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Presence of Toll Like Receptor-2 in spleen, lymph node and thymus of Swiss albino mice and its modulation by *Staphylococcus aureus* and bacterial lipopolysaccharide

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Toll-like receptors (TLR) are a family of pattern recognition receptors identifying pathogen associated molecular patterns (PAMPs). They play a critical role in the innate immune response during the initial interaction between the infecting microorganism and phagocytic cells. Here, we verified the presence of TLR-2 in spleen, lymph node and thymus of Swiss albino mice and their modulation after infection with *Staphylococcus aureus* and Lipopolysaccharide (LPS) challenge. It was seen that TLR-2 gene transcribed to its respective mRNA on *S. aureus* infection, in thymus, spleen and lymph node of mice but their levels and mode of expression varied. When challenged with LPS no prominent changes in the expression of TLR-2 receptor was observed but its expression increased gradually with time in the thymus, spleen and lymph node of *S. aureus* infected mice. TLR-2 expression was also found enhanced in infected splenic macrophages. By studying the serum cytokine profile the functionality of the receptor was measured. The results indicate the presence of TLR-2 in thymus, spleen and lymph node of Swiss albino strain of mice and that they are modulated by *S. aureus*.

Keywords: Cytokine, Expression, LPS, Splenic macrophages, Swiss albino mice, TLR-2.

Toll like receptors (TLRs) are pattern recognition receptors which bind microbial signature molecules and triggers innate and adaptive immune responses upon stimulation with their specific ligands[1]. TLRs play an important role in bridging of the innate and adaptive immune responses against infection[2,3]. Their activation not only leads to the induction of inflammatory responses but also to the development of antigen specific adaptive immunity. The first characterization of mammalian TLR dates back to 1997 and so far fourteen TLRs have been reported in vertebrate species, 10 in humans and 13 in mice[4]. TLRs are differentially expressed and regulated in many tissues and cell types[5-7]. Dr. Jules Hoffman and Dr. Bruce A. Beutler's discovery of the role of TLR in innate immunity earned them Nobel Prize in 2011[8-9]. Their work provided an insight into the pathogenesis of inflammatory diseases and the deleterious sequel associated with lymphoid organ inflammation.

Previous studies have indicated abundant expression of all known TLRs in phagocytes[5-7]. However, there has been no report so far on the expression of TLR-2 in Swiss albino mice model and its role in innate immunity. Moreover, it has been established as a receptor of gram positive bacteria including *S. aureus* and a pro-inflammatory and catabolic role of TLR-2 mediated by NF-κB pathway in septic arthritis has been suggested in previous studies[10]. As we have already explored some septic arthritis mice model induced by *S. aureus*, previously in our laboratory, we tried to correlate the modulation of TLR-2 expression after *S. aureus* infection with our previous findings in our current study. TLR-2 expression has been reported in lymphoid tissues such as spleen, lymph node, thymus and bone marrow of C3H/HeN, KM, C57BL/6 mice[11-14] except Swiss albino mice. It is predominantly expressed by monocytes, macrophages and neutrophils in C57BL/6J, C3H/OuJ and BALB/c strain of mice[15-17]. Although various *in vivo* studies by immuno histochemical staining and *in situ* hybridization have revealed the expression of TLR-2, its role *in vivo* has not been fully elucidated[18-19].

TLR-2 has been identified as the receptor responsible for immune recognition of the Gram positive bacteria *S. aureus* as well as Gram positive

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bacterial component lipoteichoic acid (LTA) and peptidoglycan (PGN) which are potential virulence factors[20-22]. *Staphylococcus aureus* can colonize the host's nostrils, upper respiratory tract, and skin by successful immune evasion strategies. It causes a variety of diseases ranging from minor skin infections to severe systemic complications such as bacteremia and septic shock, often with fatal results[23]. Bacterial pathogenicity in the case of *S. aureus* is multifactorial since a single virulence factor is not sufficient to cause a staphylococcal infection[24,25]. Among several innate immunity receptors implicated in host defense against *S. aureus*, TLR-2 has been reported as the transmembrane component involved in the identification of *Staphylococcal* LTA and phenol-soluble modulin leading to the synthesis of inflammatory cytokines by monocytes/macrophages. TLR-2 plays a significant role in systemic infection with *S. aureus*, because TLR-2 knockout but not wild type succumbs to infection within the first week after challenge. This enhanced susceptibility is correlated with much higher bacterial loads in kidney and blood compared to wild type mice C57BL/6[26]. Researchers have already demonstrated that TLR-2 is required for protection of mice from systemic *S. aureus* challenge[27-29,] and also the quantitative expression of TLR-2 can influence the disease outcome of infection[30]. For example TLR-2 deficient patient are significantly more susceptible to pneumococcal infection[31]. In order to investigate the role of TLRs in the disease, normal levels of expression in different tissues and cells must be assessed accurately. Further study was suggested to clarify the tissue distribution of each TLR[7,32].

Previous report suggests that Gram negative bacterial component lipopolysaccharide (LPS) also acts as a ligand of TLR-2 under certain circumstances[33]. There is also lack of distinction between the ligands of TLR-4 and TLR-2 which suggests that the TLRs can replace each other under certain circumstances. Direct comparison of TLR activation in different species, and even between species with a high accessibility of reagents like mouse (particularly in Swiss albino mice) or human is not reported much in literature. Expression of distinct set of TLR-2 and differences in reactivity to microbial molecules in BALB/c and C57BL/6 mice have been reported earlier[34].

In the present study, we investigated the basic expression patterns of TLR-2 in thymus, spleen and lymph node and isolated splenic macrophages of control and *S. aureus* infected Swiss albino mice and also studied its functional activity when encountered with the specific ligands.

Materials and Methods

Materials—The LPS (from *E. coli* O55: B5) was bought from Sigma Chemicals. The antibodies and substrate required for Western Blot were purchased from Abcam, and the ELISA kits for cytokine assays were purchased from RayBiotech, Inc. All other chemicals were of analytical grade.

Animals—Male Swiss albino mice, 6-8 weeks of age with body weight 20±4 g were obtained from Chittaranjan National Cancer Institute, Kolkata and immediately randomized in plastic cages with filter bonnets and saw dust bedding. Six mice were housed per cage with food and water *ad libtum* and were kept in quarantine for 8 days. Animals were maintained throughout at a temperature of 21-24 °C, 40-60% humidity and a 12 h L:D cycle. Animals were fed with normal rodent diets. All experiments performed in this study were approved by the Institutional Animal Ethical Committee (IAEC) [Registration Number: 820/GO/AC/04/CPCSEA) of the Department of Physiology, University of Calcutta] as per guidelines of the CPCSEA, Ministry of Environment and Forests, Govt. of India.

Preparation of bacteria and culture conditions—The coagulase positive *S. aureus* isolate (P-1145) was obtained from Calcutta Medical College and Hospital, Kolkata and was maintained in our laboratory. As certified by the hospital, this strain was obtained from the blood of an adult male patient suffering from septic arthritis.

Characterization of bacterial strain—Generation time of the bacterial isolate was determined and antibiotic profiling of the strain was done by Minimum Inhibitory Concentration (MIC)[35] and Disk Agar Diffusion (DAD) method[36] for the antibiotics studied earlier on different other strains of *S. aureus* in our laboratory.

Treatment of mice with bacterial LPS and infection with S. aureus—The bacterial cells were obtained from log phase of its growth and mice were infected intravenously (I.V.) in the tail vein under sterile condition with effective dose (5×10^6 cells/ml) as done earlier[37]. Another set of mice was challenged intraperitonialy (I.P.) with LPS (5µg/mouse)[38]. Control mice were injected only with sterile saline. The mice infected with *S. aureus* were sacrificed on

3rd, 9th and 15th days of post infection (dpi)[39] whereas all mice challenged with LPS were sacrificed 24 h after treatment. Mice were euthanized painlessly as per guidelines.

Determination of number of viable S. aureus in blood—Blood (0.5 ml) was obtained on day 3, 9 and 15 after *S. aureus* infection before sacrifice by retro-orbital sinus bleeding at specific intervals. Blood from each infected mice were diluted, plated in triplicate on nutrient agar and incubated overnight at 37 °C and the number of colony forming unit (CFU) per ml were counted.

Collection of tissue, *blood and preparation of Serum*—After the animals were sacrificed blood, spleen, thymus and lymph nodes were collected aseptically. The tissues were store at −80 °C until used.

Blood collection and preparation of serum—Blood samples were collected by venipuncture into 1-ml sterile syringes and were separated by centrifugation at 1200 × *g* for 20 min at 4 °C. Separated serum samples were aliquoted and stored at −80 °C for quantification of produced cytokines.

DNA extraction from spleen thymus and lymph nodes—Portion of the tissues were lysed in the lysis buffer and incubated overnight in shaking water bath at 55 °C. The supernatant from tissues were extracted by spinning the tubes at 10-13000 rpm for 5 min. DNA was made to precipitate with chloroform-isopropanol. The DNA mass was fished out with the tip of a glass pipette and dipped into 70% ethanol. After washing with ethanol the pellet was allowed to air dry for at least 10 min. The dry pellet was then dissolved in 200 µl of de-ionized filter water and after estimating the concentration of DNA according to A_{260} values, it was dissolved in Tris EDTA buffer for long term storage.

PCR amplification and agarose gel electro phoresis—The specific primers for TLR-2 were as follows: forward: 5′-AAGAGGAAGCCCAAGAA AGC-3′, reverse: 5′-CGATGGAATCGATGATGT TG-3′. PCR was performed in a total volume of 25 µl containing 20-90 ng/µl DNA, 1X PCR buffer, 3 mM $MgCl_2$, 200 µM dNTPs, 20 pmol primers and 1.25 IU Taq polymerase. An initial cycle at 94 °C for 4 min was followed by 35 cycles at 95 °C for 45 s, 54 °C for 45 s and 72 °C for 1 min. Final extension was performed at 72 °C for 10 min. PCR was performed in Biorad MJ-mini thermocycler. After amplification PCR products were visualized on a 2% agarose gel stained with ethidium bromide by electrophoresis.

Reverse transcriptase (*RT*)-*PCR*—Total RNA was isolated using the standard TRIzol method (Gibco BRL). One µg of total RNA was used to reverse transcribe into cDNA by One step Access RT-PCR kit (Promega, Madison, WI), followed by the amplification of the gene of interest using gene specific primers for TLR-2 and GAPDH. PCR was performed after AMV RT inactivation and RNA/cDNA/primer denaturation for 4 min at 94 °C, and repeating the cycles at 94, 55, and 72 °C successively. Amplified products were separated by agarose gel electrophoresis (2%) and visualized by ethidium bromide staining. The primer sequence and product size have already been mentioned above. Here, GAPDH was used as reference gene for quantitative real time RT-PCR as certain appropriate genes were chosen and used as housekeeping genes for accurate quantitative RNA expression in real time RT-PCR technique. The expression levels of reference genes remain constant between the cells of different tissues and under different experimental conditions[40].

Isolation of Splenic macrophages—Spleens were excised from killed mice and immediately placed in Alsever's solution and then macerated using frosted glass slides. Cells were repeatedly aspirated with a sterile Pasteur pipette until a single cell suspension was obtained and transferred to sterile tubes, and kept in ice for cell debris to settle. The supernatant was layered over 3 ml Histopaque 1077 (Sigma, USA), centrifuged at 1500 rpm for 30 min[41] and the band of leukocyte enriched fraction at the interface was collected and washed with DPBS. The cell pellet was re-suspended in RPMI-1640 containing 20 mM HEPES (pH 7.2), 1 mg/ml BSA and were allowed to adhere on plastic surface for 1 h in 37 °C incubator. The non adherent cells were removed and adherent cells were collected by aspiration with Pasteur pipette. Cells were then washed and finally re-suspended in culture media (RPMI+BSA) at a density of 10^6/ml. More than 95 % cells were found viable as determined by Trypan Blue dye exclusion technique[42].

Infection of murine splenic macrophages with S. aureus (*in-vitro*)—Equal volumes of a suspension of 10^7 macrophages /ml and 10^7 bacteria /ml in Hank's balanced salt solution (HBSS)-gelatin were incubated for 1 and 2 h at 37 °C, respectively under

slow rotation. The non–ingested bacteria were removed by differential centrifugation for 4 min at 110 × g and two washes at 4 °C. Intracellular killing was terminated by transferring the tubes in crushed ice and spinning the cells at 4 °C. The macrophages were disrupted with 9 mM CHAPS in DPBS. A cocktail of protease inhibitors containing PMSF (200 µM), leupeptin (1 µM), pepstatin (1 µM), EDTA (100 µM) was added to each tube. After keeping 10 min in ice, the cells were vortexed vigorously, centrifuged for 15 min at 10000 × g at 4 °C, and the cell free extract of macrophages was resolved in 10% SDS-PAGE under reducing condition[43].

Western Blot—Western blot analysis of spleen, thymus and Lymph node TLR-2 expression was performed by standard methods. Briefly, whole tissue collected from mice were lysed with RIPA-NP40 and 60 µg of the tissue lysates were separated on an 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel and blotted onto nitrocellulose membrane. The membranes were blocked with 5 % skim milk in TBST for 3 h at room temperature, washed and incubated with primary anti-mice TLR-2 Abs in 1/1000 dilution (cat no-ab 24192, Abcam, UK) overnight at 4 °C. The membranes were washed with TBST and incubated with the appropriate HRP-conjugated secondary antibody in 1/5000 dilution (cat no-ab 6721, Abcam, UK) for 1 h at room temperature. Detection of antigen was performed using the enhanced chemiluminescent detection method (ECL-plus cat no-ab140357 Abcam, UK)[44]. We have used Beta-tubulin as loading control for western blot to ensure equal loading throughout the gel. Betatubulin is a housekeeping gene that exhibited high-level constitutive expression in the sample we were examining. Moreover, it has a different molecular weight than our protein of interest *i.e*., TLR-2, to help distinguish between both the bands.

Quantification of cytokine production—Sandwich ELISA was used to determine cytokine concentrations from serum. The levels of pro inflammatory cytokines TNF-α, IL-6 and IFN-γ along with anti inflammatory cytokine IL-10 were measured as per manufacturer's guidelines of RayBiotech, Inc, USA in a BioRad ELISA Reader.

Results

Characterization of S. aureus strain —The hospital strain of *S. aureus* (P-1145) showed doubling time at 53 min and after studying the antibiotic profile it was found to be sensitive to oxacillin, vancomycin and chloramphenicol (Table. 1).

Recovery of bacteria from blood after single in vivo injection of S. aureus (*P-1145*) *at 3*, *9* & *15 dpi*—Three dpi blood had significant growth of *S. aureus* (P <0.05) with 950 ± 35.20 bacterial colonies followed by 9 dpi (290 ±14.14). No detectable amount of bacteria was found in blood at 15 dpi.

TLR-2 gene in thymus, spleen and lymph node—Presence of TLR 2 gene in the DNA from thymus (Fig. 1a), spleen (Fig. 1b) and lymph node (Fig. 1c) of control, *S. aureus* infected male Swiss albino mice at 3, 9, 15 dpi, and LPS challenged mice at 24 h was demonstrated by agarose gel electrophoresis.

TLR-2 mRNA expression in thymus spleen and lymph node—However, from the DNA analysis it could not be ascertained whether any change in the expression of TLR-2 receptor takes place in the tissues when confronted with specific ligand. Hence, expression of TLR-2 receptor was detected from the tissues of control and *S. aureus* infected mice at mRNA level. It was seen that TLR-2 gene transcribed to its respective mRNA in thymus, spleen and lymph

Table 1—Antimicrobial susceptibility testing of *S. aureus* (P-1145) by estimating Minimum Inhibitory Concentration and zone diameter

Antibiotics	MIC (µg/ml)	Zone Diameter (mm)	Zone Diameter Standards (mm)	Interpretation
Oxacillin	0.97	36.6	18-24	S
Vancomycin	1.95	21.0	17-21	S
Chloramphenicol	0.48	38.5	19-26	S

S- Sensitive

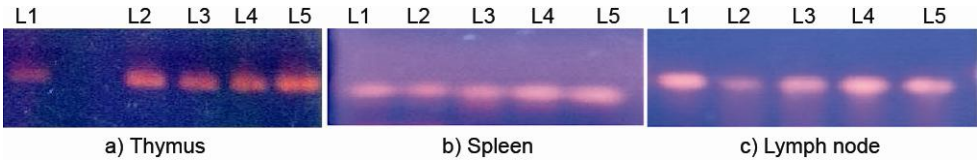

Fig. 1—Presence of TLR-2 gene in the DNA recovered from (a) thymus; (b) spleen; and (c) lymph node of control and treated mice. PCR products of DNA isolated from tissues were transferred in agarose gel to show the presence of the gene in different lanes for different time post treatment. Lane 1, TLR-2 gene from control mice; Lanes 2-4, TLR-2 gene from *S. aureus* (P-1145) infected mice at 3, 9 and 15 dpi; Lane 5, TLR-2 gene from LPS challanged mice after 24 h.

node of male Swiss albino mice but their level and mode of expression varied (Fig. 2). In thymus, TLR-2 expression was not detected prominently until 9 dpi with *S. aureus* (Fig. 2a). In case of spleen, TLR-2 expression increased at 3 dpi as compared to control and gradually decreased at 9 dpi but retained its rise on 15th day (Fig. 2b). Lymph node of control mice showed low levels of TLR-2 and their expression declined on 3 and 9 dpi. The mRNA expression was again increased on 15 dpi with *S. aureus*. (Fig. 2c). The TLR-2 mRNA was most prominent at 15 dpi in all the tissues.

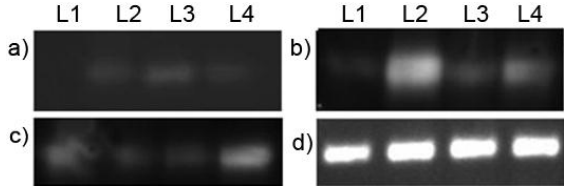

Fig. 2— Presence of TLR-2 mRNA in (a) thymus; (b) spleen; (c) lymph node; and (d) GAPDH of control and *S. aureus* (P-1145) infected Swiss albino mice. Lane 1, TLR-2 from control mice; Lanes 2-4, TLR 2 from *S. aureus* infected mice at 3, 9 and 15 dpi..

TLR-2 receptor expression by immunoblot

Effect of LPS administration on TLR-2 receptor expression in thymus, spleen, and lymph node— TLR-2 receptor protein expression in spleen (Fig. 3a) and lymph node (Fig. 3c) collected from LPS challenged male Swiss albino mice, did not show any significant change 24 h post treatment as compared to control. The fold change of TLR-2 expression in spleen and lymph node are shown in (Figs. 3b and 3d), respectively. However, no band was detected in thymus.

S. aureus infection modulates TLR-2 receptor expression in thymus, spleen and lymph node—In case of thymus, the expression of TLR-2 receptor protein was negligible in control mice as well as after *S. aureus* infection at 3 and 9 dpi, but it was noticed significantly at 15 dpi (Fig. 4a). The expression of TLR-2 increased significantly (P <0.05) in the spleen of *S. aureus* infected mice at 3, 9 and 15 dpi (Fig. 4c) with respect to control. Similarly, in lymph node a

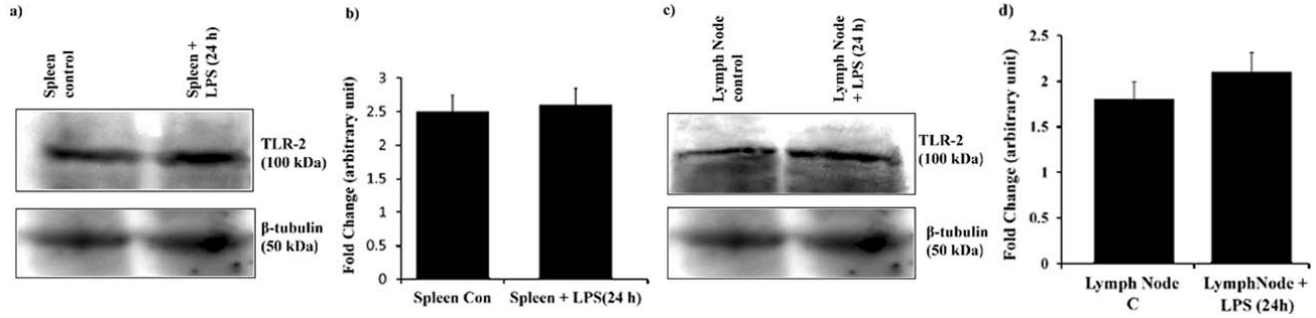

Fig. 3— Presence of TLR-2 in (a) spleen and (c) lymph node of control and LPS challanged mice. No significant change was observed in the expression of TLR-2 after 24 h of LPS administration (P <0.05). Lane 1, TLR-2 in control mice; Lane 2, TLR-2 in LPS challanged mice after 24 h. Figs. 3b and 3d shows the fold change of TLR-2 in spleen and lymph node, respectively.

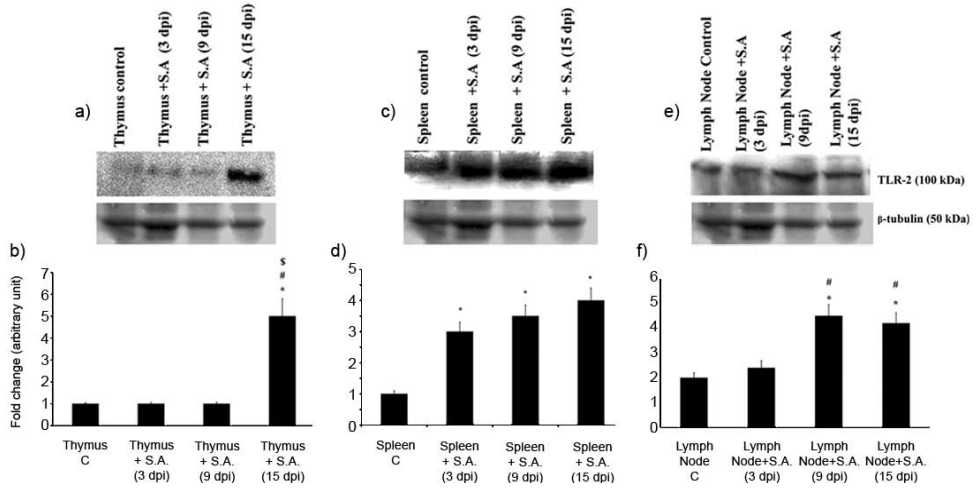

Fig. 4— Presence of TLR-2 in (a) thymus (c) spleen and (e) lymph nodes of control and *S. aureus* (P-1145) infected mice. Lane 1, Control, Lanes 2-4, TLR-2 from *S. aureus* infected mice at 3, 9 and 15 dpi. Figs. 4b, 4d and 4f shows the fold change of TLR-2 in thymus, spleen and lymph node, respectively. *without infection control *vs S. aureus* infection, $^{\#}$*S. aureus* infection at 3 dpi *vs* 9 and 15 dpi, $^{\$}$*S. aureus* infection at 9 dpi *vs* 15 dpi. Level of significance (P <0.05).

significant enhancement in the TLR-2 expression was found at 3, 9 and 15 dpi compared to control (Fig. 4e). The fold change in TLR-2 expression in thymus, spleen and lymph node are shown in (Figs. 4b, 4d and 4f), respectively.

TLR-2 receptor expression in spleen, thymus and lymph node at 15 dpi with S. aureus—In a comparative study among different lymphoid tissues of Swiss albino mice infected with *S. aureus* at 15 dpi (Fig. 5a), the expression of TLR-2 increased significantly (P <0.05) in the spleen (lane 4), thymus (lane 5), and lymph node (lane 6) of infected mice as compared to control. The expression was maximum in the infected spleen than that of thymus and lymph node. The fold change is depicted in (Fig. 5b).

TLR-2 receptor expression in murine splenic macrophages modulated by S. aureus—Expression of TLR-2 was also observed in uninfected splenic macrophages obtained from Swiss albino mice and BALB/c, used as positive control. After *in vitro* infection with *S. aureus* (P-1145) in Swiss albino splenic macrophages, the TLR-2 expression increased significantly (P <0.05) after 1 and 2 h of infection as compared to control (Fig. 6a). The fold change is depicted in (Fig. 6b).

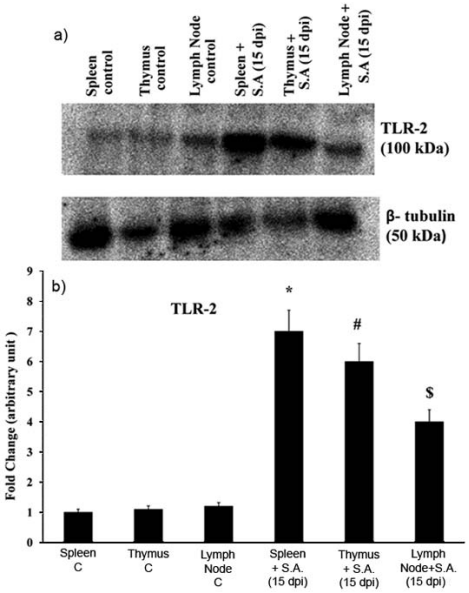

Fig. 5— Presence of TLR-2 in spleen, thymus and lymph node of control and *S. aureus* (P-1145) infected mice at 15 dpi. The expression of TLR-2 was found to increase significantly as compared to control in all the tissues. Lane 1, control spleen, Lane 2, control thymus, Lane 3, control lymph node Lanes 4-6, *S. aureus* infected spleen, thymus, and lymph node at 15 dpi, respectively. (b) depicts the fold change. *Control spleen *vs S. aureus* infected spleen, #Control thymus *vs S. aureus* infected thymus, $Control lymph node *vs S. aureus* infected lymph node. Level of significance (P <0.05).

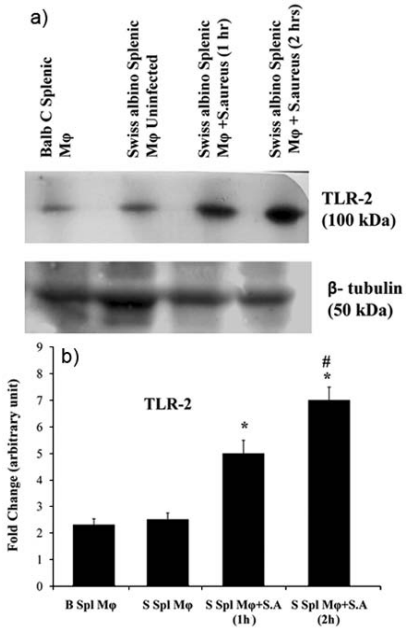

Fig. 6—(a) Presence of TLR-2 in macrophages isolated from spleen of Balb/c and Swiss albino mice after *in-vitro* infection with *S. aureus* (P-1145) at different hours. It was seen that the expression was increased significantly with time after infection. Lane 1, non-infected splenic macrophages of Balb/c (positive control). Lane 2, non-infected splenic macrophages from Swiss Albino mice. Lane 3, splenic macrophages infected with *S. aureus* for 1 hour, Lane 4, splenic macrophages infected with *S. aureus* for 2 h. (b)-depicts the fold change. *Control splenic macrophage from Swiss albino *vs S. aureus* infected, #Infection for 1 h *vs* Infection for 2 h. Level of significance (P <0.05).

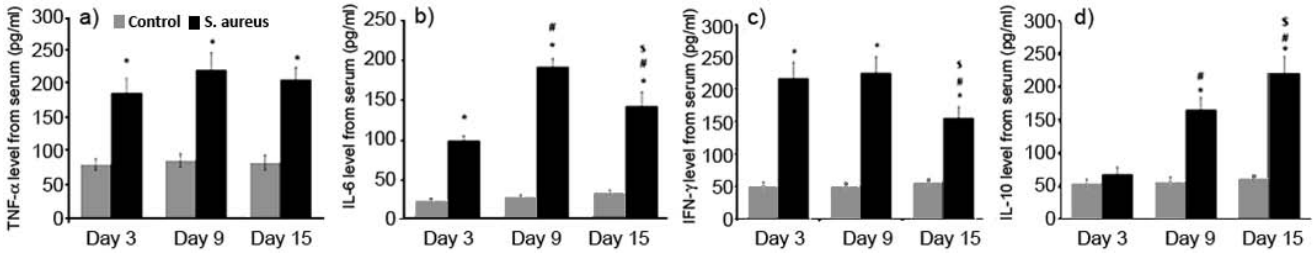

Fig. 7— Serum levels of (a) TNF-α; (b) IL-6; (c) IFN-γ and (d) IL-10 in control and *S. aureus* (P-1145) infected mice at 3, 9 and 15 days post infection. [Values are expressed as Mean±SD and are significant (P <0.05) from 6 mice in each group]. *without infection *vs S. aureus* infected, #infection for 3 days *vs* infection for 9 and 15 days, $infection for 9 days *vs* infection for 15 days. Level of significance (P <0.05).

Alteration of Serum cytokines levels at 3, 9 and 15 dpi—Serum pro inflammatory cytokines TNF-α (Fig. 7a), IL-6 (Fig. 7b) and IFN-γ (Fig. 7c) increased significantly (P <0.05) on infection at 3 dpi as compared to control with a peak at 9 dpi which returned to basal level on 15 dpi. TNF-α showed mild decrease at 15 dpi while IL-6 and IFN-γ showed significant decrease compared to 9 dpi. Anti inflammatory cytokine IL-10 (Fig. 7d) recorded a significant rise at 9 dpi that continued further at 15 dpi.

Discussion

TLR-2 plays a major role against infection by Gram positive bacteria as it binds to a wide variety of microbial products such as peptidoglycan (PGN), lipoteichoic acid (LTA), lipoproteins (LP), lipoarabinomannan and zymogen and gets activated by these pathogen associated molecular pattern (PAMP)s[6]. In mammals, proteins and immune cells which participate in host defense are distributed throughout the body and continuously recirculate in blood and lymph .However, when pathogen enters into the host or if an injury occurs, it is necessary to concentrate them and their products at the site of damage. Cells of the affected tissue and resident immune cells sense pathogens and damage through multiple TLRs that co-operate by activating a cascade of biochemical events. These in turn initiate the inflammatory responses by allowing exudation of plasma proteins and by driving selective extravasation of leucocytes from the blood into the surrounding tissue. TLRs are most extensively studied sensors of damage that participate in the initiation of inflammation.

Staphylococcus aureus has been found to gain a striking ability to acquire resistance to antibiotics, and methicillin resistance represents a growing public health problem in many parts of the world. Methicillin-resistant *S. aureus* (MRSA) has also become important outside the hospital environment as well[45]. In our study, we characterized the strain in terms of their antibiotic profile and generation time, so that a possible remedy or treatment for reducing the bacterial infection by antibiotics could be experimentally extrapolated later in a properly facilitated clinical field.

Previous studies have reported that an intravenous inoculums of 10^7 organism resulted in 90% mortality by day 14 in TLR-2 deficient mice compared to 40% in WT animals[26]. Even a larger inoculums (10^8) resulted in 100% mortality by day 5[46]. Therefore, intravenous administration of live *S. aureus* (5×10^6 cells/mouse) to Swiss albino mice is relevant and also established previously in our laboratory[47]. Previously, in an experimental model of *S. aureus* infection induced septic arthritis, we have reported that a minimum of 3 dpi was a prerequisite for induction of arthritis[47,48] and also found that the *S. aureus* were disseminated from blood to different tissues 3 dpi. We have also studied different parameters like oxidative stress, myeloperoxidase (MPO) enzyme activity, antibiotic treatment which induces the clearance of bacteria[47]. However, TLR-2 being an innate immune receptor and its ability to detect *S. aureus* might also have a role in the reduction of bacterial burden. So in the current study we sacrificed the mice infected with *S. aureus* in the same time interval so that we can coordinate our finding with previous results and understand different mechanisms involved at a time, for clearance of bacteria. On the other hand, lipopolysaccharide (LPS) of Gram negative bacteria being an endotoxin induces the release of pro-inflammatory cytokines much earlier as compared to live *S. aureus*, peaks within hours and return to basal level[49]. Thus, we studied LPS treated mice only for 24 h to verify whether it can stimulate TLR-2 expression in the lymphoid organs studied as reported earlier for LPS.

From colony forming unit (cfu) count as mentioned earlier the number of viable bacteria was found to peak at 3 dpi in blood, decreased at 9 dpi and was hardly detectable at 15 dpi. *S. aureus* being injected via vein can travel by systemic circulation and enter the tissues which might result in an increased bacterial load in tissues. Innate immune cells both in blood and tissues might play a role in the clearance of bacteria and reducing the burden by causing inflammation and triggering adaptive immune response. Thus, we opted to study TLR-2 expression after *S. aureus* infection in the mentioned organs as they could have a possible role in protection against infection. Moreover, since this is a different *S. aureus* isolate and has not been explored for arthritic model as per our earlier report, the baseline study on the induction of TLR-2 by this bacterium may be helpful for further studies in targeting the role of TLR-2 in infection induced arthritis.

The pattern recognition receptor TLR-2 show quantitatively distinct genomic DNA profiles among lymphoid tissues of Swiss albino mice and

transcription of these messengers are expressed as protein within the spleen, lymph node and thymus after *S. aureus* infection. The expression of TLR-2 mRNA was different in different lymphoid tissues with time. These variations in the mRNA level could be due to different transcription capacity, mass of the tissues studied and also on the activation of transcription factors available within the tissue. Moreover, as we challenged the mice with *S. aureus* via intravenous route the blood born bacteria will initially be trapped in the spleen leading to activation of resident cells inside the spleen and initiates transcription at an enhanced rate. Possibly, after 3 days of infection, the bacteria are cleared from the spleen, resulting in decreased mRNA at 9 and 15 dpi[47]. It is likely that TLR expression profile of individual lymphoid tissue reflects the most likely pathogen (PAMP) burden of each lymphoid tissue and its relative preparation for pathogen challenge. In addition, it may also represent the actual steady state "PAMP load" including endogenous PAMPs (such as HSP) that may reflect normal physiological functions of lymphoid tissues. Differences obtained could be explained by the earlier fact that all three lymphoid organs are not composed of similar types of cells in distinct—for instance, the spleen is the only organ with B-1 type non- re-circulating B cells.

There were reports on LPS being a ligand of TLR-2[50]. But Liu *et al*.,[34] in his studies on dose inconsistent effect of LPS on NF-kB activation and TLR-2 mRNA elevation strongly supported the hypothesis that TLR-4 but not TLR-2 is sensitive to LPS stimulation. Thus, TLR-2 may not participate in LPS signaling unless macrophages face the challenge of large amount of LPS. The consequence of TLR-2 participation is likely to be that TLR-2 gene expression is elevated in order to compensate for the consumption of TLR-2. Another explanation for insignificant changes in TLR-2 expression after LPS stimulation could be that TLR-2 and TLR-4 respond to different types of LPS. There is a divergence in LPS structure among gram negative bacteria and it is reasonable to presume that TLR-4 responds to certain types of LPS better than TLR-2 while TLR-2 responds better to others[33]. These facts could explain our result.

The bacteria might infiltrate the lymph node at a later stage leading to late activation of cells and delayed TLR-2 expression as compared to spleen. Late activation of thymic cells could also be proposed. Less inoculum size and post transcriptional modification of the transcribed mRNA might also cause this variation. We compared the expression of TLR-2 at 15 dpi among all the three lymphoid tissues studied, to find out the lymphoid organ which expresses TLR-2 maximally at that time point. It was found that spleen expresses more TLR-2 compared to thymus and lymph node, which again may be due to the large amount resident macrophages present in the spleen than thymus and lymph node. Subsequently, the *S. aureus* infected splenic macrophages of Swiss albino mice showed greater expression with respect to control. The complexity of the data that we obtained might be related to distinct route of infection in murine models such as I.P., I.V., inoculums size and differences in innate immunity between different strains mice[49,51]. The role of TLR-2 in localized infection *in vivo* have revealed a much more complex interaction between TLR-2 and microbial pathogen as macrophages play a major role in protection against extracellular and intracellular pathogens. Investigations have concentrated on the response of this cell type to live *S. aureus*[4] also supported our study in using viable *S. aureus*.

The expression and function of TLRs are different in different tissues and cell types[52]. Therefore, it is critical to understand the expression of these proteins for a better comprehension of the host response to pathogens. The expression pattern of TLR-2 could be based on anatomical localization and that they are related to the varying bacterial milieu encountered in each anatomical location also supported our study[53]. It is also possible that this expression pattern is related to the role of lymphoid organs in the maintenance of an environment hospitable to antigen trapping/ infiltration. Thus, TLR-2 expression may be maintained at a low level in the control spleen and lymph node but be up regulated in response to pathogenic challenge as a defence mechanism that can be mobilized to protect the spleen/lymph node from infection during inflammatory diseases[54]. Thus, the roles of TLR-2 in innate responses to *S. aureus* are context dependant and may include modulatory roles in infection outcome[55]. So, TLR signaling must be tightly controlled to prevent unwanted or prolonged stimulation which otherwise, might be harmful for the host[56].

TLR-2 knockout mice are known to have a pronounced TNF-α and IFN-γ cytokine response when compared to their respective wild type groups

suggesting that TLR-2 signaling may play a role in the regulation of those cytokines[57,58]. Bacterial burden in different tissues may also regulate TLR-2 dependent cytokine release. Thus, the functionality of this receptor was determined by measuring serum TNF-α, IL-6, IFN-γ and IL-10. The increased levels of proimflammatory cytokines after infection and the production of anti-inflammatory IL-10 thereafter could be correlated with TLR-2 expression patterns suggesting that it might play a role in initiation of inflammation and activation of adaptive immune response. Since TNF is a major inducer of TLR-2, the sustained higher levels of TNF-α even at 15 dpi could account for TLR-2 expression in the organs at 15 dpi, and the significantly lower levels of IL-6 and IFN-γ could be due to the anti-inflammatory effect exerted by IL-10.

Our current study is the first preliminary report for *in vivo* expression of TLR-2 within the murine lymphoid organs of Swiss albino mice and its regulation by viable *S. aureus* infection. An enhanced understanding of innate immune mechanisms within the spleen and lymph node of wild type Swiss albino mice and their role in bacterial recognition may provide insight into the pathogenesis of inflammatory diseases and the deleterious sequel associated with lymphoid organ inflammation. Although, the functionality of this receptor has been confirmed by studying the serum cytokine profile, we recognize that this data represents only a starting point and should eventually be continued using assays for TLR proteins in the presence or absence of anti-TLR-2 antibody after infection with *S. aureus* or synthetic TLR-2 ligand like pam3CSK (a synthetic triacylated lipopeptide that mimics the acylated amino terminus of bacterial LPs and activates pro-inflammatory transcription factor NF-κB). Nevertheless, these data are critical for further studies in a therapeutic approach by targeting or blocking TLR-2 and verify the role of these receptors in inflammatory diseases.

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