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Host antioxidant enzymes and TLR-2 neutralization modulate intracellular survival of *Staphylococcus aureus*: Evidence of the effect of redox balance on host pathogen relationship during acute staphylococcal infection

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ABSTRACT

Staphylococcus aureus is an important pathogen in bone disease and innate immune recognition receptor, TLR-2 is reported to be crucial for inflammatory bone loss. Role of TLR-2 in bacterial clearance and cytokine response to *S. aureus* infection in murine bone marrow macrophages has been reported but the role of host derived ROS in host–pathogen relationship still remains an obvious question. In the present study, blocking of SOD and catalase in TLR-2 neutralized fresh bone marrow cells (FBMC) with Diethyldithiocarbamic acid (DDC) and 3-Amino-1,2,4-triazole (ATZ), separately, during acute *S. aureus* infection, produces moderate level of ROS and limits inflammation as compared with only TLR-2 non-neutralized condition and leads to decreased bacterial count compared with only TLR-2 neutralized condition. In summary, host SOD and catalase modulates ROS generation, cytokine levels and TLR-2 expression in FBMCs during acute *S. aureus* infection which might be useful in the alleviation of *S. aureus* infection and bone loss.

1. Introduction

Staphylococcus aureus is a major pathogen in both community-acquired and nosocomial infections. *S. aureus* is capable of causing infections of any organ tissue. These infections may culminate in life-threatening bacteremia [1]. Despite medical advances, the frequency of both community- and hospital-acquired *S. aureus* infections has increased steadily, and the treatment of these infections is becoming even more difficult with the emergence of antibiotic-resistant strains. This increased emergence of antibiotic resistance necessitates the identification of novel therapies that are capable of interfering with the virulence of multidrug-resistant strains of *S. aureus*. While *S. aureus*, generally considered an extra-cellular pathogen, is one such bacterium that has the ability to invade and survive within different cell types, both phagocytic and non-phagocytic cells [2]. By "hiding" inside the host cells, *S. aureus* may elude host defenses and most antibiotic treatments, and may be responsible for chronic and recurrent bone infections by shifting the balance of cell repertoire. Furthermore, *S. aureus* is known to be an important pathogen in bone disease and is responsible for about 70% of cases of osteomyelitis [3] and 80% of cases of joint infections in patients with rheumatoid arthritis and is a common factor in several other bone diseases [4]. *S. aureus* infection of bone is associated with rapid, localized destruction of the tissue. *S. aureus* is a capable bone pathogen, in part, because it possesses several cell surface adhesion molecules that facilitate its binding to bone matrix. Once the bacteria adhere to and colonize bone matrix, they produce several virulence factors such as proteases which can break down matrix components. The resultant bone destruction facilitates bacterial invasiveness. *S. aureus* not only colonizes bone matrix, but is internalized in vitro by osteoblasts (bone-forming cells) isolated from the calvariae of 16-day

Abbreviation: CPCSEA, Committee for the purpose of control and supervision of experiments on animal; ELISA, Enzyme linked immunosorbent assay; LPS, Lipo poly saccharide; LTA, Lipoteichoic acid; NF-kB, Nuclear factor-kappa Beta; RANKL, Receptor activator of nuclear factor kappa Beta ligand; RPMI, Roswell Park Memorial Institute medium; ROS, Reactive oxygen species; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TLR2, Toll like receptor-2; TLRs, Toll like receptors.

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chick embryos [5]. *S. aureus* sequestered from the host immune system in the cytoplasm of the osteoblast may provide a reservoir of bacteria for recurring osteomyelitis [6] and may be more relevant to chronic disease than bacteria associated with the bone matrix.

Protection from primary staphylococcal infection is mainly dependent on innate rather than adaptive immune responses [7]. The innate immune system serves as the first line of defense against microbial infection by initiating pathways that mediate inflammation and pathogen clearance [8]. In the innate immune response, TLRs, which are predominantly expressed in cells involved in inflammatory responses, play pivotal roles in the host defense against microbial pathogens by recognizing pathogen associated molecular patterns (PAMPs) and activating intracellular signaling pathways [9]. There is evidence both in favor and against a role for TLR-2 in host defense against several Gram-positive bacteria, including *S. aureus* [10]. TLR-2 on antigen-presenting cells (APCs) enables these cells to recognize peptidoglycan-embedded lipopeptides and glycopolymers in the *S. aureus* cell wall and mount an inflammatory response to this microbe. TLR-2 signaling can also modulate immunity to *S. aureus* by inducing interleukin-10 (IL-10) responses in APCs [11]. Thus the roles of TLR-2 in innate responses to *S. aureus* are context dependent, and may include detrimental roles in infection outcome [10]. Earlier, we found that infection of macrophages with *S. aureus* resulted in the TLR-2 mediated cytokine production and increase oxidative killing of internalized bacteria which were abrogated by TLR-2 blocking [12]. But, inflammation and bone loss are intimately related. This association is seen clinically in septic arthritis, osteomyelitis, rheumatoid arthritis (RA), as well as in periodontitis. Indeed, in most of these conditions, bone loss starts early in the disease process and is a cause of considerable patient morbidity [13]. Cytokines released from macrophages such as TNF-α, IL-1β and IL-6 have been classically indicated as the major players of the severe inflammation that precedes cartilage and bone destruction during septic arthritis. These molecules stimulate osteoclast differentiation and bone resorption in a synergistic fashion [14]. TNF-α, considered the most osteoclastogenic cytokine, activates NF-kB which in turn is associated with the survival of osteoclasts [15]. However, it is important to highlight that these cytokines are also relevant to protect the host against the infectious agent. Both osteoclast differentiation and function are regulated by the molecular couple RANK (receptor activator of NF-kappa B) and its ligand, RANKL [13]. RANKL is a membrane protein expressed by osteoclast. The binding of RANKL to its receptor RANK leads to recruitment of TNF receptor-associated factor 6 (TRAF6) to the cytoplasmic domain of RANK, thereby resulting in the activation of distinct signaling cascades mediated by mitogen-activated protein (MAP) kinases, including c-Jun N-terminal kinase (JNK), p38 MAP kinase (p38), and extracellular signal-regulated kinase (ERK) [16]. On the other hand, ROS generation in response to bacterial LPS and LTA is crucial for pathogen killing by immune cells [17]. However, sustained production of ROS during immune responses and sepsis can cause damage to macromolecules, cell death and tissue injury [18]. ROS may play its role in bone loss-related diseases by two ways: suppression of born formation and stimulation of bone resorption [19]. In addition, signaling molecules such as JNK and p38, which are known to be essential for osteoclast differentiation [20], are sensitive to activation by ROSs [21].

Earlier, we reported that *S. aureus* exploits host-protective mechanisms to drive excessive inflammatory cytokine production in a TLR-2 dependent manner [12] and the accumulation of macrophages at the site of inflammation plays a critical role in driving inflammatory tissue destruction [22]. In contrast, blocking of TLR-2 in both bone marrow-derived and peritoneal macrophages showed impaired proinflammatory cytokine production and compromised bacterial clearance during acute staphylococcal infection [12].

Although several theories have been put forward, it remains unclear why the host defense response is ineffective in clearing chronic *S. aureus* infections and how the organism contributes to the maintenance of a chronic inflammatory state [23]. It is becoming increasingly apparent that *S. aureus* are able to survive engulfment by macrophages, and that the intracellular environment of these host cells, which is essential to innate host defenses against invading microorganisms, may in fact provide a refuge for staphylococcal survival and dissemination [24]. Arguably, the antioxidant enzyme system is the switch, turning on and off the signaling process. Certain pathogens used TLR-based strategies to evade host defense [25]. This regulation by TLR-2 seemed to be dependent on the host derived ROS particularly from the FBMC. The activation of TLR signaling in FBMC with these infections might promote sustained efforts to develop novel strategy to block these pathways for a variety of bone infections. Taken together, understanding antioxidant switching after induction of TLR signaling in FBMC might be helpful to develop methods of artificial manipulation of TLR signaling to modulate inflammatory diseases, overcome uncontrolled inflammation and make counter measures against *S. aureus* infection particularly in bone marrow cells. Thus, it appears that even though *S. aureus* is recognized by FBMC through TLR-2, whether *S. aureus* may utilize TLR-2 as part of its survival mechanism in FBMC, has not been studied in detail. This makes TLR-2 a very intriguing molecule to study when trying to understand the survival mechanism of *S. aureus* in FBMC as well as in relation to the ROS generated by FBMCs. Therefore, it is reasonable to assume that classical respiratory burst observed in macrophages may be attenuated in some instances, resulting in a signaling process that could induce latency, chronicity or persistence of organisms infecting macrophages. Classically, the elaboration of macrophage effector molecules [ROS, hypochloride and nitric oxide (NO)] early in microbial interaction with macrophages is supposed to kill the invading microbes [26]. It is therefore of interest to determine infection-mediated modulation of ROS signaling from the perspective of antioxidant regulators of ROS. In order to address the fate of *S. aureus* in resident bone marrow cells actively producing ROS, we have investigated the modulation of ROS by antioxidative enzymes, determined if the antioxidant enzyme reactions impact the state and survival of *S. aureus* in resident fresh bone marrow cells and the role of TLR-2 that accounts for the redox modulation in *S. aureus* infected bone marrow cells. We hypothesize that redox balance regulation in macrophages by antioxidant enzymes modulates the state and survival of *S. aureus* in murine fresh bone marrow cells during acute *S. aureus* infection and also harmonize osteoblastic/osteoclastic differentiation.

2. Materials and methods

2.1. Maintenance of animals and cells

All 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-1/2014, dated 26.08.2014], Ministry of Environment and Forest, Govt. of India. Wild type male Swiss albino mice were used throughout the study. To minimize the feeling of hypoxia or discomfort before and during mouse dissection and tissue collection, mice were anaesthetized with inhaling anaesthetics (ether) before terminal surgery. Euthanasia was performed by general anaesthesia followed by vital tissue removal using 2–3% ether for induction and 1% for maintenance. The *S. aureus* (*S. aureus*) strain AG-789 was obtained from Apollo Gleneagles Hospital, Calcutta, West Bengal, India.