ANNEXURE-IX

UNIVERSITY GRANTS COMMISSION

BAHADUR SHAH ZAFAR MARG NEW DELHI – 110 002

PROFORMA FOR SUBMISSION OF INFORMATION AT THE TIME OF SENDING THE FINAL REPORT OF THE WORK DONE ON THE PROJECT

1. Title of the Project: **Characterization, Localization and Expressions of Toll like Receptors (2, 4, 6) in Thymic, Splenic and Lymph node Lymphocytes and Macrophages of Swiss albino mice and their Role in *Staphylococcus aureus* Infection**

2. NAME AND ADDRESS OF THE PRINCIPAL INVESTIGATOR: **Professor BISWADEV BISHAYI**

3. NAME AND ADDRESS OF THE INSTITUTION:

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4. UGC APPROVAL LETTER NO. AND DATE: **F. 41-11/2012 (SR) (HRP) dated 10th July 2012.**

5. DATE OF IMPLEMENTATION: **01-07-2012**

6. TENURE OF THE PROJECT: 3 years (01.07.2012- 31.12.2015 as per sanction order)

7. TOTAL GRANT ALLOCATED: [Rs.10,64,285/- + Additional grant (Bank Interest) Rs.17,639/-]

8. TOTAL GRANT RECEIVED: Rs 9,75,537/-

9. FINAL EXPENDITURE: Rs. 10,69,644/- as per the audit report

10. TITLE OF THE PROJECT: **Characterization, Localization and Expressions of Toll like Receptors (2, 4, 6) in Thymic, Splenic and Lymph node Lymphocytes and Macrophages of Swiss albino mice and their Role in *Staphylococcus aureus* Infection**

11. OBJECTIVES OF THE PROJECT:

I. To characterize and to figure out localization and expression the of Toll like receptors (TLR- 2 , 4, 6) in lymphocytes and macrophages in the thymus, spleen and lymph node of Swiss albino mice.

II. To figure out whether *in vivo* bacterial infection (for eg. viable *S. aureus*) could also potentially predisposes the Swiss albino mice to bacterial septic shock after interacting with TLRs (TLR-2,4,6) distributed in the different localized compartments (thymus, spleen and lymph node).

III. The experiments presented here were designed to determine the extent to which the TLR (TLR-2, 4, 6) and *S. aureus* interaction (*in vivo*) regulates TH1/TH2 cytokine production in Swiss albino mice in relation to *S. aureus* infection induced inflammation.

12. WHETHER OBJECTIVES WERE ACHIEVED (GIVE DETAILS):

Expression of innate immune receptors varies among organs and species and within different strains among the same species; thus, periodic classification of different pattern recognition receptors in the available strains is necessary to initiate different therapeutic approaches to combat inflammation. 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. This study was carried out to verify 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.

Description according to the objectives:-

I. To characterize and to figure out localization and expression the of Toll like receptors (TLR- 2 , 4, 6) in lymphocytes and macrophages in the thymus, spleen and lymph node of Swiss albino mice:- In order to characterize and localize Toll like receptors 2, 4, and 6 experiments were carried out in Swiss albino mice. Animals were infected with live *S. aureus* (obtained from Calcutta Medical College and Hospital and maintained in our laboratory) and a disease model was generated by inducing septic arthritis. 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. However its expression was found to increase gradually with time in the thymus, spleen and lymph node of *S. aureus* infected mice with respect to control indicating its responsiveness. A comparative study on the expression of TLR-2 at 15 dpi among all the three lymphoid tissues revealed that TLR-2 expression is most in spleen at that time point, which again may be due to the large amount resident macrophages present in spleen as compare to thymus and lymph node. The complexity of the data that was obtained might be related to distinct route of infection in murine models such as I.V, I.P, inoculums size and differences in innate immunity between different lymphoid organs. Being the heterodimeric partner of TLR-2, TLR-6 was also found to have similar responsiveness toward the ligands of TLR-2 but no reports on its expression was found in Swiss albino strain of mice. In this study TLR-6 was characterized in the lymphoid organ along with synovial tissue of Swiss albino mice. Among the tissues studied it was found that the expression of TLR-6 in spleen and synovial tissue peaked at about 9 days post infection and in case of lymph nodes the expression peaked at about 15 days post infection. In case of thymus although the expression increased after *S. aureus* infection compared to control but no day wise variation was noticed among the infected groups. Along with TLR-2 and 6, TLR-4 was characterized because it is perhaps the only member of the

TLR family which is a sensor of LPS the major cell wall component of Gram negative bacteria. *S. aureus* is not a ligand of TLR-4, thus experimental endotoxemia was induced in order to characterize TLR-4 in the lymphoid organs of Swiss albino mice. On characterization of TLR-4 in spleen and thymus of Swiss albino mice—with no reports of TLR-4 expression—induced with endotoxemia, it was found that the mode of expression varied among the organs at both mRNA and protein level in a time-dependent manner. Then *in vitro* study was conducted to verify the presence of TLR-2, 4 and 6 on splenic macrophages and lymphocytes as lymphoid organs are the lymphocyte pool of the host and their responsiveness might give a basic insight of their functionality of inflammation. Cells from BALB/c mice were used as positive control because in this strain the expression of TLR-2 has been widely explored and this strain is responsive towards TLR-2 ligands. There are strains of mice which do not express certain PRRs due to mutation in their gene thus prior to any therapeutic approach targeting these receptors their presence in this unreported strain of mice was verified.

II. To figure out whether *in vivo* bacterial infection (for eg. viable *S. aureus*) could also potentially predisposes the Swiss albino mice to bacterial septic shock after interacting with TLRs (TLR-2, 4, 6) distributed in the different localized compartments (thymus, spleen and lymph node):- By studying the serum cytokine profile of proinflammatory cytokines TNF-α, IFN-γ, IL-6, IL-17 and anti-inflammatory cytokine IL-10 from serum and cell supernatant, the functionality of the receptor was established. Serum level of C- reactive protein indicates the onset of inflammation after *S. aureus* infection and LPS administration. Other signs of inflammation were also observed like morbidity/sickness determined by observing whether they exhibit hunched posture, decreased activity, ruffled fur and labored breathing. In case of *S. aureus* infection and induction of septic arthritis animals were sacrificed at 3, 9 and 15 days post infection because significant joint swelling and other signs were observed on those days and the expression pattern of TLR-2 and 6 were correlated with dosage of infection, mortality, bacterial burden in vital organs, inflammatory and anti inflammatory cytokines secretion, CRP and it was established that TLR-2 and 6 are responsive to *S. aureus* infection and involved in inflammation. TLR-4 is a sensor of LPS and all the receptors are modulated in presence of their respective ligands confirming their role in the stimulation of the inflammatory cascade. Variation in the pattern of TLR-2, 4 and 6 expression was observed among

spleen, thymus and lymph node. 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 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 type in distinct: for instance the spleen is the only organ with B-1 type non-re circulating B cells. Thus the expression pattern could provide an insight into the distinct cell population as well as the difference in their function.

III. The experiments presented here were designed to determine the extent to which the TLR (TLR-2, 4, 6) and *S. aureus* interaction (*in vivo*) regulates TH1/TH2 cytokine production in Swiss albino mice in relation to *S. aureus* infection induced inflammation: - In the *S. aureus* induced septic arthritis disease model we have studied the levels of TH1, TH2 as well as TH17 cytokines and tried to hypothesize their interplay to some extent. Swiss albino mice were intravenously injected with live *S. aureus* with a dose viable to induce septic arthritis. *S. aureus* home in the synovial tissue along with lymphoid organs where they are recognized macrophages and mononuclear cells as well as lymphocytes to some extent. Cell surface TLR-2/6 heterodimer which in association can recognize *S. aureus* resulting in receptor expression upregulation within 3 days of infection. Activated TLR-2/6 stimulates the adaptive immune response probably by Myd88/NF-κB dependant pathway and T-helper cells differentiates mainly to Th1, Th2 and Th17. Being an antigen presenting cell itself the immune cells can synthesize and secrete inflammatory cytokines such as TNF- α, IFN- γ, IL-6, IL-12, IL-10 and Th1 cells secretes TNF- α, IFN- γ and IL-12 mainly resulting in an increase level of these cytokines in the initial stage of the disease. With the progression of the disease Th1 response shifts to Th2 and the level of IL-6, IL-10 starts increasing more because these two are the primary TH2 cytokines. Inhibitory effect of IL-10 could inhibit APC and Th1 cytokine secretion but it is established that IL-6 and IFN- γ crosstalk and synergizes their effects, and IL-6 also helps in the differentiation of Th1 to Th17 resulting in the increased level of IL-17 at this time point. IL-17 also stimulates IFN- γ resulting in and enhanced level of IL-6, IFN- γ and IL-17 at 9 days post-infection. IL-6 stimulates CRP. IFN- γ leads to leukocyte infiltration and protease release which causes joint inflammation along with IL-17 which also targets joints for inflammation hence ROS generation takes place

which results in acute joint inflammation and septic arthritis which peaked at 15 days post-infection.

13. ACHIEVEMENTS FROM THE PROJECT:

a) Our study is the first preliminary report of *in vivo* expression of TLR-2, 6 and 4 within the lymphoid organs of Swiss albino mice. Along with the presence of TLR-2, 4 and 6 in the lymphoid organs of Swiss albino mice its modulation by viable *S. aureus* and LPS has been shown which confirms its responsiveness and also its functionality by correlating their expression with the secretion of cytokines. However as there are converging pathways in between which could be verified in future to identify other nexus points and potential targets for regulation and modification of the immune response and ameliorate inflammation in the host body. *In vitro* experiments showed Macrophages and lymphocytes to be responsive.

b) From a study designed on synovial mononuclear cells it is established for the first time that TLR-2 and 6 are interdependent in the recognition of *S. aureus* and are equally involved in induction of septic arthritis by stimulating the inflammatory cascade. Thus intracellular intake of *S. aureus* could be inhibited by neutralization of either of these receptors and restrict the microbes outside the cell surface reducing inflammation as well as potentiating *S. aureus* for antibiotic or combination therapies as they cannot use the mechanism of evading the extracellular immune responses.

c) Currently we are conducting experiments in the *in vivo* system if either of the TLR-2 or 6 could be neutralized directly by intra-articular injection of their blocker, then inflammation, swelling and pain could be reduced to a great extent at the joints. Reports are available where drugs like Etanercept - a TNF-α inhibitor is used for rheumatoid arthritis.

d)In case of endotoxemia Pre-treatment with IL-6 or IL-10 could provide a protection in endotoxemia by inhibiting tissue as well as neutralization of TNF-α and IFN-γ might also lower endotoxemia as they are found to be the primary proinflammatory cytokines found to cause the inflammation.

14. SUMMARY OF THE FINDINGS (IN 500 WORDS):

TLR-2, 4 and 6 have been characterized in the lymphoid organs along with some lymphoid cells in Swiss albino strain of mice as no reports on TLRs has been found till date in this particular mouse strain. The knowledge of the genetic background is important prior to any therapeutic approach based on molecular mechanisms because striking variations in the

susceptibility and resistance of different strains of inbred and out bred mice and rats are documented continuously against different infections. TLRs are being extensively explored because it has the ability to recognize components derived from a wide range of pathogens and so the initiation of inflammatory responses, shaping adaptive immunity are mostly TLR-mediated. Thus in order to begin from the very beginning the TLRs were required to be localized and characterized in this unreported strain of mouse in tissue specific and cell specific manner. Two disease models were also induced to study the role of three members of the TLR family both in a chronic and acute stage. Expression pattern of TLR-2 and 6 was studied after induction of chronic septic arthritis by *S. aureus* and it is found that both these receptors are modulated in response to *S. aureus* infection. *In vitro* experiments were simulated to observe the effect of *S. aureus* directly on Macrophages and lymphocytes isolated from these lymphoid organs and they are found to be responsive in expressing TLR-2 and 6 on encountering acute *S. aureus* infection. Moreover from a study designed on synovial mononuclear cells it is established for the first time that TLR-2 and 6 are interdependent in the recognition of *S. aureus* and are equally involved in induction of septic arthritis by stimulating the inflammatory cascade. In comparison another disease model was induced from Gram negative bacterial component and a different member of the TLR family was studied. LPS is a cell wall component of Gram negative *E. coli* as well as an established ligand of TLR-4. TLR-4 was characterized in this strain of mice in response to experimental endotoxemia. Similarly an *in vitro* experiment designed on macrophages and lymphocytes isolated from these lymphoid organs and treated with LPS to observe their modulation on short time acute LPS treatment.

Thus in the overall study TLR -2, 6 and 4 was characterized in the lymphoid organs and cells and their role in innate immunity was being explored both in localized and systemic inflammation to some extent. Also from this study it is established that TLR-2 and 6 are responsive to *S. aureus* in this strain of mouse and these receptors are interdependently involved in recognition, binding and intracellular intake of *S. aureus* within the lymphoid organs leading to inflammation. Thus intracellular intake of *S. aureus* could be inhibited by neutralization of either of these receptors and restrict the microbes outside the cell surface reducing inflammation as well as potentiating *S. aureus* for antibiotic or combination therapies. This fact could be implemented in *in vivo* system if either of the TLR-2 or 6 could be neutralized directly by intra articular injection of their blocker, then inflammation, swelling, pain could be reduced to a great extent at the joints. TLR-4 receptor is expressed in

both primary and secondary types of lymphoid organs of Swiss albino mice, but its time and rate of modulation differed in LPS induced endotoxemia, which could give an insight of the divergent functional activity of their distinct cell population in inflammation. Pre-treatment with IL-6 or IL-10 could provide a protection in endotoxemia by inhibiting tissue degradation Moreover neutralization of TNF-α and IFN-γ might also lower endotoxemia as they are the primary proinflammatory cytokines found to cause the inflammation.

However, an inflammatory response with excessive production of proinflammatory cytokines as seen in the early hours could induce side effects depending on the intensity of the disease progression and could even lead to multiple organ dysfunction syndrome and death. So TLR signaling must be tightly controlled to prevent unwanted or prolonged stimulation which might be harmful for the host hence regulation of TLR signaling such as receptor blocking to prevent unwanted or prolonged stimulation. Thus the time dependant expression pattern of these receptors might give a basic insight for further therapeutic approaches to provide immune potentiation or boosted immune response. This is a baseline study and all the data are critical for further studies in details for implementation.

15. CONTRIBUTION TO THE SOCIETY (GIVE DETAILS):

a) Bacterial infections are always a threat to health and life and finding remedies and other ways to counteract it are soon becoming the exigencies of medical science. The identification of MDR- bacteria has potentiated the threat even further. Antibiotics although are convenient but presently are not effective against all bacteria especially *S. aureus* which have evolved strategies to evade the host immune system by taking shelter in host cells. Hence finding new therapeutic strategies to combat these maladies has become an imperative need of medical science. An inflammatory response with excessive production of proinflammatory cytokines could induce side effects depending on the intensity of the disease progression and could even lead to multiple organ dysfunction syndrome and death. So TLR signaling must be tightly controlled to prevent unwanted or prolonged stimulation which might be harmful for the host hence regulation of TLR signaling such as receptor blocking to prevent unwanted or prolonged stimulation. Thus the time dependant expression pattern of these receptors might give a basic insight for pathogenesis in the lymphoid organs and for further therapeutic approaches to provide immune potentiation or boosted immune response. This is a baseline study and all the data are substantial and critical for further studies in details for implementation.

b) Septic arthritis has emerged as a potent disabling and life threatening disease. 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. This disease model was use to study the modulation of TLR-2 and 6 in the *in vivo* system and probable contribution in the progression of *S. aureus* induced septic arthritis. It was established that the heterodimeric partner TLR-2 and 6 are both equally involved in recognition, internalization of *S. aureus* and initiation of inflammation. In the *in vitro* study we have shown that the intracellular intake of *S. aureus* could be inhibited by neutralization of either of these receptors and restrict the microbes outside the cell surface reducing inflammatory cytokine production hypothesizing potentiation of *S. aureus* for antibiotic or combination therapies as they cannot use the mechanism of evading the extracellular immune responses. In the current study we are implementing this fact in the *in vivo* system by neutralizing either of the TLR-2 or 6 directly by intra articular injection of their blocker, to reduce inflammation, swelling, pain to some extent at the joints. Reports are available where drugs like Etanercept - a TNF-α inhibitor is used for rheumatoid arthritis.

c)We have extended the work further by initiating an endotoxemic disease model as LPS in a potent ligand of TLR-4 and our study's outcome showed that in case of endotoxemia pre-treatment with IL-6 or IL-10 could provide a protection by inhibiting tissue degradation as it has already been established in prior studies that pretreatment with IL-6 lowers liver damage in experimental endotoxemia by modulating ROS production and cell infiltration and suppressing proinflammatory cytokines due to their immune response-limiting properties. Neutralization of TNF-α and IFN-γ might also lower endotoxemia as they are the primary proinflammatory cytokines found to cause the inflammation in our study.

16. WHETHER ANY PH.D. ENROLLED/PRODUCED OUT OF THE PROJECT:

One PhD candidate, Ms. Chandrayee Ghosh ;
Registration no- 3220 Ph.D (SC) Proceed 13, Date 07.06.2013
Awarded Ph.D. degree from University of Calcutta in the year 2016
Title of the thesis: IDENTIFICATION AND CHARACTERIZATION OF TOLL-LIKE RECEPTORS (TLR-2, 4, 6) IN THYMIC, SPLENIC AND LYMPH NODE LYMPHOCYTES AND MACROPHAGES OF SWISS ALBINO MICE AND THEIR ROLE ON *Staphylococcus aureus* INFECTION AND LIPOPOLYSACCHARIDE ADMINISTRATION

17. NO. OF PUBLICATIONS OUT OF THE PROJECT (PLEASE ATTACH):

1. **Chandrayee Ghosh,** Nune Ravi Prakash, Sunil Kumar Manna & Biswadev Bishayi. 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. *Indian Journal of Experimental Biology,* Vol. 53, February 2015, pp 82-92. **[I.F- 1.165]**

2. **Chandrayee Ghosh** and Biswadev Bishayi. 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. *Immunobiology,* 2015, 220: 910-923 **[I.F- 2.814]**

3. **Chandrayee Ghosh** and Biswadev Bishayi. Characterization of Toll-Like Receptor-4 (TLR-4) in the Spleen and Thymus of Swiss Albino Mice and Its Modulation in Experimental Endotoxemia. *Journal of Immunology Research*, Hindawi Publishing Corporation Volume 2015, Article ID 137981, 13 pages, http://dx.doi.org/10.1155/2015/137981. **[I.F- 3.276]**

(PRINCIPAL INVESTIGATOR) **(REGISTRAR/PRINCIPAL) (Seal)**

(CO-INVESTIGATOR)

17. NO. OF PUBLICATIONS OUT OF THE PROJECT (PLEASE ATTACH):

1. **Chandrayee Ghosh,** Nune Ravi Prakash, Sunil Kumar Manna & Biswadev Bishayi. 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. *Indian Journal of Experimental Biology*, Vol. 53, February 2015, pp 82-92. **[I.F- 1.165]**

2. **Chandrayee Ghosh** and Biswadev Bishayi. 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. *Immunobiology*, 2015, 220: 910-923 **[I.F- 2.814]**

3. **Chandrayee Ghosh** and Biswadev Bishayi. Characterization of Toll-Like Receptor-4 (TLR-4) in the Spleen and Thymus of Swiss Albino Mice and Its Modulation in Experimental Endotoxemia. *Journal of Immunology Research*, Hindawi Publishing Corporation Volume 2015, Article ID 137981, 13 pages, http://dx.doi.org/10.1155/2015/137981. **[I.F- 3.276]**

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REGISTRAR
Calcutta University

18.08.17

ANNEXURE-X

UNIVERSITY GRANTS COMMISSION

BAHADUR SHAH ZAFAR MARG, NEW DELHI – 110 002

EVALUATION REPORT

It is certified that the final report of the UGC Major Research Project entitled "Characterization, Localization and Expressions of Toll like Receptors (2, 4, 6) in Thymic, Splenic and Lymph node Lymphocytes and Macrophages of Swiss albino mice and their Role in *Staphylococcus aureus* Infection" [vide UGC approval number: F. 41-11/2012 (SR) (HRP) dated 10th July 2012] by Professor Biswadev Bishayi, Department of Physiology, University of Calcutta, 92 APC Road, Calcutta-700009, West Bengal has been assessed by the "Evaluation committee" consisting the following members for final submission to the University Grants Commission, New Delhi.

Details of the Expert Committee:

1. Dr Manoj Kumar Chakraborty, Ex Director
   ICMR Emeritus scientist
   National Institute of Cholera and Enteric Diseases (NICED)
   Molecular Pathophysiology Division
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2. Dr Shibshankar Roy, Scientist
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   Indian Institute of Chemical Biology
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The Final report is found to be satisfactory

M Chakraborty 19/7/17

Dr Manoj Kumar Chakraborty

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18.08.17