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Altered expression of CXCR1 (IL-8R) in macrophages utilizing cell surface TNFR1 and IL-1 receptor during *Staphylococcus aureus* infection

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ABSTRACT

Currently, very few studies are available on the expression of CXCR1 in mouse macrophages having both intact TNFR1 and IL-1R or their deficiency in relation to acute *S. aureus* infection. Peritoneal macrophages from mice neutralized singly for TNFR1or IL-1R, or for both TNFR1 and IL-1R were infected with *S. aureus* in vitro and their ability to secrete cytokines and reactive oxygen species (ROS) were determined. It was observed that the release of TNF-α and IL-1β in response to *S. aureus* infection was decreased in macrophages when both TNFR1 and IL-1R were neutralized. The amount of H_2O_2, superoxide anion, nitric oxide release and bacterial CFU were significantly decreased in TNFR1 plus IL-1R blocked macrophages when compared with macrophages having intact receptors at 60 min of *S. aureus* infection. There was decrement of CXCL8 (IL-8) release and expression of CXCR1 in macrophages during dual receptor (TNFR1 plus IL-1R) blocking prior to stimulation with *S. aureus*. Expression of CXCR1 on murine peritoneal macrophages was evaluated by immunoblots from lysate at 60 min after *S. aureus* infection. It was observed that at 60 min after *S. aureus* infection in murine peritoneal macrophages, the expression of CXCR1 was increased significantly ($p < 0.05$) in comparison to the control groups. CXCR1 expression was decreased significantly ($p < 0.05$) in macrophages pre-incubated separately with *anti*-TNFR1 antibody (10 μg/ml) or IL-1R antagonist protein (240 ng/ml) at 60 min after *S. aureus* infection. However, blocking of both TNFR1 as well as IL-1R in macrophages downregulated the CXCR1expression in comparison to the groups either pre-incubated with *anti*-TNFR1 antibody or IRAP alone.

1. Introduction

Chemokine function is critical for leukocyte movement ranging from the migration required for immune cell development and homeostasis and to the pathologic recruitment of immune cells in inflammatory diseases [1]. CXCL8 (IL-8) an important pro-inflammatory chemokine, identified as neutrophil chemotactic factor, is released chiefly by macrophages [2]. The functional receptors for CXCL8 are identified as CXCR1 and CXCR2 in mammalian systems and are considered for therapeutic targets in several inflammatory diseases [3]. Chemokine receptors are differentially expressed on all leukocytes and regulate inflammation in a G protein-dependent or independent manner [4]. Engagement of pattern recognition receptors (PRR), including toll like receptor 2 (TLR-2) by *Staphylococcus aureus* (*S. aureus*) activates NF-κB and induces CXCL8 which leads to recruitment of macrophages [5]. It has also been demonstrated that interleukin-1β (IL-1β) released from stimulated macrophages facilitates CXCL8 production and aids recruitment of macrophages during bacterial infection [6]. There appears a disparity between human and rodent systems in presence of two functional CXCL8 receptors, CXCR1 and CXCR2 [7,8]. Earlier studies suggested that murine system lack CXCR1 as functional receptor, but fewer studies have reported involvement of CXCR1 during infections [9]. Live *S. aureus* infection of the murine peritoneal macrophages has been shown to increase the expression of CXCR1 [10,11].

The TNF-α and IL-1β have been shown to induce CXCL8 gene expression in macrophages, however, very few studies have been reported on the expression of CXCR1 in macrophages by them during viable *S. aureus* infection [12]. Additionally, production of CXCL8 by *S. aureus* infected leukocytes or with its cellular components have been reported [13,14]. CXCL8 responses to *S. aureus* are dependent on phagocytosis of bacteria indicating internalization of live *S. aureus* through pattern recognition receptors [15]. TNF-α induced regulation of CXCR1 expression in vitro shown that this process activates TNFR1 mediated activation of NF-κB via mitogen-activated protein kinases p38 and *c*-jun *N*-terminal kinases 1 and 2, ultimately leading to CXCL8 production [16]. Tumor necrosis factor receptor 1 (TNFR1) belongs to a superfamily of TNF receptors and plays an important role in innate host defence [17]. Binding of TNF-α toTNFR1 activates the NF-κB and JNK pathways,

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Abbreviations

CPCSEA	Committee for the purpose of control and supervision of experiments on animal
CXC	chemokine which have a single amino acid residue interposed between the first two canonical cysteines
CXCL-8	interleukin-8
CXCR1	CXC chemokine receptor-1
EDTA	ethylenediaminetetraacetic acid
ELR^+	glutamate–leucine–arginine (ELR) motif near the N terminal
FBS	fetal bovine serum
HBSS	Hank's balanced salt solution
IL-8RA	interleukin-8 receptor A
IL-1R	Interleukin 1 receptor
iNOS	inducible nitric oxide synthase
JNK	c jun *N*-terminal kinase
MAPK	mitogen activated protein kinase
MHC-II	major histocompatibility complex-II
$NaNO_3$	sodium nitrate
NaOH	sodium hydroxide
NF-κB	nuclear transcription factor kappa beta
PMSF	phenyl methyl sulfonyl fluoride
RIPA	radio immune precipitation assay buffer
SDS	sodium dodecyl sulphate
TNFR1	Tumor necrosis factor receptor 1

leading to transcriptional activation of genes encoding pro-inflammatory proteins, including CXCL8 [18]. *Staphylococcus aureus* also has been shown to bind to TNFR1 in airway epithelial cells and induce a TNFR1-related signaling pathway, leading to NF-κB activation and CXCL8 production [16] suggesting that secreted pro-inflammatory cytokines like TNF-α and IL-1β induces CXCL8 production in macrophages. However, whether *S. aureus* infected macrophages causes secretion of CXCL-8 using cell surface TNFR1 and/or IL-1R receptor was not reported. Moreover, no reports are available on the regulation of CXCR1 expression in macrophages depending on the presence of both TNFR1 and IL-1R during *S. aureus* infection.

The majority of the inflammatory effects of TNF-α occur through the activation of TNFR1 [19]. The physiological functions of TNF-α in macrophages are numerous; thus, any aberration in its production or signaling in part plays a crucial role in the pathogenesis of many inflammatory diseases [20]. In addition, over-expression of the receptors alone, can spontaneously induce TNFR signaling independent of ligand [21] prompted us to study the expression of CXCR1 when TNFR1 was blocked. However, TNF-*α* interacts with two distinct receptors, TNFR1 and TNFR2, to transduce its biologic effects whereas IL-1*β* signals through IL-1R1 [22]. Ex vivo/in vitro studies have shown that IL-1*β* induced the expression of TNFR1 [23]. Previously, it has been shown that IL-1*β* modulates TNF receptor shedding in vitro, thereby impacting cell responsiveness to TNF-*α* [24]. Since IL-1*β* signals through IL-1R and on the contrary it also regulates the expression of TNFR1, we are interested to figure out whether dual blocking of TNFR1 by antibody and IL-1R by interleukin-1 receptor antagonist (IRAP) could have any impact on the expression of CXCR1 in this study. Pretreatment of cells with IRAP reduced CXCL8 production [25]. Porat et al. demonstrated decreased levels of CXCL8 on day 5 of culture after IRAP pretreatment [26]. De Forge and coworkers [27] addressed the question of whether IRAP would suppress CXCL8 produced by bacterial lipopolysaccharide (LPS)-stimulated human blood monocytes, and found downregulation of CXCL8 by IRAP. Kaplanski and associates [28] verified a similar mechanism of CXCL8 production through IL-1 in activated platelets, and Porat and co-workers [26] showed that *Borrelia burgdorferi* induces CXCL8 by IL-1 induction, which can be regulated by IRAP. Therefore, modulation of CXCR1 in theTNFR1 blocked or IRAP pretreated macrophages during *S. aureus* infection cannot be ruled out.

S. aureus stimulated macrophages induces its high microbicidal function associated with the ability to secrete high amount of proinflammatory cytokines such as IL-1β, TNF-α, and reactive oxygen species (ROS) including superoxide anion [29]. Excessive hydrogen peroxide (H_2O_2) also promotes IκB-kinase activation and drives tyrosine phosphorylation of IκBα, leading to stimulation of NF-κB signaling [30]. Although it was reported that during *Staphylococcus aureus* infection the secreted pro-inflammatory cytokines like TNF-α and IL-1β induces CXCL8 production in macrophages, but whether it utilizes macrophage cell surface TNFR1 and/or IL-1R receptor were not shown. No reports are available on the expression of CXCR1 in macrophages when both TNFR1 and IL-1R were blocked during *S. aureus* infection. Whether expression of CXCR1 and production of CXCL8 were altered in the TNFR1 plus IL-1R blocked macrophages and in macrophages having intact TNFR1 and/or IL-1R after in vitro *S. aureus* infections were determined in this study. Whether TNFR1 alone is sufficient to promote CXCL8 release in response to *S. aureus* infection or also required the presence of IL-1R was evaluated.

2. Materials and methods

2.1. Maintenance of animals

Male Swiss albino mice, 6–8 weeks of age, average body weight 20 ± 4 gm was used for all experiments that had been approved by the Institutional Animal Ethics Committee (IAEC), Department of Physiology, University of Calcutta, [Approval Number: IAEC/IV/Proposal/BB-01/2016 dated 18.07.2016] under the guidance of CPCSEA, Ministry of Environment and Forest, Govt. of India. Upon arrival, mice will be randomized into plastic cages with filter bonnets and saw dust bedding, followed by a one-week quarantine period. Mice were housed 7 per cage with food and water *ad libitum*. Animal holding rooms was maintained at 21–24 °C and 40–60% humidity with a 12-hour light dark cycle.

2.2. Bacterial strain

The *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. Bacteria were adjusted to the desired inoculum spectrophotometrically before infection at Optical density ($(OD)_{620}$ = 0.2 for 5.0×10^6 cells/ml for *S. aureus*) and the colony forming unit (CFU) count of the desired inoculum was confirmed by serial dilution and culture on blood agar [31].

2.3. Isolation of murine peritoneal macrophages

4% sterile thioglycolate broth were injected intraperitoneally to male Swiss albino mice, and the resulting peritoneal exudate was harvested by lavage from the peritoneal cavities of mice with endotoxin-free Hanks' solution 4–5 days later. Peritoneal macrophages were suspended in 0.83% ammonium chloride solution containing 10% (v/v) Tris buffer (pH 7.65) to lyse erythrocytes. 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 plastic surface and the non-adherent cells were removed [32]. As determined by Trypan Blue dye exclusion technique and also by Flow cytometry cell sorting using macrophage specific marker (cd11b), more than 95% of peritoneal macrophages were found viable while doing experiments [33]. For in vitro study, these cells were pooled from a