NATIONAL INSTITUTE OF IMMUNOLOGY

ANNUAL REPORT
2011–2012

CONTENTS

MANDATE OF THE INSTITUTE

- To undertake, aid, promote, guide and co-ordinate research of high caliber in basic and applied immunology
- To carry out research for development of new vaccines and immunological reagents for communicable diseases
- To develop immunological approaches for regulation of male and female fertility
- To interact with industry for manufacture of vaccines and immunological reagents
- To organise postgraduate courses, workshops, seminars, symposia and training programmes of a specialized nature in the field of immunology, vaccine development and related areas
- To organise training programmes for technicians in immunological methods and related techniques
- To establish affiliation with recognised universities and institutions of higher learning for the purpose of enabling research scholars to register for postgraduate degrees
- To serve as a national reference centre for immunology and to provide consultancy services to medical and veterinary institutions, public health agencies and industries in the country
- To provide and promote effective linkages on a continuing basis between various scientific and research agencies/laboratories and other organisations working in the country in the field of immunology, vaccine development and related areas
- To collaborate with foreign research institutions, laboratories and other international organisations in fields relevant to the objectives mentioned above.

FOREWORD

The National Institute of Immunology (NII) completed its twenty five years of existence in the present campus last year. In this quarter century of research, the Institute has made notable strides in the understanding of disease processes. As the success of these explorations in terms of translational benefits are heavily reliant on an interdisciplinary approach, the Institute over the years has fostered research in multiple overlapping disciplines of modern biology including biochemistry, molecular biology, cell biology and structural biology in addition to immunology. During this reporting period many research groups, as always, have enriched the scientific pursuit of the Institute. A few of the significant advances made at the Institute demand special mention and a summary of such efforts is given below.

One of the endeavours of immunological research is to generate vaccines. Towards this goal, it is important to understand the immune regulation and development at its most fundamental level in addition to identifying potential vaccine targets in pathogens. Efforts directed towards both these aspects have yielded interesting and useful results.

VDJ recombination is the primary mechanism that generates diversity in the antigen receptor proteins that are expressed on T cells and B cells. It was shown that alteration of the chromatin architecture of the T-cell receptor locus strongly influences choice of the gene segments that undergo recombination and consequently influences the T-cell receptor repertoire. In an independent study, development of T cells was investigated. It was shown that the protein AIF plays a crucial role in this process. Interestingly, although AIF is expressed in all cell lineages and is well known for its pro-apoptotic properties, it serves an anti-apoptotic role specifically in the T cell lineage through reactive oxygen species regulation during thymic beta-selection.

In the context of immune recognition, it is intriguing to note that even though there are a limited number of possible germline immunoglobulin receptors on primary B cells, they seem to have the ability to recognize an unlimited pool of possible antigens. This fundamental discrepancy was addressed using a structural biology approach. It was revealed that there is a correlation between the degenerate specificity of an antibody and its conformational versatility.

Evaluation of the effect of antigen presentation in shaping the humoral response indicated that the intensity and duration of B cell receptor signaling may play a more important part in shaping B cell responses than an early antigen presentation for T cell help. Hence the duration of signalling might eventually govern whether or not the encounter with the antigen can generate a memory response and eventually protection against it.

With the aim of identifying potential vaccine candidates for *Streptococcus pneumoniae,* a leading cause of bacterial pneumonia and sepsis, surface-associated proteins of *S. pneumoniae* were resolved. A specific surface protein expressed during infection was identified. This protein serves as a promising candidate for inclusion in a future multi-component protein-based pneumococcal vaccine.

In this context, another study carried out at NII has substantial relevance. The housekeeping transpeptidase sortase A from *Staphyloccocus aureus* that catalyzes the covalent anchoring of surface proteins to the cell wall was utilized to generate a gamut of branched peptide oligomers and multivalent proteins. Potentially, sortase-mediated peptide ligation can be exploited as a synthetic tool for creating multiple antigenic peptides

and proteins for the purpose of multi-component vaccine development.

The well known tumor suppressor protein p53 is mutated in many cancers and hence is of considerable importance. Studies at NII have identified the basis for regulation of p53 and highlighted its importance in different cellular contexts. Metabolic stress is known to result in p53 activation, which can trigger cell-cycle arrest, reactive oxygen species clearance or cell death. However, what determines the p53-mediated decisions upon cellular metabolic stress is not well understood. It was demonstrated that the protein PGC-1α, known to be important for control of glucose, lipid, and energy metabolism can bind to p53 leading to preferential transactivation of pro-arrest as well as regulation of metabolic target genes.

In another study, role of p53 and DNA helicase RECQL4 in mitochondrial DNA was delineated. The investigations established the function of RECQL4 in mitochondria and revealed the mechanism by which p53 is regulated by RECQL4.

Investigations into specific interaction of HIV-1 viral protein U (Vpu) with SCF complex was shown to inhibit ubiquitination and proteasomal degradation of p53. These findings established a novel function of Vpu in modulating the stability of p53 protein that correlates positively with apoptosis during late stages of HIV-1 infection.

Elucidation of host pathogen interaction is paving the way for an improved management of the diseases caused by intracellular pathogens that cause many diseases. Intracellular pathogens need to avoid targeting to lysosomes of the host cell in order to survive. They are known to recruit lysosome associated membrane protein1 (LAMP1) by unknown mechanisms. It was shown in a model of enterobacteria *Salmonella* that effector protein, SipC, specifically bind with host Syntaxin6 and other accessory molecules and acquire LAMP1 by fusing with LAMP1-containing Golgi-derived vesicles. These results revealed a novel mechanism for acquisition of LAMP1 by *Salmonella* which may contribute to stabilize their niche in macrophages – a strategy that might be employed by other intracellular pathogens as well.

Mycobacteria cause serious diseases in mammals and studies revolving around the biology of such pathogens provide useful insights into combating such infections. In mycobacteria, polyketide synthases and nonribosomal peptide synthetases (NRPSs) produce complex lipidic metabolites by using a thio-template mechanism of catalysis. Exploration of mechanistic details of substrate transfer during polyketide biosynthesis using bioinformatics approaches revealed that intrinsically unstructured linker stretch preceding the ACP domain might be facilitating movement of ACP domains to various catalytic centers of PKS module. Insights into the mechanism of catalysis of mycobacterial NRPS reductase domain were also gained by analysis of structure-function interrelationships. The analysis demonstrated the unconventional recruitment of a canonical short-chain dehydrogenase/reductase family member as an off-loading domain which provides interesting clues to the evolution of enzymatic function. Together, these mechanistic studies have implications in the biosynthesis of pharmaceutically useful secondary metabolites.

Another formidable bio-medical challenge is posed by genetic disorders. While gene therapy has been speculatively considered as a corrective measure for several decades, the idea has become tangible only with recent advancements in stem cell therapy. One of the research groups at this Institute is actively engaged in such research with particular reference to Hemophilia A (HA), a genetic disorder caused by mutation in factor VIII (FVIII) gene in humans. Liver is the primary site of FVIII synthesis; however, the specific cell types responsible for its synthesis have remained controversial. By examining the cellular origin of FVIII, it was concluded that bone marrow derived hepatocytes and endothelial cells can synthesize FVIII in liver and correct bleeding phenotype in HA mice. These studies prove, in principle, that genetically engineered stem cells can be used for therapeutic purposes for mitigating Hemophilia A.

In addition to conducting cutting-edge basic medical research, the National Institute of Immunology has also contributed to the development of scientific manpower for the nation. During last year, scientists at the Institute have trained a substantial number of short-term students and summer fellows who were introduced to the realm of biological research. A large number of post-doctoral and doctoral students also worked in a collaborative atmosphere with leading scientists during the year. The Institute has also generated scientific research resources for the scientific community in terms of specific cell lines and transgenic animals that are being widely used across the country.

The National Institute of Immunology is defining an additional trajectory of research in the form of large collaborative groups addressing important disease related questions with a multi-disciplinary approach which can be asked both from a global as well as a national perspective. Such collaborative team driven efforts provide a unique value to the quest for doing world class science with continuous intellectual interactions. I believe, with the coming years NII will not only maintain its excellence in research but will also thrive in its new dimension and make significant contributions to innovation in health sciences.

Chandrima Shaha
Director

14 December 2012

RESEARCH REPORTS

IMMUNITY AND INFECTION

IMMUNITY AND INFECTION

Analysis of antigen processing and presentation

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The aim of the ongoing programmes in this group is to examine the generation and activation of T, B and antigen-presenting myeloid cells using multiple interlinked experimental systems.

A variety of experimental approaches are taken to address the theme issues. The approaches in current use examine APCs and pathways involved in antigen presentation to MHC class I and class II-restricted T cells, and analyze the consequences of intracellular signal transduction modulation for both development and responses of B cells, T cells and macrophages using genetic as well as pharmacological tools.

Molecular dissection of AIF domains in regulating T cell development and function

AIF is known to have a DNA-binding function essential for its pro-apoptotic activity, while it also has an oxidoreductase function that may be involved in its ability to regulate mitochondrial function, cell metabolism and oxidative stress. Distinct point mutations in the mouse AIF protein selectively abrogate either the DNA-binding or the oxidoreductase function; K254A & R264A for abrogation of DNA-binding and T262A & V299A for abrogation of oxidoreductase activity. We therefore examined if either/both functions were required to correct the Hq thymic defect. We generated these point mutant genes from the WT *AIF* gene by site-directed mutagenesis, and then generated transgenic mice that express these mutant AIF versions using the same heterologous promoter and testicular transgenesis protocol used above. Transgenic progeny thus obtained were bred as above with heterozygous Hq females to generate male littermates of

all four genotypes, namely, non-tg WT, non-tg Hq, tg WT and tg Hq, for both mutant AIF molecules, namely, the AIF254/264 and the AIF262/299. Both the transgenic and Hq status were confirmed by genotyping PCRs. Western blot analysis of splenic cells showed that the tg-Hq mice had significant levels of AIF in both AIF254/264-tg and AIF262/299-tg mice.

We next compared the T cell lineage phenotype in littermate mice of the four genotypes identified above, for both AIF254/264 and AIF262/299. The transgenic expression of either of these AIF mutant proteins in WT mice did not have any detectable effect in any of the readouts used. In the spleen, the reduced frequencies and numbers of naive CD4 and CD8 T cells found in Hq mice were increased in AIF254/264-tg Hq mice but not in AIF262/299tg Hq mice, although the recovery seen with AIF254/264 was not as complete as that observed with WT AIF. Similarly, the thymic hypocellularity found in Hq mice was significantly corrected in AIF254/264-tg Hq mice but not in AIF262/299tg Hq mice. The high DN thymocyte frequency found in Hq mice was reduced and the altered frequencies of DN3 and DN4 cells were substantively corrected in AIF254/264-tg Hq mice but not in AIF262/299tg Hq mice. Thus, the oxidoreductase function of AIF is required for maintaining normal thymic development, while the DNA-binding function is dispensable.

The requirement for the oxidoreductase function of AIF in correcting the Hq thymic defect indicated that the defect was due to ROS stress in AIF-hypomorphic thymocytes. We therefore tested whether treatment with a cell-permeable anti-oxidant that could mimic both superoxide dismutase (SOD) and catalase, namely Euk-134, affected the Hq thymic defect in vivo. Young (2 week-old) WT and Hq mice were treated daily with Euk-134 or vehicle alone for 14 days before the thymic phenotype was determined. Euk-134 treatment made no discernible difference to WT thymic cellularity or subpopulation frequencies, but in Hq mice, Euk-134 decreased the DN thymocyte frequency and significantly improved total thymic cellularity, particularly that of the DP population. Euk-134 treatment also increased the DN4 frequency in Hq mice. Together, these data indicate that AIF regulates the efficiency of transition through beta-selection by using its catalytic function to control oxidative stress.

We next directly tested if Hq DN thymocytes show higher levels of oxidative stress. The peroxide-sensitive dye 2´-7´-dichlorofluorescein diacetate (DCFDA) indicated higher ROS levels in Hq than in WT DN3 and DN4 cells. However, the superoxide-sensitive dye Mitosox Red did not show such a difference in DN3 and DN4 cells, suggesting that Hq thymocytes suffer from peroxide stress. Consistent with this, Hq DN3 and DN4 thymocytes showed lower levels of reduced glutathione, as reflected by lower intensities of staining by monochlorobimane (mBCl) (Hedley and Chow, 1994). While DP thymocytes from Hq mice did show an increase over WT cells in DCFDA staining levels, this was far more modest than in DN3/4 cells. Thus, low levels of AIF lead to higher oxidative stress in DN thymocytes. On the other hand, the closely related B cell lineage does not show any differences in ROS levels between Hq and WT bone marrow as indicated by DCFDA staining.

Finally, we tested the prediction that Euk-134, which rescues the Hq thymic defect in vivo, provides protection from the enhanced cell death seen in Hq DN thymocytes. When electronically sorted WT or Hq DN3 and DN4 thymocytes were put in culture with or without Euk-134 for 8 h, the enhanced death seen in Hq DN3 and DN4 thymocytes was inhibited by Euk-134.

Together, our data suggest that cells undergoing proliferative expansion during beta-selection are dying, leading to reduction in the numbers of survivors that make it through to the DP stage. The anti-apoptotic role of AIF has been very prominent in a number of cell lineages in vivo, from neurons and muscle cells to beta cells in the Islets of Langerhans. At least in neurons, there is reason to believe that AIF-hypomorphism leads to death during cell cycle and that this is related to alterations in the assembly of the mitochondrial oxidative phosphorylation apparatus and/or the generation and handling of oxidative stress. While AIF

was proposed to be a hydrogen peroxide scavenger, it has many more complex roles in mitochondrial functions, many of which lead to the generation of oxidative stress in the absence of AIF. The role of the cellular redox status in developing T cells has been reported to be a major factor in determining the efficiency of T cell development. It is therefore plausible that a similar susceptibility is created in DN thymocytes through similar mechanisms by AIF-hypomorphism. The finding that gamma/delta T cell numbers are not altered in Hq mice is consistent with this possibility, since gammadelta selection is known to induce much less proliferation than beta-selection. On this background, we have examined the mechanism by which AIF-hypomorphism caused disruption of T cell beta-selection using both genetic and pharmacological tools. Transgenic provision of DNA-binding-deficient mutant AIF rescued the Hq thymic defect, while the oxidoreductase-deficient mutant AIF did not do so. Also, treatment of Hq mice in vivo with an SOD/catalase mimic rescued both the thymic defect and the enhanced DN3 and DN4 cell death in vitro, and Hq DN thymocytes showed high ROS levels. Thus, AIF-deficient thymocytes are defective in the efficiency of beta-selection due to a failure to handle ROS stress leading to enhanced cell death and reduced proliferation.

Curiously, despite the ubiquitous expression of AIF in all cell lineages, AIF-hypomorphism causes tissue-specific defects. Similarly, while we observe a thymic defect, the B cell lineage shows no heavy-chain selection defect in Hq mice, raising the question of the cell-type specificity of this defect, since proliferation during heavy chain-selection in the B cell lineage is as substantial as during thymic beta-selection. Consistent with our model of a connection between ROS stress and tissue defects in AIFIF-hypomorphism, B lineage cells in Hq mice show no evidence of enhanced ROS stress, suggesting that the T cell lineage, which uses ROS for developmental signaling, is more susceptible to the effects of AIF-hypomoprhism than the B cell lineage is. This may address the lineage- and tissue- specificity of dysfunctional phenotypes observed in mice hypomorphic for a ubiquitously expressed protein like AIF.

Publications

Original peer-reviewed papers

1. Chatterjee P, Tiwari RK, Rath S*, Bal V*, George A*. (2012) Modulation of antigen presentation and BCR signaling in B cells of beige mice. **J Immunol 188:**695-702. [*Co-corresponding authors].

#2. Prabhu SB, Gupta P, Durgapal H, Rath S, Gupta SD, Acharya SK, Panda SK (2011) Study of cellular immune response against Hepatitis E virus (HEV). **J Viral Hepat. 18:**587-94.

In Press last year, since published

Understanding the Role of Interferon Regulatory Factors in Dendritic Cell Development and Innate Immunity

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Dendritic cells (DCs) are composed of multiple subsets that collectively provide early innate immunity, leading to subsequent adaptive immunity. Plasmacytoid dendritic cells (pDC), $CD4^+$ DC, $CD8\alpha^+$ DC and $CD4^-CD8^-$ DC are four major subtypes in the murine spleen. These subtypes of DCs express different sets of genes and assume distinct functions. We are interested in understanding the mechanisms of development of DC subsets and their functions. Members of Interferon regulatory factors (IRFs) not only play important role in DC subset development but also in their functions. Main area of research is to understand the significance of different signaling pathways and contribution of IRFs and other critical transcription factors in DC subset development and functions.

The principal aim of project is to understand the role of IRF family members in the dendritic cell (DC) development and functions. Interferon regulatory factor 4 (*Irf4)* and Interferon regulatory factor 8 (*Irf8)* plays pivotal role in generation of diverse DC subtypes. The development of $CD8\alpha^+$ DC and pDC requires *Irf8*, whereas $CD4^+$ DC subset is dependent on *Irf4*. Recent reports suggest an important role of Inhibitor of DNA binding 2 (*Id2*) and Basic leucine zipper transcription factor (*Batf3*) in the $CD8\alpha^+$ DC development. Objectives of the current study are 1] to understand the significance of *Irf8*, *Id2* and *Batf3* in DC development, 2] to understand functional significance of different domains of *Irf4* and *Irf8* in regulating diversity in DC development, 3] to understand contribution of TGF-β signaling and *Irf8* in DC development, 4] to study the *Irf4* and *Irf8* transcription complexes.

Understanding the significance of Irf8, Id2 and Batf3 in DC development

Our previous observation suggested that besides induction of *Id2* and *Batf3* genes; *Irf8* plays an essential role in development of $CD8\alpha^+$ DC development. *Id2* expression in the haematopoietic precursor cells abrogates pDC development and hence we examined the effect of *Id2* and *Batf3* expression on the pDC specific gene transcription. Co-expression of *Id2* and *Batf3* leads to the decrease in the surface expression of CD11c and pDC specific marker SiglecH. Though, *Id2* and *Batf3* when co-expressed with *Irf8* lead to synergistic increase in the $CD8\alpha^+$ DC

specific transcripts; such synergistic effect was not extended to the pDC specific gene expression. We noticed the decrease in the expression of *Siglech* gene when *Id2* and *Batf3* expressed together and also when co-expressed with *Irf8*. Co-expression of *Id2* and *Batf3* in the mouse bone marrow DC cultures lead to very low SiglecH expression and decrease in $B220^{+}MacI^{low}$ pDC population. A recent study of *Nfil3* (nuclear factor, IL-3 regulated; also called E4BP4) gene knock out mice suggested that it indirectly controls the $CD8\alpha^{+}$ DC development by regulating *Batf3* gene expression. We noticed that the expression of *Irf8* in the DC9 cells lead to modest yet reproducible 2 fold increase in the *Nfil3* transcript levels. Expression of *Nfil3* in DC9 line lead to expression of some of the $CD8\alpha^{+}$ DC specific gene expression though *Irf8* expression could induce complete profile of $CD8\alpha^{+}$ DC phenotype. Thus, taken together our data suggests that *Irf8* is a master regulator of $CD8\alpha^{+}$ DC development. To examine the interaction between *Irf8*, *Id2* and *Batf3,* we performed the mammalian two hybrid experiments and our preliminary results suggest that *Irf8* may not be directly interacting with *Batf3* and *Id2*. To further understand the regulation of *Id2* and *Batf3* gene expression in DCs, we cloned *Id2* and *Baft3* promoter driven green fluorescent protein expression in self inactivating retroviral vectors. Our preliminary data suggests that expression of *Irf8* lead to modest increase in the GFP signals from *Batf3* and *Id2* promoters. Currently, we are performing the in-depth experiments to identify the *Irf8* binding sites in these promoters.

Understanding the significance of different domains of Irf4 and Irf8 in regulation of DC development and functions

Among the IRF members *Irf4* and *Irf8* are closely related to each other; yet, together *Irf4* and *Irf8* control the diversity in dendritic cell development. Reported literature suggests that C-terminal domain of *Irf4* is inhibitory to association with other proteins whereas same domain in *Irf8* is not. Hence, to better define *Irf4/8* molecular domain important for their specific function leading to DC diversity; we exchanged the C-terminal domains between these two transcription factors. We confirmed that exchanging C-terminal domains between *Irf4* and *Irf8* does not affect their specificity in terms of transcriptional activities. Genes that are selectively up-regulated by *Irf8* are not affected by exchanging C-terminal region from *Irf4*. Exchanging C-terminal region in *Irf4* with that from *Irf8* didn't lead to change in specificity of gene transcription. We are currently performing experiments with chimeric constructs of *Irf4* and *Irf8* by exchanging DNA binding domain (DBD) and IRF association domain (IAD) to understand the effect of these exchanges on the transcriptional specificities leading to DC diversity.

Understanding the role of TGF-β signaling in Irf8 regulated DC development

TGF-β signaling plays very important role in dendritic cell development. Mice deficient for TGF-β lack Langerhans Cells (LCs), a bone marrow derived epidermal DCs. Recent reports suggest that TGF-β stimulation of the DC leads to induction of *Id2* and *Irf8;* transcription factors essential for $CD8\alpha^{+}$ DC development. Treatment of the dendritic cell cultures with different concentrations of TGF-β (1 - 20 ng/ml) for 8 hrs lead to the dose dependent increase in the TGF-β inducible gene transcripts (*Smad7*, *Tgfbi*). Transcription of TGF-β induced genes is suppressed in presence of the TGF-β signaling inhibitor SB431542; thus establishing the culture conditions to further in-depth study the contribution of TGF-β signaling and *Irf8* in DC biology. To study the role of TGF-β signalling in the *Irf8* directed DC development, we have prepared the constructs expressing *Smad7* (inhibitor of TGF-β signalling), DNsmad7 (prolonging the TGF-β signalling) and DNTGFBRII (inhibitor of TGF-β signalling). Preliminary findings from *in-vitro* cultures of DC cell line as well as mouse bone marrow DC cultures will be confirmed by using mouse model.

Biology of T lymphocytes

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The theme of the ongoing projects for this reporting year is T cell and macrophage fate decisions encompassing differentiation, proliferation, survival and death.

1. To understand the regulatory mechanisms involved in cell intrinsic differentiation of CD4 cells in Th1 and Th2.
2. To characterise the effects of *in vivo* aging on CD4 T cell function and phenotypic features.
3. To study mechanisms of Japanese encephalitis pathogenesis using murine model.

1. To understand the regulatory mechanisms involved in cell intrinsic differentiation of CD4 cells in Th1 and Th2

We further characterised our findings from last year which showed that NCD4 cells from B6 mice when activated under neutral conditions produce high levels of IFNg but very little IL-4 whereas cells from BALB.b mice produce both IFNg and IL-4. When we looked at the factors during priming conditions which could possibly affect the secondary response, it was observed that NCD4 cells from BALB.b mice produced very high levels of IL-2 during primary activation state as compared to B6 T cells. Relatively low production of IL-2 is also reflected in poorer cell yields from B6 cell cultures as compared to BALB.b. If low amounts of IL-2 [3-5U/ml] are added exogenously this defect in primary proliferative response is not only partially compensated, but it also has an effect in the secondary response outcome. Thus addition of high [10U/ml] amounts of exogenous IL-2 in priming cultures results in B6 T cells also showing a tendency to produce IL-4 unlike in the absence of IL-2. This, can possibly be correlated

to the number of cycles a given T cell can undergo before it starts producing IFNg and IL-4. But *prima facie* it appears that activated cells start producing IFNg very early, whereas larger number of cell divisions are needed before IL-4 production can take place. Availability of IL-2 can ensure continuation of cell divisions and hence possibly IL-4 secretion. The role of IL-2 described here is novel and not reported so far. We have also observed that a high frequency of BALB.b cells show presence of intracellular t-bet and GATA-3 transcription factors in contrast to B6 T cells which show very low proportions of GATA-3 - correlating with the observations on cytokine secretion reported last year.

Another aspect of Th1/Th2 differentiation studied is using CD4 cells from TCR Tg mice. CD4 T cells from DO11.10 mice recognise Ova-II peptide in association with I-Ad where as the same peptide is recognised by OT-II mice in association with I-Ab. Since DO11.10 mice are from BALB.c background and OT-II from B6 background, these T cells also provide useful tools for understanding differentiation. Similar to activation of NCD4 T cells from B6 mice, in response to peptide-pulsed dendritic cells, OT-II T cells secrete dominantly IFNg and minimal IL-4 whereas DO11.10 T cells secrete both IFNg and IL-4. We further dissected the role of peptide-MHC ligand density in the differentiation and polarisation of CD4 T cells. When DO11.10 T cells were primed with peptide doses differing by one log and recalled with identical doses of peptide, higher priming dose of the peptide brought about more IL-4 secretion than the lower priming dose, indicating that peptide-MHC ligand density may have a role to play.

We have also begun to analyse the endogenous potential of human naive CD4 T cells to response to TCR-mediated activation and the ability to secrete IFNg and IL-4,-5,-13 cytokines along lines similar to that described for mouse T cells. This is primarily attempted in the context of patients of nephrotic syndrome [NS] and appropriate healthy controls. There is a certain body of data which ascribes Th1-polarised and/or Th2-polarised T cells as contributors to pathology and hence recovery from NS. Because of lack of clarity in the published data we are attempting to see if the endogenous differentiation potential of human NCD4 cells activated with neutral stimulation can in any way correlated to the outcome of disease in NS patients. Standardisation using adult volunteer blood is done using the principles emerging from the mouse experiments. Collection of blood samples from patients is in progress.

2. To characterise the effects of in vivo aging on CD4 T cell function and phenotypic features

Last year we had shown that OT-II TCR Tg cells transferred in B6 congeneic mice and parked for more than 1 month showed phenotypic and functional features similar to NCD4 cells from aged mice. In contrast OT-II cells parked for <2 weeks in vivo showed features similar to NCD4 cells from young mice. In addition to sharing phenotypic features such as lower CD4 and TCR intensity and smaller cell size in long parked cells and in NCD4 from aged mice, functionally also long-parked OT-II cells behaved like NCD4 T cells from aged mice. Long parked OT-II cells responded poorly to their cognate-ligand based activation as compared to short-parked OT-II cells. Further longer duration of parking seemed to result in more accumulation of reactive oxygen species [ROS], as detected by DCFDA [2h-7h-dichlorofluorescin diacetate] than short-parked cells thus reinforcing the earlier finding that mitochondrial accumulation of ROS is a possible contributor to the functional senescence of T cells.

Earlier we had reported that NCD4 cells in young mice showing low CD4 intensity [gated at ~10% of the cell population] respond poorly to TCR mediated activation as compared to NCD4 cells showing highest CD4 intensity, thus allowing speculation that low CD4 expressing NCD4 cells may represent senescent fraction of the population. These data were reported in mouse population. There are reported differences in thymic output, peripheral homeostatic proliferation potential of NCD4 T cells between mice and humans. In order to see whether the differences observed in NCD4 cells with low and high intensity in mice is also evident in humans, we electronically sorted lowest and highest CD4 staining population [about 10%] in NCD4 compartment in peripheral

blood of healthy adult volunteers and activated them with anti-CD3+CD28. Preliminary results indicated that low staining NCD4 T cells in human peripheral blood also respond poorly to activating stimulus as compared to high staining cells.

3. To study mechanisms of Japanese encephalitis pathogenesis using murine model

West Nile Virus [WNV] and Japanese Encephalitis Virus [JEV] belong to the same family, flaviviridae, but there is a difference in their pathogenesis observed during the course of human infection as well as in experimental murine models. A lot of work is done on WNV in murine models but relatively limited work is done on JEV. Primary reason for lack of understanding, we feel, is absence of chronic disease model in mice. We have attempted to set this up based on the published literature that T cells play significant but not well-characterised role in JEV pathology. Using mice which lack α/β TCR bearing T cells [TCRβ-/- mice] we observe that 28 d old mice infected with JEV show a prolonged course of disease and >90% of the mice die at the end of 28 days post-infection in contrast to WT mice which survive infection well. Further work will be undertaken to characterise the role of CD4 and CD8 T cells as well as T cell cytokine producing abilities in this model.

Publications

Original peer reviewed articles

1. Chatterjee P, Tiwari RK, Rath S*, Bal V*, George A*. (2012) Modulation of antigen presentation and BCR signaling in B cells of beige mice. **J Immunol 188:**695-702. [*Co-corresponding authors].

Reviews / proceedings

1. Bal V (2011) Too much information? **Himal 24**:112-113.

Biology of Animal viruses

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The laboratory studies the biology of medically important viruses with a view to understand their replication to develop novel therapeutics and prophylactics. The viruses under investigation are: Japanese encephalitis, Chikungunya, and Rotavirus.

The broad scientific objectives of the laboratory that are currently being pursued are :

A. ***Japanese Encephalitis Virus***: Japanese Encephalitis virus (JEV), a flavivirus, is a major cause of human encephalitis and is responsible for considerable mortality and morbidity in India. We are studying virus replication and are interested in developing novel vaccine and therapies against Japanese encephalitis.

B. ***Chikungunya Virus:*** Chikungunya fever, caused by alphavirus named Chikungunya virus (CHIKV), causes substantial morbidity and economic loss. We are interested in studying CHIKV replication and development of novel vaccine and therapeutic candidates against the virus.

C. ***Rotavirus***: In India, one child in 250 will die from rotavirus diarrhea and nearly 125,000 rotavirus attributable deaths occur among children under fives annually. The development and introduction of a rotavirus vaccine, therefore, has been accorded high priority globally. We are involved in the clinical development of an oral rotavirus vaccine 116E.

Japanese Encephalitis Virus

We used electrophoretic mobility shift assay (EMSA), UV-cross linking, North-western analysis and super shift assay to demonstrate that the 55 kDa PTB interacted with both 5'-NCR and 3'-stem loop of 3'-NCR of the viral genomic RNA, albeit with different affinities. The site of proten-RNA interaction was further delineated by RNA toe-printing assay. We also validated and measured this interaction *in vivo* upon natural JEV infection in cell lines by co-immunoprecipitation followed by RT-PCR, and by colocalization of viral RNA and PTB by confocal microscopy. Interestingly, we observed relocation of nuclear PTB to cytoplasmic foci that co-localized with JEV RNA during early stages of JEV infection. Finally we demonstrated the role of PTB

during viral replication by measuring viral titers in knockdown and overexpression systems.

Chikungunya Virus

Attempts were made to develop an animal model to test the efficacy of the candidate vaccine. Several different strains of mice in different age groups were inoculated with CHIKV. C57BL/6 mice when inoculated with CHIKV intra-muscular or sub-cutaneous developed short-lived viremia and swelling of the toe joints. CHIKV could be found in the joints only during the early phase of infection.

Rotavirus

Phase III efficacy trials of 116E vaccine were initiated this year. This is a randomized, double-blind, placebo-controlled trial with the primary objective of evaluating the efficacy of three doses of ORV 116E, $10^{5.0}$ ffu, against severe rotavirus gastroenteritis, occurring at least 14 days following the 3rd dose of the test article. Three doses of ORV 116E are being co-administered with routine childhood vaccines (Pentavalent vaccine, OPV) at 6-7 weeks, ≥10 weeks and ≥14 weeks of age. 6800 subjects have been enrolled in three sites - Delhi, Pune (Maharashtra) and Vellore (Tamil Nadu) and will be followed up till the age of 2 years. Multiple trial sites are included to ensure that the vaccine works in different geographical settings in India. Efficacy outcomes are measured through ascertainment and documentation of all episodes of gastroenteritis occurring from enrollment till the age of 2 years. The vaccine immunogenicity will be assessed through a four-fold rise in rotavirus specific serum IgA antibody titers 4 weeks after the third dose in a subset of subjects. Virus shedding will also be assessed on days 0 (prior to administration), 3 and 7 in the "Immunogenicity and Viral Shedding Subset". A GLP-compliant lab with quality controlled processes has been set up with trained manpower at THSTI. ELISA for the rotavirus antigen in the stool samples and RT-PCR assay for virus genotyping have been validated and are in use. Validation of the IgA assay for the vaccine immunogenicity is ongoing.

Publications

Original peer-reviewed articles

1. Anantpadma M, Vrati S. (2011) siRNA mediated suppression of Japanese encephalitis virus replication in cultured cells and mice. **Journal of Antimicrobial Chemotherpay 67**:444-451.

#2 Vashist S, Bhullar D, Vrati S**.** (2011) La protein can simultaneously bind to both 3' and 5'-noncoding regions of Japanese encephalitis virus genome. **DNA and Cell Biology 30**:339-346.

Reviews / Proceedings

1. Bharati K, Vrati S. (2012) Viral vaccines in India : An overview**. Proc. Natl. Acad. Sci. Sect B. Biol. Sci. 82:** 181-198.

In Press last year, since published

Plasmodium proteins involved in virulence and host modulation: Host-Parasite interactions in Plasmodium Liver stages

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Plasmodium species introduce effector molecules into hepatocyte cytosol to manipulate host metabolic and /or signaling pathways for its own benefit. These could prove as good targets for drug development. Parasite kinases, phosphatases and similar molecules targeted to hepatocytes are likely candidates. The host processes affected by them could also be target for intervention. The specific aim is to identify new parasite molecules that affect the host cellular processes and possible intervention strategies. Currently primaquine is the only drug available for malaria liver stages (LS) but it can't be administered to pregnant women and people with G6PD deficiency as it causes toxicity. Alternative drugs are the need of hour. Drugs can be targeted against parasite, as was traditionally done in the past, or based on new information now even host processes may be targeted. Objective of this study is to identify new parasite derived proteins that are involved in host modulation, and are essential for parasites to grow and complete their life cycle. Using genetic, cell biology and biochemical methods, we identified that *Plasmodium* circumsporozoite protein (CSP) is introduced in the hepatocyte cytoplasm and hepatocyte nucleus, and alter thousands of host transcripts. The overall effect is improved liver schizont growth. The current project aims to identify more such parasite proteins like CSP that play role in liver schizont development. Information about the newly identified proteins interaction with host cell will provide opportunity for developing new interventions.

We are working on liver stage parasite proteins that are likely to be exported to hepatocytes cytosol. Currently, we have selected a total of nine proteins. These proteins were selected based on:- a) expressed during sporozoite /liver stage, b) the presence of pexel motif and c) conservation across the plasmodium species. These proteins are of completely unknown function and needs to be characterized for their function and localization in the parasite.

Two proteins (PB871, PB823) were produced in *E. coli* and purified to > 95% purity. These proteins were characterized by biophysical methods. Circular dichroism (CD) analysis showed presence of secondary structural elements indicating proteins are folded most likely in native state. Gel permeation chromatography (GPC) confirmed that these proteins exists as large soluble multimers. GPC data also concur with dynamic light scattering (DLS) findings.

Antibodies were raised in mouse against purified and *E. coli* produced PB871, PB 823 and PB 122500. Using these antibodies

in an IFA, we confirmed the expression of respective proteins in sporozoites (Figure-1) and liverstage parasites (not shown).

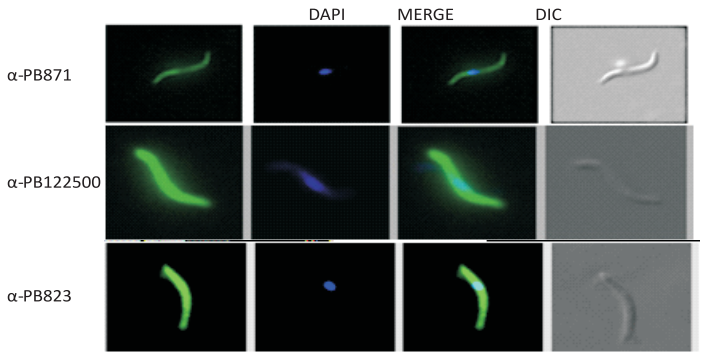

Figure-1: *Immunofluorescence assay (IFA) on wild type sporozoites using antibodies raised against respective E.coli produced proteins. Images show expression of respective proteins in the sporozoites.*

Using purified proteins Pb 871 and Pb 823, we also immunized mice three times. These proteins generated strong antibody response (titer ~3 million). Immunized mice were challenged with 10000 WT sporozoites, two week after the last booster, to access the protection. Both proteins offered protection as determined by 4 days delay in pre-patient period, which corresponds to four-log reduction in initial parasites burden. Four-log reduction in parasite burden is very significant level of protection. Interestingly, when both proteins (pb871 and pb 823) were used together to immunize mice, the protection offered against sporozoite challenge was similar to what is observed during sterile protection in whole parasite based vaccine. We followed up mice for 30 days after challenge and did not see any blood stage parasite while the control mice show blood stages by day three after challenge (Table-1).

Last year we made three parasite gene knockout lines. We have characterized two of them phenotypically in mosquito and in mouse. PB871 Knockout (KO) grew normally in mosquito in the same way as wild type (WT). Pb871KO sporozoites when injected in mouse, there was approximately 20 folds reduction in liverstage parasite growth as determined by measuring parasite 18SrRNA copy numbers Figure-2. By measuring pre-patient period we found at least 1 days delay in pre-patient period, which corresponds to one log reduction in initial parasite burden (Table-2).

Immunization	No. of Boost	Sporozoite used for challenge	Prepatient day (PPD)	Delay in PPD
None	Nil	10000	3	0
Adjuvant only	3	10000	3	0
Pb 871 full	3	10000	7	4
Pb 823 full	3	10000	7	4
823+ 871	3	10000	None till 30 days	>4 weeks

Table 1- Balb/C Mice (5 per group) were immunized three times with indicated protein (with CFA/IFA) and two weeks after last boost were challenged with 10000 wild type *P. berghei* sporozoites. Blood smears were prepared on daily basis, stained and parasitemia counted. First appearnace of blood stage parasite in each case was compared and delay in appearance of blood stage noted.

Parasite type	Sporozoite numbers used	Prepatient day (PPD)	Delay in PPD
Pb wild typr	6000	3	0
Pb 871 KO	6000	4	1
Pb 122500 KO	6000	6	2

Table 2- CD1 Mice (5 per group) were challenged with 6000 wild type P. berghei sporozoites. Blood smears were prepared on daily basis, stained and parasitemia counted. First appearnace of blood stage parasite in each case was compared and delay in appearance of blood stage noted.

PB122500 KO had defects in both mosquito stage as well as liverstage parasites. PB122500 KO produced only 25% sporozoites as compared to WT. PB122500 KO sporozoites when injected in mouse, there was approximately 100 folds reduction in liverstage parasite growth as determined by measuring parasite 18SrRNA copy numbers Figure-2. By measuring pre-patient period we found

at least 2 days delay in pre-patient period, which corresponds to two-log reduction in initial parasite burden (Table-2).

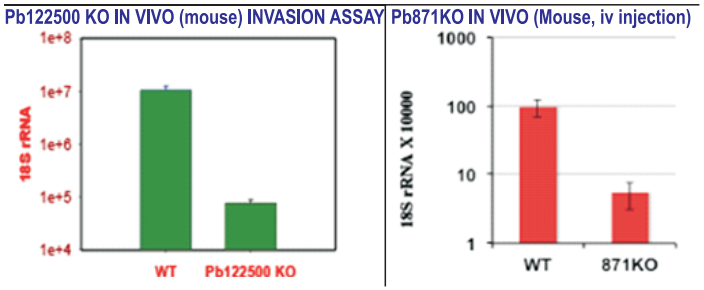

Figure-2: *Five mice in each group were injected with 6000 wild type or KO sporozoites. 48 hours later, liver was extracted and RNA prepared from liver homogenate. RNA was converted to cDNA and used for real time PCR. Parasite 18S rRNA specific primers were used for PCR. Using plasmid standards absolute copy numbers were measured. 18SrRNA is a liver stage parasite growth marker.*

Genetic and Functional Analyses of host and HIV-1 genes that affect progression of HIV-1 and development of nucleic acid based antiviral approaches

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HIV-1 /AIDS is a public health problem in India. HIV-1 infections in India is mainly due to the genetic subtype C and it is therefore important to understand how it is different than subtype B dominant regions like UK & USA. This involves constant monitoring of the nature of genetic changes in circulating HIV-1 genes from India and studying their functional implications. We also wish to develop nucleic acids and siRNA/miRNA based antiviral approaches against HIV-1 and other model virus genes.

HIV-1 infection causes global changes in variety of cellular genes. The various small ORFs like Nef, Vpu, Vif and Vpr are known to play a major role in pathogenesis. We wish to understand the role of accessory genes in modulating cellular genes important for causing pathogenesis mechanistically. To study HIV-1 protein + protein and HIV-1 protein + cellular protein interaction and their impact on overall HIV-1 gene expression and replication. We wish to design long lasting efficacious siRNAs + Ribozymes +Aptamers + DNA-enzymes with the purpose of interfering with HIV-1 gene expression that are targeted against B and C subtypes.

Novel Role of HIV-1 Vpu in pathogenesis

HIV-1 accessory protein Vpu is primarily involved with the degradation of CD4 protein and enhancement of virion release. It has also been associated with increased virulence and depletion of CD4 positive lymphocytes but the mechanism responsible for this phenomenon was not well understood. We show that Vpu stabilizes p53 protein by preventing its degradation which results in enhancement of apoptosis. Vpu expression inhibited β-TrcP dependent ubiquitination and proteosomal degradation of p53 protein which requires a functional SCF-BTRCP complex. The Vpu protein inhibited p53 ubiquitination by competitively

binding to B-TRCP. Further more, the apoptotic activity of p53 was remarkably up regulated by Vpu by introducing p53 protein in a p53 null cell line. The biological relevance of these findings was verified in T lymphocytes following HIV-1 infection. These findings establish a novel relationship between the Vpu and p53 protein which has implications in cell cycle arrest and enhanced Vpu + p53 proteins mediated apoptosis. We also carried out a comparative study on the ability of Vpu B and C with respect to its various functions. We observed that Vpu C always caused more apoptosis than subtype B Vpu which was not due to B-TrCP motif which is absolutely conserved. We constructed Vpu BC and Vpu CB –chimeric constructs to further delineate the apoptotic determinants. Our experiments related to apoptosis and Thetherin down regulation clearly suggest that they are governed by different regions. This work has been extended by genetically and functionally characterizing the natural variants of Vpu from North India. Both the N-terminal and C-terminal halves show extensive polymorphisms and functional characterization of these variants are currently being pursued. We came across a natural Vpu mutant (S61A). This mutation resulted in remarkably increasing intracellular stability. How these mutations affect virus release is currently being carried out.

Degradation of p53 by HIV-1 Nef

Nef is made early in HIV-1 infection in very large amounts and one of the remarkable properties is that it potently degrades p53. The mechanism with which it is accomplished has not been worked out. We carried out a comparative study of Nef B and Nef C with respect to its ability to degrade p53 intracellularly and if there is any difference in the kinetics. Our results clearly showed that Nef C carried out this function more efficiently. Since nothing was known about the mechanism of Nef-mediated degradation of p53, we tested several E3 ubiquitin ligases and found that E6AP ligase is recruited to carry out ubiquitination of p53 efficiently. This was concluded by several experiments including the mutant control E6AP which failed to ubiquitinate p53. We also now have evidence that Nef and E6AP ligase interact intracellularly by pull-down experiments.

Suppression of RNAi silencing & micro-RNA

We have earlier reported a sensitive mammalian cell based assay for monitoring the above activity. Using this system we have confirmed that HIV-1 Tat possesses this property. We now have preliminary evidence that even HIV-1 Rev also has this suppressive activity and currently working out the mechanism of suppression. These are important observations having direct implications for HIV-1 replication and pathogenesis. Since we have generated new information with respect to Vpu and p53, we are now asking whether p53 responsive micro-RNAS play any role in HIV-1 replication and pathogenesis.

Publications

Original peer reviewed articles

1. Kumar P, Khanna M, Kumar B, Banerjea AC (2012). A conserved matrix epitope based DNA vaccine protects mice against influenza A virus challenge. **Antiviral Res 93:** 78-85.

2. Verma S, Ali A, Arora A, Banerjea AC (2011) Inhibition of β-TrcP dependent ubiquitination of p53 by HIV-1 Vpu promotes p53 mediated apoptosis in human T cells. **Blood 117:** 6600- 6607.

Reviews/ Proceedings

1. Neogi U, Sood V, Ronsard L, Singh J, Lata S, Ramachandran VG, Das S, Wanchu A, Banerjea AC (2011) Genetic architecture of HIV-1 genes circulating in north India and their functional implications. **Indian J Med Res 134:**769-778.

Study of mucosal immune responses

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This laboratory is currently engaged in understanding events that influence the activation, differentiation and death of B lineage cells and in exploring ways of generating mucosal immunity to parenteral immunizations.

The objectives include a) dissecting signals that influence B cell differentiation into the plasma cell- and memory cell- pools, b) identifying molecules involved in plasma cell survival, c) identifying the relative roles of B cell antigen presentation and BCR mediated signal transduction in determining the final outcome of B cell triggering and d) generating IgA following systemic immunization.

Over the current reporting year, we have continued our studies on B cell mediated antigen presentation, using the *beige* mouse as a model. Using confocal microscopy, we have shown earlier that a significant fraction of endocytosed Ag-BCR complexes in Bg B cells shows poor colocalization with LAMP-1+ compartments when tracked for 2 h. We have now carried out similar experiments with LPS-activated B cells and tracked colocalization of the BCR with LAMP-1+ compartments for as long as 12 h. While 70 % of WT BCR is in lysosomal compartments by 2 h, only 32-41% of Bg BCR colocalized with LAMP-1 even at 12 h. Thus, delivery of the endocytosed BCR to lysosomes in Bg B cells does not improve even in activated cells. When antigen processing occured in the presence of the lysosomal inhibitors E64d, NH4Cl or bafilomycin A1, presentation by Bg B cells was higher than by WT cells, confirming that the bulk of endocytosed Ag is, indeed, outside lysosomes in Bg B cells. Since antigenic cargo in early endosomes can theoretically associate with recycling as well as on newly synthesized MHC-II molecules, we added the protein synthesis inhibitor cycloheximide to restrict presentation to recycling MHC-II molecules. We found that cycloheximide almost completely abrogated presentation of BCR-targeted OVA by B cells from both strains of mice indicating that while presentation by Bg B cells occurred only on newly synthesized MHC-II molecules.

Last year, we reported that the delayed antigen presentation by Bg B cells did not affect their ability to compete with wild-type B cells for T cell help in vitro. Over the current reporting year,

we have set up competition experiments in vivo. To do this, we generated a mouse strain that expresses CD45.1 and makes Ig of the "a" allotype (B6.SJL.C-20) as opposed to the Bg mouse, which expresses CD45.2 and makes Ig of the "b" allotype. Two sets of mixed bone marrow (BM) chimeras were then made where either WT or Bg BM (both CD45.2, Igh^b) was mixed 1:1 with B6.SJL.C-20 BM (CD45.1, Igh^a). This ensured that either WT or Bg cells making Igh^b would be in competition with WT cells making Igh^a in the periphery. Reconstituted mice that showed approximately 50% chimerism were immunized with NP-OVA on alum, anti-NP IgG of each allotype was tracked over time, and the ratio of the respective responses (b/a allotype) calculated and normalized for the degree of chimerism. Surprisingly, we found that Bg B cells responded better in such competition scenarios, indicating that optimal B cell antigen presentation for rapid recruitment of T cell help in vivo is not a crucial limiting factor in determining the efficacy of B cell priming to T-dependent antigens.

One possible explanation for the higher response of Bg B cells following immunization with T-dependent Ags is that delayed transport of the endocytosed Ag-BCR complex to lysosomes may allow for prolonged signaling downstream of the BCR and more than make up for any effects related to delayed antigen presentation to T cells. We tested this by looking at calcium flux and phosphorylation of signaling intermediates following BCR ligation. We found equivalent calcium flux in the two cell types and this is not surprising as the density of surface BCR is similar in the two strains and events that occur within seconds of BCR ligation are unlikely to be affected prior to receptor internalization. However, p38, JNK and ERK showed either higher phosphorylation or remained phosphorylated for longer periods in Bg B cells by Western blot assays and phosphoflow assays. No differences were seen in the phosphorylation of PLCγ. Bg B cells also proliferated better to lower levels of BCR crosslinking. These data indicate that BCR signaling is amplified in Bg B cells and that this can lead to better proliferation in the presence of limited amounts of antigen. Together with data reported last year, our findings indicate that the strength and/or duration of signaling downstream of the BCR can affect B cell differentiation events, favoring entry of activated cells into the memory-and long-lived plasma cell-pool. They also indicate that relatively small numbers of T cells can provide adequate help for B cell function and that short delays in B cell antigen processing and presentation for availing cognate T cell help do not significantly impair B cell activation or proliferation.

Over the current reporting year we have also been looking at sites of memory B cell residence in vivo, and their recruitment following challenge at distal sites. Successful recall Ab responses require recruitment of quiescent memory B cells to secondary lymphoid organs and hence entry into peripheral lymph nodes (LNs) can be crucial for secondary responses. Regardless of the route of primary immunization, most studies to date have relied on i.p. challenge with soluble Ag to elicit secondary responses - a route that likely allows Ag to access all systemic lymphoid tissues. However, whether a local challenge at a site that is distal from the initial Ag encounter and with restricted potential for systemic dissemination will evoke a secondary response has not been tested. Further, it is not clear whether memory B cells can persist in LNs draining the site of initial Ag encounter or whether systemic memory B cells are largely spleen- and BM- resident. We have addressed these issues over the last year and we report that switched memory B cells do not express CD62L, and that sorted CD62L-ve cells transferred intravenously into adoptive hosts cannot enter LNs unless LPS-mediated inflammation is induced there. Using reciprocal transfer experiments with cells from LPS-responder an non-responder strains of mice, we have shown that functional TLR4 is required on the B cells as well as on non-B cells in the LN to achieve full recruitment. Physiological significance for these findings comes from the further observation that memory B cells generated following immunization in one footpad can generate secondary Ab responses to intraperitoneal challenge with soluble Ag but not to s.c. challenge in the contralateral footpad unless LPS is co-administered. To address the issue of where B cell memory is resident, we tracked Ag-specific cells in vivo and found that while large numbers are seen in the draining

LNs 2 wks after immunization, the numbers here drop 50-fold by 17 wks. In contrast, while the spleen has smaller numbers to start with, the decline here is only 5-fold at 17 wks. Memory B cells were unidentifiable in the bone marrow and contralateral LNs and together the data indicate that the bulk of B cell memory that persists after the primary immune response has died down is spleen-resident and can be recalled following i.p. challenge with soluble Ag or following s.c. challenge in the presence of LPS at a distal site. Direct confirmation of these observations came from further experiments showing that splenectomized mice fail to respond to inflammatory s.c. challenge in contralateral footpads while lymphadenectomized mice lacking the original draining lymph nodes do. Splenectomized mice also fail to respond to i.p. challenge with soluble Ag, indicating that the bone marrow contributes very little to secondary responses in vivo. Together, our results indicate that unlike the central memory pool of T cells that circulates through resting lymph nodes, the majority of long lived memory B cells are spleen-resident and that they require inflammatory signals for mounting recall responses at distal challenge sites.

Publication

Original peer-reviewed article

1. Chatterjee P, Tiwari RK, Rath S*, Bal V*, George A*. (2012) Modulation of antigen presentation and BCR signaling in B cells of beige mice. **J Immunol 188:**695-702. [*Co-corresponding authors].

Analysis of *Salmonella typhi*-host cell interaction

Principal investigator
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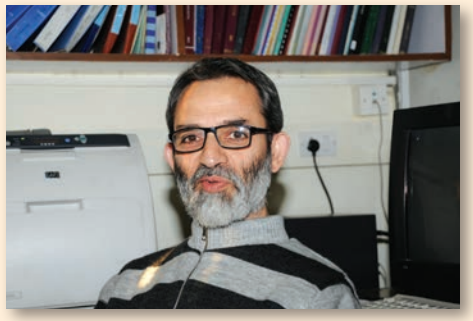

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Salmonella typhi and *Salmonella typhimurium* produce distinct clinical manifestations in humans and mice. *S.typhi* causes systemic infection, typhoid, in humans while *S.typhimurium* produces only self-limiting localized gastroenteritis. In mice, however, infection with *S.typhimurium* leads to a systemic disease that is analogous to human typhoid; *S.typhi* does not establish successful infection in mice. The reasons for different clinical manifestations produced by these two very closely related *Salmonella* serovars and the host specificity exhibited by them are not understood. Also, the mechanism by which pathogenic *Salmonella* overcomes host immune defenses in order to establish systemic infection is not clear. Work in our laboratory addresses these two important issues.

1. To decipher differences in host-pathogen interactions which ensue during infection with *S.typhi* versus *S.typhimurium*.

2. To understand modulation of immune responses during infection with pathogenic *Salmonella.*

Prohibitin-p^{56}lck-actin constitutes a dynamic trimolecular complex in T cells

We have prevsioulsy reported that the virulence polysaccharide Vi of *Salmonella typhi* can target membrane prohibitin in T cells to suppress T cell activation. Further studies revealed that prohibitin forms a complex with p^{56}lck and actin. Using Vi as a probe, we began to look at the dynamics of this complex during T cell activation. We now show that engagement of T cell receptor in the model human T cell line Jurkat with anti-CD3 antibody brings about time-dependent changes in this complex that are critical to T cell activation. Early on (15 min), signals transduced from the TCR bring about dissociation of this complex as revealed by immunoprecipitation with Vi/anti-Vi antibody complex or anti-lck antibody. This dissociation is not seen when T cells are activated with PMA suggesting that proximal intracellular signals generated specifically through the T cell receptor are required for this phenomenon. The dissociation of this trimolecular complex is accompanied by an increase in the kinase activity of lck as demonstrated by tyrosine phosphorylation at the activating Y394 and the *in vitro* kinase activity that was determined using a peptide derived from TCR ζ chain as the substrate. At 2 h post TCR ligation, prohibitin, lck and actin reassociate to form the complex. Preliminary data suggests that ligation of the TCR might bring about transient exit of prohibitin from the lipid raft. Importantly, activation of T cells with anti-CD3 antibody in the presence of Vi inhibits dissociation of the prohibitin-lck-actin complex and

prevents activation of T cells. These results provide novel insights into the mechanism of lck activation during TCR signaling. The exact mechanism which might bring about dissociation of the prohibitin-lck-actin complex is currently under investigation.

Salmonella flagellin restricts bacterial replication through a pyroptosis – independent mechanism

Flagellin brings about inflammatory and innate immune responses through activation of membrane TLR-5 and the intracellular sensor Nlrc4. Nlrc4 engagement with flagellin produces caspase-1 dependent pyroptosis resulting in death of infected macrophages accompanied by release of inflammatory cytokines IL-1β and IL-18. Pyroptosis is one of the key defence mechanisms against pathogenic *Salmonella*. We had previously shown that as the infection progresses *in vivo*, *Salmonella* loses the ability to bring about pyroptosis in freshly infected macrophages. This inability was associated with reduced expression of flagellin in the pathogen. Administration of flagellin to infected mice at the the time of flagellin downregulation resulted in significant reduction in bacterial load in the spleen although it did not prevent death of infected animals. In contrast, however, when splenic macrophages from infected mice were treated with flagellin *in vitro*, it resulted in increased bacterial replication. These results indicated that reduction in bacterial load *in vivo* following treatment with flagellin might not be due to activation of direct anti-bacterial killing mechanism in infected macrophages. We therefore investigated alternative mechanisms. Our results show that bacterial replication in splenic macrophages isolated from infected mice also increases when these cells are treated with cell culture supernatants derived from *S.typhimurium* - infected bone marrow. However, when bone marrow cells from infected mice are incubated with flagellin *in vitro*, their ability to promote bacterial growth in splenic macrophages is abrogated. Analysis of cytokines in flagellin-treated bone marrow supernatants showed upregulation of inflammatory cytokines including IFN-γ and IL-1β as well as anti-inflammatory IL-10. These data suggest that *Salmonella* might exploit host cues to sustain bacterial replication. Our findings also reveal a previously unappreciated mechanism by which flagellin might contribute to innate immunity against *Salmonella*.

lck regulates flagellin-induced inflammatory responses from human T cells

Pathogenic *Salmonella* species target intestinal epithelial cells, mononuclear phagocytes as well as lymphocytes during *in vivo* infection. These cells recognize conserved microbial entities from *Salmonella* such as LPS, flagellin, DNA through TLRs and secrete many cytokines and chemokines which participate in inflammation and immunity. The functions of TLRs and regulation of their functions have been well studied in intestinal epithelial cells and macrophages but not in T cells. Flagellin, a major proinflammatory determinant of *Salmoenlla*, readily induces secretion of neutrophil - chemoattractant CXCL8 from T cells. Here we show that secretion of this chemokine from human T cells is regulated by the src kinase $p56^{lck}$. CXCL8 secretion from Jurkat T cells in response to stimulation with flagellin is abrogated when cell stimulations are carried out in presence of src kinase inhibitor PP2. Consistent with this observation, lck deficient Jurkat cell line, JCam1.6 does not produce CXCL8 upon stimulation with flagellin. Flagellin was unable to activate many of the intracellular signaling intermediates in JCam1.6. CXCL8 secretion in response to flagellin is also not seen with Jurkat cells lacking tyrosine phosphotase CD45 which controls lck activation in T cells. On the other hand, Jurkat cells deficient in tyrosine kinase ZAP-70, which is a downstream substrate of lck, produces CXCL8 upon activation with flagellin. Preliminary analysis suggests that regulation of inflammatory responses by lck may not be restricted to TLR5 but may be seen with other TLRs as well. Lck is critical for initiating activation events through the TCR and the co-stimulatory molecule CD28. Our results suggest that in T cells, TLRs might share some of the proximal signaling events with the TCR and CD28. The involvement of lck in TLR signaling might also explain, at least in part, how TLRs might contribute to recently - proposed co-stimulatory activity of TLRs.

Molecular Basis of B cell Responses

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The theme of research is to decipher the molecular and cellular basis of immune responses against protein and polysaccharide antigens present on the cell surface of the human bacterial pathogen *Streptococcus pneumoniae* (also called pneumococcus). The other research interest is to find out how pneumococci cause disease and what interventions can be made to stop this from happening. The research is focused on the pneumococcal products and strategies that allow the pathogen to avoid being destroyed by the mammalian immune system, and the types of immune responses that can circumvent these strategies and products.

The main objectives are (a) molecular analysis of immune response to pneumococcal cell surface protein and polysaccharide antigens, (b) identification and characterization of virulence factors such as toxins and adhesins from *S. pneumoniae* that are or may be related to pathogenesis, (c) how these virulence factors interact with the immune system and host cell to alter its cellular and molecular processes, and (d) evaluating the vaccine potential of pneumococcal cell surface proteins.

SP_0845 is a conserved ribonucleoside binding protein that protects mice against *S. pneumoniae*

Immunization with SP_0845 protects mice against intraperitoneal challenge with heterologous pneumococcal strains

To evaluate the ability of SP_0845 to induce protective immunity, 12 BALB/c mice were immunized subcutaneously with SP_0845$^{23\text{-}350}$, SP_0845$^{1\text{-}350}$ or saline formulated in alum and challenged intraperitoneally with a lethal dose of heterologous virulent strain ATCC 6303 (10^5 cfu) or ATCC 6314 (5×10^6 cfu). The survival of mice was monitored for 21 days. Among the animal set challenged with ATCC 6303, 50% (6 out of 12) of mice in the SP_0845$^{23\text{-}350}$ immunized group survived for > 21 days whereas all the mice in the control group who received saline with adjuvant died within 2 days. The median survival time of the SP_0845$^{23\text{-}350}$ immunized and control groups were 15.5 and 1.5

days, respectively. Similarly, in case of the set challenged with ATCC 6314, 75% of the SP_0845$^{1-350}$ immunized mice survived for > 21 days whereas only 25% (9 out of 12) of the mice in the control group were alive at day 21. This was comparable to the data obtained with PspA where 8 out of 12 (66.7%) mice survived > 21 days. Seven of the 12 mice in the control group succumbed to the infection within 48 h. The median survival time of the SP_0845$^{1-350}$ immunized and control groups in the set challenged with ATCC 6314 were 21 and 2 days, respectively. The active mouse protection experiments suggest that SP_0845 is capable of inducing protective immunity in mice against intraperitoneal challenge with heterologous virulent pneumococcal serotypes and the protection conferred was comparable to that observed with PspA.

Intranasal immunization with SP_0845 partially reduces nasopharyngeal colonization with pneumococci

To assess whether SP_0845 can prevent nasopharyngeal colonization, 5 BALB/c mice were immunized intranasally thrice with SP_0845$^{1-350}$ or PBS with cholera toxin and colonized with pneumococcal strain D39 (10^5 cfu). Serum was obtained and SP_0845 specific serum antibody endpoint titer was determined by ELISA. Mice immunized intranasally with SP_0845$^{1-350}$ with cholera toxin induced IgA antibodies in nasal wash, and IgG1 and IgG2b in serum. The colonization data revealed that the mean cfu/ml recovered from mice immunized with SP_0845$^{1-350}$ was ~4 times less in mice that received saline with cholera toxin (132 versus 662). This experiment suggests that intranasal immunization with SP_0845 induces antibodies in both nasopharynx and serum, and partially reduces the nasopharyngeal colonization with pneumococci in mice.

Based on the accessibility of SP_0845 and its ability to elicit protective immunity, we suggest that SP_0845 may be a promising candidate for inclusion in a future multi-component protein-based pneumococcal vaccine.

Bioinformatics analysis of SP_0845 and RT-PCR based operon analysis

Having demonstrated that SP_0845 may be a potential vaccine candidate we set out to investigate its physiological role. The SP_0845 protein harbours a 22 amino acid signal peptide at its N-terminus, characteristic of lipoproteins. This signal peptide is cleaved off and the cysteine at position 22 forms a diacylglyceryl linkage with the lipid moiety on the pneumococcal cell surface. Structure-based homology modeling revealed similarity with a purine nucleoside receptor (PnrA) from the obligate bacterial pathogen *Treponema pallidum*. SP_0845 shares 39% amino acid sequence identity with PnrA. Sequence alignment of SP_0845 with PnrA protein showed that 7 out of 9 ligand (inosine) binding residues are identical in SP_0845. The other two ligand binding residues of PnrA Ser-13 and Met-143 are substituted by Thr-46 and Ile-176, respectively in SP_0845. The bioinformatic analysis suggested that SP_0845 is a lipoprotein which may have a role in binding nucleosides. Analysis of the genomic region surrounding *sp_0845* indicated that adjacent genes are either components of ABC transporter [ATPase (*sp_0846*) and permeases (*sp_0847* and *sp_0848*)] or they are involved in nucleoside metabolism [putative pyrimidine phosphorylase (*sp_0842*), deoxyribose aldolase (*sp_0843*) and putative cytidine deaminase (*sp_0844*)].

The genetic context and similarity searches indicated potential transcriptional association between *sp_0845* and its adjacent genes. In order to define the operon that encodes *sp_0845*, an RT-PCR based analysis was performed. Primers were designed to amplify the junctions of the genes in the neighbourhood of *sp_0845*. RT-PCRs were performed using total RNA isolated from D39 to determine which genes are cotranscribed with *sp_0845*. RT-PCR products were obtained for the junctions of consecutive genes from *sp_0841* to *sp_0848*. RT-PCR products were absent for junction between *sp_0840* and *sp_0841*, and *sp_0848* and *sp_0849*. These two gene pairs are contiguous in the genome as

indicated by genomic PCR. Taken together, the operon analysis suggests that *sp_0845* is a part of the operon that encodes an ABC transporter that could be involved in uptake and metabolism of nucleosides.

SP_0845 mediates import of ribonucleosides

To study the role of SP_0845 in pneumococcal physiology, a *sp_0845* deficient mutant of D39 was constructed using an overlap PCR based strategy. In order to genetically complement the mutant, *sp_0845* gene was cloned in the shuttle vector pDC123 and transformed in the *sp_0845* deficient mutant. To check for the expression of SP_0845, lysates from wild type, *sp_0845* deficient mutant, genetically complemented or vector transformed mutant strains of D39 were resolved by SDS-PAGE. The immunoblot analysis using anti-SP_0845 polyclonal sera indicated that SP_0845 was expressed in wildtype and genetically complemented mutant as indicated by the presence of a band at the expected size (~40 kDa). The expression level of SP_0845 in the wildtype and genetically complemented mutant was comparable. As expected no band was observed in lysates from *sp_0845* deficient and vector transformed mutant strains suggesting they are devoid of SP_0845 protein. The expression of another lipoprotein PpmA in the wildtype, *sp_0845* deficient, genetically complemented and vector transformed D39 strains was comparable.

In order to check the functional role of SP_0845, 5-fluorouridine, a toxic nucleoside analog was added in the culture medium. The growth of wild type, *sp_0845* deficient, genetically complemented and vector transformed strains was monitored spectrophotometrically at 6 h post-inoculation. The results demonstrated that wild type D39 was highly sensitive to 5-fluorourindine as growth was completely abolished in its presence. On the other hand, *sp_0845* deficient mutant was resistant to 5-fluorourdine as growth was not retarded in its presence. Genetic complementation of the *sp_0845* deficient mutant restored the sensitivity towards 5-fluorouridine to a significant extent but not to the wildtype level, confirming that the sensitivity to 5-fluorouridine was specifically due to SP_0845. *sp_0845* deficient mutant transformed with vector was resistant to 5-fluorouridine. The data indicates that 5-fluorouridine is mainly transported through SP_0845 in pneumococci.

To identify the natural ligands of SP_0845, a competition assay was performed wherein various nucleosides (adenosine, guanosine, cytidine, uridine, thymidine, deoxyadenosine, deoxyguanosine, deoxyuridine, deoxycytidine and inosine), nucleobases (adenine, uracil, thymine, cytosine, xanthine and hypoxanthine) and sugar (ribose) were allowed to compete with 5-fluorouridine. The sensitivity of wildtype strain to 5-fluorouridine is expected to reduce if other ligand competes for transport through SP_0845. The results indicated that cytidine, uridine, guanosine and inosine were competing better with 5-fluorouridine than other solutes tested as the growth of wildtype D39 was better in their presence. Deoxycytidine, deoxyuridine and thymidine also recovered the growth of pneumococci but to a lesser extent suggesting that they compete less efficiently with 5-fluorouridine. None of the nucleobases or sugar tested conferred resistance to 5-fluorouridine toxicity. These data indicate that SP_0845 interacts with ribonucleosides and is likely to be the substrate binding component of a multi-specificity ABC transporter. This is the first experimental demonstration of nucleoside transport by an ABC transporter in *S. pneumoniae*.

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Original peer-reviewed articles

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Patent

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Studies on immune response from antigen loaded biodegradable polymer particles and protein refolding from inclusion bodies

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The theme of the project is to evaluate polymeric particle based delivery system for improved immunogenicity of different antigens such as Tetanus Toxoid (TT), Hepatitis B surface antigen (HBsAg), viral and carbohydrate (Vi polysaccharide and *S. pneumoniae* polysaccharides) based vaccines. Another major research activity of the laboratory is the analysis of inclusion body formation and development of mild solubilization process for improved recovery of bioactive proteins.

The main objective of the project is to improve the immunogenicity of antigens entrapped in biodegradable polymer particles. High throughput refolding of inclusion body proteins into bioactive form is another objective of the research group. Research in the following areas are conducted in the laboratory to achieve the objectives :

1. Analysis of immune response from antigen loaded polymer particles and evaluation of adjuvant properties associated with polymeric particle formulation. Evaluation of memory antibody response using polymer particle based immunization.

2. Development of polymeric membrane as a scaffold for three dimensional growths of animal cells and its application as an artificial skin equivalent.

3. Solubilization and refolding of inclusion body proteins from *E. coli*. This involves analysis of inclusion body formation during protein expression and understanding of protein aggregation with an aim to recover higher amount of bioactive protein.

Immune response from polymeric particle formulations entrapping antigens

We have been reporting the improved immunogenicity of antigens by entrapping them in PLA particles. To further improve the immunogenicity of polymer particle entrapped antigens, particles were surface coated with soybean lectin (ligands for CLR) and evaluated for antibody response in mice. Soybean lectin coated particles not only improved the antibody response of polymer particle entrapped antigen but also resulted in high sustained memory antibody titer. Memory antibody titer achieved from immunization of particles coated with soybean lectin was much higher than that observed with immunization from admixture of particles and alum. This was proved using both TT and DT as model candidate vaccine antigen. Improvement in antibody response using lectin coated polymer particle was attributed to enhanced interactions of these particles with DCs. Man9 structure of soybean lectin helped in targeted delivery of antigen loaded polymer particles to DCs and resulted in enhancement of both primary and secondary antibody response from single point immunization. Surface coating of polymer particle having DC targeting ability is being currently explored for other antigens. Polymer particle formulation with surface coating of ligands for TLR/CLR provides an easy method to develop targeted delivery system without using chemical conjugation process.

Dry powder alum particle preparation for improving the immunogenicity of antigen or surface coating of polymer particles with alum has been standardized. The objective is to have single entity vaccine formulation for immunization. It was observed that dry powder alum retained almost its entire characteristic in powder form and show adjuvant activity similar to that observed with liquid alum preparation. Dry powder alum particles adsorbing antigen or entrapping antigen is being investigated to prove the suitability of such formulation for improving the immunogenicity of the antigens.

Extensive cellular interaction studies of particles formulations with DCs are being carried out in the laboratory. The purpose is to understand the presentation and processing of polymer particle entrapped antigen to APCs.

Formulation of large porous PLA particles for tissue culture and formation of polymer membranes

We have developed a process of making polymer membranes for wound healing using surfactant based fusion of polymer particles (ARTSKINII™). This involves fusion of polymer particles to form membrane like structure. To further improve the suitability of such polymer membrane for wound healing application, we have made gentamycin loaded polymer particles. Membrane made out of these polymer particles will have better wound healing application as they release antibiotic in controlled manner from the scaffold. Attempts are being made to entrap silver nanoparticle along with gentamycin so that these membranes can be evaluated for giving protection to infected wounds. These biodegradable polymer scaffolds have also been used to grow cancers cells /cell lines in three D culture *in vitro*. Scaffolds having high porosity have been formulated and under evaluation for the growth of different cell lines *in vitro*. Markers of tissue type structure such as collagen, secretion of metal proteases are being monitored from the cells grown on scaffold. Attempts are being made to grow cells inoculated on scaffold in spinner/bioreactor culture. Growth in controlled condition will provide details of metabolic activities of cells when grown in three D configuration on polymer scaffold.

Solubilization and refolding of inclusion body proteins

Our focus on inclusion bodies has been on two aspects: (1) to improve the recovery of bioactive protein and (2) to understand the nature of aggregation during inclusion body formation. Protection of native-like secondary structure during solubilization helped in lowering the aggregation and resulted in improved recovery of bioactive protein. This was achieved by solubilizing inclusion body aggregates in buffers containing β-mercaptoethanol or n-propanol. Last year we showed how different sized inclusion body aggregates are formed during expression of recombinant protein in *E. coli*. We also reported that many more recombinants proteins could be refolded from inclusion bodies after solubilizing them using propanol. Currently we are applying this solubilization process for refolding of multimeric and membrane proteins from inclusion bodies. The detail mechanism of inclusion body solubilization using propanol is also being studied using biophysical techniques.

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In Press last year, since published

Disorders of proliferation: Analysis of novel pathways and targets

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Humans are afflicted by several autoimmune diseases, the causes for many of which remain unknown. The pathological consequence of autoreactive immune responses are the subject of intense investigation. Systemic Lupus Erythematosus (SLE) is a prototypical non-organ specific autoimmune disease which generally manifests a life-long, relapsing-remitting phenotype. Multiple organs are targeted; chronic renal failure is a serious consequence, and patients can also exhibit arthritis, neurological involvement and hemolytic anemia. More than a hundred different autoantibody specificities have been described; drawing correlations between disease manifestations and autoimmune reactivity remains a high priority.

Anti-phospholipid autoreactivity, a frequent occurrence in systemic autoimmune disease, has been associated with autoimmune hemolytic anemia, thrombotic events and early pregnancy loss. The immunological and physiological sequelae arising as a result of the release of sequestered hemoglobin in animals prone to autoimmunity form a focus of current investigations. Interestingly, in both SLE patients and in animals prone to systemic autoimmunity, aberrance in apoptotic processes has been observed. Excessive spontaneous apoptosis (or excessive susceptibility to apoptotic stimuli) as well as inefficient clearance of apoptotic debris have been documented in lupus patients, and animals genetically modified to impair the uptake of apoptotic cells exhibit lupus-like pathology. Autoimmune cascades initiated by early autoantibody responses specifically directed towards apoptotic cells are being investigated; since apoptotic debris appears to constitute the original antigenic insult, it is conceivable that such events could have a bearing on pathology.

Human chorionic gonadotropin (hCG), a placental glycoprotein hormone, stimulates the release of progesterone from the corpus luteum, thereby sustaining pregnancy; successful pregnancy is believed to represent a Th2 skew. Given reports of pregnancy-associated lupus flares in humans and the ameliorating influence of hCG in murine models of organ-specific (Th1-mediated) autoimmune disease, the effects of administration of the hormone in animals genetically prone to systemic autoimmunity (a state which, like pregnancy, can also be notionally considered to constitute a Th2 skew, given associated hypergammaglobulinemia and antibody-mediated pathology) are being investigated. In

recent years, hCG has also been shown to be secreted by a variety of cancers and its presence has been associated with radio- and chemo-resistance as well as with poor patient prognosis. Understanding the molecular pathways by which hCG potentially impacts on tumor progression, as well as the development of novel immunotherapeutic anti-hCG vaccination strategies, form another focus of the laboratory.

1. To investigate the consequences of aberrant cell death in systemic autoimmune disease.

2. To delineate the mechanisms and pathways by which human chorionic gonadotropin can impact upon two disorders characterized by proliferative aberrance, systemic autoimmunity and cancer.

The influence of hCG on systemic autoimmune responses

While the disease-ameliorating effects of hCG in murine models of organ-specific (Th1-mediated) autoimmunity have been described, its influence on systemic autoimmune responses have not been reported. Such studies assume significance in view of the fact that, during pregnancy (considered to be characterized by a Th2 skew), lupus flares have often been reported. Further, the presence of hCG has been documented in male SLE patients and higher-than-normal levels have been observed in pregnant women with SLE. Previous work had established that hCG enhanced the generation of anti-self antibody responses when administered to NZB/W F1 mice. Antibody levels to a wide spectrum of lipid moieties were particularly heightened. In ensuing work, hCG was administered to NZM 2410 and FVB/J (non-autoimmune prone) female mice. While anti-self reactivity (as measured by Western blot analysis on cell lysates) appeared to be enhanced in both strains of mice, the quantum of increase was higher in NZM 2410 mice. Interestingly, while there existed considerable variability in autoreactive responses, NZM 2410 and FVB/J mice appeared to elicit responses to distinct self-moieties. In consonance with previous findings in NZB/W F1 mice, hCG treatment increased the autoantibody titres to several ribonucleoproteins and phospholipids. Interestingly, autoantibodies towards β2-glycoprotein 1, prothrombin, Protein C and Protein S were also heightened; increased levels of such auto-antibodies are strongly associated with anti-phospholipid syndrome and associated pro-thrombotic events. These findings assume additional significance in light of the fact that, in human subjects enrolled in IVF programmes, the great majority of thrombotic events occur subsequent to the administration of hCG. A comprehensive comparative analysis of the targeted moieties is being carried out by two-dimensional Western blot and mass-spectrometry analysis.

Previous work has established that hCG enhances cytokine secretion as well as the proliferation of T cells stimulated with anti-CD3 antibodies. In a logical extension of these studies, it was observed that hCG enhanced the LPS-induced proliferation of B cells derived from NZB/W F1 mice. Taken together, these studies suggest that hCG can contribute towards the generation and/or expansion of humoral autoimmune responses in systemic autoimmunity.

The role of hCG in tumorogenesis

The influence and impact of hCG on the processes of tumorogenesis has been a focus of the laboratory. A model previously employed involves use of transgenic (TG) mice expressing βhCG, which express ovarian, pituitary and mammary tumors; not surprisingly, the animals are infertile.

In ongoing work, anti-hCG immunization was shown to lead to dramatic reduction in the RNA levels of the hormone-responsive genes *Ccnd1, hmga2, e2f1, galanin* and *prolactin*. In work by other investigators, these genes have been implicated in the generation of prolactinomas. These studies will be extended to other candidate genes such as *growth hormone, pttg1, fgf2, and bmp4* and to CDK1 inhibitors involved in G1-S transition such as CDK1b (p27), CDK 2a (p16), and CDK 2c (p18). Anti-hCG immunization of TG animals was previously shown to restore ovarian and pituitary histology and dramatically reduce prolactin levels. In current work, studies were carried out to assess the

reproductive status of immunized and non-immunized TG mice. Estrus cylcicity was restored in 70% of anti-hCG immunized TG mice, as assessed by vaginal cytology; all non-immunized TG mice continued to display constant diestrus. In fertility studies, five out of seven TG immunized females experienced a normal pregnancy and delivered an average of 8 pups each. In contrast, only one out of eight non-immunized TG females conceived and delivered a litter of three pups. These results suggest that anti-hCG immunization can restore fertility in animals rendered infertile due to hCG-induced hyperstimulation of the ovary and subsequent down-stream events.

Published literature has drawn associations between the presence of hCG and radio- and chemo-resistance. Working with both human and murine tumor cells, it was determined that pre-incubation with hCG can prevent the loss of cellular viability induced by 5-flourouracil, curcumin, cisplatin, tamoxifen and etoposide; enhanced viability in the presence of hCG was attributable to a significant reduction in drug-induced apoptosis. The influence of hCG on the mRNA levels of proteins known to be associated with chemo-resistance was then assessed by PCR. Differential effects were seen, depending on cell type; while hCG increased message levels of HIF-1α, survivin, c-FLIP, KLK4, KLK10, KLK11 and TLR6 in ChaGo cells, it enhanced HIF-1α, survivin, livin, KLK10, KLK11, TLR1, TLR3, TLR8, and TLR9 mRNA in COLO 205 cells. While the physiological relevance of these findings is under investigation, the data prompted studies into the combined effects of hCG and various TLR ligands on chemo-resistance. Interestingly, while the ligand for TLR9 acted in synergy with curcumin to reduce the viability of Chago cells, hCG acted to override these effects.

IL6 has been traditionally associated with chemo-resistance, a finding that was verified and extended by our studies. While hCG was incapable of inducing significant levels of IL6 from tumor cells, macrophages incubated with supernatants of tumor cells (previously incubated with hCG) produced significant amounts of IL6. These studies elucidate a link, elaborated by hCG, between tumor cells and non-transformed cells in the production of a moiety which mediates chemo-resistance.

The immunobiology of haemoglobin

Previous work had shown an age-dependent increase in titres of anti-Hb antibodies in the sera of lupus-prone NZM 2410 mice; antibody sequestration in the kidneys and lungs followed a similar trend. Earlier observations also indicated that Hb-autoantibody immune complexes can have inflammatory effects *in vitro*, in terms of enhanced cytokine secretion from phagocytes and subsequently induced migration of endothelial cells. Studies were carried out to assess whether immunization with either murine Hb (mHb) or its dominant epitope (previously demonstrated to comprise amino acids 110-110 Hbβ) resulted in a break of tolerance to Hb as well as downstream immune events. Immunization with both Hb and Hbβ (110-119) resulted in the generation of anti-Hb autoantibody responses. Interestingly, immunization also led to the generation of autoantibodies to several other self-moieties, as was revealed by Western blot. In particular, Hb immunization resulted in heightened serum titres of anti-double stranded (ds) DNA autoantibodies and the increased sequestration of antibodies to mHb, dsDNA, Ro52, Ro60, RNP A, La and Sm in the kidneys. Immunization with Hbβ (110-119) also caused immune perturbation; antibodies to dsDNA, Sm, Ro52, Ro60 were observed in serum, and along with reactivity towards Hbβ (100-119), autoantibodies also bound Hbβ (140-146) and Hbα (80-99). Peptide immunization also induced adherence to the kidney of antibodies reactive towards mHb, dsDNA, Ro52, RNP A. These findings suggest that immunization with mHb or its dominant B cell epitope results in intra-molecular and intermolecular epitope spreading and the accumulation in the kidneys of autoantibodies associated with lupus nephritis.

Experiments were conducted in further pursuit of the etiology of anti-Hb autoimmune antibody responses in lupus. In particular, anti-mHb B cell precursor frequencies were found to be higher in autoimmune NZM 2410 mice as compared to non-autoimmune FVB mice, correlating with anti-dsDNA B cell precursor frequencies in these two strains. Further, in extension of previous work demonstrating mHb-autoantigen interaction, ferric mHb was found to interact with La with increased affinity compared with ferrous mHb. Together, these results suggest the following possible

scenario: Oxidation of Hb upon release, subsequent to erythrocyte lysis, would promote its association with autoantigens (such as La, released as a consequence of heightened apoptosis) against which activated T cells already exist; the relatively higher numbers of circulating anti-Hb B cells would then facilitate the generation of anti-Hb autoimmune antibody responses in animals prone to systemic autoimmunity.

Publication

Original peer-reviewed article

1. Purswani S, Talwar GP, Vohra R, Pal R, Panda AK, Lohiya NK, Gupta JC (2011). *Mycobacterium indicus pranii* is a potent immunomodulator for a recombinant vaccine against human chorionic gonadotropin. **J Reprod Immunol**. **91**:24-30.

Study of genetic and immune factors associated with autoimmune disorders: Type1 Diabetes and vitiligo

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The project aims to study the predisposition to develop autoimmune disorders like Type 1 diabetes (T1D) and vitiligo. In T1D it is important to diagnose a pre-diabetic and device ways to inhibit autoimmune responses since most of the insulin producing beta cells of the pancreases have been damaged by the time patients report to the clinic. Vitiligo, on the other hand, is a multifactorial disease etiology of which is not precisely understood although several hypotheses have been proposed including autoimmunity. However, it is not clear how the pigment producing melanocytes are destroyed by the autoimmune responses. So, we aim to decipher the Immunogenetics and autoimmune factors involved in the destruction of melanocytes.

1. To study the role of Human leukocyte antigens (HLA) in aetiopathogenesis of both T1D and vitiligo.
2. To study other Immune function related genes which may have a role in manifestation of T1D and Vitiligo.
3. To study the autoimmune factors associated with T1D and vitiligo.
4. To design and use peptides in-vitro to inhibit autoimmune T-cell responses.
5. To encapsulate the peptides that inhibit Th1 immune responses in-vitro, in nano-sized carriers for slow and targeted release.
6. Study delivery of peptide/vector complexes in Balb-C and *C57Bl6* mice followed by NOD mice.
7. To differentiate mouse Mesenchymal stem cells into insulin producing cells.

8. To study the role of MHC restricted auto-antigen specific $CD4^+/CD8^+$ T cells in autoimmune destruction of melanocytes in vitiligo.

9. To study the role of Cytokines increased in vitiligo patients in aetiopathogenesis of vitiligo.

Type 1 diabetes

We have established the plasticity of mouse bone marrow derived Mesenchymal stem cells as we have been able to induce adipogenesis (shown last year) and osteogenesis in these cells. Figure 1a shows the undifferentiated MSCs and figure 1b shows differentiated osteoblasts which are stained with alizarin red that stains the calcium deposits in the osteoblasts.

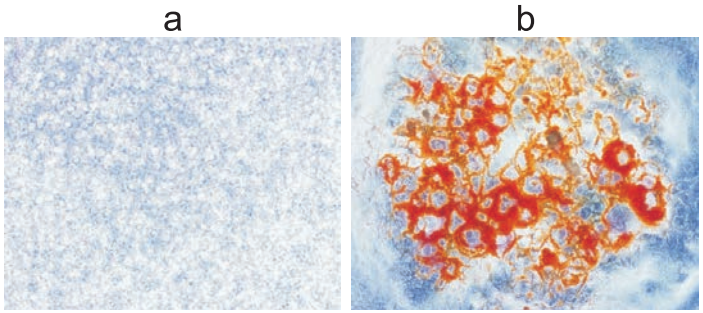

Figure 1. *Osteogenesis of Mesenchymal stem cells a. Undifferentiated Mesenchymal stem cells and b. MSCs differentiated into osteoblasts. Both cultures were stained with Alzarin red which stains the calcium deposits in the osteocytes.*

Having established the plasticity of MSCs, we further standardized the protocol to differentiate MSCs into insulin producing cells. Immunocytochemistry shows the staining with insulin antibodies (Figure 2 a, b and c), confirming that these are insulin producing cells. Preliminary results show that transcripts of Insulin 1, Insulin 2, Gucagon, Pdx1 and Beta 2 were up-regulated in the cells differentiated to produce insulin in high glucose medium (Figure. 2d). However, a very small percentage of control MSCs in the high glucose medium (which were not induced to become insulin producing cells), also showed staining with insulin antibody suggesting that they were getting differentiated into insulin producing cells. But, their numbers were much lower than the ones in which MSCs were induced to become insulin producing cells (Figure 2e). We have been able to differentiate MSCs into insulin producing cells about 5 times and have also failed to do so about 3 times following the same protocol, suggesting that some of the intrinsic factors of MSCs may have a role to play in their differentiation. So, we are looking into these factors to fine-tune the protocol

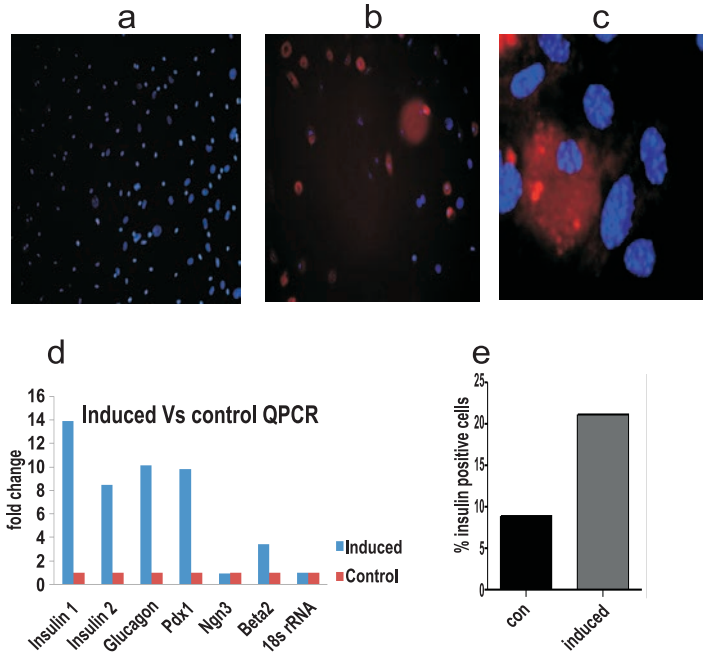

Figure 2. *Mesenchymal stem cells differentiated into insulin producing cells. a. Un-induced controls showing nuclear DAPI staining, did not stain with insulin antibody. b. Differentiated insulin producing cells stained with insulin antibody and DAPI for nuclear staining. c. Higher magnification of an insulin producing cell stained with insulin antibody and DAPI. d. Expression profile of different genes up regulated in insulin producing cells in terms of fold change in the differentiated cells as compared to controls. e. Percent insulin producing cells in control high glucose medium and induced high glucose medium.*

Vitiligo

HLA Associations in vitiligo

Our data on 1404 North Indian vitiligo patients and 902 controls shows a strong genetic factor for predisposition to develop vitiligo in the highly polymorphic MHC complex. We found three alleles, *HLA-A*33:01, B*44:03*(encoding MHC class I molecule) and *DRB1*07:01* (encoding MHC class II molecule) to be significantly increased in vitiligo population compared to healthy controls. This study was replicated in a West Indian (Gujarat) population (355 patients and 441 controls) with the same predisposing alleles suggesting that these alleles are important predisposing factors for Vitiligo. Moreover, haplotype analysis for the three loci *HLA-A, -B and -DRB1* showed *A*33:01-B*44:03-DRB1*07:01* haplotype *was* significantly increased in vitiligo patients compared to healthy controls. Association of *DRB1*07:01* ($p< 3.16x10^{-30}$) with vitiligo seems to be primary because in the absence of *A*33:01* and *B*44:03, DRB1*07:01* still remained highly significant in both the populations studied while the converse was not true. The basic predisposing alleles in both localized and generalized vitiligo are same. Generalized vitiligo is considered to be an autoimmune disease while localized vitiligo has not been considered an autoimmune disease. To our knowledge this is previously unreported that both generalized and localized vitiligo have the same predisposing MHC alleles i.e., *B*44:03 and DRB1*07:01* in both the populations studied, besides some differences in the frequencies of some other alleles. Association of *MHC* alleles with localized vitiligo clearly shows that there is a need to re-look at the aetiopathogenesis of localized vitiligo. While it may be an autoimmune disorder, similarities with unaffected controls in terms of *HLA* alleles and amino acid signature of the peptide binding pockets of DR beta chain may be contributing to the localized distribution of the lesions.

Upregulation of detoxification genes in vitiligo

Since the literature has implicated increased oxidative stress in the etiology of depigmented patches in vitiligo, we have earlier shown that the detoxification pathways regulated by transcription factor, nuclear factor E2–related factor 2 (Nrf2) and its downstream genes *NAD(P)H:quinone oxidase-1* (*NQO-1*), *g-glutamyl cystine ligase catalytic* subunit (*GCLC*), and *g-glutamyl cystine ligase modifying* subunit (*GCLM*) are significantly up regulated in lesional skin as compared to the non-lesional skin of vitiligo patients. We have further studied another family of Phase II detoxification enzymes which is regulated by *Nrf2* and is called Glutathione S-Transferase (GST) family. Several GST genes have been recognized as polymorphic. However, the most important polymorphisms are the ones where certain alleles result in impaired catalytic activity like *GSTM1 null* and *GSTT1 null* alleles. Null alleles result due to deletions of the *GSTM1* and *GSTT1* genes, causing total loss of activity of the corresponding enzymes leading to oxidative stress resulting in cellular damage. In this study we observed significant differences in frequencies of *GSTT1* and *GSTM1* alleles in vitiligo patients when compared with controls. We also observed significant differences in the expression of *GSTT1* and *GSTM1* in the lesional and non-lesional skin of vitiligo patients.

Our results strongly suggest that the cellular physiology of lesional epidermis is indeed perturbed in comparison with non-lesional skin. Our studies provide new insights into the role of phase II detoxification pathway in maintaining skin homeostasis and sustaining redox balance in vitiligo patients.

Cytokine gene polymorphism

650 vitiligo patients and 600 healthy controls have been studied for Cytokine genes polymorphisms using PCR-SSP (sequence specific primers). The SNPs in the cytokine genes which have been shown to influence the secretor status of an individual have been studied. The polymorphisms studied were IFN-γ ($A^{+874}T$), TNF-α ($G^{-308}A$), IL-6 ($G^{-174}C$), IL-10 ($A^{-1082}G$, $T^{-819}C$, $C^{-592}A$), and TGFβ1 ($T^{cdn10}C$, $G^{cdn25}C$). The data is being analysed and will be published shortly.

Cytokine profile in vitiligo

Transcripts for different cytokines were measured in the lesional and non-lesional skin of vitiligo patients using real time PCR. The

results show that expression of certain cytokines were significantly increased in lesional skin of vitiligo patients as compared to their own non-lesional or normal skins. And expressions of yet other set of cytokines were significantly higher in peripheral blood mononuclear cells (PBMCs) of vitiligo patients as compared to healthy controls. We would like to investigate what roles these cytokines are playing in destruction of melanocytes in vitiligo. To evaluate the role of these cytokines on epidermal cells, individual and a combination of cytokines are being used and alteration in transcripts of the target genes will be studied. The RNA will be isolated by trizol method, real-time assays will be performed for known regulated target mRNA, to assess the efficacy of the cytokine treatments and this will be followed by microarray analysis.

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Original peer-reviewed articles

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Study of Immunotherapeutic Potential of MIP (M.w) and the Underlying Mechanisms in Animal Models of Tuberculosis & Tumor Model

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Whole bacterial vaccines rely on multiple antigens and built-in-adjuvanticity. Mycobacterial strains which share cross-reactive antigens with *M.tuberculosis* are being considered as alternatives to *M.bovis* for vaccine use. MIP shares antigens with *M.tuberculosis* and initial studies had shown that vaccination with killed MIP induces protection against tuberculosis. Hence, we further studied the protective potential of MIP and the underlying immune responses.

The generation of antitumor immunity is often difficult in the tumor-bearing host because of various negative regulatory mechanisms. Activation of Innate and Th1 type immune response is important to overcome immunosuppression in the tumor-bearing hosts. There were indications from different clinical studies that MIP may be useful as an immunomodulatory adjunct in some cancers. In animal model of tuberculosis we had found that MIP induces Th1 type response which is also important for antitumor activity. Hence, we have started this study to evaluate the immunotherapeutic activity of MIP in mouse syngeneic tumor models.

The present study aims to investigate the protective efficacy of MIP immunisation in live or killed form, through parenteral route as well as by aerosol immunization, against subsequent infection with *M.tuberculosis* in animal models. Evaluation of immunotherapeutic efficacy of MIP along with chemotherapy in animal infection models. Study of immune response to *M.tuberculosis* in animals immunised with MIP. These responses are compared with those generated in BCG immunised mice.

Another objective is to evaluate Immunoprophylactic and Immunotherapeutic activity of MIP in mouse syngeneic tumor model. Study of MIP as an adjunct to chemotherapy in combination with commercial anti cancer drug formulation in tumor bearing mice. Simultaneous study of mechanism of *MIP* mediated host immune activation.

Protective Efficacy of MIP in Animal Models of Tuberculosis

We further evaluated the protective efficacy of MIP by monitoring the long-term protection and post-infection survival kinetics in all groups. The formation of granuloma is the hallmark of an anti TB

host immune response. Therefore we monitored and compared the level of various immune cells inside the granuloma in the lungs of the MIP or BCG-immunized / control groups. The induction of apoptosis in infected phagocytes is a host response that is believed to be helpful in the cross-presentation of antigens and killing of intracellular bacteria. We analyzed the level of apoptosis in immunized and *M.tb* infected lungs, ex-vivo by flow cytometry and also monitored the *in situ* apoptosis of the macrophages inside the granuloma. We also analyzed the *in-situ* expression of different cytokines to characterize the post challenge modulation of the immune response in MIP-immunized animals. We found reduced bacterial loads, improved pathology and organized granulomatous response at different post infection time points in the MIP-immunized group as compared to the BCG-immunized group. Combined results suggest that MIP-immunization results in heightened protective Th1 response as compared to BCG group, early after infection with *M.tb* and a balanced Th1 versus immunosuppressive response at late chronic infection.

The extended course of chemotherapy often results in poor compliance and the emergence of drug resistant strains. Therefore, improved therapeutic strategies are needed. Immunotherapy can boost protective immune responses and could contribute to the effective management of the disease when combined with chemotherapy. We sought to investigate whether MIP could be used as an adjunct to standard chemotherapy in guinea pig model of tuberculosis. The efficacy of MIP was evaluated when given by aerosol or parenteral route at sub acute or chronic stage of tuberculosis infection.

In *M.tb*-infected guinea pigs, we monitored the accumulation of immune cells in the lungs and analyzed the *in-situ* expression of different cytokine genes to characterize how adjunct immunotherapy with MIP modulated the immune response.The proportion of different immune cells and expression of cytokines genes were analysed at certain time points during the course of therapy and compared with the group treated only with drugs. We found that protection observed with MIP-treatment was associated with reduction in both bacterial loads and pulmonary pathology. Results indicate that MIP immunotherapy may not reduce the required treatment duration, but could be potentially very useful in the eradication of persistent bacteria when combined with chemotherapy. We believe that delivering immunotherapeutic agents via aerosol route can play a very important role in inducing immediate local immune response in the lung.

Immunotherapeutic Potential of *MIP* and the Underlying Mechanisms in Mouse Tumor Model

Role of the immune system in protecting the host from cancer is well established. Th1 branch of the immune system play major role in combating cancer but growing cancers actively suppress immune response. In animal model of tuberculosis we had found that MIP induces Th1 type response. Hence, we sought to analyse the immunotherapeutic potential of MIP in mouse tumor model and the underlying mechanisms for its antitumor activity.

To further understand the mechanism of MIP mediated antitumor immune response, we investigated the role of antigen presenting cells (APCs). Macrophages which constitute the major proportion of tumor infiltrating immune cells, play a crucial role in tumor prognosis. Purified macrophages were stimulated with MIP or BCG for 36 hrs at different multiplicity of stimulation. Higher level of proinflamatory cytokines and reactive nitrogen intermediates were found in the supernatants of MIP stimulated cultures. We further investigated whether such stimulated macrophages can directly kill the tumor cells. Higher killing of tumor cells was observed by MIP stimulated macrophages. A close association between tumor cells and macrophages was necessary because in transwell experiments, this cytotoxicity against tumor cells was abrogated. Culture supernatant from activated macrophages was unable to induce killing of tumor cells. This killing seems to be mediated by necrotic process as pan-caspase inhibitor did not inhibit

the killing of tumor cells. There was no toxic effect of MIP on tumor cells.

Combined chemo-immunotherapy strategies to combat cancer

It is well established that tumor suppresses the anticancer immune response at its local microenvironment to facilitate its growth, leading to cancer progression and metastasis. Immunotherapy, on the other hand, stimulates the immune system and helps to induce an effective anti-tumor response. Combined chemo-immunotherapy has multi faceted advantages. Chemotherapy induced cell death can enhance cross-priming of immune cells by providing them with important cancer specific antigens, thereby increasing the antitumor T cell response.

The prime objective of this study was to develop a combined chemo-immunotherapeutic formulation which could directly kill cancer cells as well as activate the immunosuppressed tumor microenvironment to promote killing of tumor cells. Paclitaxel (PTX) and SP-LPS (non-toxic derivative of lipopolysaccharide) were selected as anti-cancer drug and immunostimulant respectively. We prepared a chemo-immunotherapeutic compound by conjugating a chemotherapeutic drug with an immune stimulant and also prepared nanoparticles co-encasulating both. Their anticancer activity was evaluated.

Biodistribution study of nanoparticle formulation was done to evaluate the distribution of PTX when given as nanoparticles compared to commercial Paclitaxel (Taxol®). After i.v. administration of Paclitaxel-SPLPS nanoparticles (TLNP), the plasma concentration of free paclitaxel was found to be significantly less while the tumor concentration was found to be higher as compared to commercial Taxol® group. In TLNP treated group, higher amount of PTX accumulated in the tumor even at 5 minutes after injection. The drug concentration in the tumor was found to be higher at all time points studied. This signifies rapid accumulation and retention of the drug in tumor, when administered as TLNP. This provides evidence of tumor targeted delivery of nanoparticle formulation which would result in less systemic toxicity. In vivo studies in mouse model of tumor demonstrated that nanoparticles co-encapsulating both drug and immunostimulator had higher anti-cancer activity as compared to commercial Taxol® due to combined effect of chemotherapy and immunstimulation.

We have initiated work with other hydrophilic polymer having macrophage stimulating property and antitumor activity. Simultaneously we are trying to further modify the existing conjugate by incorporating the folate moiety for targeting the conjugate to folate receptor expressing tumors.

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Fine tunings of NF-kappaB signaling

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Unlike sterile cell culture condition, tissue microenvironment, owing to constituent organogenic and cell-differentiating cues, transmits a plethora of different signals to the resdient cells to concomitantly activate multiple signaling pathways. In this context, it becomes exceedingly important to understand the crosstalk between these "apparently" insulated cell signaling pathways within cellular milieu to capture fine-tuning of physiological response. For eg., pathogen sensing cells those elicit inflammatory response upon TLR (Toll like receptor) activation also receive co-stimulatory signals from organogenic LTβR (Lymphotoxin beta receptor), thereby, generating potential for crosstalk between these two receptor signaling pathways. In the proposed program, we have considered the NF-κB system as it can process signals from both pathogenic (TLRs) and developmental (LTβR) cues involving NEMO-IKK2 and NIK-IKK1 pathways, respectively. Here, we aim to explore the capacity of the NF-κB system in mediating crosstalk between inflammatory and developmental cues. In a multidisciplinary research laboratory, we are combining cellular biochemistry, mouse genetics and mathematical biology to explore the mechanism and physiology underlying cell-intrinsic signaling crosstalk between LTβR and TLRs in the context of inflammation.

1. Exploring crosstalk between LTβR and TLRs

Here, we will combine mathematical modelling and biochemical experiments to identify conditions and mechanisms underlying signalling crosstalk between TLRs and LTβR at the level of NF-κB activation. Here, we will focus on IκB-dependent mechanisms to finally construct a crosstalk deficient mutant cell.

2. Exploring cross-regulations of TLR response through PTMs

Using bioinformatics, we will predict novel post-translational modification (PTM) sites on NF-κB/RelA subunit. In cell culture, we will examine these sites for modifications and its functionality by generating modification-mutant cells. Next, we will address if signalling crosstalk through PTMs modulate inflammatory response at the level of NF-κB activation.

3. Crosstalk control of inflammatory gene expression

We will use mutant cells (generated in Aim-1 and Aim-2) to conduct gene-expression analyses to identify TLR induced inflammatory genes whose expressions are quantitatively and qualitatively dependent on signalling crosstalk.

For the past year (2011-2012), my laboratory has been deeply engaged in a multidisciplinary approach to identify and explore the elements of crosstalk within the cell-signalling network with particular focus on NF-κB signalling. The laboratory has trained students in versatile academic disciplines encompassing mathematical biology (MVR Prasad) as well as biochemistry and cell biology (B Banoth, P Roy). In a coherent group effort, we have addressed questions, as summarised below, to decipher fine-tunings of NF-κB signalling.

1. Exploring crosstalk between LTβR and TLRs:

In this aim, we have implemented a refined version of NF-κB mathematical model (NF-κB System Model *version 1.0*) to describe for the first time generation NF-κB RelA:p50 and RelA:p52 dimers as well as their signal induced activation. In this model, we wrote a system of ordinary differential equations to represent respective biochemical reactions adhering to mass action kinetics and solved them using MATLAB *ode 15s* solver to simulate NF-κB activation in response to numerical kinase inputs. As described (figure legend, **Figure-1**), in a bottom up approach we have included new biochemical reactions (pink lines, **Figure-1**) in the model, such as p100 and p52 production, p100 oligomerization to form IκBδ, generation of NF-κB1 and NF-κB2 dimers. Model represents two distinct activation mechanisms for NF-κB dimers, namely NEMO-IKK2 and NIK-IKK1 dependent degradation of classical IκBs (predominantly IκBα) and IκBδ, respectively. It also summarises two mechanisms that govern NF-κB dimer generation, namely constitutive synthesis of NF-κB1 dimer and NIK dependent signal induced generation of NF-κB2 dimer. We have conducted quantitative time course analysis (EMSA, Western blot, IP-Kinase assay) to measure cellular abundance of NF-κB/IκB proteins during TNF/LTβR signalling and used the data for parameterizing the model. We have also used kinetic EMSA data for NF-κB activation in response to TNF or LTβR treatment to evaluate model performance.

Our model-wiring diagram suggests possible crosstalk between NEMO-IKK2 and NIK-IKK1 signalling inputs. However, the conditions those would facilitate the crosstalk remain unclear. To this end, we have used an algorithm to generate a diverse library that encompasses 1044 possible theoretical NEMO-IKK2 input profiles with different durations and amplitudes. Alongside, we have also generated an activity library for theoretical 729 NIK-IKK1 kinase inputs. By iterative model simulations, using a combination of individual members from each of these two libraries, we identified that only a subset of NEMO-IKK2 and NIK-IKK1 inputs crosstalks with each other. Further mathematical explorations revealed a critical dose-duration control of both NEMO-IKK2 and NIK-IKK1 inputs in mediating signalling crosstalk.

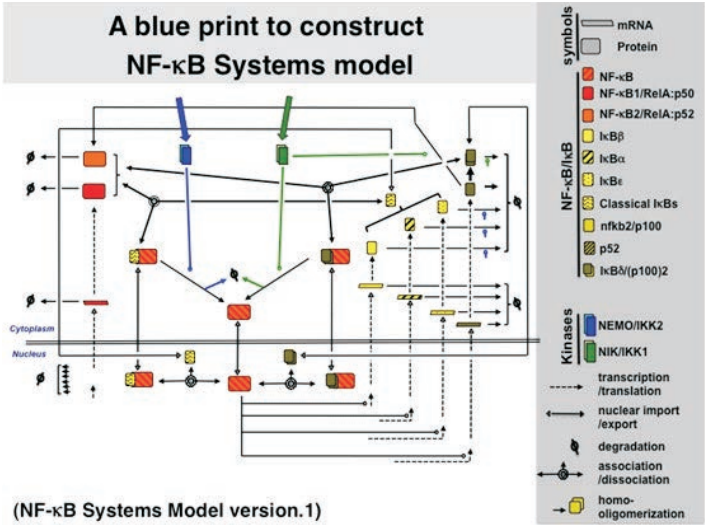

Figure-1: *A wiring diagram that describes the NF-κB signaling system: The diagram charts synthesis and degradation of NF-κB/IκB mRNA and proteins. Here, mRNAs are synthesized constitutively (dashed arrow) as well as in NF-κB induced manner (open circle) and degraded. NF-κB/IκB proteins produced from mRNAs via translation (dashed arrow) associate to form NF-κB/IκB complexes. Four different degradation pathways* **(i-iv)** *control IκB stability, namely* **i)** *kinase independent free IκB degradation,* **ii)** *NEMO-IKK2 (for IκBα) or NIK-IKK1 (for IκBδ) dependent free IκB degradation,* **iii)** *kinase independent NF-κB bound IκB degradation,* **iv)** *NEMO-IKK2 (for IκBα) or NIK-IKK1 (for IκBδ) dependent NF-κB bound IκB degradation. This liberates NF-κB dimer into the nucleus. Besides, free and IκB bound NF-κB dimers shuttle between nucleus and cytoplasm through import/export reactions. Two NF-κB dimer generation mechanism has been indicated. NF-κB1 (mimicking RelA:p50) dimers are produced constitutively from p50 mRNA. However, NIK-IKK1 signals p100 processing into p52 that then generates NF-κB2 dimers (mimicking RelA:p52). In the wiring, p100 also oligomerizes to form IκBδ that then inhibits NF-κB dimer. This wiring diagram was used as a blue print for the mathematical model. New reactions in the model have been indicated by pink line. Notations are used following Kitano et. al., Nature Biotechnology 23(8), 2005.*

We have also examined our results experimentally in cell culture system using primary mouse embryonic fibroblast cells (MEFs) to capture the NF-κB pathway activation. We used a variety of different stimuli, such as TNF (acts through TNFR1), IL-1 (acts through IL-1R), PolyI:C (acts through TLR3), LPS (acts through TLR4), Pam3Cys4 (acts through TLR2), Flagellin (acts through TLR5) and so forth those utilize the NEMO-IKK2 pathway. Similarly, we have used RANKL (acts through RANK), BAFF (acts through BAFFR) and LTβR (acts through LTβR) those activate NIK-IKK1

signalling. We have optimized an immunoprecipitation based NEMO-IKK2 and NIK-IKK1 kinase assay to experimentally explore dose-duration control. In a combinatorial treatment regime, we have further examined to establish crosstalk conditions in cell culture system.

2. Exploring cross-regulations of TLR response through PTMs

In this aim, we have generated a panel of phosphorylation defective mutants of NF-κB/RelA protein those are based on our bioinformatics based analysis, as described earlier. One of our constructs expresses a "phosphorylation-dead" mutant NF-κB/RelA protein with site directed alanine mutations at all four well-documented phosphorylations sites, namely S276, S468, T505 and S536. We used this four-residue mutant (4M) as a negative control in our assay. In addition, we have also generated both phosphorylation-null (alanine mutation) and phospho-mimicking constructs those displace critical residues as identified by our bioinformatics analysis. In particular, we have generated a phosphorylation-null AA mutant and a phospho-mimicking DD constructs by displacing two critical residues identified by our bioinformatics based analysis. In a co-transfection based analysis, we have used a kappaB-luciferase construct to quantify transcriptional activity of these mutants as compared to wild type NF-κB/RelA.

Our preliminary analysis revealed that DD mutant, as compared wild type RelA, is completely inactive in driving transcription from kappab promoter, whereas AA mutant was hyper-active (**Figure-2**). Our results suggest a negative role of phosphorylation of these residues in NF-κB mediated transcription. These mutants were also cloned into retroviral backbone. Subsequently, we have generated a panel of stable cell-lines those express wild type or mutant NF-κB/RelA protein by infecting *rela*$^{-/-}$ cell lines with retrovirus. Currently, we are characterizing these reconstituted cell-lines to understand the mechanism underlying defective gene regulation by DD mutant.

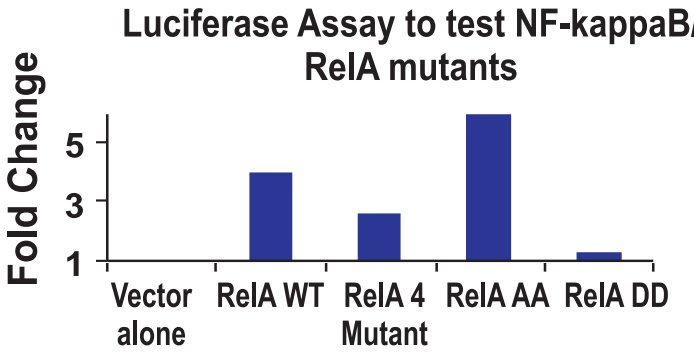

Figure-2: *Luciferase assay reveals anomalous NF-κB activation in bioinformaticallly identified RelA mutants: HEK-293T cells were co-transfected with a construct with luciferase gene under kappaB promoter and wild type or different mutant versions of RelA. Luciferase activity was measured 42h post-transfection and plotted as fold change values as compared to vector alone transfection. Our analysis revealed increased transcription in AA and reduced transcription in DD mutant suggesting a suppressive role of phosphorylation of these two residues in NF-κB mediated transcription*

3. Crosstalk control of inflammatory gene expression

In this aim, we are utilizing conditions optimized in Aim-1 to treat cells in a combinatorial regime. Subsequently, we are analyzing expression profile of a select set of inflammatory genes by Real Time PCR. Our preliminary analyses identify TNF as a crosstalk dependent gene.

Publication

Original peer-reviewed article

1. Murray SE, Polesso F, Rowe AM, Basak S, Koguchi Y, Toren KG, Hoffmann A, Parker DC. (2011) NF-κB–inducing kinase plays an essential T cell-intrinsic role in graft-versus-host disease and lethal autoimmunity in mice. **J Clin Invest 121:** 4775-86.

REPRODUCTION AND DEVELOPMENT

REPRODUCTION AND DEVELOPMENT

Studies of Sertoli cells and spermatogonial stem cells of the testis and other endocrinology related research

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We use testis as an organ of multiple research interest 1) exploiting spermatogonial stem cells for propagation of transgene; i.e. for generation of transgenic animals, 2) analyzing differential gene expression by Sertoli cells (during active vs. inactive phase of spermatogenesis) to identify factors regulating germ cell division and differentiation with an intent to divulge unknown (inborn or environmentally induced) non hormonal causes of idiopathic male infertility and 3) undertaking germ cell transplantation studies to restore fertility upon chemotherapy. In addition, we also participate in other endocrinological research as collaborators.

1. To exploit spermatogonial stem cells of testis for insertion and propagation of transgene through several generations in an attempt to over express or knock down specific genes

2. To study paracrine and endocrine modulation of signal transduction in Sertoli cells (Sc) from spermatogenetically inactive (infant) and active (adult-like) monkey and rat testis.

3. To undertake gene expression studies of rat, mice and monkey Sc to identify factors important for induction of spermatogonial stem cell division and differentiation in the testis.

4. To study biology of spermatogonial stem cells and to use germ cell transplantation technique for restoration of fertility following chemotherapy.

Differential genomics by DNA microarray in rhesus monkey Sertoli cells

Microarray analysis of RNA isolated from hormone stimulated Sertoli cells cultured from infant and pubertal monkeys were undertaken because we believe that genes differentially appearing at onset of puberty are the starter genes necessary for induction of productive spermatogenesis by the testicular Sc which bears receptors for FSH and testosterone. For this purpose RNA was isolated from cultured Sertoli cells (Sc) of infant and pubertal monkeys. Prior to RNA isolation the Sc were stimulated with Testosterone and Follicle Stimulating Hormone for 24 hours. The quantity and quality of isolated RNA was determined by obtaining electropherogram from Agilent Bioanalyser. RNA samples with RIN score above 7 and yield of above 1000 ng of RNA were used for RNA labelling and hybridisation to microarray chip.

Samples were labeled using the Agilent Low Input RNA amplification Kit. RNA was first converted into single strand cDNA by reverse transcription. The single strand cDNA was used as template for *in vitro* transcription and fluorophore was coupled to CTP to generate fluorolabelled (Cy5 or Cy3) cRNA. Specific activity of each labelled cRNA was calculated using Thermo Scientifics NanoDrop instrument.

Labelled cRNA were hybridised onto Agilent's Human Whole Genome (4x44k) slide. The microarray slides were analysed on Agilent platform. In each array, 44,000 probes were screened which together accounted for the entire expressed transcriptome in humans.

Data analysis was done using GeneSpring GX version 7.3 and Microsoft Excel. The normalization of the microarray data was done using GeneSpring GX software using the recommended Per Spot and Per Chip: Intensity dependent (Lowess) normalization. On the microarray slide there were control spots for technical quality control and there were non-control spots each of which has large number of 60mer oligonucleotides which hybridise to specific RNA only. Non-control spots with ratio 2 and above were identified as upregulated in pubertal animals. Non-control spots with ratio 0.5 and below were identified as upregulated in infants.

Fold change was assigned in log2 scale. Fold change of >1 was taken as up regulated in puberty and fold change of <-1 was taken as up regulated in infancy. The two sets of microarray analysis (Expt#1 and Expt#2) were compared amongst each other and signals from each gene were cross checked between both experiments. Genes which were up regulated over 1.8 fold in both pubertal animals as opposed to both infant animals were identified.

The crucial difference between Sc in pubertal animals and Sc in infant animals is that while both are exposed to same gonadotrophic milieu, only Sc in pubertal animals has the property to induce germ stem cell's division and differentiation. Real time PCR validation of important genes of primate origin are underway.

Causes underlying lack of differentiation of repopulating spermatogonia in post natal testis

While circulating levels of LH, FSH and testosterone (T) in infant monkeys (< 4 months-old) and boys (<6 months old) are similar to that found during puberty, testis fails to support spermatogenesis during infancy. Sertoli cells (Sc) in testis have a crucial role in the initiation and maintenance of spermatogenesis. Infant germ cells from various species are found capable of undergoing differentiation when grafted in conducive environment, suggesting that infant germ cell is amenable to differentiation and that the functions of infant Sc are probably suboptimal or defective during infancy. Lack of spermatogenesis in the phase of high hormones during infancy is a situation similar to that found in certain categories of male infertility. To investigate this intriguing situation, Sc were isolated and cultured from various age groups of monkeys. Androgen receptor (AR) and FSH receptor (FSHR) signaling in Sc isolated from testes of infant monkeys were compared with those in Sc from spermatogenically active testes of pubertal monkeys. Although AR and FSHR mRNA expressions were comparable at these two stages of development, androgen binding ability of AR and FSH mediated cAMP production by Sc were extremely low during infancy. Testosterone (T) and FSH failed to augment the expression of T responsive gene, claudin11, and FSH responsive genes, inhibin-β_B, stem cell factor (SCF) and Glial cell line derived neurotrophic factor (GDNF) by infant Sc as opposed to those by pubertal monkey Sc. However, intracellular stimulation of FSHR by cholera toxin (CT) augmented cAMP production in infant Sc indicating defective membrane bound FSHR. Similarly in rats, infancy is associated with sufficient circulating hormones but lack of spermatogenesis. Rat Sc studies suggested that AR activity is limited in neonatal life but improves with Sc maturity after 12-days of age whereas, responsiveness of Sc towards FSH changes in between 9 to 12-days of age due to improved binding of FSH to FSH-R. We have compared FSH and T signaling during four developmental phases of rat Sc by

culturing them from 5-days (neonatal), 9-days (immature), 12-days (maturing) and 19-days-old (mature) rats. Both FSH-R and AR mRNA and protein levels remained uniform in Sc of all age groups. However FSH signaling was low during immediate postnatal ages (5 and 9 days) and increased with an augmented cAMP response in 12 and 19-days-old Sc. This was because of enhanced ligand(FSH) binding by FSHR. This also increased the expression of stem cell factor (SCF) and glial cell line derived neurotrophic factor (GDNF) in Sc cultured from 12 days onwards.

Immunocytochemical studies demonstrated that the although nuclear localization of AR was evident in Sc from all age groups, T mediated relocation of AR to the plasma membrane and augmentation of claudin11 (a component of blood-testes barrier) expression was seen only in Sc cultured from by 19-days-old rats. Maturational failure in such switching of Sc from hormone resistant (during infancy) to hormone responsive (during prepubertal phase) state may play a crucial role in certain forms of hormone resistant idiopathic male infertility.

From these observations, we believe that compromised AR and FSHR mediated signaling in infant Sc might be responsible for the azoospermia in the infantile testes in spite of adequate hormonal milieu, a situation similar to that in certain forms of idiopathic male infertility.

Establishment of testicular spermatogonial Stem cell cultures

We have successfully isolated spermatogonial stem cells (Thy1.2 positive and α1 integrin negative), from testis of mice, through FACS and cultured on mouse embryonic fibroblast. Cells made mounds and are being characterized for stemness.

Establishment of germ cell transplantation in mice

Simultaneous to establishment of spermatogonial Stem cell cultures, we have standardized procedure for germ cell depletion of the testis so that cultured stem cells can be transplanted in the prepared recipient mice.

Cellular and molecular biology of human cancer

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Over the last three decades, knowledge on the molecular biology of human cancers has vastly expanded. A host of genes and proteins involved in cancer development and progression have been identified and many mechanisms at the molecular, cellular and even tissue level have been, at least partly, elucidated. In fact, cancer research has now reached a critical stage, in which the accumulated knowledge on molecular mechanisms needs to be translated into improved prevention, diagnosis, and treatment. The hallmarks of cancer comprise six biological capabilities acquired during the multistep development of human tumors. The hallmarks constitute an organizing principle for rationalizing the complexities of neoplastic disease. They include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis.Understanding the mechanisms involved in tumorigenesis has wide ranging implications for targeting the treatment of cancer. Tumor specific antigens (TSA) represent a unique class of tumor antigens, which are expressed in a variety of cancerous tissues and are silent in normal tissues. Cancer testis (CT) antigens represent a unique class of tumor antigens under this category, which are expressed in a variety of cancerous tissues and are silent in normal tissues, except for the testis. A characteristic commonly shared by cancer testis antigens is, aside from the highly tissue-restricted expression profile, their likely correlation with tumor progression and immunogenicity in cancer patients. Also the differential expression of germ cell specific genes in various cancer tissues reveals the important link between the two complementary disciplines of cell survival i.e. developmental and cancer biology.

Numerous candidate cancer associated genes have been identified to date. However, for the vast majority of these

genes, neither the expression pattern of the protein product, nor its localization and function in the tumor tissues has been investigated. The identification of specific genetic markers that are associated with tumor progression and aggressiveness may prove to be useful to assess the progression of disease. We are focusing on tumor associated proteins for the assessment of disease risk, early detection of disease, therapeutic prognosis and response to treatment as well as disease recurrence. The application of such gene products (biomarkers) to cancer will lead the way because of the unique association of genomic changes in cancer cells with the disease process. Most importantly, cancer biomarkers for prognostic, prediction and pharmacodynamics may aid in the rational development of anti-cancer drugs. In addition, our goal is to delineate in greater detail the gene-expression pathways involved in cellular growth, cell migration, and invasion for the treatment of cancer.

Colorectal carcinoma (CRC) is the third most common cause of cancer and is the second leading cause of cancer related death. Surgical resection is the primary treatment modality for CRC while chemotherapy and/or radiotherapy may be recommended depending on the individual's patient staging and other medical factors. The current chemotherapy for advanced CRC results only in a modest improvement in survival. However, it has been reported that CRC patients have higher survival rate if detected at early stages before lymphovascular invasion. Therefore, there is an urgent need for the identification of tumor biomarkers that may be used for the early detection and diagnosis of CRC. A unique class of testis proteins known as cancer-testis (CT) antigens has been found to be expressed in various malignancies. Based on their restricted expression in cancerous tissues and in lieu of their immunogenicity in cancer patients, CT antigens are the ideal targets for cancer immunotherapy and serum based biomarkers.

In the present study, we systematically investigated *SPAG9* mRNA and protein expression, *in vivo* immunogenicity in human CRC patients and its role in the tumorigenicity of colon cancer.

SPAG9 mRNA Expression in CRC Specimens and Cell Lines

Our RT-PCR analysis showed that 58 of 78 (74%) CRC patients expressed *SPAG9* mRNA. Both COLO 205 and HCT 116 colon cancer cells also expressed *SPAG9* mRNA. However, no *SPAG9* expression was detected in ANCT specimens. *SPAG9* expression was detected in 23 of 26 (89%) early stage (I & II) and in 35 of 52 (67%) late stage (III & IV) categories of CRC specimens. Based on histopathological grading categories, *SPAG9* mRNA expression was detected in 28 of 37 (76%) well differentiated, 26 of 33 (79%) moderately differentiated and 4 of 8 (50%) mucinous type of CRC specimens. Statistical analysis revealed a significant association between *SPAG9* expression and various stages; stage I & II (P = 0.020), stage II & III (P = 0.009) and early and late stages (P = 0.044) tumors of CRC patients using Pearson's Chi-Square test suggesting a tight relationship between *SPAG9* expression and tumor stages. However, *SPAG9* mRNA expression was independent of tumor histological grades indicating no correlation between tumor grades (P = 0.239). These results suggest that SPAG9 may participate in early cancer growth.

SPAG9 protein expression in CRC Specimens

SPAG9 protein expression was validated by immunohistochemistry in serial tissue sections of CRC and ANCT specimens. Importantly, the cytoplasmic localization of SPAG9 protein expression was observed in 74% (58/78) CRC specimens expressing *SPAG9* mRNA. However, no SPAG9 expression was detected in 26 paired ANCT specimens and 40 control colon tissue specimens. A significant association was found between SPAG9 expression and various stages; stage I & II (P = 0.020), stage II & III (P = 0.009) and early and late stages (P = 0.044) using the Pearson's chi-square test, suggesting that SPAG9 expression was strongly associated with the tumor stages. Furthermore, based on SPAG9 IRS in colorectal tumors, 2 groups were formed and analyzed which included; a) moderate group representing less than 50% tumor cells expressing SPAG9 protein and b) high group representing more than 50% tumor cells expressing SPAG9 protein. Our results indicated that 79% of CRC patients (46 of 58) revealed higher SPAG9 IRSs (70.48

+ 1.8) as compared with 20% of CRC patients (12 of 58) having moderate SPAG9 IRSs (38.58 + 2.28). Further, when we compared moderate and high SPAG9 IRS groups using Mann-Whitney U test, a significant difference ($P < 0.0001$) was found with a higher number of CRC patients demonstrating SPAG9 expression suggesting that majority of CRC patients exhibited higher SPAG9 protein expression during the early stages of the disease. Thus, our analysis on SPAG9 immunoreactivity scores in tumors of early stages indicates that it may have good predictive value for the early spread of cancer.

CRC patients generate humoral response against SPAG9

The circulating anti-SPAG9 antibodies were determined in 54 CRC patients' sera using an ELISA. ELISA titres above the mean + 2 SD (0.240 + 0.06) of the 50 normal healthy sera were considered as positive. Antibodies against SPAG9 were detected in 70% (38 of 54) in various stages and histological grades of CRC patients. The presence of autoantibodies against SPAG9 in the sera of CRC patients found positive in ELISA were further examined by Western blotting which exhibited immunoreactivity against SPAG9 protein irrespective of tumor stages as shown in representative Western blot. Sera from 50 normal healthy donors revealed no reactivity by Western blotting.

Down-regulation of SPAG9 decreases cell growth, colony formation, migration and invasion of colorectal carcinoma cells

Two independent siRNA target sequence directed against SPAG9 (SPAG9 siRNA I; SPAG9 siRNA) were used for gene silencing studies. Our data indicated that SPAG9 siRNA target treatment resulted in ablation of SPAG9 protein expression, whereas the other target SPAG9 siRNA-I didn't ablate the SPAG9. Therefore, we used SPAG9 siRNA target for all our subsequent experiments, although there was always a weak protein band observed representing residual SPAG9 protein expression. We observed that 72 hours post transfection with SPAG9 siRNA, cell growth was significantly ($P < 0.0001$) reduced to 31 and 33% for COLO 205 and HCT 116 respectively. In addition, significant reduction in colony-forming ability was also observed in SPAG9 siRNA transfected cells. Statistical analysis revealed that colony-forming ability was significantly decreased for cell numbers seeded for COLO 205 ($P < 0.0001$; range of 29-31% for 400-1000 cells) and HCT 116 ($P < 0.0001$; range of 29-30% for 400-1000 cells) cells. This data show that SPAG9 expression apparently is involved in cell growth and colony-forming ability of colon cancer cells.

To determine whether SPAG9 is involved in early spread of colorectal carcinoma, migration and invasion abilities of COLO 205 and HCT 116 cells were evaluated. Our results demonstrated that SPAG9 knockdown suppressed migration and invasion abilities of COLO 205 and HCT 116 cells. The histogram shows 69% & 70% inhibition in migration abilities for COLO 205 ($P < 0.0001$) and HCT 116 ($P < 0.0001$) respectively (histogram; $P < 0.0001$). Decreased SPAG9 expression also led to the inhibition of invasion by 71% and 68% in COLO 205 and HCT 116 respectively ($P < 0.0001$).

SPAG9 siRNA inhibited the growth of COLO 205 cell xenograft. Our in vitro data showed that SPAG9 siRNA reduced SPAG9 protein expression and resulted in suppression of cellular proliferation of COLO 205 and HCT 116 cells. We further validated the effect of SPAG9 siRNA on inhibition of cellular growth of COLO 205 cell xenograft in athymic mice. We observed that there was significant reduction in the average size and volume of tumors in SPAG9 siRNA treated animals as compare to control siRNA treated animals by day 44. On average, SPAG9 siRNA treatment in experimental animals resulted in decreased tumor growth and mass by 70% and 71% respectively at day 44 ($P < 0.0001$).

In conclusion, SPAG9 represents a new member of CT antigen with high expression in CRC. To best of our knowledge, this is the first report demonstrating CT antigen playing pivotal role in CRC cellular growth, migration and invasion. Our study has put forth a proof of concept wherein SPAG9 as a novel gene product participating in various malignant features could represent a candidate molecule target for CRC and could eventually provide

new research leads for smart therapeutics approaches against CRC. Most importantly our study on CRC early detection & diagnosis based on serum biomarker will be of great benefit for new modalities of cancer treatment. In conclusion, our study lays a foundation for further investigation into validation and manipulation of SPAG9 in CRC treatment.

Publication

Original peer-reviewed article

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Study of expansion and plasticity in Bone marrow stem cells

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Bone marrow (BM) niche controls self-renewal and differentiation of HSCs. To understand hematopoietic niche, it is important to dissect adult BM and fetal liver microenvironment in mouse. It has been hypothesized that artificial BM niche in culture may allow *ex vivo* expansion of HSCs without compromising with engraftability of the cells. Expanded cells not only facilitate transplantation for the purpose of hematological reconstitution, they are also expected to contribute in healing of many diseased organs. No suitable method for *ex vivo* expansion of stem cells is available. It is also not clearly understood how BM cells are involved in the regeneration of diseased organs other than BM tissue. Thus, themes of our research are study on cellular plasticity, stem cells niche, reprogramming of adult cells and origin of cancer stem cells.

We intend to pursue molecular analysis of HSCs niche, role of BM cells in normal physiological processes and in diseased organs, and adult stem cells plasticity. The objectives are:

1. Molecular control of self-renewal and engraftibility of HSCs in adult and fetal mice.
2. Regeneration of liver and other tissues by BM- and fetal liver-derived progenitor cells.
3. Molecular control for differentiation of BM progenitor cells into hepatocytes.
4. Role of BM cells in ovarian carcinogenesis.

A. Hematopoietic stem cells: Marrow niche and regulation

We have shown that during marrow regeneration donor LSK cells can multiply by a factor of about15-folds. To understand corresponding BM niche, we isolated stromal ($CD45^-$) cells from BM and carried out global gene expression analysis using Agilent microarray platform. Further, these microarray data were validated by real-time PCR analyses for few selected genes, expressions of which were changed beyond the threshold values. We selected three genes, named as Rac1, Areg and Clec11a. In order to study the effect of these gene products in self-renewal and differentiation of LSK cells, we have cloned shRNA of Rac1

and Areg genes in retrovial based expression system for silencing corresponding gene expressions. Preliminary results showed that transduction of above shRNA genes in a mouse BM stroma cell line (M210B4) may result in about 80% down-regulation of the target genes, as determined from the real-time PCR data. Currently, we are working for stable clones, expressing above shRNA genes. These modified M210B4 cell line will be used in testing LSK cells in culture.

We also conducted expansion of cord blood derived HSCs under hypoxic culture conditions, as high oxygen tension is potentially detrimental to the cells. It has been revealed that hypoxic culture can induce 27-fold expansion of $CD34^{+}CD38^{-}$ cells, which was significantly ($P < 0.01$) higher than that obtained in normoxic culture. Proliferation assay suggested that normoxic culture (21% O_2) suppress cell cycle activation as significantly few cells expressed nuclear antigen Ki-67 than hypoxic culture. Together, these results suggest that either symmetric self-renewing divisions of some progenitor cells or asymmetric self-renewing divisions (differentiation) of others are favored by hypoxia. Myeloid colony-forming potential of cells has been significantly ($P < 0.05$) improved in 5% O_2 compared with 21% O_2 culture. Another interesting observation was that hypoxia not only improved colony-forming potential of the cells, but also helped to improve the recovery of colony-forming cells from normoxic culture. This has been concluded as CFU-Mix and BFU-E potential of normoxic culture were found to be significantly ($P < 0.05$) improved in hypoxic than in normoxic colony assay. SCID-repopulation efficiency seems to be better preserved in the cells cultured under hypoxic conditions. Hypoxia significantly ($P < 0.05$) induced the expression of HIF-1a, VEGF, and ABCG2 genes and also upregulated CXCR4 receptor expression.

B. Plasticity in BM cells

To avoid immune rejection of allogeneic Lin^{-} cells ($H2K^{q}$ haplotype) by hemophilic A mouse ($H2K^{b}$ haplotype), we studied the immunomodulatory role of donor antigen-specific T_{reg} cells of the recipient mice. Transplantation of sensitized T_{reg} along with allogeneic Lin^{-} cells was found to significantly lower mortality rate than the control group, and further analysis of the recipient mice revealed phenotypic changes closely resembling to those associated with syngeneic transplantation. The plasma FVIII concentration of these mice increased to about 20% of the wild type mouse and did not significantly decrease over the period of 6 months. Similar sustainable increase was seen *in vitro* plasma FVIII activity.

Liver fibrosis occurs due to progressive accumulation of extracellular matrix proteins (especially collagen types I and III) on the liver parenchyma that used to alter the normal hepatic architecture by forming a fibrous scar. The main clinical consequences of advanced liver fibrosis are impaired hepatocellular function and increased intrahepatic resistance to blood flow, which in turn result in hepatic insufficiency and portal hypertension. Hepatic stellate cells are believed to have a major role in fibrosis. A mouse liver fibrosis model has been established (Ishak stage IV). The fibrotic liver will be treated by transplanting three groups of cells (MSCs, hematopoietic cells, and a mixture of macrophages and granulocytes) through intra-spleenic route.

In the third project, we have further characterized MSCs, isolated from fetal liver of 13.5 dpc. We followed gene expression, immuno-cytochemistry and flow-cytometric analyses of the cells cultured at various passage numbers. The sorted cells expressed all known MSC markers and were negative for hematopoietic markers. We further carried out PCR analyses for various genes expressed by MSCs, osteogenic, adipogenic and neuronal cells. The isolated cells were found to express all MSC genes and also expressed hepatogenic markers like albumin and AFP. Upon culture, the expression of albumin and AFP were completely down-regulated in the third passage (P3). Immunocytochemical studies confirmed the expression of Vimentin, α-SMA in the cultured MSCs. MSCs at P6 were tested for tri-lineage differentiation and showed that they had osteogenic, adipogenic, and condrogenic differentiation potentials. Further, targeted differentiation of cells confirmed that some genes and proteins of dopaminergic neurons are expressed in the cells.

C. Ovarian cancer stem cells

In past one year, we have focused our attention in understanding the origin of cancer stem cells (CSCs) and its role in progression of ovarian carcinoma. Some research groups have established the presence of a small population of highly tumorigenic cells in human ovarian cancer which express certain combination of stem cell markers like $CD133^+$, $CD44^+$/c-Kit^+. These cells are more popularly described as cancer stem cells (CSC). It was found that a mouse ovarian tumor cells (ID8) do not significantly express such markers when grown *in vitro*, however were found to generate highly metastatic tumors in orthotopic tumor model. Upon isolating the tumor cells from the mice we observed that the GFP^+ ID8 cells gain certain hematopoietic stem cell markers like CD34, c-Kit and Sca-1 which are not observed *in vitro*. Also, we have observed that 4.13 ± 0.57% of primary tumour cells and 2.6 ± 0.34% of cancer cells in the ascites express the hematopoietic marker CD45. Upon further investigation we have concluded that the host bone marrow cells seem to play an important role in tumour progression and in ascites formation. Not only HSCs, expressing the typical Lin^-Sca1^+c-Kit^+ (LSK) phenotype, recruited to the primary tumor stroma, they are also present in the ascitic fluid at later stages of tumor development. The percentage of these HSCs was found to be 0.3 ± 0.04% which was comparable to the HSC population present in the bone marrow. It was observed that within the GFP^+CD45^+ population there also presence of a sub population of cells expressing the hematopoietic stem cell markers CD34, c-Kit and Sca-1. Of the tumor population in the ascites 0.58 ± 0.14% was $CD45^+$LSK and 1.23 ± 0.14% was $CD45^+CD34^+$. Further investigation by quantitative real time PCR revealed that the expression of the hematopoietic phenotype in epithelial ovarian cancer cells was the result of cell-cell fusion which is probably occurring because of the close interaction between the hematopoietic system and the solid tumor. Apart from the hematopoietic phenotype these fused cells also over-express CXCR4 which could render a superior invasive property to these cells when compared to the other cells. The existence of a stem cell compartment within this fused population could implicate fusion as one of the mechanisms responsible for the origin of cancer stem cells. We are trying to dissect this phenomenon and functionally characterize the cancer stem cell-like population to determine its significance in metastasis.

Publications

Original peer-reviewed articles

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Characterization of proteins important for cell death regulation

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As like survival, cell death is one of the fundamental biological processes that is commonly required to maintain normal development and homeostasis and is firmly related to etiology, pathogenesis and disease treatment. Essentially all animal cells have the ability to kill themselves by activating a gene-encoded cell suicide program. The decision of whether a particular cell will live or die is tightly regulated by many different signals originating both from the intracellular compartment as well as the extracellular environment. The overall goal of our research program is to elucidate the precise mechanisms by which cells die and how these processes are regulated by diverse signaling pathways in various unicellular and multicellular model systems.

Broadly, we aim to explore the underlying mechanisms that induce a cell in a given situation to survive or die and identify the molecular players. This molecular insight will help us to understand the kind of alteration of a pathway is required to induce a cell to die or survive. One of our experimental model systems is the protozoan parasite *Leishmania* spp. that branched early during eukaryotic evolution and survives in disparate biological environments during its life cycle. It provides an interesting system for studying a broad range of cellular pathways leading to cell death, some of which may be universal features of eukaryotic cells and would help us understand how more complex higher-eukaryotic regulatory systems evolved. Other cellular models that we use to pursue our interests on mechanisms associated with cell death include mammalian cancer cells where we try to understand how the vital pathways of cell death may be linked to cancer.

Biology of cell survival and death in protozoan parasites

Most of the intracellular pathogens evade the microbicidal responses of the macrophages by subverting or escaping from the phagocytic pathway. The protozoan parasite *Leishmania donovani* survives and proliferates within the mature phagolysosomal compartment of macrophages. Our earlier studies have shown that oxidative stress induces cell death in *Leishmania donovani* within and outside the phagolysosomal compartment. The parasites use defensive enzymes like

peroxidases which destroy the ROS and their products to establish a successful infection. Our earlier studies showed that following ROS exposure, intracellular [Ca^{2+}] increases and these unicellular protozoan parasites die by apoptosis if the defensive enzyme repertoire is compromised. It was also shown that tryparedoxin peroxidases (TXNPx), one of the major peroxidases in *Leishmania*, are critical for the parasite survival and fall in the levels of enzyme leads to increase in [Ca^{2+}] resulting in cell death. As a query arising out of the previous data, we attempted to find if there is any link between the tryparedoxin peroxidases and proteins related to [Ca^{2+}] signaling such as calmodulin (CaM) as [Ca^{2+}] levels appeared to be crucial for cell survival. Bioinformatic techniques used to analyze TXNPx showed the presence of CaM binding sites on both forms of TXNPxs. Interestingly, the mitochondrial targeting signal (MTS) of the mTXNPx harbored a CaM binding site. Docking studies of protein structures of CaM and the mTXNPx and cTXNPx substantiated the probable interaction sites. To determine the possible importance of the MTS located CaM binding site, mutants were generated by PCR mutagenesis and recombinant proteins were obtained. It was observed through *in vitro* studies that CaM interacted with the native protein but not to some of the mutant proteins. Subsequently, parasites were generated expressing the native as well as the mutant proteins. Localization studies showed that the native protein was able to localize to mitochondria whereas the mutants failed. In summary, CaM binds to mTXNPx using the predicted interaction sites at the N-terminus. The functional consequences of expression of mutant proteins instead of the native protein are being worked out. From an evolutionary point of view, the CaM dependence of the function of the MTS would be an interesting observation as it would indicate if this process was selected in this first mitochondrial eukaryote.

Continuing our work on CYP450s, we have generated half knockouts of CYP710 which appears to be a desaturase in the *Leishmania* parasite. These cells are unable to infect as efficiently as their wild-type counterparts and show lesser growth rate as well. These parasites are also susceptible to drugs.

Modulation of cellular apoptosis in mammalian cells

Teratocarcinoma is a germ cell tumor that is sensitive to cisplatin. We have used this model to study how the balance between autophagy and apoptosis determines cell fate post chemotherapy, as it is important from the point of view of improvements in chemotherapeutic treatment. Prior studies from this laboratory with high dose of cisplatin have shown that embryonal carcinoma cells die by apoptosis. Subsequent studies with preferred lower doses suggest that both autophagy and apoptosis are induced by lower doses of the drug. Autophagy is a lysosome-dependent degradative pathway frequently activated in tumor cells treated with chemotherapy. Whether autophagy observed in treated cancer cells represents a mechanism that allows tumor cells to survive therapy or a mechanism for initiating a non-apoptotic form of programmed cell death remains controversial. To address this issue, we established an experimental model in which embryonal carcinoma cells were treated with cisplatin, a widely used anticancer agent. LC-3 protein which is incorporated in the phagosomal membrane was used as a marker for autophagic vesicle formation. A dose- and time-dependent induction of autophagy was observed in tumour cells following cisplatin treatment, as demonstrated by up-regulation of autophagy-inducing proteins and subsequent appearance of autophagic vesicles. Robust autophagy was observed in the cells treated with lower dosage of cisplatin at early time point; it was characterized by the increase of punctate LC3 dots, the cellular morphology, and the increased levels of LC3B-II protein. Knockdown of autophagy regulatory proteins Beclin-1 and ATG5 with shRNA, or co-treatment with the autophagy inhibitor, bafilomycin A1 a lysosomal inhibitor, or PI3kinase inhibitors Wortmannin and 3-methyladenine markedly augmented cisplatin-triggered PARP cleavage, leading to an increase in DNA damage and apoptotic cell death. This suggests that the autophagy caused by lower

dose of cisplatin played a protective role and delayed apoptotic cell death. Furthermore, activation of p53 was detected in cisplatin treated cells. Downregulation of p53 by shRNA promote cell survival through augmentation of apoptosis. p53 knockdown cells also induced the basal level of autophagic proteins like ATG5, LC3-IIB and Beclin-1. These data can help unravel the underlying molecular mechanism of autophagy in DNA-damaged embryonal carcinoma cells and also provide a rationale for clinical evaluation of autophagy inhibitors in combination with DNA-damaging chemotherapy in human embryonal carcinoma.

Publication

Original peer-reviewed article

1. Verma S, Mehta A, Shaha C (2011). CYP5122A1, a novel cytochrome P450 is essential for survival of *Leishmania donovani.* **PloS One** 6:e25273

Cellular and molecular aspects of reproduction and viral infections

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One of the themes of our group is to understand the structure and function of zona pellucida glycoproteins, with special reference to mammalian fertilization. An extension of this scientific pursuit is to develop contraceptive vaccines for the management of wildlife population including street dogs. Other areas of study include the evaluation of the role of cytokines and growth factors in regulating the trophoblast cell proliferation, invasion and differentiation; identification and characterization of novel anti-HIV compounds from medicinal plants or from synthetic sources; and development of flu virus neutralizing monoclonal antibodies.

1. To understand the cellular and molecular basis of mammalian fertilization
2. To develop contraceptive vaccine for the management of wildlife population with special reference to street dogs
3. To investigate the role of IL-6 group of cytokines in the regulation of proliferation, invasion and differentiation of trophoblast or trophoblast derived cancer cells
4. To discover molecules with anti-HIV activity for their use as potential microbicide
5. To develop flu virus neutralizing monoclonal antibodies

Contraceptive vaccine for the management of street dog population

Extending the previous year progress on developing the contraceptive vaccine, recombinant TT-dZP3 without His_6- tag has been cloned and expressed in *E. coli* to meet the Review Committee on Genetic Manipulation (RCGM) regulations for its application. We have been able to optimize the protocol for its purification from inclusion bodies and its refolding (an Indian Patent is being filed). Characterization of the purified recombinant TT-dZP3 by fluorescent

spectroscopy revealed blue shift (λ_{max} at 339 nm) as compared to denatured protein (λ_{max} at 353 nm) signifying the presence of folded structure. Immunization of female FvB/J mice (6-8 weeks old) with the recombinant protein (25 µg/animal) supplemented with either PetGel A or Alum as adjuvants, followed by two boosters on day 21 and day 35 led to the generation of high antibody response against dZP3 and curtailment in fertility. A higher antibody response and contraceptive efficacy was observed in group of mice immunized with PetGel A formulation. In addition, our group has also cloned, expressed and purified recombinant fusion protein encompassing promiscuous T non-B cell epitope of tetanus toxoid (TT; aa residues 830-844), followed by dilysine linker, dZP3 (aa residues 165- 346), triglycine linker and immunoglobulin domain of Izumo (aa residues 165-250)-sperm specific protein. The basic premise of expressing this recombinant protein is to generate simultaneously antibodies against egg and spermatozoa specific proteins, thereby possibly increasing contraceptive efficacy.

With an aim to develop contraceptive vaccine for management of wildlife population, our group has also cloned, expressed and purified porcine ZP3 and ZP4 with and without T non-B cell epitope in *E. coli.* Immunization studies in female FvB/J mice using permissible adjuvant as described for recombinant TT-dZP3, showed contraceptive efficacy. The antibody titre was higher in the group of mice immunized with recombinant ZP3/ZP4 incorporated with promiscuous T non-B cell epitope. Higher contraceptive efficacy was observed in the animals immunized with ZP3 as compared to ZP4. Interestingly, injection of recombinant porcine ZP3/ZP4 in mice primed with native Solubilised Isolated Zona Pellucida (SIZP) led to secondary antibody response suggesting that these recombinant proteins are competent to recall memory response generated by native protein.

Regulation of trophoblastic cell invasiveness by IL-6 family of cytokines

To establish the functional significance of MMP23B in the IL-11 mediated increase in invasiveness of JEG-3 cells, its expression was silenced by siRNA. Silencing of MMP23B expression led to a significant reduction in JEG-3 cell invasiveness even after IL-11 treatment. Last year, we had also reported that in spite of IL-11 activation of STAT3 in HTR-8/SVneo cells, there was a decrease in the expression of several molecules including *MMP2*, *MMP3*, *MMP9*, *MMP23B* and *mucin 1*. In quest to find out the possible reason, IL-11 mediated activation and localization of downstream signaling molecules have been carried out. Treatment of JEG-3 cells with IL-11 led to an increase in the activation of ERK1/2 where, a decrease in its activation was observed in HTR-8/Svneo cells. Further, IL-11 treatment to HTR-8/SVneo cells led to activation and nuclear localization of activated STAT3 but it showed extensive co-localization with PIAS1/3, which would not allow the activated STAT3 to carry out its normal function as transcription factor. So, IL-11 mediated decrease in ERK1/2 activation and p-STAT3 (tyr705) binding to PIAS1/3 could be the probable reasons for the reduction in the expression of effector molecules in IL-11 treated HTR-8/SVneo cells.

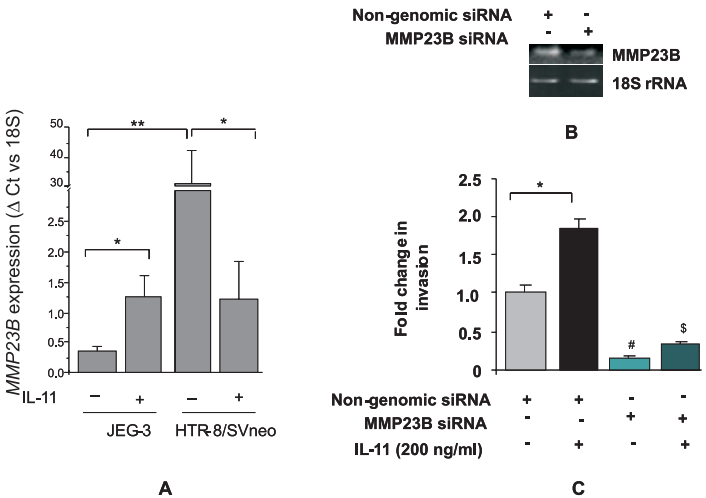

Figure 1. ***Role of MMP23B in IL-11 mediated invasion of JEG-3 and HTR-8/SVneo cells:*** *Quantitative RT-PCR was done for the expression of MMP23B after IL-11 treatment (Panel A). In another experiment, JEG-3 cells were transfected with either MMP23B siRNA or non-genomic siRNA for 72 h and end point RT-PCR was done to check the level of silencing in them, keeping 18S rRNA as internal control (Panel B). The transfected cells were used to study their invasive behavior in the presence or absence of IL-11 (200 ng/ml). *p<0.05; **p<0.001; #p<0.01 between JEG-3 cells transfected with non-genomic and MMP23B siRNA; $p<0.001 between IL-11 treated non-genomic siRNA transfected and MMP23B siRNA transfected JEG-3 cells*

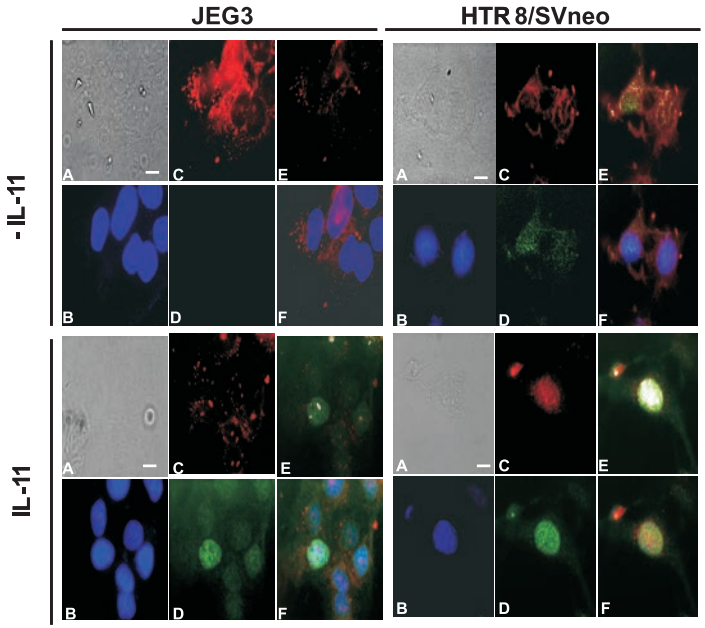

Figure 2. ***Immunolocalization of p-STAT3 (tyr705) and PIAS1/3 in JEG-3 and HTR-8/SVneo cells following IL-11 treatment:*** *JEG-3 and HTR-8/SVneo cells were treated with IL-11 (200 ng/ml) for 10 min and then checked for the immunolocalization of p-STAT3 and PIAS1/3 followed by counter staining with DAPI. In the figure sub-panels are: A; phase contrast image, B; DAPI stained cells, C; staining for PIAS1/3, D; staining for p-STAT3(tyr705), E; co-localization of p-STAT3(tyr705) and PIAS1/3, F; merge of the PIAS1/3, p-STAT3(tyr705) and DAPI images. Scale bar: 20 µm size.*

Leukemia inhibitor factor (LIF) has been shown to be important for human pregnancy as its deficiency lead to recurrent abortions. In the present investigation, we have made an attempt to decipher the role of LIF on HTR-8/SVneo cell invasiveness and the molecular mechanism associated with this event. LIF increased the invasiveness of HTR-8/SVneo cells through activation of STAT1, STAT3 and ERK1/2 (thr202/tyr204) dependent signaling pathways. This was associated with their nuclear localization as early as 10 min after LIF treatment. Microarray analysis following treatment of HTR-8/SVneo cells with LIF led to an increase in the expression of 134 genes by at least 1.5 fold while, a decrease in the expression of 103 genes by at least 0.5 fold. Amongst them, we have identified pappalysin 1, SERPINB3, podoplanin, integrin β3, ID1, CEACAM1 etc as invasion-associated genes whose, expression got significantly up-regulated upon LIF treatment. This is suggestive of the multifactorial regulation of LIF mediated invasiveness of HTR-8/SVneo cells.

Identification of natural and synthetic molecules with anti-HIV activity

Medicinal plants which showed anti-HIV activity in initial rounds of screening process have been taken further to isolate active compounds. Silica gel column Chromatography of NBRH-10 led to isolation of five compounds; identified as corosolic acid, gallic acid, ellagic acid, quercitin and rutin by NMR and MS. Corosolic and ellagic acid showed anti-HIV activity through a dose dependent inhibition in HIV-1 protease activity whereas; gallic acid did so by inhibiting the HIV reverse transcriptase activity. From NBRH-04, three compounds (SMH-27, SMH-30 and SMH-31) showed a dose-dependent inhibition in HIV infection. SMH-30 and SMH-31 inhibited the HIV-protease activity whereas; SMH-30 also inhibited the tat-LTR transactivation for HIV gene expression. Further, in collaboration with Dr. Sujata V. Bhat, Mumbai University, various synthetic derivatives of Andrographolide-1 have been evaluated for anti-HIV activity. We have identified two synthetic derivatives with potent anti-HIV activity (TI>500) which possibly act by binding to the critical residues of V3 loop region of gp120.

Neutralizing monoclonal antibodies (MAbs) against influenza virus

So far, we have been able to generate 20 MAbs against the pandemic H1N1 virus and 35 MAbs against HA proteins of seasonal influenza virus. One of the MAb showed potent neutralization of the pandemic H1N1 virus (A/California/07/2009) in an *in vitro* microneutralization assay with IC_{50} of 0.08 µg/ml.

The IgG1 isotype antibody showed high affinity (Kd = ~10^{-9}M) interaction with the HA protein, reacted with the *E. coli* expressed HA1 (52-272 aa) protein and also showed hemagglutination inhibition (HI titre < 0.53 µg / ml) with the pandemic virus using guinea pig RBCs. In order to humanize the antibody, CDRs and important framework residues from the murine MAb were grafted onto highest identical human germline framework region. Two such humanized variants were expressed in *E. coli* in single chain variable fragment (scFv) format. Preliminary studies indicate that the humanized antibody is able to neutralize the pandemic H1N1 virus. In addition, 2 mouse MAbs showed neutralization of the seasonal H1N1 (A/Solomon Islands/3/2006) virus.

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Patent

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In Press last year, since published.

*In Press on 31st March, 2012.

MOLECULAR DESIGN

MOLECULAR DESIGN

Structural studies on proteins, dynamics and ligand interactions using NMR

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The theme of the research is to study the structure and dynamics of proteins using NMR, and other biophysical techniques and relate them to their function. Our lab is involved in studying a number of proteins suitable for NMR, *viz*. B-cell linker protein, ubiquitin, acyl carrier protein, acyl CoA binding protein *etc*., all involved in protein-protein interactions.

The main objectives of the project are to clone, express, purify and structurally characterize various proteins using NMR and study their interaction with the naturally occurring partners to understand their biological function.

As proposed in the previous report, we have extended the studies on ubiquitin to understand the changes in its structure and dynamics upon binding to its naturally occurring partners STAM-I and Hrs, two important components of the endosomal sorting complex transport machinery. The most remarkable feature of ubiquitin is its ability to make innumerable non-covalent interactions that play a vital role in the regulation of a multitude of cellular processes. Comparison of the interaction surface of ubiquitin in its complexes suggests that majority of the interactions are mediated by the hydrophobic patch. It's perplexing how a single ubiquitin molecule recognizes such a diverse range of binding partners. Given the structural diversity of its ligands, and variation in specificity, multiple structural determinants of ubiquitin recognition probably exist for each partner. Identifying the structural features of ubiquitin that play an indispensable role in its recognition is necessary to understand the regulation of ubiquitin mediated pathways. What are the structural features of ubiquitin that make it such a unique protein-protein interaction partner? To address this question, we carried out extensive studies using NMR. Our results shed light on the unusual backbone flexibility of ubiquitin; disclosed from the changes in backbone conformation, hydrogen bond lengths, and dynamics upon ligand binding. Besides, the unique hydrogen bond network that makes the surface of ubiquitin fluid like, capable of undergoing changes in response to ligand binding is yet another important factor contributing to its remarkable ligand diversity.

Apart from ubiquitin, our group is involved in understanding the type I and type II fatty acid biosynthesis pathways. Acyl carrier protein (ACP) plays a central role in both the pathways. However, the molecular machinery that mediates its function is not yet fully understood. Therefore, we have initiated studies on type I as well as type II ACP's. We have selected Type I ACP from human (*Homo sapiens*) while type II ACP from a) *Plasmodium falciparum* and b) *Leishmania major*. The goal is to understand the structure of these ACP's, as well as their interaction with the enzymes of the fatty acid pathway.

In this context, we have cloned and purified the various proteins involved in the fatty acid biosynthesis of *Leishmania major viz.* Acyl carrier protein, Fatty acid synthase and enoyl ACP reductase for which the sequence is available. Very little if any information is available pertaining to the localization or function of these proteins. In case of ACP, we have completed the backbone and side chain assignments of its holo-form and published it recently.

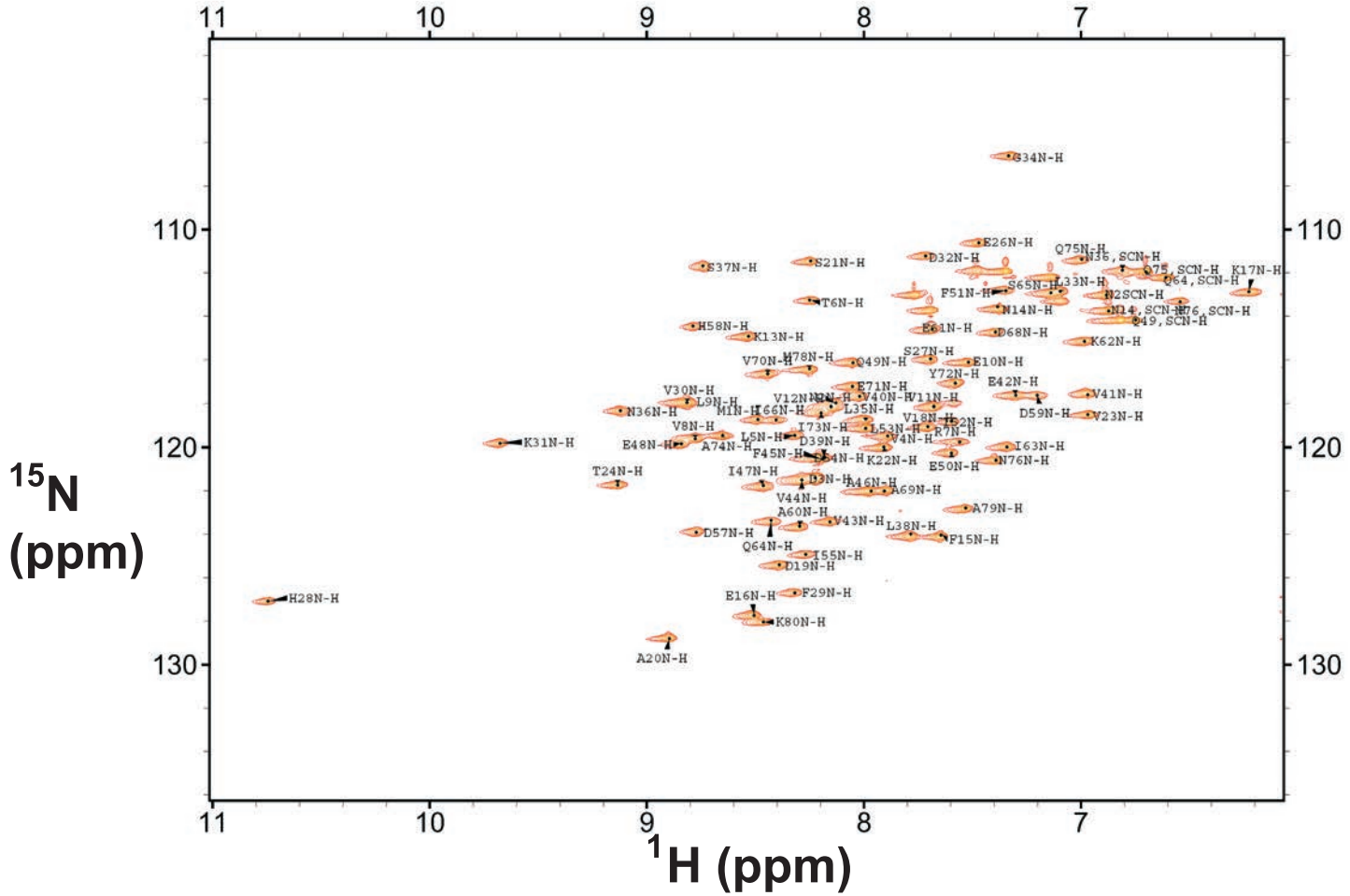

Figure 1. *A $^{1}H^{15}N$ HSQC spectrum of uniformly labeled $^{15}N/^{13}C$ Holo-acyl carrier protein of Leishmania major.*

Structure of the active form of *Leishmania* (holo-ACP) has been solved using NMR and compared to other known ACPs. Unlike *P. falciparum* ACP, *Leishmania* ACP is expressed as apo-ACP and the fatty acid synthase of *E. coli* is unable to convert this ACP into the active holo-form. Human ACP also expresses as apo-ACP and some interesting sequence similarities exist between *Leishmania* ACP and the latter. With a view to better understand the process of fatty acid biosynthesis by these ACPs, we have also prepared their intermediates *in vitro* to simulate the biosynthetic process *in vivo*.

Besides the biosynthetic pathway, we are also interested in the dispersal of fatty acids. Therefore, we have initiated studies on the proteins involved in the transport of fatty acids in *Leishmania major i.e.* Acyl CoA binding proteins that exists in six isoforms. Owing to the indispensable role of lipids in the survival of *Typanosoma*, a close relative of *Leishmania,* this protein seems to be an excellent target for drug design.

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*In Press on 31st March, 2012.

To develop strategies for making sensors and actuators for biological process

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Aim of research

To develop systems for monitoring biological processes.

To develop tools for needle free immunization.

To study the biological processes like differentiation, hybridization etc. and to develop devices and sensors based on such studies.

Immunization by the aerogenic route

We have developed a methodology to encapsulate live Mycobacterium (MIP and BCG) as a dry powder inhale-able formulation which remains viable for 4 months at 37°C. A non invasive procedure was developed to deliver this formulation to small animals.

In an important experiment we have demonstrated that the BCG encapaulated alginate particles (BEAP) and MIP encapsulated in alginate particles (MEAP) activated BMDCs show higher up regulation of CCR7 receptor on their surface as compared to MIP/BCG activated BMDCs.

This higher up regulation of CCR7 marker was further translated into enhanced migration of BMDCs in response to the chemotaxis induced by CCL19, which is an agonist for the chemokine receptor CCR7. In a similar experiment, it has been demonstrated that DCs, those were directly infected by mycobacterium migrated poorly than DCs those had acquired the mycobacterium through uptake of infected neutrophils. In our experiments, the MIP encapsulated alginate particles have an analogy with infected neutrophils and they act like a delivery module of MIP to the DCs and this mode of delivery of mycobacterium to the DCs does not impair their mobility.

Overall, we have established by a number of *in vitro* and *in vivo* experiments that this inhale-able vaccine of live Mycobacterium is more immunogenic as compared to the aerosol of bacilli and it provide better protection in mice when challenged with H37Rv. The alginate coated DPA of BCG/MIP is a very promising alternative for present day vaccination for tuberculosis; it does not require the cold chain for transportation and storage, it provide better protection than conventional intradermal or liquid aerosol and its delivery does not require the needle and a syringe.

Local hyperthermia enhanced immunization

Hyperthermia enhanced transdermal (HET) immunization is a novel needle free immunization strategy employing application of antigen along with mild local hyperthermia (42°C) to intact skin resulting in detectable antigen specific Ig in serum.

To determine the effect of local hyperthermia on dermal DC maturation and migration to regional lymph node, we subjected mice to *in vivo* local hyperthermia by placing the hyperthermia inducing patch, containing either PBS or antigen, on the right thigh of mice. After 24 hours, we prepared epidermal sheets from treated area as well as untreated area of the mice skin and stained them for CD11c+ cells. We observed a decrease in the number of CD11c+ cells in epidermal sheets taken from area of skin where hyperthermia patch was placed, suggesting that short duration hyperthermia indeed causes migration of DCs from skin to lymph node.

To further verify this, skin DCs were fluorescently labelled in vivo by the cutaneous injection of carboxyfluorescein diacetate succinimidyl ester (CFSE) and HET patch was placed at the site of CFSE injection. We prepared the lymph node suspensions after 24 hours of treatment, stained them with CD11c-PE.Cy5 antibody. The population was gated for CD11c+ cells on the basis of CD11c-PE.Cy5 staining and confirmed the the presence of CFSE+ cells, indicating the cells that have migrated from skin.

We also performed similar experiment with animals immunized by HET (along with TT antigen) and conventional needle immunization. We found that compared to control, both HET groups (with or without antigen) showed a significant increase in expression of Langerin marker, thus indicating that mild hyperthermia of the HET patch is sufficient to cause an enhanced migration of DCs from skin to draining lymph node.

To distinguish epidermal DCs (LCs) among the Langerin+ dermal DCs present in the lymph node, we utilized the selective expression of cell adhesion molecule EpCAM on epidermal DCs and results are summarized in Figure 1. This experiment reinforce that heat alone leads to enhanced migration of epidermal, EpCAM+ DCs to the draining lymph node. The presence of an antigen (TT in our case) in the HET patch further added to this enhancement. Further, the enhanced migration remain localized to the nearest draining lymph node.

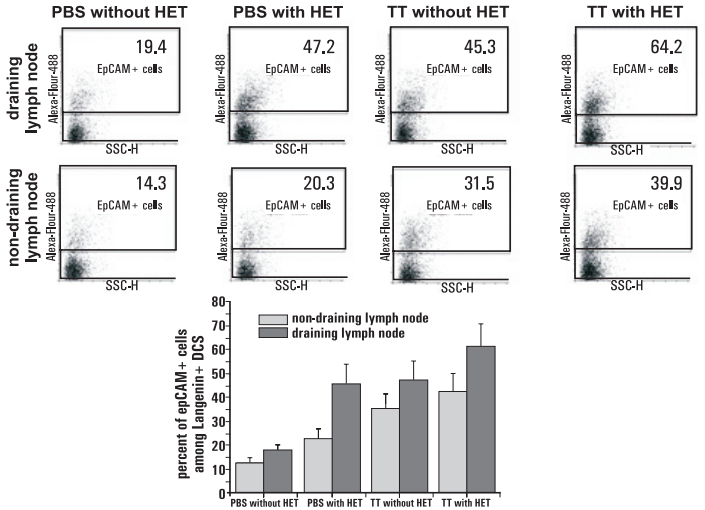

Figure 1. *Migration of EpCAM+ epidermal DCs to the lymph node. Mice were immunized by TT, with and without HET. Unimmunized (PBS without HET) animals served as control. Cell suspension from draining and non-draining lymph nodes were prepared after 24 h and stained with Langerin (CD207)-PE and EpCAM-Alexa Flour 488 antibodies. Stained cells were analyzed by flow cytometry, Langerin+ cells were gated and the expression of EpCAM was analyzed. Number on the selected area indicates percent of EpCAM+ cells among Langerin+ cells in total lymph node cells. The bar graph shows average percentages of three experiments.*

In summary, we have developed a tools for HET immunization and have shown that mice immunized by tetanus toxoid using

HET route exhibited protection from challenge with a lethal dose of tetanus toxin.

Diagnostics

We examined relatively new TB diagnosis technologies; nucleic acid detection of two target strands, IS6110 and devR, by PCR and microscopic observation drug susceptibility (MODS). The LJ culture was the gold standard. This evaluation was done on 463 sputum samples of tuberculosis suspects at a specialized tuberculosis clinic in Delhi, India.

None of the tests we evaluated can accurately detect the presence or absence of Mycobacterium tuberculosis in all the samples and smear microscopy was found to be the most reliable assay in this study. The PCR assay could detect down to 2pg of H37Rv DNA. Sensitivity, specificity was 0.40, 0.60 and 0.19, 0.81 for smear positive (n=228) and negative samples (n=235) respectively. In the MODS assay, sensitivity, specificity of 0.48, 0.52 and 0.38, 0.76 was observed for smear positive and negative samples. Sputum smear microscopy had sensitivity of 0.77 and specificity of 0.70.

Ex-vivo 3D liver culture

Hematopoietic stem cells (HSCs) are known into differentiate to hepatocyte *in-vitro* but it is difficult to get significant number of HSCs from adults. Human umbilical cord blood has higher density of CD14+ monocytes. There have been a few reports where monocytes were dedifferentiated so that they achieve some stem cell characteristics. The re-differentiation of the de-differentiated cells was then done to generate hepatocyte-like cell *in-vitro.*

We are able to 'de-differentiate' peripheral blood mononuclear cells (PBMCs) to 'hematopoietic stem like cells'. In the de-differentiate' cells the Nanog, which is a transcription factor critically involved with self-renewal of undifferentiated embryonic stem cells, has been detected at transcription level. Additionally these cells have enhanced expression of CD117.

These 'hematopoietic stem like cells' were re-differentiated to the epithelial lineage, the hepatocyte-like cells and the expression of albumin, HNF-4 alpha and Cytokeratin 18 were confirmed in hepatocyte-like (neo-hepatocytes) cells. Further, as a functional confirmation, the neo-hepatocytes were able to detoxify 7-pentoxy resorufin to resorufin.

Hepatocyte-like cells from HBV infected PBMCs

Successful generation of hepatocyte-like cell *in-vitro* will have a worthy clinical application as it may give a cell based therapeutic option for hepatitis B infected patient who are suffering from liver cirrhosis. The magnitude of liver damage caused due to cirrhosis cannot be managed by the bone marrow derived cells as very few hepatocyte-like cell can be generated by this process. Therefore the generation of hepatocyte-like cells from HBV infected PBMCs will be of remarkable utility.

We have 'de-differentiated' and further 're-differentiated' HBV+ PBMCs to neo-hepatocytes and these cells have been characterized and found to be similar to healthy neo-hepatocytes.

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Protease-catalyzed splicing of peptide bond

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We study the principles underlying peptide ligation reactions catalyzed by proteases and transpeptidases with a view to apply them to the semisynthesis of proteins, assembly of well defined bio-conjugates and protein dendrimers that may be useful in a variety of biotechnological applications. Currently we are utilizing the peptide ligation propensity of transpeptidase sortase as a versatile tool for peptide/protein engineering. Sortase enzymes recognize a pentapeptide LPXTG motif in bacterial surface proteins cleave the scissile T-G peptide bond and engage the threonyl carboxyl group in a new peptide bond with the α-amine of pentaglycine sequence present in peptidoglycan leading to covalent anchoring of the proteins to the cell wall.

A. Peptide ligation reactions catalyzed by transpeptidase sortase: Protein labeling and conjugation to a dendritic scaffold.

B. Studies on sortases from *Streptococcus pneumoniae.*

Objective A

During the reporting period, a straightforward utility of the Sortase-Click method was envisaged in the construction of multivalent vaccines. For this, surface exposed domain of pneumococcal surface protein A (PspA) from *Streptococcus pneumoniae* corresponding to residues 98-286 was considered. The choice of PspA was inspired by the fact that it is a lead candidate for a protein-based pneumococcal vaccine. PspA thus can be a good model antigen with which to construct a protein dendrimer for evaluating the effect of multivalency on immunogenicity. Accordingly, PspA was engineered to obtain a PspA-LPNTG-His_6 protein construct. PspA-LPNTG-His_6 was efficiently converted into PspA-alkyne or PspA-azide using alkyne or azide derivatized aminoglycine in the presence of SrtA. Conjugation of PspA-azide with complementary dendrons proceeded smoothly and produced the respective dendrimers in high yields. Likewise conjugation of PspA-alkyne yielded divalent or tetravalent PspA dendrimers. The high yields of dimeric and tetrameric species facilitated easy isolation of the respective protein dendrimers by size exclusion chromatography. That the dendrimers were obtained in excellent purity was established by SDS-PAGE, tryptic peptide mapping and mass spectrometry.

To demonstrate the effect of multivalency on immunogenecity, three groups of mice were immunized with PspA dendrimers (monovalent, divalent and tetravalent) in alum. A fourth group was administered PspA-tetravalent dendrimer (without alum) to gauge the intrinsic effect of multivalency on immunogenicity. The serum anti-PspA end point titre was determined for individual mice following three immunizations with various PspA dendrimer preparations using ELISA with PspA-LPNT-alkyne as capture antigen. The median PspA-specific total IgG titre revealed a hierarchy of immunogenicity as tetravalent > divalent > monovalent. The subtying of antibody response exhibited the same trend with preponderance of IgG1 indicating a bias toward Th2 response as observed previously. Interestingly, immunization with PspA-tetravalent dendrimer without alum also elicited an immune response comparable to that observed when PspA-tetravalent dendrimer was administered in the presence of alum. Thus multivalent presentation of PspA elicits enhanced immune response, both in the presence and absence of alum. The results demonstrate the high potential of Sortase-Click method in the construction of homogeneous multivalent vaccines facilitating structure-activity studies as well as easy adherence to regulatory quality control norms.

In summary, a bioorthogonal Sortase-Click reaction suite was developed by combining two extremely specific and reliable reactions that occur under very mild conditions. The method is accessible to all proteins equipped with a LPXTG sortase-recognition sequence and is easily applicable to more common His_6-tagged proteins. Besides, lysine dendritic wedges with orthogonal handles are amenable to easy synthesis by standard solid phase peptide chemistry. Both reactions, labelling as well as CuAAC, produce little or no side products. His_6-tagged unlabelled protein and SrtA are easily removed by capture on Ni-NTA beads and pure protein dendrimers, after the click reaction, are obtained by routine size exclusion chromatography. Sortase-Click approach provides enormous synthetic flexibility for incorporation of diverse proteins in the dendrimer.

Objective B

Preliminary analyses of the crystal structure revealed the presence of four molecules of pSrtA in the asymmetric unit which could be visualized as a dimer of dimers. We carried out biophysical and biochemical studies to investigate if pSrtA behaved as a dimer in solution. Accordingly we investigated the oligomeric status of pSrtA by size exclusion chromatography and sedimentation equilibrium analysis. The results showed that the protein predominantly existed as a dimer in solution corroborating the biological relevance of the crystal structure. We also attempted to map the distance between the active site Cys residues in the subunits by thiol-specific cross-linkers. Using several bifunctional maleimide-compounds separated by defined distances, Cys-Cys distance was mapped to 13 Å. Further analyses of the structure would shed more light on the participation of one or both catalytic residues and other mechanistic intricacies of pSrtA catalysis.

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Patent

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Therapeutic Interventions in Chronic Diseases

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My group is a multi-disciplinary group adapting an integrated approach in drug discovery that combines medicinal chemistry and biochemistry principles for efficient drug design process.

Interests of the group lie in identifying underlying principles in a disease pathogenesis, designing molecular intervention strategies, synthetic medicinal chemistry, and confirming the biological/therapeutic activities of the designed compounds. The small molecule regulators contribute to both drug development and understanding biological systems in human body.

1. Supramolecular calcitonin assembly for a lasting treatment of osteoporosis.

Osteoporosis affects many million people all over the world. It is a disease of skeleton system with low bone mass (osteopenia) and impaired bone micro architecture leads to an enhanced bone fragility and increased risk of bone fracture. To date several therapeutic interventions were approved for treatment of this disease. Most of them are anti-resorptive agents that targets osteoclast activity. Though they found to be a strong suppressor of osteoclast activity, these anti- resorptive agents were found to reduce the secondary bone formation except calcitonin, a natural peptide hormone produced by thyroid glands in mammals. Calcitonin has two important attributes 1) Suppress the Osteoclast activity rather depleting its number and secondary bone formation 2) Strong analgesic effect over bone fractures. But antigenecity of approved salmon-calcitonin (40-50 times active than human calcitonin) and bioavailability and stability of human calcitonin prevents its way of being the first line therapy to osteoporosis. We developed a safe and long acting supramolecular assembly of human calcitonin (SCA) for sustained treatment of postmenopausal osteoporosis in animals.

To verify anti-resorptive effect and also determine an optimal dose for therapeutic application, different doses of SCA were injected subcutaneously/intradermally in osteoporotic animals. It was observed that SCA-I at a single injection of 75 and 150 µg significantly reduced body weight. Its effect was most prominent at second week post therapy and this effect was achieved for about ~21 days. As at higher doses SCA-I lowered body weight at an accelerated pace, 75 and 150 µg of SCA-I were chosen as a therapeutic dosage for the detailed prospective studies. Figure 1a shows the superior effect exerted by SCA-I over other therapies employed. A point to be highlighted is that supramolecular form of human calcitonin imparted effects equivalent to that of salmon calcitonin and higher compared to human calcitonin. It should also be taken into contemplation that salmon calcitonin exerted protective effects following multiple injections whereas a single dose was enough for SCA-I to exert its effects for prolonged periods of time.

Serum concentration of calcitonin released from SCA administered was in the physiologically relevant range of 15-20 pg/ml for over a period of 21 days (Figure 1b). In addition to normalising body weight, SCA-I had considerable effect in reducing TRAP5b activity and CTX levels in serum (Figure 1c & d).

Bone X-ray images revealed differences in femur sensitive regions of interest (FROI: proximal, middle and distal) in ovariectomized control and SCA-I treated groups (Figure e & f). Ovariectomised rats exhibited lower bone density compared to normal rats. FROI in the SCA-I treated rats exhibited significant increase in bone density compared to ovariectomised control. Deformity observed in the epiphysis region of ovariectomized rats was not seen in SCA-I treated rats. Histomorphological analysis of the femur bone samples showed a higher order arrangement of trabeculae in SCA-I treated rats as compared to ovariectomised rats that exhibited profound degenerative changes in femur.

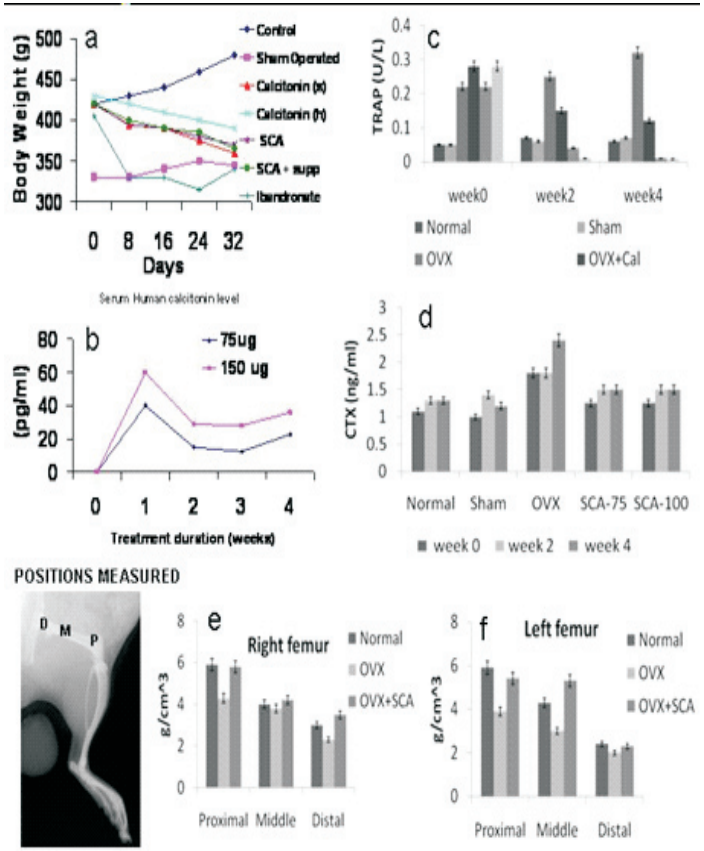

Figure 1. *(a) Anti-resorptive efficacy of SCA and other treatments in ovariectomized rat, (b) in vivo released level of calcitonin in SCA administered rats, level of (c) TRAP (d) CTX in the serum of SCA treated rats. X-ray image of femur of SCA-I treated rats. (e & f)) Changes in femur bone density at proximal, middle and distal regions of femur of normal and SCA-I treated rats.*

Thus we have improved the efficacy of human calcitonin by formation of SCA, which upon single injection had shown effect for at least 21 days. Therefore this may open the way to use human calcitonin for clinical application to avoid the limitations associated with salmon calcitonin.

2. Design, synthesis and biological evaluation of benzothiophene carboxamide derivatives as analgesics and anti-inflammatory agents

Non-steriod anti-inflammatory drugs (NSAIDs) are chemically heterogenous group of molecules used to treat symptoms of acute pain and chronic inflammatory and degenerative joint diseases, which mainly act through the inhibition of COX-2 dependent prostanoids. However, they are associated with adverse side effects on gastro-intestinal and cardiovascular systems, which is due to the inhibition of both COX-1 and COX-2 respectively. Thus novel NSAIDs with improved pharmacokinetic features and safe metabolism is needed. To address this we have design and synthesized some new benzothiophene carboxamide derivatives (Compounds **1-11**) and checked their efficacy as analgesic and anti-inflammatory agents. We studied the anti-nociceptive response of synthesized derivatives of Benzothiophene carboxamide by hot plate, tail flick latency and by mechanical allodynia (pressure withdrawal threshold) assays. First, we evaluated the anti-nociceptive response of some classic/standard NSAIDs (50 mg/kg b. wt.) i.e. Ibuprofen, Naproxen, Ketoprofen, Aspirin, Nimuslide, Indomethacin by hot plate latency assay in 60 minutes post administration and it was observed that ibuprofen showed the best anti-nociceptive effect amongst the used NSAIDs. Hence, ibuprofen (50mg/kg b. wt.) was chosen for comparison in further studies. The anti-nociceptive efficacy of benzothiophene carboxamide compounds (**4-11**) was dose dependent and no significant difference was observed at dosage > 15mg/kg b.wt. It was observed that all the animals injected with benzothiophene carboxamide derivatives at 15 mg/kg of b. wt exhibited significant increase in MPE index except for compounds 10 and 11 which showed less latency in comparison to other derivatives (Figure 2a-c). Thus, the above results reveal that compounds **4**, **6** and **8** have potent analgesic activity at much lower concentration than Ibuprofen. Anti-inflammatory potential of benzothiophene carboxamide derivatives was evaluated in animal model of inflammatory pain (induced by intra-plantar injection of 1% λ- carageenan in hind paw). Again, the anti-inflammatory effect of these compounds was much better than classical NSAID (e.g. Ibuprofen) (figure 2d).

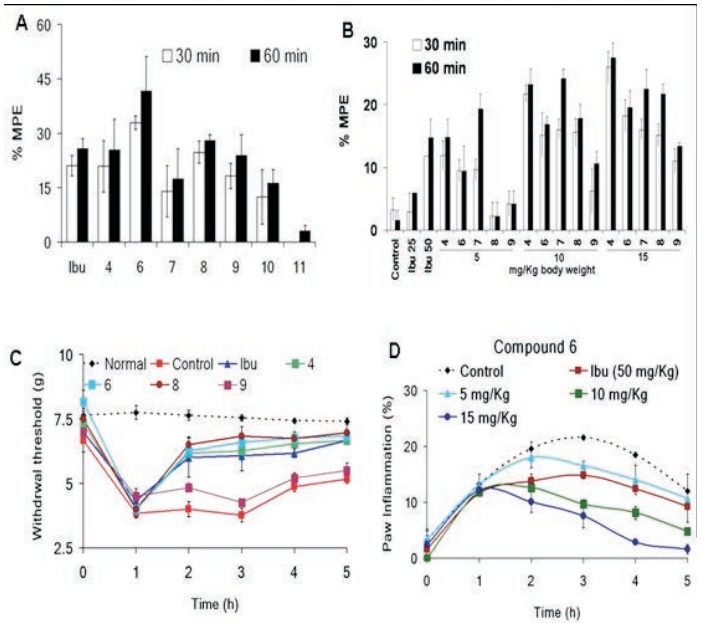

Figure 2. *Comparative analysis of anti-nociceptive latency response of Benzothiophene carboxamide compound **4-11, (a)** The latency response is assessed by Hot plate latency test and expressed in terms of maximum possible effect in percent (% MPE). **(b)** Tail Flick latency test at a constant temperature 54 ±1 °C and response is expressed in terms of maximum possible effect in percent (% MPE). **(c)** Reversal of mechanical hyperalgesia by compounds (15 mg/kg b. wt.) compared with Ibuprofen (50 mg/kg b. wt.) at different time intervals. Paw withdrawal threshold to mechanical hyperalgesia induced by intraplantar injection of 0.1 ml of 1% (W/V) λ-carrageenan assessed by Randall-selitto meter. **(d)** Effect of compounds 6 on inflammatory hyperalgesia at different dosage and time intervals (0-5hr). Inflammation was induced in right hind paw of rats by λ-carrageenan and contra lateral hind paw without injection was used as control. Paw edema was monitored by plethysmometer and expressed in percentage of paw volume edema. Results are means ± S.E.M, n = 6 each group and p < 0.05.*

In addition, we validated the mechanism of action of these compounds in regulation of hyperalgesia and inflammation by observing the inhibition of inflammatory mediators like cytokines, chemokines, neutophill accumulation, prostaglandin (PEG2) synthesis and COX-2 enzyme. Our findings demonstrate that newly synthesized bromo-benzothiophene carboxamide derivatives 4, 6 and 8 showed a good potential in attenuating nociception and inflammation at lower concentration than ibuprofen. These compounds were shown to inhibit the activation of cytokines, chemokines, neutrophil accumulation, synthesis of prostaglandin-E2 and expression of cyclooxygenase-2 (COX-2) at lower concentration than indomethacine. Molecular docking and in vitro COX isozyme inhibition studies showed that these compounds have selectivity for COX-2 and practically no affinity to COX-1. Moreover, toxicological study also reveals that these compounds were well tolerated and metabolized to avoid any toxicity. Thus, these newly synthesized molecules hold a potential to develop as an analgesic and anti-inflammatory agents. The efficacy of these compounds and their mechanism of action in neuropathic pain are currently going on.

Publication

1. Banerjee T, Singh RR, Gupta S, Surolia A, Surolia N (2012) 15-Deoxyspergualin hinders physical interaction between basic residues of transit peptide in PfENR and Hsp70-1. **IUBMB Life 64:**99-107.

In vitro reconstitution of intracellular transport: Role of GTPases

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Major theme of the project is to understand the regulation of intracellular trafficking and its modulation by intracellular pathogens as well as in different pathological conditions. One of the main goals of this project is to understand the mechanism of survival of pathogens in macrophages by modulating the host trafficking pathway. We are also trying to understand how intracellular pathogen like *Leishmania* acquires heme through endocytosis and intracellular degradation of hemoglobin and its importance in the biology of the parasites. We have also initiated the studies on cytokine mediated modulation of intracellular trafficking.

Phagocytosis is an important process in host defense and is mediated by complex interactions between defined intracellular compartments. The final fate of the nascent phagosomes usually culminates with the fusion of lysosomes. But some invading microorganisms modulate this central process for their survival in the phagocytic cells. The major objectives of the present investigations are:

a. Modulaton of phagosome maturation by intracellular pathogens.

b. Determination of the role of various cytokines in the modulation of phagosome trafficking.

Evidences from a variety of sources, have established that transport of cargo along the endocytic pathway requires a series of highly coordinated and specific vesicle fusion events regulated by small GTP binding proteins of the Rab family. Not much is known about the regulation of endocytosis and intracellular trafficking in protozoan parasites. The major objective of the project is to understand how *Leishmania* generate heme from the intracellular degradation of endocytosed hemoglobin.

c. Mechanism of intracellular trafficking of hemoglobin in *Leishmania*.

Mechanism of survival of Salmonella in macrophages

Previously, we have shown that *Salmonella*-containing phagosomes recruit Syntaxin 6 through their effector molecule SipC. Therefore, in the reviewing period, we have tried to map the interaction of these two proteins. Our results have shown that Syntaxin6 (2.5 µg) binds with SipC in a concentration dependent way and optimal binding is detected with 1 µg of

SipC. Therefore, we have used equimolar amount of respective proteins in subsequent binding experiments. Interestingly, our results have shown that SipC specifically binds with truncated Syntaxin6$^{176\text{-}230}$, the SNARE motif of Syntaxin6, but not with Syntaxin6$^{1\text{-}76}$. Subsequently, we have tried to map the region of SipC important for the interaction with syntaxin6. Topological analysis of SipC has shown that first 120-amino-acid of N-terminus and last 209-amino-acid of C-terminus extends into the host cytoplasm. Therefore, we have determined the binding of Syntaxin6 with N- or C-terminal fragment of SipC. We have found that both Syntaxin6 and Syntaxin6$^{176\text{-}230}$ interact specifically with the C terminal end of SipC, SipC$^{200\text{-}409}$ but not with the N terminal end SipC$^{1\text{-}120}$ (Figure. 1F). Taken together, our results have demonstrated that the C-terminal end of SipC specifically interacts with Syntaxin6 through its SNARE motif.

To confirm the importance of C-terminal region of SipC in the Syntaxin6 mediated recruitment of LAMP1, we have used two *Salmonella* mutants; sipC^{R315Z} and sipC^{M389K}. Subsequently, we have found that mutated SipC secreted from sipC^{R315Z} and sipC^{M389K}:*Salmonella* do not bind with Syntaxin6. Interestingly, we have also observed that phagosomes containing sipC^{M389K} and sipC^{R315Z} *Salmonella* do not recruit significant amounts of Syntaxin6 as compared to WT:SCP. Consequently, we have determined the recruitment of LAMP1 by these SipC mutants which are deficient in the Syntaxin6 binding. Our results have shown that both sipC^{M389K}:*Salmonella* and sipC^{R315Z}:*Salmonella* are unable to bind significant amounts of Syntaxin6 in comparison to WT:SCP and thereby fail to recruit LAMP1 on their phagosomes. These results clearly demonstrate the specific role of C-terminal of SipC in the LAMP1 recruitment.

It has been postulated that some effector molecules from bacteria might mimic the structure of SNARE proteins and bind with cognate SNARE partners in host cells. The C-terminus of SipC has a large number of hydrophobic residues interspersed by 2 to 3 polar residues. Such sequence pattern is characteristic of coiled coil motifs which also resemble SNARE motifs. In silico structure prediction studies of SipC also have suggested that its C-terminal stretch adopts a coiled coil helix. Hence, interaction of SipC with the SNARE motif of Syntaxin6 is likely to involve interactions between coiled coil regions of both proteins through formation of helix bundles. Such interactions involving helix bundles are known to be stabilized by hydrophobic packing. Therefore, mutation of hydrophobic methionine at position 389 in the C-terminal region to positively charged lysine is likely to destabilize helix bundle interaction between coiled coil regions of SipC and Syntaxin6 and thereby, sipC^{M389K}:SCP are unable to recruit Syntaxin6. Hence, it is possible that the coiled-coil region of C-terminus of SipC binds with SNARE motif of Syntaxin6 on the phagosomal membrane by mimicking a cognate SNARE.

Mechanism of hemoglobin trafficking in *Leishmania*

In the reporting period, we have tried to understand the role of Rab1 homologue in the regulation of newly synthesized hemoglobin receptor trafficking from ER-Golgi network to the cell surface. Therefore, we have cloned and expressed Rab1 homologue from *Leishmania.* The cloned protein has shown significant sequence homology with Rab1 sequences from different organisms and also reveals the presence of Rab signature sequences involve in C-terminal prenylation, GTP binding and GTP hydrolysis. Interestingly, we have found that LdRab1 has 18 amino acid insertion which is also reported in trypanosomatidae parasites. In order to investigate the role of LdRab1, we have generated GDP locked and GTP locked mutants of Rab1 and subsequently, we have stably expressed LdRab1 and its mutants as a GFP fusion protein in *Leishmania*. We have found that both endogenous and overexpressed LdRab1 is localized in Golgi of *Leishmania* suggesting that overexpression does not alter the localization of Rab1 in *Leishmania*.

Subsequently, we have dissected the role of LdRab1 in the regulation of secretory pathway of *Leishmania* using different secretory proteins. Our results have shown that overexpression of GTP locked and GDP locked mutants of LdRab1 block the normal

secretion of secretory acid phosphatase (SAP) and also trap gp63 in Golgi preventing its transport to cell surface in *Leishmania* clearly demonstrating that both GTP binding and hydrolysis of LdRab1 are required for appropriate targeting of SAP and gp63 to the cell surface in *Leishmania*. These results indicate that the Rab1 function is well conserved in *Leishmania* like its mammalian homologue.

After establishing well conserved function of Rab1 in *Leishmania*, we have tried to understand the regulation of transport of newly synthesized HbR from ER to the cell surface by coexpressing the LdHbR-RFP in cells previously overexpressing LdRab1:WT-GFP or mutants. Our results have shown that LdHbR traffic from ER to the flagellar pocket is not blocked by Rab1 mutants demonstrating LdHbR trafficking to the flagellar pocket is a Rab1 independent process in *Leishmania*. These results suggest that possibly HbR is traffick from ER-Golgi region to cell surface by unconventional pathway. Recent reports have shown the role of GRASP65 homologue in regulating the traffick of different secretory proteins by unconventional pathway. Currently, we are trying to understand the trafficking of LdHbR through unconventional pathway in *Leishmania*.

Publication

Original peer-reviewed article

1. Madan R, Rastogi R, Parashuraman S, Mukhopadhyay A (2012) *Salmonella* acquires lysosome associated membrane protein 1 (lamp1) on phagosomes from Golgi via sipc mediated recruitment of host syntaxin6. **J. Biol. Chem. 287:** 5574-5587.

Molecular mechanism of enzymatic reactions and enzyme-ligand interactions

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The aim of this project is to understand molecular mechanism of different classes of GTPases induced by immunomodulatory cytokine interferon-γ (IFN-γ) and to compare the mechanistic similarities and differences with other GTPases within the same as well as different classes. The study has been currently focused on human guanylate binding protein-1 (hGBP-1) and other proteins in the same family. The mechanism along with the structural data may provide an insight to design drug candidates on novel GTPases and their effectors involved in the disease.

A. IFN-γ induced GTP-binding proteins and their mechanism of GTP hydrolysis

To study the molecular mechanism of IFN-γ induced guanylate binding proteins p67 (hGBP-1 and hGBP-2) and to understand their similarities and differences within the same family as well as same and different classes.

B. Understanding the function of arginine metabolic enzymes in *Helicobacter pylori*

The aim is to investigate a detailed molecular mechanism of two arginine metabolic enzymes arginase and ADC in *H. pylori*. The mechanism along with structural data from other organisms may provide a novel strategy to develop new inhibitors with greater efficiency against *H. pylori* infection.

α-helix of the connecting region gets exposed upon nucleotide binding and mediates dimerization

To determine whether the binding of GppNHp to the wild type hGBP-1 increases the total hydrophobicity of the protein, fluorescence measurements were carried out using a hydrophobic dye 1-8-anilinonaphthalene sulfonate (ANS) in the absence and presence of the analogue GppNHp. An increase in fluorescence was observed with increasing concentrations of ANS in the wild type protein with and without the substrate analogue. But the increase in fluorescence with the nucleotide-bound protein is higher than the unbound indicating that the nucleotide-bound protein (dimer) exhibits higher surface hydrophobicity than that of the unbound (monomer). Similar experiments were carried out with the double mutant D103L.D108L to understand whether the hydrophobicity increases in the presence of the analogue. As observed, the fluorescence of ANS is almost same both in the nucleotide-bound and free forms of the double mutant indicating

that the exposed hydrophobicity did not alter upon binding with the nucleotide. The circular dichroism measurement on the wild type hGBP-1 showed that the protein undergoes a conformational change upon binding with the analogue. Similar experiments were carried out with the double mutant in the presence of the analogue but it did not show any change in the molar ellipticity. The structure of the full-length hGBP-1 showed that the α-6 helix of the connecting region is buried inside the protein. All these data suggest that the binding of the nucleotide induces a conformational change in the wild type protein, which increases the total hydrophobicity upon exposure of the α-6 helix and thus mediates dimerization.

α-helix has a role in dimerization

To directly investigate if the helix is involved in dimerization, a tryptophan was introduced in the place of Leu298 of the α-6 helix which is facing outside so that after binding with the analogue it can be in the dimeric interface. Intrinsic tryptophan fluorescence measurements of the wild type and L298W in the absence and presence of the analogue GppNHp were carried out. The wild type protein has four tryptophan residues and showed similar fluorescence in the absence and presence of the analogue indicating that the environment of tryptophans in the wild type protein did not alter upon dimerization. As expected, L298W showed higher fluorescence than the wild type without the analogue. In contrast to the wild type, L298W showed a decrease in fluorescence upon nucleotide binding. This may be due to the energy transfer from the tryptophan residue (L298W) of one monomer to the same residue in the other monomer (homo transfer), when these are present in close proximity. Similar experiments were carried out by taking an equimolar mixture of the wild type and L298W in the presence of the analogue. In this case, homos as well as heterodimer are expected to be formed. The increase in the fluorescence clearly indicates the absence of homo transfer in heterodimer. These results suggest that L298W of the two monomers are in close proximity in the presence of the analogue and thus the helix is involved in dimerization.

Role of a disulphide bond in H. pylori arginase

Previous study reported that DTT strongly inhibits the *H. pylori* enzyme activity suggesting that a disulphide bond is critical for the catalysis. To examine the role of a disulphide bond in the *H. pylori* enzyme mutational studies were carried out. *H. pylori* arginase contains six cystenine residues and has a disulphide bond (Cys66-Cys73). Individual mutants Cys66Ala and Cys73Ala were made. DTNB assays were employed to determine the number of cysteine residues. The number of free cysteine residues in the wild type protein was determined to be four. Cys66Ala and Cys73Ala mutants showed that the mutant proteins contain approximately 5 cysteine residues. Steady-state kinetic assays of the wild type as well as mutant proteins Cys66Ala and Cys73Ala were carried out. The catalytic efficiency for the mutant proteins showed similar results to the wild type suggesting that the disulphide bond does not play a significant role in the catalysis. To investigate how DTT affects the activity of the wild type protein, similar assays with the Co^{2+}-reconstituted wild type as well as mutant proteins were carried out in the presence of DTT. Surprisingly, both the wild type and mutant proteins show loss of activity with DTT in a concentration dependent manner with Co^{2+} as a metal cofactor. This suggests that the loss of activity in these proteins could be due to the interaction of DTT with Co^{2+} ions. To investigate whether this is specific for Co^{2+}, similar assays with Mn^{2+}-reconstituted protein with and without DTT were carried out. Interestingly, Mn^{2+}-reconstituted protein showed a marginal loss of activity (~ 20%) with DTT compared to Co^{2+}. The mutant proteins showed similar result to the wild type confirming that the loss of activity with DTT is due to the interaction with Co^{2+} ions.

To understand whether the disulphide bond has any role in the overall secondary structure, circular dichroism (CD) measurements of the wild type and mutant proteins were carried out with and without metal ions. Cys73Ala showed results similar to the wild type indicating that the disulphide bond does not play important role in the secondary structure of the protein. Cys66Ala showed a marginal loss in the structure in the presence of the metal ions

compared to the wild type. To investigate whether the disulphide bond is important for the stability, heat-induced denaturation studies for the wild type and mutant proteins with and without metal ions were done by monitoring the changes in CD at 220 nm from 30-90 ^{0}C. The T_m of the wild type is 7 degree higher than the mutant proteins without the metal ions indicating that the disulphide bond plays an important role in the stability of the apo-protein. The T_m of the wild type holo protein is ~ 2 degree higher than that of the apo suggesting that the metal ions marginally contribute in the stability of the wild type protein. Furthermore, the disulphide bond is found to be important for the overall stability and folding of the protein. The presence of the metal ions and the disulphide bond together contribute in the overall stability of the protein than alone.

Mutational studies on ^{88}SSEHA^{92} motif and identification of critical residues for catalytic activity and metal binding

To examine the role of a unique ^{88}SSE**H**A^{92} motif in the activity, a series of single mutants was made with the residues that are analogous to the *B. caldovelox* enzyme. The steady-state assays show that the apparent catalytic efficiency for Ser89Gly in the presence of Co^{2+} ions decreased by ~ 1.5 fold than the wild type but for Ser88Gly it is increased by about 5 fold suggesting that Ser88 in the *H. pylori* enzyme plays a role in lowering the catalytic activity compared to other arginases. Like Ser88Gly, the Co^{2+}-reconstituted Ala92Ser mutant protein also showed an increase in the apparent catalytic efficiency by about 5 fold compared to the wild type. To examine whether mutations of both Ser88 and Ala92 of this motif lead to further increase in the activity, a double mutant Ser88Gly.Ala92Ser was made. The apparent catalytic efficiency for the double mutant is approximately 6.2 and 1.3 folds higher than the wild type and single mutants Ser88Gly or Ala92Ser respectively. Interestingly, Glu90Ala mutant showed ~ 15 fold decrease in the apparent catalytic efficiency compared to the wild type indicating that this residue plays an important role in the catalytic activity. Similar results were obtained with the His91Ala mutant, where mutation caused ~11 fold loss in the catalytic efficiency.

Publication

Original peer-reviewed article

1. Srivastava A, Dwivedi N, Samanta U, Sau AK (2011) Insight into the Role of a Unique SSEHA Motif in the Activity and Stability of *Helicobacter pylori* Arginase **IUBMB Life. 62:** 1027–1036.

Ribonucleases and heat shock proteins: involvement in host defense

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The classical functions of ribonucleases are the processing, turnover and degradation of specific RNA. However, some RNase A homologues of the pancreatic ribonuclease family express diverse activities like anti-tumor, immunosuppressive and angiogenic activities. Two human ribonucleases, eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) are among the toxic proteins secreted by activated eosinophils, whose role in host defense remains enigmatic. The main theme of the study is to investigate role of human ribonucleases in host defense. Human ribonucleases of the RNase A family, and natural protein toxins targeting RNA or ribosomes are being analyzed for structure-function relationships to understand their molecular mechanism of action. Also, human ribonucleases, and ribosome inactivating proteins are being explored to design knowledge-based recombinant toxins.

Caseinolytic proteases (Clps), members of the heat shock protein family, in many pathogenic bacteria have been shown to be involved in their virulence, survival in the host and dormancy. This is because Clp proteases have been found to regulate the expression of virulence genes, whereas Clp chaperones are involved in countering the stress faced by the pathogens giving it a chance to survive under stressful conditions encountered in the host. A similar mechanism may be operative in *M. tuberculosis* also and the members of the Clp protease family will be attractive drug targets. The current study is aimed to understand the functioning of Clp protease machinery in *M. tuberculosis* by *in vitro* biochemical characterizations and by inactivating these proteins *in vivo*.

- Investigation of molecular mechanism of biological actions of human ribonucleases and their role in host defense
- Construction and evaluation of recombinant toxins as potential therapeutics
- Investigation of involvement of Clp proteases in pathogenic mechanism of *Mycobacterium tuberculosis*
- Structure-function analysis of ribonuclease P of *Mycobacterium tuberculosis*

Investigation of molecular mechanism of biological actions of human ribonucleases and their role in host defense

Human eosinophil RNases ECP and EDN are very similar in primary sequences yet they differ in their several biological activities

namely antibacterial, antiviral and antiparasitic activities. In an attempt to identify specific determinants responsible for the differential activities of these proteins a library of clones was generated using DNA shuffling with ECP and EDN DNAs. Recombinant clones were confirmed for full length inserts by whole cell PCR. All positive clones were immunoscreened for the expression of shuffled gene by colony lift assay using antibodies against ECP and EDN. Several clones which were positive by immunoscreening were sequenced, however most of the clones were of wild type DNA. A few clones were found with shuffled genes at their termini. Currently, these clones are being used to make proteins for further characterization.

Major basic protein (MBP) is the major protein of eosinophil granules and is shown to be responsible for tissue damage seen in eosinophil associated diseases. The DNAs encoding human mbp1, mbp2 and pro-mbp were isolated by PCR from cDNA made from HL60-clone 15 RNA and cloned in pQE30Xa vector. Expression of MBP1, MBP2, Pro-MBP proteins were confirmed by SDS PAGE and western blotting. These proteins are being currently purified to investigate their biological activities.

Investigation of involvement of Clp proteases in pathogenic mechanism of *M. tuberculosis*

Earlier, we expressed and purified ClpP1 and P2 of *M. tuberculosis* in *E. coli*, however the proteins were not found to be catalytically active. The DNAs encoding ClpP1 and ClpP2 of *M. tuberculosis* were cloned in the plasmid pYUB1062 and expressed in *M. smegmatis* mc^2 4517. The Clp P2 protein was purified to homogeneity from the soluble fraction, however it did not manifest any proteolytic acitivity on different substrates.

M. tuberculosis ClpX is a 428-amino acid protein and has one AAA+ module. The monomeric protein has three distinct domains namely, the N-terminal domain, D1 large domain and D1 small domain. *M. tuberculosis* ClpX wild type protein was expressed in *E. coli* and purified to homogeneity. The wild type ClpX showed hexamer formation in the presence of ATP and magnesium. Six internal deletion mutants, ClpX 316-399, ClpX 316-336, ClpX 336-356, ClpX 356-376, ClpX 376-399 and ClpX 399 were prepared to delineate the role of different domains of *M. tuberculosis* ClpX in its function. The ClpX mutants were expressed and purified as was done for the wild type protein. The mutants are being analyzed for their properties to understand the role of deleted regions in the function of *M. tuberculosis* ClpX.

To investigate the involvement of heat shock proteins of Clp family in the survival and virulence of *M. tuberculosis*, the regulation of stress response in the pathogen is being studied. The heat shock response in *M. tuberculosis* appears to be regulated both positively and negatively. There are two heat shock protein repressors, HspR and HrcA annotated in the genome of *M. tuberculosis*. We have isolated the genes encoding HspR, HrcA and ClpB of *M. smegmatis* and *M. tuberculosis*, and cloned and expressed them in *E. coli*. The recombinat proteins have been purified and antisera against them have been raised in rabbits. The recombinant ClpB has been shown to contain ATPase activity. Upstream region of Hsp60 operon contains the CIRCE DNA binding element which is the probable binding site for HrcA. A 200bp region has been cloned into pGEMT-easy vector. Binding properties of HrcA is being investigated. Similarly, upstream region of Hsp70 operon which contains HAIR motifs, the putative binding sites for HspR was cloned into pGEMT-easy vector. By gel shift assays the DNA binding ability of HspR has been established.

Structure-function analysis of ribonuclease P of *M. tuberculosis*

Mycobacterial RNase P, involved in pre-tRNA processing, is composed of one RNA and one small protein component. Although the RNA component can catalyze the pre-tRNA-processing reaction *in vitro* in the absence of protein component under non-physiological conditions, the P protein is required for function *in vivo*. The protein interacts with the 5Φ leader of pre-tRNA substrates thereby increasing the affinity of holoenzyme for substrates and also enhancing the rate of pre-tRNA cleavage. Some determinants in the protein component have been identified to

be critically involved in the function of RNase P holoenzyme. A multiple sequence alignment was performed on RNase P protein components of *M. tuberculosis*, *E. coli* and *B. subtilis*. On the basis of alignment, five residues, Val27, Ala70, Ala77, Arg93 and Asp124 were identified which are not conserved in the protein component of RNase P of *M. tuberculosis*. These unique residues were chosen to investigate their role in the catalytic activity of RNase P of *M. tuberculosis*. Five mutants, V27F, A70K, A77F, R93A and D124S substituting the selected residues to those conserved in *E. coli* and *B. subtilis* proteins at the equivalent positions were constructed by site directed mutagenesis. All mutant proteins localized in the inclusion bodies like the native protein. Currently the proteins are being purified for further characterization.

Construction and evaluation of recombinant toxins

Ribonucleases can be engineered to generate cytotoxic variants of therapeutic potential. We have earlier generated ribonuclease inhibitor resistant variants of human pancreatic ribonuclease (HPR) which were cytotoxic to mammalian cells. The efficiency of internalization is a key determinant for cytotoxicity for a toxin. Residues Glu49, Asp53, Asp83 and Glu103 form an anionic patch on the surface of HPR. In order to increase the surface basicity of HPR which inturn might increase its interaction with cell surface we mutated these acidic residues with arginine individually and in various different combinations. A total of ten variants were constructed. All the HPR variants were expressed in *E. coli* and the proteins were purified to homogeneity from the inclusion bodies. The mutations did not affect the RNase activity as all the HPR variants showed RNase activity similar to that of the wild type protein. The cytototoxicity of HPR variants is being evaluated on different cell lines.

We have initiated studies to investigate anti-HIV activity of fungal ribotoxin, restrictocin; plant ribosome inactivating protein, saporin; and human ribonucleases, HPR, ECP and EDN. Saporin, restrictoicn, ECP and EDN were found to inhibit the activity of HIV integrase *in vitro* in a dose dependent manner, whereas HPR had poor inhibitory activity towards integrase. RNase A did not show any inhibitory activity. Further, using a number of saporin mutants the anti-integrase activity of saporin was found to be independent of its N-glycosidase activity.

Publications

Original peer-reviewed article

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Reviews/Proceedings

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Structural and Functional studies of *Mycobacterial* proteins

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My laboratory aims to understanding the structure-function relationships of membrane associated serine proteases (MASPs) and of enzymes involved in the histidine biosynthesis from *Mycobacterium tuberculosis* (*Mtb*).

(i) Determine the structures of the apo- and holo-forms these enzymes mainly using X-ray crystallographic technique and elucidate the mechanisms of their action.

(ii) Identify the physiological substrates of MASPs through proteomics approach.

(iii) Perform site-directed mutagenesis studies of catalytic residues.

(iv) Dissect the roles of these enzymes in *Mtb* pathogenesis through functional studies.

(v) Design inhibitors for these bio-molecules through a structure based inhibitor design approach and determine the 3D structures of enzyme/inhibitor complexes.

(vi) Examine the efficacies of these inhibitors in *Mtb* infected macrophages.

A. Solubilization and purification Over-expression of MASPs

Three recombinant MASPs (Rv2223c, Rv2224c and Rv2672) were over-expressed in *Mycobacterium smegmatis* (*Msg*) expression system using the protocol standardized in the laboratory. Briefly, the ORF corresponding to each MASP was amplified from the *Mtb* H37Rv genomic DNA using gene specific forward and reverse primers with appropriate recombination sites. The double-digested PCR product was cloned into an *E. coli* and *Msg* shuttle expression vector pYUB1062. The protein was expressed in *Msg* strain $mc^2$4517.

The cells were disrupted in lysis buffer (20 mM Tris base, 300 mM NaCl, 1mM, PMSF and 4 mM β-mercapto ethanol) at high pressure (25,000 PSI). Inclusion bodies, unbroken cells, and cellular debris were pelleted down by centrifugation at 10,000 x g. The cell wall (outer membrane) and cell membrane (inner membrane) components were fractionated by centrifugation at 30,000g and 100,000g respectively. Importantly, mass spectrometry analysis of the proteins extracted from the gel confirmed that Rv2223c is localized in the outer membrane where as Rv2224c and Rv2672 are localized in the inner and as well as in the outer membranes. For the extraction of proteins from the membranes and identification of

detergent(s) that keep(s) the proteins in solubilised form, cell membranes were homogenized in solubilisation buffer (20 mM Tris base, 100 mM NaCl, 20% glycerol, 1mM PMSF and 4 mM β-mercapto ethanol) containing detergents n-dodecyl-β-D-maltoside (DDM), octyl-glucoside (OG), octaethylene glycol monododecyl ether ($C_{12}E_8$), dimethyldecyl-amine-N-oxide (DDAO), cyclohexyl-β-D-maltoside (CYMAL), etc. by magnetic stirring at 4°C for 24 hrs. The homogenate was ultracentrifuged at 200, 000 g and the supernatant containing the solubilised proteins was preserved. Large scale over-expression, protein extraction, and purification are currently in progress.

B. Crystal structures of HisB and HisC2

Of the 11 enzymes of His pathway we have over-expressed recombinant form two more enzymes imidazole glycerol phosphate dehydratase (HisB) and histidinol phosphate aminotransferase (Hisc2), in addition to PRPP, PRAC, and HD, in *Msg*. Importantly, we have grown diffraction quality crystals of HisB and HisC2 and their structures have been determined using molecular replacement phasing method.

The polypeptide chain of HisB, which catalyses the conversion of imidazole glycerol phosphate (IGP) to imidazole acetol phosphate, is comprised of 210 residues and folds as a single domain. Like the structure of HisB from *Arabidopsis thaliana*, *Filobasidiella neoformans*, and *Staphylococcus aureus*, the *Mtb* HisB is pseudo symmetric and is made up of a four-helix bundle sandwiched between two four-stranded mixed β-sheets (Figure. 1a). The overall dimensions of the molecule are approximately 50x40x25 Å. The two fold symmetry relates the strands β1, β2, β3, and β4 of the N-terminal β-sheet with the respective strands β5, β6, β7 and β8 of the C-terminal β-sheet. Similarly, helices α1 and α3 and α2 and α4 are symmetry mates (Figure. 1a). The regions of the structure that are not related by the 2-fold symmetry are the two loops which connect α1 and β3 and α3 and β7. The biological functional unit exhibits a quaternary assembly which is composed of 24 identical subunits and possesses as many catalytic centers (Figure. 1b). The structure of the enzyme/substrate complex reveals the exact disposition of IGP in the active site and elucidates its mechanism of action.

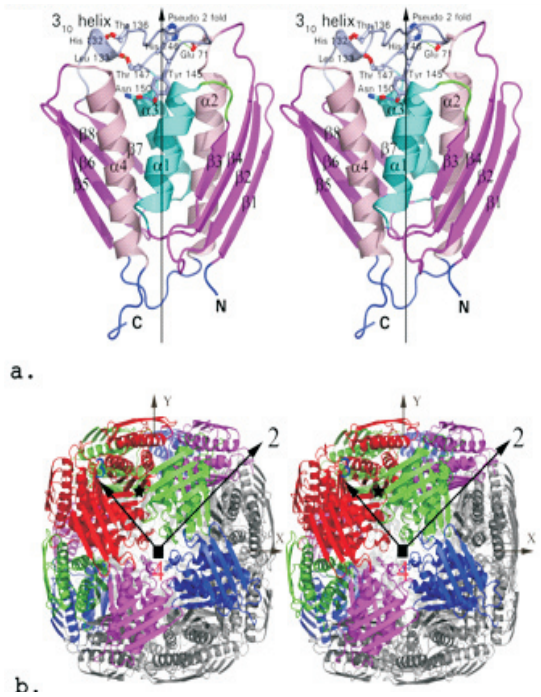

Figure. 1. *(a) Stereoview of the tertiary structure of Mtb HisB. The secondary structural elements are shown in ribbon representation. The pseudo 2-fold symmetry axis is shown by a vertical arrow. The residues protruding from the long loop that interact and make hydrogen with the other residues are shown in stick representation. Hydrogen bonds are shown by the dotted lines.*

(b) Stereoview of the quaternary structure of Rv1601. The 4, 3 and 2 fold symmetry axes of the 432 point group symmetry which generates the 24-subunit molecular assembly are shown.

In addition, we also have determined the crystal structure of HisB/aminotriazole inhibitor complex. The 1,2,4-triazole ring of the inhibitor occupies the same spatial position as that of the imodazole moiety of the substrate. The 2- and 4-nitrogen atoms interact with Mn1 and Mn2 respectively. The inhibitor inhibits the activity of HisB competitively as revealed by the crystal structure and kinetics studies.

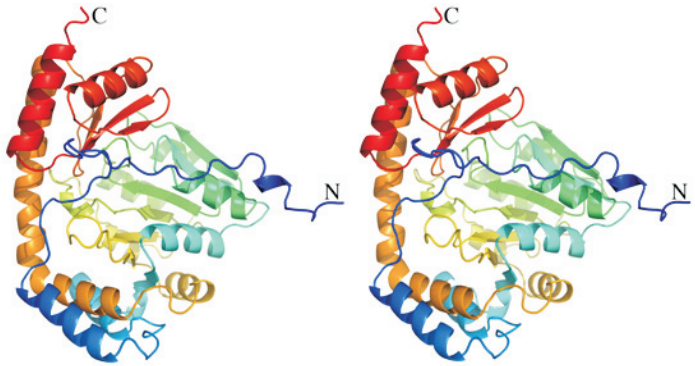

Figure.2. *Ribbon representation of the HisC2 structure. N and C termini of the structure are labeled.*

HisC2 contains 353 residues (mol wt. 38007 Da) and like other PLP-dependent enzymes, it consists of two domains, a larger PLP-binding domain having an α/β/α topology and a smaller domain (Figure. 2). Detailed structural analysis is in progress.

Publications

Original peer-reviewed articles

1. Nasir N, Vyas R, Chugh C, Ahangar MS, Biswal BK (2012) Molecular cloning, overexpression, purification, crystallization and preliminary X-ray diffraction studies of histidinol phosphate aminotransferase (HisC2) from Mycobacterium tuberculosis. **Acta Crystallogr Sect F Struct Biol Cryst Commun 68:**32-36.
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Molecular modelling of proteins and protein-ligand complexes using knowledge-based approaches and all atom simulations

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The main theme of the research project is to understand the structural principles that govern folding of peptides/proteins to stable conformations and binding of various ligands to proteins, and use these principles for developing novel computational approaches for prediction of the structures of peptides/proteins and specificities of protein-ligand complexes. Theses prediction approaches for structure and substrate specificity are being used to assign functions to proteins in the various genomes for identifying novel biosynthetic and protein interaction networks.

The specific objective of the various projects are to investigate, whether the combination of knowledge-based and *ab initio* approaches can be used for predicting the (1) substrate specificity of proteins involved in biosynthesis of secondary metabolites, (2) substrate specificity of various peptide recognition modules like MHCs, kinases, PTB, PDZ and WW domains etc, (3) structure and stability of glycoproteins and glycopeptides and (4) identifying new biosynthetic and signaling networks in various genomes.

A. In silico analysis of enzymes involved in biosynthesis of secondary metabolites

Modeling holo-ACP:DH and holo-ACP:KR complexes of modular PKSs

Modular polyketide synthases biosynthesize a variety of secondary metabolites using various combinations of dehydratase (DH), ketoreductase (KR) and enoyl-reductase (ER) domains. Even though three dimensional structures are available for DH, KR and ACP domains, no structures are available for DH or KR domains in complex with ACP or substrate moieties. A novel combination of protein-protein and protein-ligand docking has been used to first model complexes involving apo-ACP and then dock the phosphopantetheine and substrate moieties using covalent connectivity between ACP, phosphopantetheine and substrate moiety as constraints. The holo-ACP:DH and holo-ACP:KR complexes obtained from docking have been further refined by restraint free explicit solvent MD simulations to incorporate effects of ligand and receptor flexibilities. The results from 50 ns MD simulations reveal that substrate enters into a deep tunnel

in DH domain while in case of KR domain the substrate binds a shallow surface exposed cavity. Analysis of the conservation profile of binding pocket residues in homologous sequences of DH and KR domains indicated that, these results can also be extrapolated to reductive domains of other modular PKS clusters.

Identification of novel CDPS enzymes in genomes and prediction of their substrate specificity

Cyclodipeptide synthase (CDPS) is a newly discovered enzyme which adopts class I aminoacyl tRNA synthetase (AARS) fold, but shares no detectable sequence similarity with class I aminoacyl tRNA synthetases. They have been shown to catalyze biosynthesis of cyclodipeptides by using amioacyl-tRNAs as substrates and the cyclodipeptides are further modified by additional tailoring enzymes to produce diketopiperazine (DKP) class of secondary metabolites. In view of their extremely high sequence divergence, identification of CDPSs by conventional sequence searches has been a difficult task and only 9 CDPSs have been biochemically characterized so far. We have carried out profile as well as structure based searches for identification of novel CDPSs in genomes of various organisms. Since no ligand bound structure is available for any CDPS, we have also attempted to dock various known substrates to available CDPS structures for identifying the substrate binding pocket and putative specificity determining residues.

B. Analysis of protein-protein interaction networks involving kinases and PDZ domains

Substrate specificity of PDZ domains & inhibitors of PDZ-peptide interactions

PDZ domains constitute a major class of peptide recognition modules (PRMs) in protein interaction networks. Even though high throughput studies using phage display, protein microarrays and quantitative fluorescence polarization experiments have unraveled the substrate selectivity space of PDZ domains, the conventional C-terminal motifs X-S/T-X-ϕ-COOH, X-ϕ-X-ϕ-COOH and X-D/E-X-ϕ-COOH cannot explain selectivity of PDZ-peptide interactions. Analysis of the available crystal structures of PDZ-peptide complexes in PDB suggested that despite the high sequence divergence, the overall structural fold as well as the peptide binding site remains conserved across the family. Multiple explicit solvent all atom simulations of 5 nano second duration were carried out on 28 PDZ-peptide complexes with experimentally known binding affinities. Interestingly, binding free energy values calculated from these explicit solvent MD trajectories using MM-PB/SA approach showed good correlation with the dissociation constants reported by experimental studies. The various binding pocket residues which make dynamically stable contacts with different residues of the substrate peptides were also identified based on analysis of these MD trajectories. We have also attempted to investigate whether the specificity determining residues (SDRs) of PDZ domains identified by our MD simulations can be used to develop genome scale approaches for predicting differential specificities of various PDZ domains for their interaction partners. We benchmarked this multi-scale approach on mouse PDZ data set by calculating the binding energies for 217 different substrate peptides in binding pockets of 62 different mouse PDZ domains using residue based statistical pair potentials. Receiver operating characteristic (ROC) curve analysis indicates that, the area under curve (AUC) values for binder *vs* non-binder classification by our structure based method is 0.779. This multi-scale approach can potentially be applied to human genome as well to understand the binding selectivity of PDZ domains and reconstruct the PDZ interaction networks.

We have also attempted to develop a computational protocol for identification of modulators of PDZ-peptide interactions. Docking studies have been carried out on a set of 27 known inhibitors of PDZ3 domain of PSD-95 protein. The comparison of the binding free energies of these docked complexes with those of cognate PDZ-peptide complexes clearly showed higher binding affinity of inhibitors compared to the native peptide. Work is currently in progress to compute binding free energies for these complexes by MM-PB/SA analysis and compare the results with experimentally determined Ki values for these PDZ-peptide inhibitors.

Analysis of conserved features in protein phosphorylation networks

The currently available sequence as well as structure based methods for prediction of substrates for kinases have been benchmarked primarily as predictors of phosphosites on a given phosphoprotein in the context of different kinase families. Such methods have major limitations in identification of phosphorylation networks in genomic context which require distinguishing cognate kinase-substrate pairs from all other non-cognate combinations. Analysis of the evolutionary conserved patterns in protein phosphorylation networks can potentially help in formulation of prediction rules for *in silico* reconstruction of kinase-substrate interaction networks. Therefore, we have analyzed kinase-substrate interaction networks to understand whether different kinase families show any preference for recognition of specific protein families as substrates. Interestingly, classification of protein kinases from PhosphoSitePlus based on the PFAM domains they phosphorylate reveals distinct correlation between kinase families and PFAM domains of their substrate proteins. In fact preferential phosphorylation of a given PFAM domain by certain protein kinases, is statistically significant compared to the noise representing phosphorylation of that domain by all other kinases. Our benchmarking studies indicate that, ~47% of the human phospho-proteins can be correctly assigned their cognate kinases by incorporation of these observations as prediction rules.

C. *In silico* analysis of enzymes associated with novel post-translational modifications

Eliminylation is a novel PTM which is mediated by phosphothreonine lyase enzymatic domains in different bacterial effector families. Phosphothreonine lyase irreversibly converts phosphothreonine into dehydrobutyrine, which cannot be phosphorylated again. Such PTMs involving phosphothreonine lyase activity have also been shown to be involved in biosynthesis of lantibiotics by LanL like enzymes in bacterial organisms. Even though it has been proposed that eukaryotes might employ eliminylation to regulate their signaling pathways, no eukaryotic homologues of phosphothreonine lyase have been found yet. Therefore, we have carried out structure as well as profile based search for identifying phosphothreonine lyase catalytic domains in various organisms and analyzed their possible biological functions. Interestingly, fold based searches using Dali server revealed eukaryotic translation initiation factor 4E (eIF4-E) and human basophilic leukemia expressed protein (BLES03) as proteins sharing phosphothreonine lyase fold. Analysis of the conservation profile of catalytic residues indicated that, BLES03 had 4 out of 7 active site residues conserved and two other arginine residues located in the loop regions can also flip into the catalytic pocket upon conformational rearrangements. Substrate docking studies showed movement of one of these arginines (R215) towards the catalytic pocket. Similar conformational rearrangement upon binding of substrate is known to occur in case of phosphothreonine lyase, SpvC. Thus our *in silico* analysis suggests that BLES03 might have phosphothreonine lyase activity. Available micro array data in BioGPS server indicate that, expression levels of BLES03 is among the top 10 % of all genes in many tumorogenic cells. However, the molecular basis of the disease association of BLES03 is not known. Our bioinformatics analysis suggest that BLES03 can potentially affect MAP kinase signaling pathways because of the putative eliminylation enzymatic activity.

Publications

Original peer-reviewed articles

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Reviews/Proceedings

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Structure, interaction and design studies involving regulatory peptides and proteins

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The structural aspects of molecular recognition and its applications in analyzing the mechanisms associated with specific regulatory events and in rational molecular design.

1. Understanding the protein architecture and the structural biology of various regulatory events.
2. Analysis of the structural principles of immune recognition and molecular mimicry.
3. Rational molecular design studies based on the above.

The gene-encoded peptides possessing antimicrobial activity are among the principal effecter molecules of innate immune system. The structure-activity relationship of two such peptides–tritripticin (VRRFPWWWPFLRR) and indolicidin (ILPWKWPWWPWRR), has been subject of investigations in this laboratory. Naturally found in porcine and bovine leukocytes, respectively, these peptides belong to the cathelicidin family of peptide antibiotics. We have explored the bioactive conformations of tritripticin in the context of monoclonal antibody as model receptor. Anti-tritripticin hybridoma clone 6C6D7 was generated and produced in large quantities by raising ascites in mice and subjecting it to conventional purification. The purified mAb was then used to characterize the peptide-antibody interaction. The mAb 6C6D7 was found to bind tritrpticin at nanomolar concentrations with a K_D of 350nM. To establish that mAb 6C6D7 recognizes an active conformation of tritripticin and thus is a valid model receptor, purified mAb was used to pan a library of random 12mer peptides and a distinct peptide sequence was identified showing about 35% antibacterial activity as that of the native tritrpticin indicating that mAb 6C6D7 may recognize a structural antibiotic motif mimicking tritripticin, and thus can act as a receptor for such structural motif with antimicrobial properties.

To determine the mode of interaction of mAb with the motif represented by tritripticin, the binding kinetics and thermodynamics of this receptor-ligand system was analyzed. The enthalpy and entropy contributions towards the free energy showed entropy was highly favorable suggesting that the binding is primarily driven by hydrophobic interactions. To gain insights to the conformational properties of the antimicrobial motif in the context of mAb 6C6D7, *in-silico* methods were used to model the complex of the antibody with the peptide. The free energy for binding of the final selected structure was in accordance with the experimental energy. The three tryptophans and two phenyl

alanines could equivalently interact with multiple tyrosines that line the grooves and pockets on the surface of the antibody CDRs. Thus, a receptor equivalent of tritripticin was generated to explore motifs associated with its antimicrobial activity. The receptor, in the form of a mAb 6C6D7, evidently recognized tritripticin in its active conformation. Both thermodynamics and *in-silico* analyses suggest that antibiotic-antibody interaction is driven primarily by aromatic residues, abundant in both epitope and paratope.

The mode of binding of the tritrypticin with antibody is consistent with the possible involvement of membrane disruption for the mode of antibacterial activity of tritripticin. Yet, short cationic antimicrobial peptides of this family have been shown to bind ATP and inhibit activity of ATP dependent enzymes that could contribute to the killing of microbes and they have also been reported to bind DNA, which may have physiological relevance if the peptides were to interfere at the transcriptional/translational level. In order to compare the mechanism of action of tritrypticin, the peptide and its designed analogs were tested for their ability to disrupt the membrane potential by compromising membrane integrity and it was observed that membrane is indeed a primary target. Tritripticin does inhibited ATP driven conversion of luciferin to oxyluciferin by firefly luciferase at high concentrations. However; this inhibition is not physiologically relevant as the inhibition observed at physiological ATP levels was less significant. Moreover this inhibition is not driven by ATP hydrolysis. This made clear that ATP is unlikely to be the intracellular target for tritrypticin. The DNA binding ability of tritrypticin was confirmed by changes in the electrophoretic mobility of a DNA fragment. To examine whether the binding had any physiological consequences a cell-free coupled transcription and translation reaction coding for the firefly enzyme luciferase was set up. Native tritripticin was found to inhibit the reaction completely. Therefore, it appears that the antimicrobial activity of tritripticin might involve multiple targets, which include cell membrane and DNA.

Continuing the studies concerning the structural proteomics of plant seed allergens, the seed proteome of eggplant *(Solenum melongena)* was subjected to allergy screen towards carrying out crystallographic analyses of food allergens. The seeds were delipidified and proteins separated based on ammonium sulphate fractionation. The abundantly present proteins were then subjected to N-terminal sequencing in order to identify the protein molecule through homology based computational searches. The screening of non-redundant database assisted in correlation of these proteins with allergy. Two of the segregated proteins from *S. melongena,* EP1 and EP2, which showed sequence relationship with known allergenic proteins were purified and subjected to crystallization attempts.

Crystals of EP1 could be obtained that diffracted at 1.5Å resolution at synchrotron source (BM14, ESRF, Grenoble). The preliminary characterization showed that the crystals belonged to space group R32 with cell parameters a = 119.4 Å, c = 158.0 Å, respectively. As no crystal structures of closely related proteins are available, structure determination was pursued by *ab initio* phasing. Single wavelength anomalous diffraction (SAD) using sulphur was carried out and structure was refined at 1.5 Å. The final refinement cycle gave an overall R factor of 0.197 (R free = 0.218). The overall crystal structure of EP1 consists of 393 residues of which residues 180-198 and 274-293 are structurally disordered and show broken density whereas the core is clearly resolved. The structure of EP1 contains one monomer in the asymmetric unit. A monomer subunit is composed of two similar domains further subdivided into a core and a loop subdomain. Each domain consists of 2 elements, a compact eight-stranded beta barrel having the “Swiss roll” topology and an extended flexible fragment containing several short alpha helices.

Publication

Original peer-reviewed article

1. Khan T, Salunke DM(2012). Structural elucidation of the mechanistic basis of degeneracy in the primary humoral response. **J Immunol. 188:**1819-1827.

Chemical Glycobiology: Glycoproteomics and carbohydrate-based drug design

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To advance understanding of fundamental importance of glycosylation in biological processes through design, development, and application of powerful chemical tools. Design and development of carbohydrate-based small molecules for glycan engineering and inhibitor design.

Carbohydrates are one of the four major building blocks of life, in addition to genes, proteins, and lipids. Glycosylation is the most abundant and complex post translational modification of proteins and almost all mammalian membrane proteins are glycoproteins. Dynamic spatiotemporal changes of glycosylation play important roles in cell-cell, cell-pathogen, and cell-matrix interactions. Challenges in study of functional glycomics necessitated development of unique tools including metabolic glycan engineering (MGE). Depending on the nature of non-natural analog, MGE could modulate glycoforms of cell surface antigens and consequent responses such as T-cell activation. Currently, (**A**) we seek to apply MGE for engineering of mucin-type *O*-glycans using appropriately designed non-natural analogs of monosaccharides, (**B**) identification of cell surface antigens subjected to MGE and modulation of their glycoforms, (**C**) development of carbohydrate-neurotransmitter hybrid molecules for MGE across blood brain barrier (BBB), and (**D**) design and synthesis of glycopeptidomimetics (GPM) for inhibition of matrix metalloproteinases (MMP).

A. Engineering of thiols on N-acetyl-D-galactosamine (GalNAc) of cell surface glycans.

Mucin-type *O*-glycosylation of proteins is initiated by addition of *N*-Acetyl-D-galactosamine (GalNAc) (Tn-antigen) on Ser/Thr by UDP-GalNAc:polypeptide galactosaminyltransferases (pp-GalNAc-T) (EC 2.4.1.41). Peracetylated non-natural GalNAc analogs such as *N*-azidoacetyl-D-galactosamine (Ac_4GalNAz) could be processed by GalNAc salvage pathway resulting in expression azide functionality. To test susceptibility of *O*-glycan biosynthesis to thiol-MGE, we explored a panel of non-natural GalNAc anlaogs carrying pendant thiol moiety.

In order to study effect of *N*-acyl side chain length on cell surface thiol (CST) levels following peracetylated analogs were synthesized: *N*-thioglycolyl-D-galactosamine (Ac_5GalNTGc, **1**), *N*-3-thiapropanoyl-D-galactosamine (Ac_5GalNTPr, **4**), and *N*-4-

thiabutanoyl-D-galactosamine (Ac_5GalNTBut, **5**); *N*-glycolyl-D-galactosamine (Ac_5GalNGc, **2**), *N*-acetyl-D-galactosamine (Ac_4GalNAc, **3**), and dimethyl sulfoxide (DMSO, vehicle) were employed as controls. Additionally, *N*-methylthioacetyl-D-galactosamine (Ac_4GalNMeTA, **6**) was synthesized as control to discriminate sulfhydryl specific effects. Jurkat cells were incubated with GalNAc analogs **1** – **5** at 100 μM for 48 h. Estimation of CST by flow cytometry revealed that **1** induced an increase of three-fold in accessible native CST levels, while no change was observed with **2** – **5**. Further, treatment with tris-(2-carboxyethyl)-phosphine (TCEP) prior to biotinylation revealed a 16-fold increase with **1**, but only a four-fold increase with **2** – **5**, confirming that **1** is processed efficiently through GalNAc salvage pathway and that majority of CST on GalNAc exist in oxidized disulfide form. Analogs with longer chain length **4** and **5** were not metabolically processed presumably due to steric limitations of enzymes of salvage pathway.

In order to assess toxicity of GalNAc analogs **1** – **6**, Jurkat cells were incubated at a concentration of 0 – 500 μM using previously reported methodology. Cells treated with **1** exhibited growth inhibition initially at day 3, but recovered robustly by days 5 and 15; whereas, cells treated with **5** showed acute growth inhibition. Further, apoptosis assay on Jurkat cells incubated for 48 h with 200 μM of **1** or **2** revealed minimal toxicity compared to controls.

B. Modulation of glycoforms of cell surface antigens by non-natural monosaccharide analogs.

We reasoned that thiol-selective biotinylation of GalNTGc followed by immunoprecipitation of cell surface *O*-glycoproteins should establish proof-of-concept of thiol-MGE on *O*-glycans. However, thiol-selective biotinylation would suffer from background of cysteine-thiols on polypeptides. A bioinformatics based search of Uniprot database to identify membrane glycoproteins that lack 'cysteine' in polypeptide was performed. One of the candidates was found to be CD43 (leukosialin/ sialophorin) which is a heavily glycosylated type I membrane protein known to carry one *N*-glycan along with 80-90 *O*-glycans and lack cysteine. Extensive studies by Fukuda and others have shown that glycan content of CD43 varies widely among various cells. CD43 from resting T-cells were found to carry tetrasaccharide *O*-glycans, while, those from activated T-cells carry hexasaccharide *O*-glycans. Studies of CD43-/- knockout mice have established that lack of CD43, while did not impede T-cell activation, prevented viral clearance.

First, we assessed effect of GalNAc analogs on expression of CD43 using a panel of CD43-specific antibodies that recognize (i) neuraminidase-sensitive (clones 1G10, L60, and DFT-1), (ii) neuraminidase-resistant (L10), and (iii) glycosylation independent C-terminal (C-20). Upon treatment with **1**, but not **2**, neuraminidase-sensitive L60 and 1G10 epitopes were down regulated drastically in a dose-dependent and analog-specific manner. Neuraminidase resistant L10 epitopes were reduced only moderately. SDS-PAGE and Western blotting using glycosylation independent C-20 showed significant faster mobility and appearance of multiple bands (100-75 *kDa*) in cell treated with **1** compared to 125 *kDa* observed for cells treated with **2** or DMSO. Under the conditions, CD43 mRNA was found to be unaffected thus suggesting that **1** interferes in a post-translational manner at *O*-glycan initiation step. Treatment of Jurkat cells with **3**, **4**, **5**, and **6** did not show any effect on CD43 glycosylation status or mobility shifts in SDS-PAGE showing analog-structure specific effects induced by **1** with critical steric requirements and free '-SH'. Observation of multiple glycoforms of CD43 upon treatment with **1** could arise due to hypo-glycosylation of CD43 in multiple steps such as (a) hyposialylation, (b) hypo-glycosylation (c) reduction in site occupancy, and (d) reduction in site occupancy as a consequence of incorporation of GalNTGc.

Next, we established Jurkat-E6.1 cell lines stably expressing CD43 myc/FLAG-tagged at *C*-terminus, as anti-CD43-C-20 was not effective for immunoprecipitation. Jurkat-E6.1-CD43-FLAG cells were treated with **1**, **2**, or DMSO for 48 h and CD43-FLAG was immunoprecipitated. Thiol-selective biotinylation of CD43-FLAG

after treatment with TCEP followed by far-western blotting with HRP-avidin revealed strong biotinylation of CD43 incubated with **1**, but not **2**, thus establishing 'proof-of-concept'. Mechanistically, incorporation of GalNTGc in CD43 *O*-glycans might abrogate decoration of GalNAc on neighbour sites leading to reduced site occupancy. It is known that pp-GalNAc-T utilize binding to pre-existing GalNAc through their lectin domain thus facilitating *O*-GalNAc addition on neighbouring Ser/Thr sites.

C. Carbohydrate-neuroactive (CH-NA) hybrids for MGE across BBB.

Sialic acid biosynthetic pathway, wherein *N*-acetyl-D-mannosamine (ManNAc) is a committed precursor, has been shown to be amenable to MGE in small animals by Reutter, Bertozzi and others. Treatment of mice with peracetyl *N*-azidoacetyl-D-mannosamine (Ac_4ManNAz, **7**) for seven days resulted in efficient expression of NeuAz in various tissues such as heart, kidney, liver, and blood, but not in brain. It is possible that due to small size and polar nature **7** is incapable of crossing the BBB. In order to overcome this hurdle and to gain access to study role of glycans in development and diseases in CNS we applied prodrug approach for development of CH-NA hybrid molecules for MGE across BBB.

CH-NA hybrids of the formula, Ac_3ManNAz-NA, where in NA = choline ester, theophylline acetate, and nicotinate, were synthesized employing selective protection and deprotection strategies, purified, and characterized (^{1}H and ^{13}C NMR, MS and UPLC). First we conducted control experiments using **7** with multiple modes of administration. Treatment of C57BL/6j and BALB/c mice with **7** either (a) intraperitoneally, (b) intraperitoneally along with hyper-osmotic mannitol (2.0 M), a known disruptor of BBB, or (c) intravenously (via tail vein) showed strong expression in heart but not in brain, thus confirming the inability of **7** to cross BBB. On the contrary, direct delivery of **7** by intracranial injection showed robust expression of azide in brain thus indicating that sialic acid pathway in brain is amenable to MGE. Finally, treatment of mice with CH-NA hybrids intravenously at various dose and time points showed successful expression of NeuAz in brain validating that delivery of non-natural monosaccharides could be accomplished by piggy-backing on neuroactive molecules.

D. Synthesis of glycopeptidomimetics as potential inhibitors of MMPs.

MMPs play critical roles in tissue remodeling, homeostasis, and disease, particularly in cancer metastasis. Concept of 'jail break' of primary tumor cells confined to a single tissue to escape and establish a secondary tumor is known to be facilitated by MMP activation leading to degradation of collagen of basement membrane (BM). First generation MMPi based on peptidomimetics and collagen failed due to pleiotropic and broad-spectrum activities. Our approach for development of anti-metastatic agents involves design, synthesis, characterization, and biochemical and cell biological evaluation of glycopeptidomimetic small molecules to achieve differential MMP inhibition. We have synthesized representative molecules with three different scaffolds carrying a zinc binding group (hydroxamic acid moiety), namely, (a) peptides with ZBG on side chain residue, (b) variously protected monosaccharides with ZBG on C-6 position, and (c) glycopeptides with ZBG on carbohydrate moiety. The small molecules will be screened for inhibitory activity against thermolysin and selected panel of MMPs by fluorogenic assays. In parallell, we have established zymography and invasion assays as a means to assess metastatic behaviour of cancer cells.

Publication

Original peer-reviewed article

1. Du J, Che PL, Aich U, Tan E, Kim HJ, Sampathkumar SG, Yarema KJ (2011). Deciphering glycan linkages involved in Jurkat cell interactions with gold-coated nanofibers via sugar displayed thiols. **Bioorg. Med. Chem. Lett. 21:** 4980-4984.

Role of carbohydrates in modulating the structure and function of glycopeptides

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The project is aimed for understanding the differential roles of carbohydrate domains in modulating the structure and function of glycopeptides by involving model systems such as antimicrobial and thrombin-inhibitory glycopeptides.

1. Synthesis and structural characterization of glycosylated amino acids

2. Structure-function analysis of the synthetic glycoconjugates

We had shown the influence of different sugars and their linkage with antimicrobial peptides, on their function. Indeed, these effects of glycosylation are found to be sequence-specific and depend in the milieu of amino acid residues. The consequences of the unique differences among the sugars of glycosylated analogs were apparent on their antibacterial activities but not evident structurally by circular dichroism studies. The bioactive-conformational studies of these glycopeptides by crystallography in complex with their receptors is hindered because the receptors for this class of glycopeptides are yet unknown. We therefore selected another model system of a recently discovered thrombin inhibitory glycopeptide – variegin for which the receptor, thrombin, is structurally well characterized to address the precise structural effects of glycosylation on the structure and its correlation with the function.

Thrombin is a serine protease, and is the ultimate enzyme in the coagulation cascade. It has a central role in maintaining blood homeostasis during vascular injury. A potent antihemostatic compound, variegin was isolated from the salivary gland extract of the tropical bont tick, *Amblyomma variegatum*, and characterized as a peptide containing glycosylated threonine. However, the exact nature of hexose and its linkage to threonine is still unknown. It has been shown that the glycopeptide, variegin, possesses the higher thrombin inhibitory activity than its synthetically prepared non-glycosylated form. To explore the nature of sugar present and to study its structural and functional effects, we have synthesized two glycosylated and a non-glycosylated analogue of variegin. The synthesis of glycosylated analogues of variegin was achieved in two steps, a) Synthesis of glycosylated threonines, N^{α}-Fmoc-Thr (Ac_4-β-D-Glc)-OH, and N^{α}-Fmoc-Thr (Ac_4-β-D-Gal)-OH and b) Incorporation of the glycosylated amino acid into the peptide.

The galactosylated and glucosylated threonines were synthesized successfully by following the methodologies standardized in the

lab. All the peptides were synthesized, purified and characterized by mass spectrometry. The secondary structure analysis of glycosylated and non-glycosylated variegin analogues was done by circular dichroism spectroscopy in phosphate buffer (pH 7.4). All the three peptides showed a characteristic dip at around 200nm wavelength which is indicative of random conformation. Thus, all the three peptides did not possess any regular secondary conformation in phosphate buffer. Presence of glucosyl or galactosyl residue in its β-linkage did not induce any characteristic change in the secondary structure of glycopeptide.

The thrombin inhibitory abilities of the synthesized peptides were studied by performing the thrombin-time (TT) assay. Thrombin enzymatically activates fibrinogen in plasma to form fibrin, which gets polymerized to form a fibrin mesh and results in clot formation. In the presence of a thrombin inhibitor; the time for formation of clot is prolonged. In TT assay, the time required for clot formation was monitored in the absence (control) and presence of variegin analogues at different concentrations starting from equimolar concentration (with respect to thrombin) to 100 times excess of equimolar concentration. The clot formation in control was observed as early as at 16.6 sec which is in accordance with the normal thrombin-time. As the concentration of the peptide increased from equimolar concentration to 100 fold equimolar concentration, an increase in the time taken for clot formation was observed. All the three peptides efficiently prolonged the TT indicating that they are functionally active.

All the peptides were assayed for their abilities to inhibit thrombin amidolytic activity with chromogenic substrate S2238, a small peptidyl substrate that binds only to the active site. Typically, different concentrations of peptides and thrombin were preincubated before the addition of substrate S2238. The rates of formation of coloured product *p*-nitroaniline were followed at 405nm for 10min with an enzyme-linked immunosorbent assay plate reader. Percentage inhibition was calculated by taking the rate of increase in absorbance in the absence of inhibitor as 0%. All the peptides inhibited the amidolytic activity and showed the comparable IC_{50} values. The progress curves of inhibition showed that steady-state equilibrium was achieved upon mixing.

Publications

Original peer-reviewed articles

1. Talat S, Thiruvikraman M, Kumari S, Kaur KJ (2011) Glycosylated analogs of formaecin I and drosocin exhibit differential pattern of antibacterial activity. **Glycoconju J 28:**537-555.

2. Shabareesh PRV, Thiruvikraman M, Kaur KJ (2011) Synthesis and preliminary comparative structure-function studies of glycosylated and non-glycosylated analogues of a thrombin inhibitory peptide, variegin. **Trends in Carbohydr Res. 3:**37-43.

Biophysical and biochemical characterization of *Leishmania* phosphoglycerate kinase: an enzyme in the glycolytic pathway of parasitic protozoa

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It is known that *Leishmania* sp. has only two PGK genes encoding proteins PGKB and PGKC. Both these isoenzymes are simultaneously present in the cytosol and glycosome in a ratio of 80/20, in both promastigotes and amastigote forms. Therefore in *Leishmania* sp. the ratio 80/20 of glycosomal and cytosolic PGK reflects possibly the specific activities of different routes of sugar breakdown; glycolysis, leading to pyruvate and, the formation of malate via PEPCK and MDH respectively. Cytosolic PGK may in addition also be involved in other activities such as gluconeogenesis that has been claimed to be a cytosolic process. It is found that *Leishmania* PGK has some distinct structural features, as PGKB and PGKC differ in a handful of internal residues and in the presence of a long extension at the C-terminus of PGKC. Even though the *Leishmania* sp PGKC extension does not contain the glycosomal targeting tripeptide found in the similar extension of *T. brucei* PGKC it is possible that the former is responsible for the targeting of PGKC to the glycosome by some other unknown mechanism. Alternatively since it contains discontinuous stretches of hydrophobic and charged residues, it may associate with, either soluble or membrane associated glycosomal proteins, or ligands which modulate its enzymatic properties. Using previously published glycolytic reaction-based assays, the activities of PGKB and PGKC form *L. mexicana* can be compared.

Organism of the genus *Leishmania* lead to debilitating diseases in humans. According to current estimates hundred thousand individuals are infected yearly in the third world with a quarter of the world population living under threat of infection. Problems such as drug resistance and toxicity necessitate the development of better more effective drugs. Since glycolysis is very important for the generation of metabolic energy, many research efforts are underway to inhibit the parasite through the inhibition of the glycolytic pathway. One of the unique features of kinetoplastida glycolysis is its partial sequestering in intracellular organelles called glycosomes. Thus drug development efforts can be targeted, either at the glycosome itself or at the enzymes present within them, as has been demonstrated by other workers in the field. In this context it is useful to compare structurally and functionally the glycolytic enzymes present in the glycosome with those present in the cytoplasm.

1. Expression, purification and determination of specific activities of PGKB and PGKC.
2. Steady state kinetics by spectroscopic method; determination of the effect of high substrate concentration on enzyme activity.
3. Comparison between PGKB and PGKC of, pH optimum of activity and enzyme inhibition by salt and suramin.
4. ^{31}P NMR studies using substrate / enzyme (PGKB or PGKC) mixtures, with, either no metal, $MgCl_2$, $CaCl_2$, $MnCl_2$ or $CoCl_2$ to determine the change in the dissociation constant of substrate with metal ions. Comparison with data from similar experiments in literature with yeast PGK using Mg-ADP and Mg-ATP.

5. Peptide based studies ofglycosomal membrane association of PGKC. The peptides used in these studies will be evaluated as useful models to understand the structural basis of the biochemical differences between PGKC and PGKB.

Therefore, looking at the previous reports we took the glycolysis reaction for testing the activities of PGKB and PGKC from *Leishmania mexicana*.

$$\text{ATP + 3-PG} \xrightarrow{\text{PGK}} \text{ADP+ 1,3-PG} \xrightarrow[\text{GAPDH}]{\text{NADH}} \text{3-PGA+Pi+NAD}^{+}$$

The recombinant clones of *L. mexicana*PGK (PGKB and PGKC) in *E.coli* available to us have been checked by isolating the plasmid and sequencing to confirm the presence of the PGK genes in the correct reading frame. The enzymes were characterized in terms of kinetic parameters.

Phosphoglycerate Kinase Assay for ADP binding followed a protocol using pyruvate Kinase/PEP for ATP generation.

$$\text{ADP} \xrightarrow[\text{Pyruvate kinase}]{\text{PEP}} \text{ATP + 3-PG} \xrightarrow{\text{PGK}} \text{ADP + 2,3-PG} \xrightarrow[\text{GAPDH}]{\text{NADH}} \text{2-PGA+Pi+NAD}^{+}$$

PGK in leishmania cultures: The PGK activity was measured using the standard assay, in the amastigote cultures. Suramin dependent inhibition of activity was also observed as expected from a known inhibitor of PGK.

The specific activity of PGKB and PGKC was found to be different, PGKB being more active than PGKC. The ATP binding of PGKC is stronger as compared to PGKB where 3-PG binding is stronger in the case of the latter. ADP binding is stronger in the case of PGKB. When compared with data published by others on yeast PGK we find the ATP affinity is higher with the *Leishmania* enzymes whereas yeast has a higher affinity for the other ligands 3-PG and ADP. Although 3-PG is actually a product of the PGK reaction it is the preferred ligand in the assays as the substrate 1,3-BPG is not stable.

When trying to understand the basis of protein function one is inevitably led to the structure of the protein (if it is known) or biochemical studies based on structure or sequence of the protein. No structure of *Leishmania* PGK is available so far and only two sequences published, that of *L. major* and *L.mexicana*.

Based on the results of computational analysis 3 synthetic peptides derived from the C-terminal sequence of *L.mexicana* PGKC, were complexed with lipids or micelles and studied by circular dichroism spectra and NMR. Proton NMR spectra of the peptide complexes reconstituted in SDS micelles were recorded. Preliminary estimation of the secondary structure in the micelles was made from circular dichroism spectroscopy before recording the NMR spectra in deuterated SDS micelles.

Due to the presence of micelles the spectral overlap in the proton region was worsened by the line-width. Fortunately a solution was found by recording the spectra in deuterated MeOH at 500 MHz. This sample gave good resolution. Meanwhile conditions were found for obtaining high resolution proton spectra of peptide in deuterated SDS at 40C using field strength of 700 MHz. The structure of the peptide has been solved.

GENE REGULATION

GENE REGULATION

Molecular Analyses of the Human and Animal Genomes

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Our focus has been on the analyses of human and animal genomes in the context of functional and comparative genomics. Present work relates to the molecular mining of *RsaI* repeats in water buffalo *Bubalus bubalis* and characterization of the *ACOT11* gene.

1. Delineation of *RsaI* repeats in water buffalo *Bubalus bubalis* and related bovid species.
2. Expression analysis *RsaI* tagged transcriptomes in water buffalo across various somatic tissues and the spermatozoa, their copy number analysis and chromosomal distribution

***RsaI* repetitive DNA in Buffalo *Bubalus bubalis* representing retrotransposons, conserved in bovids, are part of the functional genes**

Different families of repetitive DNA contribute towards architectural organization of the mammalian genomes. They represent both, tandemly arranged and interspersed sequences. Interspersed elements replicate and proliferate themselves employing a "copy and paste" mechanism called retrotransposition.

Retrotransposons have gained novel functions, providing alternative splice sites and/or polyadenylation signals or modifying gene expression. The impact of the interspersed repeats on the genomes of human, dog, cow, mouse and opossum has been studied. However fate of these interspersed elements and their association with mRNA transcriptomes in buffalo remains still unclear. Here, we report *RsaI* family repetitive DNA in the genome of water buffalo "*Bubalus bubalis*" and their copy number status. We also studied their expression in somatic tissues and spermatozoa. The repeat fractions pDp1, pDp2 and pDp3 and were used for fluorescence *in situ* hybridization (FISH) with buffalo metaphase chromosomes. In addition, we

isolated and sequenced full length *ACOT11 (*Acyl-coenzyme A thioesterase 11) gene harboring part of pDp1 repeat.

RsaI enzyme digestion uncovers four repeat fractions

Digestion of buffalo genomic DNA with RsaI enzyme, besides minor ones, showed four prominent bands ranging from 1331 base pairs, pDp1; 651, pDp2; 603, pDp3; to 339, pDp4. Five-six clones from each fragment were sequenced. All the four major repeat elements were AT rich but sequence-wise, were different from one another. No inter-clonal variations were detected in these sequences. However, random repeats were present in the nucleotide sequences. Repeat Masker programme revealed presence of LTR LINE, SINE elements within the four fragments. Blast search for each clone showed 69-98% homology with genomic DNA/ contigs and 68-93% with transcribing genes in the database mostly in UTRs.

Buffalo derived RsaI Open Reading Frame (ORFs) has amino acid similarity to LINE reverse transcriptase

Most striking feature of pDp1 sequence was the presence of 489 bp, +1 ORF (nucleotide position, 841-1329) (Figure 1). BLASTP search with GenBank sequences using conceptual translation of this ORF (162aa) gave matches to putative reverse transcriptase's domain. This region corresponds to central position of the Reverse transcriptase ORF. Similarly, pDp2, 111 bp, +3 ORF (nucleotide position, 135-245, 65 aa) and pDp4, 135 bp, +3 ORF (nucleotide position, 81-215, 44aa) showed homology to endonuclease reverse transcriptase. These ORFs corresponded to central endonuclease reverse transcriptase of LINE1 ORF2. pDp3 sequence,112 bp, +3 ORF (nucleotide position, 492-602, 37 aa) showed no similarity with endonuclease reverse transcriptase.

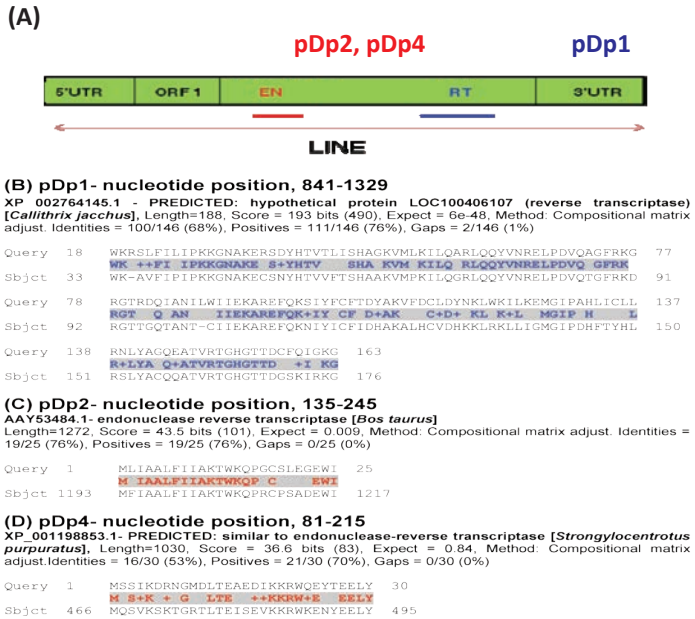

Figure 1. *Amino acid alignment of buffalo derived RsaI sequences showing positions of pDp1, 2 and 4 based on ORF search. Panel (A) is a diagrammatic illustration showing 5'UTR, ORF1, Endonuclease, Reverse transcriptase and 3'UTR of LINE. Position of pDp1 (Blue), pDp2 and pDp4 (Red) within LINE are shown on top of the figure. The aligned sequences in the panel (B) shows region with homology to reverse transcriptase domain (Blue). Panel (C) and (D), shows region with homology to endonuclease domain (Red).*

RsaI Repeat status among bovids

Independent cross hybridization of pDp1, pDp2, pDp3 and pDp4 with genomic DNA from different species under high stringent conditions showed signals only in bovids. PCR conducted using region specific primers amplified bands in buffalo, cattle, goat and sheep genomic DNA (Figure. 2).

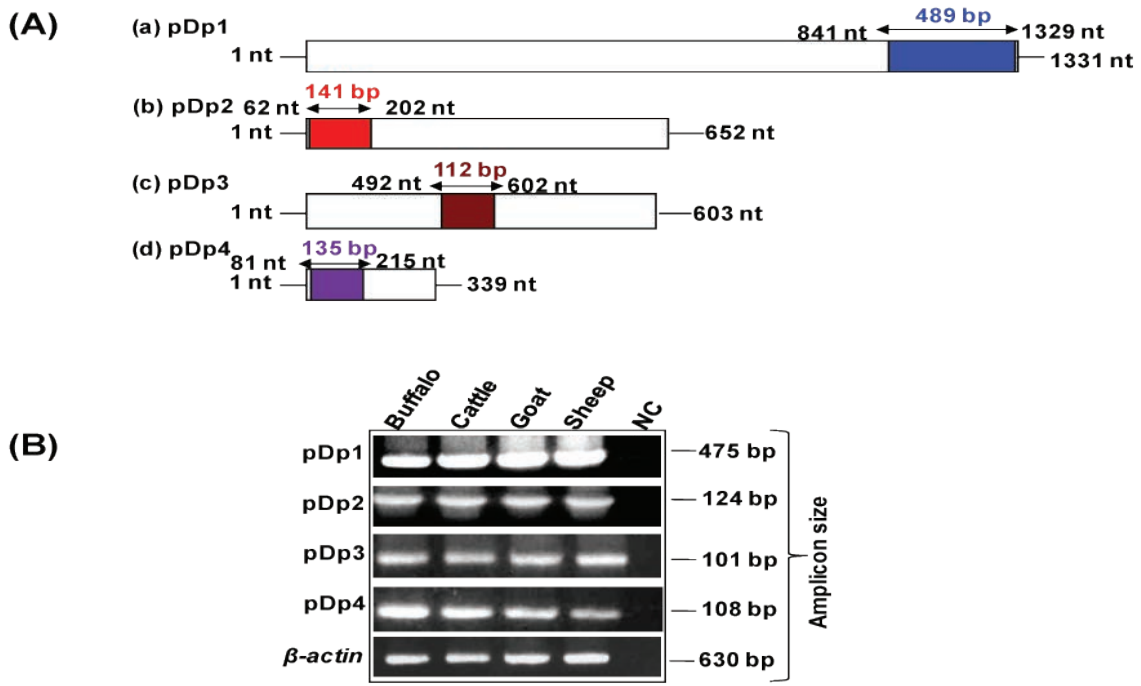

Figure 2. *PCR amplification of bovine genomic DNA using internal primers designed from the ORF of buffalo RsaI sequences. Schematic representation* ***(A)*** *shows PCR strategy used for amplication of ORF regions of RsaI sequences corresponding to 489, 149, 112 and 135 bp, respectively* ***(a-d)****. Panel* ***(B)*** *shows PCR amplification of RsaI ORF regions and β-actin as control. The corresponding position of each PCR product is shown in Panel* ***(A)****. Sequence IDs are indicated on left, amplicons size on right and species are mentioned on top of the lanes.*

Differential expression of RsaI fragments in somatic tissue and spermatozoa of buffalo

Quantitative Real Time PCR analysis of these sequences showed differential expression of *RsaI* related transcripts across somatic tissues and spermatozoa. pDp1 showed highest expression in lung, pDp2 and pDp3 in Kidney and pDp4 in ovary.

Multiple copies of RsaI fragments in bovids

Copy number analysis of these fragments in cattle buffalo, goat and sheep using Real Time PCR showed ~2×10^4 copies in pDp1, ~ 3000 copies each in pDp2 and pDp3, and ~1000 copies in pDp4.

pDp1, pDp2 and pDp3 sequences are dispersed throughout the buffalo genome

FISH mapping of pDp1, 2, 3 spectrum red labeled cloned probes showed ubiquitous discernible signals over buffalo metaphase chromosomes.

Full length cDNA sequence of ACOT11 gene in Buffalo

Blast search using reference mRNA sequence revealed *RsaI* repeats to be part of Acyl-coenzyme A thioesterase 11(*ACOT11*), *V*acuolar Protein Sorting 24 (*VPS24*) and Solute carrier organic anion transporter family member 1A2 (*SLCO1A2*) genes. Full

length Buffalo *ACOT11* cDNA was generated using end point PCR and gene specific (*B. taurus ACOT11)* primers. Assembled cDNA sequence of 2592 base pair fragment lacking poly A tail representing six exons were deposited in the GenBank (HQ848649 and HQ848650).

As shown above, we analyzed four (pDp1, pDp2, pDp3 and pDp4) *RsaI* fragments from the buffalo genome which are AT rich, though buffalo genome on the whole is GC rich (40.69%, NC_006295). Database searches with the repeat-maskers revealed the presence of several LTR, LINE and SINE element in the four sequences. Presence of partial reverse transcriptase and endonuclease domains in pDp1, pDp2 and pDp4 reported herein led to the hypothesis that *RsaI* repeats might be related to a novel retrotransposable element.

Interspersed repeats get inserted into a new genomic location through the process of retrotransposition. This is reflected by our FISH results of pDp1, 2 and 3 showing signals on all over the chromosomes with varying intensity. *In silico* analysis of pDp3 on reference cattle genome showed fewer distribution suggesting its poor characterization in the cattle genome. This is supported by the fact that using real time PCR, we detected similar copy number of pDp3 in the bovids. Startlingly, these four repeat elements were not detected in any of the non-bovid species. It is likely that these repeats are collectively involved in the evolution and sustenance of bovid chromosomes. Reports suggest that new retrotransposons are conserved within the same group of species. This is corroborated by our Slot blot and PCR results. Real time PCR results showed approximately similar copy numbers for pDp1, 2, 3 and 4 in cattle, goat and sheep genomes as mentioned earlier.

RsaI elements were found to be part of the three functional genes *(ACOT11*, *VPS24 and SLCO1A2*) mostly present in 3' UTR. Our work corroborates recent reports that most part of retrotransposons inserts themselves in first and last exons and in untranslated regions (UTRs). In human, this type of insertion has been shown to create new non-conserved polyadenylation signals, influencing the level of gene expression. However, how these insertions affect expression of buffalo transcriptomes is still a matter of speculation.

In conclusion, buffalo has several known and non-descript breeds of which a few are considered to be superior with respect to productivity and economic return. Whether, *RsaI* repeat in different breeds of buffalo would show similar organization and expression pattern is not known. However, if informative in breed delineation, these would prove to be useful bio-markers.

Publications

Original peer reviewed articles

1. Saini M, Jha AN, Abrari A, Ali S (2012) A subset of human gliomas shows over-expression of KIT without its amplification. **Gene 497**:155-163.

2. Rawal L, Ali S, Ali S (2012) Molecular mining of GGAA tagged transcripts and their expression in water buffalo *Bubalus bubalis.* **Gene 492:** 290–295.

3. Kumar S, Gupta R, Kumar S, Ali S (2011) Molecular mining of alleles in buffalo *Bubalus bubalis* and characterization of *TSPY1* and *COL6A1* genes, **PLoS One 6**: e24958.

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Deciphering the role of cell signalling in M. tuberculosis biology & in the function and dynamics of nucleoporins

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The laboratory has two parallel themes of research

I. Cell signalling & host-pathogen interaction

The *M. tuberculosis* genome encodes for eleven eukaryotic-like serine/threonine protein kinases. Though all the *M. tuberculosis* STPKs have been biochemically characterized and a number of substrates identified, many questions remain to be answered. The role of the extracellular environment on activation/inactivation of kinases has not yet been investigated. We aim to decipher the signalling pathways in *M. tuberculosis*. We also aim to investigate the role of these cascades in modulating the host signalling network and the survival of pathogen in the host.

II. The role of cell signalling events in modulating the functions and dynamics of nucleoporins

In eukaryotes, Nucleopore complex (NPC) forms a major channel of communication between the nucleus and the cytoplasm, which helps in the import and export of proteins and messenger RNA. The NPC is a large protein assembly comprising of over 30 different proteins called nucleoporins (Nup's) which are arranged as sub complexes. Nucleoporin Tpr, present at the nuclear phase of the NPC, is a large 270 kDa protein with a bipartite structure. Our aim is to decipher the role of phosphorylation on the functions of nucleoporin Tpr.

I. Cell signalling & host-pathogen interaction

a) To identify novel downstream targets and determine the role of kinase mediated phosphorylations of these targets.

b) Generate phospho-specific antibodies and gene replacement mutants that can recognize activated kinases and utilize them to investigate their activation status under various growth conditions and upon infection.

c) Investigate modulation of host signalling pathways upon infection with *M. tuberculosis* H37Rv wild type or kinase gene replacement mutants.

II. The role of cell signalling events in modulating the functions and dynamics of nucleoporins

a) Identification and validation of *in vivo* phosphoryaltion sites on Tpr

b) Investigating the role of Tpr in the export of intron containing (unspliced) mRNA.

c) Determining the functional significance of identified phosphorylation sites

I. Cell signalling & host-pathogen interaction

a) Deciphering signalling pathways in *M. tuberculosis*

Generation of gene replacement mutants in *M. tuberculosis* is a challenging task due to low transformation efficiency and high rates of illegitimate recombination. Since PknA and PknB are essential genes, it is necessary to provide an inducible integrated copy of these genes. We have created integrating shuttle vectors where *pknA* or *pknB* genes have been cloned under a tetracycline/ acetamide inducible promoter. We have successfully generated conditional gene replacement mutants of *pknA* and *pknB* in *M. smegmatis* using two step recombination approach. Efforts are underway to generate conditional gene replacement mutants of *pknA* or *pknB* genes in *M. tuberculosis*. These mutants will be used to investigate the role of various domains in the function of PknA and PknB, and in the pathogen's survival in host macrophages.

The activation loop comprises the region that includes the β9 helix of the kinase domain and is sandwiched between conserved DFG and APE motifs. Activation loop residues that are phosphorylated have been identified in PknB, D, E, F and K. Unlike other STPKs, activation of PKnG is not through activation loop phosphorylation, which was shown to be maintaining stable conformation. To date, tools are not available to investigate the *in vivo* activation status of kinases. Since the activation of these kinases requires phosphorylation of activation loop threonine residues, we have generated phospho-specific antibodies for the residues in the activation loop. Preliminary data indicates that these antibodies are capable of recognizing activation loop phosphorylation on PknA and PknB. We are in the process of further characterizing these antibodies and once characterized they will be used investigate the activation status of PknA & PknB under various growth conditions, and upon infection in host macrophages.

M. tuberculosis resides in macrophages, where it encounters limited nutrient supply, hypoxia, and prolonged oxidative and nitrosative stress. TCA cycle plays essential roles in cell metabolism, providing reducing equivalents for energy generation and biosynthetic reactions, along with precursors for lipids, amino acids and heme. PknG mediated phosphorylation of GarA was shown to regulate glutamate metabolism in mycobacteria. Phosphorylation of GarA by PknG abrogates its binding to KGD, GDH and GS. In order to investigate role of PknG in regulating the TCA cycle, we have generated *M. smegmatis and M. tuberculosis* Δ*garA* mutants. We plan to investigate the growth rates of wild type and mutant strains on different carbon sources. We also paln to perform metabolomics, which should provide insights into the role of PknG and GarA in regulating the TCA cycle and glutamine metabolism.

b) Development of Convenient Shuttle Vectors for expression of genes, gene replacements and for investigating protein-protein interaction studies in Mycobacteria

E. coli-mycobacteria shuttle vectors are great tools for gene expression and gene replacement studies in mycobacteria. However, most of the vectors available are limited by the lack of extended multiple cloning sites (MCS) and convenient epitope tags. We generated series of constitutive, regulatable ectopic and integrating expression shuttle vectors containing MCS with several routine restriction sites for cloning of target genes, flanked by 6xHis and FLAG Tag sequences in frame before and after the MCS, respectively, for N- and C- terminal tagging of proteins. We demonstrated the applicability of these vectors by showing the constitutive and controlled expression of *M. tuberculosis* serine-threonine protein kinase gene, *pknK*. We have also developed a suicide delivery plasmid with expanded MCS that allows efficient generation of gene replacement mutants. Further, the shuttle vector was modified by combining the expanded features with hypoxia indcible promoter narK2 to convert it into hypoxia responsive vector. In order to explore the possibility of crosstalk between proteins, we have developed a shuttle vector for co-expression of two target ORFs, with two

MCS, each of which is preceded by a promoter and ribosome binding site. Novel and user-friendly shuttle vectors developed by us would be excellent tools for the gene function analysis in mycobacteria.

II. The role of cell signalling events in modulating the functions and dynamics of nucleoporins

Nucleoporin Tpr is a component of the nuclear pore complex (NPC) that localizes exclusively to intranuclear filaments. Tpr functions as a scaffolding element in the nuclear phase of the NPC and plays a role in mitotic spindle checkpoint signalling. Export of intron-containing mRNA in Mason Pfizer Monkey Virus is regulated by direct interaction of cellular proteins with the cis-acting Constitutive Transport Element (CTE). In mammalian cells, the transport of Gag/Pol-CTE reporter construct is not very efficient, suggesting a regulatory mechanism to retain this unspliced RNA. Recently, we reported that the knockdown of Tpr in mammalian cells leads to a drastic enhancement in the levels of Gag proteins (p24) in the cytoplasm, which is rescued by siRNA resistant Tpr. Tpr's role in the retention of unspliced RNA is independent of the functions of Sam68 and Tap/Nxf1 proteins, which were reported to promote CTE dependent export. Further, we investigated the possible role for nucleoporins that are known to function in nucleocytoplasmic transport in modulating unspliced RNA export. Results showed that depletion of Nup153, a nucleoporin required for NPC anchoring of Tpr, plays a role in regulating the export, while depletion of other FG repeat-containing nucleoporins did not alter the unspliced RNA export. Results suggested that Tpr and Nup153 both regulate the export of unspliced RNA and they are most likely functioning through the same pathway. Importantly, we found that localization of Tpr to the NPC is necessary for Tpr mediated regulation of unspliced RNA export. Collectively, the data indicated that perinuclear localization of Tpr at the nucleopore complex is crucial for regulating intron containing mRNA export by directly or indirectly participating in the processing and degradation of aberrant mRNA transcripts.

Publications

Original peer-reviewed articles

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Reviews

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Elucidating the molecular mechanisms of aging and innate immunity using *Caenorhabditis elegans* as a model system

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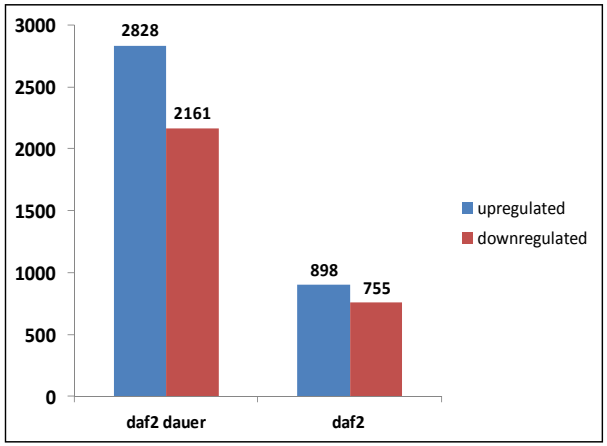
Our laboratory is trying to understand the underlying mechanisms of organismal aging. We are also interested in identifying the molecular link between aging and innate immunity. *Caenorhabditis elegans*, a nematode that has played a pivotal role in longevity research is being used as a model system in the lab, backed by a powerful combination of molecular genetics, molecular biology, genomics and Next Generation sequencing (NGS). The Insulin-IGF-1 Signalling (IIS) pathway is a major signalling cascade that controls aging as well as pathogen resistance. We are trying to decipher the complex interplay between transcription factors (TF), downstream of the IIS pathway, to determine life span and pathogen resistance. On the other hand, Dietary Restriction (DR) is the only known intervention that can extend life span in all organism tested. We are interested in understanding how dietary restriction works and its relation to innate immunity.

We would like to understand how HSF-1 and DAF-16 recruit to target gene promoters following different physiological inputs. A NGS platform will be setup that can perform transcriptomics, small RNA expression as well as chip-sequencing analysis to help characterize regulation of IIS pathway. In the second objective, we will try to understand how DR works by characterizing several novel genes that work in the DR pathway. In our pursuit to find novel proteins that regulate IIS pathway, we have identified 15 candidates in a reverse genetic screen. In the third objective, we will study the interaction of these genes with IIS pathway. We have discovered a novel model of DR where the worms are internally dietary restricted (iDR). Using this system, in the fourth objective, we will study the effect of iDR on pathogen response and innate immunity.

Investigating the regulations of the IIS pathway using Next Generation Sequencing

We purchased an Illumina Next Generation Sequencing (NGS) platform using funds provided by DBT. We also received a complete upgrade package from Illumina which was installed and validated using control samples. Following that, we first standardized sample preparation protocols for transcriptomics and small RNA digital gene expression experiments. Taking advantage of the powerful technology, we asked whether we can identify genes and microRNAs that are regulated by IIS. For example, we compared the transcriptome of *daf-2(e1370)* and *daf-16(mgdf50)* and found 800-900 genes up- or down-regulated (Figure 1). Many of the genes were common to published microarray data, but we found novel and alternatively spliced ones due to the depth and quantitative nature of the method. We also compared *daf-2(e1370)*



Up regulated			Down regulated		
Gene I.D	Fold change	P value	Gene I.D	Fold change	P value
cel-miR-38	2.1	1.98E-07	cel-miR-34*	-7.77	0.04
cel-miR-237	2.38	4.61E-09	cel-miR-51	-7.28	1.92E-14
cel-miR-784	2.56	8.91E-07	cel-miR-1022	-7.17	2.89E-12
cel-miR-227	2.56	8.54E-06	cel-miR-788	-7.04	2.78E-15
cel-miR-79	3.79	5.90E-07	cel-miR-243	-6.17	0
cel-miR-236	3.84	1.55E-15	cel-miR-90	-5.87	0
cel-miR-246	4.09	1.87E-14	cel-miR-230	-5.52	0
cel-miR-62	4.13	0	cel-miR-124*	-5.47	4.58E-03
cel-miR-355	5.93	8.88E-16	cel-miR-245	-5.4	6.04E-05
cel-miR-1820	6.57	1.15E-07	cel-miR-52*	-5.19	3.00E-04
cel-let-7	6.71	0	cel-miR-35	-4.36	8.88E-16
cel-miR-240*	7.65	0	cel-miR-44-5p	-4.22	3.03E-03
cel-miR-84	8.66	0	cel-miR-52	-3.98	0
cel-miR-239a	8.99	0	cel-miR-57	-3.91	2.72E-14
cel-miR-1	9.37	0	cel-miR-42	-3.69	0
cel-miR-85	14.85	0	cel-miR-64	-3.57	0
cel-miR-60	18.65	0	cel-miR-790	-3.42	4.88E-08
cel-miR-59	19.73	0	cel-miR-54-3p	-3.15	8.77E-15
cel-miR-785	31.16	4.97E-04	cel-miR-49	-3.08	8.88E-16
cel-miR-239b	50.33	0.04	cel-miR-73	-3.07	1.11E-16

Figure1. *Excerpts from Deep sequencing experiments- (Left panel) Left: Number of genes whose expression change in daf-2(e1370) dauers compared to non-dauer; Right: genes whose expression change in daf-2(e1370) vs daf-16(mgdf50) mutant; (P≤ 0.05 and fold change ≥ 2.0). (Right panel) MicroRNAs that change expression in daf-2(e1370) mutant*

transcriptome to that of dauer, a developmental stage controlled primarily by the IIS pathway. In subsequent experiments, we are planning to perform transcriptomic analysis to identify genes downstream of *daf-16* and *hsf-1* as well as determining how they are modulated by stress and developmental cues. This will complement our study using ChIP-sequencing. We also performed an in-depth analysis of microRNAs that are regulated by the IIS pathway. We compared small RNA profiles of wild-type, *daf-2(e1370)*, *daf-16(mgdf50)* and *daf-16(mgdf50);daf-2(e1370)* worms. We have identified several miRNA species that are differentially regulated. We have also discovered several novel small RNA species that are differentially regulated in these mutants (Figure 1). In another study, we compared the microRNA profiles of 6 different long-live strains. These strains live long because they have mutations in either IIS, TOR, mitochondrial electron transport, neuronal genes or are calorically restricted. We are currently analysing the data to identify common as well as unique microRNAs.

We commercially prepared two sets of antibodies against DAF-16 and HSF-1 using purified denatured proteins. However, none of the antibodies worked in western and IP analysis. We are currently making antibodies in-house at NII animal facility against purified and soluble proteins. As an alternative strategy, we have obtained different DAF-16 isoforms tagged to GFP variants (discussed below) those are being used for ChIP-seq experiments.

A novel kinase knockdown initiates dietary restriction

We have isolated several kinases that work either in the IIS pathway or in a genetically parallel pathway. The serine-threonine kinase, *eddf-1*, works not in the IIS pathway but in the dietary restriction pathway. EDDF-1 knockdown using RNAi leads to dramatic increase in life span that is not dependent on the downstream regulators of IIS pathway (*daf-16* and *hsf-1*). However, it showed synergistic increase in life span when combined with a knockdown of the IIS pathway (*daf-2* mutant). This suggested that *eddf-1* works in a separate pathway. The increased life span is also independent of TGF-beta pathway that functions genetically upstream of the IIS pathway in *C. elegans*, controlling life span. Knocking down *eddf-1* results in dramatic decrease in fat storage in both wild-type worms or in high fat-storing *daf-2* mutants. The *eddf-1* knockdown fails to increase the life span of a genetic model of DR, *eat-2(ad1116)* and is dependent on the FoxA transcription

factor PHA-4. Using extensive genetic analysis, we found that *eddf-1* knockdown extends life span through the p38 MAPK pathway. On the other hand, *eddf-1*does not require the p38 pathway to control fat storage. A microarray analysis to identify genes that have altered expression in *eddf-1* knockdown worms showed distinct signature of upregulation of beta-oxidation and phase detoxification genes. We showed that the decreased fat storage in *eddf-1* RNAi is due to increased

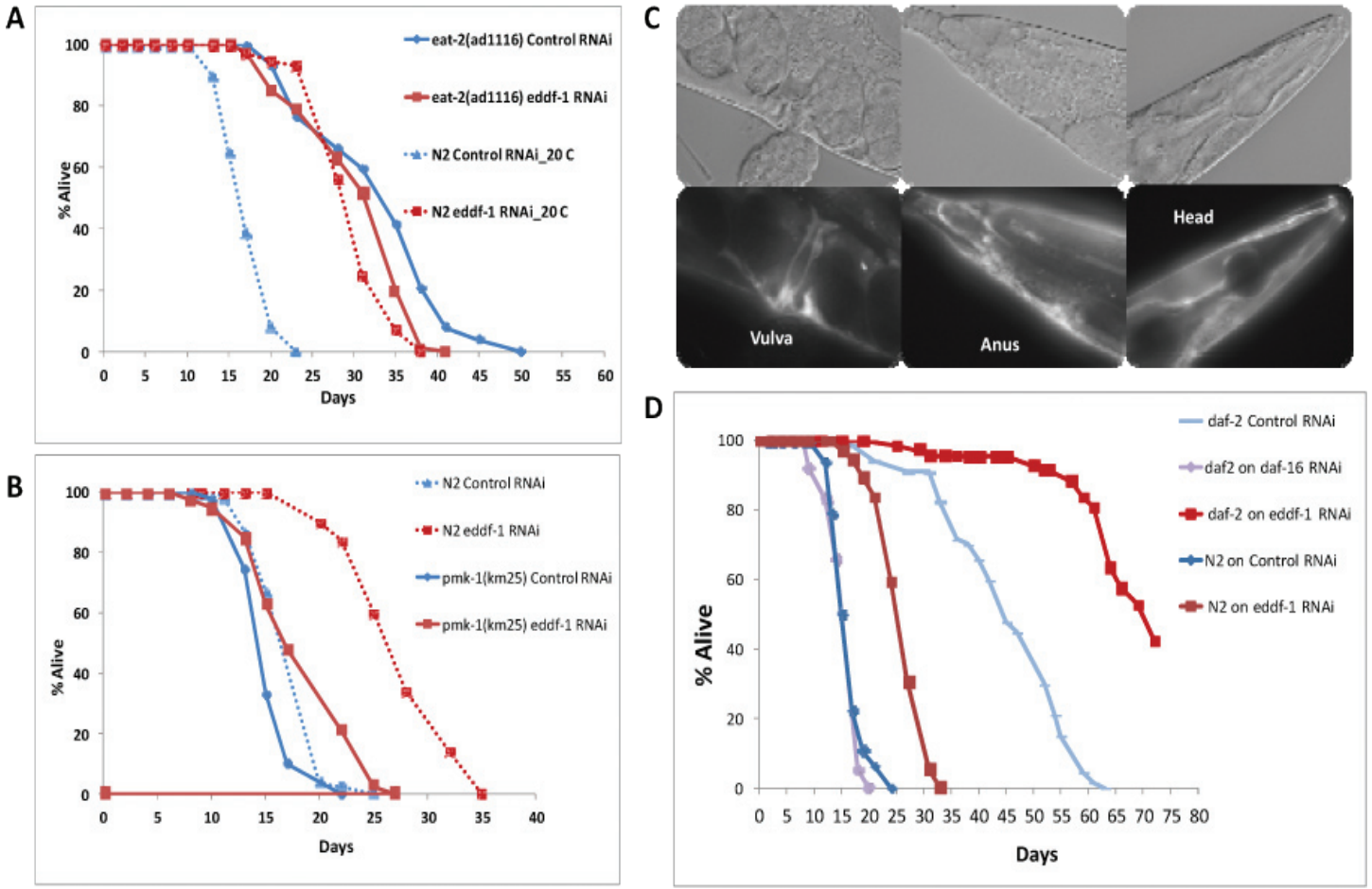


Figure 2. *EDDF-1 is a novel gene in the DR pathway. (A) The eddf-1 RNAi fails to extend life span of eat-2(ad1116) mutant, that is a model of DR. (B) The increased longevity of eddf-1 RNAi is completely abrogated in p38 MAPK gene (pmk-1) mutant background. (C) Eddf-1 promoter drives GFP expression in the head, anus and vulva region. (D) RNAi of eddf-1 synergizes with daf-2(e1370) life span. A daf-16 RNAi completely suppresses long life of daf-2(e1370) (Top).*

beta oxidation and generation of low ROS due to use of the complex II of electron transport chain. EDDF-1 expresses primarily around pharynx, vulva and anus along with some expression in the neurons. We hypothesize that *eddf-1* influence life span by upregulating cellular detoxification machinery as well as by shifting the metabolism towards respiration.

We have also started in-depth characterization of the *eddf-1* ortholog, *eddf-B*. Knockdown using RNAi or genetic mutation in *eddf-B* also extend life span dramatically. Genetically, *eddf-1* and *eddf-B* may work in the same pathway as *eddf-1* knockdown cannot further extend the life span of *eddf-B* mutant. Interestingly however, *eddf-B* shows opposite fat storage phenotype on different bacterial sources. We are currently investigating this.

Relation between caloric restriction and innate immunity

We wish to understand whether DR has any effect on innate immunity and pathogen resistance in *C. elegans*. The well known genetic model for DR, *eat-2* mutants, cannot be used for such studies due to significantly reduced ingestion of pathogenic bacteria. However, we have now discovered a new genetic model of dietary restriction (*eddf-1* gene) where the worms consume same amount of bacterial food but are internally calorically restricted. The issue of different levels of ingestion of pathogen does not arise in this model. Using this model, we have performed some preliminary experiments. We exposed the worms, in which the *eddf-1* gene has been knocked down, to different pathogenic bacteria. We used both gram negative (*Pseudomonas aeruginosa* and *Salmonella typhimurium*) as well as gram positive bacteria (*Staphylococcus aureus*). In multiple experiments, we find that the response to gram negative bacteria is diametrically opposite to that in gram positive bacteria. While the DR worms are susceptible to gram negative bacteria, they are resistant to gram positive bacteria (Figure 3). We are in the process of understanding this observation in details. Preliminary experiments using a microarray analysis suggests that several genes involved in innate immunity and xenobiotic detoxification pathway are upregulated on internal dietary restriction. We have obtained a pathogenic fungus (*Drechmeria coniospora*) and will evaluate the resistance of IDR worms towards the fungus.

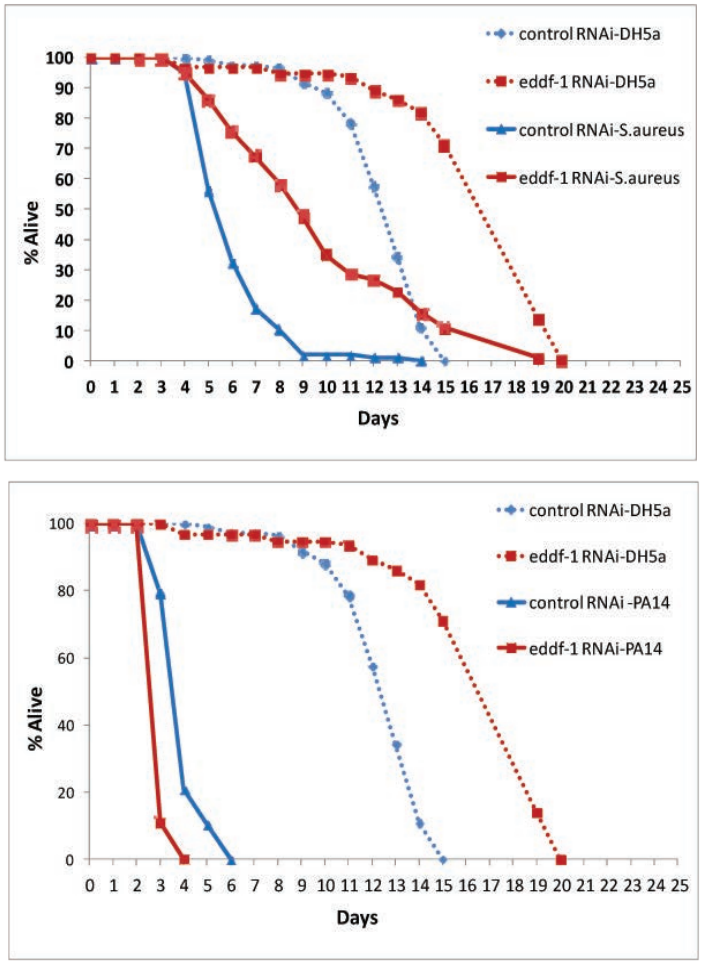


Figure 3. *Differential response to gram positive and gram negative bacteria following dietary restriction. (Upper panel). The eddf-1 RNAi worms are resistant to S. aureus (Lower panel). The eddf-1 RNAi worms are susceptible to Pseudomonas aeruginosa PA14 infection. DH5a is taken as control non-pathogenic bacteria in each case.*

Molecular biology of infectious diseases

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In global surveys, infectious diseases rank among the leading causes of death of both humans and animals. Vaccination against infectious agents continues to be one of the most effective methods of limiting the cost of management of many infectious diseases. The goal of this study is to clone and express genes of biomedical importance with an emphasis on the development of vaccines against pathogens and to unravel the molecular mechanisms of infectious diseases to explore new drug targets.

(A) Development of recombinant ε-toxin and DNA based vaccine against *Clostridium perfringens*

Gram positive *Clostridium perfringens* is a major cause of human and veterinary enteric diseases largely because this bacterium can produce several toxins when present inside the gastrointestinal tract. Epsilon toxin (Etx), produced by *C. perfringens* types B and D, is the key antigen implicated in the Enterotoxaemia and Pulpy kidney disease of domestic animals. Being the most common causes of cattle mortality, it is of great economic importance. Also, it is considered a major potential bioterrorism agent. The project aims at cloning and expression of Etx and its mutants for the development of recombinant and DNA vaccine against *C. perfringens.* Further, we aim to study the role of various residues within the toxin for its toxicity and immunogenicity.

(B) Studies on the enzymes of metabolic pathways and functional characterization of PE_PGRS and PE_PPE proteins of *Mycobacterium tuberculosis* H37Rv

Targeting the proteins/enzymes that are unique to *M. tuberculosis (Mtb)* is an effective approach to control *M. tuberculosis*. The branched-chain amino acid biosynthetic pathway is essential for the growth and virulence of *Mtb*. The

absence of this pathway in humans, and its essentiality in *Mtb*, make the enzymes of this pathway attractive targets for the development of antituberculosis drugs. Therefore, we plan to produce these enzymes through recombinant route and carry out their detailed characterization.

Sequence analysis of the *Mtb* H37Rv genome resulted in identification of novel multigene families- the PE (proline-glutamic acid) and the PPE (proline-proline-glutamic acid). These families account for much of the genomic difference between *Mtb* and nonpathogenic mycobacterial genomes. Therefore, they may play role in *Mtb*'s virulence and host-specificity. However, their exact role in *Mtb* biology is not clearly understood. We aim to explore the possible role these proteins may play in the biology of *Mtb*.

(A) Development of recombinant ε-toxin and DNA based vaccine against *Clostridium perfringens*

We have earlier reported characterization of three of the five non-toxic mutants of Etx. During the current year, further studies on the remainder non-toxic mutants of Etx have been carried out. The mutants were analyzed for their binding and heptamerization capability on MDCK cells membrane. Cell surface staining and Western blot analysis confirmed that both the mutants were able to bind to the MDCK cells membrane. SDS-PAGE analysis revealed that like the wild type Etx, these mutants were also able to heptamerize. The pore formation analysis was evaluated with membrane impermeable nucleic acid stain (propidium iodide) influx in the MDCK cells treated with mutant proteins. The data suggest that although the mutants were able to bind, and heptamerize, they failed to form pores on the membrane.

The type of the immune response generated against the mutants was analyzed by assessing the levels of antigen specific IgG subclass and secreted cytokines in the antigen-induced *in vitro* culture of lymphocytes isolated from immunized BALB/c mice. The data suggested that mutants generated mixed Th1 and Th2 immune responses. To check the vaccine potential of non-toxic mutants, *in vivo* protection studies were carried out in mice immunized with the mutants followed by challenge with wild type Etx. One hundred percent protection was observed.

The wild type Etx requires C-terminal region to be cleaved by trypsin for activation. Therefore, in order to reduce purification steps, C-terminal deletion mutants were generated with or without the 6x-Histidine tag at the C-terminus. The C-terminal deletion variants of Etx were expressed and purified. Both the deletion variants were found to be cytotoxic *in vitro.*

For the development of DNA-based vaccine, we had earlier reported construction of DNA constructs targeting the ε-toxin (etx) for cytosolic (pcEtx), membrane anchored (EtxpDSm) and secretory expression (EtxpDsec) and evaluation of the immunogenic potential of the first two. To assess the immunogenic potential of the construct expressing the Etx as a secretory protein, BALB/c mice were immunized with this construct after establishing its expression in CHO-K1 cells *in vitro*. Immunization of mice with the DNA constructs gave basal level antibody titers at early time intervals. However, the titers showed a significant increase with time. To get higher response, prime boost strategy was performed in which mice were immunized with DNA alone followed by one booster of heat inactivated Etx. This resulted in significant increase in the antibody titers when compared to DNA immunization alone.

Cell mediated immunity was checked by T cell proliferation assay from the splenocytes of DNA immunized and prime boosted mice. Analysis of cytokines in the supernatant of the T cell proliferation assay indicated a Th_1 type of response. *In vitro* protection analysis of sera from DNA immunized, DNA immunized and prime boosted with heat inactive Etx was done by pre-incubation of Etx with anti-sera prior to its addition to the MDCK cells. The results indicated that the anti-sera of these mice exhibited neutralization potential *in vitro*.

In vivo protection efficacy was evaluated by challenge with purified epsilon toxin (approximately 50 LD_{50} doses). The immunized mice survived after challenge. These mice were further observed for an additional two weeks to ensure that they survived the challenge. In order to develop sub-unit vaccine against epsilon toxin, it was envisaged to clone the potential immunogenic epitopes in fusion with LTB. The potential surface exposed regions of the ε-toxin were predicted using bioinformatics tools. Oligonucleotides corresponding to the five potential immunogenic regions with dominantly hydrophilic character thus identified were cloned at the C-terminus of LTB. The final construct carried the *E. coli* LTB gene under the control of T5 promoter in C- terminal translational fusion with the Etx epitope with 5-glycine residues in between. One of the five Etx epitope-LTB fusion was subcloned in pMMB vector for secretory expression in *E. coli* DH5 α cells. The construct was then conjugally transferred to *Vibrio cholerae* cells.

Expression of the gene fusion protein in E. coli and *V. cholerae* under the control of the tacP promoter was done by inducing the cells with IPTG. Periplasmic and extracellular expression of the fusion protein was obtained in *E. coli* and *V. cholerae* cells, respectively. The recombinant fusion protein was purified from the *Vibrio* culture supernatant by ammonium sulfate precipitation followed by cation exchange chromatography. Female BALB/c mice were immunized with the fusion protein emulsified in alum followed by two booster doses. Western blot analysis and ELISA using the anti-sera against the fusion protein indicated that the fusion protein retained the antigenicity of both the fusion partners and was able to detect the Etx as well as LTB. Neutralization analysis of sera generated against the fusion proteins was carried out using MDCK cells.

(B) Studies on the enzymes of metabolic pathways and functional characterization of PE_PGRS and PE_PPE proteins of *Mycobacterium tuberculosis* H37Rv

To understand the role of PE_PGRS proteins in the biology of *M. tuberculosis*, several PE_PGRS genes were expressed in *M. smegmatis* which naturally lacks these genes. Out of 61 genes known, 22 were selected on the basis of previous literature about their expression pattern during *Mtb* infection.

Of the 22 genes selected, 05 genes were successfully PCR amplified and cloned in fusion with green fluorescent protein in *E.coli*/*Mycobacterium* shuttle vector pVV16. The recombinant plasmids were transformed in *M. smegmatis* by electroporation. Expression of the proteins in *M. smegmatis* was confirmed by western blotting with anti-GFP antibody. Two of the PE_PGRS proteins resulted in phenotypic changes in *M. smegmatis* with altered colony morphology and growth profile. Scanning electron microscopy studies indicated that the change in colony morphology was not due to changes in the morphology of individual cell. Growth kinetic studies were performed which suggested that the slow growth of the recombinants was due to prolonged lag-phase. Sub-cellular fractionation of the whole cell lysates of recombinant *M. smegmatis* indicated that the two proteins are associated with the cell wall.

One of these proteins, PE-PGRS30 has been further characterized. Fluorescence microscopy studies showed that the protein is concentrated at the cell pole. Cell wall localization of the protein was confirmed by immuno-electron microscopy. Proteinase K sensitivity assay revealed that the protein was exposed on the mycobacterial surface. Owing to the observed phenotypic changes, recombinant *M. smegmatis* were studied for any change in sensitivity to hydrophobic drugs (antibiotics). However, there was no significant change in its sensitivity either to antibiotics or to detergent such as SDS. Triton X-114 fractionation of the whole cell lysates of recombinant mycobacteria fractionated the PE_PGRS protein to the detergent fraction, indicating that either the protein is attached to a lipid moiety or shows his property because of its hydrophobicity. Further, the protein did not show labeling when the recombinant *M. smegmatis* were grown in the presence of ^{14}C-acetate. Infection of THP-1 macrophages with the recombinant *M. smegmatis* resulted in the down-regulation of pro-inflammatory cytokines, IL-6, IL-12 and TNF-α.

Publications

Original peer-reviewed articles

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Patents

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In Press last year, since published

Epigenetic regulation of the eukaryotic genome : Role of transcriptional insulators in organizing chromatin

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The current understanding of the mechanisms by which *cis*-acting regulatory elements interact with each other in context of chromatin is rather limited even though such interactions are crucial for appropriate regulation of nuclear processes like transcription and VDJ recombination. In this context, CTCF dependent insulators play an important role in the functional organization of the mammalian genome as they can coordinate intrachromosomal and interchromosomal contacts and thus influence *cis*-DNA interactions. A large number of CTCF binding sites have been identified genome-wide suggesting their extensive involvement in governing *cis* DNA interactions among regulatory elements. However, the mechanisms by which they organize independently regulated domains remain only partially understood. Our efforts are directed towards understanding how the mammalian insulators influence chromatin domain organization and contribute to regulation of nuclear processes. A combination of genetic, molecular and biochemical approaches are being utilized for investigations.

To explore the mechanisms underlying insulator activity, antigen receptor loci like IgH, TCRα/δ, TCRβ etc, are particularly interesting. By exhibiting long range interactions between different types of elements, they present a useful framework to explore the role of CTCF in defining independently regulated chromatin domains. Enhancer-promoter interactions are necessary for defining the developmental stage specificity of RAG mediated VDJ recombination at these loci. Additionally, recombination requires physical interaction between RSS elements associated with the V, D and J segments. These segments are located at large distances from each other on the chromosome and higher order chromatin reorganization is necessary to bring them together prior to recombination. Taking advantage of this, we are currently investigating the chromatin structure and organization of the wild type and genetically manipulated TCRβ loci to address the following:

1. Ability of a CTCF dependent insulator to organize chromatin structure and establish a functional insulator in a heterologous context that might impact enhancer-promoter interactions at the TCRβ locus

2. Ability of the CTCF dependent insulator to effect higher order chromatin organization that might influence VDJ recombination by modulating other cis–DNA interactions

Our functional analysis of TCR-ins mutants in the previous years clearly showed that normal chromatin interactions for ordered VDJ recombination can be disrupted simply by the ectopic insertion of non-locus specific CTCF binding sites. The inserted *H19-ICR* interfered not only in promoter-enhancer interactions but also reorganized the chromatin landscape such that Vβ-to-DβJβ interactions were affected leading to enhanced usage of TRBV31 and concomitant reduction in the usage of other Vβ segments for VDJ recombination. Currently, investigations are in progress to find molecular correlates of our functional observations.

A. Importance of CTCF for the observed effects of *H19-ICR* at TCRβ locus

It was important to establish that the alterations in the transcription and recombination patterns at the TCRβ under the influence of inserted *H19-ICR*, were due to CTCF binding rather than insertion of a 2.2kb DNA fragment. To address this issue, we generated mouse mutants (TCRmut) which were similar to TCR-ins mutant mice but carried a mutated version of *H19-ICR* that could not bind CTCF. Analysis of TCRβ locus transcription and VDJ recombination in the thymocytes of these mutants proved beyond doubt that CTCF binding by *H19-ICR* is crucially important for enhancer blocking as well as for interference in VDJ recombination.

B. Altered chromatin structure of TCRβlocus due to insulator insertion

At the TCRβ locus, the enhancer Eβ is responsible for generating chromatin accessibility of about 25 kb region. Some of these chromatin alterations are dependent on Eβ-PDβ interactions while others are independent of it. We carried out a detailed allele specific analysis of histone modifications in this region in wild type and TCR-ins mutant mice by ChIP. The *H19-ICR* insulator insertion in the TCR-ins mice prevented the acquisition of activating histone modifications H3K4me3 and H3K9Ac at the PDβ1 promoter in concordance with the loss in transcription observed earlier. Additionally, the insulator blocked the acquisition of these modifications in the gene segments Jβ1 and Cβ1 that are known to be independent Eβ-PDβ1 interaction. Consistent with the position dependence of insulator activity, PDβ2 and the associated gene segments (Jβ2 and Cβ2) continued to acquire Eβ dependent activating histone modifications in the thymocytes of TCR-ins mice. We are currently investigating the alterations in chromatin accessibility measured *via* restriction enzyme digestion assays. This analysis is likely to corroborate our functional observations. Also, it may provide insights into the enhancer-promoter-insulator interactions and their relevance for chromatin organization.

C. Identification of CTCF binding sites at the TCRβ locus

Antigen receptor loci like IgH and TCRα/δ have been reported to have multiple CTCF binding sites. As predicted, some of the sites exhibit enhancer blocking at the IgH locus. An additional role of CTCF, in concert with cohesin, has been postulated in locus contraction. Consistent with this, Pro-B cells exhibit multiple interactions between the distantly placed CTCF binding sites at IgH locus. Knockdown of CTCF led to a partial reduction in these long range interactions thereby supporting the role of CTCF in higher order chromatin organization. Our analysis demonstrated that introduction of the few ectopic CTCF binding sites from an unrelated locus was sufficient to drastically alter the transcription and recombination patterns at the TCRβ locus and suggests a role of CTCF in defining the choice of Vβ segments for Vβ-to-DβJβ recombination.

To analyse the specific long-range interactions that CTCF mediates at TCRβ locus and the manner in which the ectopic CTCF binding sites alter these interactions with functional consequences, it is important to determine the specific CTCF and cohesin binding sites at this locus. Towards this goal, we have identified 8 CTCF binding sites on the murine TCRβ locus and have validated CTCF and cohesin binding by several of them as analysed by chromatin immunoprecipitation in thymocytes derived from Rag deficient mice i.e. prior to initiation of VDJ recombination. In the next stage of investigations, we plan to use chromosome conformation capture analysis that will help in delineating the higher order chromatin organization at TCRβ locus in the wild type and *H19-ICR* carrying mutant allele.

Role of cell signaling in eukaryotic development

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It is well known that extracellular signals control biological responses in most eukaryotic cells by regulating specific intracellular signaling cascades. We are interested in signaling and trafficking events in two diverse cell types; 1) malaria parasite *Plasmodium falciparum,* 2) mammalian neurons.

1. Dissection of intracellular signaling and trafficking cascades of *Plasmodium falciparum*.

Characterization of signaling pathways that operate in malaria parasite may help unravel novel mechanisms involved in its development. We are interested in the role and regulation of phosphoinostides, their effectors and calcium in the life cycle of *Plasmodium falciparum*.

2. Role of cyclins and cyclin dependent kinases in neuronal apoptosis

Apoptosis of neurons is important for proper brain development and can also lead to neuronal loss in neurological disorders. During apoptosis, terminally differentiated neurons attempt to re-enter into the cell cycle, which is reflected by the aberrant modulation of cell cycle proteins like cyclins and cyclin dependent kinases (cdks). Molecular mechanisms underlying Cell Cycle Related Neuronal Apoptosis (CRNA) are poorly understood. We are interested in how cell cycle proteins are regulated and in turn control CRNA.

1. Dissection of intracellular signaling and trafficking cascades of *Plasmodium falciparum*.

Role and regulation of protein kinases PfPKB and PfCDPK1 in malaria parasite life cycle.

Calcium mediated signaling is crucial for the development of malaria parasite *Plasmodium falciparum*. Phospholipase C (PfPLC) has emerged as a key player in this process as calcium release triggered by it results in the activation of protein kinases like PfPKB and PfCDPK1. Inhibition of PfPLC prevents RBC invasion by the parasite and PfPKB and PfCDPK1 may contribute

to this process. Our attempts to disrupt PfPKB gene suggested that it may be essential for asexual parasite growth. Previously, we have reported that pharmacological inhibitors that block PfPKB activity block invasion. PfPKB and PfCDPK1 phosphorylate a critical member of the glideosome assembly, PfGAP45. The actomyosin motor complex of the glideosome provides the force needed by apicomplexan parasites such as *Toxoplasma gondii* and *Plasmodium falciparum* to invade their host cells and for gliding motility of their motile forms. Glideosome Associated Protein 45 (PfGAP45) is an essential component of the glideosome complex as it facilitates anchoring and targeting of the motor. PfGAP45 is phosphorylated in response to Phospholipase C and calcium signaling by protein kinases PfPKB and PfCDPK1. Mass spectrometry and phosphopeptide mapping studies revealed that S89, S103 and S149 are targeted by these kinases. A PfPKB inhibitor blocked the phosphorylation of S103 in the parasite suggesting that it may be the candidate kinase for S103 in the parasite. Several parasite lines expressing GFP-fusion proteins of GAP45 and its phosphorylation site mutants were generated. Using phosphorylation site specific antibodies and ^{32}P-inorganic phosphate labeling, we could demonstrate that S89, S103 and S149 are phosphorylated in the parasite. The phospholipase C pathway influenced the phosphorylation of S103 and S149 and the phosphorylation of PfGAP45 at these sites was differentially regulated during parasite development. PfGAP45 regulation in response to calcium fits in well with the previously described role of calcium in host cell invasion by the malaria parasite. To assess the effect of phosphorylation on IMC and GAP45 targeting, immunofluorescence assays (IFA) and live cell imaging studies were performed. These studies suggested that the localization of PfGAP45 and its glideosome association may not entirely be dependent on the phosphorylation of these sites. Since recent proteomic studies have suggested that PfGAP45 may be phosphorylated at additional sites in the parasite, it will be worth evaluating if phosphorylation of the other sites in combination with the sites identified by us has an impact on PfGAP45 function.

Regulation of Calcium Dependent Protein Kinases (PfCDPKs).

Calcium Dependent Protein Kinases (CDPKs) are major effectors of calcium signaling in apicomplexan parasites like *Toxoplasma* and *Plasmodium* and control important processes of the parasite life cycle. Despite recently reported crystal structures of *Tg*CDPKs, several important questions about their regulation remain unanswered. PfCDPK1 has emerged as a key player in the life cycle of the malaria parasite as it may be involved in the invasion of the host cells. Calcium binding to the EF hand motifs causes major domain rearrangement leading to key interactions between the CH1 helix/J-domain and the CLD. Molecular modeling and site directed mutagenesis studies of PfCDPK1 suggested that several residues in the regulatory domain play a dual role as they may contribute to the stabilization of both the active and inactive kinase. Mass spectrometry revealed that PfCDPK1 was autophosphorylated at several sites, some of these were placed at strategic locations, therefore were found critical for kinase activation. The N-terminal extension of PfCDPK1 was found to be important for *Pf*CDPK1 activation. We identified a novel nucleotide binding Walker A like motif in the NTE of PfCDPK1, which is not conserved in CDPK1 of other *Plasmodium* spp. Since there is a reasonable sequence similarity between PfCDPK1 and other CDPKs, our studies may help explain the regulation of the CDPK family. However, variations in regions like the NTE or in putative phosphorylation sites suggest that subtle differences may exist in their cellular regulation. Typically, protein kinase inhibitors are generated by targeting the ATP binding pocket or the substrate binding cleft. Our studies also led to the identification of two new pockets, which can be targeted to inhibit PfCDPK1 activity.

2. Role of cyclin/cyclin dependent kinases in neuronal apoptosis

We had reported earlier that neurotoxic $A\beta_{42}$ peptide causes aberrant MEK-ERK signaling in cortical neurons and neuronal PC12 cells, which results in CRNA. These results were surprising as the MEK-ERK Map Kinase pathway is also important for neuronal differentiation and survival under physiological conditions.

We further dissected the molecular mechanisms which may contribute to cell cycle related neuronal apoptosis (CRNA) by this pathway. We confirmed these findings in the neurons from mouse model of Alzheimer's disease (AD), wherein cyclin D1 was expressed at higher levels in comparison to the wild type animals. Cyclin D1 is a key cyclin, which is necessary for the S-phase entry and its levels are low in neuronal cells. The $A\beta_{42}$ triggered aberrant MEK-ERK signaling resulted in increased production of cyclin D1 levels via transcription factor STAT3, which contributed to CRNA. Cyclin D1, in turn, amplifies the MEK-ERK signaling via a positive feedback loop which involves cyclin dependent kinase 5 (cdk5). Cdk5 is an atypical CDK which is active mainly in neurons as a result of its interaction with its neuron-specific cyclin like activators p35 and p39. Cyclin D1 prevented the activation of cdk5 by its regulator p35 leading to the hyperactivation of MEK-ERK. $A\beta_{42}$ induced CRNA was reversed by p35 confirming that cyclin D1 mediated down regulation of p35-cdk5 may contribute to CRNA. These data also reveal a novel mechanism via which p35-cdk5 may promote neuronal survival.

Publication

Original Peer-reviewed article

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Chemical Biology of *Mycobacterium tuberculosis*: Deciphering the Role of Polyketide Synthases in *Mycobacteria* and *Dictyostelium*

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Our laboratory is interested in genome-based approaches to identify and exploit the microbial metabolic pathways that are involved in the biosynthesis of various natural products. The primary focus is to understand the importance of various polyketide synthase gene clusters from *Mycobacterium tuberculosis*. We are now extending these studies to other organisms like *Dictyostelium discoideum* to understand how polyketide synthases generate metabolic diversity.

The genome sequence of *M. tuberculosis* (Mtb) and *Dictyostelium* (Dicty) has revealed a remarkable array of genes that are homologous to polyketide synthases. Our study attempts to understand and exploit the role of polyketide synthases in the biology of Mtb and Dicty.

To summarize, the objectives of the studies proposed are:

1. Identification and biochemical analyses of enzymes that are involved in the biosynthesis and/or degradation of lipidic metaboilites
2. Characterization of molecular mechanisms mediating the crosstalk between various polyketide synthases (PKSs) and fatty acid synthases (FASs) in *M. tuberculosis*.
3. Genetic and/or Chemical knock-out of PKS genes to synthesize novel polyketides and to study the effects of these changes on mycobacterial pathogenecity and Dictyostelium development.
4. Delineate mechanisms that generate metabolic diversity, particularly by using versatility of polyketide biosynthetic machinery.

Molecular basis of the functional divergence of Fatty Acyl-AMP Ligase biosynthetic enzymes of Mycobacterium tuberculosis

Fatty acid metabolism in nature involves conversion of fatty acids to their corresponding acyl-CoA thioesters. This mechanism of fatty acid activation is conserved throughout evolution. To date only known variants of this activation is the formation of fatty acyl-ACP and fatty acyl-adenylate. Our group in Mtb first reported acyl-adenylate formation in 2004 by a new family of enzymes called FAALs. We have now traced the evolutionary origin of FAAL class of enzymes from the omnipresent FACLs. The study shows a classic example of enzyme evolution, wherein, two enzymes with a conserved structural scaffold, catalytic site and substrate binding cavity have diversified to perform two distinct functions in the biology of Mtb. Our studies demonstrate that this new evolutionary mechanism involved in biosynthesis of lipidic metabolite has occurred by

incorporation of an insertion sequence in the omnipresent FACLs. To elucidate how the insertion was able to modulate the catalytic function, the interaction between the insertion and the N- and C-ter domain of the protein was systemically investigated. The lower part of the insertion was found to be stabilized by strong hydrophobic interaction between the insertion and N-terminal domain of the protein. Strikingly, in the structural models generated for all other Mtb FAAL homologues similar hydrophobic interaction could be mapped. Homology model of FAAL32 suggested a hydrophobic patch with a possibility of aromatic interactions between Phe383 and Phe481.The two spatially adjacent phenylalanines were mutated to alanine residues. Mutagenesis of the two interacting Phe to Ala converted FAAL32 to FACL32, establishing the importance of a stabilized insertion in effectively arresting the acyl CoA catalysis in FAALs. Next based on the presence of the insertion stabilized by hydrophobic anchorage in other AAEs homologues we succeed in fishing out FAALs homologues from close to 40 different genomes establishing that formation of acyl adenylate is indeed is a generalized mode of fatty acid activation. We thus propose similar mode of activation across various *Actino-*, *Cyano-* and *Proteobacteria* and in eukaryotes, establishing that formation of acyl-adenylate is indeed an important generalized mechanism for biosynthesis of lipidic metabolites.

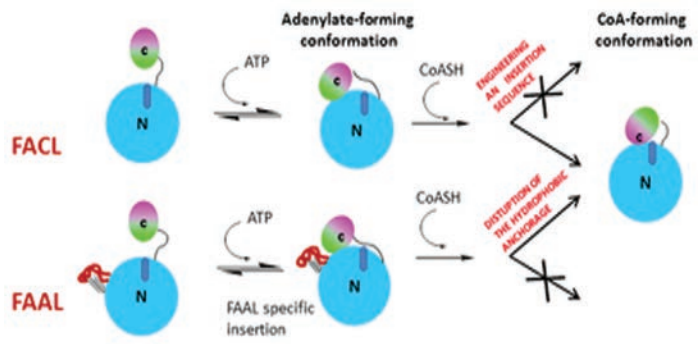

Figure 4: *An insertion motif stabilized by hydrophobic anchorage abrogates acyl CoA catalysis by FAALs.*

To investigate the striking non-redundant roles of FAALs in Mtb biology, we decided to investigate into the fatty acid binding pockets of these proteins. The comparative analysis of the substrate binding pocket with those of other members of AAEs superfamily suggested an interesting possibility of broadly dividing AAEs into four categories of small, medium, large and very large substrate binding proteins: 1) Acetyl-CoA synthetase (ACS) from Salmonella enterica as small 2) Phenylalanine activating subunit of gramicidin synthetase 1 (PheA), DhbE adenylation domain (May et al., 2002) and 4-chlorobenzoate-CoA ligase (CBL) as medium, 3) FAAL21, FAAL23, FAAL25, and FAAL28 as large and 4) FAAL31, FAAL32 and FAAL34 as very large. It is interesting to note that subtle variations in amino acid side chains lining the substrate-binding pocket show correlation with the volume of the substrate. The predicted substrate-binding pocket was then validated by generation G330W and I227W FAAL28 mutant. Unlike FAAL28WT (wild type) the G330W FAAL28 mutant protein catalyzed activation of C2 fatty acid as acetyl-AMP. The other FAAL28 I227W mutant protein showed substantial decrease (~69%) in activity with C12 substrate but showed 4-fold higher activity with C10 long fatty acyl chain. Our biochemical assays with several Mtb FAALs and FACLs homologues revealed overlapping substrate specificity the two enzyme families. We therefore decided investigate if FACLs and FAALs share similar determinants of substrate specificity. Structurally analogous to I227 of FAAL28 a Threonine residue is present at position 214 in FACL13. T214W FACL13 mutant protein showed marked decrease in activity with longer acyl chains of C16 and C12 with the simultaneous increase in the activity with C8 fatty acid, suggesting mechanistic similarity in terms of substrate binding across FAALs and FACLs .

Nonprocessive [2+2]e- Offloading Reductase Domains from Mycobacterial Non-ribosomal Peptide Synthetases

An important feature of the assembly-line multifunctional non-ribosomal peptide synthetase (NRPS) and polyketide synthase

(PKS) biosynthetic enzymes is the presence of chain releasing domain. Apart from performing the crucial offloading step to maintain enzymatic turnover, both thioesterase (TE) and Reductase (R) domains mediate different outcomes of the final product. While TE domains belong to α/β hydrolase fold and involve mandatory formation of oxyester intermediate, R domains have been proposed to reductively release acyl chains directly from the ppant arm of the upstream T domain. We have characterised R domains from two mycobacterial NRPS proteins (glycopeptidolipid producing R_{GPL} and uncharacterized R_{NRP}) that perform 4e- reductions to release lipidic metabolites as alcohols. Our studies suggest a nonprocessive mechanism of catalysis for 4e- reductases, wherein at the end of each reductive cycle the product extrudes out from the active site pocket to facilitate exchange of cofactor. Biochemical studies using a lipopeptidyl- and fatty acyl-thioester and aldehyde substrates revealed that R domains possess broad substrate tolerance. The catalytic mechanisms employed for reduction has been delineated by biochemical and fluorescence studies using WT and mutant RGPL proteins. Our studies revealed that two step reduction of R_{GPL} lipopeptidyl thioester involves NAD(P)H-dependent hydride transfer with the concomitant proton transfer from the conserved Thr-Tyr-Lys triad of the SDR catalytic motif. The structural studies of R_{NRP} provide first insights into this mechanism of chain releasing reductase domains. Modeling of substrate and the cofactor indicate that the aldehyde intermediate generated during the first reductive step could physically obstruct the exchange of the oxidized cofactor. This makes it mandatory for the aldehyde to be extruded in the 'free form'. Biochemical studies also strongly support such a mechanism – 1) both R_{GPL} and R_{NRP} overwhelmingly catalyze reduction of lipopetidyl aldehyde over the cognate lipopetidyl thioester substrate and 2) the relative ratio of abundance of 4e- reduction product to 2e- reduction product increases with increasing amount of enzyme. Crystallographic studies in conjunction with SAXS and fluorescence data suggest an important role of protein conformational changes in response to cofactor NAD(P)H. In the apo-structure of R_{NRP}, the NAD(P)H binding site is occupied by a loop S153 to F166. We propose that this loop will have to move out to accommodate NAD(P)H, as observed for other homologous structures crystallized with this cofactor. The correlation of this loop movement to the [2+2]e- reduction cycle could in future provide further insights into this nonprocessive mode of catalysis. The functional analysis of R_{GPL} also clarifies the crucial step in GPL biosynthesis, as the formation of this terminal alcohol is mandatory for the subsequent glycosylation. In conclusion, our structural and biochemical studies reveal an unconventional two-step catalytic mechanism of reduction, which provides interesting insights into evolution of enzymatic functions.

Publications

Original peer reviewed articles

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Determining the signaling and repair pathways that are altered in human cancer using RecQ helicases as the model system

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Our research program evolves around the understanding of the cellular processes that are altered in neoplastic transformation leading to human cancer. Towards this aim, we focus my research endeavor on the RecQ helicases. BLM and RECQL4 are members of the RecQ family of DNA helicases. Germline mutations in both BLM and RECQL4 helicase result in autosomal-recessive disorders, Bloom syndrome (BS) and Rothmund-Thomson syndrome (RTS) respectively. BS afflicted individuals are predisposed to almost all types of cancers while RTS individuals are predominantly predisposed towards osteosarcomas. Since RecQ helicases are intimately involved in the many vital cellular processes, they are ideal candidates to investigate the reasons for neoplastic transformation.

In the current year the work in the lab was aimed to dissect the *in vivo* functions of RecQ helicases. Specifically the aims were:

1. Dissect the roles of RECQL4 and p53 in the regulation of mitochondrial DNA replication

2. Decipher the role of BLM in regulating c-Myc functions

Aim #1: Role of RECQL4 and p53 in the regulation of mitochondrial DNA replication

Rescue of RTS fibroblasts by expression of RECQL4

Having earlier established the mechanism of the mitochondrial localization of RECQL4 and p53 in untreated normal human cells, we hypothesized that the transcriptionally active nuclear p53 observed in RTS patient cells can be relocated to the mitochondria by stably overexpressing wildtype RECQL4. Hence we rescued RTS patient cell line, AG05013 with AcGFP RECQL4 (1-1208) to generate AG05013 AcGFP-RECQL4 (1-1208) Clone 1. Very few AG05013 AcGFP-RECQL4 (1-1208) Clone 1 cells had spontaneous 53BP1 foci formation. AcGFP-RECQL4 localized to

mitochondria as it co-localized with Mitotracker Red. In contrast to the parental AG05013 cells, p53 colocalized with AcGFP RECQL4 in the mitochondrial nucleoids of AG05013 AcGFP-RECQL4 Clone 1 cells, thereby proving that accumulation of p53 to the mitochondria under untreated conditions depended on RECQL4. In AG05013 AcGFP-RECQL4 (1-1208) Clone 1 cells a decrease in the level of endogenous p53 was observed under untreated conditions. The levels of p53 target genes (like p21, Bax, PUMA, MDM2) and the N-terminal phosphorylation of p53 at Ser15 and Ser46 were also decreased. By immunfluorescence the high level of transcriptionally active nuclear p53 observed in AG05013 under untreated condition was reduced in AG05013 AcGFP-RECQL4 Clone 1 cells. Clonogenic assays carried out on AG05013 and AG05013 AcGFP-RECQL4 (1-1208) Clone 1 cells after exposure to camptothecin and neocarzinostatin indicated that the hypersensitivity of RTS fibroblasts could be rescued due to the re-expression of wildtype RECQL4. Altogether it can be summarized that endogenous RECQL4 in normal cells relocates p53 to the mitochondria under untreated conditions, thereby preventing its nuclear activation in absence of exogenous DNA damage. Finally clonogenic assays with AG05013-RECQL4 (Δ84) cells indicated that the nuclear localization of RECQL4 could not rescue the high sensitivity of RTS fibroblasts to camptochecin and neocarzinostatin, especially at higher concentrations of the drug. The lack of mitochondrial localization of RECQL4 caused the accumulation of nuclei p53 in a majority of AG05013-RECQL4 (Δ84) cells. However in a percentage of RECQL4 (Δ84) cells, p53 was still mitochondrial. This indicated that mechanisms not mediated by RECQL4 could also lead to p53 accumulation in the mitochondria.

Aim # 2: Decipher the role of BLM in regulating c-Myc functions

BLM enhanced the binding of Fbw7 with c-Myc

We hypothesized that interaction of BLM with c-Myc may be a prerequisite for the enhanced c-Myc turnover in cells expressing BLM. To determine whether the two proteins physically interact, GST pull-down assays were carried out with equalized BLM and c-Myc fragments. *In vitro* interaction assays indicated that the N-terminal region of BLM interacted with c-Myc. Reciprocally the C-terminal region of c-Myc2, spanning amino acids (300-410), encompassing the basic region and the helix loop helix (HLH), interacted with BLM. It was interesting to note that two c-Myc fragments which overlap only partially in this region [i.e. c-Myc2 (368-439) and c-Myc2 (238-368)], also interacted with BLM. This indicated that while the entire Myc2 (300-410) region contributed to the interaction, amino acids (368-410) was probably obligatory for the process. Reciprocal immunoprecipitations using either c-Myc or BLM antibodies indicated that *in vivo* the two endogenous proteins constitutively interact in both asynchronous cultures or after replication stress. Both BLM and c-Myc have been separately reported to localize to the nucleoli, the sub-nuclear compartment implicated for c-Myc degradation. Immunofluorescence staining with ice-cold methanol:acetone fixation indicated that overexpressed Myc and BLM were present in both nucleoplasm and nucleolus (identified by DAPI exclusion). BLM and c-Myc colocalized extensively in the nucleolus and to a limited extent in the nucleoplasm. We next wanted to determine whether the colocalization observed with the overexpressed proteins also extended to their endogenous counterparts. Endogenous c-Myc and BLM, as stained by using a prelysis protocol and ethanol based fixation, colocalized in both nucleolus and nucleoplasm. However Myc-BLM colocalization was not observed in the PML nuclear bodies where BLM also localized.

Based on above results we hypothesized that BLM may be directly affecting the interaction of c-Myc with its E3 ligase, Fbw7. First we wanted to test whether BLM and its fragments also interacted with the Fbw7 isoforms. *In vitro* interaction assays carried out with all the three isoforms of Fbw7 indicated that the E3 ligase interacted with GST-tagged BLM (1-1417) but not to GST alone. Ability of BLM to interact with Fbw7 (ΔN) indicates that BLM-Fbw7 interaction occurred via the common C-terminal dimerization, F box and WD40 domains of the Fbw7 isoforms. Since BLM is a nuclear protein, we were more interested with BLM-Fbw7α and

BLM-Fbw7γ interactions. Hence to determine the subnuclear location where BLM could interact with Fbw7, we carried out immunofluorescence after overexpression of the tagged version of the two proteins (as endogenous Fbw7 could not be detected by the Fbw7 antibody during immunofluorescence). BLM colocalized with both Fbw7α and Fbw7γ under asynchronous conditions in the nucleoplasm and nucleolus, respectively.

The above results led to the hypothesis that BLM may simultaneously bind to both c-Myc and Fbw7. We found that except the N-terminal (1-212) amino acids, the rest of BLM interacted with both Fbw7α and Fbw7γ. Since two distinct regions of BLM interacted with c-Myc and Fbw7, we hypothesized that may be BLM was acting as a "clamp/adaptor" protein, thereby bringing c-Myc in close proximity to its E3 ligase, Fbw7. Indeed addition of wildtype BLM (1-1417) enhanced the interaction between c-Myc and Fbw7γ. The N-terminal (1-212) amino acids of BLM also enhanced c-Myc-Fbw7 interaction but to a lesser extent. This is to be expected as the region of BLM that binds to Fbw7 is not present in BLM (1-212), thereby reducing the efficiency of the proposed "clamp/adaptor" function of BLM. *In vivo,* increased levels of endogenous Fbw7 isoforms were complexed with endogenous c-Myc in A-15 cells, in spite of the presence of more endogenous c-Myc in BS cell. These results indicated that BLM enhanced the binding of c-Myc to its E3 ligase, Fbw7.

BLM enhanced Fbw7-mediated K48-linked c-Myc ubiquitylation

Based on the above results we wanted to determine whether full-length recombinant BLM had any effect on Fbw7-dependent c-Myc ubiquitylation. For these assays we intentionally used the lowest amount of Fbw7 which showed detected barely visible c-Myc ubiquitylated forms so that the effect of BLM could be visualized. The amount of *in vitro* translated c-Myc used for the ubiquitylation reactions were equal. BLM (1-1417) enhanced Fbw7-mediated c-Myc ubiquitylation *in vitro*, as detected by a c-Myc antibody. The extent of enhancement for both Fbw7γ and Fbw7α was equal at the higher concentration of BLM. The blot was subsequently probed with anti-FK2 antibody, which recognized both mono-and poly-ubiquitylated conjugates. BLM enhanced both mono and poly-ubiquitylation of c-Myc. Though BLM (1-212) alone could enhance c-Myc ubiquitylation, the degree of the enhancement with wildtype BLM (1-1417) was much higher. This indicated that the full-length BLM enhanced the physical interaction of c-Myc with its E3 ligase (i.e. Fbw7), thereby leading to increased c-Myc ubiquitylation. A helicase dead BLM (K695A) mutant also stimulated the ubiquitylation to the same extent as wildtype BLM, indicating that this function of BLM was not dependent on its DNA unwinding activity. BLM stimulated Fbw7γ-dependent c-Myc ubiquitylation even in presence of exogenously added recombinant GSK3β and GSK3β inhibitor, thereby indicating that two independent modes of enhancement of c-Myc ubiquitylation. *In vivo*, although more endogenous c-Myc was present in the BS cells, K48-linked ubiquitylated forms of c-Myc were enhanced in A-15 cells, irrespective of the presence or absence of stalled replication forks. However depletion of Fbw7 in A-15 cells by the corresponding siRNA (as also validated by the stabilization of c-Myc) led to a loss of c-Myc ubiquiylation in A-15 cells, indicating that the presence of BLM enhanced Fbw7-mediated c-Myc degradation *in vivo*.

Publication

Original peer-reviewed article

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Understanding the regulation of DNA replication

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We are studying DNA replication and our objective is to understand the regulation of DNA replication and cell-cyclemachinery during normal and stressed conditions.

DNA replication is a vital process of life and must be completed precisely each time a cell divides. When mammalian cells experience DNA damage, they activate protective mechanisms to stall the progression of cell cycle and DNA replication. We want to understand the regulation of replication machinery during stress. Wewant to identify the mechanism that is responsible for inhibition of essential replication proteins during stress. We want to understand how replication is regulated by ubiquitination under normal and stressed conditions. We want to understand the cellular response to aberrations in replication complexes. The objective is to identify yet unknown checkpoint pathways that monitor the replication machinery. Summing up, we are attempting to unravel the protective regulatory control of mammalian cells, failure of which is likely to lead to genomic instability.

Identification of substrate recognition subunit that targets Mcm10 post-UV irradiation

After identifying that Cul4-Roc1-DDB1 ubiquitin ligase mediates Mcm10 downregulation post UV-irradiation, we wanted to identify the substrate recognition subunit that targets Mcm10 and therefore we depleted the known DDB1 interacting proteins by RNAi and assayed the effect on UV-triggered Mcm10 degradation. We observed that depletion of a DDB1 interactingprotein calledVprBP (HIV Vpr-binding protein) stabilized Mcm10 (Figure.1a). We observed HA-tagged Mcm10 in anti-VprBPimmunoprecipitate, confirming an association between Mcm10 and VprBP (Figure.1b). Using purified His_6-tagged Mcm10 and GST-tagged VprBP we demonstrated that Mcm10 directly binds with VprBP, providing strong evidence that VprBP serves as a substrate recognition subunit targeting Mcm10 to CRL4 ubiquitin ligase. Using deletion mutants we concluded that VprBP binds to the CTD domain of Mcm10, causing its degradation. Using a cell free ubiquitination system we demonstrated that VprBP-associated complex ubiquitinates Mcm10 in vitro(Figure.1c).

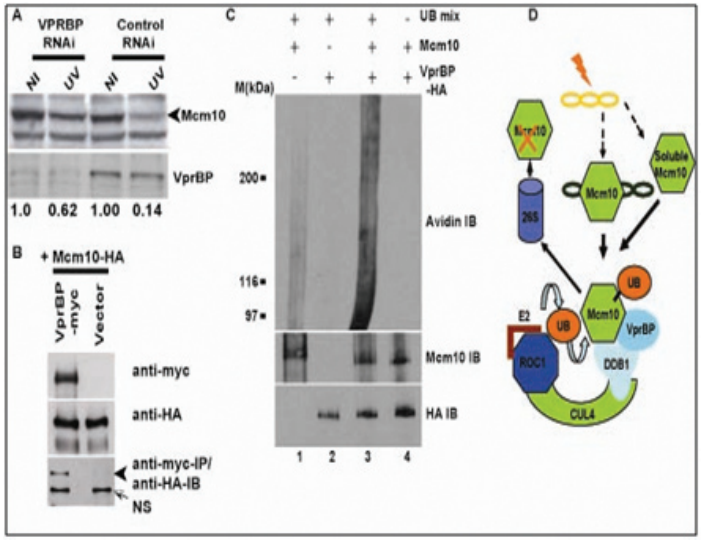

Figure 1. *Levels of Mcm10 protein in non-irradiated (NI) and UV-irradiated (UV) cells after depletion of VprBP. (B) HA-tagged Mcm10 co-immunoprecipitates with myc-tagged VprBP (C) VprBP-associated complex ubiquitinates Mcm10 in vitro.(D) A model depicts that CRL4-DDB1 ubiquitin ligase mediates the UV-triggered proteolysis of Mcm10.*

Regulation of Mcm10 during the M-phase

Since Mcm10 is essential for replication initiation and elongation, it is proteolyzed naturally during the M-phase to ensure a single round of replication. We investigated if Cul4-based E3 ligases are involved in the natural degradation of Mcm10. As Mcm10 levels are low in M-phase, we hypothesized that if Cul4-based E3 ligases is involved in the cell-cycle regulation of Mcm10 levels, their depletion would result in enhanced levels of Mcm10 during the M-phase. The siRNA duplexes designed to target *CUL4A+B* significantly reduced the mRNA levels (Figure. 2b). After the release from nocodazole, we observed a significant increase in the levels of Mcm10 in *CUL4A+B*siRNA transfected cells(Figure. 2a). Thus, our study provides evidence that Cul4-based E3 ligases are required for the cell-cycle regulation of Mcm10 levels.

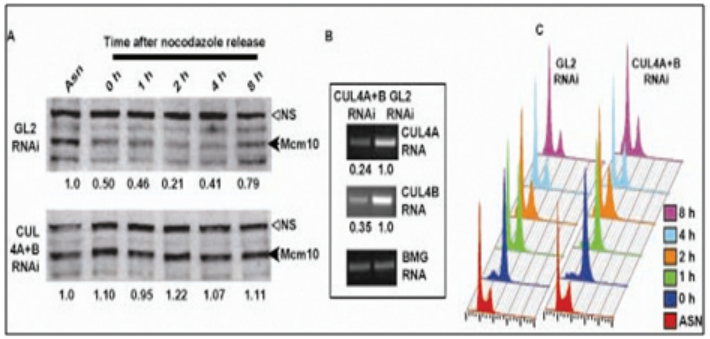

Figure2. *M-phase proteolysis of Mcm10 is dependent on Cul4. (A) CUL4A+B or GL2siRNA transfected cells were released from nocodazole block and then harvested along with asynchronous cells (Asn) at the indicated time-points for analysis of Mcm10 protein. (B& C) The decrease in CUL4A and CUL4B mRNA levels and cell cycle profile after siRNA depletion.*

Regulation of replication and cell-cyclemachinery by E3 ubiquitin ligases

We wanted to identify the E3 ligases that regulate the stability of replication factors. We transfected HeLa cells with either control *GL2* or specific siRNA against various E3 ligase components such as ring finger proteins, scaffold proteins, adaptor proteins and newly identified substrate recognition subunits. After the siRNA transfection, cells were arrested with nocodazole or hydroxyurea to evaluate the stability of replication factors in different cell cycle phases after depletion of a specific E3 ligase. The asynchronous and blocked cells were lysed and immunoblotted to determine the levels of 23 different replication and cellcycle factors. Stabilization in the levels of Cyclin B was observed after the depletion of cell cycle E3 ligase (CC E3 ligase)(Figure. 3). Since Cyclin B protein decreases immediately after anaphase (NOC+2), our data indicates that CC E3 ligaseregulates the natural turnover of Cyclin B. We are now studying the interaction between Cyclin B and CC E3 ligase and trying to identify the role of different E3 ligases like APC in the regulation of Cyclin B.

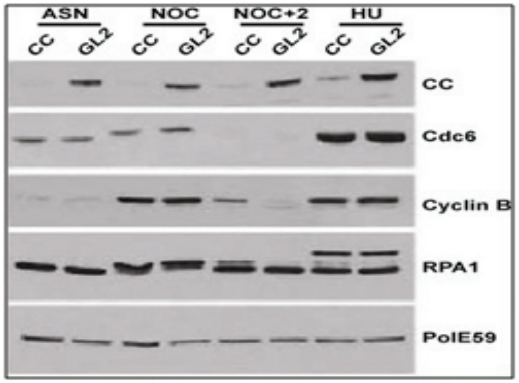

Figure 3. *Effect of siRNA mediated depletion of CC E3 ligase on the stability of different replication and cell-cycle proteins. ASN, NOC and HU refer to asynchronous, nocodazole and hydroxyurea arrested cells respectively. Immunoblot shows protein levels of Cdc6, Cyclin B, RPA1 and DNA polymerase epsilon p59 in control cells (GL2) or after depletion of CC E3 ligase.*

Understanding the cellular response to aberrations in replication complexes

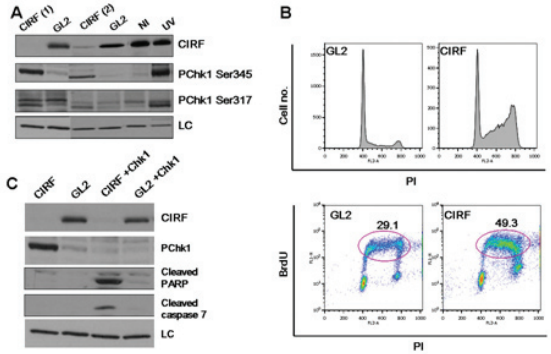

Figure 4. *Activation of checkpoint pathway after silencing of replication protein CIRF.(A) After depletion of CIRF by two different siRNA duplexes, the phosphorylation of Chk1 (P-Chk1)was assayed. Non-irradiated (NI) and UV-irradiated (UV) cells served as controls for activation of Chk1. (B) BrdU incorporation was analyzed by flow cytometry after depletion of CIRF. Top panels depict the incorporation of propidium iodide while the bottom panels show BrdU incorporation. (C) Chk1 and CIRF were depleted alone or in combination and the cleavage of PARP was analyzed.*

In order to identify the protective checkpoint mechanisms monitoring the replication apparatus; the checkpoint signaling was studied after depletion of individual replication proteins with small inhibitory RNA in HeLa cells. We observed that depletion of a replication factor, checkpoint inducing replication factor (CIRF),lead to a progressive S and G2/M block with an increase in the inhibitory phosphorylation of CDK1, a marker for a G2/M block (Figure. 4b). We co-depleted CIRF with different checkpoint factors in order to identify the factors involved in regulation of the cell cycle block observed after the loss of CIRF. The results demonstrate the role of Chk1 mediated response on sensing low levels of CIRF (Figure. 4a). Absence of Chk1 alleviates the S phase accumulation as revealed by the decrease in BrdU incorporation on co-depleting Chk1 with CIRF. Though the checkpoint response was suppressed after the loss of Chk1, it lead to massive genomic aberrations, DNA damage and finally apoptosis, indicated by increased levels of apoptotic marker cleaved PARP (Figure. 4c). Thus, we conclude that the state of replication complexes is monitored by protective checkpoint mechanisms in mammalian cells.

Role of tumor suppressor p53 in stress response

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In response to various intracellular and extracellular stresses p53 is rapidly stabilized and activated thereby inducing cell cycle arrest, apoptosis or senescence depending upon the extent of cellular damage. Recent reports suggest that p53 plays an important role in regulating cellular metabolism. Our interest is to comprehend the role played by p53 in metabolic stress response. We would like to unravel the plethora of genes regulated by p53 in response to metabolic stress. Microarray, RNAi, and functional assay based approaches would be used to identify and characterize novel p53 target genes.

In addition we would like to identify other proteins with which p53 interacts to initiate the stress response process using protein-protein interaction based approaches like yeast two hybrid and immunoprecipitation/mass spectroscopy. Thus the overall goal is to understand what and how the final cellular outcome of survival, senescence or apoptosis is achieved in response to metabolic stress.

1. Characterization of PGC-1 as a p53 co-activator

PGC-1 (**P**PARγ **c**oactivator**-1**) coactivator plays a key role in transcriptional regulation of cellular metabolism. In recent times p53 has been reported to play a role in regulating cellular metabolism. Our preliminary data show that PGC-1 can serve as a p53 coactivator. We propose to investigate in depth the role of PGC-1 in p53-mediated transactivation. We also plan to investigate the effect of this interaction on p53 function under metabolic stress condition.

2. Identification and characterization of novel p53 target genes involved in metabolic stress response

Here we propose to carry out microarray based experiments using cells subjected to glucose starvation induced metabolic stress to identify novel p53 target genes involved in metabolic stress response.

3. Identification and characterization of novel p53 interacting partners involved in stress response

We plan to use a proteomics based approach to identify novel p53 interacting proteins. For this we intend to generate a recombinant adenovirus expressing HA and Flag tagged p53.

1. Characterization of PGC-1α as a p53 co-activator

Previously we had observed that upon glucose starvation both p53 and PGC-1 are upregulated and PGC-1 functions as a p53

coactivator resulting in induction of pro-arrest and metabolic target genes of p53. However, prolonged starvation results in PGC-1α degradation concomitant with induction of apoptosis. Further experiments suggested that the PGC-1α protein is targeted for degradation during prolonged exposure to metabolic stress due to activation of an ubiquitin-proteasome pathway. To identify the ubiquitin ligase responsible for stress induced PGC-1α degradation we carried out a yeast two hybrid screen. One of the clones obtained from the screen was RNF2. RNF2 is a Polycomb group protein that possesses E3 ubiquitin ligase activity. To test if RNF2 interacts with PGC-1α, we transfected HepG2 cells with Flag-tagged RNF2 construct followed by infection with Ad-PGC-1α and immunoprecipitation was carried out using PGC-1α antibody. Our results show that RNF2 interacts with PGC-1α. Similar results were obtained by reverse co-IP experiment using Flag antibody. To study the PGC-1α-RNF2 interaction under physiological conditions we carried out immunoprecipitations over the time course of starvation using PGC-1α antibody. Here we found that only at extended periods of starvation (48 and 72 hours) RNF2 co-immunoprecipitated with PGC-1α. Similar results were obtained in the reverse co-IP experiment using anti-RNF2 antibody. To investigate the effect of RNF2 on PGC-1α protein we looked for PGC-1α protein levels over the duration of starvation period in presence or absence of RNF2. For this purpose we stably transfected HepG2 cells (pooled puromycin-resistant population) with control (luciferase) or RNF2 shRNA. Transfection of RNF2 shRNA resulted in the suppression of endogenous RNF2 but not so in control shRNA transfected cells. In HepG2 control cells PGC-1α was induced at 24 hours but reduced drastically at extended periods of starvation. On the other hand, in HepG2 RNF2 knockdown cells PGC-1α was induced at 24 hours and remained steady throughout the rest of time course. These results indicate that RNF2 targets PGC-1α for degradation at extended periods of starvation. To examine the effect of RNF2 on PGC-1α-mediated cell fate decision upon metabolic stress, we subjected the HepG2 control and RNF2 knockdown cells to glucose starvation and carried out cell cycle analysis. Here we found that in HepG2 RNF2 knockdown cells where PGC-1α degradation was abrogated; there was no significant apoptosis unlike control cells at 72 hours of starvation. We also looked at the transcript levels of *p21*, *TIGAR*, *Bax*, *PGC-1α* and *RNF2* over the time course of starvation in these cells. In HepG2 control cells *p21* and *TIGAR* levels were induced at early time points up to 36 hours but subsequently their levels decreased rapidly but in RNF2 knockdown cells *p21* and *TIGAR* levels were induced at early time points and remain steady over the rest of the time course of starvation. *Bax* levels in HepG2 control cells were induced only at extended periods of starvation (48 and 72 hours) but in RNF2 knockdown cells *Bax* levels were not induced at any period of starvation. *PGC-1α* levels were induced at 24 hours of starvation and remained steady over the rest of the time course of starvation in both HepG2 control and RNF2 knockdown cells. *RNF2* levels remain unchanged over the course of starvation in HepG2 control cells while its levels in RNF2 knockdown cells were substantially reduced. To exclude off-target effects of shRNA, two other RNF2 shRNAs were used, and similar results were obtained. In conclusion, RNF2 targets PGC-1α for degradation at extended periods of starvation leading to induction of apoptosis. RNAi-mediated knockdown of RNF2 expression prevents PGC-1α degradation at extended periods of starvation which protects the cells from undergoing apoptosis.

2. Identification and characterization of novel p53 target genes involved in metabolic stress response

Based on the results of DNA micro array and RT-qPCR confirmation, *Caspase-10* was selected as one of the genes up regulated by p53 for further study. *Caspase-10* is a member of the cysteine-aspartyl protease (caspase) family. Caspase-10 is involved in the intrinsic pathway of apoptosis. Death effector domain of Caspase-10 interacts with death effector domain of FADD (Fas associated death receptor) to initiate death receptor signaling. During *in silico* promoter analysis of *caspase 10*, one putative p53 binding site was found 2.2kb upstream region of the Transcription start site. This region was then cloned in a Luciferase reporter vector and p53 transactivation function at this site was confirmed. *In vivo* binding of p53 to this site was also confirmed by ChIP analysis under glucose starvation conditions. During the promoter analysis of Caspase-10, a c-Myc binding site was found in the vicinity of

p53 binding site. By carrying out luciferase reporter assay c-Myc was found to act as a repressor and opposes the effect of p53. It has been previously reported that Myc undergoes proteasomal degradation upon glucose starvation. Thus we believe that there is competition between p53 and c-Myc for occupancy of *Caspase-10* promoter.

3. Identification and characterization of novel p53 interacting partners involved in stress response

Recombinant adenovirus is one of the most efficient exogenous overexpression systems. To construct a replication deficient recombinant adenovirus which expresses p53 tagged with HA and FLAG epitope we plan to follow the strategy as described previously (He et al., 1998). Briefly, the gene of interest (in this case p53 tagged with HA and FLAG epitope) has been cloned into a shuttle vector called pAdTrack-CMV. p53 expression from this vector was checked by transfection of this construct into a p53 null cell line followed by western blotting. This construct was then linearised with PmeI and along with the adenoviral vector pAdEasy-1 was electroporated into *E.coli* strain BJ5183 wherein homologous recombination is highly efficient. The recombinant DNA was isolated from individual colonies, linearised with PacI and transfected into packaging cell line 293 to get adenoviral particles. Ad-p53 virus was amplified. p53 expression from this adenovirus was confirmed by western blotting. This virus would be used to infect p53 null cell lines followed by tandem immunoprecipitations using HA and FLAG antibodies and mass spectroscopy to identify the p53 interacting proteins.

Publication

Original peer reviewed article

1. Sen N, Satija YK, Das S (2011) PGC-1α, a key modulator of p53, promotes cell survival upon metabolic stress. **Mol. Cell 44**: 621-634.

ANCILLIARY ACTIVITIES

Production of transgenic animals and development of new transgenic technologies

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Suresh B Gokhale, BAIF,Pune

Theme of the research is to produce transgenic animals for using them as a system for the study of functional genomics and mammalian development.

To develop transgenic animal models using genes relevant to human health and diseases as well as to use this technology for making large animals expressing therapeutic products in their milk for drastically cutting down the cost of such therapeutics to make them easily available. The other objective is to develop techniques for making transgenic mice expressing ShRNA against specific gene with an objective to knock down its function in vivo with an objective to provide an alternative for difficult to do Knock-out technique for studies of functional genomics.

Studies with SG2NA

SG2NA is a cell cycle regulated WD-40 repeat protein with potential scaffolding function. It is conserved in evolution from lower eukaryotes to human. Dr. Goswami's laboratory at JNU has for the first time demonstrated that it has multiple variants arising out of differential splicing. Amongst various isoforms, at least one that is 87 kDa is present in brain but not in heart, liver and muscle. Also, one isoforms (35 kDa) is devoid of carboxyl terminal WD-40 repeat and might have dominant negative functions. Studies done in his laboratory suggests that isoforms of SG2NA are present in various subcellular membranes and interacts with various proteins. The most ubiquitous isoform that is 78 kDa interacts with Parkinson associate protein DJ-1 and SG2NA-DJ-1 interaction protects cells from redox stress. To understand the role of SG2NA in mammalian biology, we planned overexpressing 78 and 35 kDa variants in mice under CMV promoter. The transgenic mice were made using testicular transgenesis established at NII. Fore founder mice were given to JNU for the propagation of transgenic line. The F1 generation mice overexpressed 35 kDa SG2NA . Further propagation is being carried out. The work to overexpress 87 kDa SG2NA in brain only (under a brain specific promoter) is underway.

SMAR1 mediated susceptibility to infection by *M. tuberculosis*

Studies involving animal models and human infections have implicated T cells as an essential module of immune response to Mycobacterium tuberculosis. Infection with M. tuberculosis invokes an inflammatory host response associated with increased secretion of IFN-γ, symptomatic of a TH1 type response. In Mycobacterium tuberculosis infected SMAR1 transgenic mice, the number of fields showing acid fast bacilli are 2 fold more than the control infected mice which shows that SMAR1 transgenic mice are more susceptible than the control mice. Moreover, histological studies performed on the lung tissue sections obtained from both set of mice also demonstrated extensive lymphocyte infiltration in SMAR1 Tg mice. Thereafter, cytokine analysis was performed in target organs of these mice; it revealed that SMAR1 transgenic had lower production of IFNg compared to control in response to infection. These data are indicative of a defective TH1 induction in SMAR1 transgenic mice, making them more predisposed to disease where Th1 type immune response is crucial.

Attempts to generate transgenic buffalo expressing therapeutic protein in the milk

Analysis based on bioinformatics and available literature showed that functional β-Casein promoter of cow along with its regulatory elements is of approx 3.7 - 4 kb in length, which spans up to Exon 2 of β-Casein gene. β-Casein promoter along with the regulatory elements from the buffalo genome was isolated using long PCR. Isolated βCasein promoter was cloned upstream of pIRES2-EGFP (pBucsn2-IRES2-EGFP) for this promoter driven expression of EGFP in the udder cells.

***In Vitro* efficacy of βCasein Promoter**

pBucsn2-IRES2-EGFP construct was digested with PstI and SfoI enzyme to generate fragment for characterization. This fragment contain functional cassette of Buffalo β-Casein promoter along with EGFP as reporter gene followed by SV40 PolyA signal. This construct was transfected into MCF7 cells (from human breast cancer cell line) using electroporator to check the promoter efficiency of buffalo β casein promoter *in vitro*. This construct successfully expressed EGFP in these cells in vitro. For the first time, we have isolated, sequenced and characterized buffalo beta casein promoter displaying its activity. This is an important achievement for the country and we have applied for patent in India and U.S.A.

Generation of transgenic (Tg) mice with β casein promoter driven EGFP construct to test *in vivo* efficacy of the promoter.

After in vitro confirmation of the promoter activity, transgenic mice carrying this functional cassette was generated using testicular transgenesis method developed in our laboratory. Screening for the presence of transgene in Tg animal was done by PCR and Slot Blot analysis. On day 7 of lactation, mammary gland of F1 progeny female mice, which were positive for the transgene, were surgically exposed and observed under UV using stereozoom microscope. This construct successfully expressed EGFP in the cells of mammary glands *in vivo.* The mammary gland of Wild Type (WT) mice did not show any expression of EGFP. This proved the *in vivo* bioactivity of the isolated buffalo β casein promoter. Additionally, immuno Histochemical analysis was performed on tissue sections of the mammary gland (Tg mice and WT Mice) using Antibody against EGFP. EGFP Expression was found to be present in the udder cells of transgenic mice.

Gene electroporation in buffalo testis:

We have initiated electroporation of the transgenes in the testis of buffalo (at BAIF, Pune research station and farm) for insertion of the desired genes in buffalo genome via germ cells. Results are awaited.

Attempts to generate transgenic monkeys

Preliminary studies have not been successful in generating live transgenic offspring of monkey. However, slot blot analysis using ejaculated sperm have indicated our ability to introduce

the transgene into sperm. Establishing methods for selection of transgenic sperm seems to be necessary before extending this work further.

AIF mediated circumvention of T cell defects

In another study, from laboratory of Satyajit Rath, NII it was seen that in harlequin (Hq) strain of mice, naive T cell numbers were low in circulation. The CD4-CD8- 'double-negative' (DN) T cells lineage in thymocyte stage was less in thymus. These mice are hypomorphic for Apoptosis-inducing factor (AIF). Apoptosis-inducing factor is a mitochondrial flavoprotein with multiple roles, both pro- as well as anti-apoptotic, in mitochondrial functions. It is well known that apoptosis decisions via intrinsic and extrinsic pathways are vital during T lymphocyte development. Hence, a study was undertaken collaboratively to evaluate whether replenishment of AIF can circumvent the T cell developmental defects in Hq mice. Transgenic mice over-expressing AIF were generated using testicular transgenesis established at NII. Transgenic mice were cross bred with Hq mice. Interestingly, as hypothesized by Dr.Satyajit Rath, T cell developmental defects were rescued in progeny with Hq background having AIF over-expression.

Transgenic mice to study dendritic cell development

This study is initiated from the laboratory of Prafulla Tailor to generate transgenic animals for intervention of different signal transduction pathways involved in dendritic cell subtype development. This will allow to asses role of these molecules in dendritic cell subtype development and function.

Publications

#1. Datta S, Alam MP, Majumdar SS, Mehta AK, Maiti S, Wadhwa N,Brahmachari V (2011) Nucleosomal occupancy and CGG repeat expansion: A comparative analysis of triplet repeat region from mouse and human fragile X mental retardation gene1. **Chromosome Res 19:**445-455.

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In Press last year, since published.

*In Press on 31st March,2012.

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PATENTS & TECHNOLOGY TRANSFER

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TECHNOLOGY TRANSFER

Technology for Composition useful for the treatment of inflammatory disease or disorders transferred to M/s Extended Delivery Pharmaceuticals, USA.

RTI ANNUAL RETURN INFORMATION SYSTEM (2011-2012)

ANNUAL RETURN FORM

Ministry /Department /Organization: Department of Bio-Technology (National Institute of Immunology), New Delhi-110067

Year 2011-2012 (upto March 2012)

Insert Mode (New Return)

		Progress in 2010-11			
	Opening Balance as on 01/04/2011	Received during the year (including cases transferred to other Public Authority)	No. of cases transferred to other Public Authority	Decisions where request/appeals rejects/appeals rejected	Decision where requests/appeals accepted
Request	109	29	0	0	29
First Appeals			0	0	

No. of Cases where disciplinary action taken against any Officer	0

No. of CAPIOs designated	No. of CPIO designated	No. of AAs designated
1	0	1

No. of times various provisions were invoked while rejecting request													
Relevant section of RTI Act 2005													
Section 8 (1)										Sections			
a	b	c	d	e	f	g	h	i	j	9	11	24	Others
0	0	0	0	0	0	0	0	0	0	0	0	0	0

Amount of Charges Collected (in Rs.)				
Registration Fee Amount		**Additional Fee & Any other charges**	**Penalties Amount**	
Rs. 280/-		**812/-**	**0**	

Last date of Uploading the Pro-active Disclosures on the website of PA	**(Format dd/mm/yyyy)**

Name of the person who is entering/updating data	**Dr. Sagar Sengupta**
Designation of the person who is entering/updating data	**CAPIO**

National Institute of Immunology

New Delhi

Report on Monthly Disposal of Cases

2011-2012

Year	Month	Opening Balance	Receipt	Disposal	Closing Balance	Cumulative Disposal
2011	April	109	1	0	110	110
2011	May	110	3	3	113	112
2011	June	113	1	1	114	113
2011	July	114	3	1	117	114
2011	August	117	2	3	119	117
2011	September	119	5	4	124	121
2011	October	124	1	2	125	123
2011	November	125	4	3	129	126
2011	December	129	6	5	136	131
2012	January	135	1	4	136	135
2012	February	136	0	1	136	136
2012	March	136	2	1	138	137

Ph.D Degrees Awarded to NII Scholars

Twelve scholars of the Institute were awarded the degree of Doctor of Philosophy by the Jawaharlal Nehru University on the completion of their work for the said degree during the under report. The details are as under:

Sl.	Student's Name	Topic of Research	Guide
1.	Ms. Arush Chabra	Investigation of downstream modification enzymes involved in the assembly of polyketides and non-ribosomal peptides	Dr. Rajesh S Gokhale
2.	Mr. C.K. Anish	Formulation and Evaluation of polymer particle based delivery system for polysaccharide and protein	Dr. A.K. Panda
3.	Ms. Neha Singh	Regulation and role of Cell Cycle Proteins in Neuronal Apoptosis	Dr. Pushkar Sharma
4.	Ms. Nitika Kaushal	Molecular analysis of immune response to Streptoccus pneumonia capsular polysaccharide	Dr. Devinder Sehgal
5.	Ms. Sakshi Gupta	Deciphering the role of Supressors of Cytokine Signalling (SOCS) in Regulation of Neuronal Cell Signalling	Dr. Avadhesha Surolia
6.	Ms. Shazia Khan	Biochemical and Functional investigations of Mycobacterium tuberculosis Serine/Threonine Protein Kinases PknA and PknB	Dr. Vinay K. Nandicoori
7.	Ms. Suruchika Chandra	Molecular determinants that modulate the expression and function of c-MYC protoocogne by tumor supressor BLM helicase	Dr. Sagar Sengupta
8.	Mr. Tarique Khan	Deciphering the Mechanisms of Primary Antibody Pluripotency	Dr. Dinakar M. Salunke
9.	Mr. Hidesh Banerjee	Role of AIF in T cell development and function	Dr. Satyajit Rath
10.	Mr. Indrashis Bhattarcharya	Evaluation of developmentally regulated gene expression by rat sertoli cells and its association with spermatogonial stem cell differentiation	Dr. Subeer S. Majumdar
11.	Mr. Ruchir Rastogi	Differential role of Rab 5 isoforms in endocytosis in Leishmania	Dr. Amitabha Mukhopadhyay
12.	Mr. T. Krishnamurthy	Studies in T Cell signal transduction pathways	Dr. Satyajit Rath

LECTURES DELIVERED ON INVITATION/PAPERS PRESENTED

TITLE	SPEAKER	DATE AND TIME
Alteration of metabolic pathways driven by pro-apoptotic Bax and Bak in early thymopoiesis initiates the stage specific T-cell Leukemia	Dr. Subhrajit Biswas, Department of Medicine Vanderbilt University Medical Center,USA	18th April, 2011. Monday 11:00 A.M.
Treatment of Type 1 Diabetes by epigenetic modulation of the genome	S. Jayaraman, Ph.D. Research Associate Professor Institute for Personalized Respiratory Medicine Dept. of Medicine The University of Illinois at Chicago,USA	23rd May, 2011. Monday 11:00 A.M.
Diagnostic and Therapeutic Implications of EPH Receptors in Breast Cancer"	Dr. Raj Kandpal Laboratory of Molecular Biology and Functional Genomics Western University of Health Sciences Pomona, CA 91766, USA	5th July, 2011. Tuesday 11:30 A.M.
Strategies to modify leukocyte-endothelium interactions: Gene silencing, metabolic inhibitors, and systems glycobiology	Prof. Sriram Neelamegham Chemical and Biological Engineering University at Buffalo State University of New York (SUNY) Buffalo, NY, USA	4th August, 2011. Thursday 3:00 P.M.
Tumour suppressor mechanisms in the control of chromosome stability	Prof. Ashok Venkitaraman, University of Cambridge, UK	16th September, 2011. Friday. 11:00 A.M.
A well orchestrated cellular network protects the proteome during stress	Swasti Raychaudhuri Cellular Biochemistry, Max Planck Institute for Biochemistry, Martinsried, Germany	22nd September, 2011. Thursday 3:00 P.M.
Capturing functional motions of membrane transporters using MD simulations	Dr. Saher Afshan Shaikh, University of Illinois at Urbana-Champaign	25th October,2011. Tuesday 3:00 P.M.

TITLE	SPEAKER	DATE AND TIME
Cytokine regulated novel tumor suppressors: a GRIM story	Prof. Dhan Kalvakolanu Professor of Microbiology & Immunology, University of Maryland, School of Medicine Baltimore, USA	1st November, 2011. Tuesday 4:00 P.M.
Organelles Biogenesis in Apicomplexans	Prof. Dominique Soldati-Favre, Department of Microbiology and Molecular Medicine, University of Geneva, Switzerland	2nd November, 2011. Wednesday 3:30 P.M.
Managing Innovations	Dr. Ravinder Jain Sprott School of Business and Canada-India Centre Carleton University Canada	3rd November, 2011. Thursday 4:00 P.M.
Salmonella: from diarrhea to typhoid fever	Dr. B. Brett Finlay, OC, OBC, FRSC, FCAHS UBC Peter Wall Distinguished Professor Michael Smith Laboratories, University of British Columbia, Vancouver, B.C., Canada	17th November, 2011. Thursday 4:00 P.M.
Regulation of Histon Occupancy in Coding Regions during Pol II Elongation	Dr. Chhabi Govind Biological Sciences Oakland University USA	14th December, 2011 Wednesday 4:00 P.M
Interweaving microRNA, p53 and innate immunity networks in human cancer	Dr. Curtis C. Harris, Chief Laboratory of Human Carcinogenesis National Cancer Institute, NIH USA	20th December, 2011 Tuesday 4:00 P.M.
*Regulation of Autophagy and its Implications in Neurodegenerative Diseases	Dr. Sovan Sarkar Whitehead Institute for Biomedical Research MIT,USA; Hughes Hall, Univ. of Cambridge,UK	20th December, 2011. Tuesday 2:30 P.M.
Regulation of synaptic vesicle transport	Dr. Sandhya P. Koushika National Centre for Biological Sciences (NCBS), Bangalore	29th December, 2011. Thursday 3:00 P.M.
Immunology taught by viruses	Prof. Rolf Zinkernagel, Noble Laureate, University of Zurich, Zurich	5th January, 2012. Thursday 3:30 P.M.

TITLE	SPEAKER	DATE AND TIME
Chemistry, Biology and the Interface	Dr. Jeet Kalia National Institute of Health, Bethesda, MD, USA.	23rd January, 2012. Monday 4:00 P.M
Age-dependent effects of AIRE on the susceptibility to experimental autoimmune myasthenia gravis	Prof. Miriam Souroujon Department of Natural Science, The Open University of Israel, Raanana, Israel	1st February, 2012. Wednesday 11:00 A.M.
T cell based therapy of autoimmune diseases	Dr S.P. Roychaudhary, Prof. & Head, Rheumatology University of California, USA	6th February, 2012 . Monday 4:00 P.M
Perpetual crisis: The way to dusty death	Dr. Sorab Dalal Advanced Centre for Treatment, Research & Education in Cancer, Tata Memorial Centre, Kharghar, Navi Mumbai	7th February, 2012. Wednesday 11:00 A.M.
DNA damage signaling and DNA repair	Prof. Junjie Chen, Chairman, Department of Experimental Radiation Oncology, MD Anderson Cancer Center, USA	7th February, 2012. Wednesday 4.00 P.M.
Horror autotoxicus: T cell receptor recognition of self and foreign antigens".	Dr Dhruv K. Sethi, Dana-Farber Cancer Institute, Harvard Medical School	9th February, 2012. Thursday 4:00 P.M.
Connecting DNA damage to the initiation of Cell Cycle Checkpoints	Peter M. Burgers, Brenneke Professor of Biologicals Chemistry, Washington University in St. Louis, USA	10th February, 2012. Saturday 11:00 A.M.
The role of hnRNP proteins in regulating DNA double strand break end-resection	Dr Grant Stewart, Lister Fellow, School of Cancer Sciences, University of Birmingham, Vincent Drive, Edgbaston, Birmingham, United Kingdom	10th February, 2012. Saturday 9:30 A.M.

TITLE	SPEAKER	DATE AND TIME
Mechanobiology of cell-substrate interactions	Dr. Michael Sheetz Director, Mechanobiology Institute & Distinguished Professor of the Department of Biological Sciences, National University of Singapore Professor of the Department of Biological Sciences at Columbia University, USA	14th February, 2012. Tuesday 11:15A.M.
Silencing and antisilencing in Salmonella pathogenesis	Dr. Linda Kenney Principal Investigator, Mechanobiology Institute, Singapore Professor of Microbiology & Immunology, Adjunct Professor of Bioengineering, University of Illinois- Chicago, USA	14th February, 2012. Tuesday 11:45A.M .
Cellular Geometry and Genome Regulation	G.V.Shivashankar Deputy Director Mechanobiology Institute, Singapore Associate Professor Department of Biological Sciences National University of Singapore	14th February, 2012. Tuesday 12:15 P.M.
Molecular Architecture of the Exocyst Complex and its Function in Exocytosis	Dr Mary Munson, Associate Professor, Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester MA USA	1st March, 2012. Thursday 11:30 A.M
Deep sequencing methods to investigate transcriptomes and proteomes	Dr Ramesh Vaidyanathan, Senior Staff Manager, Scientific Research Epicentre (an Illumina Company), Madison, Wisconsin, USA	12th March, 2012. Monday 4:00 P.M.
Vaccine Design: Innovative Approaches and Novel Strategies	Dr. Seyed M. Faisal, Research Associate, College of Veterinary Medicine, Cornell University, USA	12th March, 2012. Monday 10:00A.M.

CONFERENCES/SYMPOSIA/WORKSHOPS ORGANIZED

Second International Symposium on Cancer Biology (ISCB), November 14–16, 2011, at the National Institute of Immunology, New Delhi, India

Participants of the Second International Symposium on Cancer Biology (ISCB), November 14–16, 2011, at the National Institute of Immunology, New Delhi, India.

The second International Symposium on Cancer Biology (ISCB) was organized from November 14-16, 2011 by National Institute of Immunology (NII), New Delhi, India, in collaboration with Queen's University, Belfast, UK. The symposium also coincided with the Silver Jubilee celebration of National Institute of Immunology. The symposium was mainly focused on the sharing of knowledge and dissemination of research data among renowned cancer biologists, clinicians and immunologists across the world. The symposium addressed the multi-faceted issue of cancer with particular emphasis on the discovery of novel tumor antigens, tumor immunity, immunotherapeutic strategies, drug-resistance mechanisms and clinical trials. The symposium was aimed at providing a common platform to facilitate the interaction of scientific elite of today and tomorrow to promote the exchange of research and ideas for future benefits in the field of cancer biology. The symposium had active participation from 200 young researchers, postdoctoral fellows and clinicians. The symposium was structured in six scientific sessions: Immunity, chemotherapy and cancer, cell cycle/ checkpoints and cancer, cancer immunotherapy, cancer biomarkers and bioinformatics, cancer vaccines and translational research in cancer.

Biotechnology and Biological Sciences Research Council (BBSRC,UK)-Department of Biotechnology (DBT, India) Livestock Health and Disease Workshop

Participants of Biotechnology and Biological Sciences Research Council (BBSRC,UK)-Department of Biotechnology (DBT, India) Livestock Health and Disease workshop held at National Institute of Immunology, New Delhi during 6-8 February 2012 , interacting with the Director, Dr. Chandrima Shaha, National Institute of Immunology, New Delhi, India.

A joint Biotechnology and Biological Sciences Research Council (BBSRC,UK)-Department of Biotechnology (DBT, India) Livestock Health and Disease workshop supported by Science and Innovation Network (SIN) and Research Councils UK (RCUK), India was held in New Delhi during 6-8 February 2012, hosted by the National Institute of Immunology. This workshop brought together Indian and UK researchers with interest and activities in this area. The scope of the workshop was broad, covering both BBSRC's and DBT's research interests in livestock health including biological aspects of key infectious animal diseases, underpinning control measures and generic technologies.

DISTINCTIONS/HONOURS/FELLOWSHIPS

Dr Sher Ali received the J.C. Bose National Fellowship from Department of Science and Technology, Government of India. He was elected by VC, JMI, as a visiting Professor, Department of Computer Sciences, Jamia Milia Islamia.

Dr Akhil C Banerjea was the recipient Shakuntala Amir Chand National Prize of ICMR. Dr Banerjea received the National Bioscience Award for Career Development of DBT 2011-2012. He is the fellow of the National Academy of Sciences, Allahabad, UP, & fellow of the Indian National Science Academy. He is the member of the Editorial Boards of several Journals including l AIDS Journals. DBT-National Overseas Fellowship. He received several Full International Scholarships to attend and present work in Conferences in USA, UK, Canada, Japan, South Africa etc. He is the member of several DBT-ICMR task forces on HIV. Thomson Reuters recognized Dr Banerjea as a distinguished expert in his field and was included in the Thompson Pharma KOL experts database.

Dr Soumen Basak is the Intermediate fellow of the Welcome Trust DBT India Alliance, for the year 2010-2015.

Dr J K Batra was elected as a fellow of the Indian Academy of Sciences, Bangalore and the National Academy of Sciences, Allahabad.

Dr Sarika Gupta received the NASI-Platinum Jubilee Young Scientist Award in biological sciences from National Academy of Sciences, Allahabad for the year 2011.

Dr Satish K Gupta received the Tata-Innovation Fellowship of Department of Biotechnology , Govt. of India 2011-2014. Dr. Gupta is the fellow of the Indian National Academy, Delhi, the Indian Academy of Sciences, Bangalore, National Academy of Sciences, Allahabad, National Academy of Medical Sciences (India)and Punjab Academy of Sciences, Patiala. Dr Gupta is the member of Editorial Board of Indian Journal of Medical Research, Journal of Reproductive Immunology, Reproductive Biology and Endocrinology, and Reproductive Medicine and Biology. He is also elected Secretary-General, International Society for Immunology of Reproduction.

Dr Subeer S Majumdar is the recipient of Tata Innovation Fellowship 2011 given by DBT for application oriented innovative research carried in India. Dr Majumdar is awarded National Academy of Science-Reliance Industries Platinum Jubilee Award for application oriented innovations in biological sciences-2011. He received the P. Govindarajalu Gold Medal Oration Award for Reproductive sciences by Society of Reproductive Biology and Cellular Endocrinology 2012

Dr Debasisa Mohanty was elected fellow of Indian Academy of Sciences Banglore, 2012.

Dr Arnab Mukhopadhyay received the Ramalingaswami Fellowship from the Department of Biotechnology, Government of India for the year 2010-2012.

Dr Sagar Sengupta was the recipient of National Bioscience Award for Career Development by Department of Biotechnology, Government of India. He was elected as a member of Guha Research Conference at 51st Annual Meeting of Guha Resaerch Conference held at Khimsar, Rajasthan from 1-5 December 2011. Dr. Sengupta is the member of Editorial Board of IRSN Cell Biology.

Dr Chandrima Shaha received the Ranbaxy Research Award 2010.

Dr Pushkar Sharma was elected fellow of the National Academy of Sciences, India.

Dr Agam P Singh received the Ramalingaswami Fellowship from the Department of Biotechnology, Government of India for the year 2009-2013.

Dr Sudhanshu Vrati was a recipient of Tata Innovation Fellowship of DBT, Government of India. Dr Vrati is the fellow Indian Academy of Sciences, Bangalore, National Academy of Sciences, India.

VISITORS TO THE INSTITUTE

Hon'ble Union Minister of Science & Technology Sh Late Vilas Rao Deshmukh visited the Institute on September 21, 2011. He held discussions with Director and scientists of the Institute.

Sh Late Vilas Rao Deshmukh, Hon'ble Union Minister of Science & Technology, with Prof Avadesha Surolia, NII Director, during his visit to NII.

Sh Late Vilas Rao Deshmukh, Hon'ble Union Minister of Science & Technology, with Prof Avadesha Surolia, NII Director, and Dr Asok Mukhopadhyay, NII Scientist, during his visit to NII.

The Hon'ble Minister of Health, Mr. Michael De Jong Q.C, British Columbia, Canada visited the Institute on November 10, 2011. During this visit he held discussions with Director and scientists of the Institute.

Mr. Michael De Jong Q.C, Hon'ble Minister of Health, British Columbia, Canada with Prof Avadesha Surolia, NII Director, during his visit to the Institute.

Dr Stefan Kibstantinov, Hon'ble Minster of Health, Bulgaria visited the Institute on November 28, 2011. He had a discussion with Director and scientists of the Institute.

Dr Stefan Kibstantinov, Hon'ble Minster of Health, Bulgaria, interacting with Dr Rajni Rani, NII Scientist, during his visit to the Institute.

Noble Laureate Prof Rolf Zinkernagel of University of Zurich, Switzerland, visited the Institute on January 5, 2012. During his visit he held discussions with Director and scientists of the Institute.

Noble Laureate Prof Rolf Zinkernagel of University of Zurich, giving a seminar in the National Institute of Immunology.

SUPPORTING UNITS

SMALL ANIMAL FACILITY

The Small Animal Facility of the Institute is committed to ensure the humane care of animals used in approved research and cater defined strains of mice and rats to the scientific community of the institute.

The propagation of all defined strains is done in a three- tier system i.e., the Foundation Stock (FS), Pedigreed Expansion Stock (PES) and Production Stock (PS). Mice in Foundation Stock consist of pedigreed identified pairs which are reared in restricted area under barrier facility. The animals from this colony provide breeding pairs for the pedigreed expansion stock colony for expansion purpose as well as replace those pairs in Foundation Stock which have completed their breeding life. The animals in production stock consist of randomly mixed animals from Foundation as well as Expansion colonies which are raised in the conventional manner.

The selection of sibling pairs at these three levels is done in such a way that all the descendants of each strain can be traced to a common ancestral breeding pair within seven generations of inbreeding. In addition to the above, yet another kind of mice stock namely Non-Pedigreed Identified Pairs are also raised. These are brother x sister pairs which are bred without maintaining their pedigree records. The progeny thus produced from such mice is used only for the experimentation. Apart from this Mutant animals are bred either by 1. Homozygous mutant (-/-) x homozygous mutant (-/-) 2. Heterozygous mutant (-/+) x homozygous mutant (-/-) 3. Heterozygous mutant (-/+) x heterozygous mutant (-/+) to maintain our mutant colonies.

Defined breeding protocols and careful management and husbandry procedures are followed to ensure the purity of each strain of mice. To maximize genetic purity and uniformity of mice, inbred strains are propagated in such a manner that minimizes the genetic drift and the number of generations which separate breeding stock in production colonies from their ancestors in foundation colonies. A random sample of few retired breeders of Foundation, Expansion and Production stock are monitored with the help of genetic markers to assess their genetic purity. Commonly used inbred strains like BALB/cJ, C57BL/6J, C3H/HeJ, CBA/J, CBA/CaJ, FVB/NJ, SJL/J are evaluated periodically using biochemical markers (Idh-1, Car-2, Es-1, Hbb, Gpi-1, Es-3, Pgm-1, Apoa-1, Trf and Mod-1) using Cellulose Acetate Electrophoresis and/ or informative microsatellite markers. The facility also gets support from various Principal Investigators in the molecular genotyping of transgenic and knockout mice strains to confirm the genetic purity based on presence or absence of the selected gene of interest. Congenic mice strains differing at MHC locus are screened by Principal Investigators using immunological tools to confirm the H-2 haplotypes. The animals are examined routinely by a veterinarian and trained animal care technicians to assure that they are free of contagious diseases.

Health monitoring procedures includes microbiological examination of feed, water, bedding material, stool samples and animal organs from moribund animals. Every six months serum samples from sentinel animals are screened for rodent pathogens such as Sendai virus, Rodent corona virus, Mycoplasma pulmonis, PVM, MHV, MVM, TMEV and MPV using serological based Elisa kits from Charles River Laboratories. For Clinico-pathology surveillance, skin/hair is monitored for presence of ectoparasites. Fecal samples are randomly selected for the presence of endoparasites by sedimentation method. The health quality procedures are implemented to prevent the transmission of infection between cages, which include careful handling of animals, use of sterilized corn cob bedding, autoclaved cages, and acidified autoclaved drinking water. Procedures for barrier facility include personnel to take shower prior to entry and wear sterilized clothing and use of masks. The immuno-compromised mice are maintained in either IVC systems or animal isolators of international standards. Necessary action based on clinical signs is taken by the veterinarian concerning the necropsy/ autopsy of the infected animals. Preventive and recommended schedule of medication is strictly followed to prevent the infection/s. The sick animals are euthanized immediately.

PRIMATE RESEARCH CENTER

The Primate Research Centre of the National Institute of Immunology provides services to various investigators of the Institute. Rhesus monkeys (Macaca mulatta) are bred and maintained in the Primate Research Centre for generation of in house animals of known ages for approved basic, pre-clinical and toxicological research.

Under the breeding program, group mating is done for the production of healthy animals. This helps in providing animals of known age and parentage. We have large open pens which are used for group mating under semi-natural conditions where food and water is provided ad libitum. Infants are weaned at the age of six months after which they are transferred to open semi-natural housing for over-all growth and better development of bones, muscles and coordination. Monkeys are housed in independent cages at around pubertal age. To prevent cross-cage contamination strict procedures are followed. All cages are washed routinely by scrubbing with soap and are painted once a year. Deworming of the colony is done at least once a year. To check outbreak, the routine TB tests are performed because non-human primates are susceptible to this infection. The chest x-ray of animals, doubtful of the infection, is performed using x-ray machine and dark room of the Centre. The sick animals are isolated and treated properly after pathological investigations and veterinary consultation according to international norms. To treat the minor injuries, gastrointestinal disorders and to revive animals during acute cardio-pulmonary crisis, a stock of medicine is maintained at the Centre.

Pellets rich in protein, fat, carbohydrate and vitamin are provided to monkeys ad libitum. In addition to this, bread, germinated gram, vegetables and/or fruits are also given daily. For change of taste, occasional feast like bread with sauce or jaggary coated groundnuts are given. Breast feeding mothers and pregnant females are given calcium and vitamin supplements on bread. Care is taken to provide excess feed to such females. Drinking water is provided to the animals by pipelines behind monkey cages, which are connected to flexible protective hose-pipe at the top of each cage. For drinking water, steel nozzles with Teflon interior are fitted at the tip of these hose pipes for the continuous access.

The attendants are provided with overall, jacket, pajama and foot wears for use during animal handling and cleaning. Use of gloves and mask is mandatory during work. Booster of TT is given once every year. The staff also receives boosters of anti rabies vaccine when required. TB test and chest x-ray of staff and the security personnel are performed occasionally. As a preventive measure, persons having injury are given non-animal work. Every precaution is taken to prevent cross species infection; monkey to human and vice versa. High-grade sanitary norms are followed for cleaning in the monkey rooms and area surrounding the building by using disinfectants and insecticides. To prevent colonization of microbes the sewer channels and tiles of room are routinely cleaned. To make the staff aware of or to remind preventive measures for health safety, occasional meetings are held with the staff and they are mentored very often.

Technical expertise for surgery, immunization, bleeding, biopsy, electro ejaculation and fertility studies is extended in addition to maintaining and providing primates free of microbial pathogens. Major surgeries are performed in the well-equipped operation theatre whereas minor surgeries involving cuts and wounds are performed in the animal prep room adjacent to it. Surgical linen is washed using a washing machine. Autoclaving facility for surgical equipments and accessories is provided within the building. A research laboratory is situated in the centre for the research related to primates and the samples obtained from them. This provides basic services to various investigators involving primary processing of biological samples in the Centre. Remote blood sampling and infusion unit is successfully working at the centre. These catheters are used for continuous or pulsatile administration of hormones and drugs using a set of pump and chrontrol (a time setting device) to the ambulatory animals. This is a great asset for physiological mimicry and pharmaco-kinetic studies where experiments can be performed without causing any stress to the animals.

Clearance of the research proposals by CPCSEA after primary clearance from the Institutional Animal Ethics Committee, comprising of scientists from various fields of expertise and member of CPCSEA is a necessary requirement for conducting research on primates at the Centre. The macaques at this Centre are used for research related to infectious diseases, reproduction, endocrinology, immunology and contraception. The staff of Centre makes sure that all the procedures involved in animal handling are pain-free and involve minimum stress to the animal. Where ever unavoidable, proper medication is given to reduce the pain. Experimental animals are provided with special feed, whenever needed. A constant effort is made to keep the animals in comfortable and stress free environment as per the available guidelines. There are seventeen open enclosures with swings and shelters, some of these are used for rotation of monkeys and some for rehabilitation and or socializing.

OTHER SERVICE UNITS

Establishment, Personnel & General Administration Services

The Division has been providing effective administrative support in terms of manpower employment, liaison, secretarial assistance and infrastructure to meet its goal of coordination of human and material resources. The activities include service matters, policy implementation, preparation and submission of periodic reports to nodal Ministry, dak, foreign visits of scientists for training, conferences, exchange visits etc. The Institute also conducts periodical trainings for its Administrative & Technical Cadre on subjects of relevance to them. The activities are performed through the use of modern gadgets like fax machines, computers, e-mails etc. In addition to the routine jobs, the staff of Administration has also been involved in other activities like maintenance of building, residential complex, swimming pool, guest house, organization of various seminars, workshops, training courses, lectures etc.

Financial & Accounting Services

The Division has been responsible for preparation of annual budget, management of funds utilization, receipt and disbursement of all payments, internal auditing, getting accounts audited by statutory and CAG auditors, sending reports to funding agencies and recovery and remittance of TDS from salary and contractors, filling institutional income tax return, obtaining required exemptions of the Income Tax department, maintaining bank accounts, management of trust for CPF, Gratuity Fund, and recovery and remittance of subscriptions of NPS.

Estate and Transport Services

The Institute has a Guest House to cater to the boarding/lodging requirements of scientists who visit the Institute from within and outside the country. The requirements of the Department of Biotechnology, Government of India, the Jawaharlal Nehru University and sister scientific/research organizations are also met. In addition, the Institute has a residential campus having accommodation for core as well as project-based scientific staff and research scholars, This Group takes care of allotment of accommodation and provision of related basic facilities for the project-based scientific staff, short term and long term trainees etc. The Transport Service Group has the prime responsibility of providing transport service to the Institute's personnel for carrying out various assignments/movements smoothly. It also helps in coordination of transportation needs during the national/ international conferences organized by the Institute.

Stores & Purchase Department

The Stores & Purchase Department of the Institute is responsible for all purchases such as chemicals, consumables, research equipment and instruments, glassware and other items. It acts as lifeline for research activities. Special emphasis is laid on economic and timely procurement of stores and supplies from local as well as international sources. The important function of purchase is overseen by various purchase committees comprising of three or more scientists, Finance & Accounts Officer and Stores & Purchase Officer. The officials of the Stores Department carry out the processing of orders and procurement of materials of different types for the Institute and distribute them to the concerned labs on receipt.

Engineering, Maintenance & Instrumentation Services

The Engineering department of the Institute has been entrusted with all the engineering activities involving maintenance, services and capital works. It has always been the endeavor of the department to provide the best of services with use of the latest/ modern technology; as a result systems are being continuously modernized. Major activities under taken during the reporting year are as follows:

i) Providing New open enclosures for monkeys at terrace level PRC. ii) Work for installation of New Informatory/Direction boards in bilingual in entire campus. iii) Replacement of Air handling Units

and laboratory tables for various laboratories. iv) Renovation of HPC clusters Room. v) Up gradation of BMS system at NII. vi) Duco painting of BSL-3 Facility.vii) Repairing /servicing of 11 KV HT systems. viii) Reactivation of water softening plant.

The department is currently working on the following projects:

i)Renovation of main building toilets. ii) Installation of rain harvesting system. iii) Installation of sewage treatment plant. iv) Renovation of air handling units in various laboratories. vi) Segregation of power supply system i.e. separate H.T. Metering system for residence at NII. v) Creation of Parking space for official vehicles. vi) Creation of yard for scrap items. vii)New Electrical Control Panel for Pump House. viii) Automatic Fire Detection & alarm System for Auditorium.

Library & Documentation Services

The Library & Documentation Services Unit of the Institute is involved in procurement of books (print & electronic version) and journals (print as well as online version), document delivery services (journals, articles, reprints), photocopy services, binding services, purchase of reprints, newspaper clippings, preparation and distribution of annual reports of the Institute, etc. The Library subscribes to more than 1,000 journals (mainly online) directly from the publishers and through DeLCON Consortium. The Library also subscribes to book series, annual reviews, current protocols. Due to easy accessibility and increasing rate of usage, the library selection policy mostly emphasizes on subscribing online version of resources. All routine functions of the Library are automated for effective services. The total book collection has crossed 20,000 (approx). A Hindi Library has been set up for popularizing the official language amongst staff of the Institute.

Academic and Training Services

The activities of the Academic & Training Department can be grouped under three major groups viz. Students Affairs, Outside Training, and In-House training. The unit has been involved in Ph.D. admissions, Pre-Ph.D registration courses, Doctoral Committee meetings, Academic Committee meetings, Fellowship of scholars, Indian Institute of Science. Department of Bio-technology- Post Doctoral Fellowship (DBT-PDF), Indian Council of Medical Research- Research Associates (ICMR- RAs), Wellcome Trust Fellow and Department of Science and Technology Women Scientist (DST-WOS). The unit has been involved in arranging the participation of scientific, technical and administrative officials of the Institute in the training courses, workshops and seminars organized by outside organizations in different parts of the country.

Vigilance Cell

The Institute has a Vigilance Cell headed by a Scientist nominated as part – time Chief Vigilance Officer (CVO) by the Chief Vigilance Commissioner (CVC). The CVO and the support staff perform vigilance functions as adjunct duties in addition to their responsibilities. The Cell has effectively followed various instructions issued by the CVC from time to time to ensure effective implementation of the measures outlined in the instructions and strengthening vigilance and anti-corruption work. Emphasis has been laid primarily on preventive vigilance since such vigilance, if properly conceived and executed aids in plugging weak and vulnerable areas. The Institute has been reviewing existing procedures to identify corruption prone areas, making policies more transparent to avoid ambiguity and streamlining procedures to achieve a corruption free environment. Plans for rotation of staff employed in sensitive areas prone to corruption have been implemented. Sizable purchases of chemicals, consumables and instruments are handled by various purchase committees of the Institute, thus eliminating the possibility of collusion detrimental to quality and price of purchases. The Institute has thus far been able to maintain a clean slate in corruption matters. The Cell has been rendering the periodical reports and returns on vigilance activities to the administrative ministry and the CVC.

KEYNOTE LECTURES

FOUNDATION DAY LECTURE

The 25th Foundation Day Lecture of the Institute was delivered by Prof Sir Leszek Borysiewicz, Vice-Chancellor, University of Cambridge on "The Problem with Vaccines" on 12th September 2011. The function was presided over by Dr Chandrima Shaha, Dy. Director, National Institute of Immunology, New Delhi.

Prof Sir Leszek Borysiewicz, Vice-Chancellor, University of Cambridge with NII Deputy Directors, Dr Chandrima Shaha and Dr Satish K Gupta at Foundation Day Lecture.

NII SILVER JUBILEE LECTURE

Prof Goverdhan Mehta, National Research Professor and Lily-Jubilant Chair School of Chemistry, University of Hyderabad delivered the NII Silver Jubilee Lecture on "Chemistry for sustainable future: Serenading a great legacy for a better world" on 23 September 2011. The function was presided over by Prof Avadhesha Surolia, Director, National Institute of Immunology, New Delhi.

Prof Goverdhan Mehta, National Research Professor and Lily-Jubilant Chair School of Chemistry, University of Hyderabad with NII Director, Prof Avadhesha Surola, at Silver Jubilee Lecture.

NOTABLE ACTIVITIES

ACADEMIC COURSES AND TRAINING PROGRAMMES

Ph.D Programme

The Institute imparts long term residential training leading to Ph.D. Degree of the Jawaharlal Nehru University, New Delhi. Every year 30-35 scholars are admitted to this Programme on competitive basis after an examination and interviews amongst a large number of applicants from all over the country. For the Ph.D. Programme in the academic year 2011-2012, 2002 candidates applied for admission, out of which 1421 candidates took the National Level Test at New Delhi, Ahemdabad, Kolkata & Bangalore centres. On selection 32 scholars joined the programme. The Ph.D. Programme of the Institute was launched in the academic year 1986-87. Since then the Institute has admitted a total of 504 students in 26 batches. Out of these 228 students have successfully completed the course and have been awarded Ph.D. degree by the Jawaharlal Nehru University, New Delhi. Out of the remaining 276 students, 87 have left the course in between and the rest 189 students are at various stages of their research work for the degree.

ACADEMIC INTERACTION WITH UNIVERSITIES AND OTHER INSTITUTIONS

The Institute accepts students from various universities/ institutions as Summer Research Fellowship Awardees and provides them facilities and guidance. Besides, the Institute also accepts students for the project work during the last semester of the Post Graduation course. The facilities are also provided for short term training to the students and faculty members from various universities/research institutions.

PUBLICATIONS

Eighty eight papers by the scientists and scholars of the Institute were published this year in different areas of research being conducted at the Institute. Of these publications seventy two were published in journals as peer-reviewed research papers and remaining sixteen papers were published as reviews/proceedings. The complete details of these papers including author(s), title, journal name, volume, year, pagination or name of the conference, name of the book, their publishers etc. (Annexure-I).

PATENTS & TECHNOLOGY TRANSFER

The Institute has a policy of protecting the intellectual property rights on inventions made within its laboratories. Early research leads are evaluated for commercial viability and patentability. The Institute files applications first in India and when necessary, at patent officers in other countries. During the year under report, the Institute has fourteen applications and two patents were granted. (Annexure-II).

A technology for 'Composotion useful for the treatment of inflammatory disease or disorder' has been transferred to M/s Extended Delivery Pharmaceuticals, USA.

LECTURES DELIVERED ON INVITATION/PAPERS PRESENTED

The scientists of the Institute continued to deliver lectures including 'Keynote Addresses and 'Inaugural Addresses' 'Serial Lectures' etc at various institutions, conferences, symposia, workshops and training programmes in India and abroad.

SEMINARS BY VISITING SCIENTISTS/GUEST INVESTIGATORS

The Institute continued to receive visiting scientists and guest investigators from all over the world. Thirty three seminars were organized during their visit to the Institute on topics related to the areas of research being carried out at the Institute. Out of these two seminars were delivered by investigators from India and the rest thirty one seminars were presented by the scientists from abroad. These seminars were attended not only by the scholars and scientists of the Institute but also by the investigators from other institutions. Fruitfull discussions usually followed these seminars.

FOUNDATION DAY 2011

The twenty fifth Foundation Day of the Institute was celebrated on 6th Oct 2011 During the preceding fortnight, in-house competitions like Quiz, Antakshri, Rock show, Treasure hunt, Mixed bag, Rangoli, Marathon, Street play were organised for scholars and staff of the Institute whose participation was very enthusiastic. The special feature of the activities included the sports events and fancy dress competitions by the children of staff members.

IMPEMENTATION OF OFFICIAL LANGUAGE POLICY

The Official Language policy of the Govt. of India is followed in the Institute in letter & spirit:

The Committee of Parliament on Official Language had carried our inspection in connection with implementation of Official Language policy in the Institute on 5/11/2011 and appreciated our efforts made on implementation of Official Language team, Department of Biotechnology also inspected this Institute on 16/3/2012 and was satisfied with the efforts made by the Institute on implementation of Official Language policy in official work.

To promote Official Language Hindi in official work, the Hindi Pakhwara (Hindi Fortnight) was celebrated in the Institute with great zeal from 1st to 14th September 2011. During this period, various Hindi competitions via Hindi Sulekh (Hindi Writing), Hindi Shrutlek (Hindi dictation), Hindi Nibandh (Hindi Essay), Hindi Anuvad (Hindi Translation), Hindi Vaad-vivad (Hindi Debate) and Hindi Kavita pathan (Hindi Poetry recitation) were organized in the Institute, in which a large numbers of faculty members, staff members and students participated. Hindi Diwas (Hindi Day) was celebrated on 14th September, 2011 on the culmination of Hindi Pakhwara. To sort out the difficulties and to remove the hesitation of the staff while doing their official work in Hindi, Hindi workshops had also been organized in this Institute on 1/7/2011 and 6/2/2012 respectively

NII Staff and PhD students participating in Hindi Sulekh, during Hindi Pakhwara.

INDEPENDENCE DAY CELEBRATION

Independence day was celebrated in the Institute on 15 Aug 2011. The event was marked by Independence Day Message from the Director followed by the national anthem by the students and children of the staff of the Institute.

The Singing of the National Anthem during the Independence Day celebration at the Institute. 15th August 2011.

FAREWELL TO PhD STUDENTS

Farewell function of the 2006 batch of PhD students was marked by planting of a tree sapling in the Institute premises by the students.

ANTI-TERRORISM DAY, SADHBHAVNA DIWAS AND COMMUNAL HARMONY WEEK

Anti-Terrorism Day was observed by all employees of the Institute on 21 May 2011 by taking anti-terrorism/violence pledge stating: 'We, the people of India, having abiding faith in our country's tradition of non-violence and tolerance, hereby solemnly affirm to oppose with our strength, all forms of terrorism and violence. We plege to uphold and promote peace, social harmony and understanding among all fellow human beings and fight the forces of disruption threatening human lives and values'.

With the theme to promote national integration and communal harmony among people of all religions, languages and regions, 'Sadhbhavna Diwas' was observed in the Institute on the birth anniversary of late Shri Rajiv Gandhi on 20 Aug 2011 by taking pledge by each staff that 'I take this solemn pledge that I will work for the emotional oneness and harmony of all the people of India regardless of caste, region, religion of language. I further pledge that I shall resolve all differences among us through dialogue and constitutional means without resorting to violence'. To promote the idea further a fortnight from 20 Aug to 3 Sep 2011 was observed as Communal Harmony Week. A Communal Harmony Campaign and Fund Raising Week was observed in the in the Institute from 19 to 25 Nov 2011 and Flag Day on 25 Nov 2011, when the funds for rehabilitation of child victims of communal violence were collected and sent to the National Foundation for Communal Harmony.

REPRESENTATION OF SCHEDULED CASTES, SCHEDULED TRIBES & OTHER BACKWARD CLASSES

The Institute follows reservation orders as per directives of Goverment of Inida, while maing appointments, to ensure representation of Scheduled Castes, Scheduled Tribes, Other Backward Classes and Physically Handicapped persons as per the prescribed percentage. Existing orders in force and as amended from time to time by the Government of India, are compiled with.

COMMITTEES OF THE INSTITUTE

(as on 31.03.2012)

NII SOCIETY

Prof G Padmanaban
(President)
NII Society & NASI Chair/ Hon. Professor
Department of Biochemistry
Indian Institute of Science
Bangalore

Prof M Vijayan
DAE Homi Bhabha Professor
Molecular Biophysics Unit
Indian Institute of Science
Bangalore

Prof M K Bhan
Chairman, G.B. NII & Secretary
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Director General Health Services
Ministry of Health & Family Welfare
Nirman Bhawan
New Delhi

Prof R C Deka
Director
All India Institute of Medical Sciences
Ansari Nagar
New Delhi

Dr V M Katoch
Secretary, DHS & Director General
Indian Council of Medical Research
Ansari Nagar
New Delhi

Dr S Ayyappan
Director General
Indian Council of Agricultural Research
Krishi Bhawan
New Delhi

Prof Ved Prakash
Chairman
University Grants Commission
Bahadur Shah Zafar Marg
New Delhi

Prof Sudhir K Sopory
Vice-Chancellor
Jawaharlal Nehru University
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Dr Chandrima Shaha
Director
National Institute of Immunology
Aruna Asaf Ali Marg
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GOVERNING BODY

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(Chairman)
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Dr Jagdish Prasad
Director General Health Services
Ministry of Health & Family Welfare
Nirman Bhawan
New Delhi

Ms Anuradha Mitra
Joint Secretary & Financial Advisor
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New Delhi

Prof R C Deka, Director
All India Institute of Medical Sciences
Ansari Nagar
New Delhi

Dr V M Katoch
Secretary, DHS & Director General
Indian Council of Medical Research
Ansari Nagar
New Delhi

Dr S Ayyappan
Director General
Indian Council of Agricultural Research
Krishi Bhawan
New Delhi

Prof Ved Prakash
Chairman
University Grants Commission
Bahadur Shah Zafar Marg
New Delhi

Prof Sudhir K Sopory
Vice-Chancellor
Jawaharlal Nehru University
New Delhi

Prof M Vijayan
DAE Homi Bhabha Professor
Molecular Biophysics Unit
Indian Institute of Science
Bangalore

Prof K Vijay Raghavan, Director
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Tata Institute of Fundamental Research
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