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Toll-like receptor 2 and 6 interdependency in the erosive stage of *Staphylococcus aureus* induced septic arthritis mediated by IFN-γ and IL-6 – A possible involvement of IL-17 in the progression of the disease

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ABSTRACT

Staphylococcus aureus induced septic arthritis has emerged as a potent disabling and life threatening disease; hence combating this malady has become an imperative need of medical science. Role of TLR-2 in innate recognition of *S. aureus* and activation of inflammatory cascade by the interplay of some proinflammatory cytokines, resulting in joint inflammation has been established. Variation in the reports suggesting both functional dependency and independency of TLR-2 on its heterodimeric partner TLR-6 in response to ligands exists, thus this study was postulated to observe the expression pattern of TLR-6 in synovial tissue and lymphoid organs after inducing septic arthritis by *S. aureus* in Swiss albino mouse model and the instigated cytokine profile could affirm its plausible role in SA. The functional relation of TLR-2 and 6 was verified by simulating an *in vitro* study design on synovial mononuclear cells, blocking TLR-2 and 6, and it was found that they are required to co-express for generating cytokine, NO and H_2O_2 on infection. IFN-γ, IL-6 and IL-17 were identified to play a distinguished role in SA from their secretion pattern in both *in vivo* and *in vitro* study. IFN-γ and IL-6 remained high throughout the infection possibly by the shift of response from Th1 to Th2 and Th17 and contribute in various converging pathways of inflammation. IL-17 increased with the onset of the disease but reduced on the late period. Hence IFN-γ, IL-6, IL-17 along with TLR-6 can be a potent target for therapeutic approach because of their significant contribution in SA.

Introduction

Septic arthritis has emerged as a potent threat to life and limb, and the ways to counteract it are soon becoming the exigencies of medical science (O'Shea et al. 2003). Among the other pathogens *Staphylococcus aureus* is a crucial causative agent of septic arthritis which causes inflammation of the joints leading to permanent limb disability or fatal outcomes all over the globe (Bukharie 2010; Arnold et al. 2006). On invasion of *S. aureus*, the host body implicates many factors for the innate detection of *Staphylococcal* components among which some members of the Toll-like receptor (TLR) family play the cardinal role (Fournier and Philpott 2005; Hultgren et al. 1998). These are single transmembrane cell-surface pattern recognition receptors found on immune cells such as macrophages, lymphocytes, mast cells, dendritic cells and are activated by molecules which are highly specific towards evolutionary conserved entity on microbes, leading to a downstream inflammatory signaling pathway. Hence characterizing the differential expression of such receptors in different strains and organs in septic arthritic model may give an insight of their role in the onset of the disease and how they can be targeted for therapeutic applications in near future.

Till date TLR-2 seems to be the most profligate member of the TLR family as it has the ability to recognize the most diverse set of pathogen associated molecular patterns (PAMPs) alone or in association with TLR-1 and TLR-6. Its proinflammatory and catabolic role

Abbreviations: CPCSEA, Committee for the Purpose of Control and Supervision of Experiments on Animals; CRP, C-reactive proteins; DPI, days post-infection; DST-PURSE, Department of Science and Technology-Promotion of University Research and Scientific Excellence; FLS, fibroblast-like synoviocytes; GPI, glucose-6-phosphate isomerase; IFN, interferon; IL, interleukin; LP, lipoproteins; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MALP-2, macrophage-activating lipopeptide-2; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; Pam2CSK4, palmitoyl-2-Cys-Ser-Lys-4; PAMPs, pathogen associated molecular patterns; PGN, peptidoglycan; RA, rheumatoid arthritis; SA, septic arthritis; sBLP, synthetic bacterial lipopeptide; SF, synovial fibroblast; TLR, Toll-like receptor; TNF, tumor necrosis factor.

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mediated by the NF-κB pathway in septic arthritis has also been explored (Papathanasiou et al. 2011). The TLRs generally exist as homodimers except TLR-2/6 and TLR-2/1 who act as heterodimers. Prior reports suggested that the expression of both TLR-2 and 6 is higher in septic arthritic chondrocytes compared to control. TLR-2 was blocked in order to investigate its crucial mediation in the proinflammatory cytokine production and it was identified to be a potent target for modulation in therapeutic strategies. However in this study TLR-6 was not blocked to find whether it is indispensible for the initiation of the inflammatory cascade along with TLR-2 (Papathanasiou et al. 2011). Moreover in other arthritis like rheumatoid arthritis (RA), TLR-2 was found to be involved (Seibl et al. 2003) whose expression increase in the synovial fibroblast (SF) of RA patients after treatment with IL-1β, TNF-α, LPS and SBLP and this elevated expression of TLR-2 was assumed to be a consequence of direct exposure to microbial compounds or of the inflammatory mediators in the joints. TLR associated signaling pathways has been found to contribute to the pathogenesis of RA either by initiating or perpetuating activation of SFs. Other than TLR-2, TLR-4 is involved in inflammatory and joint destructive pathways in collagen-induced arthritis in DBAIJ mice (Pierer et al. 2011). Young et al. in the year 2009 have demonstrated synergism of TLR-2, 4 and 6 ligation on the production of TNF-α in a spontaneous arthritic animal model of IL-1 receptor antagonist deficient mice (Jung et al. 2009). Zymogen when used as a specific ligand for TLR-6 was found to be involved in the production of TNF-α from the Fibroblast-like synoviocytes (FLS), suggesting their role in the perpetuation of spontaneous arthritis. In another study in 2005 it was exhibited that the expression of TLR-3 and 7 in rheumatoid arthritis synovium increase and costimulation of TLR-3, 4 and 7/8 results in synergistic cytokine production by dendritic cells (Roelofs et al. 2005). Even the role of some endosomal TLRs has been explored but till date no reports on the direct contribution of TLR-6 in septic arthritis in Swiss albino strain of mice has been found. Studies on *S. aureus* induced SA has been conducted in our laboratory before and anti oxidant and antibiotic treatments have been suggested to ameliorate the inflammation, but the direct interaction of *S. aureus* with specific TLRs and their contribution in the inflammation still were left for investigations.

However as already discussed, TLR-2 is known to form functional pairs with 6 and 1 to discriminate among large number of pathogen associated molecular patterns and literature on its interaction with *S. aureus* was explored as that was the concern of this study. In 2001, Hajjar et al. confirmed that TLR-2 transduce the response to phenol soluble modulin which is enhanced by TLR-6 but inhibited by TLR-1, indicating a functional interaction between the former pair in innate recognition of pathogens with the above mentioned structural feature (Hajjar et al. 2001). As *Staphylococcus epidermidis* is close to the structure of *S. aureus*, similar functional interaction could be hypothesized to take place between TLR-2 and 6 in the presence of *S. aureus.* It is already established beyond any confusion that TLR-2 is the most potent receptor for recognizing *S. aureus*, and that the heterodimeric partner may be TLR-6 is also hinted to some extent (Takeuchi et al. 2000a,b; Kopp and Medzhitov 2003). *Staphylococcal* lipoproteins, Panton-Valentine toxin and Phenol soluble modulins have been ascertained as potent TLR-2 ligands but the ligand function attributed to peptidoglycan (PGN) and Lipoteichoic Acid (LTA) remained controversial and could be ascribed to the heteromeric partner TLR-6 or TLR-1. Although not of live *S. aureus*, but some reports directly stated the involvement of TLR-6 in recognition of endogenous ligands which are mostly pattern recognition proteins of *S. aureus* in other species as well. Nakao et al. in 2005 stated that surface expressed TLR-6 participates in the recognition of diacylated LP and PGN in human cells (Nakao et al. 2005). In similar contemporary studies TLR-6 has been shown to form a heterodimer with TLR-2 while recognizing diacyl lipopeptides MALP2, zymosan, lipoteichoic acid and lipopeptides derived from mycoplasma, then activating the NF-κB signaling cascade in synchrony with TLR-2 (Okusawa et al. 2004; Takeuchi et al. 2000a,b). Nonetheless there are variations in this regard as whether or to what extent they co-express on incursion of various pathogens including *S. aureus*. Another uncertainty prevailed regarding the structural variation of LP and its interaction with TLR-2/6 heterodimer. The fact that TLR-6 could recognize bacterial diacylated lipopeptides in association with TLR-2 but could not participate in cytokine production in response to triacylated lipopeptides which was conducted by TLR-2/1 heteromer was also emphasized. Furthermore identification of ligands like Pam2CSK4 as well as MALP2-SK4 which were reported to be recognized by TLR-2 in a TLR-6-independent manner challenged a new debate questioning the role of TLR-6 in the functional recognition of diacyl LP as well (Buwitt-Beckmann et al. 2005). But later it was justified that distinct lipopeptides could be recognized by TLR-2 even in a TLR-1 and TLR-6 independent manner (Buwitt-Beckmann et al. 2006) indicating that in native cells, TLR-2 might be able to signal as homomers under certain circumstances. In the midst of these contradictions an interesting report from Grabiec et al. in 2004 suggested that both human and murine cells shows a species-specific difference in the TLR-2-dependent recognition of distinct LP structures (Grabiec et al. 2004) and as new strains are generated and others become extinct it is useful to review and characterize immunological aspects periodically of what strains are available and how are they related to the others (Whitehead and Crawford 2006) for a better approach of therapeutic strategies at genetic level.

Regarding the structure of *Staphylococcal* lipoproteins also, a variation in the opinion exist. Some suggest that it is triacylated which somehow underrate the contribution of TLR-6 in the commencement of septic arthritis, contemplating with the previous reports (Asanuma et al. 2011). However others identified TLR-2 activating lipoproteins from *S. aureus* cells and characterized the N-terminal lipopeptides structure of a lipoprotein as a diacylated one (Tawaratsumida et al. 2009). It was argued that because these lipoproteins devote to the virulence of *S. aureus*, hence studies on protein expression both of ligand and receptor should be carried out to resolve their immunobiological properties. Jin Young Kang et al. explored the crystal structure of TLR-2/6 heteromer and identified that the lipid channel of TLR-6 is blocked by two phenylalanines and sometimes simultaneous mutation of these phenylalanines takes place which makes TLR2–TLR6 fully responsive not only to diacylated but also to triacylated lipopeptides. These facts reestablish the need to explore the expression pattern of TLR-6 on *S. aureus* infection and whether its presence is conspicuous or insignificant in the recognition of the ligand (Kang et al. 2009).

S. aureus enters the body of the host by direct inoculation or by contiguous spread from other infections, then through hematogenous route they enter the joints and "home" there because of the lack of a limiting basement plate in synovial tissues, causing an acute inflammatory response (Smith and Piercy 1995). Cytokine profile on inflammation give an insight of the functionality of the receptor involved. Cytokines release from mononuclear cells and macrophages such as TNF-α, IL-1β, IL-6 and IFN-γ play major roles in severe inflammation that precedes cartilage and bone destruction during septic arthritis. The receptor signal gets conducted in sequence *via* a myeloid differentiation marker (MyD88), IL-1R-associated kinase, TNFR-associated factor 6, and TGF-β-activated kinase, leading to nuclear translocation of NF-κB activating factor (Kwan et al. 2004). IL-17 has been a well-established mediator of rheumatoid arthritis in both, humans and mice (Nakae et al. 2003) but its role in *S. aureus*-triggered septic arthritis is largely unknown. Among the proinflammatory mediators TNF-α play an important role in the pathogenesis of septic arthritis (Tarkowski et al. 2002;

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