of TNFR1, IL-1R, CXCR1, sIL-1R, iNOS, and COX-2

Western blot analysis for TNFR1, IL-1R, CXCR1, iNOS, and COX-2 expressions were performed from the cell lysate, but only for soluble IL-1R (sIL-1R) cell culture supernatant was used. The cells were lysed with radioimmunepreciptation assay (RIPA) buffer (RIPA-NP40) and protein content quantified by the Lowry method. One hundred micrograms of the lysate proteins from different groups of macrophages was taken for TNFR1, IL-1R, CXCR1, iNOS, and COX-2 expressions and 100 μl of the concentrated cell culture supernatant were used only for soluble IL-1R blot. It was separated on a 10% sodium dodecyl sulphate-polyacryllamide gel

electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane and then blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline with Tween 20 (TBST) for 3 h at room temperature. It was then washed and incubated separately with primary anti-mouse TNFR1 antibody (Cat No. orb27627; Biorbyt Ltd) [45], IL-1R antibody (Cat No. orb6227; Biorbyt Ltd), CXCR1 antibody (Orb 10,487; Biorbyt Ltd), iNOS antibody (Cat No. orb13614; Biorbyt Ltd), and COX-2 antibody (Cat No. orb106537; Biorbyt Ltd) in 1/500 dilution overnight at 4 °C [9, 10]. The membranes were washed with TBST solution and incubated with the appropriate HRP-conjugated secondary antibody in 1/5000 dilution for 1 h at room temperature. The antigen was detected by the enhanced chemiluminescent detection method (Thermoscientific, USA). Beta-tubulin (Cat No. orb11537, Biorbyt Ltd) was used as the loading control for western blot to ensure that equal loading was done in the lanes throughout the gel as it is a housekeeping gene that exhibit high level of constitutive expression in the sample. Images were analysed with ImageJ software.

Flow cytometric analysis of CXCR1expression

FACS analysis was performed in peritoneal macrophages subjected to various treatments before infection. Each sample was then labelled with 5 μl of phycoerythrin (PE)-conjugated monoclonal anti-chemokine receptor CXCR1 (Cat No. FAB8628P from R and D systems) and then incubated for 30 min in the dark. The cells were centrifuged and washed with FACS buffer. It was suspended in 2% paraformaldehyde and incubated for 15 min in the dark. It was then resuspended in 500 μl to 1 ml of ice-cold PBS. The samples were kept in the dark on ice until the scheduled time for analysis. Flow cytometry was performed using BD FACS verse flow cytometers (BD Biosciences). Ten thousand events were collected and analysed by FlowJo software [46].

Assay of superoxide anion free radical production

Superoxide anion release assay was determined from the collected supernatant of peritoneal macrophages 1 h post *S. aureus* infection as per different treatment groups. The cell supernatant was incubated in presence of Cytochrome c (100 μl at 2 mg/ml) for 1 h at 37 °C. It was then centrifuged for 5 min and supernatant was collected. The production of superoxide anion was then monitored spectrophotometrically at 550 nm in reference to the blank [47]. The amount of superoxide production was calculated by the following formula:

$$\textit{Micromoles of superoxide anion} = (\textit{mean absorbance} \text{ at } 550 \text{ nm} \times 15.87)$$

Assay of nitric oxide production

Nitric oxide assay was determined from the collected supernatant of peritoneal macrophages 1 h post-*S. aureus* infection as per different treatment groups. The cell supernatant were incubated with 40 μM Tris (pH 7.9) containing 40 μM of the reduced form of β-nicotinamide adenine dinucleotide phosphate, 40 μM flavine adenine dinucleotide, and 0.05 U/ml nitrate reductase at 37 °C for 15 min. Then, reduced samples were incubated with an equal volume of Griess reagent consisting of sulphanilamide (0.25%, *w*/*v*) and *N*-1-naphthylethylenediamine (0.025%, *w*/*v*) for 10 min and the absorbance at 550 nm was measured. The total nitrate/nitrite concentration was determined by comparison with a $NaNO_3$ standard curve [48].

Assay for quantification of hydrogen peroxide production

Hydrogen peroxide (H_2O_2) assay was determined from the collected supernatant of peritoneal macrophages 1 h post-*S. aureus* infection as per different treatment groups. The 70 μl of cell supernatant was treated with 20 μl Horse Raddish peroxidase (HRP) (500 μg/ml) and 70 μl of Phenol red (500 μg/ml) and was allowed for incubation for 2 h at 37 °C [49]. The reaction was stopped by adding 25 μl of 2 N NaOH and the absorbance was taken at 620 nm. A standard H_2O_2 curve was plotted, and H_2O_2 release in supernatants and lysate was evaluated and expressed in $\mu M/10^6$ cells.

Cytokine assay by ELISA

TNF-α, IL-1β, CXCL8, and soluble TNFR1 (sTNFR1) were quantified from the cell culture supernatant by Sandwich ELISA technique. The supernatants from different groups were normalized to the protein content by Lowry method before the assay and the levels of TNF-α (Cat No. ELM-TNFa), IL-1β (Cat No. ELM-IL1b), and CXCL8 (Cat No. MBS261967) along with sTNFR1 (Cat No. ELM-TNFR1) were determined in accordance with the manufacturer's guidelines in a BioRad ELISA Reader at 450 nm. The minimum detectable levels for TNF-α is 60 pg/ml, IL-1β is 5 pg/ml, and CXCL8 < 3 pg/ml as given in the manual.

Statistical analysis

The peritoneal macrophages were isolated from the mice that were pooled together to obtain the requisite amount of cells (5×10^6 cells/ml) and various parameters were measured by triplicate experiments performed independently. The mean value and standard deviation value of these triplicate experiments were taken for calculation. Data was expressed as mean ± S.D. Significant differences of the means between the groups were performed by One-Way ANOVA. In ANOVA observed variance is partitioned into components due to different explanatory

variables. A level of $p < 0.05$ was considered significant. Scheffe's F test had been done as post hoc test for multiple comparisons of means of different groups when significant F value was found.

Results

For our convenience, the effects of BFA and TAPI-1 have been separately focussed to justify their individual role in inhibition of receptor mobilization and receptor shedding respectively under surface neutralization and for better understanding of their signalling mechanisms in combating inflammatory responses.

Inhibition of receptor mobilization and receptor shedding during surface receptor neutralization downregulates *S. aureus* infection-induced TNFR1, IL-1R, and CXCR1 expressions

The effects of (i) TNFR1-mediated CXCR1 expression, (ii) IL-1R-mediated CXCR1 expression, and (iii) CXCR1 expression under infected condition, were determined by western blot from the recovered cell free lysate obtained from murine

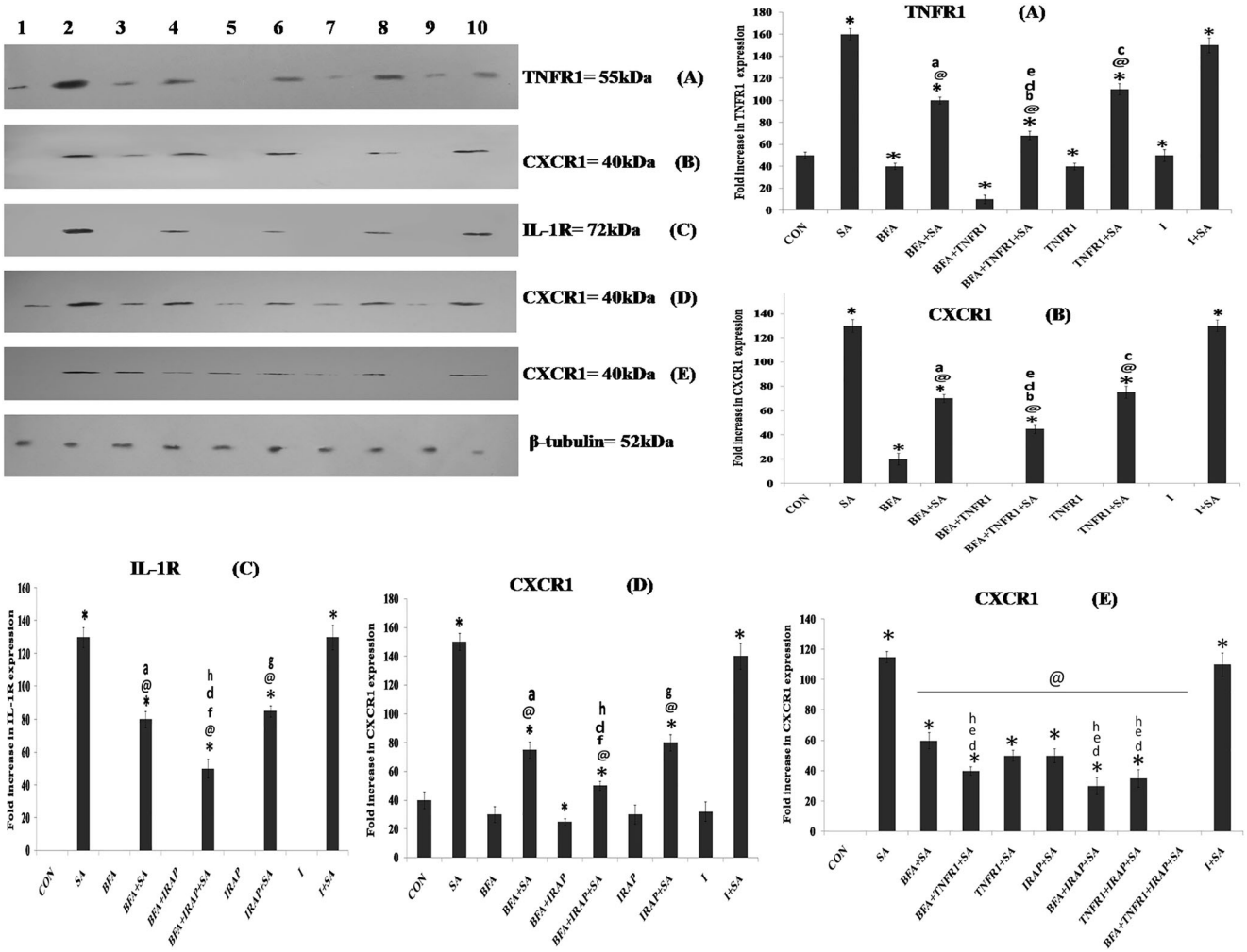


Fig. 2 Western blot analysis of TNFR1, IL-1R, and CXCR1 pre-treated with brefeldin A, anti-TNFR1 antibody and IRAP. Expressions of **a** TNFR1, **b** TNFR1-mediated CXCR1, **c** IL-1R, **d** IL-1R-mediated CXCR1, and **e** CXCR1 under infected condition, were evaluated by western blot in murine peritoneal macrophages during *S. aureus* infection. The respective fold changes were shown in arbitrary units. The values were shown as mean ± SD ($n = 5$/group) from three independent experiments. All the samples were probed with β-tubulin to show equal amount of protein loading. The symbols denote: $^{*}p < 0.05$, significant difference with respect to CON; $^{@}p < 0.05$, significant difference with respect to SA; significant difference ($p < 0.05$) between BFA and BFA+SA (a); significant difference ($p < 0.05$) between BFA+TNFR1 and BFA+TNFR1+SA (b); significant difference ($p < 0.05$) between TNFR1 and TNFR1+SA (c); significant difference ($p < 0.05$) with respect to BFA+SA (d); significant difference ($p < 0.05$) with respect to TNFR1+SA (e); significant difference ($p < 0.05$) between BFA+IRAP and BFA+IRAP+SA (f); significant difference between IRAP and IRAP+SA (g); and significant difference ($p < 0.05$) with respect to IRAP+SA-infected macrophages (h)

peritoneal macrophages. The receptor expression was studied at 60 min post-infection to obtain propitious information of the ultimate effects.

Effects of BFA The control cells (without any treatment or infection) displayed few or almost negligible receptor expression. A manifold increase in the expressions of TNFR1, IL-1R, and CXCR1 was observed after *S. aureus* infection compared with the uninfected control cells ($p < 0.05$) (Fig. 2). BFA+*S. aureus* infected macrophages also showed significant rise in the expressions of TNFR1, IL-1R and CXCR1 ($p < 0.05$) compared with BFA alone treated macrophages. Sole pre-treatment of macrophage with BFA-reduced CXCR1 expression under infected condition which is suggestive of the ability of this antibiotic to inhibit chemokine expression. However, dual treatment with both BFA and anti-TNFR1 antibody reduced TNFR1 expression (Fig. 2a), and consequently, further reducing CXCR1 expression (Fig. 2b) during *S. aureus* infection which was significant at $p < 0.05$ in comparison with the infected groups either pre-incubated with anti-TNFR1 antibody or BFA. Similarly, dual treatment with both BFA and IRAP reduced IL-1R expression (Fig. 2c) and ultimately IL-1R-mediated CXCR1 expression (Fig. 2d) prior to *S. aureus* infection, which is significant at $p < 0.05$ in comparison with the groups either pre-incubated with IRAP or with BFA during infected condition. CXCR1 expression in murine peritoneal macrophages was almost undetectable near to control values when cells were pre-incubated

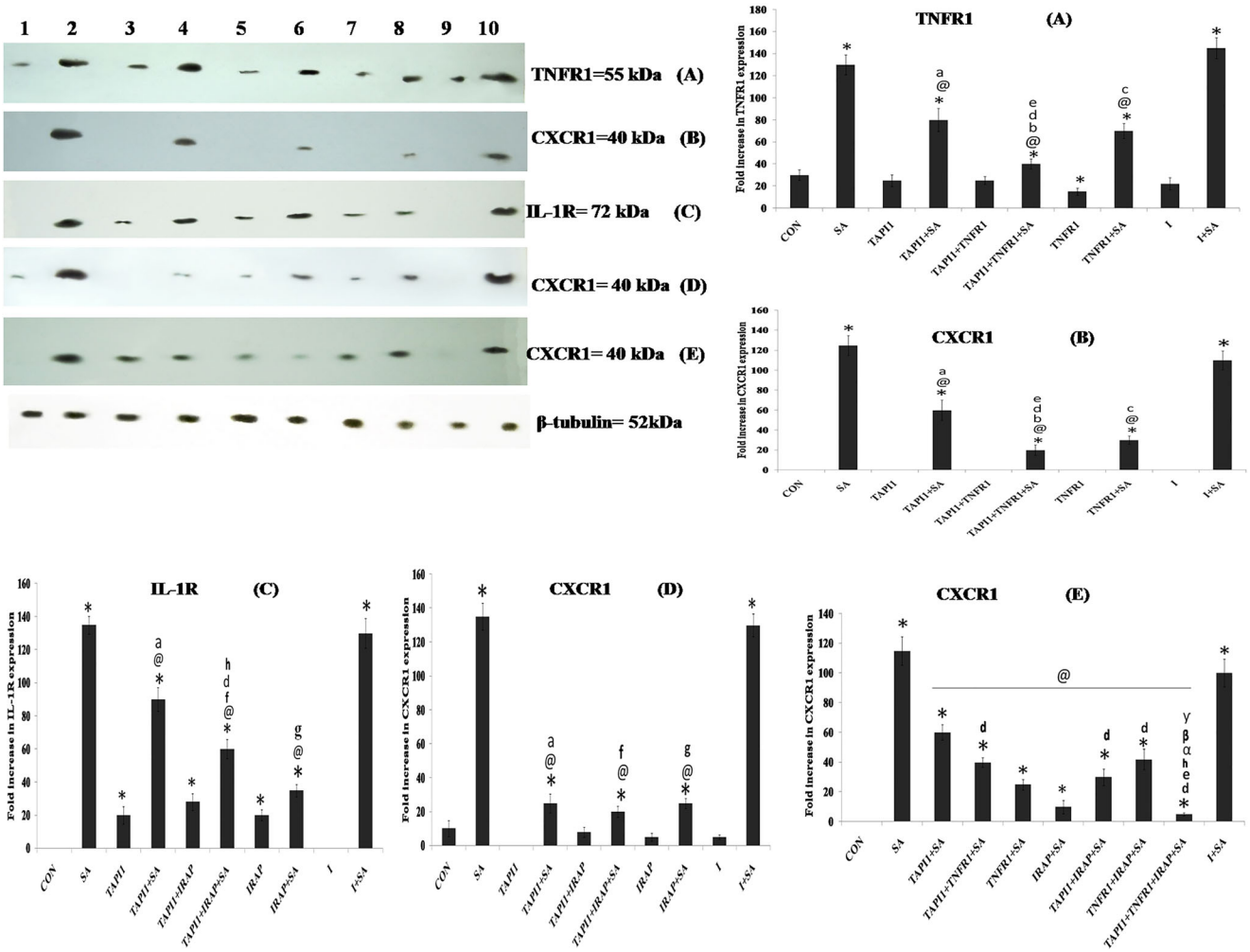


Fig. 3 Western blot analysis of TNFR1, IL-1R, and CXCR1 pre-treated with TAPI-1, anti-TNFR1 antibody, and IRAP. Expressions of **a** TNFR1, **b** TNFR1-mediated CXCR1, **c** IL-1R, **d** IL-1R-mediated CXCR1, and **e** CXCR1 under infected condition, were evaluated by western blot in murine peritoneal macrophages during *S. aureus* infection. The respective fold changes were shown in arbitrary units. The values were shown as mean ± SD ($n = 5$/group) from three independent experiments. All the samples were probed with β-tubulin to show equal amount of protein loading. The symbols denote: $^{*}p < 0.05$, significant difference with respect to CON; $^{@}p < 0.05$, significant difference with respect to SA; significant difference ($p < 0.05$) between TAPI-1 and TAPI-1+SA (a); significant difference ($p < 0.05$) between TAPI-1+TNFR1 and TAPI-1+TNFR1+SA (b); significant difference ($p < 0.05$) between TNFR1 and TNFR1+SA (c); significant difference ($p < 0.05$) with respect to TAPI-1+SA-infected macrophages (d); significant difference ($p < 0.05$) with respect to TNFR1+SA (e); significant difference ($p < 0.05$) between TAPI-1+IRAP and TAPI-1+IRAP+SA (f); significant difference ($p < 0.05$) between IRAP treated and IRAP+SA (g); significant difference ($p < 0.05$) with respect to IRAP+SA (h); $^{\alpha}p < 0.05$, significant difference with respect to TAPI-1+TNFR1+SA; $^{\beta}p < 0.05$, significant difference with respect to TAPI-1+IRAP+SA; $^{\gamma}p < 0.05$, significant difference with respect to TNFR1+IRAP+SA

with BFA in the presence of anti-TNFR1 antibody and IRAP prior to *S. aureus* infection at 60 min at $p < 0.05$ (Fig. 2e).

Effects of TAPI-1 The data showed an elevated peak in TNFR1, IL-1R, and CXCR1 expression after *S. aureus* infection compared with the control groups, which is significant at $p < 0.05$. As shown in the figure, TAPI-1-treated macrophages showed a prominent increase in TNFR1 expression during *S. aureus* infection compared with only TAPI-1-treated group ($p < 0.05$) (Fig. 3). Similar expressional changes were observed in the case of receptors IL-1R and CXCR1. In this study, dual treatment with TAPI-1+anti-TNFR1 antibody and TAPI-1+IRAP prior to *S. aureus* infection reduced TNFR1 (Fig. 3a), TNFR1-mediated CXCR1 expression (Fig. 3b) and IL-1R (Fig. 3c) and IL-1R-mediated CXCR1 expression, respectively (Fig. 3d), under infected condition. Furthermore, CXCR1 expression was almost negligible (Fig. 3e) when pre-treated with TAPI-1 in the presence of both anti-TNFR1 antibody and IRAP under infected condition compared with the single or above-mentioned dual-treated groups ($p < 0.05$). This can be suggested that the pre-treatment of macrophages with both BFA and TAPI-1 during surface TNFR1 and IL-1R-neutralized condition led to downregulation of *S. aureus* infection-induced CXCR1 expression in murine macrophages.

Inhibition of receptor mobilization and receptor shedding during surface receptor neutralization downregulates *S. aureus* infection-induced CXCR1 expression by FACS

The peritoneal macrophage cells expressing CXCR1 receptors were quantified by FACS analysis from the collected cell free lysate of peritoneal macrophages and the median fluorescence intensity (MFI) was measured.

Effects of BFA Few western blot data showed negligible receptor expression in uninfected or control samples. This might be probably due to experimental or human error, but the more sophisticated technique FACS data clearly suggest that the control cells exhibited detectable CXCR1 receptors on their cell surface (Fig. 4). CXCR1 expression increased abruptly under infected condition compared with the control group, which is significant at $p < 0.05$ (Fig. 4a). Pre-treatment of macrophages with BFA or anti-TNFR1 antibody or IRAP reduced the number of

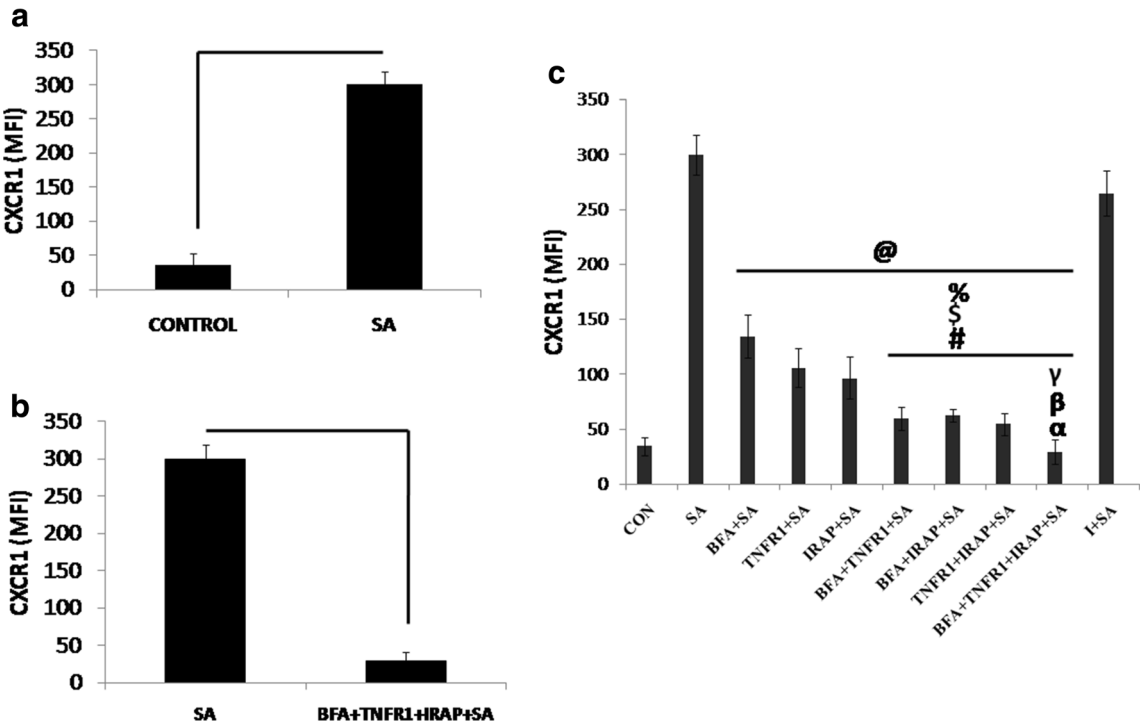


Fig. 4 FACS analysis of CXCR1 expression pre-treated with brefeldin A, anti-TNFR1 antibody, and IRAP. Phycoerythrin (PE)-conjugated CXCR1 expression (CXCR1 PE-A) in peritoneal macrophages during *S. aureus* infection was measured by FACS analysis, and median fluorescence intensity (MFI) measured was shown in arbitrary units. **a** CXCR1 (MFI) comparison between control and SA group, **b** CXCR1 (MFI) comparison between SA and triple-treatment group, BFA+TNFR1+IRAP under infected state, **c** CXCR1 (MFI) of different groups under infected condition. The values were shown as mean ± SD ($n = 5$/group) from three independent experiments. The symbols denote: $^{@}p < 0.05$, significant difference with respect to SA; $^{\#}p < 0.05$, significant difference with respect to BFA+SA; $^{\$}p < 0.05$, significant difference with respect to TNFR1+SA; $^{\%}p < 0.05$, significant difference with respect to IRAP+SA; $^{\alpha}p < 0.05$, significant difference with respect to BFA+TNFR1+SA; $^{\beta}p < 0.05$, significant difference with respect to BFA+IRAP+SA; and $^{\gamma}p < 0.05$, significant difference with respect to TNFR1+IRAP+SA

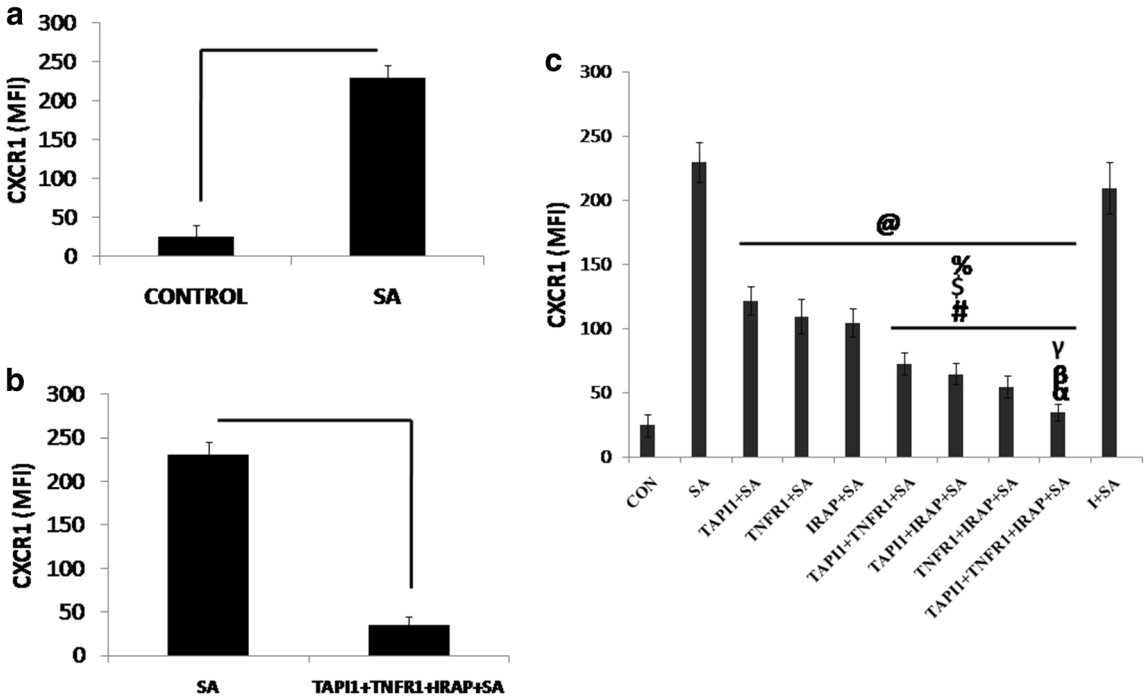


Fig. 5 FACS analysis of CXCR1 expression pre-treated with TAPI-1, anti-TNFR1 antibody, and IRAP. Phycoerythrin (PE)-conjugated CXCR1 expression (CXCR1 PE-A) in peritoneal macrophages during *S. aureus* infection was measured by FACS analysis, and median fluorescence intensity (MFI) measured was shown in arbitrary units. **a** CXCR1 (MFI) comparison between control and SA groups, **b** CXCR1 (MFI) comparison between SA and triple-treatment group, TAPI-1+TNFR1+IRAP under infected state, and **c** CXCR1 (MFI) of different groups under infected condition. The values were shown as mean ± SD ($n = 5$/group) from three independent experiments. The symbols denote: $^{@}p < 0.05$, significant difference with respect to SA; $^{\#}p < 0.05$, significant difference with respect to TAPI-1+SA; $^{\$}p < 0.05$, significant difference with respect to TNFR1+SA, $^{\%}p < 0.05$, significant difference with respect to IRAP+SA; $^{\alpha}p < 0.05$, significant difference with respect to TAPI-1+TNFR1+SA; $^{\beta}p < 0.05$, significant difference with respect to TAPI-1+IRAP+SA; and $^{\gamma}p < 0.05$, significant difference with respect to TNFR1+ IRAP+SA

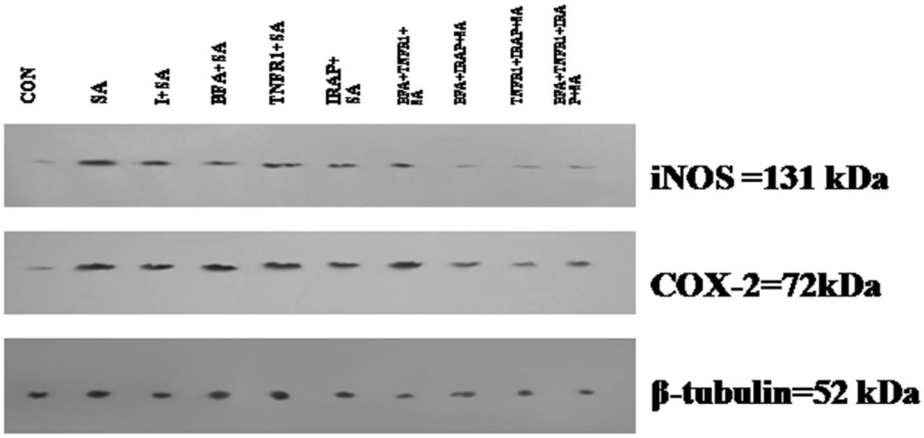
cells expressing CXCR1 under infected state, which is significant at $p < 0.05$. Furthermore, CXCR1 expression further declined significantly at $p < 0.05$ comparable with control values when treated with BFA in the presence of anti-TNFR1 and IRAP compared with the dual-treatment groups (BFA+anti-TNFR1 antibody, BFA+IRAP, or anti-TNFR1 antibody+IRAP) prior to *S. aureus* infection (Fig. 4b, c).

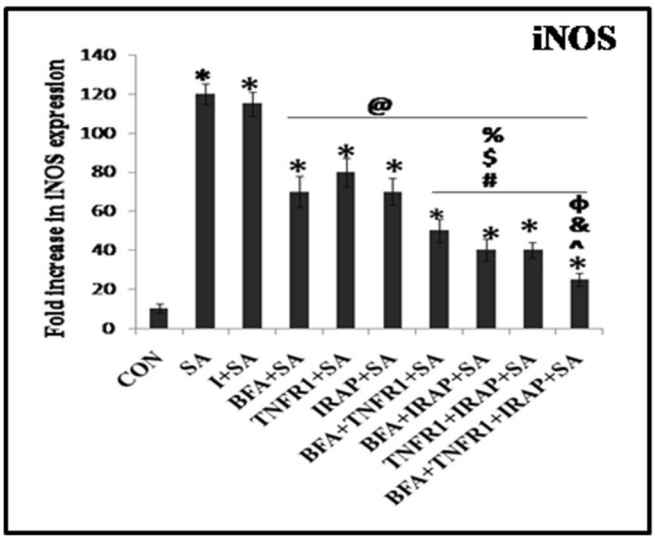
Effects of TAPI-1 As parallel with the western blot data, infection with live *S. aureus* raised CXCR1 expression on macrophage surface significantly at $p < 0.05$ that dropped when pre-treated with TAPI-1 or anti-TNFR1 antibody or IRAP (Fig. 5a, c). Pre-treatment with TAPI-1 further diminished the macrophage cell count expressing CXCR1on surface post-*S. aureus* infection significantly at $p < 0.05$ when the surface receptors TNFR1 and IL-1R were neutralized by anti-TNFR1 antibody and IRAP, respectively (Fig. 5b), compared with the groups pre-treated with TAPI-1+anti-TNFR1 antibody or TAPI1+IRAP or anti-TNFR1 antibody+IRAP under infected condition.

Inhibition of receptor mobilization and receptor shedding during surface receptor neutralization also down regulates *S. aureus* infection induced inflammatory markers—iNOS and COX-2

Expressions of inflammatory markers like iNOS and COX-2 might help provide a clear view about the molecular pathways involving TNFR1-IL-1R-CXCR1 crosstalk and hence, their expressions were evaluated from the cell free lysate in macrophages which were pre-treated with brefeldin A and TAPI-1 in presence or absence of either anti-TNFR1 antibody alone or IRAP or both anti-TNFR1 antibody+IRAP prior to *S. aureus* infection.

Effects of BFA A multifold rise in iNOS and COX-2 expression was observed after infection with *S. aureus* compared with the control cells, which are significant at $p < 0.05$ (Fig. 6). Macrophages that received dual-blockade treatment, that is, BFA+anti-TNFR1 antibody, BFA+IRAP, or anti-TNFR1 antibody+IRAP prior to *S. aureus* infection showed a significant decrease in both iNOS and COX-2 expressions compared with





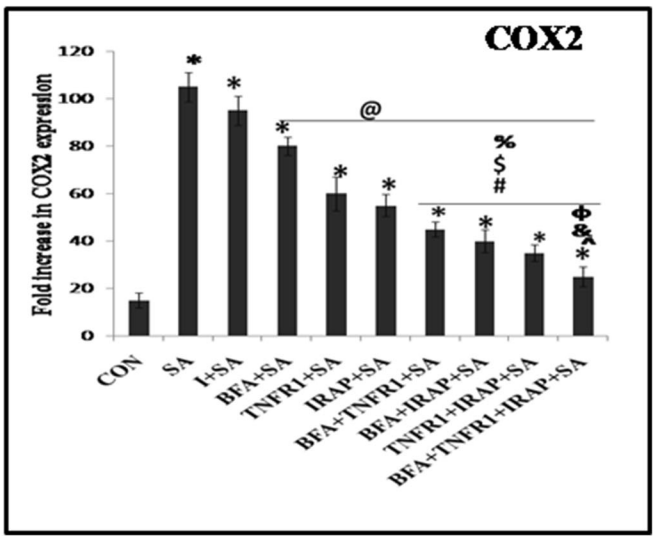


Fig. 6 Western blot analysis of iNOS and COX-2 pre-treated with brefeldin A, anti-TNFR1 antibody, and IRAP. Expressions of inflammatory mediators, iNOS and COX-2 were evaluated by western blot in murine peritoneal macrophages during *S. aureus* infection. The respective fold changes were shown in arbitrary units. Beta-tubulin added to ensure equal protein loading (might show poor image quality). The values were shown as mean ± SD ($n = 5$/group) from three independent experiments. The symbols denote: $^{*}p < 0.05$, significant difference with respect to CON; $^{@}p < 0.05$, significant difference with respect to SA; $^{\#}p < 0.05$, significant difference with respect to BFA+SA; $^{\$}p < 0.05$, significant difference with respect to TNFR1+SA, $^{\%}p < 0.05$, significant difference with respect to IRAP+SA; $^{\wedge}p < 0.05$, significant difference with respect to BFA+TNFR1+SA; $^{\&}p < 0.05$, significant difference with respect to BFA+IRAP+SA, and $^{\varphi}p < 0.05$, significant difference with respect to TNFR1+IRAP+SA

the groups pre-treated singly with BFA, anti-TNFR1 antibody or IRAP alone ($p < 0.05$). Furthermore, expressions of iNOS and COX-2 reduced drastically in macrophages that were pre-incubated with BFA in the presence of both anti-TNFR1 antibody+IRAP prior to *S. aureus* infection at 60 min as compared with the above-mentioned dual-blockade treatment groups at $p < 0.05$ after *S. aureus* infection.

Effects of TAPI-1 *S. aureus*-infected macrophage cells displayed tremendous rise in iNOS expression that was significantly higher compared with the untreated cells ($p < 0.05$) as shown in Fig. 7. Pre-treatment with TAPI-1, anti-TNFR1 antibody, or IRAP prior to *S. aureus* infection reduced the iNOS expression. Similar expressional changes were observed in the case of COX-2. Both iNOS and COX-2 expressions reduced substantially (COX-2 expression almost negligible) when treated with TAPI-1+anti-TNFR1 antibody+IRAP compared with the dual-treated groups (TAPI-1+anti-TNFR1 antibody or TAPI-1+IRAP or anti-TNFR1 antibody+IRAP) under infected condition ($p < 0.05$).

Inhibition of receptor mobilization and receptor shedding during surface receptor neutralization also downregulates *S. aureus* infection-induced NO production, superoxide anion release, and H_2O_2 release by murine peritoneal macrophages

The amounts of H_2O_2, NO and superoxide anion (major ROS) released by murine peritoneal macrophages serve as measure of

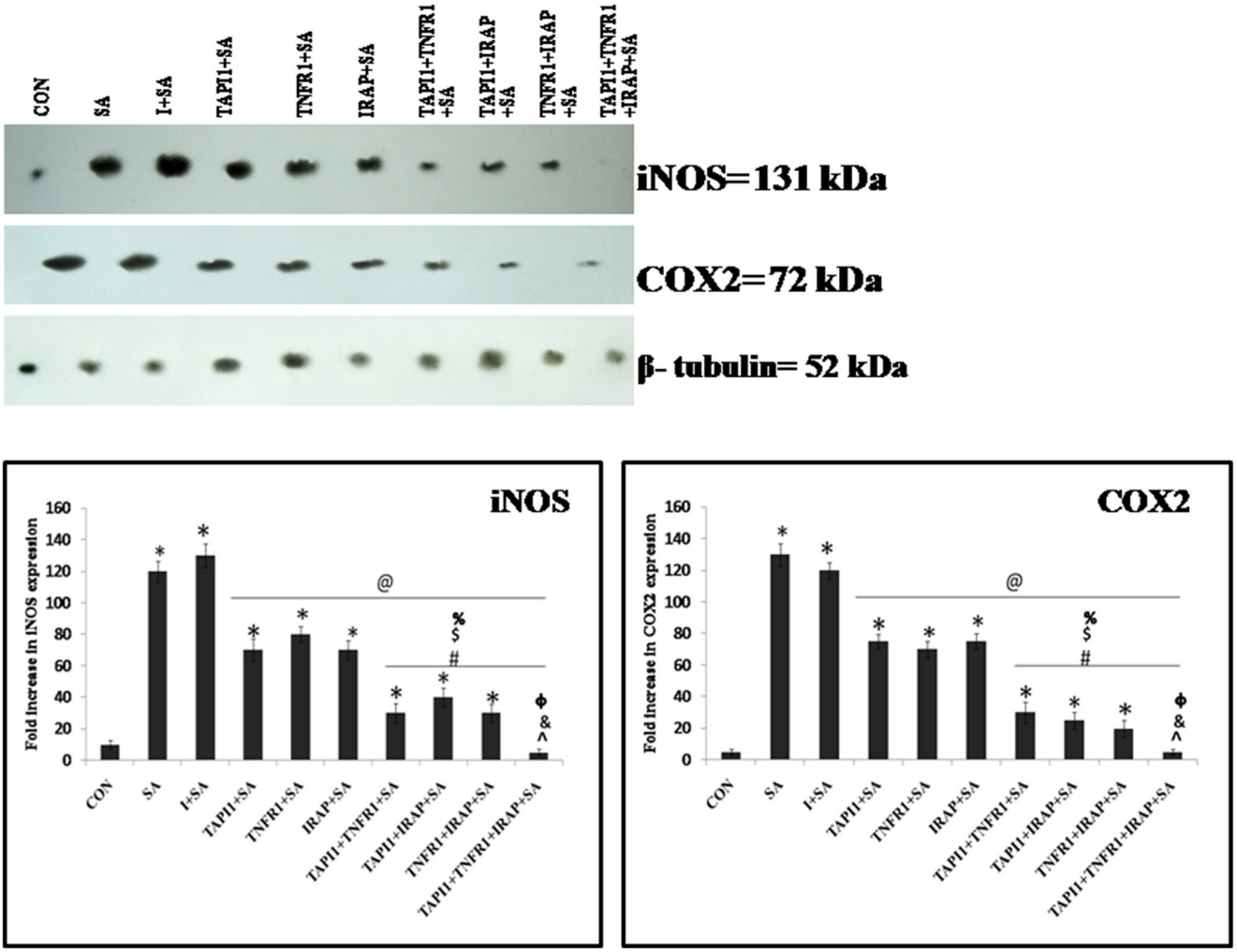


Fig. 7 Western blot analysis of iNOS and COX-2 pre-treated with TAPI-1, anti-TNFR1 antibody, and IRAP. Expressions of inflammatory mediators, iNOS and COX-2 were evaluated by western blot in murine peritoneal macrophages during *S. aureus* infection. The respective fold changes were shown in arbitrary units. The values were shown as mean ± SD ($n = 5$/group) from three independent experiments. The symbols denote: $^{*}p < 0.05$, significant difference with respect to CON; $^{@}p < 0.05$, significant difference with respect to SA; $^{\#}p < 0.05$, significant difference with respect to TAPI-1+SA; $^{\$}p < 0.05$, significant difference with respect to TNFR1+SA; $^{\%}p < 0.05$, significant difference with respect to IRAP+SA; $^{\wedge}p < 0.05$, significant difference with respect to TAPI-1+TNFR1+SA; $^{\&}p < 0.05$, significant difference with respect to TAPI-1+IRAP+SA; and $^{\varphi}p < 0.05$, significant difference with respect to TNFR1+IRAP+SA

functional activity of macrophages especially for phagocytosis and bactericidal and ROS were estimated from the collected supernatant at 60 min post-infection in macrophages which were pre-treated with brefeldin A and TAPI-1 in the presence or absence of either anti-TNFR1 antibody or IRAP or both anti-TNFR1 antibody+ IRAP.

Effects of BFA Our data (Fig. 8) showed that the superoxide anion (Fig. 8a), NO production (Fig. 8b), and H_2O_2 release (Fig. 8c) in terms of μm/10^6 cells increased significantly ($p < 0.05$) after *S. aureus* infection in peritoneal macrophages compared with the control or treatment groups. Apparently, pre-treatment with BFA or anti-TNFR1 antibody or IRAP significantly reduced the release of NO, superoxide anion, and H_2O_2 under infected condition ($p < 0.05$) compared with the amount released by macrophages during *S. aureus* infection. Additionally, their release from macrophages further reduced substantially when pre-incubated with BFA in presence of both anti-TNFR1 antibody+IRAP during the infection course ($p < 0.05$).

Effects of TAPI-1 From Fig. 9, it was observed that pre-treatment with TAPI-1 prior to *S. aureus* infection significantly reduced the amount of superoxide anion (Fig. 9a), nitric oxide (Fig. 9b), and H_2O_2 (Fig. 9c) secreted by macrophages compared with the robust amount produced when the cells were infected with live *S. aureus* ($p < 0.05$). Similarly, pre-treatment with anti-TNFR1 antibody or IRAP reduced the ROS production, thus, reducing bacterial killing. The ROS production in macrophages further reduced significantly at $p < 0.05$ when pre-treated with TAPI-1

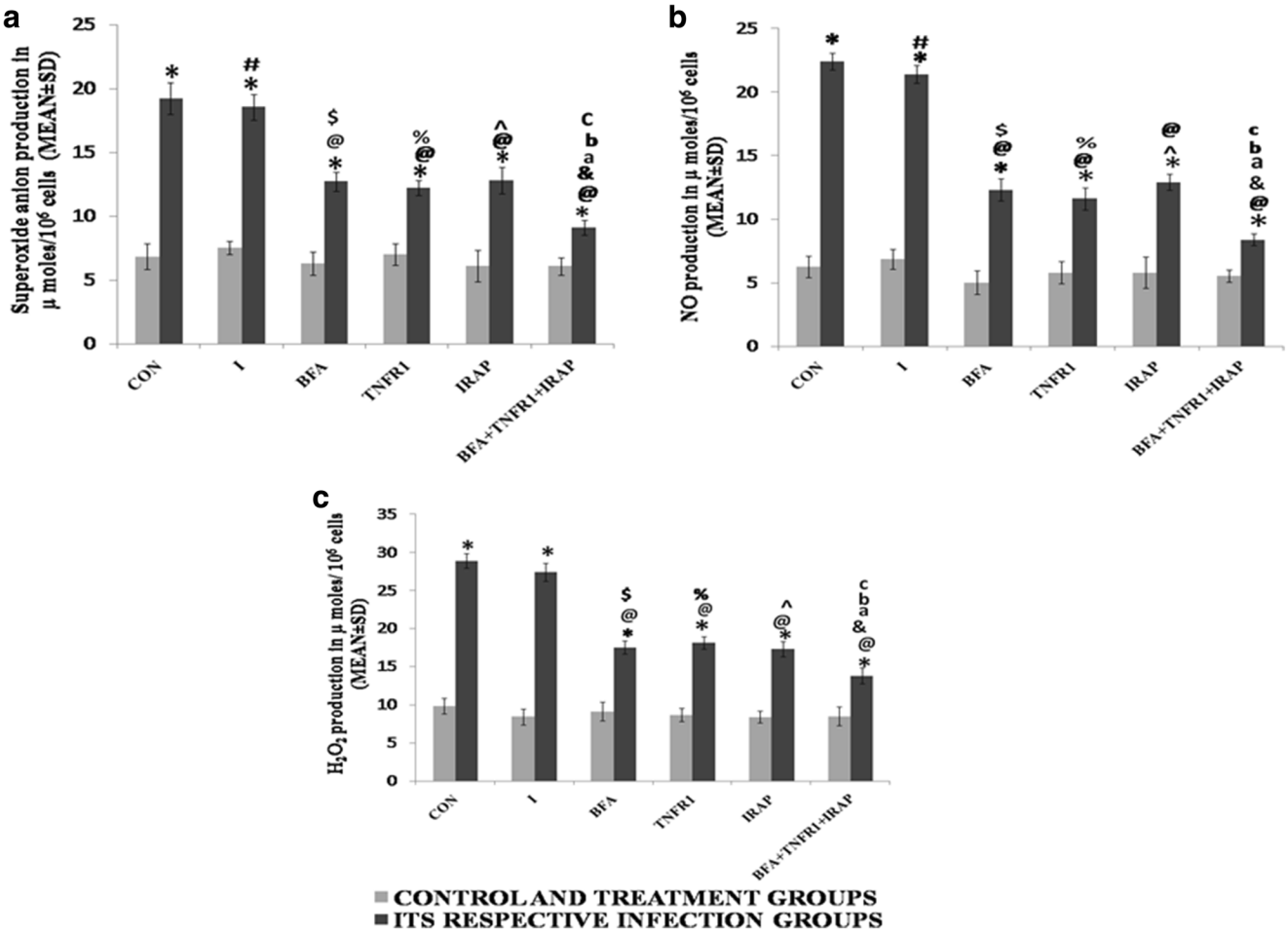


Fig. 8 *S. aureus* infection induced superoxide anion release, NO production, and H_2O_2 release by murine peritoneal macrophages pre-treated with brefeldin A, anti-TNFR1 antibody, and IRAP. **a** Superoxide anion release, **b** nitric oxide (NO) release, and **c** hydrogen peroxide (H_2O_2) release were evaluated by standard assays in murine peritoneal macrophages during *S. aureus* infection. The values were expressed in μm/10^6 cells and were represented as mean ± SD ($n = 5$/group) from three independent experiments. The light grey shade denotes uninfected or treatment groups and the dark grey shade denote *S. aureus* infected macrophages when treated with the corresponding uninfected or treatment groups. The symbols denote: $^{*}p < 0.05$, significant difference with respect to CON; $^{@}p < 0.05$, significant difference with respect to SA; $^{\#}p < 0.05$, significant difference between I and I+SA; $^{\$}p < 0.05$, significant difference between BFA and BFA+SA; $^{\%}p < 0.05$, significant difference between TNFR1 and TNFR1+SA; $^{\wedge}p < 0.05$, significant difference between IRAP and IRAP+SA; $^{\&}p < 0.05$, significant difference between BFA+TNFR1+IRAP and BFA+TNFR1+IRAP+SA; significant difference ($p < 0.05$) with respect to BFA+SA (a); significant difference ($p < 0.05$) with respect to TNFR1+SA (b); and significant difference ($p < 0.05$) with respect to IRAP+SA (c)

in presence of anti-TNFR1 antibody+IRAP prior to *S. aureus* infection, thus, exhibiting reduced phagocytosis. It would be worthwhile to note that the ROS production calculated during BFA and TAPI-1 study might show high variability. This would be probably due to the experiments being conducted at different times.

Inhibition of receptor mobilization and receptor shedding during surface receptor neutralization also downregulates *S. aureus* infection-induced TNF-α, IL-1β, CXCL8, and sTNFR1 release by murine peritoneal macrophages

Levels of pro-inflammatory cytokines TNF-α, IL-1β, chemokine CXCL8, and sTNFR1 were estimated from the cell culture supernatant at 60 min in macrophages which were pre-treated with brefeldin A and TAPI-1 in the presence or absence of either anti-TNFR1 antibody or IRAP or both anti-TNFR1 antibody +IRAP prior to *S. aureus* infection.

Effects of BFA As shown in Fig. 10, *S. aureus*-infected macrophages stimulated the production of pro-inflammatory cytokines and there was a significant increase in the levels of TNF-α (Fig. 10a), IL-1β (Fig. 10b), and CXCL8 (Fig. 10c) compared with the control macrophages ($p < 0.05$). Even the concentration of sTNFR1 (Fig. 10d) increased abruptly under infected condition compared with the untreated group ($p < 0.05$). Treatment with BFA or anti-TNFR1 antibody or IRAP prior to *S. aureus* infection reduced the levels of TNF-α and IL-1β and consequently,

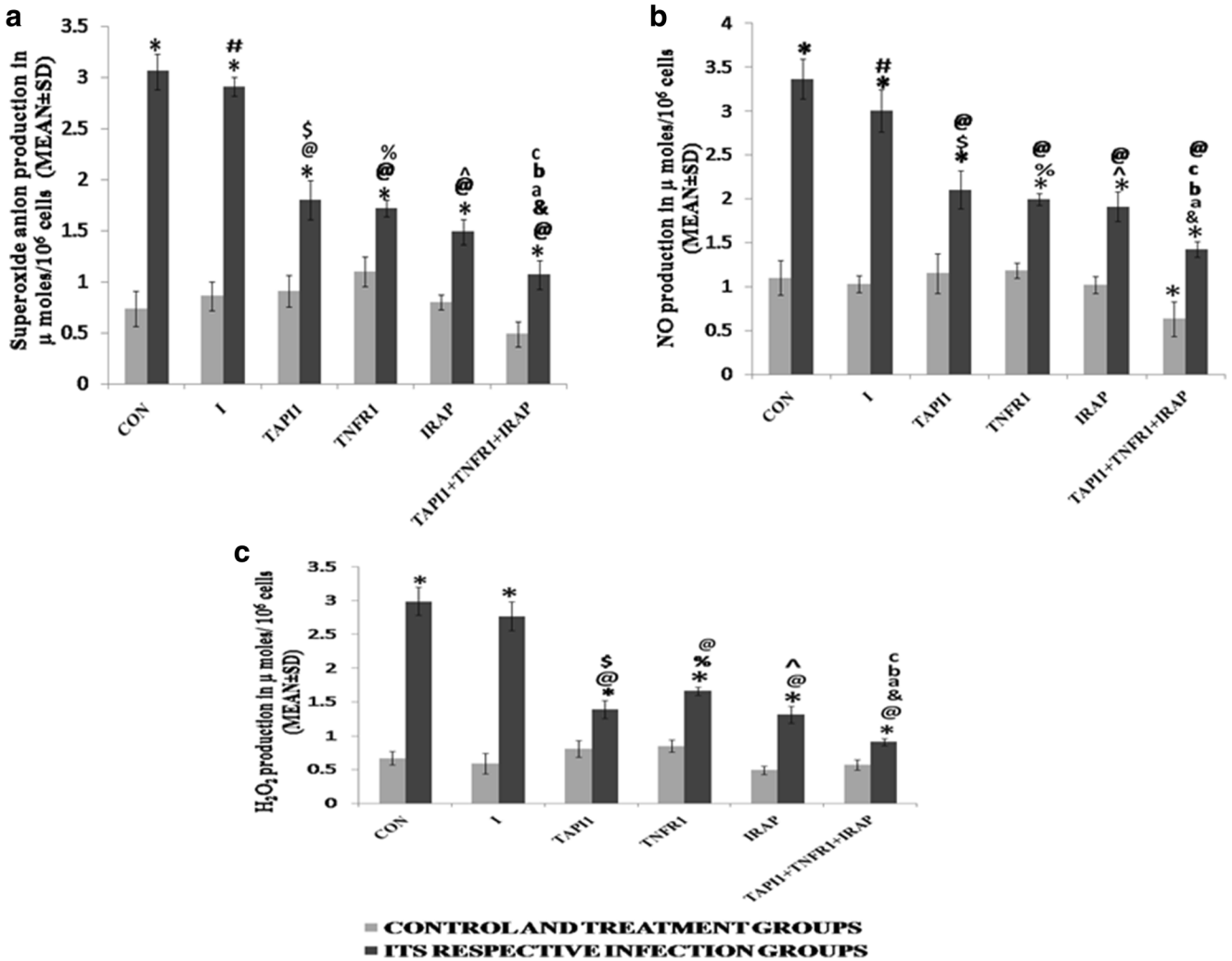


Fig. 9 *S. aureus* infection induced superoxide anion release, NO production, and H_2O_2 release by murine peritoneal macrophages pre-treated with TAPI-1, anti-TNFR1 antibody, and IRAP. **a** Superoxide anion release, **b** nitric oxide (NO) release, and **c** hydrogen peroxide (H_2O_2) release were evaluated by standard assays in murine peritoneal macrophages during *S. aureus* infection. The values were expressed in μm/10^6 cells and were represented as mean ± SD ($n = 5$/group) from three independent experiments. The light grey shade denotes uninfected or treatment groups and the dark grey shade denote *S. aureus* infected macrophages when treated with the corresponding uninfected or treatment groups. The symbols denote: $^{*}p < 0.05$, significant difference with respect to CON; $^{@}p < 0.05$, significant difference with respect to SA; $^{\#}p < 0.05$, significant difference between I and I+SA; $^{\$}p < 0.05$, significant difference between TAPI-1 and TAPI-1+SA; $^{\%}p < 0.05$, significant difference between TNFR1 and TNFR1+SA; $^{\wedge}p < 0.05$, significant difference between IRAP and IRAP+SA; $^{\&}p < 0.05$, significant difference between TAPI-1+TNFR1+IRAP and TAPI-1+TNFR1+IRAP+SA; significant difference ($p < 0.05$) with respect to TAPI-1+SA (a); significant difference ($p < 0.05$) with respect to TNFR1+SA (b); and significant difference ($p < 0.05$) with respect to IRAP+SA (c)

reduced CXCL8 level. The cells which were pre-incubated with BFA in presence of both anti-TNFR1 antibody+IRAP prior to *S. aureus* infection led to tremendous decrease in levels of TNF-α, IL-1β, CXCL8, and even significantly reduced sTNFR1 compared with the groups pre-incubated with BFA+anti-TNFR1 antibody, BFA+IRAP, and anti-TNFR1 antibody+IRAP during infection ($p < 0.05$).

Effects of TAPI-1 TAPI-1, a TACE inhibitor, inhibited the release of sTNFR1 in the medium and thus, the level of sTNFR1 (Fig. 11d) under infected condition reduced significantly when pre-treated with TAPI-1 ($p < 0.05$) as shown (Fig. 11). Even the amount of TNF-α (Fig. 11a), IL-1β (Fig. 11b), and CXCL8 (Fig. 11c) that arose prominently after *S. aureus* infection reduced when treated with TAPI-1 or anti-TNFR1 antibody or IRAP, which is significant at $p < 0.05$. Moreover, the amount of cytokines TNF-α, IL-1β, and CXCL8 and amount of sTNFR1 further diminished when subjected to pre-treatment with TAPI-1+anti-TNFR1 antibody+IRAP significantly compared with the amount released when pre-treated with the dual-blockade treatment groups (TAPI-1+anti-TNFR1 antibody, TAPI-1+IRAP, and anti-TNFR1 antibody+IRAP

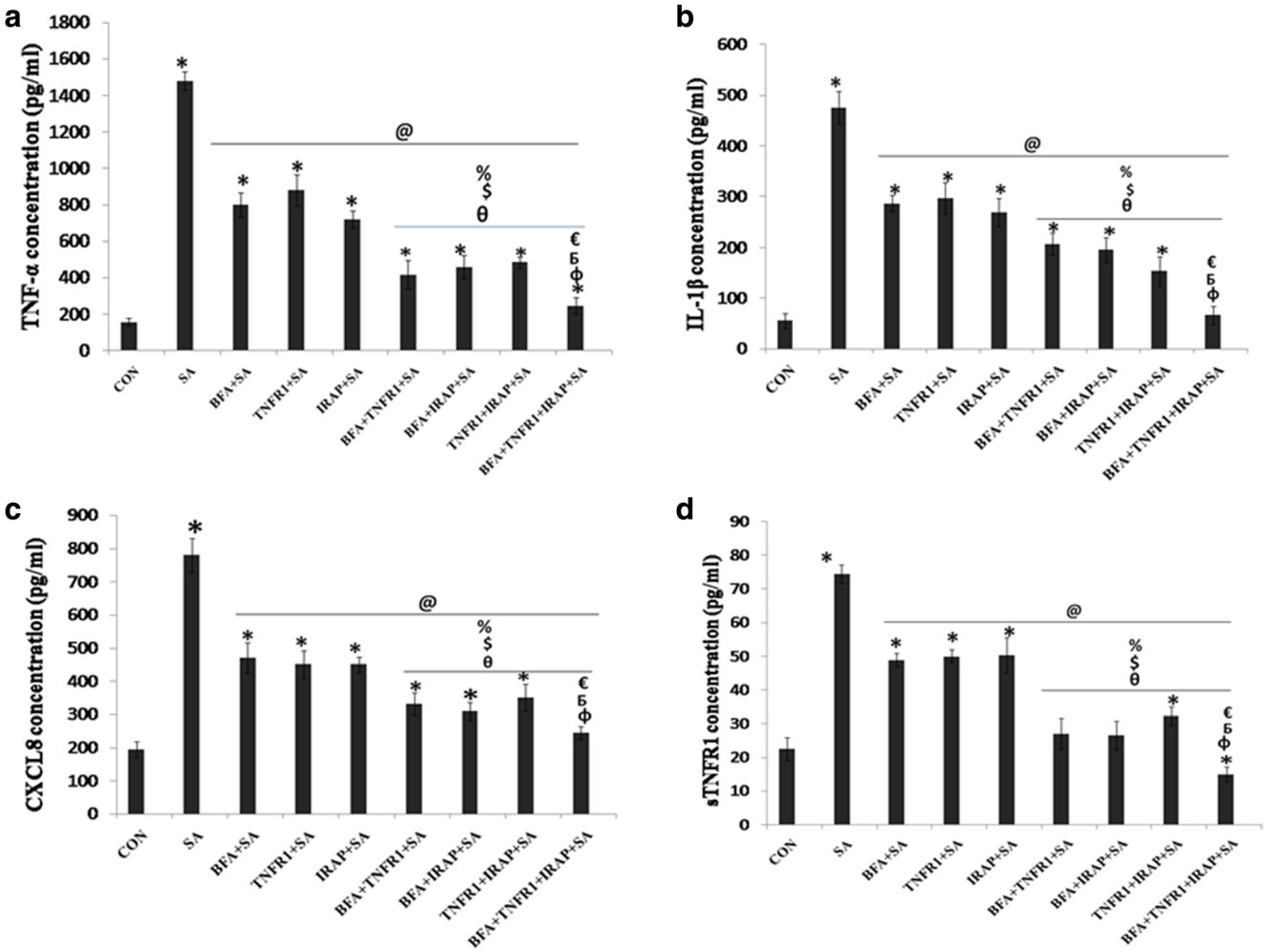


Fig. 10 Estimation of TNF-α, IL-1β, CXCL8, and sTNFR1 production by ELISA when pre-treated with brefeldin A, anti-TNFR1 antibody, and IRAP. Release of **a** TNF-α, **b** IL-1β, **c** CXCL8, and **d** sTNFR1 were evaluated by ELISA in murine peritoneal macrophages after *S. aureus* infection. The values were expressed in picograms per milliliter and were represented as mean ± SD ($n = 5$/group) from three independent experiments. The symbols denote: $^{*}p < 0.05$, significant difference with respect to CON; $^{@}p < 0.05$, significant difference with respect to SA; $^{\theta}p < 0.05$, significant difference with respect to BFA+SA; $^{\$}p < 0.05$, significant difference with respect to TNFR1+SA; $^{\%}p < 0.05$, significant difference with respect to IRAP+SA; $^{\varphi}p < 0.05$, significant difference with respect to BFA+TNFR1+SA; $^{Б}p < 0.05$, significant difference with respect to BFA+IRAP+SA; and $^{€}p < 0.05$, significant difference with respect to TNFR1+IRAP+SA

treated groups) prior to *S. aureus* infection ($p < 0.05$). This is suggestive of the ability of TAPI-1 and BFA to curtail the release of these major cytokines and hence, reducing the inflammatory responses under surface receptors neutralization condition.

Inhibition of receptor mobilization and receptor shedding during surface receptor neutralization also downregulates *S. aureus* infection-induced sIL-1R release by murine peritoneal macrophages

The concentration of sTNFR1 was measured by ELISA to estimate the amount of receptor shed in the culture medium. However, to determine whether TACE also functions to cleave IL-1R, the supernatants were concentrated and sIL-1R expression studied by western blot (Fig. 12). It was observed that *S. aureus*-infected cells exhibited maximum sIL-1R expression compared with untreated cells, which is significant at $p < 0.05$. Pre-treatment with TAPI-1, anti-TNFR1 antibody, or IRAP prior to *S. aureus* infection significantly reduced sIL-1R expression on cell surface compared with the expression during infected condition ($p < 0.05$). Its expression was almost undetectable when pre-treated with TAPI-1 in the presence of anti-TNFR1 antibody and IRAP prior to *S. aureus* infection compared with the dual-treated groups, that is, TAPI-1+anti-TNFR1 antibody, anti-TNFR1 antibody+IRAP, or TAPI-1+IRAP under infected state ($p < 0.05$). Undoubtedly, this signifies the role of TACE in shedding IL-1R as TAPI-1, a potent TACE inhibitor, inhibiting the receptor shedding and

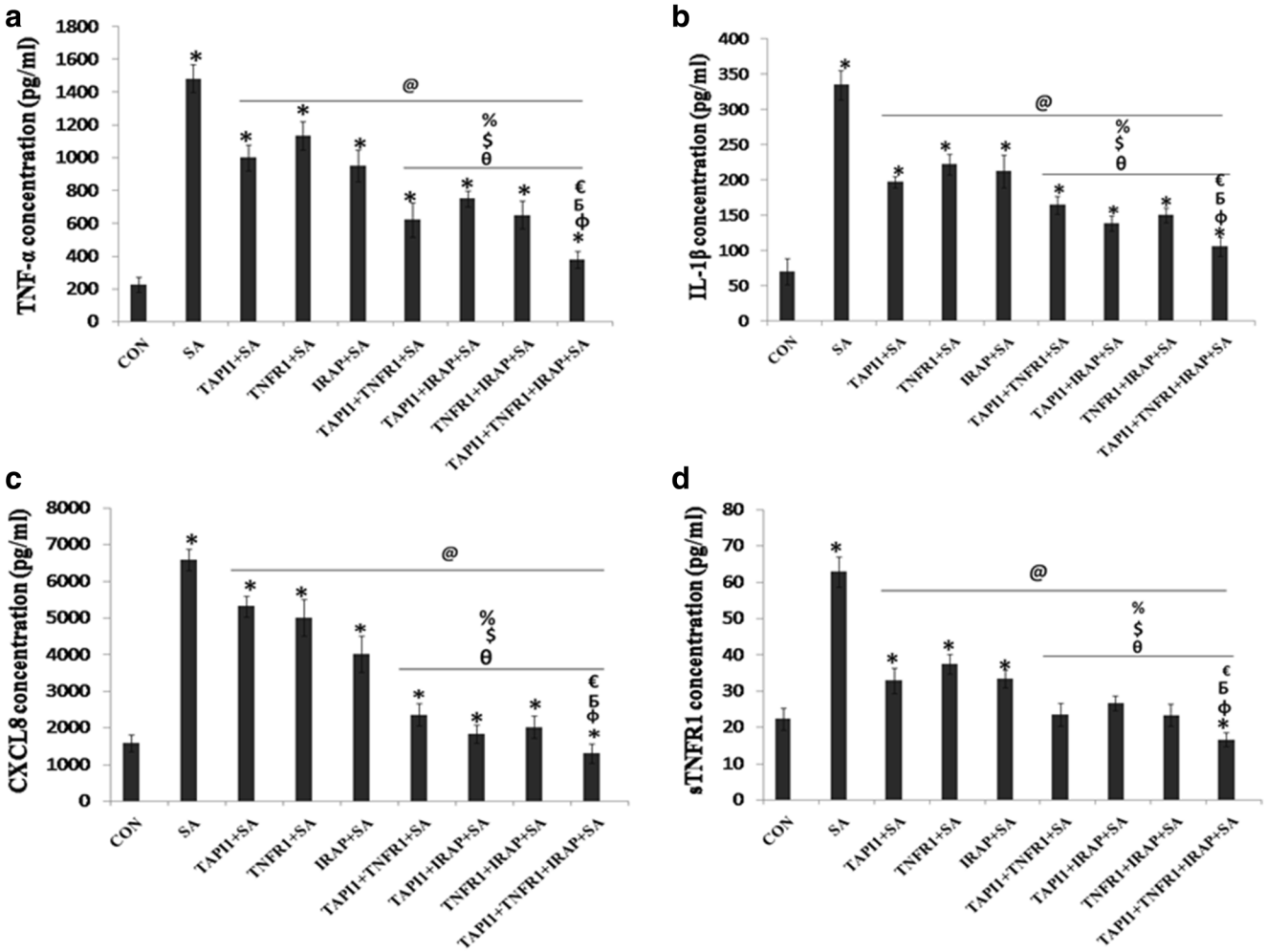


Fig. 11 Estimation of TNF-α, IL-1β, CXCL8, and sTNFR1 production by ELISA when pre-treated with TAPI-1, anti-TNFR1 antibody, and IRAP. Release of **a** TNF-α, **b** IL-1β, **c** CXCL8, and **d** sTNFR1 were evaluated by ELISA in murine peritoneal macrophages after *S. aureus* infection. The values were expressed in picograms per milliliter and were represented as mean ± SD ($n = 5$/group) from three independent experiments. The symbols denote: $^{*}p < 0.05$, significant difference with respect to CON; $^{@}p < 0.05$, significant difference with respect to SA; $^{\theta}p < 0.05$, significant difference with respect to TAPI-1+SA; $^{\$}p < 0.05$, significant difference with respect to TNFR1+SA; $^{\%}p < 0.05$, significant difference with respect to IRAP+SA; $^{\varphi}p < 0.05$, significant difference with respect to TAPI-1+TNFR1+SA; $^{Б}p < 0.05$, significant difference with respect to TAPI-1+IRAP+SA; and $^{€}p < 0.05$, significant difference with respect to TNFR1+IRAP+SA

henceforth reducing the expression of sIL-1R in the supernatant.

Discussion

CXCL8/CXCR1 axis play an important role in resolving infection by inducing chemotaxis, ROS production, and phagocytosis [50], and any sort of manipulation or disturbance in its production might lead to cellular damage or severe inflammatory diseases. This study have been designed to highlight the role of BFA and TAPI-1 in modulation of CXCL8/CXCR1 axis and also provide mechanistic insights into changes in ROS production, cytokine profile, surface expression of TNFR1, IL-1R, CXCR1, soluble form of receptors (sTNFR1 and sIL-1R), and the role of inflammatory mediators (iNOS and COX-2) involved in TNFR1-IL-1R-CXCR1 axis during the infection.

When the peritoneal macrophages were infected with live *S. aureus* in vitro, the bacteria bind to the dimeric receptor TLR-2, leading to excessive ROS production (superoxide and hydrogen peroxide) and nitric oxide generation that are responsible for cellular damage, oxidative stress, and intracellular killing of bacteria (Fig. 13a). Besides generating ROS, the stimulated macrophages release a tremendous production of TNF-α, IL-1β, and CXCL8 along with upregulated expression of cytokine and chemokine receptors (TNFR1, IL-1R, and CXCR1). Innumerable papers suggest that the mitogen-activated protein kinase (MAPK) signalling cascade might

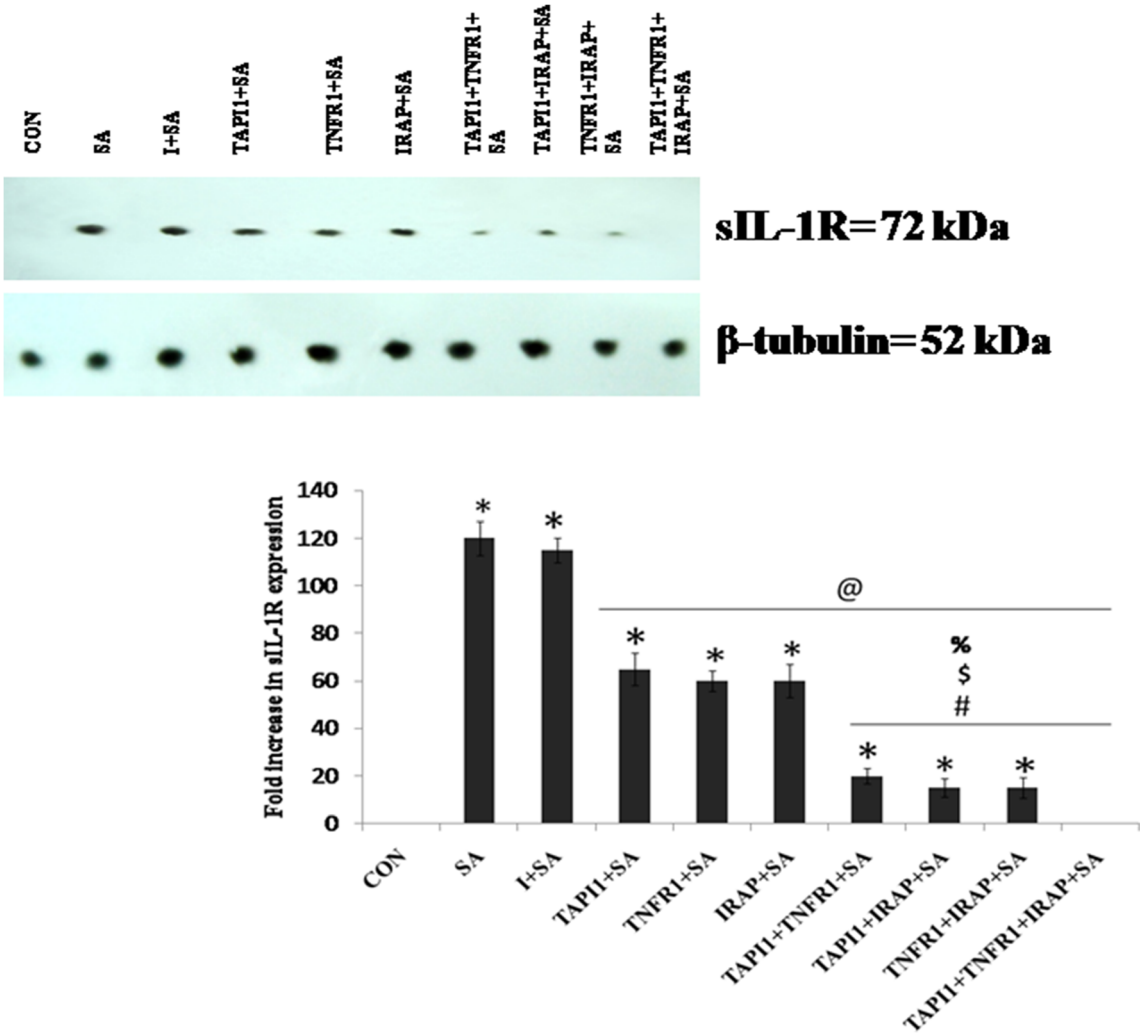


Fig. 12 Western blot analysis of soluble IL-1R (s IL-1R) pre-treated with TAPI-1, anti-TNFR1 antibody, and IRAP. Expressions of sTNFR1from concentrated supernatant were evaluated by western blot in murine peritoneal macrophages during *S. aureus* infection. The respective fold changes were shown in arbitrary units. The values were shown as mean ± SD ($n = 5$/group) from three independent experiments. All the samples were probed with β-tubulin to show equal amount of protein loading. The symbols denote: $^{*}p < 0.05$, significant difference with respect to CON; $^{@}p < 0.05$, significant difference with respect to SA; $^{\#}p < 0.05$, significant difference with respect to TAPI-1+SA; $^{\$}p < 0.05$, significant difference with respect to TNFR1+SA; and $^{\%}p < 0.05$, significant difference with respect to IRAP+SA

regulate IL-1β-induced CXCL8 expression [51]. NF-κB and JNK (though data not shown) play an apex role in regulating ROS, cytokine secretion, and modulating cytokine-receptor negotiation. Even the virulence factors of *S. aureus* itself potentiate IL-1β secretion through activation of inflammasome signalling [52]. Moreover, TNF-α creates an autocrine network by inducing production of IL-1β, CXCL8, and TNF-α itself, thus, maintaining the continuous pool of pro-inflammatory cytokines [3]. *S. aureus* infection in macrophage is followed by robust increase in expression of TNFR1, IL-1R and consequently, CXCR1 on cell surface as apparent from the blot data. The basic mechanism involved binding of TNF-α to TNFR1 and IL-1β to IL-1R induces translocation of NF-κB factors and mediates transcription of many genes, like that of pro-inflammatory chemokine CXCL8 as well as inflammatory mediators—iNOS and COX-2. Increased iNOS production leads to excessive NO generation that further aggaravates ROS generation. COX-2 induced prostaglandin secretion from its precursor arachidonic acid is also stimulated by bacterial lipopolysaccharide (LPS) or proinflammatory cytokines like TNF-α, IL-1β, and IL-6 during inflammation [53]. ROS generated also act as a direct causative agent for COX-2 expression and consequently raise prostaglandin level that mediates various inflammatory responses like hyperalgesia, fever, pain, increase in vascular permeability, and edema [54]. Hence, this continuous genesis of inflammatory signalling induced due to TNFR1-IL-1R-CXCR1 crosstalk might prove to be a havoc on the life of normal cells and responsible for varied inflammation-related diseases.

Numerous studies took the support of specific Golgi markers like golgin GM130 to verify the effects

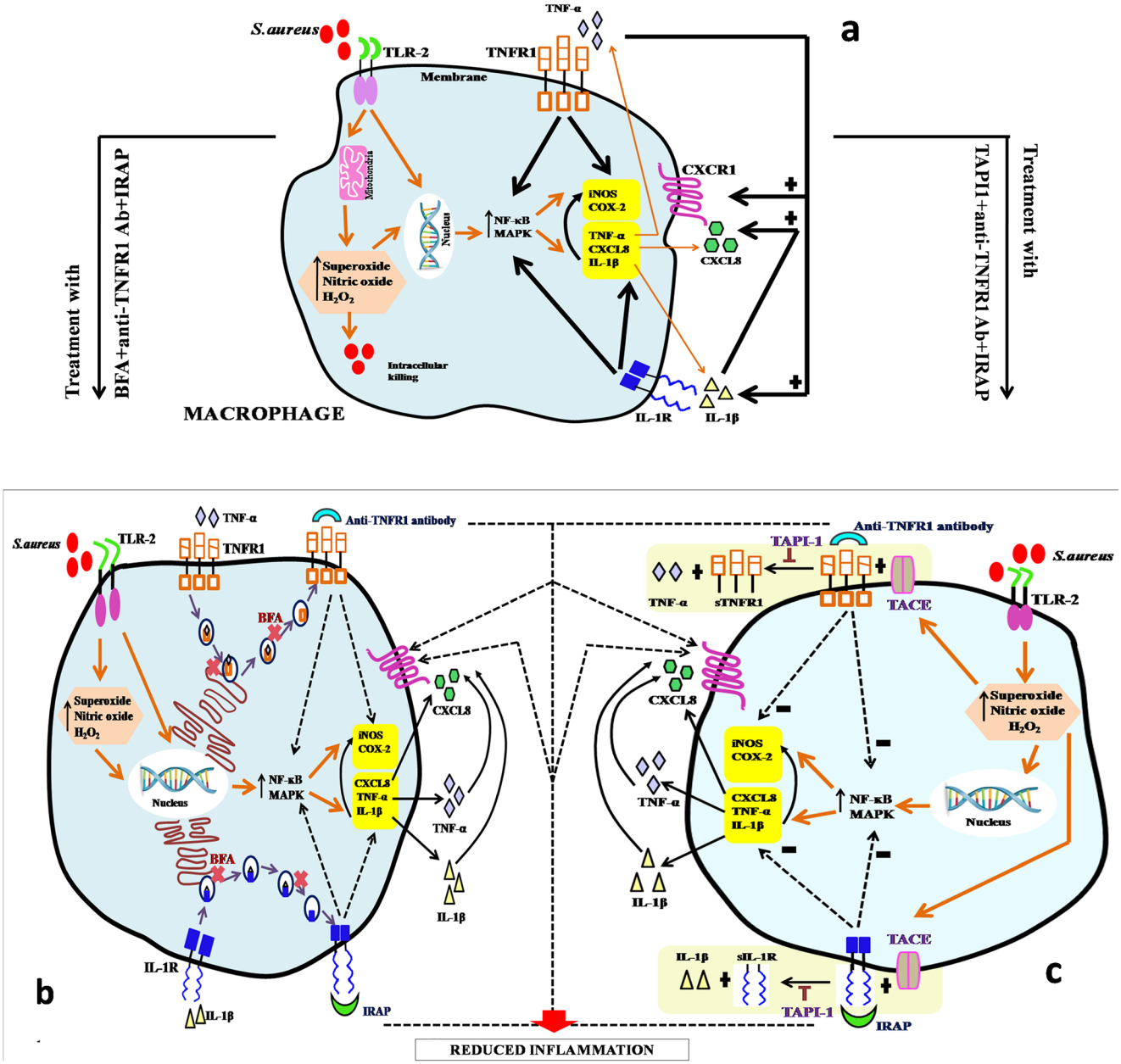


Fig. 13 Schematic representation of effects of brefeldin A (BFA) and B: TAPI-1 in inhibition of receptor mobilization and receptor shedding, respectively, during *S. aureus* infection in the presence of anti-TNFR1 antibody and IRAP in reducing inflammation. Scheme representing **a** *S. aureus*-induced inflammatory pathway involves TNFR1-IL-1R-CXCR1 crosstalk in peritoneal macrophages, **b** role of BFA in inhibition of CXCR1 expression, and **c** role of TAPI-1 in inhibition of CXCR1 expression and downregulation of inflammatory responses. The straight arrow indicates positive stimulation, and the dotted arrow indicates inhibition

of BFA on Golgi structure [55]. According to various morphological data, incubation of cells with BFA (2.5 μg/ml) disrupted the typical tubular network of Golgi structure whereas higher doses of BFA (10 μg/ml) further led to dilation of ER (due to protein accumulation) along with fragmented Golgi bodies [56]. Though the detailed intracellular study showing site of action of BFA and its effects on Golgi-mediated TNFR1 and IL-1R transport have not been shown (with basic focus on surface receptor expression study), the experiments conducted and data presented suggest that BFA have inexorable role in blocking both TNFR1 and IL-1R intracellular trafficking pathway. Both TNF-α and IL-1β bind to their respective receptors followed by internalization of the receptors. BFA disrupted this receptor recycling phenomenon, reducing the number of cytokine receptors on the macrophage surface. BFA also have substantial

role in inhibiting cytokine secretory pathways as TNF-α, IL-1β, and CXCL8 production (that elevated during infection) reduced significantly when pre-treated with BFA prior to *S. aureus* infection. Various reports suggest the positive role of IL-1β in inducing TNFR1 expression [57]. This implies that not only reduced TNF-α secretion but also reduced IL-1β production plays a substantial role in downregulation of TNFR1 signalling pathway. According to our data, BFA have probable role in diminution of CXCR1 expression (as obtained in western blot and FACS data). Now the question arises whether BFA directly modulate CXCR1 or mediated through TNF-α/TNFR1 and IL-1β/IL-1R signalling pathways. FACS analysis suggests that drastic reduction in cell count expressing CXCR1 was due to combination treatment of BFA+anti-TNFR1 antibody+IRAP rather than BFA or anti-receptor antibodies alone. Furthermore, pre-treatment with BFA also led to evident drop in ROS production, almost negligible iNOS and COX-2 expression, along with reduced production of sTNFR1 (obtained from ELISA data) due to reduced TNFR1 expression on surface. For the very first time, we showed that BFA have inhibitory role on iNOS and COX-2, and thus further research needs to be done to explore this phenomenon to use it as anti-inflammatory strategy. This leads us to the conclusion that though BFA might solely regulate the above factors, its efficiency in presence of anti-TNFR1 antibody and IRAP increased manifold in abatement of inflammatory effects (Fig. 13b). Hence, blockage of intracellular receptor endocytic pathway (route from Golgi body to ER to cell membrane) might serve as one of prominent tools to combat inflammation.

TACE is well known for TNFR1 shedding in activated macrophages [58]. This metalloprotease is activated in response to ROS production during bacterial infection [59, 60]. Even hydroxyl radical formed through H_2O_2-dependent Fenton reaction serves as an important messenger in shedding of TNFR1 [61]. Various studies suggest that TACE mRNA expression is significantly elevated as early as 1 h on stimulation with *S. aureus* or *Pseudomonas aeruginosa* [62]. As shown in Fig. 13c, the increased availability of TNF-α, IL-1β, and ROS generated during infection induce TACE expression that cleaves TNFR1 present on the macrophage surface and raise sTNFR1 level in the circulation. However, treatment with TAPI-1 leads to a substantial drop in concentration of sTNFR1 in supernatant, hence, inhibiting the ectodomain shedding. In one side, TAPI-1 inhibited receptor shedding, keeping intact the TNFR1 and IL-1R on the cell surface. On the other hand, this inhibitor is also associated with processing of TNF-α and IL-1β as pre-treatment with TAPI-1 reduced their concentrations in the culture medium, leading to reduced pool of cytokines and consequently reduced CXCL8 production. This dual role of TAPI-1 can be explained with probable mechanism—at 60 min post-infection, concentration of TNF-α, IL-1β, and sTNFR1 rose abruptly in the medium, where TNF-α and IL-1β showed an almost sevenfold increase and sTNFR1 showed a threefold rise during *S. aureus* infection compared with untreated or control macrophages. This tremendous pool of cytokines becomes the dictating factor rather than sTNFR1 in the circulation. Furthermore, pre-treatment with TAPI-1 reduced superoxide, nitric oxide and hydrogen peroxide release along with suppression of iNOS (that further diminish NO level) and COX-2. Even FACS analysis data supports the fact that TAPI-1 has a probable role in the reduction of PE-conjugated CXCR1 expression under infected condition. However, for better understanding of this controversial role of TAPI-1, when macrophages were treated alongside with anti-TNFR1 antibody and IRAP, there was drastic reduction in all the above parameters, leading to downregulation of CXCL8/CXCR1 axis.

Our experimental study has certain limitations. Since our primary focus involved cell surface expression study, the structural changes in Golgi bodies observed under confocal microscopic studies due to BFA and its effect on TNFR1, and IL-1R intracellular trafficking needs to be studied for better understanding of intracellular mechanisms. Besides TACE, there might be other metalloproteinases that are responsible for ectodomain shedding of TNFR1. Despite these shortcomings, the results presented in this experimental study underscore the role of two promising inhibitors, brefeldin A and TAPI-1 in the presence of anti-TNFR1 antibody and IRAP in de-escalating *S. aureus*-induced CXCR1 expression by the two possible mechanisms (that is, by inhibiting receptor mobilization and receptor shedding, respectively) as mentioned above.

Conclusions

The two potent inhibitors, brefeldin A and TAPI-1 have been brought in the forefront alone or in the presence of anti-TNFR1 antibody and IRAP, and an experimental study have been designed to justify their role in diminution of *S. aureus*-induced CXCR1 expression in macrophages. In general, we can conclude that inhibition of receptor trafficking and

receptor shedding under receptor (TNFR1 and IL-1R) neutralized condition have following implications: (a) reduced ROS generation as well as pro-inflammatory cytokine secretion, (b) reduced secretion of inflammatory mediators (iNOS and COX-2), (c) reduced TNFR1 and IL-1R expression on cell surface, and hence (d) downregulated CXCL8/CXCR1 expression, resulting in abrogation of inflammatory responses. Hence, targeting the intracellular receptor mobilization pathway or receptor shedding mechanism to curb the cytokine-receptor interaction might serve as a promising therapy to reduce inflammation and its associated responses.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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