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A novel CCR-2/TLR-2 triggered signaling in murine peritoneal macrophages intensifies bacterial (*Staphylococcus aureus*) killing by reactive oxygen species through TNF-R1

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ABSTRACT

Macrophages are remarkably versatile in their ability to recognize and respond to a wide range of stimuli by expressing a variety of surface and intracellular receptors and triggering multiple signal transduction pathways. The onset of microbial infection is primarily determined by the initial contacts made by the microbes with the host macrophages. Although there prevail a relationship between the chemokine receptor and Toll like receptors during disease, particularly TLR-2 and CCR-2 signaling interdependence on each other has not been yet investigated during acute staphylococcal infection. Thus, the present study was aimed to trace possible interaction between CCR-2 and TLR-2 in peritoneal macrophages during acute *Staphylococcus aureus* infection. We found that neutralization of CCR-2 attenuates TLR-2 expression and restricts *S. aureus* burden but TLR-2 neutralization augments CCR-2 expression in macrophages, along with compromised host-derived reactive oxygen species production. *S. aureus* infection to CCR-2 intact but TLR-2 neutralized macrophages triggered production of IL-1β, TNF-α, IL-6, IFN-γ, MCP-1 and expression of iNOS, TNFR-1 and GPx with concomitant decrease in IL-10 production. Further, study with NG-monomethyl-L-arginine (L-NMMA) [iNOS blocker] and buthionine sulfoximine (BSO) [GPx blocker] revealed that *S. aureus* infection enhanced TLR-2 expression in CCR-2 intact and TLR-2 neutralized macrophages possibly via iNOS and TNFR-1 up regulation and GPx down regulation. Overall, our data indicate that targeting CCR-2 with neutralizing antibody in the early phase of *S. aureus* infection could restrict excessive inflammation with less compromised bacterial killing. It certainly would be a therapeutic strategy in *S. aureus* induced inflammatory and infective diseases.

1. Introduction

The onset of microbial infection is primarily determined by the initial contact made by the microbes with the host macrophages. This association between microbes and host cell membrane is essentially mediated by interaction between host receptors and their specific ligands exposed on the surface of both the host cell and bacteria. In this regard, particularly chemokine receptors and Toll like receptors have gained tremendous impetus [1]. The selective recruitment of monocytes to sites of inflammation is critical for establishing host defense against infectious agents, with chemokines and chemokine receptors pivotal to orchestrating an effective inflammatory response to TLR activation by pathogens [2]. There is increasing evidence for crosstalk between TLRs and chemokine receptors on cells from the innate immune system, suggesting a coordinated action of these receptors for microbial recognition. Whether such communication provides protective immune response or reflects the ability of microbes to hijack the system remains controversial and may depend on the microbial component considered and also the host receptors involved.

To date, the majority of interest in regulation of monocyte chemokine receptor expression has focused on CCR-2, and it has been shown previously that cytosolic lipopolysaccharide (cLPS) causes internalization and degradation of CCR-2 [3]. It has also been reported that TLR engagement by cLPS decreases steady-state CCR-1 RNA levels in a monocytic cell line [4]. Retrospective studies also demonstrated that CCR-5 and TLR-2 are two significant receptors involved in the early

Abbreviations: CCR 2, C-C chemokines receptor-2; CPCSEA, Committee for the purpose of control and supervision of experiments on animal; EDTA, ethylene diamine tetraacetic acid; ELISA, enzyme linked immune sorbent assay; FBS, foetal bovine serum; IL, interleukin; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor kappa beta; NO, nitric oxide; RIPA, radio immune precipitation assay; ROS, reactive oxygen species; RPMI, Roswell park memorial institute medium; SDS-PAGE, sodium dodecyl sulfate- poly accrylamide gel electrophoresis; SOD, super oxide dismutase; TLR-2, toll like receptor-2

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host parasite interaction during leishmanial pathogenesis and that silencing of TLR-2 and CCR-5 in vitro as well as in vivo have the potential to regulate the initial entry of the leishmania parasites through the induction of pro-inflammatory cytokines like IL-12, IFN-γ and TNF-α along with the subsequent decrease in IL-10 and TGF-β [5]. Moreover, the outcome of TLR-2 activation in response to distinct microbial molecules may be influenced by differential TLR-2 association with accessory receptors, also during *Porphyromonus gingivalis* infection. [6].Therefore, *P. gingivalis* appears to exploit its interaction with CXCR-4 as a mechanism of immune evasion. Furthermore, crosstalk between TLR2 and chemokine receptor pathways can occur in human monocytes within minutes of TLR2 stimulation by LTA from *S aureus*. LTA induces progressive down-modulation of these receptors rather than functional inactivation of CCR1, CCR2, and CCR5 at the cell surface. PLC- and Rac1-mediated events are reported to be involved in this TLR2-dependent cross-regulation of the chemokine receptors and leads to activation of the intracellular machinery used to support chemokine-dependent receptor endocytosis in the case of CCR5 [7]. Though recent finding showed a strong relationship between the chemokine receptor and Toll like receptors during progression of disease [6] but particularly, TLR-2 and CCR-2 signaling interdependence on each other has not been yet investigated during acute staphylococcal infection.

TNF-α is considered to be the key inflammatory cytokine in rheumatoid arthritis and is found in high levels in patients with the disease. Tumor necrosis factor receptor 1 (TNFR-1) belongs to a superfamily of TNF receptors and plays an important role in innate host defense [8]. Binding of TNF- α to TNFR-1 activates the NF-kB and JNK pathways, leading to transcriptional activation of genes encoding pro-inflammatory proteins, including IL-8 [9]. Although activation of TNFR-1 plays a critical role in host defense, pathogenic bacteria have evolved to utilize and subvert this pathway to their advantage in susceptible hosts. Thus, bacteria can also use host TNFRs to mediate pathogenicity. *Staphylococcus aureus* also has been shown to bind to TNFR-1 in airway epithelial cells and induce a TNFR-1 related signaling pathway, leading to NF-κB activation and IL-8 production [10]. A recent study has demonstrated that TNFR-1 is a receptor for protein A from *Staphylococcus aureus*, a pathogen associated with pneumonia and sepsis. Activation of TNFR-1 by protein A induces TNF-like responses which are associated with the pathogenesis of staphylococcal pneumonia [10].

The majority of the deleterious effects of TNF-α is related to the activation of TNFR-1, and includes short term negative inotropic effects and long term TNF-α induced cell death [11]. In contrast, activation of TNFR-2 appears to exert protective effects [12]. TNF-α has also been shown to induce oxidant stress and to cause a drop in glutathione levels, which precedes and regulates its cytotoxic effects [13]. However, the expression levels of TNFR proteins can be regulated by cytokines, especially by interferons which explain in part, the noted synergy between TNF and interferons [14]. Both TNF-α and IFN-γ exhibits a crosstalk at the level of TNFR-1 to induce activation of macrophages. It has been shown that TNF-α induces a stronger activation of NF-κB in the presence of IFN-γ [15].

Activated macrophages can migrate to sites of inflammation, where they encounter pathogens and lyse them. This is accomplished by an increased production of toxic oxygen species and via induction of inducible nitric oxide synthase (iNOS) to produce nitric oxide (NO). In a recent study, it was demonstrated that the control of *Trypanosoma congolense* infection is dependent upon macrophage and neutrophil-derived soluble TNF-α [16]. They also showed that intact TNFR-1 signaling via nitric oxide pathway was essential for this event. Additionally, LPS induced iNOS expression in the liver is, in part, dependent on TNFR signaling in the macrophages [17] but there was no report on the expression of TNFR-1 and iNOS when cell surface receptors especially CCR-2 and TLR-2 were neutralized with either anti CCR-2 antibody or anti TLR-2 antibody during acute staphylococcal infection.

Furthermore, earlier report has shown that Pretreatment with GSH, or another −SH antioxidant compound, N-acetyl cysteine (NAC), resulted in a marked inhibition of TNF production, whereas the inhibitor of GSH synthesis, buthionine sulfoximine (BSO), had an opposite effect [18]. Furthermore, treatment of animals with NAC, a precursor of GSH synthesis and an antioxidant itself, protected against the toxicity of LPS and TNF thus supporting the idea of an oxidant-mediated regulation of LPS and TNF [18]. Treating either animals or cells with BSO induces the expression of a variety of stress-responsive genes, including those for the heat shock protein HSP-32 and metallothionein-1[19,20]. Further, NO directly inactivates glutathione peroxidase (GPx), resulting in an increase in intracellular peroxides, which in turn are responsible for cellular damage or gene expression [21]. GSH depletion pre-disposes the cell due to compromised redox balance [22]. During the enzymatic reaction catalyzed by GPx, GSH is oxidized to GSSG which is reduced back to GSH by glutathione reductase (GRx). Therefore, GRx plays a central role in maintaining the cellular GSH level, protecting cells against ROS mediated injury by detoxification of lipid hydro peroxides formed due to oxidative damage [23,24]. Even though any cell express superoxide dismutase (SOD) in order to provide protection from NO toxicity, the inactivation of GPx may nevertheless occur in certain cells that produce NO or in surrounding cells. This is because other antioxidative enzymes, such as SOD or catalase do not inhibited by NO [21].

Therefore, the study with iNOS blocker NG-monomethyl-L-arginine (L-NMMA), and GSH blocker buthionine sulfoximine (BSO) may better define the interdependency of CCR-2 and TLR-2 crosstalk signaling in response to *S. aureus* infection as opposed to macrophage derived NO and ROS. Upon encounter with micro-organisms or stimulation by cytokines, they produce and release various sets of effector molecules aimed at destroying the foreign agents. These highly diffusible products exert strong cytotoxic activities against micro-organisms. Therefore, these redox stress blocker compounds are widely used to potentiate the combating ability against microbial infection. However, it is not yet clear now whether the combined actions of L-NMMA and BSO would play a prime impact on the intracellular survival of *S. aureus* within peritoneal macrophages.

Therefore, the aims of the study are to characterize specific interdependency in between TLR-2 and CCR-2 signaling in peritoneal macrophages during acute staphylococcal infection and find the possible link to intracellular survival of *S. aureus* and host defense via the involvement of TNFR-1 and other redox signaling pathway. We report here an alternative mechanism by which CCR-2 regulates bacterial killing and TLR-2 induced inflammatory responses and demonstrates the significance of iNOS and GP_x blocker in defining TLR-2 and CCR-2 interdependent mechanism.

2. Materials and methods

2.1. Materials

The antibodies, substrates and molecular weight marker required for Western blot were purchased from Abcam (Cambridge, UK), Biorbyt (Cambridge, UK). The kits for cytokine assays were brought from Raybiotech (Norcross, GA, USA). All other chemicals used for the experiments were of analytical grade.

2.2. 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, under the guidance of CPCSEA [Approval Number: 820/04/ac/CPCSEA dated 26.08.2014], 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 6 per