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SELECTION OF OPTIMAL PARAMETERS AND REVISITING FUNCTIONAL CHARACTERIZATION OF CXCR1 IN RESPONSE TO *STAPHYLOCOCCUS AUREUS* INFECTION

Puja Dutta[1] and Biswadev Bishayi[1]*

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

***Corresponding Author: Biswadev Bishayi**
Department of Physiology, Immunology Laboratory, University of Calcutta, University Colleges of Science and Technology, 92 APC Road, Calcutta-700009, West Bengal, India.

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ABSTRACT

Staphylococcus aureus, a major opportunistic pathogen, can cause spacious range of infections in animals and humans. Treatment of *S.aureus* induced sepsis and inflammatory diseases have become the need of the hour. *S.aureus* stimulated murine peritoneal macrophages releases a major chemokine, CXCL8 and aggravate CXCR1 expression on the macrophage surface. CXCL8/CXCR1 axis is pivotal for leucocyte recruitment and bacterial clearance from the site of infection. Extrapolating our previous research works, the effect of graded bacterial inoculum size and peritoneal macrophage cell count on CXCL8, ROS generation, and CXCR1 expression had been evaluated. Secondly, based on the optimum doses obtained, kinetic-dependent *S.aureus* infection on CXCR1 expression, its subsequent effect on bacterial phagocytic phenomenon had also been studied. The bacterial inoculums size and macrophage quantity had a regulatory effect on CXCR1 expression on the macrophage surface. Increasing bacterial inoculum or macrophage quantity in the culture medium does not elevate CXCR1 expression on macrophage surface beyond a certain point, suggesting a reconcilable interaction between bacterial components and macrophage essential for receptor expression. According to the results obtained, maximum intracellular phagocytosis occurred at 60 minutes post infection. The magnitude of phagocytic activity wasfound roughly proportional to the surface CXCR1 expression on macrophage surface. Hence CXCL8/CXCR1 axis has a significant role in intracellular bacterial phagocytosis and serves as a potential therapeutic target to control *S.aureus* infection.

KEYWORDS: CXCL8; CXCR1; peritoneal macrophage; phagocytosis; *Staphylococcus aureus*.

INTRODUCTION

Depending on the type of strain involved and site of infection, the human pathogen, *Staphylococcus aureus* can cause an extensive range of clinical infections, from mild to severe, life-threatening diseases. It contains a wealth of virulent or pathogenic factors that target the host cellular membrane or are involved in host tissue colonization for its survival.[1] The continuous emergence of this multi-drug resistant bacterium and its induced inflammatory diseases has fuelled the researchers to opt for non-drug alternative strategies to treat these deadly diseases. CXCR1 and CXCR2 are two well-constituted G-protein coupled receptors (GPCRs) for CXCL8 (or Interleukin-8), a chemokine released by the immune cells in response to pathogenic infection.[2] Comparative studies between these two receptors indicate CXCR2 can bind to various chemokines, whereas CXCR1 binds specifically to CXCL8 to mediate its major function of chemotaxis. Interestingly, both respond to bacterial infections through their distinct pathways, but the fundamental inflammatory-cytotoxic-regulatory signalling is propagated through CXCR1.[3] CXCR1 is basically used as an inflammatory marker in varied inflammatory and pathological conditions.[4] Thus our topic of interest is centered on CXCR1. CXCL8/CXCR1 axis performs the following functions in response to pathogenic insult- a) chemotactic migration of leucocytes, b) activation of cytotoxic function of leucocytes, c) calcium ion (Ca^{++}) mobilization, cell proliferation, migration, d) regulation of angiogenesis, e) activate Nuclear factor kappa light chain of B cells (NF-κB) and Mitogen activated protein kinase (MAPK) signaling to alter gene expression and inflammatory responses.[4] Any disruption or overexpression of this axis has a dreadful effect on the host immune mechanism against infection and welcomes severe fatal diseases.[5] However, research on chemokine receptors in murine models is limited. These might be probably due to more chemokines been detected than its corresponding receptors or due to the redundancy property of the chemokines[6] Murine macrophages are one of the active immune cells that release CXCL8 when attacked by any pathogen.[7] Our previous experimental works spotlighted the significance of CXCR1 during *S.aureus* (strain AG-

789) infection in peritoneal macrophages obtained from the swiss albino mice model.[8- 10]

The bacterial inoculums size is one of the considerable parameters to test the efficacy of antibiotics or antimicrobial agents.[11] The selection of bacterial doses to be injected or administered is important for the development of substantial inflammation in animal disease models. The graded effect of bacterium inoculums size on CXCL8 secretion and CXCR1 expression had been studied in vitro in cultured peritoneal macrophages. The recognition of bacteria and its binding to specific receptors on macrophages is of utmost importance not only for bacterial engulfment but also for the development of inflammatory signaling.[12] Hence the effect of increasing peritoneal macrophage count and selection of optimum cell count on Reactive oxygen species (ROS) generation, CXCL8 production and CXCR1 expression against a specific *S.aureus* dose had also been studied. The coordinated cooperation among ROS, cytokines and chemokines is the essence for *S.aureus* phagocytosis in activated macrophages.[13] However, despite the well-programmed phagocytic process to kill the bacteria within the cell, *S.aureus* has developed various evading strategies to combat this process.[14] Very few noticeable reports are available showing association between phagocytosis and surface CXCR1 expression. Extrapolating our previous works, the kinetic dependent *S.aureus* infection on bacterial engulfment by peritoneal macrophages and corresponding CXCR1 expression had been evaluated in this study.

Thus the major objective of this short, experimental study was to redetermine the graded effect of bacterial inoculum size and peritoneal macrophage count on CXCL8/CXCR1 expression (using the *S.aureus* strain AG-789) to standardize the optimal dose to stimulate its production. As a second important point, we also hypothesized the role of CXCL8 in the modulation of intracellular bacterial phagocytosis through the induction of CXCR1 in murine peritoneal macrophages. CXCR1 had been functionally characterized along with ROS and its ligand, CXCL8 production in response to *S.aureus* infection.

MATERIALS AND METHODS

Isolation of peritoneal macrophages

The peritoneal macrophages were collected from specific, pathogen-free, 6-8 weeks old male Swiss albino mice kept in proper animal-holding rooms. The mice were pre-injected intraperitoneally with 2 ml of 4% sterile thioglycolate broth, and the resulting peritoneal exudates were harvested by lavage of the peritoneal cavities with endotoxin-free Hanks' solution 4 to 5 days later. The erythrocytes, if present, were lysed using 0.83% ammonium chloride solution containing 10% (v/v) Tris buffer (pH 7.65). The peritoneal macrophages were suspended 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 plate surface. The nonadherent cells were removed by washing three times with 0·9% sodium chloride (NaCl). The adherent macrophages were then scraped and suspended into fresh phosphate buffer saline (PBS). By Trypan Blue dye exclusion method and CD11b marker-based FACS analysis, the peritoneal macrophages can be used for the experimental study if more than 95% cells were found viable.[15]

Bacterial strains

S.aureus strain AG-789 was used in all experiments, obtained from the Apollo Gleneagles Hospital, West Bengal, India. For all these experiments, the bacteria were grown for 18 hours on Luria- Bertani broth, diluted with fresh broth and cultured until the mid-logarithmic phase of growth at 37°C with shaking. The bacteria were harvested, washed with cold PBS to adjust the inoculum spectrophotometrically before infection (OD_{620}=0.2 corresponding to 5×10^7cells/ml). The colony forming unit (CFU) count of the desired inoculum was confirmed by serial dilution and cultured on blood agar. From the biochemical analysis, *S. aureus* (AG- 789) was found to be methicillin resistant and catalase and coagulase positive.[16]

Experimental design

In one set of experiments, peritoneal macrophages (5×10^6 cells/ml) from Swiss albino mice were treated and incubated with an increasing inoculum size of live *S.aureus* (by serial dilution of 1:100) at doses of 5×10^2, 5×10^4, 5×10^6 and 5×10^8 CFU/ml in the culture medium. In the second set, increasing peritoneal macrophage cell count in the culture medium (by serial dilution of 1:10) were stimulated with a specific optimum *S.aureus* dose that will be obtained from the previous experiment and allowed to incubate. In the third set, the optimum dose of macrophage and bacteria obtained from the previous set of experiments were mixed in the culture medium and allowed to incubate for different periods (30, 60, 90 and 120 minutes post infection) at 37°C in 5% CO_2 incubator. In these three experimental conditions, the incubation period was followed by centrifugation; the supernatants and lysates were prepared from varied groups and stored in aliquots at -20°C to carry out further experiments.

Measurement of ROS generation

The intracellular ROS level in different groups had been measured by specific assays as well as FACS analysis. To detect the effect of increasing inoculum size and macrophage count on ROS level, the superoxide anion assay, nitric oxide (NO) production and hydrogen peroxide production had been performed separately. The superoxide production was quantified by cytochrome c reduction method.[17] and NO production by the colorimetric Griess assay.[18] To quantify H_2O_2 production, the cultured supernatant was incubated with 20 µl Horse Raddish peroxidase (HRP) (500 µg/ml) and 70 µl of Phenol red (500 µg/ml) for 2 hours at 37°C.

Later the reaction was stopped by adding 2 N sodium hydroxide (NaOH) and the absorbance was taken at 620 nm using a spectrophotometer.[19] The intracellular ROS level in different groups has been analyzed by 2',7'-dichlorodihydrofluorescein diacetate (DCFHDA) assay. Different groups of macrophages were incubated with freshly prepared 20 mM DCFH-DA stock solution made in Dimethyl sulfoxide (DMSO) medium and incubated for 90 min in dark at 37ºC. Finally, cells were suspended in PBS and ROS generation was measured by fluorescence intensity at 530 nm.[20]

Measurement of CXCL8 production by ELISA

Sandwich ELISA was performed to detect CXCL8 (Cat No.: MBS012027; MyBioSource) following the procedure as described in the manual and the readings were taken using a BioRad ELISA Reader at 450 nm.

Analysis of CXCR1 expression by western blotting and flow cytometry

Western blotting was used to study CXCR1 expression using the CXCR1 antibody (orb215479; Biorbyt)[10] and FACS analysis using Phycoerythrin (PE)-conjugated monoclonal anti-chemokine receptor CXCR1 (Cat No.: FAB8628P from R and D systems)[21] respectively according to the standard protocols described earlier in our previous studies. 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. The densitometric data were analyzed using ImageJ software.

Confocal analysis of intracellular bacterial engulfment by peritoneal macrophages

The peritoneal macrophages were grown on coverslips in 6-well culture plates, containing RPMI-1640 medium supplemented with 10% FBS, 100 IU/ml of penicillin, and 100 µg/ml of streptomycin). The macrophage nuclei were stained using Nuclear yellow (Hoechst S769121, ab13903) at 250 µg/ml concentration and incubated for 2 hours. For the preparation of Fluorescein Isothiocyanate (FITC)-labeled *S. aureus*, the bacteria were grown overnight in Mueller-Hinton broth (MHB) at 37°C. Then, the bacteria were diluted with fresh broth and were cultured until the mid-logarithmic phase of growth (OD_{620} = 0.2). Bacterial pellets of required CFU (as obtained from results) were resuspended in carbonate buffer (pH = 9.0) containing 100 µg/ml FITC isomer I (catalog no: ab145325) for 1 hour at room temperature in the dark. The preparation was extensively washed with PBSE (PBS+ 5 mM EDTA). The FITC-labelled *S. aureus* were added in stained macrophage containing well and incubated for 30, 60, 90 and 120 minutes post infection. The 30 minutes post *S. aureus* infected macrophages were taken out from the incubator, rinsed with cold PBS and fixed with 4% paraformaldehyde for 15 minutes. The same procedure was followed for other groups of infected macrophages. The specimens were mounted on microscope slides with 10% glycerol and covered with a coverslip. The images were analyzed using Olympus FV1000 viewer software and the co-localization was accessed by the Pearson correlation coefficient.[22]

Statistical Analysis

Data were presented as the Mean ± SD, unless otherwise indicated, across at least three independent experiments. For experiments where individual cells were assayed by microscopy, at least 100 cells were examined in each experiment. Comparisons were performed employing a two-tailed student t-test, and $P<0.05$ was considered significant.

RESULTS AND DISCUSSION

Graded effect of *S.aureus* inoculum size on ROS, CXCL8 and CXCR1 expression in murine peritoneal macrophages CXCL8 and CXCR1 are potent biomarkers of inflammatory sequelae and hence, serve as major attractive targets for the development of therapeutics to treat inflammatory diseases.[4] The effect of increasing *S.aureus* inoculum size on ROS generation, CXCL8 and CXCR1 expression in murine peritoneal macrophages had been analyzed (Fig. 1). A significant increase in superoxide, NO, H_2O_2 generation (Fig. 1a) and CXCL8 release (Fig. 1b) in the medium was observed in the consecutive doses (of 5×10^2 CFU/ml and 5×10^4 CFU/ml) until 5×10^6 CFU/ml *S.aureus* dose. On further increasing the inoculum size to 5×10^8 CFU/ml, no further significant differences in the above parameters were observed. The results were indeed in co-parallel with CXCR1 expression obtained on the macrophage surface (Fig. 1c), wherein the band intensity was maximum at 5×10^6 CFU/ml dose and remained insignificant till 5×10^8 CFU/ml *S.aureus* dose. This implies that CXCR1 expression became stagnant or remained desensitized at 5×10^8 CFU/ml, despite further increase of inoculum size. This might be accountable for a limited ratio of macrophage surface availability despite increasing bacterial count in the medium. Contrary to our previous research works, CXCR1 expression, though less or very minute, had been observed in resting peritoneal macrophage surface. The possible explanation could be-the current antibody been used was highly species-specific compared to the previous one. Or else the theoretical possibility claims the receptors might remain in a dormant or aphasic state on the cell surface unless attacked by the pathogen due to which it was undetectable in resting macrophages. Thus besides chemokine-receptor expression, ROS-CXCL8 also showed synchronized changes in their production in response to graded bacterial doses against a specified macrophage count in the cultured medium. The ROS released in response to infection further elevated its production through a positive feedback mechanism. Uncontrolled ROS generation or oxidative stress might have a detrimental effect on cellular integrity due to changes in cellular redox environment.[23] The continuous genesis of free radicals, CXCL8 and other pro-inflammatory cytokines like TNF- α and IL-1β with surface receptors (TNFR1 and IL-1R respectively)

interaction were prime factors responsible for elevating CXCR1 expression during optimum *S.aureus* infection (5×10^6 CFU/ml).[10] Hence we can conclude that 5×10^6 CFU/ml could be utilized as an optimum bacterial dose for maximal CXCL8 and CXCR1 expression in macrophages.

Graded effect of peritoneal macrophage count on ROS, CXCL8 and CXCR1 expression in murine peritoneal macrophages during *S.aureus* infection

Using this obtained standard bacterial inoculum dose (5×10^6 CFU/ml), the effect of increasing peritoneal macrophage cell count on ROS (that includes superoxide, H_2O_2 and NO production), CXCL8 and CXCR1 expression had been measured during *S.aureus* infection (Fig. 2). The peritoneal macrophages (5×10^6 cells/ml) were used as a control to scrutinize the difference in parameters under normal and infected conditions. Both ROS generated (Fig. 2a) and CXCL8 production (Fig. 2b) was maximum at 5×10^6cells/ml and remained unaltered till 5×10^7 cells/ml (no significant difference). In this experiment, ROS-CXCL8 production showed similar changes with respect to graded macrophage cell count in the medium. But slight variations were observed in case of CXCR1. According to western blot data, CXCR1 expression was highest at *S.aureus* stimulated 5×10^6 cells/ ml of macrophage, with a significant decrease at 5×10^7 cells/ml dose (Fig. 2c).This might be possible because the rate of CXCR1 internalization was faster compared to the rate of changes in intracellular free radicals and chemokine production in *S.aureus* stimulated peritoneal macrophages. Thus we corroborated that peritoneal macrophage count of 5×10^6 cells/ml could be considered as standard cell strength in the medium for maximal CXCR1 expression against *S.aureus* infection (5×10^6 CFU/ml). A dynamic interaction between the surface receptor and its ligand is the essence for the initiation of any signalling cascade. *S.aureus* generally bind to Toll-like receptor2 (TLR2) and initiate signalling to activate NF-κB and JNK pathways, leading to enhanced cytokines, chemokines and receptor expression.[24] Hence the optimum concentration of bacterium inoculums (5×10^6 CFU/ml) or peritoneal macrophage strength (5×10^6 cells/ml) is essential for optimal CXCL8 secretion. Through this experiment, we can affirm increasing macrophage quantity in the culture medium does not warrant proportionate CXCL8 release and CXCR1 expression, suggesting a reconcilable interaction between bacterial components and macrophage essential for receptor expression on macrophage surface for specific response. These simplified experiments might help develop more accurate pharmaceutical drugs or anti-CXCR1 targeted drugs for the treatment of inflammatory diseases. We also unveiled the oxidative stress pathway in bacterial infected macrophages could be modulated through the regulation of CXCR1.

The effect of time-dependent *S.aureus* infection on bacterial engulfment, ROS, CXCL8 and CXCR1 expression in murine peritoneal macrophages

Using the optimal doses of macrophage count and bacteria obtained from the previous two experiments, in the next step, the process of intracellular bacterial engulfment by murine peritoneal macrophages (5×10^6 cells/ml) at different infection (5×10^6 CFU/ml) time points and the percent colocalization of FITC-labelled *S.aureus* with Hoechst dye stained macrophage nuclei had been studied by confocal microscopy. The intracellular bacteria were found within the cytoplasm of macrophages. The confocal image analysis suggests that maximum bacterial phagocytosis occurred at 60 minutes post *S.aureus* infection, resulting in a drastic reduction in bacterial count. However, post 60 minutes, the intracellular bacterial count significantly increased from 90 to 120 minutes (indicating reduced phagocytosis) (Fig.3a-b). To explain this phenomenon, the two major parameters- ROS and CXCL8 production had also been measured. The CXCL8 production and total ROS level (detected from DCFDA Intensity) by macrophages increased significantly from 30 to 60 minutes, till 90 minutes, followed by a significant dwindling at 120 minutes post infection, the last time point in this experiment (Fig. 3c-d). To determine whether CXCR1 had any role in this phenomenon, its expression had been detected by both western blot and FACS analysis. *S.aureus* stimulated peritoneal macrophages exhibited maximum CXCR1 expression at 60 minutes (results coparallel with our previous studies) with a significant decrease at 90 and 120 minutes of infection. These findings were further confirmed by FACS analysis where CXCR1-PE signalling was highest at 60 minutes, which further saliently decreased at 90 to 120 minutes post infection (Fig. 3e-f). Collectively, we can conclude that both ROS and CXCL8/CXCR1 axis reduced bacterial count at 60 minutes; with decrease in CXCR1 expression, the macrophages fail to clear *S.aureus* from the intracellular compartments, resulting in increased bacterial count at 90 to 120 minutes infection points.

The maximal bacterial phagocytosis obtained at 60 minutes post infection was contributable by ROS, chemokine CXCL8 and CXCR1. CXCL8/CXCR1 axis regulates ROS level and major pro-inflammatory cytokines (TNF-α and IL-1β) through NF-κB and JNK pathways.[22] Among the ROS, superoxide and H_2O_2 are most potent in bacterial membrane damage. Nitric oxides, a major part of Reactive Nitrogen Species (RNS) produced by activated macrophages perturb bacterial respiration, affect membrane integrity as well as bacterial DNA replication.[25] Excessive NO generation contributes to the inflammatory process and thus used as a liable marker to detect inflammatory activity. The pro-inflammatory cytokine portfolio (TNF-α, IL-1β, IL-6, IFNγ) was found to be highest at 60 minutes in *S.aureus* stimulated macrophages (though not shown here). The activated macrophages orchestrate all these factors to potentiate *S.aureus* killing. The bacterial engulfment

process is associated with CXCR1 expression, confirmed by Western blot and FACS analysis data. More the CXCR1 expression on macrophage surface, more is the bacterial engulfment by activated macrophages. But how the expression varies with time-dependent infection events without any treatment needs to be reasoned out. *S.aureus* has its own tactics to turn the host environment into its favor or growth.[25] The continuous process of phagocytosis provides negative signals to retard chemotactic responses of migrating cells at later stages of infection, resulting in diminished CXCL8 mediated cellular functions and consequent decrease in CXCR1 expression prominent at 90 and 120 min post infection.[26-27] Rapid receptor internalization due to endogenous signals could be another major reason for the downregulation of CXCR1 on the macrophage surface. The balance between receptor internalization and degradation might dictate the availability of receptor level on the cell surface.[28] The bacterial components and cytokines like TNFα also reduce the CXCR1 level through proteolytic cleavage by metalloproteinases.[29] The concentration of CXCL8 is also a significant parameter to determine CXCR1 activation or desensitization on the macrophage surface. Consequently, decreased CXCR1 reduces ROS and chemokine release through its signaling pathways, resulting in increased replication of bacteria within macrophages. Thus we can critically conclude that CXCR1 is essential for *S.aureus* clearance in peritoneal macrophages through a mechanism involving CXCL8 and ROS production. CXCR1 stands at a swivel point between inflammation and resolution of infection. The uncontrolled bacterial colonization or growth inside the host tissues might lead to chronic *S.aureus* mediated infections. CXCR1 expression that decreased in the kinetic study might re-express itself contributable by other cytokine or chemokines signaling, which if unstopped, can aggravate the inflammatory cascade. Thus the proper selection of antibiotic or anti- CXCR1 therapeutics or their combination would be wise to control *S.aureus* infections.

LEGENDS TO THE FIGURES

Fig. 1. Effect of increasing *S.aureus* inoculum size on ROS generation (superoxide, nitric oxide, and hydrogen peroxide), CXCL8 and CXCR1 expression in murine peritoneal macrophages

Effect of increasing *S.aureus* inoculum size on the release of **a** Superoxide, Nitric oxide (NO) and hydrogen peroxide (H_2O_2), **b** CXCL8 (pg/ml), and **c** CXCR1 expression was measured in murine peritoneal macrophages. The fold changes for CXCR1 were shown in arbitrary units. All the samples were probed with β-tubulin to show an equal amount of protein loading. The values were shown as mean ± SD (n = 5/group) from three independent experiments. P <0.05 was considered significant.PM = Peritoneal Macrophages, SA = *S.aureus* infection. The symbols denote: $^{@}$ Significant difference ($p<0.05$) with respect to control peritoneal macrophage (PM), $^{\#}$ Significant difference ($p<0.05$) with respect to PM+SA1 (5×10^2 CFU/ml), $^{\$}$ Significant difference ($p<0.05$) with respect to PM+SA2 (5×10^4 CFU/ml).

Fig. 2. Effect of increasing peritoneal macrophage cell count on ROS generation (Superoxide, Nitric oxide, and hydrogen peroxide), CXCL8 and CXCR1 expression during *S.aureus* infection

Effect of increasing peritoneal macrophage cell count on the release of **a** Superoxide, Nitric oxide (NO) and hydrogen peroxide (H_2O_2), **b** CXCL8 (pg/ml), and **c** CXCR1 expression during *S.aureus* infection was measured in murine peritoneal macrophages. The fold changes for CXCR1 were shown in arbitrary units. All the samples were probed with β-tubulin to show an equal amount of protein loading. The values were shown as mean ± SD (n = 5/group) from three independent experiments. P < 0.05 was considered significant.PM = Peritoneal Macrophages, SA = *S.aureus* infection. The symbols denote: $^{@}$ Significant difference ($p<0.05$) with respect to control macrophages (PM), * Significant difference ($p<0.05$) between PM and SA+PM3 (both PM and PM3= 5×10^6 cells/ml), $^{\#}$ Significant difference ($p<0.05$) with respect to SA+PM1 (5×10^4 cells/ ml), $^{\$}$ Significant difference ($p<0.05$) with respect to SA+PM2 (5×10^5 cells /ml), $^{\wedge}$ Significant difference ($p<0.05$) with respect to SA+PM3 (5×10^6 cells /ml).

Fig. 3. Effect of time-dependent *S.aureus* infection on bacterial phagocytosis, ROS, CXCL8 and CXCR1 expression in murine peritoneal macrophages

Effect of time-dependent *S.aureus* infection on **a** Bacterial phagocytosis analyzed by confocal microscopy: Hoechst dye used to stain the nucleus of macrophages (blue) and FITC dye to stain *S.aureus* (green). The merged images represent the engulfment of *S.aureus* by peritoneal macrophages (n=5; scale bar=10-15µm), **b** Percent co-localization (%)**c** CXCL8 production (pg/ml), **d** ROS level (by FACS analysis), **e** CXCR1 expression by FACS analysis and **f** CXCR1 expression (by western blot) have been evaluated in murine peritoneal macrophages. The fold changes for CXCR1 were shown in arbitrary units. All the samples were probed with β-tubulin to show an equal amount of protein loading. The values were shown as mean ± SD (n = 5/group) from three independent experiments. P < 0.05 was considered significant. CTL = Control peritoneal macrophages, SA = *S.aureus* infection. The symbols denote: $^{@}$ Significant difference ($p<0.05$) with respect to control macrophage (CTL), $^{\#}$ Significant difference ($p<0.05$) with respect to CTL+SA30, $^{\$}$ Significant difference ($p<0.05$) with respect to CTL+SA60, $^{\wedge}$ Significant difference ($p<0.05$) with respect to CTL+SA90.

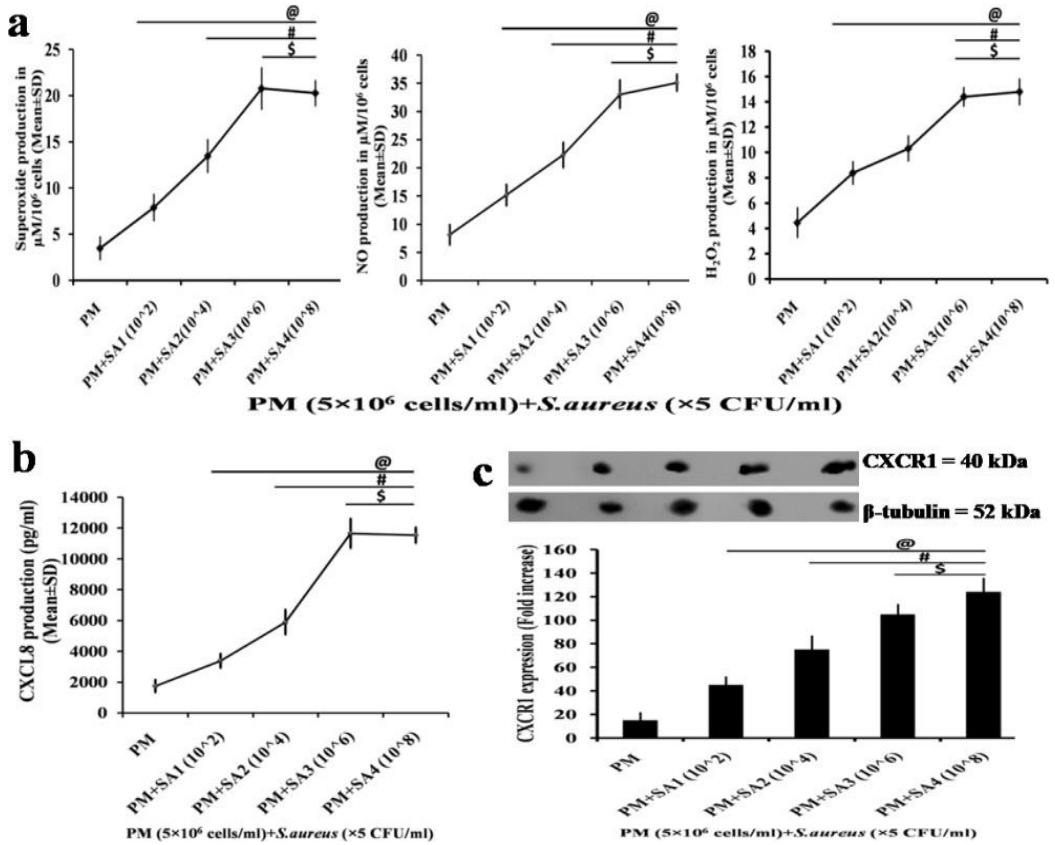
a
Superoxide production in µM/10⁶ cells (Mean±SD)
NO production in µM/10⁶ cells (Mean±SD)
H2O2 production in µM/10⁶ cells (Mean±SD)
PM
PM+SA1 (10^2)
PM+SA2(10^4)
PM+SA3(10^6)
PM+SA4(10^8)
PM (5×10⁶ cells/ml)+S.aureus (×5 CFU/ml)
b
CXCL8 production (pg/ml) (Mean±SD)
c
CXCR1 = 40 kDa
β-tubulin = 52 kDa
CXCR1 expression (Fold increase)

Fig. 1.

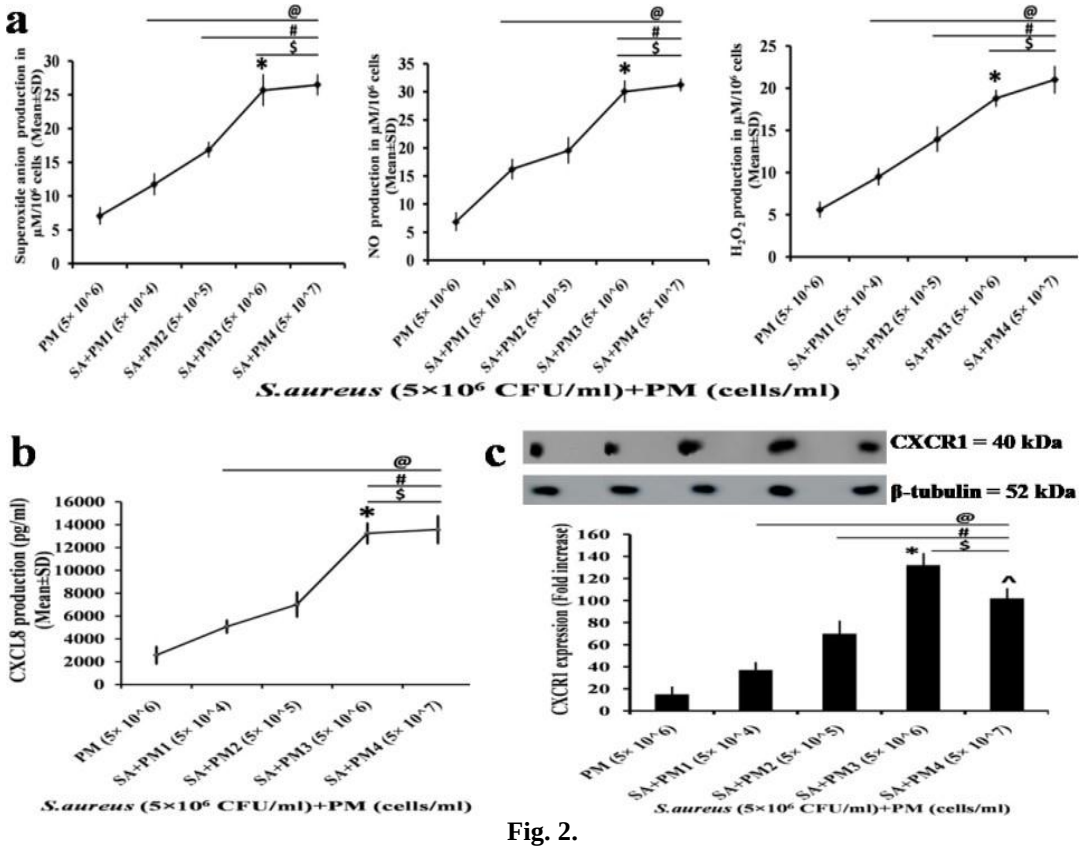
a
Superoxide anion production in µM/10⁶ cells (Mean±SD)
NO production in µM/10⁶ cells (Mean±SD)
H2O2 production in µM/10⁶ cells (Mean±SD)
PM (5× 10^6)
SA+PM1 (5× 10^4)
SA+PM2 (5× 10^5)
SA+PM3 (5× 10^6)
SA+PM4 (5× 10^7)
S.aureus (5×10⁶ CFU/ml)+PM (cells/ml)
b
CXCL8 production (pg/ml) (Mean±SD)
c
CXCR1 = 40 kDa
β-tubulin = 52 kDa
CXCR1 expression (Fold increase)

Fig. 2.

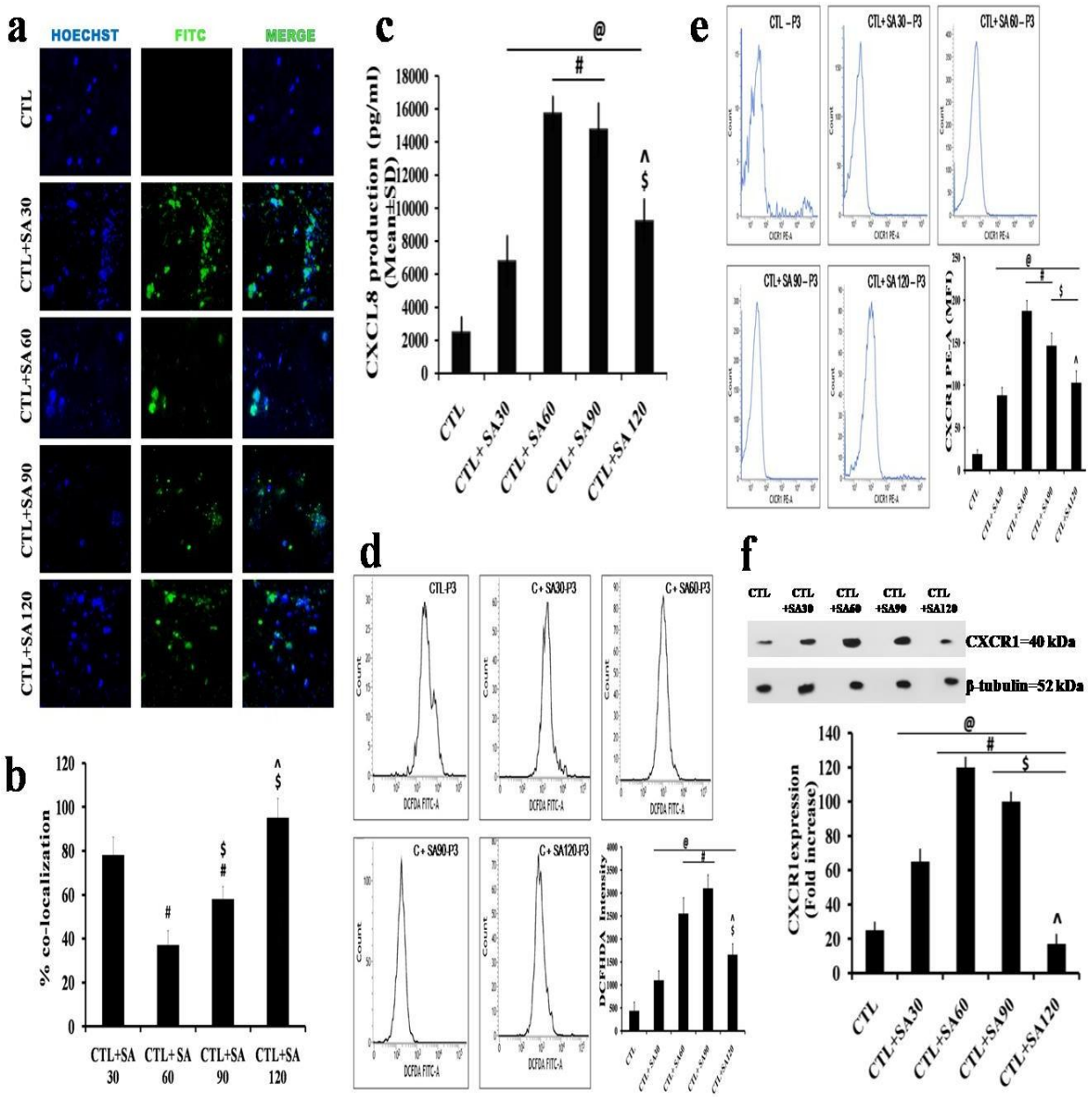

Fig. 3.

CONCLUSION

Nevertheless, we can conclude that murine CXCR1 is a characterized receptor of peritoneal macrophages that have an important role in a) ROS regulation, b) inflammation, c) bacterial phagocytosis during *S.aureus* infection. We anticipate that more studies on CXCR1 sequencing and comparison between murine and human CXCR1 sequences are essential for better exploration of its functional properties. More comprehensive knowledge of the role of CXCR1 in bacterial phagocytosis along with the multitude of complex factors, cytokines and signaling systems needs to be studied. Hopefully, certain newer concepts obtained from this experimental study could be converted into clinical outcomes and more designing of therapeutics with antibiotics or a combination of anti-chemokine receptor and antibiotics be developed for effective bacterial killing and reducing inflammation in the host system.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. 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 Number: IAEC/IV/Proposal/BB- 01/2016 dated 18.07.2016], Ministry of Environment and Forest, Govt. of India.

AUTHORS' CONTRIBUTIONS

Biswadev Bishayi (BB) and Puja Dutta (PD) designed the experimental study. PD performed all the experiments related to the study. BB and PD managed the literature searches and analyses. PD undertook the statistical analysis and interpretation of the study data. PD wrote the manuscript. All the authors read and approved the final manuscript.

REFERENCES

1. Bien, J., Sokolova, O. and Bozko, P. (2011). 'Characterization of virulence factors of *Staphylococcus aureus*: novel function of known virulence factors that are implicated in activation of airway epithelial proinflammatory response', *Journal of Pathogens*, 2011; 601905.
2. Turner, M.D., Nedjai, B., Hurst, T. and Pennington, D.J. (2014). 'Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease', *Biochimica et Biophysica Acta*, 1843(11): 2563–2582.
3. Nasser, M.W., Raghuwanshi, S.K., Grant, D.J., Jala, V.R., Rajarathnam, K. and Richardson, R.M. (2009). 'Differential activation and regulation of CXCR1 and CXCR2 by CXCL8 monomer and dimer', *Journal of Immunology*, 183(5): 3425– 3432.
4. Ha, H., Debnath, B. and Neamati, N. (2017). 'Role of the CXCL8-CXCR1/2 axis in cancer and inflammatory diseases', *Theranostics*, 7(6): 1543–1588.
5. Russo, R.C., Garcia, C.C., Teixeira, M.M. and Amaral, F.A. (2014). 'The CXCL8/IL- 8 chemokine family and its receptors in inflammatory diseases', *Expert Review of Clinical Immunology*, 10(5): 593–619.
6. Zlotnik, A., Yoshie, O. and Nomiyama, H. (2006). 'The chemokine and chemokine receptor superfamilies and their molecular evolution', *Genome Biology*, 7(12): 243.
7. Jia, J., Sun, Y., Hu, Z., Li, Y. and Ruan, X. (2017). 'Propofol inhibits the release of interleukin-6, 8 and tumor necrosis factor-α correlating with high-mobility group box 1 expression in lipopolysaccharides-stimulated RAW 264.7 cells', *BMC Anesthesiology*, 17(1): 148.
8. Bishayi, B., Adhikary, R., Sultana, S., Dey, R. and Nandi, A. (2017) 'Altered expression of CXCR1 (IL-8R) in macrophages utilizing cell surface TNFR1 and IL-1 receptor during *Staphylococcus aureus* infection', *Microbial Pathogenesis*, 113: 460– 471.
9. Bishayi, B., Nandi, A., Dey, R. and Adhikary, R. (2017). 'Expression of CXCR1 (IL- 8 receptor A) in splenic, peritoneal macrophages and resident bone marrow cells after acute live or heat killed *Staphylococcus aureus* stimulation in mice', *Microbial Pathogenesis*, 109: 131–150.
10. Dutta, P., Sultana, S., Dey, R. and Bishayi, B. (2019). 'Regulation of *Staphylococcus aureus*-induced CXCR1 expression via inhibition of receptor mobilization and receptor shedding during dual receptor (TNFR1 and IL-1R) neutralization', *Immunologic Research*, 67(2-3): 241–260.
11. Li, J., Xie, S., Ahmed, S., Wang, F., Gu, Y., Zhang, C *et al*.(2017). 'Antimicrobial activity and resistance: influencing factors', *Frontiers in Pharmacology*, 8: 364.
12. Taylor, P.R., Martinez-Pomares, L., Stacey, M., Lin, H.H., Brown, G.D. and Gordon, S. (2005). 'Macrophage receptors and immune recognition', *Annual Review of Immunology*, 23: 901–944.
13. Koziel, J., Maciag-Gudowska, A., Mikolajczyk, T., Bzowska, M., Sturdevant, D.E., Whitney, A.R et al.(2009). 'Phagocytosis of *Staphylococcus aureus* by macrophages exerts cytoprotective effects manifested by the upregulation of antiapoptotic factors', *PLoS One*, 4(4): e5210.
14. Lacoma, A., Cano, V., Moranta, D., Regueiro, V., Domínguez-Villanueva, D., Laabei, M. *et al*.(2017). 'Investigating intracellular persistence of *Staphylococcus aureus* within a murine alveolar macrophage cell line', *Virulence*, 8(8): 1761–1775.
15. Wang, C., Yu, X., Cao, Q., Wang, Y., Zheng, G., Tan, T.K *et al*.(2013). 'Characterization of murine macrophages from bone marrow, spleen and peritoneum',*BMC Immunology*, 14: 6–16.
16. Yao, L., Berman, J.W., Factor, S.M. and Lowy, F.D. (1997). 'Correlation of histopathologic and bacteriologic changes in experimental murine model of bacteremic *Staphylococcus aureus* infection', *Infection and Immunity*, *65*(9): 3889– 3895.
17. Absolom, D.R. (1986). 'Basic methods for the study of phagocytosis', *Methods in Enzymol*ogy, 132: 95–180.
18. Watanabe, I., Ichiki, M., Shiratsuchi, A. and Nakanishi, Y. (2007). 'TLR2-mediated survival of *Staphylococcus aureus* in macrophages: a novel bacterial strategy against host innate immunity', *Journal of Immunol*ogy, 178(8): 4917–4925.
19. Alves-Filho, J.C., Freitas, A., Souto, F.O., Spiller, F., Paula-Neto, H., Silva, J.S et al.(2009). 'Regulation of chemokine receptor by Toll-like receptor 2 is critical to neutrophil migration and resistance to polymicrobial sepsis', *Proc Natl Acad Sci USA*, 106(10): 4018–4023.
20. Sekhar, S., Sampath-Kumara, K.K., Niranjana, S.R. and Prakash, H.S. (2015). 'Attenuation of reactive oxygen/nitrogen species with suppression of inducible nitric oxide synthase expression in RAW 264.7 macrophages by bark extract of *Buchanania*

lanzan', *Pharmacognosy magazine*, 11(42): 283–291.
21. Sherry, B., Dai, W.W., Lesser, M.L. and Trachtman, H. (2008). 'Dysregulated chemokine receptor expression and chemokine-mediated cell trafficking in pediatric patients with ESRD', *Clin J Am Soc Nephrol*, 3(2): 397–406.
22. Dutta, P., Bishayi, B., Neutralization of TNF-α and IL-1β regulates CXCL8 production through CXCL8/CXCR1 axis in macrophages during *Staphylococcus aureus* infection, *Immunological Investigations*, 50(6): 700–725.
23. Zorov, D.B., Juhaszova, M. and Sollott, S.J. (2006). 'Mitochondrial ROS-induced ROS release: an update and review', *Biochimica et Biophysica Acta*, 1757(5-6): 509– 517.
24. Fournier, B. (2013). 'The function of TLR2 during staphylococcal diseases', *Frontiers in cellular and infection microbiology*, 2: 167.
25. Flannagan, R.S., Heit, B. and Heinrichs, D.E. (2015). Antimicrobial mechanisms of macrophages and the immune evasion strategies of *Staphylococcus aureus*, *Pathogens*, 4(4): 826–868.
26. Doroshenko, T., Chaly, Y., Savitskiy, V., Maslakova, O., Portyanko, A., Gorudko, I *et al*.(2002). 'Phagocytosing neutrophils down-regulate the expression of chemokine receptors CXCR1 and CXCR2', *Blood*, 100(7): 2668–2671.
27. Richardson, R.M., Marjoram, R.J., Barak, L.S. and Snyderman, R. (2003). 'Role of the cytoplasmic tails of CXCR1 and CXCR2 in mediating leukocyte migration, activation, and regulation', *Journal of immunology*, 170(6): 2904–2911.
28. Sorkin, A. and von Zastrow, M. (2009). 'Endocytosis and signalling: intertwining molecular networks', *Nature reviews. Molecular cell biology*, 10(9): 609–622.
29. Khandaker, M.H., Mitchell, G., Xu, L., Andrews, J.D., Singh, R., Leung, H et al.(1999). 'Metalloproteinases are involved in lipopolysaccharide- and tumor necrosis factor-alpha-mediated regulation of CXCR1 and CXCR2 chemokine receptor expression', *Blood*, 93(7): 2173–2185.