NATIONAL INSTITUTE OF IMMUNOLOGY

ANNUAL REPORT 2010-11

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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

Rapid strides in consonance with NII's mandate have taken place during the silver jubilee year of its foundation. To commemorate the occasion, three international symposia were organized in the areas of infectious diseases, aging, and cancer biology which attracted leading researchers in these areas. It is satisfying to note that the jubilee year began with the recognition of NII as a major hub in pharma sector by Thomson Reuters.

A novel role of CD4 T cells in aging was unraveled and CD4 staining was identified as a potential new marker for identifying age of individual cells. The role of iNOS in B cell differentiation was deciphered. In another study, the role of Apoptosis Inducing Factor (AIF) in mediating its various functions in the T cell lineage was dissected. The finding that administration of hCG to lupus-prone mice leads to an increase in autoreactivity may help delineate the causes of pregnancy-associated flares of systemic autoimmune diseases. Local immune response in the lung of *MIP* immunized / BCG immunized guinea pigs was characterized and a higher number of MHC-II expressing cells and CD8+ve cells were found in the granuloma of *MIP* immunized group at early chronic phase.

Structural studies directed at understanding antibody responses against neutralizing epitopes of an immune evading pathogen revealed the involvement of CDR flexibility and differential polar interactions in multispecificity of the antibody response. Novel antibiotic peptides with therapeutic potential were generated and characterized. Long molecular dynamics studies on mycobacterial and human protein kinases provides insight on how phosphorylation on primary autophosphorylation site facilitates the phosphorylation of secondary autophosphorylation site. Role of dimerization in regulation of human guanylate binding proteins was elucidated.

Studies on *Salmonella typhi,* which causes typhoid in humans, indicated that this pathogen switches to a phenotype which is very poor at activating macrophage cell death. This may involve down regulation of flagellin expression. InhA, the primary target for the first-line anti-tuberculosis drug isoniazid, a key enzyme of the Fatty Acid Synthase-II, was identified as a substrate for mycobacterial serine/threonine protein kinases. The phosphorylation of InhA resulted in a decreased in its enzymatic activity. A novel Splicing Related Protein Kinase (PfSRPK1) was characterized, this enzyme may regulate mRNA splicing in the parasite by phosphorylating splicing factors like PfSR1. Benzothiophene carboxamides have been developed as a new class anti-malarial scaffolds. A role for polyketide synthetase, DiPKS1, in inducing spores in *Dictyostelium* was delineated.

A novel physical form of insulin, SIRF, developed recently at NII is likely to provide a lasting treatment for diabetes. Natural compounds derived from a plant possessing anti-HIV activity, were isolated and are being developed further. Novel genetic elements with possible implications in evolution of the buffalo and related genomes were identified.

The parallel regulatory roles of two tumor suppressors p53 and RECQL4 helicase were elucidated during mitochondrial DNA replication. The involvement of E3 ubiquitin ligases with replication machinery was elucidated which may provide important insights into genomic instability which often leads to human cancers. Hsp70-2 was identified as a potential target for early diagnosis and the treatment of cancer. A highly effective combination treatment for teratocarcinoma was discovered, this involves combination of cisplatin and plant flavonoid fisetin.

A large number of students and young researchers from around the country received training at NII in various areas. They also benefited from lectures and symposia organized at the institute.

Professor Avadhesha Surolia
Director

12 August 2011

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 analyse 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.

Potential role of immunoproteasomal components in regulating signal transduction threshold in APCs

The proteasome is a multi-subunit protein degrading system involved in cellular protein turnover. Proteosomal activity thus regulates many biological functions, including cell cycle control, cell stress responses, intracellular signaling, and antigen processing. In the constitutively expressed proteosomal 20S catalytic core, the inner catalytic rings are composed of beta-type subunits (beta1, beta2, beta3, beta4, beta5, beta6 and beta7), of which three (beta1, beta2 and beta5) have catalytic activity. These three subunits can be replaced by their interferon-gamma (IFNγ)-inducible homologues; beta1i or LMP2, beta2i or LMP10, and beta5i or LMP7, leading to changes in cleavage specificities with increased cleavage activity after hydrophobic, basic and asparagines residues, of the resultant immunoproteasomes. We have begun asking if such changes in cleavage properties also cause changes in APC signaling thresholds.

Transfection-based expression of LMP2, LMP7 and LMP10 together into murine monocytic cell lines caused enhanced responsiveness to LPS-mediated induction of MHC class II (MHCII) levels. Individually, while LMP7 and LMP10 caused some MHCII induction, the combined effect was synergistic. Another outcome of TLR-4 signaling, tumor necrosis factor-alpha (TNFα) production, was also enhanced by simultaneous expression of LMP2/7/10. Since nuclear factor-kappa B (NFkB) activation is a major signaling outcome of TLR-4 signaling, we next tested the NFkB activation status LMP2/7/10-transfected monocytic cells. Transfected cells were flow cytometrically sorted and activated with LPS in culture. Nuclear translocation of the p65 subunit of NFkB was enhanced in immunoproteasome-expressing cells, as was the degradation of the NFkB inhibitor protein IkB. Interestingly, the total cellular p65 levels were also enhanced in these cells over sham-transfected cells upon LPS activation. Thus, these data suggest that immunoproteasomal cleavage

patterns alone can probably modify the biological behaviour of APCs. Since the crucial inflammatory cytokine IFNγ is the major inducer of LMP2/7/10 expression, these data on the role of immunoproteasomes in TLR-4 signaling as a threshold modulator have interesting implications for infections in vivo, where the rapidity and extent of cellular immune responses are important parameters deciding the outcome.

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

We have previously reported that apoptosis inducing factor (AIF), a phylogenetically conserved mitochondrial flavoprotein with NADH redox activity and apoptosis-inducing capability, has a significant role in T cell development and function by playing both a pro- and an anti-apoptotic role. The *aif* gene is located on the X chromosome. The Aif protein has a DNA-binding capacity important for pro-apoptotic function, and a catalytic capacity important for mitochondrial function and redox regulation. For these studies, we have been using the natural mutant harlequin (Hq) mouse strain hypomorphic for *aif* expression. Activated peripheral Hq T cells show reduced susceptibility to cytokine withdrawal-induced death, but show increased susceptibility to activation-induced cell death (AICD). Hq mice also show thymic hypocellularity and developmental retardation of the T cell lineage in the thymus during the stage of beta-selection-mediated cell proliferation at the DN3 to DN4 transition.

We have initiated a genetic approach to studying the roles of DNA-binding and oxidoreductase catalytic properties of AIF in mediating its various functions in the T cell lineage. For this, we have cloned the wild-type *aif* cDNA, generated point-mutants of two residues each, known to be deficient in DNA-binding activity (K255A and R265A) and catalytic activity (T263A and V300A), and built expression plasmids carrying these cDNAs along with an IRES-separated *egfp* gene for generating transgenic mice.

We have used the testicular transgenesis method to generate transgenic mice of the FVB strain. Male mice given intratesticular linearised plasmid were mated with wild-type FVB females. Transgenic male progeny were then bred with heterozygous Hq females. The four genotypes of resultant male progeny, - non-transgenic WT, non-transgenic Hq, transgenic WT and transgenic Hq, - were then used for further analysis. We have analysed control mice transgenic for the empty vector, and mice transgenic for native AIF, and we observe that transgenic expression of AIF substantially corrected all the defects we have reported in the thymus as well as in the peripheral T cells of Hq mice. Thus, the stage is now set for us to examine the effects in vivo of point mutants of AIF deficient in specific functions.

Publications

Original peer-reviewed articles

1. Varanasi V, Mattoo H, Tupperwar NC, Thyagarajan K, Das A, Kumar R, Bal V*, Vaidya T*, George A* and Rath S* (2010) A superantigen interacts with leishmanial infection in antigen-presenting cells to regulate cytokine commitment of responding CD4 T cells. **J Infect Dis 202**: 1234-1245.

2. Satpathy S, Shenoy GN, Kaw S, Vaidya T*, Bal V*, Rath S* and George A* (2010) Inhibition of terminal differentiation of B cells mediated by CD27 and CD40 involves signalling through JNK. **J Immuol. 185**: 6499-6407.

3. Khare A, Viswanathan B, Gund R, Jain N, Ravindran B, George A*, Rath S* and Bal V* (2011) Role of Bruton's tyrosine kinase in macrophage apoptosis. **Apoptosis 16**: 334-346.

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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 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*. Inhibitor of DNA binding 2 (*Id2*) and *Irf8* are essential transcription factors for $CD8\alpha^+$ DC development and recent report suggest that TGF-β signaling regulates expression of *Id2* and *Irf8*. Objectives of the current study are 1] to understand the regulation of *Id2* gene transcription by *Irf4* and *Irf8*, 2] to study the functional significance of *Id2* gene expression in DCs, 3] to understand functional significance of different domains of *Irf4* and *Irf8* in regulating diversity in DC development, 4] to understand contribution of TGF-β signaling and *Irf8* in DC development, 5] to study the *Irf4* and *Irf8* transcription complexes.

Understanding the functional significance of Id2 gene expression in DCs

Id2 is reported to be pivotal in DC development. *Id2* expression in bone marrow progenitor cells inhibits pDC development; whereas *Id2* expression is essential for development of $CD8\alpha^+$ DCs. As mentioned earlier our unpublished preliminary data suggested that expression of *Irf8* in DC9 cell line leads to increase in *Id2* transcripts. Previous reports have shown that *Irf8* and *Id2* knockout mice show the lack of $CD8\alpha^+$ DC subtype. Hence it would be interesting to examine whether induction of *Id2* by *Irf8* plays central role in $CD8\alpha^+$ DC development or does *Irf8* has larger role besides regulation of *Id2* gene. To understand the effect of individual gene expression and synergistic/antagonistic effect (if any); we expressed *Irf8*, *Id2* individually and together in DC9 cells. Our data clearly shows that in absence of *Irf8*, expression of *Id2* alone is not sufficient to direct the $CD8\alpha^+$ DC development. Besides *Irf8* and *Id2*, recent report suggests essential role of Basic leucine zipper transcription factor *(Batf3)* in $CD8\alpha^+$ DC development. We extended our experiments by including *Batf3* gene expression in our experiments so that we can study the relation between *Irf8*, *Id2* and *Batf3*.

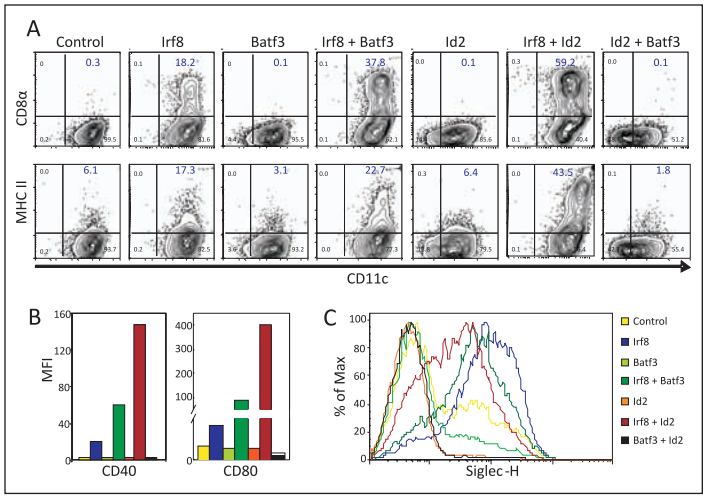

Figure 1: *Understanding the role of transcription factors Irf8, Batf3 and Id2 in the CD8α+ DC development. DC9 cells were transduced with retroviral constructs expressing Irf8, Batf 3, and Id2 individually and co-expressed with each other. Different populations were treated with CpG oligonucleotide (TLR9 ligand) for 24 hour and analysed by flow cytometry.* **A]** *Expression of Id2 or Batf3 alone or co-expressed together didn't induce CD8α surface marker. Id2 or Batf3 when co-expressed with Irf8 showed synergistic effect on expression of surface markers CD8α and MHC II.* **B]** *DC maturation markers CD40 and CD80 also showed synergistic increase when Id2 and Batf3 were co-expressed with Irf8.* **C]** *Siglec-H, pDC specific surface marker was down regulated on unstimulated DC9 cells by Id2 and Batf3 alone or by co-expression. Expression of Irf8 rescues down regulation by Id2 and Batf3 and increases Siglec-H on cell surface.*

Our data suggests that in absence of *Irf8*; *Id2* and *Batf3* expressed alone or co-expressed, can not drive differentiation of CD8α$^+$ DCs as analysed by flow cytometry as well as subset specific gene quantitative PCR. We did observe decrease in the CD11c and pDC specific Siglec-H gene expression when *Id2* and *Batf3* were expressed alone or co-expressed in absence of *Irf8*. *Id2* and *Batf3* expression has a synergistic effect specifically on CD8α$^+$ DC development when expressed along with *Irf8* (Figure 1). DC activation markers CD40, CD80 and MHC II were up regulated by *Irf8* and co-expression of *Id2* and *Batf3* along with *Irf8* had a synergistic effect (Figure 1). The synergistic effect is not extended to pDC specific gene expression, thus demonstrating CD8α$^+$ DC specific synergism between transcription factors *Irf8*, *Id2* and *Batf3*. Together our results suggest that *Irf8* is a master regulator of CD8α$^+$ DC differentiation.

Regulation of Id2 gene transcription by Irf4 and Irf8

To understand the regulation of *Id2* gene by *Irf4* and *Irf8*, we started with standardization of Chromatin Immunoprecipitation (ChIP) technique. In the first step we standardized the sonication conditions in Mysonix 4000 instrument to obtain the formalin-fixed DNA shearing in the regions of 200-2000bp with the majority of the DNA in the regions of 200-500bp. We performed ChIP assay for IRF8 binding in the control vector and *Irf8* transduced DC9 cells. Our positive control TLR gene promoter showed a very good binding and negative control HPRT gene did not show any binding for IRF8. Similar chromatin samples were subjected to ChIP assay to study IRF8 binding to *Id2* gene promoter. Our preliminary ChIP assay result suggests a relatively higher binding of IRF8 to *Id2* promoter as compared to negligible binding seen in control cells. Currently we are performing in-depth ChIP assay to study pattern of IRF8 and IRF4 binding to *Id2* gene promoter.

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. Our preliminary data suggests 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 preparing chimeric proteins of *Irf4* and *Irf8* by exchanging DNA binding domain (DBD) and IRF association domain (IAD) of these proteins.

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;* both these transcription factors have been shown to play essential role in $CD8\alpha^{+}$ DC development. Published report further demonstrated that treatment of DCs with TGF-β signaling inhibitor blocks TGF-β induced *Irf8* gene transcription. We have standardized DC cultures from mice bone marrow and have carried out preliminary experiments to check our DC cultures. 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.

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

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

3. To study the regulation of macrophage survival and death in response to various stimuli.

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

We had observed earlier that naive CD4 [NCD4] T cells with lower staining intensity for CD4 show poorer proliferative potential as compared to similar cells with high CD4 intensity. We hypothesised that CD4 intensity may go down with prolonged survival of NCD4 cells *in vivo* and such cells may indeed show poor functional potential. In order to evaluate this we transferred T cell receptor [TCR] transgenic [Tg] OT-II cells in congeneic recipients [i.e. C57Bl.6 with CD45.2, referred as B6.SJL] and allowed them to age. OT-II TCR recognises I-Ab loaded with ovalbumin [Ova] derived peptide. It is unlikely that these cells would be exposed to the cognate MHC-peptide complex *in vivo*. Cells were allowed to age for varying periods of time [from 7 days to 3 months] *in vivo*, at the end of which mice were euthanised, transferred cells identified by the phenotypic markers using flow cytometry and their ability to proliferate and upregulate activation markers were analysed.

We observed that between 1 week to 3 months of *in-vivo* aging the proportion of transferred cells decreased. When activated with Ova peptide *in vitro*, and examined for the upregulation of CD69 as an early activation marker, cells aged for $\geq$ 1 month *in vivo* showed poor upregulation of CD69 as compared to those aged for $\leq$ 2 weeks. The proliferative response of these cells as measured either by 3H-thymidine incorporation or CFSE dilution showed that Tg cells aged for $\geq$ 1 month proliferated poorly as compared to those which were aged for $\leq$ 2 weeks. It was also observed that higher proportion of cells which have aged *in vivo* for $\geq$ 1 month show lower levels of CD4 as compared to cells which have aged for lesser duration. These data together suggest age of an individual NCD4 cells after its exit from the thymus is important for its functional potential. Longer it survives as a naive cell less is it likely to function optimally.

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

We have set up experimental systems where NCD4 T cells from two mouse strains [C57Bl.6 (B6) and BALB.b] with identical

MHC genes but different background genes are used to analyse consequences of neutral stimulus-based activation. Purified NCD4 cells from the two strains are activated with plate-coated antiCD3+anti-CD28 antibody cocktail in the absence of exogenous antigen-presenting cells or cytokines, rested and restimulated to evaluate the intrinsic potential of these cells to secrete Th1 and Th2 cytokines in culture supernatants. Interferon-gamma [IFNg] and IL-4 are the representative cytokines used as a read out. We observed that at various titrating doses of anti-CD3+anti-CD28 used during recall response NCD4 cells from B6 strain produced high levels of IFNg, and little of IL-4, as has been reported in the literature for B6 strain. Surprisingly, the NCD4 cells from BALB.b strain produced high levels of IFNg as well as IL-4. While BALB/c mice are known to have predilection for a Th2-response contribution of APC-derived cytokines in this polarisation has been shown. The cell intrinsic ability of NCD4 from BALB.b mice to secrete high levels of IFNg has not been reported so far. Which factors govern the cell intrinsic differentiation and how the simultaneous production of IFNg and IL-4 by activating cells modulates further differentiation pathway remains to be explored. Using similar approaches on human NCD4 cells are also likely to provide meaningful information.

3. To study the regulation of macrophage survival and death in response to various stimuli.

Some of our findings on the role of Bruton's tyrosine kinase [Btk] in macrophage apoptosis were extended during the reporting period. We had shown that in response to lipopolysaccharide [LPS] and IFNg, Btk deficient macrophages from Xid mice die more easily. While dissecting the role of various kinases we had observed that Erk/MEK kinases, which are extensively reported to play an anti-apoptotic role in many situations of insult to the cell, in the absence of Btk play a pro-apoptotic role. We further characterised this finding to show that in Btk deficient state, Erk/MEK kinases are possibly responsible for early degradation of Bxl-xL protein, which results in enhanced macrophage death after stimulation by LPS+IFNg.

Publications

Original peer-reviewed articles

1. Varanasi V, Mattoo H, Tupperwar NC, Thyagarajan K, Das A, Kumar R, Bal V*, Vaidya T*, George A* and Rath S* (2010) A superantigen interacts with leishmanial infection in antigen-presenting cells to regulate cytokine commitment of responding CD4 T cells. **J Infect Dis 202**: 1234-1245.

2. Satpathy S, Shenoy GN, Kaw S, Vaidya T*, Bal V*, Rath S* and George A* (2010) Inhibition of terminal differentiation of B cells mediated by CD27 and CD40 involves signalling through JNK. **J Immuol. 185**: 6499-6407.

3. Khare A, Viswanathan B, Gund R, Jain N, Ravindran B, George A*, Rath S* and Bal V* (2011) Role of Bruton's tyrosine kinase in macrophage apoptosis. **Apoptosis 16**: 334-346.

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Biology of animal viruses

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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 therapeutic candidates against JE.

Several siRNAs were tested that targeted both coding and non-coding parts of JEV genomic RNA. Results demonstrated that siRNAs targeting various regions in viral genome inhibited JEV replication to different extent. Importantly, we found that siRNAs targeting coding regions of the ORF were much more effective in bringing down JEV titers than those targeting the non-coding regions. Out of the several siRNAs that were tested, shN7660 that targeted the NS5 coding sequence was most effective. It may be noted that NS5 is the RNA-dependent RNA polymerase that is necessary for virus replication.

To address issues related to siRNA delivery and and to test its therapeutic efficacy, adenoviral and retroviral delivery systems were employed. Compared to plasmid transfections, better efficiencies of delivery and virus titer knockdown were achieved using the viral delivery methods. Thus, retrovirally-delivered shN7660 provided 100% protection to mice in a lethal JEV challenge model. Adenovirally-delivered shN7660 while enhanced the life span of JEV-infected mice, it failed to protect mice from the leathal JEV challenge.

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.

A site preparation and disease burden study for the planning and implementation of the phase III trials for the oral rotavirus vaccine 116E was initiated last year in association with the SAS. These studies continued this year. Three sites were selected, one each in the three states of Delhi, Haryana and Uttar Pradesh. Two cohorts of infants were recruited at the Delhi site, Cohort-I was enrolled at birth and followed up weekly till 1 year of age (n=100) and Cohort-II was enrolled at 12 months of age and followed up till 24 months of age (n=100). When events of gastroenteritis (GE) were identified, information was collected to estimate Vesikari score and stool specimens collected for rotavirus detection by ELISA and RT PCR.

Total number of GE episodes in Cohort-I was 125 whereas 68 GE episodes were recorded in Cohort-II. The mean duration of GE episode in Cohort-I was 5.01 days compared to 3.09 days for Cohort-II. Thus duration of GE episodes was longer in younger children. Incidence of GE was higher in younger age group of <1 year-olds. The incidence rate was 1.43 GE episodes/child/year in <1 year old cohort whereas it was 0.74 GE episodes/child/year in 1-2 year old children. In Cohort-I, 25 out of 125 episodes

had rotavirus association. This number was 19 out of 68 in the case of Cohort-II. Thus, ~20% of GE episodes were associated with rotavirus infection. The mean number of days the rotavirus antigen was detectable in stool samples was 3.1.

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 candidates against the virus.

Blind passaging of CHIKV in BHK cells resulted in small plaque morphology virus. The virus was plaque purified twice to obtain a homogenous preparation. Growth characteristics of the small plaque virus (SPV) differed from that of the wild type large plaque virus (LPV). Thus SPV grew slower in Vero cells but it finally reached similar or higher titers compared to LPV. In one-week old mice SPV was avirulent whereas the LPV was highly lethal. Nucleotide sequencing of the section of viral genome coding for SPV structural proteins has identified mutations when compared with LPV.

Publications

Original peer-reviewed articles

1. Anantpadma M, Stein DA and Vrati S (2010) Inhibition of Japanese encephalitis virus replication in cultured cells and mice by a peptide-conjugated morpholino oligomer. **J Antimicrob Chemother 65**: 953-961.

2. Appaiahgari MB and Vrati S (2010) IMOJEV®: a Yellow fever virus-based novel Japanese encephalitis vaccine. **Expert Rev Vaccines 9**: 1371-1384.

Reviews / Proceedings

1. Bharati K and Vrati S (2010) Japanese encephalitis vaccines: current status and future prospects. **Proc Natl Acad Sci India 80**: 179-89.

Patents

1. Vrati S (2010). Recombinant vaccine against Japanese encephalitis virus (JEV) infection and method thereof. Indian Patent no. 243547.

2. Vrati S (2010). Recombinant vaccine against Japanese encephalitis virus (JEV) infection and method thereof. Singapore Patent no. 123824.

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. The newly identified proteins detail 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 (PB000823.03.0, Pb000871.00.0, Pb000268.02.0, Py-S10, Pb-S23, Pb-LSA3, PB300768.00.0, PB000779.02.0 and PY 07420) for analysis. 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 (except LSA-3) in the parasite. As a first step to verify that these genes are transcribed, we used sporozoite cDNA as template to amplify these genes, and found that the selected genes are transcribed as we get an expected size amplicon from the amplification reaction.

Since these proteins contain putative pexel motif, to check whether these motifs are functional or not, we constructed a plasmid expressing the N-terminal 150 amino acid of the test protein (containing the pexel motif and signal sequence). Test protein was fused with GFP at the C- terminus. Constitutive promoter EF1alpha drives the expression of fusion protein. Using transient transfection system we show here the presence of functional pexel motif in these proteins. Last year we have already showed the presence of pexel motif in PB000823.03.0.

This year we demonstrate presence of active pexel motif (Figure 1) in the remaining eight proteins (Pb000871.00.0, Pb000268.02.0, Py-S10, Pb-S23, Pb-LSA3, PB300768.00.0, PB000779.02.0 and PY 07420).

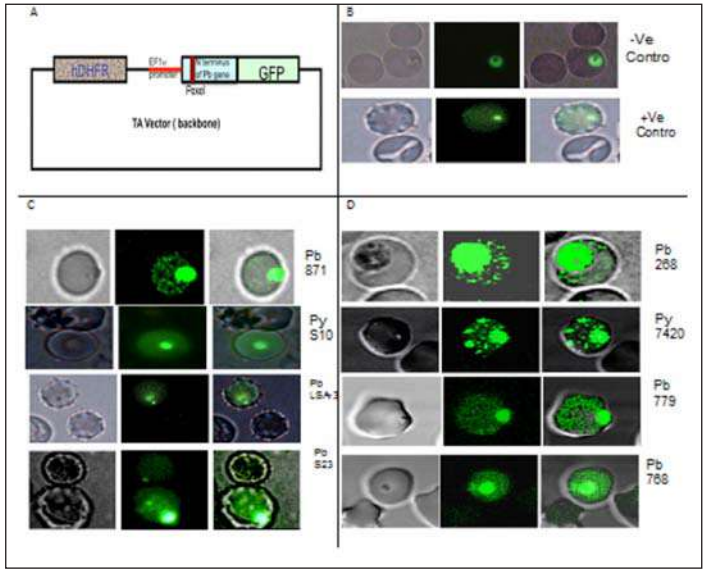

Figure 1: A) *Plasmids details showing various regions. These plasmids were used for transient transfection of blood stage parasites. DHFR present in vector gives pyrimethamine resistance and is used for selection of plasmid containing parasites. GFP is used for microscopic visualization of parasite. Test gene is expressed under the control of constitutive EF1alpha promoter. Test gene was fused (in frame) at the C-terminus with GFP.* **B)** *Microscopic examination of the transiently transfected P.berghei ANKA blood stage parasites. Negative control was vector alone. Positive control was from a known protein with active pexel motif. In case of positive control green fluorescence was visible in the cytoplasm. Green fluorescence detected beyond parasite PV membrane, indicates presence of a functional pexel motif.* **C&D)** *Microscopic examinations of the transiently transfected P.berghei ANKA blood stage parasites. Images show presence of active pexel motif in respective proteins as mentioned next to each panel.*

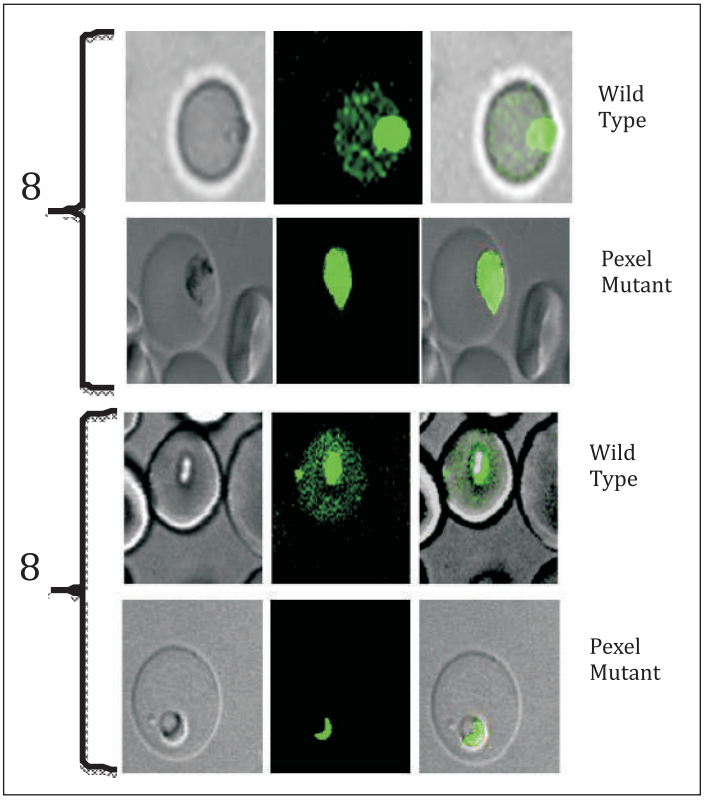

Figure 2: *Microscopic examinations of the transiently transfected P.berghei ANKA blood stage parasites. Images show active pexel motifs in wild types, while inactive pexel motif in mutant proteins. In case of pexel mutant, arginine and leucine, the critical amino acids of motif were mutated to alanine. Results also confirm presence of a single motif in respective proteins.*

So far we have mutated pexel motif in two genes (PB000823.03.0, Pb000871.00.0) and observed that these proteins are no longer exported, confirming presence of a single export motif in these proteins (Figure 2). Work is in progress to mutate pexel motif in the remaining seven proteins.

Towards understanding the role of these proteins in parasite we are knocking out these genes from parasite. Till date, we have obtained knock out clonal population of two genes (PB000823.03.0, Pb000871.00.0). Phenotypic characterization has just begun and results are awaited.

To find-out the host interacting proteins, we performed co-immunoprecipitation using the normal liver lysate and the *E. coli* produced purified Pb871 protein. We obtained one interacting partner with PB871. Mass-spectrometry based analysis of the interacting protein (single band) was performed but results were not conclusive. To know the identity, we plan to do the N-terminal sequencing of the interacting protein.

Publication

Original peer-reviewed article

1. Singh AP, Zhang Y, No JH, Docampo R, Nussenzweig V, Oldfield E (2010) Lipophilic bisphosphonates are potent inhibitors of Plasmodium liver-stage growth. **Antimicrob Agents Chemother 54**: 2987-2993.

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 infection causes global changes in the susceptible cell and role of individual HIV-1 genes in this process needs to be understood in details to understand the mechanistic details. The major focus of my laboratory is to understand the unique features of HIV-1 epidemic in North India and also to develop nucleic acids and siRNA/miRNA based antiviral approaches against HIV-1.
HIV-1 pathogenesis is governed by multiple factors and we wish to explore the role of various HIV-1 genes and host genes to understand the dynamics of HIV-1 epidemic.

To understand the role of accessory genes in modulating cellular genes and causing global changes. To genetically characterize the HIV-1 genes from strains that are circulating in North India and to determine the functional implications of these changes. To study HIV-1 protein + protein and HIV-1 protein + cellular protein interaction and their impact on overall HIV-1 gene expression. Designing long lasting efficacious siRNAs + Ribozymes +Aptmers + DNA-enzymes with the purpose of interfering with HIV-1 gene expression specifically.

Genetic and functional characterization of HIV-1 Tat variants from North India.

Approximately 50 Tat sequences (exon 1) were amplified from the infected individuals. Evidence for B/C recombinant was observed in few of the HIV-1 Tat samples. The most remarkable feature about this recombinant is the presence of a break point in the middle of the Open reading frame. We observed region-specific changes and some unique changes. We cloned some of the variants into HA-tag vector. We obtained varying phenotypes with respect to their ability to activate the LTR- reporter gene. The activity of the natural variants of Tat correlated with their ability to bind to the Tar RNA – a stem loop structure present at the 5'-end of all HIV-1 transcripts. Recently Tat has been shown to act as a RNAi suppressor. We will carry out this study in comparison with subtype B and C.

Diversity of HIV type 1 long terminal repeat (LTR) sequences following mother-to-child transmission in North India.

We genetically characterized the extent of variation in HIV-1 LTR sequences from 11 mother-to-child transmission (MTCT) pairs from HIV-1-infected individuals from North India. Nine pairs were found to be infected with subtype C virus whereas two pairs were

infected with subtype B virus. They harbored the characteristic three and two NF-κB sites, respectively. The analysis of intra-subtype divergence between B and C revealed greater diversity with subtype B LTR sequences than subtype C ($p < 0.005$). Significant evolutionary divergence of subtype C and subtype B was found in NFAT-III ($p < 0.000001$), NFAT-II ($p < 0.0001$), and USF ($p < 0.005$) transcription factor binding sites (TFBS). NF-κB-I, Sp I and II, Ets-I, AP-I and II, and TATA Box TFBS were highly conserved in both the subtypes. An alternate secondary structure of Tar was detected in the VT5 sample due to the point mutation from G to C (position +21) and T to C (position +38).

Novel siRNA+ Rz + AS, aptamer constructs that are effective against HIV-1 B and C genes

Very potent siRNAS were obtained that worked equally well against Vif and Vpr genes. When used in combination with antisense or catalytic DNA, further reduction in the target gene was obtained. Efficient down regulation of target gene expression was observed from the cell surface (by FACS analysis) and some of these constructs showed protection against HIV-1 challenge. The down regulation of genes showed good correlation with reduced RNA synthesis and then confirmed by western blot analysis.

HIV-1 Vpu induces p53 protein

By using a number of experimental approaches we observed the stabilization/accumulation of p53 protein in response to Vpu which correlated with increased apoptosis. This p53 induction depended upon the presence of B-TrcP motif present in Vpu.

Publications

Original peer-reviewed articles

1. Sharma Y, Neogi U, Sood V, Banerjee S, Samrat S, Wanchu A, Singh S and Banerjea AC (2010) Genetic and functional analysis of HIV-1 Rev Responsive Element (RRE) sequences from North India. **AIDS Res & Ther 7**: 28.

2. Karjee S, Minhas A, Sood V, Ponia SS, Banerjea AC, Chow TKV, Mukherjee SK and Lal SK (2010) The 7a accessory protein of SARS-CoV acts as a RNA silencing suppressor. **J Virol 84**: 10395 –10401.

3. Kumar P, Sood V, Vyas R, Gupta N, Banerjea AC and Khanna M (2010) Potent inhibition of Influenza virus replication with novel siRNA-chimeric-ribozyme contructs. **Antiviral Res. 87**: 204-212.

4. Neogi U, Sharma Y, Sood V, Wanchu A and Banerjea AC (2010) Diversity of HIV-1 Long Terminal Repeat (LTR) Sequences following Mother to-Child transmission in North India. **AIDS Res & Human Retroviruses 26**: 1299-1305.

5. Neogi U, Sood V, Choudhry A, Das S, Ramachandran VG, Wanchu A and Banerjea AC (2010). First report of human immunodeficiency virus type 1 circulating recombinant form 02_AG strain of African origin from North India. **American Medical J 1**: 94 -99.

Study of mucosal immune responses

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This laboratory is engaged in understanding various aspects of B cell biology and mucosal immunology that influence the generation and duration of immune protection at systemic and mucosal sites.

The objectives comprise dissecting signals that influence the terminal differentiation of activated B cells, the relative roles of antigen presentation and signal transduction following BCR engagement in determining the final outcome of B cell triggering, factors that determine plasma cell longevity and the generation of immunity at mucosal surfaces.

Role of iNOS in B cell differentiation: Free radicals, notably reactive oxygen species and reactive nitrogen intermediates and their products such as peroxynitrite are major inducers of mitochondrial apoptosis. Inhibitors of some of these pathways have been shown to inhibit T cell death and previous work from our group has indicated a role for iNOS in determining the persistence of T cell memory. We have been analyzing the role of iNOS in B cell responses and have reported that while B cell maturation and induction of activation, proliferation and death by polyclonal stimuli like LPS and anti-IgM in vitro is similar in iNOS-null and wild-type B cells, iNOS-null B cells differentiate relatively poorly into antibody secreting plasma cells in vitro, and also that antibody responses following immunization are lower in iNOS-null mice. Over the past year, we have been trying to dissect the possible mechanisms involved. We report that the lower frequency of live CD138+ plasmablasts in iNOS-null cultures is not due a defect in differentiation, but is accounted for by their greater susceptibility to death. Thus, the frequency of plasma cells in both cultures is similar, but a larger fraction of iNOS-null plasma cells are non-viable and take up propidium iodide. We have used L-NAME, L-NIL, aminoguanidine, L-NMMA and C-PITO for pharmacological inhibition of iNOS in wild-type cells and have seen that at least with some inhibitors, there is a dose-dependent decrease in plasma cell frequency. Our attempts to rescue the phenotype in iNOS-null cells by addition of the NO-donor NONOate have not been successful so far, partly because of its short half-life. We have also carried out flow cytometric analyses for active caspases in B cell cultures stimulated for 3 or 4 days with LPS, and we report that very few B cells or plasma cells in wild-type cultures show caspase activity. Interestingly, while this pattern is seen in the stimulated iNOS-null B cell fraction, the plasma cell fraction shows demonstrable presence of caspases 8,9,12,3 and 6 by day 3 and the proportions increase significantly on day 4. We have also shown that when CD138+ cells purified by MACS- or FACS- sorting are put back into culture, iNOS-null cells die more rapidly than wild-type cells. Together, our data indicate that, unlike in many other cell lineages where NO generated from iNOS is associated with enhanced cellular death, it appears to be associated with survival in plasma cells.

Analysis of B cell antigen presentation: We have been trying to assess the relative contribution of signal transduction downstream of BCR engagement versus antigen presentation for recruitment of T cell help in determining the final outcome of B cell triggering. In this context, we have reported previously that B cells from the *beige* mouse strain that is defective in endosome

biogenesis show hyperresponsiveness to BCR engagement on the one hand and delayed antigen presentation to T cells on the other. Over the past year, we have assessed the ability of *beige* B cells to avail of T cell help when put in competition with wild-type B cells. For this, ovalbumin was targeted to B cells from *beige* mice and CD45-congenic wild-type mice and they were cultured 1:1 with titrating numbers of activated CD4 cells from the OT-II transgenic mouse. B cell activation and proliferation were then read out at 12h and 24h respectively. We report that as B cell:T cell ratios decreased in culture from 3:1 to 3: 0.01, the intensity of CD69 staining and the proportion of cells that had upregulated CD69 decreased, but the staining profiles were similar in WT and *beige* B cells whether present alone or in competition with each other. Further, when CFSE-labeled B cells presenting OVA were cultured with OT-II cells, the overall CFSE dilution decreased with deceasing T cell help, but the proliferation was equivalent in WT and Bg B cells cultured alone or in competition. We also report that following immunization with NP-OVA, WT and *beige* mice showed equivalent primary responses as determined by serum Ab levels as well as by the frequency of plasmablasts in draining lymph nodes and there was no significant difference in the affinity maturation profiles. However, adoptive transfer experiments indicate that memory B cell frequencies are higher in *beige* mice. Further, the frequency of long-lived bone-marrow resident plasma cells was also higher in *beige* mice especially 65 days after challenge. Together, the data indicate that the strength of signaling downstream of the BCR may be more important than rapid recruitment of T cell help in shaping B cell responses.

Publications

Original peer-reviewed articles

1. Varanasi V, Mattoo H, Tupperwar NC, Thyagarajan K, Das A, Kumar R, Bal V*, Vaidya T*, George A* and Rath S* (2010) A superantigen interacts with leishmanial infection in antigen-presenting cells to regulate cytokine commitment of responding CD4 T cells. **J Infect Dis 202**: 1234-1245.

2. Satpathy S, Shenoy GN, Kaw S, Vaidya T*, Bal V*, Rath S* and George A* (2010) Inhibition of terminal differentiation of B cells mediated by CD27 and CD40 involves signalling through JNK. **J Immuol. 185**: 6499-6407.

3. Khare A, Viswanathan B, Gund R, Jain N, Ravindran B, George A*, Rath S* and Bal V* (2011) Role of Bruton's tyrosine kinase in macrophage apoptosis. **Apoptosis 16**: 334-346.

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Analysis of *Salmonella typhi*-host cell interaction

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Salmonella typhi causes systemic infection, typhoid, exclusively in humans while non-typhoidal *Salmonella* serovar *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. During systemic infection, *Salmonella* disseminates throughout the reticulendothelial system and bacteria can be isolated from spleen, liver, bone marrow and gall bladder. The reasons for different clinical manifestations produced by *S.typhi* and *S.typhimurium* in humans, and the host specificity exhibited by these two closely related *Salmonella* species 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.

i) To identify and characterize host-pathogen interactions that might be responsible for producing different manifestations with *S.typhi* and *S.typhimurium*.

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

Flagellin administration reduces *Salmonella* burden in infected mice

We had previously demonstrated that *S.typhimurium* switches to a non-pyroptotic (unable to produce cell death and bring about IL-1β secretion from macrophages) form with the progression of infection in mice. This switch was associated with downregulation of flagellin expression, one of the key *Salmonella* effectors responsible for bringing about pyroptosis in macrophages. The reduction in flagellin expression did not occur during infection of intestinal epithelial cells with *S.typhimurium in vitro* suggesting that the switch in the phenotye might take place in response to specific cues that the pathogen senses inside mononuclear phagocytes. Considering that caspase 1 deficient mice have been shown to have higher bacterial load during infection with *S.typhimurium*, our results suggested that transformation into a non-pyroptotic phenotype might enable this pathogen to establish a niche inside macrophages. The induction of pyroptosis in infected cells might therefore interfere with establishment of infection with *Salmonella*. To explore this possibility, we first investigated if *Salmonella*-infected cells were amenable to flagellin-induced pyroptosis. Treatement of splenic adherent monocytic cells from *S.typhimurium*-infected mice with flagellin *in vitro* induced caspase-1 – dependent secretion of IL-1β but did not bring about cell death as analysed by release of lactate dehydrogenase (LDH). Unexpectedly, stimulation with flagellin resulted in a dramatic increase in bacterial replication. This effect was not specific to TLR5 activation with flagellin as infected cells stimulated with LPS (TLR4 ligand) or Pam3csk (TLR2 ligand) also showed similar results suggesting that signals generated through TLRs might promote bacterial replication. Significantly, however, administration of flagellin *in vivo* around the time when bacteria begin to switch to a non-pyroptotic phenotype resulted in significant reduction in bacterial numbers in the spleen but did not prevent death of infected animals. Increasing the dose of flagellin did not reduce bacterial burden further and did not change survival of mice. In fact, higher amounts of flagellin generated enhanced inflammatory responses and these mice succumbed faster to infection with *S.typhimurium*. We are currently investigating various modalities by which flagellin could be efficiently delivered inside infected cells in order to bring about potent secretion of IL-1β and more importantly, activate cell death of infected macrophages.

Prohibitin regulates IL-2 secretion from T cells

Vi targets membrane probitin complex to inhibit IL-2 secretion from activated T cells as suggested by our previous studies. Our analysis also showed that in human T cell line, Jurkat, the Vi - recognition complex comprising of membrane prohibitin and its closely related homolog BAP-37 was non-covalently associated with the src kinase $p56P^{lckP}$ (lck). Engagement of this complex with Vi during T cell activation inhibited dephosphorylation of lck at the regulatory Y505 and consequently prevented activation of its kinase activity. Since most of the lck in T cells is bound to co-receptors CD4 and CD8, we explored if prohibitin also remains associated with the CD4-lck complex. Immunoprecipitation of cell lysates from mouse CD4 T cells and mouse T cell hybridoma with anti-CD4 or anti-lck antibody showed that CD4, lck and prohibitin form a trimolecular complex. The complex was not seen with human moncytic cell line THP-1 which lacks lck. These results established the presence of lck-prohibitin complex in normal T cells. Preliminary data suggests that this complex might be dynamic in nature and its composition might get modulated during T cell activation. Defining the mechanism by which this complex regulates T cell responses might offer new insights into TCR signaling.

To understand the role prohibitin in T cell activation, the expression of this molecule was knocked down in Jurkat T cells by RNA interference. The reduction in the expression of prohibitin in these cells was established by reactivity with specific anti-prohibitin antibodies generated in the laboratory. Consistent with the findings from other laboratories, knock-down of prohibitin also led to reduced expression of BAP-37 as the two proteins are present as a heterodimer in cells. The prohibitin knock-down Jurkat produced considerably reduced amounts of IL-2 upon stimulation with anti-TCR antibodies as compared to cells that had been tranfected with vector alone. The reduction was not due to different levels of membrane TCR nor was it due to activation-induced cell death. The inhibition in IL-2 secretion was also observed in response to stimulation with PMA and ionophore.

We have also knocked down expression of prohibitin from humany monocytic cell line U937. The results obtained so far suggest that these cells might secrete higher amounts of chemokine CXCL8 following activation with flagellin indicating that prohibitin might negatively regulate TLR-induced inflammatory responses from monocytes. The mechanism of this regulation is being investigated.

Publication

Original peer-reviewed article

1. Garg R and Qadri A (2010) Hemoglobin transforms anti-inflammatory *Salmonella typhi* virulence polysaccharide into a TLR-2 Agonist. **J. Immunol 184**: 5980-5987.

Molecular basis of B cell responses

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B cells critically control infections caused by extracellular bacteria. We use the extracellular bacterial pathogen *Streptococcus pneumoniae* as a model system to understand B cell responses against antigens in the context of the pneumococcal cell surface. *S. pneumoniae* has several immunogenic protein (T-cell dependent) and polysaccharide (T-cell independent) antigens on its cell surface. Immunization with certain cell surface determinants has been shown to confer antibody mediated protective immunity against *S. pneumoniae*. The research theme of my laboratory is to decipher the molecular and cellular basis of immune responses against protein and polysaccharide antigens in the context of the pneumococcal cell surface.

The main objectives are:

(1) Molecular analysis of immune response to pneumococcal surface antigens

(2) Identification and evaluation of *S. pneumoniae* derived proteins for inclusion in a protein-based pneumococcal vaccine

Molecular analysis of immune response to pneumococcal capsular polysaccharide

All virulent strains of *S. pneumoniae* are encapsulated and loss of capsule renders pneumococci avirulent thus indicating its importance as a virulence factor. The objectives of this study include analysis of interaction of PCP1 with different immune cell types and study their downstream effects. PCP1 is zwitterionic in nature and has been shown to activate T cells. We were interested in investigating whether production of anti-PCP1 antibodies was affected by its zwitterionic nature. BALB/c mice were administered PCP1 in two forms: one, as a soluble molecule and two, in the context of whole heat killed pneumococci (strain ATCC 6301, serotype 1). We observed that the kinetics of anti-PCP1 antibody production were different. Anti-PCP1 antibody responses in PCP1 immunized mice peaked at day 8 while in animals immunized with heat killed ATCC 6301 it peaked at day 28. Both the formulations yielded IgM and IgG anti-PCP1 antibodies. Zwitterionic polysaccharides have been demonstrated to activate T cells in an MHC class II dependent manner. We were interested in finding out whether processing and MHC class II dependent presentation of PCP1 had any effect on the production of PCP1-specific antibodies. We took Tap1-deficient, Ii-deficient and C57BL/6 (as genetic background control) mice for this analysis. Immunization of Ii-deficient mice with PCP1 did not lead to production of anti-PCP1 antibody response while antibody titers were comparable in C57BL/6 and Tap1-deficient mice indicating that processing and MHC class II dependent presentation is important for PCP1 specific antibody production. Acetyl groups have been shown to be important for generating an antibody response against certain polysaccharides. We wanted to know whether this was also true for PCP1. We deacetylated PCP1 by treating it with NaOH and observed that unlike PCP1, deacetylated PCP1 did not induce TNFα production from RAW264.7 cells. In addition, unlike PCP1, deacetylated PCP1 did not bind RAW264.7 cells. To test whether acetyl groups were important for the immunogenicity of PCP1, we immunized BALB/c mice with PCP1 and deacetylated PCP1. Mice immunized with PCP1 made antibodies that recognize PCP1 and deacetylated PCP1. On the other hand, mice immunized with

deacetylated PCP1 did not make antibodies against PCP1 and deacetylated PCP1. These data indicates that deacetylation of PCP1 makes it non-immunogenic.

Identification and characterization of pneumococcal surface proteins as potential protective antigens

The currently licensed polysaccharide-based pneumococcal vaccines have limitations. Alternate strategies are being explored to overcome these limitations. As a result of their serotype-independence, protein-based vaccines could represent the best strategy to prevent pneumococcal infections. Since several pneumococcal virulence factors have been implicated for different diseases caused by *S. pneumoniae*, a vaccine strategy based on using a combination of these proteins is most promising. The aim of this study is to identify and evaluate the protection eliciting ability of pneumococcal proteins that can be included in a protein-based pneumococcal vaccine. An ideal pneumococcal vaccine candidate should be conserved across different serotypes, accessible on the pneumococcal surface for antibodies and should be able to protect against pneumococcal infections. Our group has been making efforts to identify and characterize proteins present on the pneumococcal surface that could serve as protective antigens. One such novel antigen is SP_0845. Sequence analysis of SP_0845 homolog from 36 diverse pneumococcal strains (29 from online databases and 7 from our laboratory) suggested that it is 98.71 ± 0.30% identical at the amino acid level. Western blot analysis of pneumococcal lysates from different strains suggested that it is expressed in *in vitro* and *in vivo* conditions. Immunofluorescence microscopy and whole cell ELISA using anti-SP_0845 antibodies suggested that SP_0845 was present on the pneumococcal surface. Immunization of mice with SP_0845 elicited high titer antibodies. Anti-SP_0845 antibodies promoted uptake of pneumococci by murine peritoneal macrophages in an *in vitro* opsonophagocytic killing assay. Active immunization with SP_0845 protected mice against intraperitoneal challenge with a heterologous pneumococcal strain. These data suggest that SP_0845 is a highly conserved surface exposed pneumococcal protein which protects mice against pneumococcal sepsis. We propose SP_0845 as a potential candidate for development of protein-based pneumococcal vaccine.

Publication

Reviews/Proceeding

1. Madhavi Y, Puliyel JM, Mathew JL, Raghuram N, Phadke A, Shiva M, Srinivasan S, Paul Y, Srivastava RN, Parthasarathy A, Gupta S, Ranga U, Lakshmi VV, Joshi N, Nath I, Gulhati CM, Chatterjee P, Jain A, Priya R, Dasgupta R, Sridhar S, Dabade G, Gopakumar KM, Abrol D, Santhosh MR, Srivastava S, Visalakshi S, Bhargava A, Sarojini NB, Sehgal D, Selvaraj S, Banerji D (2010) Evidence-based national vaccine policy. **Indian J Med Res 131**: 617-628.

Vaccination against oncofetal antigen human chorionic gonadotropin (hCG): A potential immunotherapy for hormone dependent cancers

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Human chorionic gonadotropin (hCG), normally produced by the placental trophoblast during pregnancy, is also aberrantly secreted by a variety of malignant tumors. The presence of hCG (or its subunits) has been linked to chemo- and radio-resistance, increased invasiveness and poor prognosis. Neutralization of hormonal activity may be of potential benefit; anti-hCG vaccination is therefore an attractive option for immunotherapy of tumors secreting or sensitive to gonadotropin.

Potentiation of immune responses against hCG, and assessment of the effect of anti-hCG responses/antibodies on tumor growth parameters. Delineation of the biochemical pathways involved in the hCG-stimulated up-regulation of molecules implicated in tumorigenesis, and investigation of the effects of hCG on immune cells in the tumor microenvironment.

We had previously demonstrated that hCG can act as a growth factor for tumor cells *in vitro*. To assess whether it exhibited similar properties *in vivo*, hCG was exogenously administered to C57BL/6 mice implanted with LL cells; enhanced tumor growth was observed in animals receiving 1µg hCG. As earlier studies had suggested the up-modulation of angiogenic factors by hCG, experiments were designed to delineate the biochemical pathways involved; the MAPK and PKA pathways were found to induce the up-regulation of IL-8 and VEGF in human cells (Colo205 and ChaGo), while the PI3K pathway was implicated in murine LL cells. hCG, as well as hCG-secreting tumor cells, induced the migration of T cells and of macrophages (but not of B cells), a finding whose physiological relevance remains to be established.

Previously, we demonstrated that inclusion of *Mycobacterium w (Mw)* as an adjuvant in anti-hCG vaccine formulations led to enhanced immunogenicity in mice of diverse genetic backgrounds. The elicited antibodies exhibited high affinity to bind hCG and could prevent hCG-receptor interaction; antibody isotyping indicated a Th1 shift. Inclusion of *Mw* also led to enhanced T cell recall responses to the carrier TT as measured by both proliferative responses as well as the secretion of IFNγ. In addition, *Mw* was found to induce the secretion of pro-inflammatory cytokines (TNFα and IL-6) from purified murine macrophages. In addition to TNFα and IL-6, IL-12p70 and KC (murine IL-8) were also elevated in *Mw*-stimulated bone marrow derived dendritic cell cultures from BALB/C and C57BL/6 mice; surface expression of activation markers such as CD83 and CD86 was also up-regulated. These results indicate that *Mw* induced the maturation of antigen presenting cells, which could conceivably result in the observed enhanced levels of T cell activation and the consequent stimulation of antibody responses.

Anti-hCG antibodies generated during these studies specifically inhibited the growth of the tumor cells *in vitro*; addition of hCG induced a reversal of this effect. Using two syngenic murine models (SP2/O myeloma cells in BALB/c mice and B16F10 melanoma cells in C57BL/6 mice), it was demonstrated that immunization with either βhCG-TT or *Mw* caused a reduction in tumor volume. Significantly, the combination of βhCG-TT and *Mw* acted synergistically to enhance survival and reduced tumor incidence, re-iterating results previously obtained with LL cells in C57BL/6 mice.

Transgenic (TG) mice expressing βhCG (obtained under a collaborative program with the Imperial College, London) were also employed to assess the potential of anti-hCG vaccination as an immunotherapeutic strategy. These animals express ovarian, pituitary and mammary tumors with high penetrance. The ovaries appear enlarged and demonstrate the presence of extensive corpora lutea, enlarged vessels and a few hemorrhagic cysts. We had previously demonstrated that anti-hCG immunization in transgenic mice was able to break the tolerance against the "self" molecule. The age-dependent increase of body weight (resulting from the deposition of fat in the abdominal region) seen in non-immunized animals was prevented. In addition, ovarian morphology appeared normal, with follicles of different sizes present along with relatively fewer corpora lutea and an absence of cysts. Immunization also led to a decrease in prolactin levels, indicating the capability of elicited anti-hCG antibodies to reverse hCG-induced pituitary adenomas (caused by high progesterone levels) in TG mice.

Tumor cells incubated with sera from βhCG TG mice demonstrated enhanced transcription and expression of VEGF (an angiogenic factor), KC (murine IL-8; an angiogenic and autocrine growth factor) and MMP-9 (a protease associated with matrix degradation). Addition of anti-hCG antibodies negated these effects and sera from hCG-immunized TG mice were incapable of eliciting these molecules, as were sera from non-TG animals.

While these results further corroborate the role of βhCG in tumor progression, emerging data also indicates a role for hCG in chemo-resistance; the molecule appeared to protect tumor cells from drug-induced apoptosis, possibly by up-modulating levels of suvivin, livin, Bcl2 and c-FLIP.

Our results indicate novel mechanisms by which hCG can promote tumor progression and provide, for the first time, a scientific basis for observations linking the presence of hCG with poor patient prognosis. Immunization studies indicate that multi-pronged anti-tumor vaccination protocols activating both innate and adaptive immunity have potential as clinically relevant strategies for immunotherapy of gonadotropin-secreting tumors.

Publications

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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 varieties of antigens such as Tetanus Toxoid (TT), Hepatitis B surface antigen (HBsAg), viral and carbohydrate (Vi polysaccharide and *S. pneumonia* 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 substitute.

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.

(A) 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 flagellin (ligands

for TLR5) or soybean lectin (ligands for CLR) and evaluated for antibody response in mice. It was observed that coating of the polymeric particles with ligand recognizing different PRR resulted in improved antibody response. Detailed investigations on the interaction of these modified particles with macrophages and dendritic cells are currently under investigation. Attempts were being made to formulate polymer particles using spray drying. Immunogenicity studies with spray dried polymer alum particle and alum particles entrapping antigen are in progress.

PLA polymeric particles entrapping Typhoid vaccine (Vi polysaccharide) and capsular polysaccharide from *S. pneumoniae* (PCP) were evaluated for antibody titer in mice. Surprisingly nanoparticle based immunization gave rise to better antibody titer and protected the mice in challenge experiments. Co-entrapment of TT along with Vi polysaccharide did not induce better antibody response. Anti –TT antibody titer from these formulation were low. Interaction studies of these particles with macrophages indicated that presence of Vi inhibits phagocytosis. Antibody titer observed with immunizing particle entrapping polysaccharide was better than that achieved from particle entrapping PCP and PsaA/PspA.

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

Last year we reported a process of making polymer membranes for wound healing using surfactant based fusion of polymer particles (ARTSKINII™). Large porous polymeric particles (> 200 µm) made from PLA polymers were formulated for growth of animal cells *in vitro*. Due to the biodegradable nature, these polymeric scaffolds support three dimensional growths of animal cells *in vitro* and thus can be used as a model for cytotoxic evaluation of a drug or drug delivery system *in vitro*. Different varieties of animal cells such as MCF-7, NIH-3T3, fetal fibroblast, Vero cells, mouse melanoma (B16 F10) have been successfully grown on the porous macroparticles or on scaffold made from polymer particles. Degradation of polymer scaffold implanted in mice has been analyzed. Polymer particles entrapping antibiotics have been standardized to be fused to form membrane for wound healing.

(C) 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.

In spite of the availability of a large amount of literature on inclusion body formation, the mechanism of inclusion body formation and the nature of intermediates involved in different types of inclusion body aggregates are still not clear. We tried to elucidate the kinetics of inclusion body formation inside the *E. coli* cells using bacterial L-asparaginase II and Human Growth Hormone (hGH) as model proteins. Kinetics of inclusion body formation and their morphological and structural characteristics were studied by transmission electron microscopy, size distribution analysis, proteolytic degradation, solubilization, Congo Red and Th-T dye binding assays and FTIR spectroscopy of inclusion bodies isolated from culture harvested at different time points after induction. Results obtained showed stark differences in kinetics of inclusion body formation and structural properties of hGH and asparaginase inclusion bodies. The seeding and growth behavior of hGH inclusion bodies were found to be sequential, kinetically stable and more organized in comparison to those of asparaginase inclusion bodies. They also showed higher resistivity to denaturants and proteinase K degradation. Both hGH and asparaginase inclusion bodies showed binding with amyloid specific dyes and higher proportion of β-sheet in comparison to respective native proteins. It was also observed that for classical inclusion bodies, the size of inclusion bodies

increases with induction time where as for non-classical inclusion bodies the size remain unchanged. Non-classical inclusion bodies were soft and could be solubilized using low concentration of chaotropes. Thus depending on the nature of inclusion body aggregates, they need different solubilization process to improve the recovery of bioactive protein.

Publications

Original peer-reviewed articles

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2. Madhusudan S, Panda AK, Dhanapal CK, Mohanta GP, Manavalan R and Manna PK (2010) Formulation, in vitro and in vivo evaluation of glipizide loaded ethyl cellulose microparticles. **Journal of Chemical and Pharmaceutical Sciences. 3:** 55-61.

3. Rafi M, Singh SM, Kanchan V, Anish CK and Panda AK (2010) Controlled release of bioactive recombinant human growth hormone from PLGA microparticles. **J Microencapsul 27:** 552-560.

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Review/Proceeding

1. Panda AK (2011) Induction of anti-tumor immunity and T-cell responses using nanodelivery systems engrafting TLR-5 ligand. **Expert Rev Vaccines 10**: 155-157.

Patent

1. CK Anish, Dinesh G Goswami and Amulya K Panda, Spray dried formulation of antigen containing alum or antigen entrapped polymer particles with surface coating of alum (2256/DEL/2010).

Analysis of anti-lymphocyte autoimmune antibody responses

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Humans are plagued by a large variety of autoimmune diseases. Though etiologies remain unknown for most diseases, the pathological implications of autoreactive immune responses are the subject of intense investigation. Systemic Lupus Erythematosus (SLE) is a prototypical non-organ specific autoimmune disease. The disease 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, with some reactivities associated with specific pathologies. Drawing correlations between distinct disease manifestations and individual autoimmune reactivities remains a high priority.

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. Apoptotic debris may therefore be the source of antigenic material that initiates autoimmune disease. It is conceivable that early immune responses to apoptotic cells in individuals genetically prone to enhanced autoreactivity could influence pathological outcomes.

This project seeks to investigate the consequences of the aberrant recognition of cell death in systemic autoimmune diseases. Apoptotic cells would serve as targets for the development of monoclonal antibodies from SLE patients and autoimmune-prone mice. Antigenic targets would be determined. The impact of such antibodies on the uptake of apoptotic debris and their influence on elicited cytokines would be assessed, and effects on the migratory behaviour of phagocytes would be evaluated. Antibody variable regions would be sequenced in order to determine whether the humoral recognition of death in a pathological context is necessarily an antigen-driven process. The postulate that anti-idiotypic antibodies could contribute to epitope spreading would be investigated. The potential influence of hemoglobin (a potentially pro-inflammatory molecule released into an environment already inflammatory as a result of autoimmune processes) and gonadotropin (administration of which have been shown to ameliorate organ-specific autoimmune diseases) on systemic autoimmune diseases would be investigated.

Previous work had established that immunization with apoptotic cell-specific antibodies in autoimmune-prone animals led to an expansion in the autoantibody repertoire. Significantly in the context of lupus, antibodies non-reactive to double-stranded DNA (dsDNA) could elicit antibodies that bound dsDNA. Results suggest that the purified anti-idiotypic fraction represents a sub-population of anti-self autoantibodies present in serum, a finding strongly indicative of the possibility that further down-stream activation of the idiotypic network contributed to anti-self responses. For example, while antibodies in the serum of animals immunized with Antibody P1B3 bound predominantly Sm, La, Ro60 and the RNP A protein, purified anti-idiotypic antibodies bound only Sm. IgG antibodies recognizing apoptotic cells also induced the generation of inflammatory cytokines from phagocytic cells, a characteristic that could add to their pathogenic potential. Immunizations carried out using anti-self

monoclonal antibodies that did not recognize antigens/epitopes exposed on the surface as cells died too resulted in antigen spreading; interestingly, elicited antibodies exhibited specificity for apoptotic cells, further supporting observations that antibody immunization impacts upon the specificity of elicited auto-antibody responses.

Previous work had demonstrated that lupus-prone NZB/W (F1) animals harboured antibodies to murine Hb (mHb) in sera and an age-related sequestration of such antibodies was observed in the kidneys and lungs. Similar kinetics of anti-mHb humoral reactivity was also demonstrated in the sera and organs of lupus-prone NZM 2410 mice. The peptide representing amino acids 110-119 of Hbβ appeared to be a dominant epitope in murine sera and organ eluates, as well as in the human autoimmune sera obtained from the Center for Disease Control and Prevention previously shown to be Hb-reactive. Additionally, the peptide representing 110-119 of Hbα chain appeared to be preferentially recognized by antibodies in murine sera.

The presence of IgG anti-Hb antibodies in the serum and organ eluates of autoimmune prone animals was strongly suggestive of T cell involvement. However, a significant or preferential loss of T cell tolerance to Hb or individual peptides representing its primary sequence was not observed. Previous work had established that anti-Sm and anti-Hb autoantibody responses were linked in autoimmune-prone animals; immunization with the former led to the appearance of autoantibodies to the latter. Experimental evidence was sought for the postulate that association between Hb and various autoantigens might be a mechanism by which T cell help could be recruited for the generation of anti-Hb antibodies. In solid phase binding assays, Hb was demonstrated to interact with Sm, Ro52, Ro60, La and the RNP-A protein to varying degrees, while Surface Plasmon Resonance analysis revealed that murine hemoglobin interacted with the autoantigens La (Kd $\cong 4.84 \times 10^{-8}$ M), RNP A (Kd $\cong 4.58 \times 10^{-8}$ M) and RNP 68k (Kd $\cong 1.16 \times 10^{-8}$ M). The functional consequences of these associations are being determined.

Experiments were initiated to assess whether the presence of free Hb in diseases characterized by erythrocyte lysis can impact upon immune homeostasis in other ways. Much like LPS, Hb induced the maturation of bone marrow-derived dendritic cells (BMDCs) from NZB/W (F1) mice as measured by the enhanced appearance of cell surface CD80 and CD86. Equivalent Hb-induced enhancements were not observed in BMDCs derived from non-autoimmune prone BALB/c mice. Additionally, unlike LPS, Hb induced the increased secretion of some signature pro-inflammatory cytokines from BMDCs derived from autoimmune mice. Whether the preferential effects of Hb in autoimmune-prone mice leads to a modulation in antigen presentation capability is currently being investigated.

While the ameliorating effects of human chorionic gonadotropin (hCG) have been described in the context of organ-specific auto-immune disease, its effects on systemic autoimmunity have not been evaluated. When hCG was administered to autoimmune-prone animals, a modulation of anti-self reactivity was observed, associated with an increase in reactivity towards cell-surface moieties. While evidence suggested some increases in reactivity towards commonly-targeted ribonucleoprotein autoantigens, reactivity towards a panel of lipids was significantly enhanced in treated animals; reactivity towards dsDNA was not significantly altered. Most of these effects appeared to be partially dependent on the presence of the ovaries. Further, non-autoimmune prone animals either transgenic for the hCGβ subunit or exogenously administered the hormone did not exhibit these responses.

Due to its primary role in the sustenance of pregnancy, hCG has been traditionally viewed as an immunosuppressive molecule. However, its roles in mitogen-induced proliferation and cytokine synthesis in the context of systemic autoimmunity have not been evaluated. While no consistent effects were observed in the LPS, concanavalin A-induced or mixed leucocyte-associated splenic proliferative responses, hCG appeared to synergize with plate-bound anti-CD3 antibody-induced proliferation. Such synergy was also observed in the secretion of IFNγ, TNFα, IL6 and IL10, cytokines associated with lupus pathology.

In an effort to discern the immunomodulatory effects of hCG, its effects on dendritic cell maturation (induced in response to TLR ligands) will be ascertained on cells derived from autoimmune and non-autoimmune murine strains and from humans. The consequences of hCG induced anti-phospholipid responses will be ascertained.

Publications

Original peer-reviewed article

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Patent

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The 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

The basic problem with type 1 diabetes is that by the time patients report to the clinic, most of their insulin producing beta cells are already destroyed. So, to inhibit autoimmune responses, we had reported earlier *in-vitro* and *ex-vivo* sensitivity of potentially immunotherapeutic/prophylactic peptides encapsulated microparticles on cell lines, Balb/C and C57Bl6 mice. We tagged the microparticles with a targeting moiety and reported the targeting of the microparticles to pancreatic cells /pancreas in

in-vitro, ex-vivo and in-vivo animal model. While this approach would help in inhibiting autoimmune responses, there may not be enough insulin producing beta cells left in the pancreas. So the patients need to be replenished with Mesenchymal stem cells (MSCs) or MSC differentiated to produce insulin.

Treatment of streptozotocin (STZ) induced diabetic mice with MSCs.

To check whether MSCs or bone marrow cells (BMCs) could take care of the insulin production in mice suffering from diabetes, we induced diabetes in Balb/c mice using streptozotocin and treated them with MSCs or BMCs. Mesenchymal stem cells were cultured from bone marrow of Balb/C mice. Three groups of mice were subjected to STZ induced diabetes and one group was kept as control. One group of STZ induced diabetic mice was kept as diabetic control, second group got bone-marrow cells and the third group was given MSCs at a concentration of $1x10^6$ cells per week for four weeks, ten days after establishment of diabetes. Blood sugar levels were monitored routinely till 58^{th} day when none of the diabetic controls survived i.e. the last one died. Survival curve was plotted for all the groups including controls. Normal healthy controls survived through out the period i.e., beyond 58 days as was expected. 66% of the STZ controls who did not get MSCs or BMCs survived till day 29, 33% survived till day 56 and none survived beyond day 58. However, 75% of the BMC treated mice survived even after day 58 and 33% of the MSC treated mice survived beyond day 58. Even though the mice treated with BMC or MSC survived, their blood glucose levels fluctuated in a cyclic manner, suggesting that treatment with BMCs or MSCs is not enough and that we need to differentiate MSCs into insulin producing cells.

Characterization of mesenchymal stem cells for further manipulations

Mesenchymal stem cells were characterized by flow cytometry using markers for mesenchymal and hematopoetic stem cells. Results showed Positive staining for CD29, CD73 And CD44 and negative staining for hematopoetic markers CD11b, CD34 and CD45. To further characterize these cells at different passages, expression of stem cell transcription factors were checked using real time PCR for 15 consecutive passages. We checked for the expression of: Oct4 (marker for self renewal and undifferentiated stem cells and tumor cells), Nanog (marker for self renewal and pluripotency of stem cells), FoxD3 (marker for undifferentiated stem cells), FoxC2 (marker involved in cancer metastases) and Sox2 (marker for self-renewal of undifferentiated stem cells). Figure 1 shows the expression of these transcription factors at different passages of MSCs

As is clear from figure 1 most of the markers for pluripotency and stemness are enhanced at passage 3, FoxC2 which is a marker involved in cancer metastases, is not enhanced during any of the passages. We first wanted to check if we could differentiate MSCs into adipocytes (Figure 2), and we could indeed do so. We are now standardizing the protocol to differentiate MSCs into insulin producing cells. Preliminary results show that transcripts of Insulin 1, Neurogenin 3, and Beta 2 were upregulated in the cells differentiated to produce insulin in high glucose medium. However, we still need to fine-tune these protocols to get insulin production in the medium.

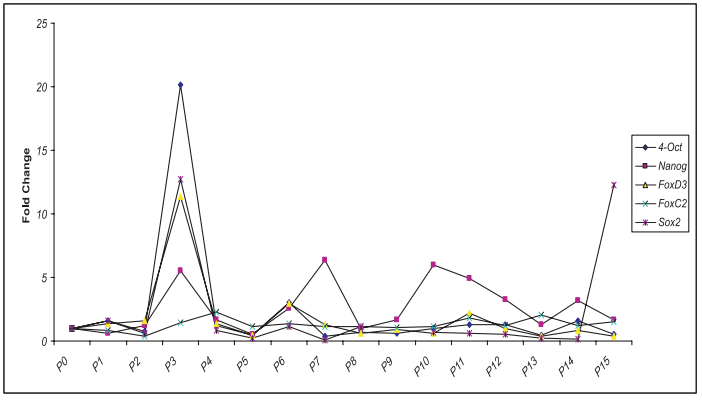

Figure 1. *Expression of Oct4, Nanog, FoxD3, FoxC2 and Sox2 in Mesenchymal stem cells for 15 consecutive passages.*

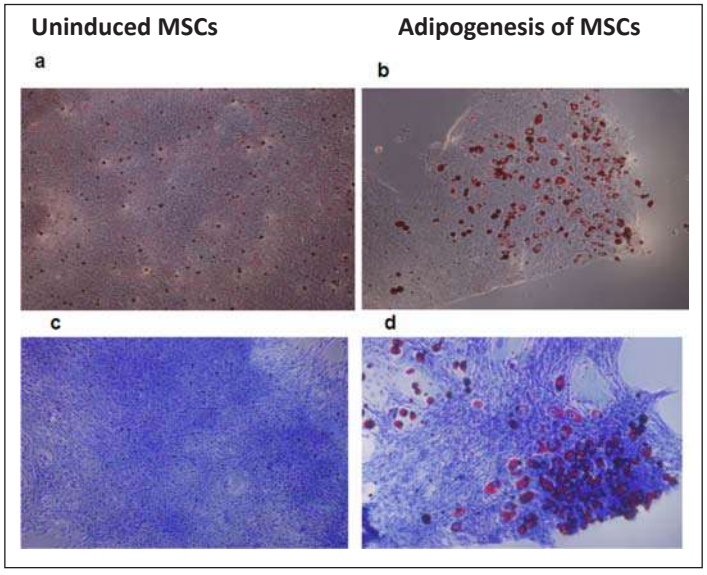

Figure 2 Mesenchymal stem cells **a**.*&* **c.***control MSCs not induced to differentiate into adipocytes,* **b. & d.** *MSCs induced to differentiate to adipocytes. Red blobs (lipids) are stained with Oil red O dye. Pictures without counter stain in* **a. & b**. *and counter stained with methylene blue in* **c. & d**.

Vitiligo

Vitiligo is a depigmenting disorder of the skin characterized by the loss of functional melanocytes. Although the exact aetiology is not understood, autoimmunity is thought to be a crucial deterministic factor. A recurring theme of several autoimmune disorders is the aberrant presentation of self antigens to the immune system that triggers downstream perturbations. Here in this study, we have examined role of alleles of Human Leukocyte Antigen (HLA) class-I and class-II loci (using molecular methods) to delineate vitiligo manifestation in two distinct populations. Our studies on 1404 vitiligo cases from North India and 902 controls is the largest study reported so far. We have also done a replication study in Gujarat population on 355 cases and 441 controls. The data is being analysed currently and will be reported.

Publication

Original peer-reviewed article

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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 not only with *M.leprae* but also 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 type-1 acquired 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

To characterize the local immune response in the lung in *MIP* immunised / BCG immunised / Control group at different time points after infection with *M.tb* (acute phase / early chronic phase / late chronic phase), infiltration of different type of immune cells in the infected lungs were analysed by phenotypic analysis of single cell suspension of lungs. Up regulation of MHC-I expression and higher infiltration of lymphocytes was observed in the *MIP* treated group at early acute phase of the disease as compared to the other groups. To analyse and compare the immune cell composition of the granuloma in different groups, lung specimens were collected at different stages of the disease and studied by immunohistochemistry. Higher number of MHC-II expressing cells and CD8+ve cells were found in the granuloma of *MIP* immunized group at early chronic phase. To investigate the functional status of immune cells in infected lungs of different experimental groups, expression of different cytokine genes was evaluated by RT PCR. Higher expression of IFN-γ was observed in early acute phase of infection in immunized groups as compared

to control. Presently total cytokine profiling at different stages is being done.

Immunotherapeutic potential of *MIP* and the underlying mechanisms in mouse tumor model

Immune response in the tumor microenvironment is most crucial factor to decide the prognosis of the disease. In general higher infiltration of immune cells correlates with better prognosis. Four week after tumor cell implantation, immune cells from the tumor mass were isolated and analysed from MIP treated and control group. We observed higher frequency as well as higher expression of phenotypic activation markers on macrophages and T cells of MIP treated mice. Upregulation of activation markers also reflected in their enhanced functional activity. In addition to induction of IFN-γ, TNF-α and IL-12 secretion, higher NK cell and CD8+T cell cytotoxic activity was observed. An interesting finding of this study was that along with activation of antigen presenting cells, NK cells and T cells, peritumoral injection of MIP also resulted in significantly less number of tumor infiltrating regulatory T cells. Proportion of regulatory T cells in the spleen was not significantly different in the MIP treated and control group and was about 20% of the total CD4+ cells. Strategy that combines modulation of suppressive factors along with the activation of pro-inflammatory Th1 type of response within the tumor micro environment could be a potentially effective approach which could be combined with chemotherapy to combat cancer successfully. Hence, we explored the complementary approach of combination of immunotherapy and chemotherapy to improve the anticancer efficacy of the both. We observed synergistic activity of cyclophosphamide and MIP treatment.

Synthesis and evaluation of chemical conjugate and nanoparticles of paclitaxel and LPS derivative

Chemotherapy sometime fails to achieve its goal. Hence, multi-dimensional treatment modality is desired. Tumor suppresses the immune response at its local microenvironment to facilitate its growth, leading to cancer progression and metastasis. Immunotherapy stimulates the immune system and helps to induce an effective anti-tumor response. Combined chemo-immunotherapy approach could exert a synergistic effect. Cell death induced by chemotherapy enhances cross-priming of immune cells.

Paclitaxel, a promising anti-cancer drug was conjugated with LPS derivative in an attempt to make the drug water soluble and at the same time possibly increasing the anti-tumor efficacy by combining the chemotherapeutic and immunotherapeutic activities.

We also prepared PLGA nanoparticles (NP) encapsulating paclitaxel and SPLPS by double emulsion method. NP dosage form improved the therapeutic index of the drug by modifying its pharmacokinetics. Encapsulation of SP-LPS was found to be in suboptimal amount in NPs prepared by double emulsion method hence, NPs were prepared by o/w single emulsion method using P-LPS. By above modification encapsulation of LPS derivative increased from 21% to 62%. In the conjugate and in the nanoparticles, bioactivity of paclitaxel and SP-LPS was found to be intact as confirmed by the direct cytotoxicity and immunostimulatory studies. In the co-culture experiments which mimic the *in-vivo* situation, fluorescently labeled cancer cells were incubated with unlabeled splenocytes and given treatment with commercial taxol, paclitaxel-LPS conjugate and nanoparticles. Significantly higher amount of cancer cell death was detected with conjugate and nanoparticle treatment as compared to taxol alone, confirming combined chemo-immunotherapeutic activity. *In-vivo* anti-tumor activity of both conjugate and nanoparticles was studied in mice implanted with B16F10 melanoma cells. Tumor growth was significantly less in both conjugate and NPs treated mice as compared to equivalent amount of taxol treated group. To understand the mechanism of tumor regression and to analyze the involvement of immune cells, tumor infiltrating mononuclear cells were isolated and analyzed for their frequency and activation status. Higher infiltration of macrophages, DCs and T cells were seen in the conjugate and nanoparticle treated

mice. These infiltrated immune cells were also found to be in an activated state as confirmed by expression of different activation markers.

Publication

Original peer-reviewed article

1. Roy A, Singh MS, Upadhyay P*, Bhaskar S* (2010) Combined Chemo-immunotherapy as a Prospective Strategy to Combat Cancer: A Nanoparticle Based Approach. **Mol. Pharmaceutics 7**: 1778–1788. . * Corresponding authors

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

We found that key genes necessary for spermatogenesis like stem cell factor, GDNF etc. began robust expression at 12-days of age in rat, presumably leading to the initiation of spermatogenesis. We wanted to find out some other less known genes with Sc specific expression and probable role in regulation of spermatogenesis.

Since hormones are known to be secreted in a pulsatile manner *in vivo*, we treated cultured rat Sc with pulses of FSH and T together to mimic the *in vivo* situation. The initial objective of this pulsatile treatment was to find out a particular regime of hormonal treatment (both FSH and T in combination) where we would be able to detect the maximal transcription of hormone responsive genes in a reproducible manner. For this purpose, Sc were cultured from rat testes before and after the initiation of Gc differentiation (i.e. 5-days and 12-days of age respectively) and were treated with hormones by periodic pulses for the same period of time.

Differentially expressed genes from Sc of these two age groups were identified by microarray analysis. Feature extracted data was analyzed using GeneSpring GX v 10.0.1 software from Agilent terchnologies. 5A and 5B (two separate cultures of 5-days-old Sc treated with pulsatile FSH and T (FT) for 11hr) and 12A and 12B (two separate cultures of 12-days-old Sc treated with pulsatile FT for 11hr). In Experiment I , gene expression of 12A and 12B were

normalized against 5A and in Experiment II , gene expression of 12A and 12B were normalized against 5B. Exp. I and Exp. II were compared amongst each other and commonly up or commonly down regulated genes were listed; total 1429 and 1584 genes were found to be up and down regulated in 12-days-old rat Sc compared to that of the 5-days-old rat Sc, respectively.

We, for the first time have, done a microarray analysis between undifferentiated and differentiating Sc (5-days and 12-days-old rats) in order to identify genes responsible for Sc differentiation followed by the initiation of spermatogenesis. A single color experiment, (cRNA labeled with Cy3) was performed in order to evade dye bias. Signals generated from 5-days-old Sc were used for normalization to measure the level of gene expression. One fold change (in log2 scale) in the level of expression were considered as the minimal limit. Further quality control of normalized data was done using correlation based condition tree to eliminate bad experiments. Normalized data from 5-days Sc RNA was used as control for normalizing data from 12-days Sc. Genes showing > 1 fold change in log2 scale were compiled as differentially regulated genes. Some of the genes (up or down regulated in either 5-days or 12-days of age) were selected from the array list on the basis of a probable indication that they might have a role either in Sc differentiation or directly linked with Gc development.

Genes like ABP, inhibin β-B, transferrin, Dmrt-1, Pde4d etc were detected by microarray to be upregulated in 12-days-old Sc. SCF and GDNF, essential for Gc differentiation were found to be upregulated in 12-days-old Sc compared to younger ages as revealed by *real time PCR analysis*.. The expression of these genes was also found to be high in 12-days-old Sc in the array list, alluding to the authenticity of the microarray data.

Occludin and Claudin-11 (essential components of Sc-Sc tight junctions to establish blood testis barrier) were also upregulated in the array data. We found claudin11 to be progressively upregulated with age in rats. Sox-9 which is known to be essential for early testicular differentiation and development was also found to be upregulated in 12-days of age in the microarray data. In down regulated list of genes, MIS and GATA-4 which are known to be down regulated in differentiated Sc, were missing in the array which used 12 days old Sc mRNA.

Transgenic sperm selection in the ejaculates of large animals

Although we are planning to use testicular route of transgenesis in large animals (monkeys, buffaloes etc.), we envisage a major problem in getting a transgenic offspring because several of the sperm in ejaculate are normal and these animals give birth to only one offspring at a time. Hence, we have designed constructs where the desired gene is cloned under specific promoter along with a marker gene (like GFP or RFP) having mitochondrial localization signal or nuclear localization signal. This would help in sorting transgenic sperm using FACS sorter and use fluorescent sperm for artificial insemination with an objective to assure production of a transgenic animal.

Other Endocrinological research

We had supportive role in some studies of signal transduction on glucose metabolism by insulin. These are related to NF-κB mediated lipid-induced fetuin-A expression in hepatocytes that impairs adipocyte function effecting insulin resistance and some studies establishing involvement of PKCε in lipid induced insulin resistance.

Publications

Original peer-reviewed articles

1. Dasgupta S, Bhattacharya S, Biswas A, Majumdar SS, Mukhopadhyay S, Ray S and Bhattacharya S (2010) NF-κB mediates lipid-induced fetuin-A expression in hepatocytes that impairs adipocyte function effecting insulin resistance. **Biochem. Journal 429**: 451–462.

2. Dasgupta S, Bhattacharya S, Maitra S, Pal D, Majumdar SS, Datta A and Bhattacharya S (2011) Mechanism of lipid induced insulin resistance: Activated PKCε is a key regulator. **Biochimica et Biophysica Acta 1812**: 495-506.

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. Therefore, our study sought to determine the functional genomics of germ cell associated proteins in cancer biology. 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.

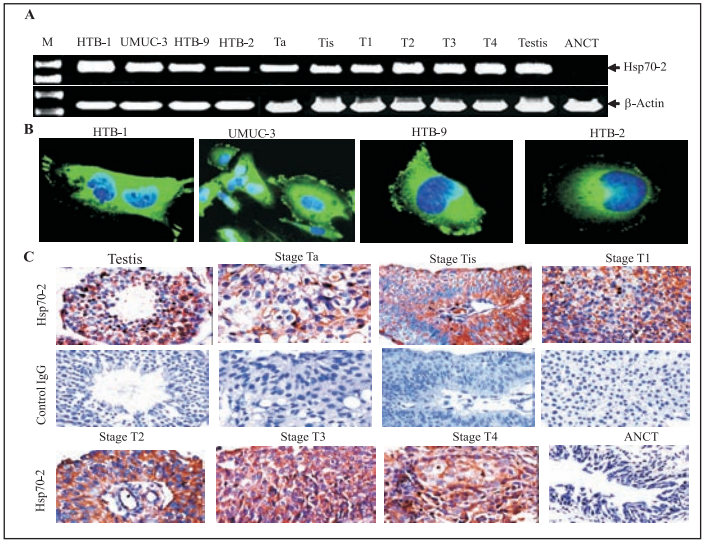
Bladder cancer is the second most common malignancy of the genitourinary tract and the fourth major cause of death among men. Most urothelial carcinomas (~80%) present as superficially invasive tumors, which include Ta (non-invasive) or T1 (lamina propria invasive) tumor. Muscle-invasive cancer (T2-4) has a much less favourable prognosis than superficial cancer, despite aggressive multimodal therapy. Due to the unfavourable prognosis of muscle-invasive cancer, there is a need for developing markers that can identify superficial cancers with a high risk of progression. The characterization of such type of marker will help to detect the life-threatening invasive bladder cancer and thus improve the prognosis of this disease.

Heat shock proteins (Hsps) are a group of evolutionarily highly conserved chaperone proteins induced by a variety of environmental and pathophysiological stresses and are over-expressed in a wide range of human cancers. The human Hsp70 family has at least eight homologous proteins having different cellular localization and expression pattern. Hsp70-2 (HspA2) is abundantly expressed in testis and shows rare or no expression in other tissues. In the present study, we report the expression of Hsp70-2 in superficially invasive and muscle-invasive urothelial carcinoma of bladder cancer patients, correlating its expression with the pathologic and clinical data. We further evaluated its potential role and provide evidence for its association in cell migration, invasion, and tumor growth in urothelial carcinoma cells.

Hsp70-2 expression in urothelial carcinoma cells and clinical specimens

RT-PCR analysis revealed *Hsp70-2* expression in all urothelial carcinoma cell lines tested (Fig. 1A). However, higher *Hsp70-2* expression was found in HTB-1 and UMUC-3 as compared with HTB-9 and HTB-2. The size of the PCR product was same as in testis (Fig.1A)

Figure 1



RT-PCR analysis further revealed that *Hsp70-2* mRNA expression was detected in 70% of the patients with superficially invasive tumors, and 90% of the patients with muscle-invasive tumors not in ANCT as shown in representative tissue specimens in Figure 1A. Statistical analysis revealed that a significant association was found between *Hsp70-2* mRNA expression and tumor stages by Pearson Chi-square test (P = .008). *Hsp70-2* expression was detected in 72% patients with low grade and 88% patients with high grade urothelial tumors. Interestingly, significant association was also found between *Hsp70-2* mRNA expression and histological grades (low and high grade) by Pearson Chi-Square test (P = .028). It is important to note that *Hsp70-2* mRNA expression was significantly correlated with tumor stages and grades.

All urothelial carcinoma cells revealed strong cytoplasmic Hsp70-2 protein localization in fixed and permeablized cells (Fig. 1B). Further, western blot analysis revealed higher Hsp70-2 protein expression in HTB-1 and UMUC3 cells as compare to HTB-2 and HTB-9 cells.

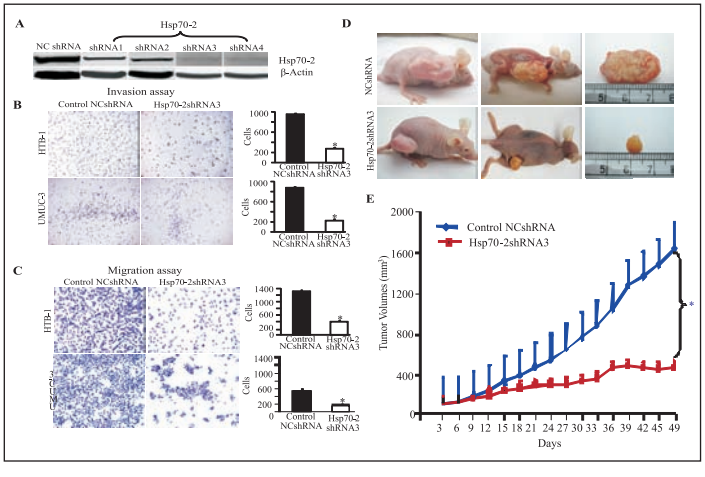
Hsp70-2 protein expression in human bladder cancer specimens

In bladder cancer specimens, Hsp70-2 expression was observed 70% of superficially invasive urothelial carcinoma and 90% of muscle-invasive urothelial carcinoma but not in ANCT as shown in Figure 1C. No reactivity was observed in serial tissue sections incubated with control IgG and in ANCT specimen. In addition, Hsp70-2 expression was detected in 72% patients with low grade and 88% patients with high grade urothelial carcinoma.

Down-regulation of Hsp70-2 decreases cell growth, colony formation migration and invasion of urothelial carcinoma cells

To determine the role of Hsp70-2 in urothelial carcinoma cells, we used RNAi strategy to down-regulate the Hsp70-2 expression. Four independent sets of Hsp70-2-specific shRNAs were evaluated and Hsp70-2shRNA3 and Hsp70-2shRNA4 showed a greater impact as compare to Hsp70-2shRNA1 and Hsp70-2shRNA2 on ablation of Hsp70-2 protein expression in Western blot analysis (Fig. 2A). These results indicate that Hsp70-2shRNA3/Hsp70-2shRNA4 effectively down-regulated Hsp70-2 protein expression in HTB-1 as compared to control NCshRNA. Hence, the subsequent experiments were restricted to Hsp70-2shRNA3.

Figure 2



Subsequently, we examined the effect of Hsp70-2shRNA3 on the growth of HTB-1 and UMUC-3 cells. Knockdown of Hsp70-2 protein resulted in growth retardation and colony forming ability of both HTB-1 and UMUC-3 cells. Further, knockdown of Hsp70-2 led to the inhibition of invading potential of HTB-1 and UMUC-3 cells (Fig. 2B) by 71% and 70% respectively and histogram shows that a significantly lower number of cells ($P < .0001$) migrated through inserts. Subsequently, transwell migration assay revealed 70% and 75% inhibition in motility of Hsp70-2shRNA3 transfected HTB-1 and UMUC-3 cells respectively (Fig. 2C) and histogram shows that a significantly lower number of cells ($P < .0001$) migrated through inserts. Subsequently, Hsp 70-2 knockdown also revealed decreased motility of Hsp70-2shRNA3 transfected HTB-1 cells in wound healing assay. The wound was not closed in Hsp70-2shRNA3 transfected HTB-1 cells even after 48 h, whereas cells transfected with control NCshRNA successfully closed the wound within 24 h. Our findings revealed an essential role of Hsp70-2 in the tumor cell motility and invasion, a key property of the aggressive cancer phenotype.

Growth inhibition of HTB-1 urothelial carcinoma cell xenograft with Hsp70-2 shRNA

We examined the effect of Hsp70-2shRNA3 treatment on the *in vivo* growth of bladder tumor in xenograft-nude mouse model. Interestingly, mean tumor volumes and weight in Hsp70-2shRNA3 treated mice were substantially reduced by day 49 ($P < .0001$) in comparison with those mice treated with control NCshRNA (Fig. 2D & E).

Our results revealed that silencing the Hsp70-2 by siRNA resulted in inhibition of cellular proliferation, colony forming ability, migration, and invasion. These results further confirm the significant role of Hsp70-2 expression in bladder tumor growth and indicate that Hsp70-2 may be a molecular target for effective treatment for urothelial carcinoma of bladder.

Publications

Original peer-reviewed articles

1. Garg M, Kanojia D, Saini S, Suri S, Gupta A, Surolia A and Suri A (2010) Germ Cell Specific Heat Shock Protein 70-2 is expressed in Cervical Carcinoma and is Involved in Growth, Migration and Invasion of Cervix Cells. **Cancer 116**: 3785-3796.

2. Kanojia D, Garg M, Saini S, Agarwal S, Kumar R and Suri A (2010) Sperm associated antigen 9 expression and humoral response in chronic myeloid leukemia. **Leukemia Research 34**: 858-863.

Study on 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 damaged 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 damaged organs besides bone marrow tissue. Thus, themes of the research in Stem Cell Biology Laboratory are study on (i) BM niche and expansion of HSCs, (ii) adult stem cells plasticity, tissue regeneration, study on reprogramming and (iii) ovarian cancer stem cells and metastasis.

We intend to pursue molecular analysis of HSCs niche, its migration during development and in other physiological processes, and adult stem cells plasticity. The specific objectives are as follows:

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 mechanism for differentiation of BM progenitor cells into hepatocytes.
4. Study of metastasis of ovarian cancer cells in syngenic mouse model.

A. Hematopoietic stem cells: Marrow niche and regulation

During regeneration of bone marrow in mouse following irradiation and transplantation, it was revealed that donor cells proliferated rapidly than the recipient cells. Donor-derived HSCs (LSK) number was found to increase with time, indicating that they can also divide symmetrically. This was further analyzed by BrdU uptake by LSK cells; in donor compartment more cells incorporated the analogue. Again, the cell cycle analysis of donor cells confirmed that LSK population was highly proliferating in the initial phase of marrow regeneration, as the number of quiescent cells (G_0) was significantly declined. Interestingly, 15 days of post-transplantation G_0 cells fraction was increased. At the niche, HSCs are supposed to be maintained in quiescent state. The reason for withdrawal of a significant fraction of LSK from cell cycle, later in marrow regeneration phase, was probably the consequence of their localization in the endosteal niche. The location of donor derived cells was identified by IHC of the bone marrow sections. The maximum proliferation of donor-derived LSK cells was observed between 10 and 15 days of marrow regeneration. So, we have analyzed global gene expression profile of the donor-LSK and stromal cells ($CD45^-$) on day 10 for identifying activated signaling pathways and the gene expressions of the corresponding ligands, respectively.

B. Plasticity in BM cells:

Our earlier study showed that a specific population of BM cells not only differentiated into liver cells, but also expressed active

factor FVIII protein in hemophilia A (HA) mice. The cellular source of factor FVIII synthesis remains controversial, according to the literature it is synthesized by either hepatocytes or liver sinusoidal endothelial cells. In HA-K/O experimental model, for the first time we have shown that both BM-derived hepatocytes and endothelial cells synthesize FVIII protein. This conclusion has been drawn on the basis of analysis of real-time PCR, IHC and TEM results.

It has been shown by many investigators that BM-derived mesenchymal stem cells (MSCs) has propensity of differentiation into the cells across the germ layers. We have been trying to exploit the benefit of MSCs of fetal liver origin for differentiation into neuronal cells. FL-derived MSCs has been targeted as they are less matured than BM-derived cells. We are working on Parkinson's disease (PD) as a model for the replacement of degenerating dopaminergic neurons by transplanting fetal liver-derived MSCs. We have been able to isolate a phenotype of cells from mouse fetal liver, which was shown to transdifferentiate into dopaminergic neurons in support culture system. Further, we have established a PD mouse model by unilateral injection of 6-hydroxydopamine (6-OHDA) in substantial nigra (SN) region of the brain with the help of a steriotaxic apparatus. It was found that this injection may cause damage to the neurons of the striatum region of the brain. The damage of dopaminergic neurons was identified by analysis of IHC results. To confirm PD, 6-OHDA injected mice were further examined for apomorphin-induced rotation test.

C. Ovarian cancer stem cells:

In last year we showed the development of a metastatic mouse model of ovarian cancer by surgical orthotopic implantation of ID8 cell line. To tract-down tumor cells, we have transduced ID-8 cells for stable GFP expression by lentiviral based delivery system. GFP-expressing ID8 cells were found to initiate primary tumor, ascites with tumor cells and solid secondary tumors in the intestine, omentum and spleen. ID-8 cells do not express specific cell surface markers by which stem and non-stem (differentiated) population can be distinguished. Generally stem cells have a robust cellular machinery to efflux drugs (side population, SP); we have used this property to identify cancer stem cells (CSC) in heterogeneous ID8 cells. It was found that normal ovarian surface epithelium contains 0.32 ± 0.12% SP cells; the similar fraction of SP cells has been detected in culture ID-8 cell line. To prove that SP/ID-8 cells are like CSC, we have initiated the induction of ovarian tumors by implanting different doses of SP/ID-8(GFP) and non-SP/ID-8(GFP) cells. We have found that the metastatic cells are highly proliferating and contain lesser proportion of SP cells than that present in the primary and secondary solid tumors. Though in normal culture conditions non-SP cells appear from SP cells, under stress (e.g. hypoxia) non-SP cells give rise a significant number of SP cells. This result suggest that hypoxic inducing factor-1α (HIF-1α) might have certain role in pushing back the differentiated non-SP cells into CSC-like SP cells.

Publication

Original peer-reviewed article

1. Pati S, Kalra OP and Mukhopadhyay A (2011) Foe turned Friend: Multiple functional roles attribute to hyper-activating stem cell factor receptor mutant in regeneration of hematopoietic compartment. **Cell Proliferation 44**: 10-18.

Characterization of proteins important for cell death regulation

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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. 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 macrophages and male germ cells. In these cell systems, stress induced by drugs or hormones is used as stimulus to create altered situations and the effects on the signaling pathways are explored with a view to understand the complexities underlying the survival and death response of cells to various agents.

Biology of cell survival and death in protozoan parasites

CYP450s of the kinetoplastid parasites could play an incisive role in the ability of the parasites to infect and withstand drug pressure. There are no reports on the function of CYP450s in *Leishmania* although potentially these proteins could be of significant importance in *Leishmania* biology. Three cytochrome P450s (CYP5122A1, CYP710C1 & CYP5123A1) were identified in *Leishmania donovani* by our laboratory. Continuing our investigations on CYP5122A1, we established a link between the CYP5122A1 and the parasites ability to grow, withstand drug pressure and infect. Parasites expressing only one allele of the CYP5122A1 gene (HKO) were generated as reported in 2010 progress report. When the parasites were analyzed for ergosterol content, the HKOs showed 3.5 times lower ergosterol than the WTs and supplementation of ergosterol in culture or complementation of CYP5122A1 levels through episomal expression improved the growth rate and their ability to withstand drug exposure. These parasites were resistant to amphotericin B treatment presumably because the drug acts through association with membrane ergosterol and the low ergosterol could be responsible for this resistance. Although *Leishmania* parasites can survive with lower sterol levels, normal supplement of sterol is required for optimal parasite function which is reinforced by this study. The study also indicates that amphotericin B resistance could be linked to alterations in ergosterol levels. CYP710C1 is currently under investigation and parasites expressing one allele of CYP710C1

have been generated for further studies. Preliminary studies show that the number of parasites expressing one allele of CYP710C differentiating to infective metacyclic forms is less as compared to the wild types.

Tryparedoxin peroxidases in the *Leishmania* parasite are important detoxification enzymes. In continuation of our work on the cytosolic and mitochondrial TXNPxs (Mol. Microbiol. 68: 372-391, 2008), last year we reported the generation of constructs with mutations in the calmodulin binding sites of the mTXNPx. Different mutants had varying abilities to be transported to the mitochondria. The effect of the various mutations on the transportability of the protein is being explored further. In addition to the above studies, the effects of overexpression or deletion of both alleles of mTXNPx in *Leishmania* cells on their defense abilities are being studied.

Modulation of cellular apoptosis in mammalian cells

We have earlier shown that estrogen induces apoptosis in mammalian macrophages if estrogen induced Bcl-2 increase is suppressed (J. Immunol. 179:2330-2338, 2007; J. Cell. Mol. Med. 13: 2317-2329, 2009). Investigating the mechanism of Bcl-2 upregulation, we now show that Bcl-2 increase is dependent on downregulation of miR181b. MiR 181b overexpression resulted in downregulation of estrogen induced Bcl-2 inducing increased cell death. Further studies on miR based regulation of estrogen induced Bcl-2 is now being further explored.

In a model of DNA damage induced apoptosis in embryonal carcinoma cells arising out of male germ cells, role of a flavonoid in manipulating cisplatin induced apoptosis was investigated (Mol. Cancer. Ther. 10:255-268, 2011). Addition of the plant flavonoid fisetin to cisplatin enhanced cisplatin cytoxicity *in vitro* at four times lower dose than that required by cisplatin monotherapy for similar cytotoxic effects. Cisplatin, fisetin monotherapy and addition of fisetin to cisplatin in a combination increased FasL expression. Cisplatin and fisetin as single agents activated caspases-8 and -3 and caspases-9 and -7 respectively while combination treatment activated all four caspases. Increases in p53 and p21 and decreases in cyclin B1 and survivin occurred, all effects being more exaggerated with the combination. Fisetin, with or without cisplatin, increased expression of proapoptotic protein Bak and induced its mitochondrial oligomerization. Bid truncation and mitochondrial translocation of Bid and p53 was induced by fisetin in the presence or absence of cisplatin. Downregulation of p53 by shRNA during drug treatment decreased p21 levels but caused survivin increase thus reducing cell death. Upstream to p53, inhibition of p38 phosphorylation reduced p53 phosphorylation and cell death. In a NT2/D1 mouse xenograft model, combination therapy was most effective in reducing tumor size. In summary, findings of this study suggest that addition of fisetin to cisplatin activates both the mitochondrial and the cell death receptor pathway and could be a promising regimen for the elimination of embryonal carcinoma cells. In similar models, studies are also ongoing on the balance between apoptosis and autophagy in response to a given stimuli and how they determine the ultimate fate of the cell.

Publications

Original peer-reviewed article

1. Tripathi R, Samadder T, Gupta S, Surolia A and Shaha C (2011) Anti-cancer activity of a combination of cisplatin and fisetin in embryonal carcinoma cells and xenograft tumors. **Mol Cancer Ther 10**: 255-68**.**

Reviews/Proceeding

1. Shaha C, Tripathi R and Mishra DP (2010) Male germ cell apoptosis: regulation and Biology. **Philos Trans Roy Soc B 365**: 1501-1515.

Cellular and molecular aspects of reproduction and viral infections

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One of the themes of our group is to understand the molecular basis of mammalian fertilization and to develop a contraceptive vaccine for humane control of street dog population. 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 as potential microbicide; 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 controlling street dog population
3. To investigate the role of IL-6 group of cytokines in the regulation of trophoblast cell proliferation, invasion and differentiation
4. To discover molecules with anti-HIV activity for their use as potential microbicide
5. Develop flu virus neutralizing monoclonal antibodies

Contraceptive vaccine for street dog population management

To manage street dog population, who are the major carriers of rabies virus, dog zona pellucida glycoprotein-3 (23-348 aa residues) was expressed in E. coli as a fusion protein with a 'promiscuous' T non-B cell epitope of tetanus toxoid (aa residues 830-844) (TT-dZP3). In collaboration with Indian Immunologicals Limited, Hyderabad, two groups of female dogs (n = 4) were immunized with the purified recombinant fusion protein adsorbed on aluminum hydroxide either at 200 µg or 1 mg per injection on days 0, 21 and 35. Immunization led to the generation of high antibody titers against recombinant dog ZP3 in both the groups. Subsequently, immunized animals were mated with the male dogs of proven fertility along with the control unvaccinated group of female dogs (n = 4). All female dogs immunized with recombinant fusion protein failed to conceive,

whereas unvaccinated female controls became pregnant and delivered normal pups. The immunized animals were followed-up and additional booster of 1 mg recombinant fusion protein/animal was given on day 273 in both the above groups. In the subsequent breeding season, none of the immunized animals from both the groups accepted the males for mating and thus did not conceive. These initial observations are encouraging and needs validation by undertaking large field trials to ascertain the utility of recombinant dog ZP3 based contraceptive vaccine for management of the population of street dogs.

Role of cytokines on the proliferation, invasion and differentiation of the trophoblast cells

Effect of IL-11 on the invasion of the trophoblastic cells

The significance of IL-11 in controlling the invasion of the trophoblastic cells through expression of invasion associated markers was reported during the last year. Stimulation of JEG-3 cells by IL-11 led to an increase in the invasion whereas, a decrease in the invasion of HTR-8/SVneo cells was observed in the presence of IL-11. To investigate the differential response of these two cell lines towards IL-11, cDNA Microarray using GeneChip® Human Exon 1.0 ST (Affymetrix, Santa Clara, CA) revealed that HTR-8/SVneo cells have 1176 genes showing at least 2 fold increase while, about 1334 genes showing about 2 fold decrease in their expression as compared to JEG-3 cells. In addition, HTR-8/SVneo cells had higher levels of expression of molecules of signaling pathways associated with cancer, cytokine signaling etc. This basic information suggests that these two cell lines have remarkable differences in terms of the basal level of expression of signaling pathways and genes which could be essential for controlling their invasiveness. Following IL-11 stimulation to JEG-3 and HTR-8/SVneo cells, some distinct set of genes got up-and down-regulated. In JEG-3 cells, following IL-11 stimulation, 314 genes got up-regulated by at least 1.5 fold while, 313 got down-regulated by 0.5 fold. In contrast to this, in HTR-8/SVneo cells, 75 genes showed up-regulation by at least 1.5 fold while, 54 showed down-regulation by at least 0.5 fold following IL-11 stimulation. IL-11 stimulation to JEG-3 cells influences biological processes like cell signaling, cell division, cell organization and secretary proteins. IL-11 increased the expression of cytokines like *IL-17B*, *IL-32*, *IL-1*, *CSF* and certain chemokines. The signaling appears to be affected through lipid metabolism as genes associated with peroxisome biogenesis and metabolism was enriched in JEG-3 stimulated with IL-11. In contrast to this, in HTR-8/SVneo cells, following IL-11 stimulation only two processes were found to be specifically affected. These included olfaction and sensory perception and G-protein coupled receptor (GPCR) mediated signaling. Genes involved in RNA processing and translation, though not statistically significant were also found to be affected following IL-11 stimulation in HTR-8/SVneo cells. Following IL-11 stimulation mitochondrial functions also seem to be affected with relatively more number of genes getting affected in JEG-3 cells as compared to HTR-8/SVneo cells. There were several genes which showed differential expression in the two cell lines following IL-11 stimulation. To further validate the Microarray findings, qRT-PCR for the expression of *MMP23B* and *MUC1* was performed. A significantly high ($p<0.05$) level of expression of MMP23B and MUC1 was observed in HTR-8/SVneo cells as compared to JEG-3 cells. Following IL-11 stimulation, there was a significant increase in the expression of both *MUC1* and *MMP23B* in JEG-3 cells while, there was a decrease in HTR-8/SVneo cells. These results demonstrate that IL-11 can stimulate the invasion of less invasive JEG-3 cells by up-regulating the expression of invasion associated genes as well as inhibit the invasion by down-regulating the expression of same set of genes in highly invasive HTR-8/SVneo cells (Fig 1). Hence, IL-11 may function as critical regulator for the spatial and temporal regulation of trophoblast invasion.

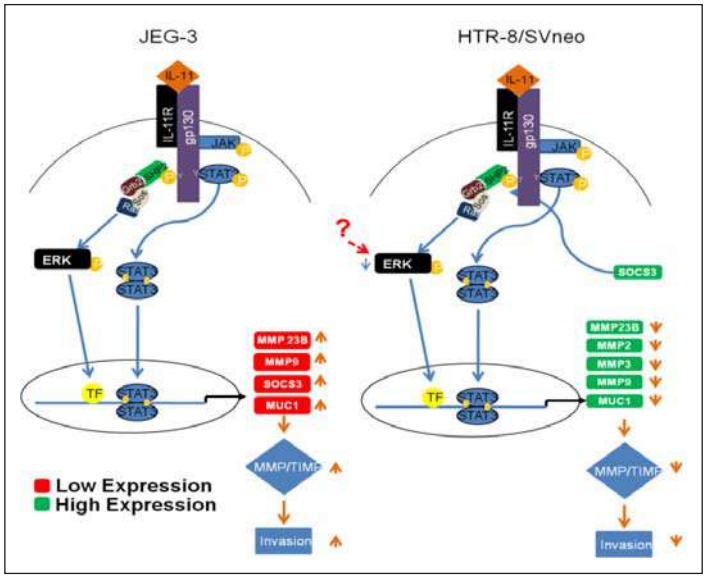

Fig 1. *Schematic representation of the activation and expression of molecules following IL-11 stimulation in JEG-3 and HTR-8/SVneo cells*

Relevance of syndecan-1 in the trophoblastic BeWo cell syncytialization

To study the role of syndecan-1 during syncytilization of the cytotrophoblasts, trophoblastic BeWo cells were used as experimental model. *In vitro* syncytialization was induced by forskolin treatment. Syncytia formation was assessed by desmoplakin I+II immunostaining (Fig. 2A). Syncytialization was associated with an increase in the expression of syndecan-1 with a concomitant decrease in the expression of desmoplakin I+II. Further, silencing of the syndecan-1 expression by siRNA in BeWo cells led to a significant decrease in the cell fusion, both in the absence or presence of forskolin (Fig. 2b). It was associated with a significant decrease in hCG level in the conditioned medium. These findings suggest that syndecan-1 is critical for the syncytia formation of the trophoblastic cells.

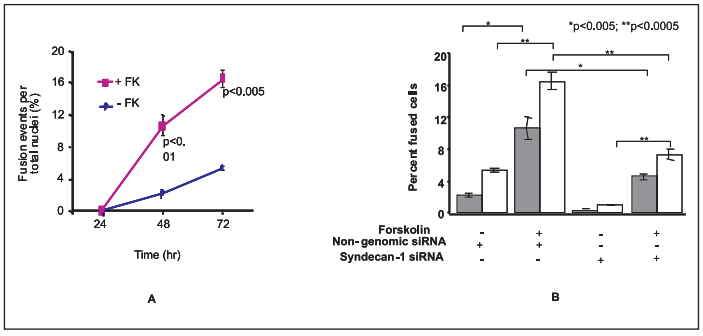

Fig 2. Role of syndecan-1 in BeWo cell fusion. *Panel A, shows the time dependent increase in cell fusion in response to forskolin (FK). Panel B, shows the extent of cell fusion upon syndecan-1 silencing in BeWo cells after 48 (filled bar) and 72 hours (open bar) of treatment with forskolin.*

Identification of plant based molecules with anti-HIV activity as potential microbicides

Till now, a total of 150 extracts prepared using different solvents from various parts of 30 plants have been screened for anti-HIV activity using reporter-gene based *in vitro* cell assay systems. Extracts prepared from 8 plants showed anti-HIV activity. Out of these 8 plants, extracts prepared from 4 plants showed TI values greater than 10. The extracts from 6 out of 8 plants did not show a significant increase in pro-inflammatory cytokines (IL8, IL1β, IL6, IL10, TNF, IL-20p70), when incubated with vaginal keratinocytes (Vk2/E6E7). The extracts/fractions from different parts of 3 plants also inhibited reverse transcriptase activity. One of the active principles in the extract of NBRH-10 has been identified and an Indian Patent filed. Initial experiments have shown that the extracts prepared from NBRH-4 and NBRH-16 act synergistically to inhibit HIV infection.

Generation of the monoclonal antibodies against the influenza virus

In natural influenza virus infection, antibody mediated immune response is sufficient for the protection. Neutralizing antibodies;

which prevent either the entry of the virus into the cells or the post-entry events, are directed against the hemagglutinin (HA) protein of the influenza virus. In line of this, we aim to generate influenza virus heterosubtypic neutralizing monoclonal antibodies (MAbs) for exploring the possibility of passive antibody based treatment for severe influenza infections. In collaboration with the Indian Institute of Science, Bangalore and Serum Institute, Pune; we have initiated generation of murine as well as human MAbs against the pandemic and seasonal influenza viruses.

So far, we have been able to generate 14 MAbs against the pandemic H1N1 strain (provided by Serum Institute, Pune). Eight MAbs also showed cross-reactivity with the seasonal vaccine strain of the H1N1 (A/Brisbane/59/2007) in ELISA. In addition, we have generated MAbs against 3 recombinant HA proteins provided by Dr. Raghavan Varadarajan, Molecular Biophysics Unit, IISc, Bangalore. Twelve MAbs were generated against recombinant HA protein (H3HA6) of H3N2 (A/HK/1/68). The recombinant HA proteins contain conserved regions of the HA molecule; mainly HA2 region (1-172 aa) which shows overall 90-95% similarity within the subtype and also contain smaller regions of the HA1 (1-46 aa, 290-325 aa). Using similar strategy recombinant HA protein (H1HA6) corresponding to H1N1 (A/PR/8/34) was also designed and made available to us by Dr. Varadarajan. Eleven murine MAbs have been generated against H1HA6. In addition, 12 MAbs have also been generated against recombinant HA (H1HA10_IZ) corresponding to H1N1 (A/PR/8/34) which differs from H1HA6 by having HA1 from 18-41 aa and 290-323 aa and HA2 from 41-113 aa. Three of the MAbs against the H3N2 recombinant HA react with the seasonal vaccine strain of H3N2 (A/Brisbane/10/2007) whereas, 10 of the MAbs generated against the H1N1 recombinant HA proteins showed significant reactivity with the seasonal vaccine strain of H1N1 (A/Brisbane/59/2007) in ELISA.

Publications

Original peer reviewed articles

1. Prakash GJ, Suman P, Prieto DMM, Markert UR, Gupta SK (2011). Leukemia inhibitory factor mediated proliferation of HTR-8/SVneo trophoblastic cells is dependent on extracellular signal-regulated kinase 1/2 activation. **Rep Fert Dev 23**:714-724

2. Godbole G, Suman P, Gupta SK and Modi D (2011) Decidualized endometrial stromal cell derived factors promote trophoblast invasion. **Fertil Steril 95**: 1278-1283.

3. Zimmerman SW, Manandhar G, Yi Y-J, Gupta SK, Sutovsky M, Odhiambo JF, Powell MD, Miller DJ and Sutovsky P (2011) Sperm proteasomes degrade sperm receptor on the egg zona pellucida during mammalian fertilization. **PLOS One 6**: e17256.

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7. Ganguly A, Bukovsky A, Sharma RK, Bansal A, Bhandari B and Gupta SK (2010) In humans, zona pellucida glycoprotein-I binds to spermatozoa and induces acrosomal exocytosis. **Hum Reprod 25**: 1643-1656.

Reviews/Proceedings

1. Gupta SK, Gupta N, Suman P, Choudhury S, Prakash K, Gupta T, Sriraman R, Nagendrakumar SB and Srinivasan VA (2011) Zona pellucida-based contraceptive vaccines for human and animal utility. **J Reprod Immunol 88**: 240-246.

2. Gupta SK and Bhandari B (2011) Acrosome reaction: relevance of zonapellucida glycoproteins. **Asian J Andrology 13**: 97-105.

3. Gupta SK and Bansal P (2010) Vaccines for immunological control of fertility. **Reprod Med Biol 9**: 61-71.

Patent

1. Gupta SK, Modi M, Nutan, Malik S, Tuli R, Malhotra S, Suri S, Rawat AKS and Srivastava SK (2010). Plant extract for anti-human immunodeficiency virus (HIV) activity and preparation thereof. Indian Patent filed; Application # 242/DEL/2010.

MOLECULAR DESIGN

MOLECULAR DESIGN

Structural studies on proteins, dynamics and ligand interactions using NMR

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The theme of 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 understanding the structure and dynamics of a number of proteins, *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.

This year, we report studies on the dynamics of ubiquitin, a protein of intense biological and mechanistic interest because of its expanding number of functions. Slow conformational fluctuations were identified on its surface a long time ago, *i.e*. more than 15 years, and the relevance of these motions to ubiquitin function has been highlighted by a number of studies. However, the molecular basis for these motions has remained a mystery. The present work reveals the molecular basis for the slow dynamics in ubiquitin.

A unique side chain to backbone hydrogen bond exists in ubiquitin

Two residues display extreme line broadening in the NMR spectrum: Glu 24 and Gly 53. Their chemical shifts are found downfield *i.e*. 10.14 and 9.77 at neutral pH that move upfield by 1.25 ppm and 1.13 ppm upon pH titration from neutral to the acidic range, indicating hydrogen bonds involving a side chain carboxyl group. Based on the relative position of these amides with respect to nearby carboxylates (PDB entry 1UBQ), and comparison of the pK_a values reported by them to the previously determined carboxyl pK_a values by NMR, we identified the putative hydrogen atom acceptor as the Glu 24 carboxylate.

Mutation of Glu 24 or Gly 53 to an Ala perturbs the hydrogen bond

In order to test the hypothesis that the Glu 24 side chain is indeed the hydrogen bond acceptor, Glu 24 was changed to an alanine by site directed mutagenesis. A large upfield change in HN chemical shift was observed at two different sites in the protein spectrum of the mutant protein relative to wild type, suggesting perturbation of the hydrogen bond. Mutation of Gly 53 to an Ala yielded similar results suggesting that Gly 53 is a prerequisite for the hydrogen bond

Glutamate 24 and Glycine 53 amides undergo conformational exchange

The amides of Glu 24 and Gly 53 display line broadening in NMR, which could be a reflection of conformational exchange. This possibility was confirmed by studying the temperature dependence of the amide chemical shifts using 2mM unlabeled ubiquitin sample at pH 6.0. The peaks for Glu 24 and Gly 53 amides in a 1D spectrum get more intense as the temperature is raised, supporting a scenario of multiple interchanging conformers, each present sparsely and transiently.

Hydrogen bond interactions are coupled to the ms motions

$^{1}H^{15}N$ heteronuclear relaxation studies on wild type ubiquitin and the mutants E24A and G53A were carried out to explore the relation between slow motions and the hydrogen bond. In wild type ubiquitin, a few residues Ile 23, Asn 25, Thr 55, and Val 70 display significant R_{ex} value. However, in the mutant E24A, all residues except for Val 70 do not show a statistically significant

R_{ex} term. Similar results were obtained in the G53A. Notably, the slow dynamics observed in the amides of Ile 23, Asn 25 and Thr 55 are quenched in the mutants E24A and G53A, suggesting a strong dependence of ms motions on the hydrogen bond interactions.

Hydrogen bond and the crystal structures of ubiquitin

With the large number of PDB structures available for ubiquitin, we decided to explore the conformation of Glu 24 side chain in those structures. Interestingly, two discrete conformations based on distance were identified, conformer (a), distances between Glu 24 OE1/OE2 to its own amide and amide of Gly 53 were fairly short, within 2.8-4.8 Å, and conformer (b), all four distances were much longer, between 4.0-9.2 Å, displaying a wide variation in conformation as shown in Fig. 1.

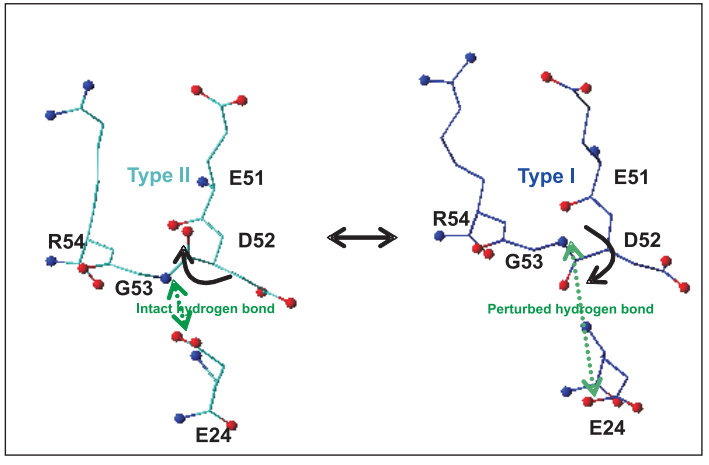

Fig. 1 Slow motions regulated by a hydrogen bond by altering a β-turn equilibrium

Dihedral angles from the X-ray structures vs. the NMR data

Backbone phi and psi values were calculated for Asp 52 and Gly 53 for the 155 ubiquitin molecules in the 59 crystal structures using the web interface 'VADAR' (Volume, area, dihedral angle reporter) version 1.8. Dihedral angles were also calculated from our NMR experimental data using TALOS+ for the backbone of Asp 52 and Gly 53 in wild type ubiquitin and in the mutants. Judging from the NMR data, wild type ubiquitin appears to adopt the (a) conformation in solution while the two mutants E24A and G53A exist in the (b) conformation. Notably, these data are in accord with the pK_a and mutagenesis data.

Comparison of these dihedral angle values to the standard values suggest that the (a) conformer exists as a type II β-turn and the (b) conformer exists as a type I β-turn. Thus, the hydrogen bond supports the high energy conformer, the type II β-turn and thus regulates slow motions by modulating the β-turn.

To develop strategies for making sensors and actuators for biological process

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The theme of research is to develop systems for monitoring biological processes. The objectives are to (i) develop tools for needle free immunization (ii) study the biological processes like differentiation, hybridization etc. and (iii) develop devices and sensors based on such studies.

Local hyperthermia enhanced immunization

We have established that heat component of Hyperthermia Enhanced Transdermal (HET) Immunization leads to phenotypic and functional maturation of dendritic cells *in vivo* as well as *in vitro*. Upon activation by hyperthermia, DCs migrate from skin to draining lymph node due to activation associated migration, in this process DCs present the antigens encountered in the skin to the lymphocytes in draining lymph node. Thus compared to normal immunization, HET causes an enhanced migration of DCs to lymph node. Since this effect is independent of presence of antigen, our study establishes that hyperthermia alone is an efficient adjuvant in enhancing DC migration and maturation in lymph nodes.

We have shown that upon hyperthermia exposure, the cytokine secretion profile of DCs gets altered with higher levels of IL-10 and lower IL-12 levels being detected. This suggests how DC may be altering the Th response *in vivo* by favouring the development of Th2 cells.

The success of any vaccine therapy depends on its ability to confer protection against actual biological challenge. Thus to determine the value of HET immunization as a novel transdermal vaccine delivery technique, we evaluated its protective efficacy. We followed multiple immunization regimen using higher concentration of antigen (200µg TT in the HET patch). Mice (n=4) were immunized with 4 doses of TT via HET (Day 0, Day 14, Day 28 and Day 42). On day 49, mice were challenged with LD50 of biologically active toxin and observed for a period of 5 days for mortality or paralysis. All animals in the control group (unimmunized) were found to be dead by the end of Day 5, whereas in the test group no death or paralysis was observed and all the animals survived. The mice in the test group did not show any symptoms of disease even after 2 weeks of challenge, thus establishing the protective efficacy of vaccine delivered through HET.

These observations support HET as a very attractive mode of immunization especially for human use combining the obvious benefits of needle fee immunization (painless and safety) along with immune modulatory properties of standard adjuvants.

Immunization by the aerogenic route

We encapsulated live MIP and BCG in 2- 4 micron sized alginate micro particles using a novel laboratory made assembly.

We found that MEAP significantly increased the surface expression of activation marker like CD80, CD86, MHCII and CCR7 on BMDCs compared to the activation by BCG, MIP and blank particles. Further, the allogenic mixed lymphocyte reaction confirmed that MEAP activated BMDC and lung DCs had enhanced ability of antigen presentation. Also we observed that MEAP were engulfed by BMDCs and co-localize with lysosome.

The dry powder formulation (2000-4000 *Mycobacterium*/mice) was directly delivered to mice lung and immunogenic potential analyzed. The *in vitro* recall response of MEAP immunized mice compared in terms of the proliferation index and IFN- gamma released by spleenocytes and mediastinal's cells was found to be much higher than any of the groups. Different groups of immunized mice were challenged with H37Rv and after 16 weeks the CFU in lung and spleen estimated. The MEAP (BCG/MIP) immunized mice had 3.4/3.7 and 1.8/2.1 log CFU in lung and spleen respectively. The liquid aerosol immunized animals (BCG/MIP) had 5.0/4.9 (lung) and 4.2/3.7 (spleen). In the control group the log CFU load was 6.5 (lung) and 5.4 (spleen).

In summary, the *Mycobacterium* encapsulated formulation when delivered through the aerosol route generated more robust immune response compared to aerosol of only *Mycobacterium*. Hence, vaccination by dry powder aerosol of BCG or MIP would be more effective, safer and economical.

Ex-vivo 3D liver culture

In this study decellularization of rat livers was performed by portal vein perfusion and we plan to perform recellularisation with neo-hepatocyte generated from PBMCs of rat.

Publication

Original peer-reviewed article

1. Roy A, Singh MS, Upadhyay P*, Bhaskar S* (2010) Combined Chemo-immunotherapy as a Prospective Strategy to Combat Cancer: A Nanoparticle Based Approach. **Mol. Pharmaceutics 7**: 1778–1788. * Corresponding authors

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 bio-conjugates and protein dendrimers that may be useful in a variety of biotechnological applications.

A. Peptide ligation reactions catalyzed by protease transpeptidase 'sortase'

Studies on peptide ligation specificity of sortases.

The focus of the work in the current reporting period was to develop a sortase-mediated strategy for assembly of protein dendrimers. For this, we utilized simple lysine-based scaffold carrying two or four branched arms terminated with Gly residues as receptors for LPXTG carrying polypeptide substrates. The ability of Sortase A (Srt A) of *Staphylococcus aureus* to transfer a model LPXTG peptide substrate to the dendrons was tested. Analyses of the product by reverse-phase HPLC and mass spectrometry revealed poor efficiency of transfer and product heterogeneity. Thus alternative chemo-enzymatic options were explored to improve the yields. The coupling of sortase activity with azide-alkyne 'click' chemistry appeared quite attractive in this endeavor. Accordingly, alkyne-terminated aminoglycine peptides were coupled to LPXTG substrates *via* sortase action and the product of this reaction (alkyne terminated) was coupled to azide functionalized branched dendrons. The sortase-mediated reaction of alkyne and LPXTG peptides produced almost quantitative yields of the alkynated derivative. Subsequent coupling of this derivative to the azide dendron resulted in high yields of the dimeric or tetrameric peptide dendrimers.

The feasibility of this approach for the assembly of dendrimeric proteins was tested using green fluorescent protein (GFP). GFP was engineered with a LPNTG sequence at the C-terminus and an alkyne group was installed by sortase-mediated ligation. The alkynated product was then reacted with the azide dendron by click chemistry. HPLC and mass spectrometric analyses revealed the formation of GFP dendrimers in good yields.

B. Studies on sortases from *Streptococcus pneumoniae*

Studies on the substrate specificity of pSrt A from *S. pneumonia* had revealed rather stricter specificity for LPXTG substrate as compared to Srt A of *S. aureus.* Unlike Srt A which recognizes any amino acid at position X, pSrt A recognized only those LPXTG peptide substrates that contain Asn at the X position. Moreover, pSrt A could recognize LPNTG sequence only when it was placed in a YAQLPNTGA frame. Further dissection of the substrate specificity indicated that pSrt A could not process LPNTG pentapeptide with free termini. Instead required a peptide in which both the N- and C-terminus were protected with acetyl and amide groups respectively. Extending the substrate peptide at N-terminus increased the yield of transpeptidation product but presence of a charged residue such as lysine at the C-terminus of LPNTG was refractory to pSrt A action.

Attempts were also made to crystallize pSrt A. The pSrt A construct that lacked 59 N-terminal residues (Δ59pSrt A) and contained a hexa-histidine tag at the N-terminus was used in initial crystallization experiments. However, crystals obtained from this construct diffracted poorly. To obtain better quality crystals, a new construct was designed by deleting additional

residues from the N-terminus. The Δ81pSrt A construct yielded good quality crystals that diffracted at 2.8 Å. Currently, work on the solution to the crystal structure is in progress.

Publication

Original peer-reviewed article

1. Khan S, Nagarajan SN, Parikh A, Samantaray S, Singh A, Kumar D, Roy RP, Bhatt A and Nandicoori, V.K (2010) Phosphorylation of enoyl-ACP reductase InhA impacts mycobacterial growth and survival. **J. Biol. Chem 285**: 37860-37871.

Dissecting the mechanism of fibril disruption by BPE-conjugated CdSe/ZnS core/shell quantum dots.

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It is well documented that proteins frequently alter their native conformation in response to different external stimuli. Some of these conformations may lead to pathogenic states. Under the conditions which destabilize their native structure, a number of proteins aggregate into the characteristic β-sheet rich fibrillar assemblies known as amyloid fibers. The extracellular deposition of these amyloid fibrils is a hallmark of many devastating diseases which include Alzheimer's disease, prion disease, dialysis-related amyloidosis, familial amyloid polyneuropathy and type II diabetes. Currently, ~40 different proteins and peptides are known to cause human amyloid disease. Although these diseases have been identified long ago, their cure still remains elusive. The actual mechanism of amyloid formation is not yet fully understood, due to which prevention or treatment of disease is still far from satisfactory. Our lab is involve in identifying the important modifications in the protein sequence that leads to change in its folding and interactions with other protein and ultimately leads to its misfolding and aggregation in to amyloid fibril. These studies may help understanding the intracellular events that leads to cell death on extracellular deposition of protein fibril or intermediates of fibril formation. Research in my lab is focused on understanding the molecular mechanism of amyloid formation, toxicity of intermediate oligomers in the fibrillogenesis pathway and disruption of fibers by small molecule inhibitors. The detailed understanding of the mechanism and inhibition of protein fibrillogenesis as well as fibril disruption will shed insight into the disease pathogenesis and, in turn, can potentially lead to innovative therapeutics against such conformational diseases.

a. Dissecting the mechanism of fibril disruption

Transthyretin, a tetrameric serum protein has been implicated in the systemic senile amyloidosis (SSA), familial amyloid polyneuropathy (FAP) and familial amyloid cardiopathy (FAC). In our previous report we designed some potent biphenyl ether (BPE) based molecules as inhibitors of TTR fibrillogenesis and fibril disrupters. We proposed the mechanism of inhibition by these potential BPE inhibitors. However, the mechanism of fiber disruption by these compounds still remains under-explored. As the process of fibril disruption is very slow, a stable probe is required. To gain insight into the mechanism, we designed and synthesized a new BPE derivative and coupled it with Quantum dots (QDs) and used this novel QD labeled BPE to track the process of fibril disruption. To compare the specificity of these conjugates, the unconjugated QDs and sugar-conjugated QDs have been used.

We found that these new BPE derivatives are the potent inhibitors of TTR fibril formation. We selected one derivative represented here as P8 and conjugated it with QDs. The BPE conjugated QD also inhibited the process of TTR fibril formation with IC50 value of 11 ± 3.11 and 29 ± 4.21 μM for P8 and P8-QDs respectively. The P8 and P8-QDs inhibited the fibril formation by stabilizing the TTR tetramer. Next we check whether P8 and P8-QDs are able to bind TTR fiber. Our studies showed that the new BPE-QDs bind to the fiber uniformly and has affinity and specificity for TTR fiber (Fig 1). After confirming their binding to TTR fibers we went ahead to study the process of fibril disruption by them. We found that P8 was an efficient fiber disrupter however, P8-QDs disrupted the

preformed fiber at a relatively slow rate. Further to understand the mechanism of fiber disruption by these compounds the process was observed by atomic force microscope (Fig. 2) and observed that disruption of fibers by P8-QDs is mainly from termini. Based on these studies we put forth the probable mechanism of fiber disruption by BPEs. Also, we explored the specificity of this BPE-QD for other amyloid fibers and found that the BPE-QDs interact with high affinity to the amyloids of $A\beta_{42}$, lysozyme and insulin. The potential of BPE-QDs in the detection of senile plaque in the brain of transgenic Alzheimer's mice has also been explored.

The use of technology such as QD labeled BPE, fluorescence and Atomic Force Microscopy provided us with the opportunity to study the very slow process of fibril disruption in great detail. To the best of our knowledge we present here the first attempt to show the binding of small molecule inhibitors to amyloid fibers directly and study the mechanism of fiber disruption using BPE-QDs (P8-QDs). Thus we believe that the synthetic BPE-QDs may open a new approach for studying the morphology of amyloid fibrils and their detection as well as for the screening of inhibitory molecules to prevent assembly of amyloid providing an avenue for treating these incurable ailments.

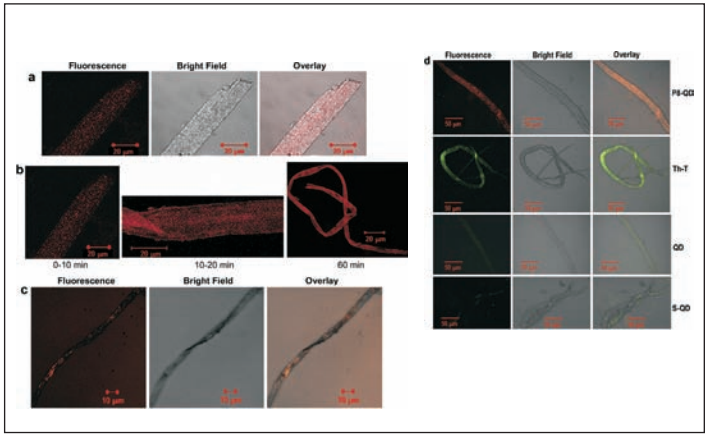

Fig. 1. *Binding propensities of P8-QDs observed under confocal microscope (a) Pre-formed TTR fibers were incubated with 10 mM of P8-QDs in PBS at room temperature just before imaging. (b) Time dependent labeling of TTR fiber with P8-QDs. Samples removed at 0, 20 and 60 min were observed for labeling with P8-QDs using confocal microscope. (c) Competitive binding assay of P8-QDs to TTR fibers in the presence of P8 compound. TTR fiber was incubated with 10 mM of P8 for 5 min and than P8-QDs (5 mM) was added. 5 ml samples after 1 h of incubation were used for microscopic observations. (d) Specificity of P8-QDs for TTR fiber. TTR fiber was incubated separately with P8-QDs, Th-T, QDs and S-QDs for 1 h and than observed under confocal microscope.*

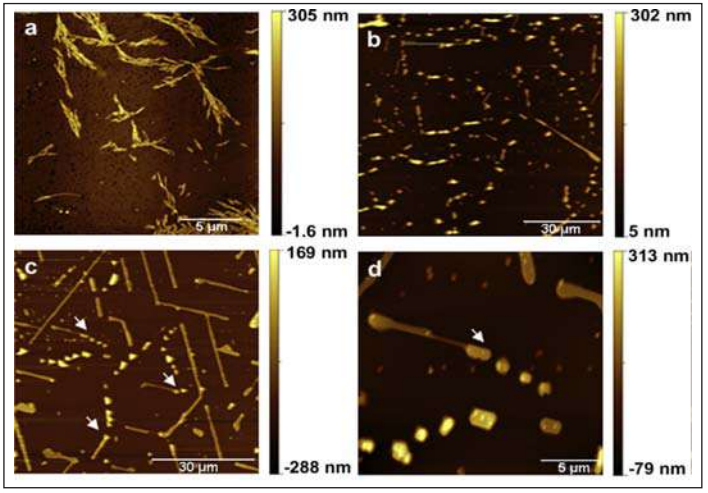

Fig. 2. *TTR fiber disruption studies using Atomic Force Microscopy: (a) Fully grown TTR fibers at pH 4.4, 37°C. To study the fiber disruption using AFM liquid cell, fully grown fibers were incubated with either P8 or P8-QDs (50 mM) in PBS for 96 h at 37°C under sterile condition. After 96 h the buffer in liquid cell was replaced and cell was imaged using Mac Mode of AFM, (b) Almost >80% long fibers were broken into small fragment by BPE (P8) from different sites of fiber simultaneously, (c) The presence of significant amount of fiber due to their slow disruption by P8-QDs and disruption was mainly from termini, (d) a part of (c) showing disruption from termini. Arrow shows the disruption from fiber termini in the presence of P8-QDs.*

b. 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. Osteoporosis can affect both men and women at any age but women are more prone to this disease, especially those have gone through menopause. To date several therapeutic interventions were approved for treatment of this disease. Most of them are antiresorptive agents that targets osteoclast activity include biphosphonates, estrogen replacement therapies, selective estrogen receptor modulators (SERMs), calcitonin and strontium ranelate. 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 salmom-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. In this study, we report a safe and long acting supramolecular assembly of human calcitonin (SCA) for sustained treatment of postmenopausal osteoporosis in animals. 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.

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Original peer-reviewed articles

1. Tripathi R, Samadder T, Gupta S, Surolia A and Shaha C (2011) Anti-cancer activity of a combination of cisplatin and fisetin in embryonal carcinoma cells and xenograft tumors. **Mol Cancer Ther 10**: 255-68.
2. Gupta S, Chattopadhyay T, Pal Singh M and Surolia A (2010) Supramolecular insulin assembly II for a sustained treatment of type 1 diabetes mellitus. **Proc Natl Acad Sci USA 107:** 13246-13251.
3. Gupta S, Babu P and Surolia A (2010) Biphenyl ethers conjugated CdSe/ZnS core/shell quantum dots and interpretation of the mechanism of amyloid fibril disruption. **Biomaterials 31**: 6809-6822.

Development of treatment modalities for infectious and chronic human diseases.

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The theme of my research is divided into two major groups. On one hand work related to understanding the basic physiology of disease causing organisms such as *Mycobacterium tuberculosis* and *Plasmodium falciparum* is carried on, the other focus is on innovation of various treatment modalities for chronic diseases such as Diabetes, Arthritis and Osteoporosis. Tuberculosis is an infectious bacterial disease, currently affecting one-third of the world's population and an immense burden on the economic and health-care policy for all countries. The emergence of extensively drug-resistant (XDR) TB poses a serious threat to TB control, and confirms the urgent need to strengthen basic TB control. Thus major scientific efforts are required for the development of alternative drug molecules, which will be more directed and efficient against these pathogens. Working in that direction, research in my laboratory is focused on targeting the lipid biosynthetic pathway and the pathway for the synthesis of vitamins and co-factors, which are crucial for the survival of this intracellular parasite.

On an entirely different note, research is also carried out to find alternative treatment protocols for chronic diseases. Diabetes is emerging as a global epidemic, with India, China and the USA topping the list of countries being affected by both Type I and Type II diabetes. Diabetic patients need to administer themselves with insulin frequently to prevent high blood glucose excursion after ingestion of meals. The fear of pricking oneself multiple times a day, leads to low patient compliance and therefore rise in complications such as diabetic cardiopathy and nephropathy. This has prompted interest in developing alternative, less invasive routes of delivery. In an attempt to address the issue of multiple injections and patient compliance, we have developed a novel concept wherein a Sustained Insulin Release Formulation (SIRF) is used for treatment of DM-I.

The objectives of my research work are:

a) Molecular and biophysical characterization of the proteins of the metabolic pathway of pathogens, b) design of synthetic inhibitors against biotin and CoA biosynthetic proteins, c) development of new treatment modalities for chronic diseases such as diabetes, arthritis, osteoporosis and multiple sclerosis.

a. Identification of critical residues of the mycobacterial dephosphocoenzyme a kinase by site-directed mutagenesis.

Dephosphocoenzyme A kinase performs the transfer of the γ-phosphate of ATP to dephosphocoenzyme A, catalyzing the last step of coenzyme A biosynthesis. Differences in the enzymatic organization and regulation between the human and mycobacterial counterparts, have pointed out tubercular CoaE as

a high confidence drug target (HAMAP database). Systematically mutating the residues from the P-loop and the nucleotide-binding site, identified Lys14 and Arg140 in ATP binding and stabilization of the phosphoryl intermediate. Mutagenesis of Asp32 and Arg140 showed catalytic efficiencies less than 5-10% of the wild type. Leu114 is a critical residue from the hydrophobic cleft involved in leading substrate, DCoA binding.

b. Mannose-binding dietary lectins induce adipogenic differentiation of the marrow-derived mesenchymal cells via an active insulin-like signaling mechanism.

We have recently demonstrated that the mannose-binding lectins, namely banana lectin (BL) and garlic lectin (GL), interact with insulin receptors on M210B4 cells and initiate mitogen-activated protein kinase kinase (MEK)-dependent extracellular signal-regulated kinase (ERK) signaling. In this study, we showed that this lectin-mediated active ERK signaling culminates into an adipogenic differentiation of these cells. Experiments carried out with pharmacological inhibitors show that MEK-dependent ERK and PI3K dependent AKT pathways are positive regulators of the lectin and insulin mediated adipogenic differentiation. Since both lectins could efficiently substitute for insulin in the standard adipogenic induction medium, they may perhaps serve as molecular tools to study the mechanistic aspects of the adipogenic process that are independent of cell proliferation.

c. Supramolecular insulin assembly II for a sustained treatment of type 1 diabetes mellitus.

Diabetes is a chronic disease requiring continuous medical supervision and patient education to prevent acute secondary complications. In this study, we have harnessed the inherent property of insulin to generate a form that exhibits controlled and sustained release for extended periods. Administration of a single dose of the insulin oligomer, defined here as the supramolecular insulin assembly II (SIA-II), to experimental animals rendered diabetic by streptozotocin or alloxan, released the hormone capable of maintaining physiologic glucose levels for >120 days.

d. Diversity in Functional Organization of Class I and Class II Biotin Protein Ligase.

The cell envelope of *Mycobacterium tuberculosis* is composed of a variety of lipids including mycolic acids, sulpholipids, lipoarabinomannans, etc., which impart rigidity crucial for its survival and pathogenesis. Biotin Protein Ligase (BPL/BirA) activates apo-biotin carboxyl carrier protein (BCCP) by biotinylating it to an active holo-BCCP. A minimal peptide (Schatz), an efficient substrate for Escherichia coli BirA, failed to serve as substrate for *M. tuberculosis* Biotin Protein Ligase (MtBPL). MtBPL specifically biotinylates homologous domain of MtBCCP, but not EcBCCP. This is a unique feature of MtBPL as EcBirA lacks such stringent substrate specificity. This suggests that when biotin is limiting, EcBirA preferentially catalyzes, biotinylation of BCCP over self-biotinylation. R118G mutant of EcBirA showed enhanced self and promiscuous biotinylation but its homologue, R69A MtBPL did not exhibit these properties. Holo-MtBPL is protected from proteolysis by biotinyl-5' AMP, an intermediate of MtBPL catalyzed reaction. In contrast, apo-MtBPL is completely digested by trypsin within 20 min of co-incubation.

e. *M. tuberculosis* pantothenate kinase: dual substrate specificity and unusual changes in ligand locations.

Kinetic measurements of enzyme activity indicate that type I pantothenate kinase from *Mycobacterium tuberculosis* has dual substrate specificity for ATP and GTP. A possible explanation is based on two critical substitutions in the amino acid sequence and the local conformational change resulting from them. The structures also provide a rationale for the movement of ligands during the action of the mycobacterial enzyme. Dual specificity of the type exhibited by this enzyme is rare. The change in locations of ligands during action, observed in the case of the *M. tuberculosis* enzyme, is unusual, so is the striking difference between two homologous enzymes in the geometry of the binding site, locations of ligands, and specificity.

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In vitro reconstitution of intracellular transport: Role of GTPases.

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Pathogenesis is related to the survival of pathogens in macrophages. The main goal of this project is to understand the mechanism of survival of pathogens in macrophages. We are also trying to understand the hemoglobin trafficking in *Leishmania* 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 (i)Modulaton of phagosome maturation by intracellular pathogens,(ii) 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.] and (iii) Mechanism of intracellular trafficking of hemoglobin in *Leishmania*.

Mechanism of survival of *Salmonella* in macrophages

In the reporting period, we have tried to understand the mechanism of fusion of *Salmonella*-containing phagosomes with LAMP1-containing Golgi derived vesicle. Therefore, we have analyzed the recruitment of Syntaxin6 and its interacting partners on these phagosomes. Thus, we have compared the recruitment of VAMP2, Vti1a, Vti1b between WT:*Salmonella* and s*ipC*$^{-}$:SCP. We have found that that WT:SCP progressively acquire higher amounts of Syntaxin6, VAMP2 and Vti1b during its maturation inside the macrophages and maximum amount of these proteins on these phagosomes are found at 90 min post internalization. Whereas, Vti1a levels peak early and gradually decline as they move near the Golgi. In contrast, s*ipC*$^{-}$:SCP have shown reduced levels of Syntaxin6, VAMP2, Vti1b and enhanced content of Vti1a particularly at later time point of their maturation. These results have shown that SCP at later stages of their maturation recruits Syntaxin6, VAMP2 and Vti1b to promote the fusion with LAMP1-containing Golgi-derived vesicles. Specificity of membrane fusion is also regulated by Rab GTPases, therefore, we have also compared the recruitment of two Golgi associated Rabs like Rab6 and Rab8 by respective SCP. Our results have shown that WT:SCP recruit significantly higher levels of both Rab6 and Rab8 at later time point of their maturation in macrophages supporting the fact that SCP interact with post-Golgi vesicles. On the contrary, s*ipC*$^{-}$:SCP show relatively lesser amount of these Rabs. To investigate the role of Syntaxin6 in LAMP1 recruitment on SCP, we have used specific shRNA to silence endogenous Syntaxin6 in RAW macrophages. Our results have shown that silencing of Syntaxin6 in RAW cells led to a significant reduction in LAMP1 recruitment (~50%) on WT:SCP at later stages of maturation

(120 min) when compared to untransfected or control shRNA transfected cells (85%). Taken together, our results have shown that SipC present on Salmonella-containing phagosomes fuses with Syntaxin6 positive LAMP1-containing vesicles to acquire LAMP1 from Golgi without their targeting to lysosomes.

Mechanism of hemoglobin trafficking in *Leishmania*

Initially, we have shown that early step of hemoglobin endocytosis in *Leishmania* is regulated by Rab5 where as hemoglobin transport from the early to late compartment is regulated by Rab7 homologue in *Leishmania*. However, analysis of *Leishmania* genome suggests that two isoforms of Rab5 with about 90% sequence homology are present in *Leishmania.* In the reporting period, we have tried to determine which isoform of rab5 homologue is involved in hemoglobin endocytosis in this parasite. Subsequently, we have cloned and expressed both isoforms of Rab5; namely, LdRab5a and LdRab5b from *Leishmania*. To determine the functional significance of these LdRab5 isoforms, we have generated both dominant negative (GDP form) and constitutively active (GTP form) mutants of each Rab5 isoforms. Finally, we have individually overexpressed respective Rab as GFP fusion protein in *Leishmania* and determine their role in various modes of endocytosis in *Leishmania* using either labeled hemoglobin as receptor-mediated endocytic probe or HRP as fluid phase marker. Our results have shown that Rab5a regulates the fluid-phase endocytosis in *Leishmania*. In contrast, we have found that kinetics of HB-Alexa-Red trafficking to the lysosomes is specifically enhanced in Rab5b overexpressed cells in comparison to control cells demonstrating that Rab5b specifically regulates the receptor-mediated endocytosis in *Leishmania*.

In the reviewing period, we have initiated studies to evaluate the HbR as a potential target against leishmaniasis in collaboration with Dr. Syamal Roy of IICB, Kolkata. N-terminal or full length HbR was cloned into appropriate vector and respective DNA was injected into Balb/c mouse on day 0 and 20. On day-30, each animal was challenged with pathogenic parasites via intra-cardiac route. Finally, animals were sacrificed on day-60 and parasite load was calculated from each group. Our results have shown that more than 95% protection is achieved in N-terminal or full length DNA injected mice in comparison to untreated or vector DNA injected mice. Parasite clearance is also supported by the facts that immunized animals show comparatively lower levels of IL-10 and higher levels of IFN-γ and IL-12. These results have demonstrated the possibility of HbR as a candidate for DNA vaccine for leishmaniasis which needs to be explored.

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

The objective of this project is 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.

α-helix of the connecting region has a pivotal role in dimerization

To identify which region of the multidomain protein hGBP-1 is involved in the dimerization, we made a series of truncated proteins, where the nucleotide binding domain has been removed. The analytical gel filtration assay of these proteins was carried out. Remarkably, hGBP-$1^{290\text{-}592}$ exits as a mixture of monomer and dimer. We also obtained a similar result with hGBP-$1^{279\text{-}592}$. Interestingly, hGBP-$1^{308\text{-}592}$, hGBP-$1^{312\text{-}592}$ and hGBP-$1^{318\text{-}592}$ that do not contain the α-6 helix, eluted with a single peak that corresponds to a monomer. To further examine the oligomeric state, we carried out chemical cross-linking experiments of these truncated proteins. The reaction products were analyzed by SDS-PAGE. In the presence of the cross-linking reagents hGBP-$1^{290\text{-}592}$ and hGBP-$1^{279\text{-}592}$ exist as a dimer. On the other hand, hGBP-$1^{308\text{-}592}$ and hGBP-$1^{318\text{-}592}$ exists primarily as a monomer. All these data confirm that the truncated proteins containing at least α-6 helix (hGBP-$1^{289\text{-}592}$ & hGBP-$1^{279\text{-}592}$) are able to dimerize without the globular domain. Thus, the α-6 helix of the connecting region plays a critical role in the dimerization.

GMP formation is regulated with dimerization and cross-talk between the monomers in hGBP-2

The objective of the project is to examine whether hGBP-2 follows dimerization linked GMP formation, we carried out analytical gel filtration assays in the absence and presence of GppNHp. hGBP-2 exists as a dimer in the presence of GppNHp, confirming that GMP formation occurs through dimerization of the protein. To investigate the cross-talk, we have immobilized the proteins in two different ways in the absence of the substrate analogue. In contrast to the unimmobilized hGBP-2, the immobilized protein produced only GDP, and GMP formation was abolished. The absence of GMP formation by preventing dimerization in the immobilized proteins clearly shows a cross-talk between the two monomers that allows the second hydrolysis. The data on hGBP-2 suggests that this family of protein primarily follows a common mechanism of dimerization associated GTP hydrolysis to GMP.

Cloning and transfection of mutant and truncated proteins

To understand which domain of hGBP-1 has a role in antiviral activity, we cloned the full-length and several mutant and truncated proteins of hGBP-1 into eukaryotic expression vector under the constitutive promoter containing the Flag-tag to check the expression of the protein. The recombinant clones were transfected into HeLaM cells using effectene transfection reagent. The expression of the proteins was verified by western blot analysis using anti FLAG antibody. Additionally, immuno-fluorescence microscopy was done to verify, whether the protein is expressed in the cell cytoplasm. Subsequently, the viral assays of these stable cell lines are currently under investigation.

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 antibiotics with greater efficiency against *H. pylori* infection.

Self-association and activation of protein

To examine whether the *H. pylori* enzyme exhibits a concentration dependent self-association and activation, we measured the specific activity at various concentrations of the metal reconstituted protein. The specific activity was found to increase with increasing concentrations of the protein suggesting a cooperative mechanism of substrate hydrolysis by this enzyme. The self-association and activation are unique in the *H. pylori* enzyme as it has not been reported for other arginases. Attempts to fit the data using an equation to determine K_d of dimerization with a model $2M \rightleftharpoons D$ (where M and D represent monomer and dimer respectively) provided $k_1 = 5.1\ min^{-1}$, $k_2 = 19\ min^{-1}$ and $K_d = 4.5\ \mu M$, where k_1 and k_2 represent the rate constants for hydrolysis by the monomer and dimer respectively. The k_1 is more reliable compared to other parameters due to large number of data points at lower concentrations of the protein. The simulated value of k_1 is in close agreement with the experimental data at 500 mM salt ($k_{cat} = 7\ min^{-1}$), which shows the validity of this model.

Dimerization and salt dependent activity assay

To investigate the oligomeric state of the *H. pylori* enzyme, we carried out analytical gel-filtration assay of the wild type protein in the absence and presence of the metal ions (Co^{2+} and Mn^{2+}). The wild type apo-protein eluted with two peaks at lower concentration of salt (0-4 mM) that corresponded to monomer and dimer respectively. With increasing salt concentrations the protein tends to be primarily monomer. At 150 and 500 mM salt only monomer was observed. All these data show that dimer decreases with increasing concentrations of salt indicating that electrostatic interactions play important role in dimerization of the protein. The catalytic efficiency as well as Hill co-efficient (n) was found to decrease with increasing concentrations of salt. A decrease in the catalytic efficiency of about 6 fold at 500 mM salt compared to 4 mM suggests that dimer is responsible for increasing activity. At 500 mM salt no appreciable co-operativity was observed ($n \sim 1.2 \pm 0.1$), which is consistent with the gel-filtration analysis and further supports that monomer is responsible for activity at higher concentrations of the salt. The data suggests that the monomer is less active than the dimer.

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Ribonucleases and ribosome-inactivating proteins: Role in host defense and development of recombinant toxins

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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.

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

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

To understand the effect of expression of genes encoding eosinophil granule proteins, U373 cells, which do not inherently express any eosinophil proteins, were transfected with plasmids containing DNA encoding EDN and ECP. The transcriptome of control cells and cells transfected with ECP and EDN were compared by microarray analysis using a human whole genome chip. Upon transfection with ECP, 634 genes were found to be upregulated while 451 were downregulated. EDN transfection resulted in the upregulation of 570 and down regulation of 423 genes. The pathways found to be significantly upregulated upon transfection with ECP and EDN included those involved in apoptosis signaling and oxidative stress response. Some of the results were validated by real time PCR analysis and more are in progress.

To understand the mechanism of cytotoxicity of ECP, differences in transcriptome in ECP treated and untreated U373 cells were studied by microarray analysis. The study revealed 309 genes to be upregulated and 300 genes to be downregulated upon ECP treatment. The upregulated pathways included various amino acid metabolic pathways, oxidative stress pathway marker MAP kinase-interacting serine/threonine-protein kinase 2 (MKNK2) and apoptosis marker Bcl-2 homologous antagonist/killer (BAK). JAK/STAT signalling pathway molecules were found to be downregulated upon ECP treatment.

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

M. tuberculosis ClpC1 is an 848-amino acid protein and has two AAA+ modules. The monomeric protein has five distinct domains namely, the N-terminal domain (residues 3–153), D1 large domain (residues 154–350), D1 small domain (residues

351–464), D2 large domain (residues 465–722) and D2 small domain (residues 723–848). Seven C-terminal deletion mutants were prepared and characterized to understand the role of different domains of *Mtb* ClpC1 in its function. These mutants were analyzed for ATPase and prevention of protein aggregation activities. All mutants retained full ATPase activity. However, mutants having deletions beyond amino acid 721 lost chaperonic activity. A mutant devoid of the chaperonic activity was found to be in monomeric form indicating that oligomeric form is required for the chaperonic activity of ClpC1 of *M. tuberculosis*. A C-terminal region has been identified which may be crucial for the oligomerization of ClpC1 and in turn in its chaperone activity.

To understand the involvement in virulence and survival of the pathogen in the host, constructs have been made to generate *M. smegmatis* knockouts for ClpC1, ClpX, ClpP1 and ClpP2. In addition, knockdown studies of ClpP1 and ClpP2 genes have been carried out using antisense DNA in *M. tuberculosis*. Knockdown of ClpP2 resulted in retarded growth of *M. tuberculosis*.

Comparison of amino acid sequence of protein subunit of RNase P showed that histidine 67 in mycobacterial protein subunit corresponds to asparagine of the conserved RNR motif of *E. coli* and other bacteria. RNR motif of bacterial RNase is important for binding of protein to RNA component to generate the holozyme. Similarly phenylalanine 18 in *E. coli* RNase P protein subunit has been shown to be important in the holozyme reaction. Phenylalanine 8 is the corresponding amino acid in mycobacterial RNase P. To investigate the role of His67 and Phe8 in RNase P protein function of *M. tuberculosis*, these residues were individually mutated to alanine to generate mutants H67A and F8A. The variants were over expressed in *E. coli* and purified to homogeneity. The wild type RNase P protein, F8A and H67A variants showed similar CD spectra. Holozyme activity of reconstituted RNase P with H67A and F8A variants showed reduced activity compared to that of wild type protein. H67A variant protein was found to bind more tightly to the RNA subunits as compared to the wild type protein. It appears that the targeted residues are crucial for the interaction of protein component of RNase P with the ribozyme. To further investigate the mechanism of interaction of RNase P protein component of *M. tuberculosis* with RNA, two more mutants, H67N and F23A have been generated. Currently the variant proteins are being produced recombinantly.

Construction and evaluation of recombinant toxins

Constructs have been made in which ribonucleolytic toxin, restrictocin and ribosome inactivating protein, saporin have been separately fused with a ligand, head and neck cancer peptide, HN1 specifically targeting human head and neck cancers. The recombinant chimeric toxins have been expressed in E. coli and the proteins purified to homogeneity. Currently, these chimeric toxins are being evaluated for their toxicity against target and non-target cells.

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Structural and functional biology of *Mycobacterial* proteins

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The research program aims at understanding the structure-function relationships of membrane associated serine proteases (MASPs) and enzymes involved in histidine biosynthesis from *Mycobacterium tuberculosis* (*Mtb*) mainly using X-ray crystallographic technique. The objectives of the program are (i) Over-expression and purification of MASPs and his pathway enzymes, (ii) Identification of physiological substrates of MASPs through proteomics approach, (iii) Biochemical and biophysical characterization of these proteins, (iv) Dissecting their roles in *Mtb* pathogenesis through functional studies,(v) Crystallization and 3D structure determination mainly using X-ray crystallographic technique,(vi) Understanding the molecular mechanisms of their action,(vii) Design of inhibitors for these bio-molecules through structure based inhibitor development approach.

A. Over-expression of MASPs in Mycobacterium smegmatis expression system

We have successfully over-expressed three recombinant MASPs (Rv2223c, Rv2224c and Rv2672) in *Mycobacterium smegmatis* (*Msg*) expression system using a protocol standardized in the laboratory. Briefly, 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 ligated into an *E. coli* and *Msg* shuttle expression vector pYUB1062 (a gift from Prof. William R. Jacobs, Albert Einstein College of Medicine, NY, USA) by T4 DNA ligase. The plasmid containing the insert was then transformed into *Msg* strain $mc^2$4517 by electroporation and plated on 7H10 media supplemented with OADC and antibiotics Kanamycin and Hygromycin B. *Msg* colonies grew in around 72 hours. For protein expression, a single colony was inoculated in a primary 10 ml of 7H9 liquid media culture supplemented with ADC and Kanamycin and Hygromycin B. The culture was grown for 24 hours at 37°C. The primary culture was inoculated into 1 litre 7H9 culture media, expression of the protein was induced by adding acetamide at a final concentration of 0.2% when the OD at 600 nm reached to 0.6. After 24 hours of induction the cells were harvested by centrifugation at 10,000g. To get rid of contaminants from the media such as BSA and catalase, the cells were washed twice in buffer 20mM Tris base pH 8.0, 150 mM NaCl.

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 (20,000 PSI). Inclusion bodies, unbroken cells and cellular debris were pelleted down by low-speed centrifugation (10,000g).The cytoplasmic, cell wall (outer membrane) and cell membrane (inner membrane) components of the supernatant were fractionated by centrifugation at appropriate g-forces. The subcellular localization of MASPs was examined by SDS PAGE. Importantly, mass spectrometry analysis of the tryptic digestion of proteins extracted from the gel confirmed that Rv2223c is localized in the outer membrane where as Rv2224c and Rv2672 are 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 such as DDM, OG and CYMAL 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. Experiments aiming to purify MASPs using affinity chromatography are in progress.

B. Purification and crystallization of enzymes involved in the histidine biosynthesis

In this project, we have over-expressed recombinant form of three (Rv1017, phosphoribosyl pyrophospho (PRPP) kinase; Rv1606, phosphoribosyl-AMP cyclohydrolase (PRAC); Rv1599, histidinol dehydrogenase (HD)), of the 11 enzymes required for histidine biosynthesis from α-D-Ribose 5-phosphate, in *Msg* using a similar protocol mentioned above. The expression system was changed from *E. coli* to *Msg* as the yield and stability of the over-expressed proteins in *Msg* are better. Among the three, Rv1016 and Rv1599 were purified to homogeneity using affinity and gel filtration liquid chromatography. The identity of the proteins was confirmed by the mass spectrometry analysis of protein samples extracted from SDS PAGE gel. Medium throughput crystallization screening experiments were set up using commercially available screens: Hampton crystal screens 1 and 2 and JBScreen classic kits 1-10 covering 240 different conditions. The crystallization experiments were performed in 96-well Intelli-plates with nano-volume drop size (200 nl protein plus 200 nl precipitant in the drop and 100 µl of precipitant in the reservoir) using sitting-drop vapour-diffusion technique by the Mosquito robot. Potential lead crystallization conditions showing the growth of micro crystals were obtained from few conditions.

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 catalytic domains involved in biosynthesis of secondary metabolites

Structural modeling of complete PKS modules and analysis of the interaction of ACP with other catalytic domains

We have modeled structures of complete PKS modules by superimposition of modeled structures of individual PKS catalytic domains on the structures of the corresponding domains of mammalian FAS. Since the mammalian FAS structure lacked the ACP domain, we have carried out protein-protein docking studies in combination with molecular dynamics (MD) simulations to identify the interaction site of ACP on the other catalytic domains. Similarly protein-ligand docking and MD simulations have been used to map the phosphopantetheine and substrate binding sites on various catalytic domains. We have also attempted to understand the mechanistic details of substrate channeling between catalytic centers which are separated by as far as 70Å within a PKS module. Multiple explicit solvent MD simulations on ACP domain, KS-AT di-domain and its sub-fragments suggest that, neither the intra domain conformational flexibility of ACP nor the inter domain movements in a module can facilitate substrate transfer over such large distances during polyketide biosynthesis. Based on these studies and prediction of intrinsic disorder we hypothesize that intrinsically unstructured liker stretch preceding the ACP domain might be facilitating movement of ACP domains to various catalytic centers.

Computational analysis of evolution of PKS gene clusters

We have analyzed organization of various catalytic domains and modules in 55 experimentally characterized modular PKS gene clusters to understand how diversity in chemical space is related to organization of functional domains in genomic space. For easy

comparison of insertion, deletion and alterations of PKS domains in various modular PKS gene clusters, each of the four possible PKS modules have been represented by a unique single letter alphabet. The resulting strings of alphabets corresponding to various PKS clusters have been aligned using standard sequence alignment tools like BLAST and CLUSTALW after making suitable alterations to the scoring matrices. It has been observed that PKS clusters having similar module strings in general also have similarity in the linearized forms of their corresponding polyketide chemical structures. We are currently investigating whether this approach can be used for deciphering putative polyketide products of uncharacterized PKS clusters.

B. Conformational transitions associated with activation and autophosphorylation of Ser/Thr kinases

We have carried out a series of long explicit solvent molecular dynamics simulations on active and inactive forms of CDK2 both in presence and absence of cyclin to understand the conformational transitions associated with the activation process. Comparison of intermediates generated during our simulations with known crystal structures indicate that our approach is capable of tracing biologically meaningful path of activation. Our simulations have provided insights into mechanisms by which presence of cyclin or prior phosphorylation of specific sites significantly accelerate the transitions towards the active state.

In order to understand conformational transitions associated with activation via autophosphorylation, we have carried out a series of long molecular dynamics studies on crystal structures of monomeric as well as dimeric forms of mycobacterial Ser/Thr kinase PknB. Our simulations indicate that, in the front-to-front dimmers activation loop of one monomer does not remain in proximity of the catalytic center of the other monomer for facilitating autophosphorylation. On the contrary, in the simulations on the monomeric PknB a solvent exposed activation loop moves to close proximity of its own catalytic center. Our monomer simulations also provide insight on how phosphorylation on primary autophosphorylation site facilitates the phosphorylation of secondary autophosphorylation site. These results have interesting implications for understand auto phosphorylation mechanism of PknB.

C. Identification of new enzymes and novel signaling networks in genomes

AMPylation is a newly discovered post-translational modification involving enzymatic transfer of AMP moiety from ATP, to tyrosine/threonine/serine residues of eukaryotic substrate proteins. We have attempted to identify new AMPylation/de-AMPylation domains in various organisms, understand structural details of their catalytic mechanism and decipher their potential substrate proteins. Computational analysis involving profile HMM and SVM approaches have helped in identifying new AMPylation domains from among the unannotated proteins in genomes of various organisms. We have compared catalytic and active site pocket residues of AMPylation domains to those of kinases to understand the similarity and differences in their catalytic architecture. Molecular dynamics (MD) simulations on crystal structure of Fic-cdc42 complex and modeled structures of Fic-peptide complexes have revealed the structural basis of substrate selection by AMPylation domains. We have also analyzed the known biochemical reactions catalyzed by various folds to identify other catalytic domains which might potentially carry out AMPylation and de-AMPylation reactions.

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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 rationalmolecular design.

1. Understanding the protein architectureand 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.

Continuing our endeavors in understanding the issues of specificity/degeneracy in the immune response, we decided to address the same in the context of an immune evading pathogen HIV. In that direction, two neutralizing epitopes ELDKWA and GGERDRDR were identified on the surface of HIV gp41. Extensive database search was carried out to identify some of the escape mutants of the epitopes. The degeneracy/specificity in the polyclonal immune response was studied by immunizing different groups of mice with each of the wild type epitopes and checking for crossreactivity in the polyconal sera with the variants. These studies showed that degeneracy in the immune response is observed at the polyclonal level even after the maturation of the antibodies post booster.

To further explore the behavior at the level of monoclonal antibodies and understand the physico-chemical basis of such a property, hybridomas were generated against the wild type epitopes. Clones producing multispecific monoclonal antibodies were carefully picked from a population of monoclonal hybridomas following multiple rounds of selection. MAbs KEL10 against ELDKWA and 3C6F7 against GGERDRDR were used for further studies. Both the antibodies showed remarkable binding to the immunizing as well as the variant peptides in a affinity range of 10^{-6} - 10^{-9} nM suggested it to be not non-specific crossreactivity. Further, we showed that these antibodies can recognize the respective epitopes on the surface of the whole Env proteinHIV gp160 Env-B and Env-C (obtained from NIH, USA) which were expressed on the surface of HEK cells. Flowcytometry based assays showed that both the antibodies KEL10 and 3C6F7 were able to bind with the Env protein at levels comparable to a clinical antibody T-32 against envelope, obtained from NIH. This was further confirmed using a clinical ELISA kit coated with the native env protein from subtype B and C.

In order to decipher the structural basis of crossreactivity observed in both the mAbs, we modeled and docked with the correspondiong epitopes and their varients. Extensive in-silico studies revealed a unique mechanism involving CDR flexibility and differential polar interactions, as the basis of multispecificity in the antibodies.

Our earlier success of anti-indolicidin mAb V2D2 in elucidating novel antibiotic peptides with therapeutic potential as well as deciphering the possible mechanism of action of indolicidin prompted us to generate and characterize surrogate receptor mAbs against the homologous tryptophan rich peptide tritrpticin and an anti-tritrpticin mAb T-6C6D7D6 was made, ascites generated in mice and mAb purified and characterized. It was seen that the antibody binds to tritrpticin with micro-molar affinity. Further analysis of the binding of mAb T-6C6D7D6 with the native and phage-derived peptides is being carried out to investigate nature and motif of binding. In order to further extend the studies towards atomic resolution analysis, by x-ray diffraction, scFv of the antibody was made by recombinant approach.

Structural proteiomics of plant seed allergens is being continued further with fractionaltion, purification and characterization of proteins with potenyial aggergy and medicinal properties from *Mucuna pruniens* and *Jatropha curcus*.

One of the proteins from *Mucuna pruniens*MP4, was purified and identified as kunitz type trypsin inhibitor of Delonix regia with 63% sequence homology and 48% sequence homology with water soluble chlorophyll binding protein from *Lepidium virginicum*. Earlier stagnated crystallography of this protein was successful reinitiated in a new crystal form that diffracted at better resolution of 2.1Å. Similar seed proteomics analysis of jatropa led to purification and characterization of a 13.9 kDa protein J90,which showed 48% sequence homology with a protein from*A.thalliana.* The protein has been crsytallized and diffraction data collected to a resolution of 2.5Å. Due to lack of good homologous model in protein data bank, phase problem is being addressed *ab initio.*

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Original peer-reviewed articles

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Chemical Glycobiology: Glycoproteomics and carbohydrate-based drug design.

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Our research efforts are focused on design, development, and application of powerful chemical tools to advance our understanding of fundamental roles played by carbohydrates in biological processes.

Glycosylation is the most abundant and complex post translational modification (PTM) of proteins and almost all mammalian membrane proteins are glycoproteins. Enormous diversity of cell surface glycans and their dynamic spatiotemporal changes are considered to play important roles in cell-cell, cell-pathogen, and cell-matrix interactions. Challenges in study of functional glycomics necessitated the development of unique tools including metabolic glycan engineering (MGE). Depending on the nature of analog used, MGE could modulate glycoforms of cell surface antigens and consequent responses such as T-cell activation. Currently, (I) we seek to apply MGE for engineering of mucin-type *O*-glycans using appropriately designed non-natural analogs of monosaccharide precursors, (II) identification of cell surface antigens subjected to MGE and modulation of their glycoforms, (III) development of carbohydrate-neurotransmitter hybrid molecules for MGE across blood brain barrier (BBB), and (IV) design and synthesis of glycopeptidomimetics (GPM) for inhibition of matrix metalloproteinases (MMP).

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

Peracetylated GalNAc analogs carrying *N*-thioacetyl (Ac_5GalNTGc, **1**) and *N*-glycolyl (Ac_5GalNGc, **2**) groups were synthesized with a goal to apply MGE for mucin-type *O*-glycans. Sulphur analog **1,** if processed by GalNAc salvage pathway, would result in expression of *N*-thioglycolyl-D-galactosamine (GalNTGc); whereas, **2** would result in expression of *N*-glycolyl-D-galactosamine (GalNGc) (control). Jurkat (human T-lymphoma) cells were incubated with dimethyl sulfoxide (DMSO) (vehicle), **1**, or **2** at 100 μM for 48 h and cell surface thiols (CST) were estimated by flow cytometry. Upon treatment with **1**, but not **2**, CST levels increased by three fold indicating that **1** was processed efficiently. Upon treatment with mild reducing agent (TCEP) prior to biotinylation, CST levels increased by 16-fold compared to untreated native (non-TCEP treated) thiol levels indicating that a large proportion of GalNTGc existed in disulfide form. CST levels increased in a dose-dependent manner with **1** up to 200 μM. Time course of CST expression with **1** (100 μM), showed a three-fold increase within 12 h saturating to 3.5 fold at 36 h.

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

In order to identify specific cell surface glycoproteins that may be subjected to MGE, CD43 (leukosialin / sialophorin), a heavily glycosylated type I membrane protein carrying 80–90 *O*-glycans, was chosen as a model. It is known that glycosylation of CD43 varies among cell types and upon T-cell activation. In order to ascertain effects of MGE on expression of CD43 we chose three anti-CD43 antibodies that recognize distinct glycoforms of CD43. Flow cytometry revealed that **1** (50 μM, 48 h) induced drastic down-modulation of neuraminidase-sensitive epitopes (1G10 and L60 reduced to 28 and 26 % of controls) on CD43, while

neuraminidase-resistant epitopes were reduced moderately (L10 – reduced to 54 % of control). In contrast, cells treated with **2** did not show any change indicating that apparent hyposialylation of CD43 induced by **1** is a direct consequence of incorporation of GalNTGc on *O*-glycans. Western blotting confirmed glycoform modulation of CD43 (L60) by **1** in a time and dose dependent manner. Effect of **1** could either be (a) sustained by a second dosage of **1**, (b) allowed to recover by 96 h, or (c) enhance recovery by 72 h by treatment with Ac_4GalNAc. Notably, mRNA levels of CD43 remained unaffected upon treatment with **1** (0 – 100 μM), thus indicating that the effects seen are post-transcriptional. Further studies are focussed on (i) differential MGE, (ii) effect of MGE on T-cell activation, and (iii) characterization of engineered glycans by mass spectrometry (MS).

III. Carbohydrate-neurotransmitter (CH-NT) hybrids for MGE across blood brain barrier (BBB).

MGE for expression of azide-carrying glycans has been shown to be effective in living animals. Azide moiety was expressed in majority of tissues except brain which could be due to poor permeability of BBB by monosaccharide anlaogs. In order to achieve MGE in brain, we reasoned that non-natural monosaccharide analogs could be delivered across BBB if coupled to neurotransmitter or BBB permeable molecules. Towards this end, CH-NT hybrids of peracetylated *N*-azidoacetyl-D-mannosamine (ManNAz) with nicotinic acid, caffeine, and choline (connected *via* esters either at C-1 or C-6) were synthesized and characterized by NMR (^{1}H and ^{13}C) and MS. Water soluble alkyne-biotin probes were synthesized for detection of azides on cell surface using click chemistry. Studies in Jurkat cells using CH-NT hybrids revealed that levels of azide expression was similar to Ac_4ManNAz, indicating intracellular cleavage of neurotransmitter from carbohydrate. Current studies are focused on evaluation of permeability using *in vitro* BBB models and MGE in human neuroblastoma (SH-SY5Y) cells.

IV. Synthesis of glycopeptidomimetics (GPM) as potential inhibitors of matrix metalloproteinases (MMP).

MMPs control tissue remodeling and cancer metastasis. Peptidomimetic small molecules carrying a zinc binding groups (ZBG) are potent inhibitors of MMP (MMPi). However, majority of MMPi suffered from broad-spectrum activities and pleiotropic effects. We seek to design and develop selective MMPi using glycopeptidomimetics. Towards this goal, we have synthesized multiple activated monosaccharide donors with suitable protecting groups for coupling to peptide acceptors under various glycosidation conditions. After optimizing several combinations of donors and acceptors, reaction of armed donor Bn_4Glc-trichloroacetimidate with Bz-Ser-Bn was found to give glycopeptide in good yields. Deprotection of glycopeptide, oxidation of C-6 of carbohydrate, and introduction of ZBG *via* hydroxamic acid are currently underway. In parallell, gene expression and activities of MMPs in HT-1080 (fibroblastoma) cell line are being studied using RT-PCR and zymography, respectively.

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Review / Proceeding

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Role of carbohydrates in host-parasite interactions

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The project is aimed for understanding the differential roles of carbohydrate domains in host-parasite interactions by using synthetic glycoconjugates involving model system such as antimicrobial glycopeptides of innate immune origin.

1. Synthesis and structural characterization of glycoconjugates

2. Structure-function analysis of the synthetic glycoconjugates in the context of host-parasite interactions

It has been shown that all-D-analogs of some of the cationic antimicrobial peptides are equally active as native peptides which contain L-amino acids. But in case of proline rich antimicrobial peptides, formaecin I and M-drosocin, the comparison of the antibacterial activities of D- and L-enantiomers of designed analogs resulted that D-enantiomers were completely inactive or showed very little activity. These peptides are found to be stereospecific in displaying their activities. To explore the importance of conformational or enantiomeric specificity at the sugar level, we have synthesized analogs of formaecin I and drosocin containing L-sugars. Sugars are present in D-enantiomeric form in native formaecin I and drosocin. We have standardized the synthesis of N^{α}-Fmoc-Thr(Ac_3-α-L-GalNAc)-OH at analytical scale and preparatory scale synthesis is still going on. The N^{α}-Fmoc-Thr(Ac_4-β-L-Glc)-OH and N^{α}-Fmoc-Thr(Ac_4-β-D-Glc)-OH were synthesized and used for synthesizing formaecin I as well as drosocin analogs and their structural and functional properties were compared with that of native peptides. All the above synthesized analogs were assayed for their comparative spectrum of antibacterial activities against *E. coli* ATCC 25922, and S. *typhimurium* PNP2 using Radial Diffusion Assay. Both L- and D-Glc- containing analogs of formaecin I and drosocin exhibited similar activity against Gram-negative bacteria indicating that conformational specificity is not present at the sugar level. But the pattern found in case of formaecin was not similar to drosocin analogs. Structural studies of conformationally different sugar containing analogs of formaecin I and drosocin were done by CD in different environments like water, membrane mimicking environment: SDS and helix inducing environment : TFE. In all the environments these glycopeptides show unordered structure. The glycopeptides were unable to aquire any regular structure.

Drosocin is found to contain monosaccharide (2-acetamido-2-deoxy-D-galactopyranosyl; GalNAc) or a disaccharide (D-β-galactopyranosyl-GalNAc) attached to Thr- in its sequence. It is reported that monosaccharide containing drosocin has lower level of antibacterial activity than disaccharide containing drosocin. To study the effect of disaccharide variation and carbohydrate's chain length on structure and function of antibacterial peptides, the analogs of formaecin I and drosocin containing lactose, maltose, and cellobiose are designed. The synthesis of building blocks N^{α}-Fmoc-Thr(Ac_3-β-D-Galp-(1-4)-Ac_3-β-DGlcp)-OH (β-lactosyl-Thr), N^{α}-Fmoc-Thr(Ac_3-α-D-Glcp-(1-4)-Ac_3-β-DGlcp)-OH (β-maltosyl-Thr), and N^{α}-Fmoc-Thr(Ac_3-β-D-Glcp-(1-4)-Ac_3-β-DGlcp)-OH (β-cellobiosyl-Thr) were standardized at preparatory scale.

For synthesizing β-lactosyl-Thr, the free lactose was converted to its acetylated derivative followed by hydrazinolysis of the anomeric *O*-acetyl group in the presence of hydrazine

acetate. For subsequent reaction 1-*O*-unprotected lactose derivative was transformed into trichloroacetimidate by treatment with trichloroacetonitrile in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as base. The glycosyl donor was purified by flash chromatography and then coupled with Fmoc-Thr-OBzl in the presence of catalytic amounts of trimethysilylmethanesulfonate. The product formed was purified and subsequent deprotection of the benzyl ester by hydrogenolysis yielded the final compound, N^{α}-Fmoc-Thr(Ac_3-β-D-Galp-(1-4)-Ac_3-β-DGlcp)-OH. All the intermediate compounds and the final product were characterized by ^{1}H-NMR, ^{13}C-NMR and 2D NMR and mass spectrometry. The syntheses of β-maltosyl-Thr and β-cellobiosyl-Thr were done by following the same synthetic scheme standardized for β-lactosyl-Thr.

The different disaccharide containing analogs of formaecin I and drosoicn were synthesized utilizing synthetically prepared building blocks of threonine. The glycosylated peptides were purified by HPLC and purified peptides were used for the removal of acetyl groups of glycan moiety, followed by their purification again by HPLC. Characterization of the peptides was performed by molecular mass determination.

All the above synthesized formaecin and drosocin analogs were assayed for their antibacterial activities against *E. coli* ATCC 25922, and *S. typhimurium* PNP2 and their activities were compared with their respective native glycopeptides. The pattern of antibacterial activities of different di-glycosylated analogs of formaecin was found to be different with those of drosocin glycosylated analogs but in both the cases glycosylated analogs showed more activity than their respective non-glycosylated forms. CD spectral studies for different disaccharide containing analogs of formaecin and drosocin in different environments like water, SDS and TFE, suggested that there was no much difference in the gross secondary structures even if the carbohydrate side chain was altered. All the spectra showed random coil like conformation.

Publication

Original peer-reviewed article

1. Tapryal S, Krishnan L, Batra JK, Kaur KJ, Salunke DM. (2010) Cloning, expression and efficient refolding of carbohydrate-peptide mimicry recognizing single chain antibody 2D10. **Protein Expr Purif. 72**: 162-168.

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

Principal Investigator
Vidya Raghunathan

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.

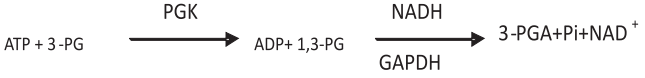
3. Comparison between PGKB and PGKC of, pH optimum of activity and enzyme inhibition by salt and suramin.

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 of, **a**) the role of the C-terminus of PGKC in, modulating enzyme activity when added to PGKB in solution and **b**) glycosomal 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.

Work reported earlier

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



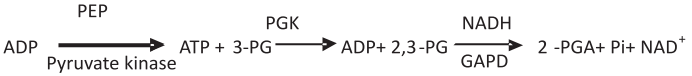
The recombinant clones of *L. mexicana* PGK (PGKB and PGKC) in *E.coli* available to us have been checked by isolating the plasmid and sequencing to confirm the presence of the PGK genes in the correct reading frame. The enzymes were characterized in terms of kinetic parameters.

Protein expression was detected by SDS-PAGE, immunoblotting and activity measurement. For immunoblotting we used rabbit anti-PGK antibody, which detects both PGKB and PGKC. Due to poor stability of PGKC, the purification has been shortened and simplified to a single step, which works for both PGKB and PGKC. This method which, has not been previously used in the case of the PGK enzymes, includes use of Cibachron Blue Sepharose chromatography followed by gel permeation chromatography, giving us pure proteins. PGKC has been purified to about 80% homogeneity by a combination of Cibachron Blue Sepharose and S-100 Sephacryl column chromatography. Purification of PGKB is about >90% after a single step Blue Sepharose chromatography.

Phosphoglycerate Kinase Assay:

ATP and 3-PG binding, assay1. The activity of the *E.coli* recombinant proteins PGKB and PGKC were measured spectrophotometrically (see reaction above) by assaying the oxidation of NADH in the back reaction as shown above (ref). The assay was performed at 20° C in a 3.04 ml reaction mixture containing 100mM triethanolamine hydrochloride buffer adjusted to pH 7.6 with KOH, 40 mM ATP, 150mM 3-P-glycerate, 12mM NADH, 100mM magnesium acetate, 40mg/ml bovine gamma globulin and glyceraldehyde-3-phosphate dehydrogenase (crystalline suspension in 3.2M $(NH_4)_2SO_4$ solution that is about 10mg/ml enzyme at 80 U/mg.) The reaction was initiated by the addition of 20µl of diluted enzyme and the conversion of NADH to NAD^+ monitored at 340nm.

ADP binding: assay2. A modified assay protocol was developed using pyruvate Kinase/PEP for ATP generation. This assay was carried out at 20° C in a 3.06 ml reaction mixture containing 200mM triethanolamine hydrochloride buffer adjusted to pH 7.7 with KOH, 60mM magnesium acetate, 500mM potassium acetate, 40mM ADP, 100mM phosphoenolpyruvate, 150mM 3-phosphoglycerate, 20mM NADH, 40mg/ml bovine gamma globulin, pyruvate kinase and glyceraldehyde-3-phosphate dehydrogenase (as above). The reaction was initiated by the addition of 50µl of diluted enzyme.



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.

Effect of peptides on substrate binding to PGKB: The peptides derived from C-terminal extension of PGKC were incubated with PGKB at the ratio of protein: peptide, 1:1, for 20min at 4ºC and tested for the effect on affinity for ATP and 3-PG using phosphoglycerate kinase assay- 1.

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. It was suggested by RAPSAC members that labeled peptide be used for recording ^{15}N, ^{13}C spectra or else deuterated amino acid be substituted in parts of the peptide. However, making a completely ^{15}N, ^{13}C labeled peptide was prohibitively expensive as the peptide was purchased commercially. Further, partial deuteration would still have left large parts of the spectra unresolved. Fortunately a solution was found by recording the spectra in deuterated MeOH at 500 MHz. This sample gave good resolution. Conditions were found for obtaining the proton spectra of peptide in deuterated SDS. High resolution spectra were recorded at 40C for the peptide reconstituted in deuterated SDS using field strength of 700 MHz. These data have been analyzed.

GENE REGULATION

GENE REGULATION

Molecular analyses of the human and animal genome(s)

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Our focus has been on the analyses of human and animal genomes. Present work relates to the germ line genetics and genome-wide search of the genes tagged with minisatellites in buffalo *Bubalus bubalis*.

1. Isolation and characterization of satellite tagged somatic and spermatozoal transcriptome in buffalo *Bubalus bubalis*.
2. Mapping and copy number analysis of the candidate genes.

Genome-wide search of the genes tagged with minisatellites in buffalo *Bubalus bubalis*

In the present study, we used two other primers 33.6 and $(TGG)_5$ to uncover tagged mRNA from the spermatozoa and different somatic tissues. The amplification resulted in 29 different transcripts tagged with 33.6 and 53 tagged with $(TGG)_5$.

A. mRNA transcripts tagged with 33.6 repeat

Of the 29 transcripts tagged with 33.6 repeat, 21 were found to be conserved across the species and 7 (Dp2, Dp5, Dp6, Dp11, Dp13, Dp24 and Dp28) were specific to bovids while one transcript (Dp25) was exclusive to the buffalo genome. RT–PCR analysis of the transcripts showed varying levels of signal among somatic tissues, gonads, and spermatozoa with respect to 13 mRNA transcripts whereas the remaining 16 showed almost uniform signals in all the tissues examined. Subsequently, 13 transcripts were subjected to quantitative expression analysis using real-time PCR. Nine showed the highest expression in spermatozoa (Table 1). Dp2 showed maximum expression in testis, Dp9 in liver, and Dp22 in lung, suggesting their specific roles in these organs.

We characterized two single-copy 33.6 tagged genes *SARS2* (Seryl-tRNA synthetase 2) and *PXMP-4* (Peroxisomal membrane protein-4) uncovered by Dp10 and Dp26, respectively. Both showed maximum expression in spermatozoa. Absence of peroxisomes (*PXMP-4*) in the cells of the liver, kidney, and brain has been associated with Zellweger syndrome; a rare congenital disorder that affects children. Dp10 showed high level of sequence homology (95%) with that of *SARS2* nuclear gene encoding mitochondrial protein. Chromosomal mapping of *SARS2* using bovine *SARS2* BAC probe showed it to be present on buffalo chromosome 18 (Figure 1A). In humans, several point mutations in *SARS* of mitochondria lead to inaccurate translation causing sensorineural deafness. In mouse mitochondrial *SARS* showed elevated expression in heart and liver having high metabolic rates. Therefore, lowest expression of this gene in

buffalo's heart and liver was found to be startling. Additional work on this line would strengthen clinical significance of this gene.

B. mRNA transcripts tagged with TGG repeat

The presence of TGG repeats in the buffalo transcriptome was ascertained as mentioned above. A total of 53 transcripts tagged with $(TGG)_5$ repeats were detected, of which 14 were confined to spermatozoa while 38 showed differential expression across the tissues. Of these 38, 18 showed significant homologies with cDNA sequences from the refseq_mRNA database. Surprisingly, none of the spermatozoal originating transcript depicted significant homology with sequences in the database, suggesting that they are yet to be characterized. The transcripts were found to exhibit varying levels of expression across the tissues.

We derived full length sequence of SR1 (3084 bp) and SR29 (975 bp) coding for 1028 and 325 amino acids, respectively. SR29 [GenBank: GU433091] originated from testis and showed maximum expression in spermatozoa (>5000 folds) followed by testis (>1000 folds) compared to that from heart used as calibrator (expression =1). Further, the *TSPY1*-like sequence (SR29) amplified from *Bubalus bubalis* showed >95% identity with that of *Bos taurus* [GenBank: XM_001254382.2] and BAC clone of *Bos taurus* Y Chromosome CH240-127C20 [GenBank: AC234853.4]. Invariably, this gene is referred to as *TSPY1*-like gene. We propose that the same may be referred to as *TSPY1* since we have detected its high level of expression in testis and localized the same onto the buffalo Y chromosome (Figure 1B).

There is a remarkable variation in the copy number of this gene among different species of mammals. Significantly, an increase in the *tspy* copies is linked with the human male infertility while a decrease is associated with prostate cancer. The *tspy* copy ranges from 20-60 in the humans and 50-200 in bovids. More copies are related with enhanced level of protein synthesis. However, it is not clear if more copies of this gene protect a human male from the prostate cancer. Even if it does, the high copy number of this

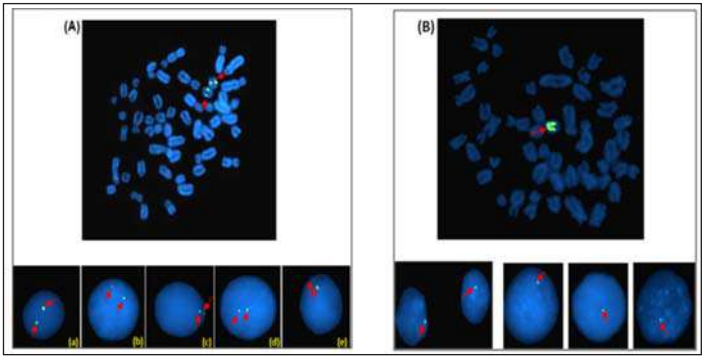

Figure 1. Chromosolnal Localization of SARS2 and TSPY1-like gene. SARS2 BAC probe showing signals on buffalo metaphase chromosome 18 and interphase nuclei **(A)** *TSPY1-like gene showing signals on the buffalo metaphase chromosome Y and interphase nuclei* **(B)**

gene in human becomes cause of infertility. Taken together, we construe that a critical balance of copy number of *TSPY1* gene needs to be maintained. The fact that some genes detected in the present study have clinical significance adds additional strength to MASA-mediated approach of genome analysis.

Table 1. Transcripts showing highest expression in different categories of the tissues

Tissue specificity	Clone ID	Total
Spermatozoa only	Dp1, Dp4, Dp8, Dp10, Dp20 , Dp27, pSRC13, pSRC15, pSRC18, pSRC19, pSRC20, pSRC27, pSRC31, pSRC40, pSRC43, pSRC44, pSRC46, pSRC47, pSRC49, pSRC51, pSRC53, pRSC1, pRSC4, pRSC5, pRSC7, pRSC8, pRSC11 & pRSC12.	28
Testis only	pSRC48.	1
Spermatozoa and Testis	Dp17, pSRC32, pSRC34, pSRC38, pSRC45 & pSRC55.	6
Spermatozoa, Somatic and Testis	Dp2, Dp19, Dp22, Dp26, pSRC1, pSRC8 , pSRC16, pSRC23, pSRC24, pSRC26, pSRC28, pSRC30, pSRC36, pSRC39, pSRC41, pSRC42, pSRC50, pRSC3, pRSC6, pRSC10, pRSC13 & pRSC14.	22
Excluding Spermatozoa	Dp9, Dp16, pSRC17, pSRC22, pSRC37 & pSRC54,	6

Detailed characterization of mRNA transcripts from different somatic tissues and spermatozoa (Table 1) is envisaged to be useful for (1) ascertaining their involvement in regulation of in/fertility, (2) molecular basis of delineation of buffalo breeds, if any, and (3) identification of "superior" germplasm enriching

prospects of animal biotechnology. Novel part of the present approach is that several functional, structural, and regulatory genes have been accessed without screening the cDNA library.

Publications

Original peer-reviewed articles

1. Raina A, Yadav B, Ali S and Das Dogra T (2010) Misinterpretation of Results in Medico-legal cases due to Microdeletion at Y chromosome. **Molecular and Cellular Probes 24**: 418-420.
2. Ali S and Ali S. (2010) Genetic Integrity of the human Y chromosome exposed to groundwater arsenic. **BMC Medical Genomics 3**: 35.
3. Pathak D, Srivastava J, Samad R, Parwez I, Kumar S and Ali S (2010). Genome-wide search of the genes tagged with the consensus of 33.6 repeat loci in buffalo *Bubalus bubalis* employing minisatellite-associated sequence amplification. **Chromosome Research 18**: 441-458.

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

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I. Cell signalling & host-pathogen interaction

M. tuberculosis serine/threonine protein kinases (STPKs) have been implicated to play a role in a number of cellular processes. However, the exact mechanisms as to how they bring about these effects and the existence of signalling cascades, if any, have not yet been identified. In this project 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.

Nucleocytoplasmic transport between the nucleus and the cytoplasm occurs through nuclear pore complexes (NPCs), ~ 60 MDa macromolecular structures that span from cytoplasm to nucleus across the lipid bilayers of the nuclear envelope. NPCs are suggested to be dynamic structures which undergo conformational changes during transport and cell cycle progression. Phosphorylation of nuclear transport proteins was shown by various groups to either up-regulate or down regulate transport machinery. However, the precise role of these phosphorylations in the nuclear pore function have not been elucidated. We are interested in addressing the role of phosphorylation in the function of nucleoporins and their dynamics.

While reports from various groups have shown that the STPKs modulate activities of proteins involved in various cellular processes, much work remains to be done towards understanding how they respond to extracellular environment and how the signals are transferred from one kinase to the other. PknA and PknB are essential proteins that play a role in modulating cell wall syntheis. The activity of PknG has been reported to be necessary for modulating the survival of the pathogen in the host. Thus far our endeavours have been directed towards the biochemical characterization of these kinases, the identification of their substrates, and the characterization of the role of the substrate phosphorylations mediated by these kinases. We aim to examine the *in vivo* signalling mediated by the *M. tuberculosis* STPKs, and the modulation of host signalling pathways.

I. Cell signalling & host-pathogen interaction

The characteristic nature of the cell envelope of *M. tuberculosis* is linked to its pathogenicity. The thick layer of lipids on the outer surface of mycobacteria is protective in nature, and mycolic acids comprise the bulk of this layer. Mycolic acids also constitute structural components of the cell wall and envelope. Mycobacteria are unique in having two fatty acid synthase (FAS) systems, FAS-I and FAS-II, and both these pathways are involved in the synthesis of mycolic acids. Eukaryotic- like FAS-I is a single multi-domain enzyme, while FAS-II comprises discrete monofunctional enzymes, which carry out the various sequential steps involved in the process of synthesis. We have identified InhA, the primary target for the

first-line anti-tuberculosis drug isoniazid, a key enzyme of the Fatty Acid Synthase-II, as a substrate for mycobacterial serine/threonine protein kinases. Using a novel approach to validate phosphorylation of a substrate by multiple kinases in a surrogate host (*Escherichia coli*), we have demonstrated efficient phosphorylation of InhA by PknA, PknB and PknH, and to a lower extent by PknF. Additionally, the sites targeted by PknA/PknB have been identified and shown to be predominantly located at the C-terminus of InhA. Results demonstrate in vivo phosphorylation of InhA in mycobacteria and validate Thr266 as one of the key sites of phosphorylation. Significantly, our studies reveal that the phosphorylation of InhA by kinases modulate its biochemical activity, with phosphorylation resulting in decreased enzymatic activity. Co-expression of kinase and InhA alters the growth dynamics of *Mycobacterium smegmatis*, suggesting that InhA phosphorylation in vivo is an important event in regulating its activity. An InhA-T266E mutant, that mimics constitutive phosphorylation, is unable to rescue an *M. smegmatis* conditional inhA gene replacement mutant, emphasizing the critical role of Thr266 in mediating post-translational regulation of InhA activity. The involvement of various serine/threonine kinases in modulating the activity of a number of enzymes of the mycolic acid synthesis pathway, including InhA, accentuates the intricacies of mycobacterial signalling networks in parallel with the changing environment.

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. Nucleoporin Tpr was originally identified as the oncogenic activator of the met, raf, and trk protooncogenes. Tpr is a large 270 kDa protein with a bipartite structure consisting of a ~1600-residue α-helical coiled-coil N-terminal domain and a highly acidic noncoiled ~800 amino acid carboxy terminus. In a previous study we have identified Tpr as a substrate of MAP kinase ERK2 and identified the ERK2 mediated phosphorylation sites on Tpr. Tpr has been demonstrated to act as an ERK2 scaffold in NPC, in turn resulting in phosphorylation of substrates that interact with Tpr. Conventionally in eukaryotes, unspliced RNA is retained in the nucleus, and only processed mRNA is exported through the NPC. However, retroviruses have developed mechanisms to overcome this regulation, thus enabling unspliced genomic RNA to be exported and finally packaged. These mechanisms can be classified into two types, Rev dependent and Rev independent. The Rev dependent pathway, employed by the Human Immuno deficiency Virus (HIV), utilizes retroviral Rev protein bound to the Rev response element. The Rev independent mechanism to export unspliced RNA is used in Mason Pfizer Monkey Virus (MMPV). This is mediated by a *cis* – acting element present in the unspliced transcript, known as the Constitutive Transport Element (CTE), which directly recruits host cellular factors for exporting intron containing RNA. We have performed a comprehensive analysis of nucleoporin Tpr's role in modulating protein import, export, mRNA export and unspliced RNA export. We found that Tpr does not seem to play any significant role in regulating protein import or protein and mRNA export. However, it plays a definitive role in modulating CTE- mediated export of intron containing RNA. Depletion of Tpr results in enhancement of CTE function ensuring an increase in the export of Gag/Pol-CTE RNA, thus leading 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 are reported to promote CTE dependent export. Importantly, we find that localization of Tpr to the NPC is necessary for Tpr mediated regulation of unspliced RNA export. Collectively, our results demonstrate that nucleoporin Tpr plays a novel and critical role in modulating the export of aberrant intron containing RNA.

Publications

Original peer-reviewed articles

1. Sajid A, Arora G, Gupta M, Upadhyay S, Nandicoori VK and Singh Y (2011) Phosphorylation of Mycobacterium tuberculosis Ser/Thr Phosphatase by PknA and PknB. **PLoS One 6**: e17871

2. Khan S, Nagarajan SN, Parikh A, Samantaray S, Singh A, Kumar D, Roy RP, Bhatt A and Nandicoori, VK (2010) Phosphorylation of enoyl-ACP reductase InhA impacts mycobacterial growth and survival. **J. Biol. Chem 285**: 37860-37871.

Elucidating the molecular link between aging and innate immunity using *Caenorhabditis elegans* as a model system

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The main focus of our lab is to understand how longevity of an organism is controlled and 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. We are trying to decipher the complex interplay between two transcription factors (TF), downstream of the insulin-like signalling (IIS) pathway, to determine life span and pathogen resistance. Using a powerful combination of molecular genetics, molecular biology and genomics, we are also trying to unravel novel pathways that determine longevity. We are also working on identifying the whole complement of TFs that regulate both innate immune response and longevity in worms.

AIM 1. Deciphering the cross-talk between IIS pathway and HSF-1 in innate immunity and during aging

The mechanism by which two TFs, DAF-16 and HSF-1 control longevity, stress response and innate immunity is not well-understood. Using Chromatin immunoprecipitation (ChIP) combined to deep sequencing, we will study the differential recruitment of the TFs under different physiological conditions and pathogen stress.

AIM 2. RNAi screen to identify TFs that are regulators of innate immunity

TFs are central to all processes, including innate immunity and aging. So, understanding the contributions of TFs towards a particular phenotype is of paramount importance. Here, we will try to identify all the TFs that are part of the innate immune system in *C. elegans* using a robust RNAi screen.

AIM 3. Identify novel kinase regulators of IIS pathway using a directed RNAi screen

We are interested in identifying novel kinases that regulate the IIS pathway. An RNAi screen will be used to identify these kinases that will be further characterized extensively.

Standardization of a liquid dauer assay using *C. elegans*

In order to identify kinases that modulate the IIS pathway, we designed an RNAi screen using dauer as an output. Dauer is a developmental stage that is controlled by the IIS pathway in *C. elegans*. We used a temperature sensitive mutant in the IIS receptor, *daf-2(e1370)* that forms 100% dauer at 25 °C but no dauers at 15 °C. However, at an intermediate temperature, some of the worms form some dauers while the others mature to adulthood. We performed extensive standardization experiments by using different liquid media, volume of media, temperature, number of worms per lane, time of scoring etc. in order to device a highly reproducible assay. Worms were fed RNAi producing bacteria and increase or decrease in dauer formation was scored. As a control, we used *pdk-1* RNAi that produce 100% dauer and *daf-16* RNAi that produce no dauers and *daf-16* RNAi that produce no dauers.

Identification of novel kinases that modulate IIS pathway

After extensive screening of the kinome RNAi library, we identified 15 kinases that modulate the IIS pathway. We identified *akt-1* and *akt-2*, which are known components of the IIS pathway and

expected to increase dauer formation when knocked down in *daf-2(e1370)*. However, most of the kinases that we identified are novel and we are in the process of characterizing them.

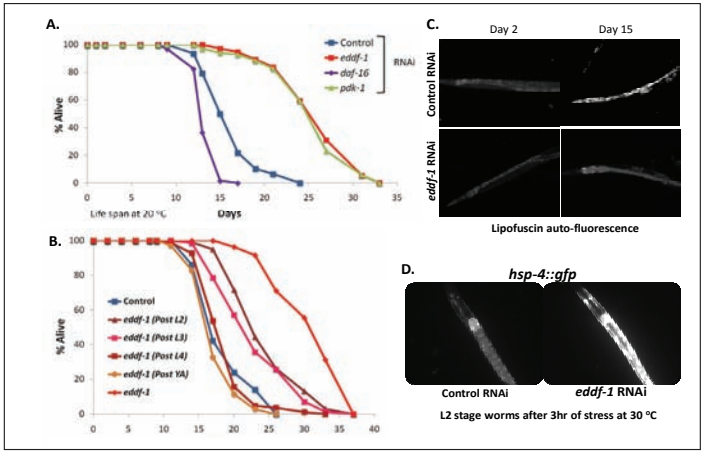

Figure 1. *eddf-1, a novel kinase that regulates longevity. (A) Knock down of eddf-1 by RNAi leads to enhanced longevity (B) eddf-1has to be knocked down early during development for maximum life span (C) eddf-1 knockdown worms have lower auto fluorescence, an indicator of heathspan (D) knocking down eddf-1 produces robust ER stress response as measured by expression of a xbp-1 target gene, hsp-4.*

eddf-1, a novel regulator of longevity

The serine-threonine kinase gene, *eddf-1* is the first gene from the screen that we characterized. Knock down of *eddf-1* resulted in dramatic extension of life span, upto 45% (Figure 1A). These animals not only live long, but have increased health span (Figure 1C) as well as extended reproductive span. Interestingly, *eddf-1* has to be knocked down very early in life in order to get increased longevity (Figure 1B), a phenomenon very different from most other long-lived mutants. Apart from that, *eddf-1* knockdown does not increase stress and pathogen resistance, a hallmark of other longevity genes. Using genetic analysis, we found that *eddf-1* works independently of the IIS pathway, TGF-beta signalling pathway, the germline signalling pathway as well as other known longevity pathways. Preliminary experiments show that *eddf-1* may be working by regulating ER homeostasis (Figure 1D) and we are in the process of further characterizing it.

eddf-2, a novel modulator of IIS pathway

Another serine-threonine kinase, *eddf-2*, was found to significantly increase dauer formation (Figure 2A), but surprisingly, decreased life span through the IIS pathway (Figure 2B). Such instances of decoupling of the two phenotypes (dauer and longevity) as well as their reciprocal regulation are quite rare. In order to better understand the functioning of this gene, we looked at its effect on the most important output of the IIS pathway, DAF-16/FOXO.

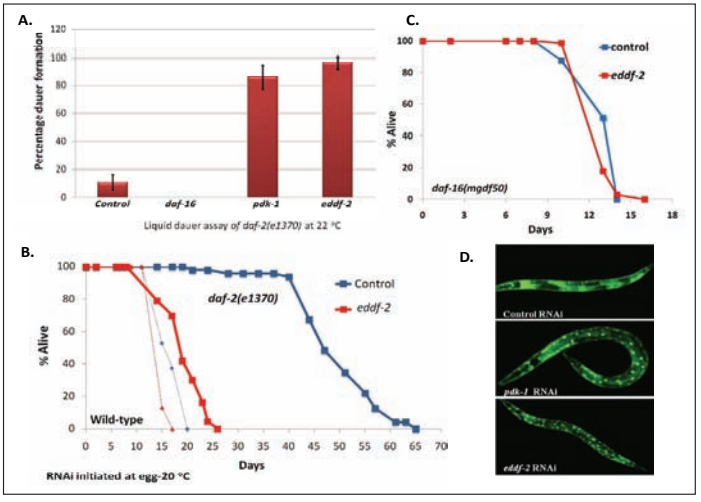

Figure 2. *eddf-2 regulates life span through the IIS pathway. (A) eddf-2 knock down results in increased dauer formation in daf-2(e1370), a mutant in the IIS receptor (B) eddf-2 RNAi dramatically suppresses the life span of daf-2(e1370) (C) eddf-2 fails to suppress the life span of daf-16 mutant worms (D) eddf-2 knock down results in DAF-16 nuclear translocation.*

We found that knockdown of *eddf-2* results in increased DAF-16/FOXO nuclear translocation (Figure 2D) and small increase in the expression of target genes, but no change in life span on a *daf-16* mutant (Figure 2C). We hypothesize that *eddf-1* may be a kinase for one of the adaptor molecules in the IIS pathway. Currently,

we are performing life span analysis with different mutants to decipher the role of *eddf-2*.

Identifying transcription factors that regulated innate immunity genes

In order to identify transcription factors those regulate innate immunity genes in *C. elegans*, we standardized an RNAi screen in liquid culture. We took a transgenic *C. elegans* that expresses a C-type lectin gene (*clec-85*) promoter driving *gfp* expression. On a vector control RNAi, the worms express GFP in the intestine. As a control for this experiment, we cloned the *gfp* gene in a RNAi vector; *gfp* RNAi completely suppress *gfp* expression in the *clec-85::gfp* transgenic lines. While the screen is ongoing, we have three putative clones that suppress *gfp* expression to varying degrees and may be important regulators. The roles of these transcription factors in innate immunity and longevity will be further characterized.

Producing antibody against DAF-16 and HSF-1

We raised antibodies against the two important transcription factors, DAF-16 and HSF-1. We performed western blot and immunoprecipitation analysis using worm lysates to check the specificity of the antibodies. Our experiments reveal that the antibodies are not of high specificity. It failed to recognize endogenous proteins but was able to detect the overexpressed proteins in worms. We performed the exercise of generating the antibody twice with the same result. Now, we will try to purify the protein from a eukaryotic system and raise the antibody. We are in the process of standardizing ChIP using tag-specific antibodies in a transgenic system that overexpresses the tagged proteins.

Molecular biology of infectious diseases

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Infectious diseases rank among the leading causes of death of both humans and animals in global surveys. 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.

(I) 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 disease 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 or 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 gene for its toxicity and immunogenicity.

Further studies on 3 of the 5 non-toxic mutants of Etx have been carried out. The mutants were analyzed for their binding and heptamerization capability on MDCK cells membrane. All the mutants were able to bind, as established by cell surface staining and Western blotting. All the 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 response.

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 one step during purification, 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.*

(II) Studies on the enzymes of metabolic pathways of *M. tuberculosis.*

One of the strategies for developing new drugs to control microbial pathogens is to utilize differences in the metabolism of the pathogenic and host organisms. Targeting the enzymes of metabolic pathways as potential drug targets would be important to control *M. tuberculosis*.The branched-chain amino acid biosynthetic pathway, for the synthesis of leucine, isoleucine and valine, is essential for the growth and virulence of *Mycobacterium tuberculosis*. The absence of this pathway in humans, and its essentiality in *M. tuberculosis*, make the enzymes of this pathway attractive targets for the development of antituberculosis drugs.

The objectives of the project are to clone, express and characterize the enzymes of metabolic pathways of *M. tuberculosis*. Biochemical characterization of the recombinant enzymes will be carried out in order to identify the differences and similarities between *Mtb* and human, which can be exploited for designing new selective inhibitors.Further, large amounts of recombinant proteins will be produced in order to carry out structural studies. We also plan to study the effect of site-directed mutagenesis of the crucial residues on the enzyme activity.

The two leuA mutants, E218W and H285A, generated on the basis of the residues that have remained conserved across the species, were investigated in detail. The mutants showed no detectable activity suggesting that these residues might be critical for catalysis.

In order to gain further insight into the specific role these residues might be playing in enzyme function, biophysical studies using CD spectroscopy, fluorescence spectroscopy, isothermal titration calorimetry and ANS surface hydrophobicity were carried out. The results obtained from these studies were correlated with the functional activity of the enzyme under similar conditions, such as variable temperature, pH and denaturants.

The far-UV and near-UV CD-spectroscopy revealed that the mutations did not affect either the ordered secondary structure or the tertiary structure of the enzyme. Steady-state intrinsic tryptophan fluorescence studies revealed that the point mutations resulted in perturbations in the metal binding sites of the protein.

The change in local conformation brought about by monovalent ions were more pronounced in the wild type protein when compared to the mutants. Although, the mutants were equally thermostable, the metal ions destabilized the overall conformation, as indicated by decrease in the TM (melting transitin temperature) in the presence of the metals. Binding of both the substrate and the inhibitor resulted in more pronounced changes in the secondary structure as compared to the overall tertiary structre. Ligand binding studies using ITC revealed that both the wild type and the mutant LeuA had moderate binding affinity for its inhibitor L-leucine.

Investigations with another enzyme of the branched chain amino acid pathway of the *M. tuberculosis* i.e. ilvE were also carried out. The ilvE showed a broad substrate specificity for amino acid and was able to transaminate L-leucine>L-isoleucine>L-valine. ITC data showed that the binding of ilvE to its co-substrate (α-KGA) has a low affinity justifying its broad specificity.

Publications

Original peer-reviewed articles

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Patent

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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 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. Our current efforts are focussed on understanding different aspects of the mechanisms by which mammalian insulators influence chromatin domain organization.

In view of the diverse mechanisms that might operate to achieve functional abrogation of enhancer-promoter interaction by the insulators, we are investigating the following aspects regarding the murine insulator at *H19-ICR* that is a well established CTCF-dependent insulator responsible for regulating the monoallelic imprinted *Igf2* expression in vivo in mammals.

1. Analysis of the mechanisms by which *H19-ICR* insulator organizes chromatin structure at the endogenous locus to prevent the enhancer-promoter interaction.
2. Evaluating the ability of the *H19-ICR* insulator to organize chromatin at the heterologous TCRβ locus and interfere in *cis* DNA interactions

The enhancer Eβ at the TCRβ locus is responsible for regulating the chromatin accessibility of nearly 25kb region that encompasses the two DJCβ clusters and the two promoters PDβ1 and PDβ2 that regulate the transcription and recombination at these clusters. It has been suggested that Eβ generates accessibility in two distinct ways; while accessibility at PDβ1 and PDβ2 requires a holocomplex formation between Eβ and PDβ, the accessibility of the rest of the 25 kb region is independent of this interaction. As the Eβ-PDβ1 functional interaction was observed to be reduced by the *H19-ICR* inserted between the two, it is of interest to determine the structural correlates of this reduction to gain insights into the ability of the insulator to prevent enhancer initiated tracking signals and/or looping interactions.

Towards this goal, allele specific chromatin immunoprecipitation (ChIP) assays against specific histone modifications were designed. Since the *H19-ICR* was shown to organize a functional insulator upon maternal inheritance, thymocytes from TCR-ins/TCRβ-del, Rag deficient mice were used for analysis. The paternally inherited TCRβ-del allele has a 15kb deletion and hence the ChIP analysis reported specifically the chromatin structure of the maternally inherited TCR-ins allele wherein the inserted *H19-ICR* acts as a functional insulator. Our analysis so far indicates that the acetyl-H3K9 and trimehtyl-H3K4, the two marks of activated and accessible chromatin, are reduced in the TCR-ins allele in the DJCβ1 region. This correlates perfectly with the observed reduction in transcription and recombination at this region. As expected, the region downstream to the inserted *H19-ICR* encompassing PDβ2 and DJCβ2 region, that did not exhibit any reduction in transcription and recombination due to position dependence of insulator activity, acquired acetyl-H3K9 and trimethyl-H3K4 to extents similar to the wild type allele. Notably, the DJCβ1 region is made accessible in the wild type by the Eβ without its interaction with PDβ1. Hence, these results suggest that *H19-ICR* has the ability to prevent enhancer initiated

tracking signals in the heterologous context of TCRβ. Since the TCRβ-del background allele has the PDβ1 promoter region, we have not yet been able to check the chromatin structure of PDβ1 in TCR-ins. However, single nucleotide polymorphisms (SNPs) have been identified between *Mus musculus domesticus* and *Mus castaneus* and a congenic strain of mice has been created that carries the *castaneus* TCRβ locus in the *domesticus* backgroud (TCR-cas) in Rag deficient mice. TCR-cas will be used for the allele specific ChIP analysis to validate our findings and to analyse the PDβ1 chromatin structure by Single Nucleotide Primer Extension (SNuPE) assays that take advantage of the SNPs.

Since antigen receptor loci like IgH and TCRα/δ have been reported to have multiple CTCF binding sites, it has been hypothesized that CTCF is important for contraction of these loci prior to recombination. Our results demonstrating preferential usage of TRBV31 for recombination suggest altered chromatin loopscape of the TCRβ locus in the mutant allele in support of this idea. Whether the inserted *H19-ICR* interacts with other CTCF binding sites or with other types of cis regulatory elements to effect an altered chromatin loopscape, needs to be investigated. 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. Their functional insulator activity is currently under investigation. Additionally, conditions are being standardized for chromatin 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 response in most eukaryotic cells by regulating specific intracellular signaling cascades. We are interested in exploring signaling events in the biology of two diverse cell types; 1) malaria parasite *Plasmodium falciparum,* 2) mammalian neurons.

The following are the major areas of interest:

I. 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 elucidating the role and regulation of phosphoinostides, their effectors and calcium mediated signaling in the life cycle of *Plasmodium falciparum*. In addition, we are also investigating the role of protein kinases that may control important events like mRNA splicing in *P. falciparum*.

II. 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 the cell cycle, which is reflected by the aberrant modulation of cell cycle proteins like cyclins and cyclin dependent kinases (cdks). Molecular mechanisms underlying this process are not understood clearly. We are investigating how levels of cyclin/ cdks are modulated during neuronal apoptosis and via what mechanisms aberrant cdk activity causes neuronal cell death.

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

a. Regulation of parasite Glideosome Assembly and Inner Membrane complex (IMC) by phosphorylation.

As reported in the previous year, we have identified PfGAP45 as one of the targets for both PfPKB and PfCDPK1. The phopshorylation sites for both these kinases on PfGAP45 were identified using mass spectrometry, site-directed mutagenesis and 2D-phsophopeptide mapping in tandem. To understand how phosphorylation at these sites regulates PfGAP45 function, parasite lines expressing GFP-fusion proteins of GAP45 and its phosphorylation site mutants were generated. In addition, phosphorylation site specific antibodies were also raised successfully. A combination of these and other tools is being used to investigate the regulation of PfGAP45 and glideosome function. We found that PfGAP45 phosphorylation is dependent on phospholipase C (PLC) mediated calcium release, which fits in well with the regulation of PfCDPK1 and PfPKB by PLC-calcium signaling. In the light of previous findings from our group *(Vaid*

et al., JBC 2008) and others (*Singh et al*. *Plos Pathogens 2010*) which proposed the role of PLC mediated calcium release in RBC invasion, the present observations hint at the possible existence of a PLC-calcium-PfPKB/PfCDPK1-PfGAP45 like cascade. Additional studies to test this model are progress.

b. Role and regulation of Calcium Dependent Kinases

It is now clear that Calcium Dependent Protein Kinases (CDPKs) are responsible for various critical events in the life cycle of malaria parasite. We have been interested in how PfCDPK1/4 are regulated and contribute to calcium signaling in the parasite. In the previous year we had reported molecular mechanisms involved in the regulation of PfCDPKs by their Junction domain (JD) and identified regulatory elements in this domain. To gain deeper insights into the regulation of PfCDPK1 by its Junction-Domain, molecular modelling studies were performed using recently reported crystal structure of *T. gondii* TgCDPK1/3. The homology model for PfCDPK1 was generated in both active and inactive conformation. Several putative residues in both J-domain as well as the kinase domain (e.g.E152, E155,) which were predicted by the model were mutated to validate their function. Biochemical studies using these mutants suggested that some of these residues (like M347, F350) may stabilize both the inactive and active conformation of the kinase. The N-terminal region of PfCDPK1 is also essential for the regulation of PfCDPK1. In addition, the autophosphorylation sites important for PfCDPK1 activation were identified.

c. Characterization of a Splicing Related Protein Kinase (PfSRPK) from P. falciparum

Recent studies indicate that post-transcriptional events like mRNA processing and splicing may regulate gene expression and thereby control the diversity of the proteome of malaria parasite *Plasmodium*. However, molecular mechanisms that regulate events like mRNA splicing in malaria parasite are poorly understood. We identified a novel Splicing Related Protein Kinase from *Plasmodium falciparum (*PfSRPK1*)*. PfSRPK1 modulated RNA splicing mediated by parasite extracts. PfSR1, a putative splicing factor from *P. falciparum*, was identified as a substrate of PfSRPK1. PfSR1 interacted with RNA and PfSRPK1 modulated its RNA binding ability. PfSRPK1 and PfSR1 associated and co-localized in the parasite and exhibited nucleocytoplasmic shuttling during parasite development. These studies provide insights into two important components of the parasite splicing machinery, which are likely to play an important role in mRNA splicing in *P. falciparum (Dixit et al. J. Biol Chem 2010)*.

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

We are interested in the regulation of cyclin D1, which is important for G1-S transition of the cell cycle, in neuronal cell death. The levels of cyclin D1 in neurons were elevated upon treatment with neurotoxic beta amyloid peptide $A\beta_{1-42}$ or DNA damaging agent like camptothecin (Cpt). In the previous year, we had reported that aberrant MEK-ERK signaling may be responsible for cell cycle reentry on neurons which results in their apoptosis. The analysis of cyclin D1 promoter suggested that $A\beta_{1-42}$ induction of cyclin D1 may be dependent on a putative STAT3 binding site on the promoter. Once over expressed via MEK-ERK pathway, Cyclin D1, contributes to aberrant MEK-ERK signaling via a positive feedback loop. The increase in cyclin D1 levels caused a significant decrease in p35-cdk5 activity. Since p35 associated cdk5 activity is important for neuronal survival, this observation may explain the induction in apoptosis caused by increased cyclin D1 levels.

Publications

Original peer-reviewed article

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Review / Proceeding

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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:

i). Identification and biochemical analyses of enzymes that are involved in the biosynthesis and/or degradation of lipidic metaboilites

ii). Characterization of molecular mechanisms mediating the crosstalk between various polyketide synthases (PKSs) and fatty acid synthases (FASs) in *M. tuberculosis*.

iii). 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.

iv). Delineate mechanisms that generate metabolic diversity, particularly by using versatility of polyketide biosynthetic machinery.

Understanding Enzymes Involved in Polyketide Metabolism during *Dictyostelium* Development

Dictyostelium discoideum (Dicty) is a lower eukaryote that undergoes a complex differentiation cycle, alternating between unicellular and multicellular phase. Starvation induces the single-celled amoeba to initiate a process of streaming and aggregation that eventually culminates in the formation of fruiting bodies. One of the notable features in Dicty genome is the existence of more than 40 putative polyketide synthases (PKSs). a new development regulatory factor – MPBD, was isolated and it was shown to have a general effect on pre-spore and pre-stalk induction during the differentiation process. The resorcinolic structure of this metabolite led us to hypothesize that it could be synthesized by DiPKS1 because this type I PKS has a type III PKS domain fused to its C-terminus. To understand the biosynthetic pathway of MPBD, we purified the 352 kDa DiPKS1 protein. The expression in *E. coli* could only be obtained after synthesizing an "*E. coli* codon-optimized" gene for this protein. Radio-TLC assay with malonyl CoA

(M-CoA) as the extender, and acetyl CoA as the starter unit clearly showed product formation at Rf of 0.83, corresponding to the Rf of synthetic MPBD. Tandem MS analysis confirmed the identity of the radioactive spot to be MPBD. The presence of a C-methyltransferase (C-Met) domain in DiPKS1 prompted us to speculate that it could be responsible for methyl group incorporation on the MPBD backbone. However, it is also possible that the methyl group is in fact due to incorporation of methyl-malonyl CoA (MM-CoA) during chain-extension. To resolve this issue, we set up assay using radiolabeled MM-CoA and unlabeled M-CoA. It was observed that radioactivity did not get incorporated into MPBD; rather, a new product was being formed. No product formation was observed if only MM-CoA was taken as the extender-substrate (M-CoA being excluded). Further, in assays which did not include S-adenosylmethionine, product formation was substantially reduced. Taken together, these results suggest that methyl group is introduced by the C-Met domain and that methylation is essential for transfer of the intermediate to type III PKS domain for further iterations.

To elucidate the significance of other DiPKSs, we generated knock-out strains of Dicty for dipks1, 2, 3, 14, 18 and 37. All these mutants completed their development cycle to form fruiting bodies, but with subtle phenotypic defects. In order to gain more insight into the genes/pathways that get affected to yield the observed phenotype, microarray studies on Agilent platform was performed. We observed that although most DiPKSs expressed at levels below detection, quite a few also showed stage-specific expression changes. Most notably, six DiPKSs were found to be maximally/solely up-regulated during the fruiting body stage. Further analysis was done with special focus on DiPKS1 knock-out, which showed defects in gene-expression pattern similar to expression profile of DiPKS1 in wild-type strain. It showed down-regulation in some of the important pre-stalk and pre-spore markers. Heat-map profile of the mutant highlighted a cluster of genes which gets specifically down-regulated in the early culminant stage. Of the seven hundred genes in this cluster, seventy of them were proteins associated with extra-cellular matrix (that forms the slug and stalk-sheath) and cellulose-binding domain containing proteins. To correlate these changes with phenotypic defects, we did a detailed structural analysis of the mutant using scanning electron microscopy. SEM images clearly showed that the DiPKS1 knock-out had much elongated and longer stalk as compared to the wild-type. In contrast the spore sac of the mutant was relatively much smaller. To determine whether the smaller size of spore-sac was due to reduced spore formation by the DiPKS1 knock-out, competition experiments were performed with the wild-type. Wild type cells were transformed with GFP vector for green fluorescence and plated in equal proportion with non-fluorescent mutant strain. Spore count using phase-contrast and fluorescence microscopy made it evident that approximately 88% spores were contributed by the wild-type. This observation suggested that MPBD might be a spore-inducer and its absence leads to a selective disadvantage to the DiPKS1 knock-out towards spore contribution when grown with the wild-type strain.

Publication

Original peer-reviewed article

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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 will 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:

Aim #1: Study the role of Chk1 in phosphorylation of BLM

Aim #2: Dissect the roles of RECQL4 and p53 in the regulation of mitochondrial DNA replication

Aim # 3: Decipher the role of BLM in regulating c-Myc functions

Aim #1: Role of Chk1 in phosphorylation of BLM

Using a newly generated phosphospecific antibody, BLM was found to be phosphorylated at Ser646 only under asynchronous conditions and this phosphorylation was lost when the cells were treated with hydroxyurea (HU, which induces stalled replication forks) or neocarzinostatin (NCS, which causes doubles strand breaks). The loss of BLM Ser646 phosphorylation after DNA damage correlated with decreased binding to nucleolin and PML nuclear bodies. These results were further validated using mutational analysis carried out with overexpressed Flag-tagged wildtype or BLM (S646A) variant in Cos cells. Altogether the results indicates that Chk1-dependent constitutive phosphorylation of BLM helicase at Serine 646 decreases after DNA damage.

Aim #2: Dissect the roles of RECQL4 and p53 in the regulation of mitochondrial DNA replication

Disruption of mitochondrial targeting of RECQL4 led to the accumulation of transcriptionally active nuclear p53

To determine the functional consequence of the loss of RECQL4 from the mitochondria, we used asynchronously grown RTS patient cell AG05013 that stably expressed either wildtype EGFP RECQL4 (1-1208) or EGFP RECQL4 (Δ13-18). In cells expressing RECQL4 (1-1208) very few cells expressed nuclear p53 and its

transcriptional target, p21 in the nucleus. Lack of RECQL4 in the mitochondria in RECQL4 (Δ13-18) cells led to increased accumulation of transcriptionally active nuclear p53 even under asynchronous conditions leading to the induction of cell cycle dependent kinase inhibitor, p21, which probably accounted for the doubling time of 29 hr and 38.7 hr for EGFP RECQL4 (1-1208) and EGFP RECQL4 (Δ13-18), respectively. Accumulation of transcriptionally active p53, along with induction of its target genes also occurred in RTS patient cells, after transient silencing RECQL4 expression with specific siRNA or by stably depleting RECQL4 with shRNA.

RECQL4 and p53 stimulate PolγA/B2-mediated mtDNA replication

To determine whether mtDNA replication rate in NHF would be altered only due to loss in expression of RECQL4, Southwestern analysis with anti-BrdU antibody was carried out in control cells or cells which lack RECQL4. Approximately 50% decrease in the rate of mtDNA replication was observed in NHF shRECQL4 compared to NHF shControl cells. RECQL4 may affect mDNA replication during initiation and/or elongation step. To investigate these possibilities, we scored for mtDNA replication in isogenic control ρ^0 cells. Lack of RECQL4 decreased BrdU-PolγA colocalization at all time points, indicating that RECQL4 was involved in the regulation of both initiation and elongation steps of mtDNA replication. Lack of RECQL4 prevented recruitment and/or maintenance of p53 at the mtDNA. However lack of p53 did not affect the localization of RECQL4 at the sites of mtDNA reinitiation.

To mechanistically dissect the role of RECQL4 and p53 in mitochondrial replication, we first determined whether the two proteins interacted with members of the mitochondrial replication machinery using *in vitro* pulldown assays with recombinant proteins. While p53 interacted with both PolγA and PolγB, RECQL4 interacted with PolγA but not with PolγB. RECQL4 interacted separately with the exonuclease and polymerase domains but not the spacer domain and the two thumbs of PolγA. The saturation curve depicting the reaction rate yielded a dissociation constant (K_D) of 114±22.5 nM for RECQL4-PolγA interaction, indicating that PolγA-PolγB interaction was almost 3-5-fold stronger than PolγA-RECQL4 interaction. To elucidate the specific role of RECQL4 and p53 during the polymerization process, the proteins were added in trans to the polymerization or primer extension assay performed with recombinant heterotrimeric PolγA/B2 holoenzyme. PolγA/B2-dependent polymerization. The helicase-dead mutant RECQL4 K508A had equivalent activity as the wildtype RECQL4, indicating that the enhancement of PolγA/B2-dependent polymerization was independent of the unwinding function of RECQL4.

Aim # 3: Decipher the role of BLM in regulating c-Myc functions

Loss of BLM enhances c-Myc stability at post-transcriptional level

To determine whether the absence of BLM affects c-Myc protein levels, we compared the relative levels of the two proteins in three isogenic pairs of cell lines, either expressing or not expressing BLM helicase. Levels of c-Myc were always higher in absence of BLM. This effect was specific as similar enhancement in the oncoprotein level was not observed in immortalized cells from WRN or in primary cells from RTS patients. Usage of three independent techniques concluded that *BLM* did not regulate the trancription of *c-myc*.

Presence of BLM enhances the degradation of c-Myc

Since c-Myc is known to undergo ubiquitylation and subsequent proteosomal degradation, it maybe possible that this process is dependent on BLM. Western analysis performed for c-Myc with two different antibodies indicated that the level of the oncoprotein in isogenic celllines either expressing or not expressing BLM were identical after treatment with proteosomal inhibitor, LLnL. The turnover of c-Myc was found to be higher in cells expressing BLM as determined either by treating the cells with protein synthesis inhibitor, cycloheximide and checking for the rate of endogenous BLM disappearance or after pulse labeling with S^{35} methionine.

Publications

Original peer-reviewed article

1. Kaur S, Modi P, Srivastava V, Mudgal R, Tikoo S, Arora P, Mohanty D and Sengupta S (2010) Chk1-dependent constitutive phosphorylation of BLM helicase at Serine 646 decreases after DNA damage. **Mol Cancer Res 8**:1234-47.

Review/Proceeding

1. Tikoo S and Sengupta S (2010) Time to Bloom. **Genome Integr 1**: 14

Understanding the regulation of DNA replication

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

We propose to identify the protective machinery that ensures precise DNA replication. It is believed that when a eukaryotic cell experiences stress such as UV and gamma irradiation, it degrades its replication proteins to prevent replication initiation. Therefore, we are trying to identify the replication proteins which are degraded in response to stress. We have initiated this effort by identifying such mammalian replication proteins and some of their modifications induced upon stress. After characterizing the modifications, we would assess its biological consequences. We believe that some of the human cancers arise from malfunctioning of these protective pathways.

Regulation of replication apparatus on replication stalling and oxidative stress

In order to impose replication stress on p53 positive U2OS cells, we utilized hydroxyurea (HU), which inhibits the ribonucleotide reductase preventing the reduction of ribonucleotides to deoxyribonucleotides impeding the S phase progression. The activation of checkpoint on treatment with HU was evident by phosphorylation of Chk1 (Fig. 1A). Cdt1, Cdc6 and Mcm10 were downregulated by UV irradiation but remained stable after replication stalling. Therefore, the replication stress induced pathway does not utilize the downregulation of these key replication molecules to inhibit replication. However, replication arrest with hydroxyurea results in phosphorylation and formation of punctuate pattern of Rpa2 reminiscent of localization to the sites of replication arrest, which is clearly absent in untreated U2OS cells. The levels and localization of other replication proteins remain unaffected after hydroxyurea block. In comparison to HeLa cells, the level of phosphorylation of Rpa2 was significantly higher in p53 positive U2OS cells underlining that the stress response has to be addressed in different genetic backgrounds. We also wanted to study the effect of oxidative stress on replication apparatus and therefore we exposed U2OS cells to 2.5 mM hydrogen peroxide (H_2O_2) for 1 h and cells were harvested 3 hours later. There was a time dependent increase in reactive oxygen species and damage to DNA as indicated by phosphorylation of Chk1 (Fig. 1B). We observed that the majority of replication proteins did not show any change in levels or localization. We report that Cdt1 and Cdc6 are downregulated after exposure to hydrogen peroxide indicating that these proteins could be possible targets for inhibiting replication following oxidative stress. Mcm10, which was downregulated following UV irradiation did not decrease; indicating that oxidative stress induced inhibition of replication machinery is independent of Mcm10. Though Cdc6 was destabilized after oxidative damage, it was stable after gamma-irradiation or exposure to DNA modifying chemicals and therefore this highlights that different genotoxic agents target specific factors for inhibiting replication.

M-phase proteolysis of Mcm10 is dependent on Roc1

We wanted to discern the components of E3 ubiquitin ligase that mediate the natural turnover of Mcm10. We wanted to determine if the cell-cycle decrease of Mcm10 is dependent on Roc1 (Fig. 1C). To test the involvement of Roc1 in cell-cycle proteolysis of Mcm10, we designed siRNA duplex to target *ROC1* which significantly reduced the respective mRNA levels. After siRNA depletion, the cells were blocked with nocodazole for 15 h, released into nocodazole free medium and collected at different time-intervals after release. The depletion of Roc1 did not affect the nocodazole induced block or subsequent release

as is evident from the flow cytometry analysis. Cells transfected with *GL2* siRNA showed a decrease in Mcm10 levels after release from nocodazole block. At all time points after release from nocodazole, we could observe a significant difference between the levels of Mcm10 in *GL2* and *ROC1* siRNA transfected cells. Therefore, our study demonstrates that M-phase degradation of Mcm10 is dependent on Roc1. This data conclusively establishes that cell-cycle degradation of Mcm10 is dependent on Roc1dependent ubiquitin ligase.

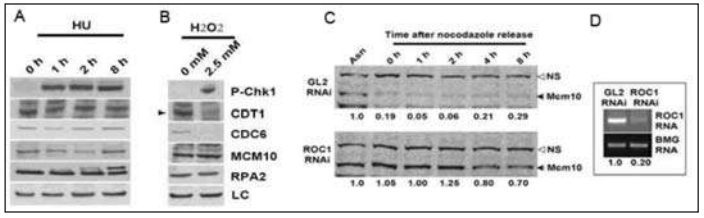

Figure 1. Regulation of replication apparatus on replication stalling and oxidative damage. *(A and B) U2OS cells were exposed to hydroxyurea (HU) (A) or hydrogen peroxide (B) harvested at indicated time-points and protein levels were analyzed by immunoblotting with specific antibody. (C) M-phase proteolysis of Mcm10 is dependent on Roc1. HeLa cells transfected with ROC1 or GL2 siRNA were released from nocodazole block. The numbers in panel indicate the levels of Mcm10 protein in nocodazole released cells relative to asynchronous cells after specific siRNA transfection. (D) The decrease in ROC1 mRNA levels in the experiment described in part (C) was confirmed by reverse-transcriptase PCR.*

Understanding the cellular response to aberrations in replication complexes

We have depleted replication proteins with small inhibitory RNA (siRNA) and investigated whether checkpoint pathways get activated when the cell senses such aberrations in replication complex. The objective is to identify yet unknown checkpoint pathways that monitor the replication machinery. We began with depleting known replication proteins in the G_1 phase of the cell cycle by RNA interference (RNAi). Our results demonstrate that after depletion of replication proteins, RP1 and RP2, a specific checkpoint is activated (Fig. 2A, B). We now aim at determining any cell-cycle arrest or replication block after the depletion of the candidate replication proteins. By evaluating other markers of DNA replication/cell-cycle progression, we would unravel the cause of cell cycle block. Therefore, in this study we attempt to identify the activated checkpoint pathway and its effectors after aberrations in replication complexes.

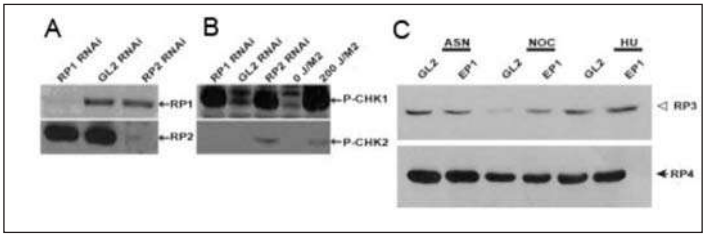

Figure 2. Activation of checkpoint pathway after silencing of replication proteins. *HeLa cells were transfected with siRNA against RP1 and RP2. (A) siRNA depletion of target proteins and activation of Chk1 and Chk2 after specific siRNA depletion (B). (C) Effect of siRNA mediated depletion of EP1 on the replication proteins. The immunoblot depicts stabilization of RP3 in the nocodazole arrested cells (NOC) but not in asynchronous (ASN) and hydroxyurea (HU) arrested cells.*

Regulation of replication machinery by E3 ubiquitin ligases

The E3 ubiquitin ligases are known to be involved in the regulation of eukaryotic DNA replication and repair through the controlled degradation of target proteins. HeLa cells were transfected with either control *GL2* or *EP1* siRNA (E3 ubiquitin ligase component) and were arrested with nocodazole or hydroxyurea and subsequently harvested. Nocodazole blocks the cells during M phase while hydroxyurea traps the cells at the G_1/S transition. The asynchronous and blocked cells were lysed and immunoblotting was performed to determine the levels of many replication proteins. RP3 was observed to be stabilized upon EP1 knockdown in nocodazole treated cells (Fig. 2C). This indicates that EP1 might play a role in the regular turnover of RP3 and it might be linked to an essential function in the stress blocked cells. Our work highlights that replication machinery is tightly regulated by E3 ubiquitin ligases. We will now identify the different E3 ligases and their targets within replication machinery.

Publications

Original peer-reviewed articles

1. Sharma A, Kar A, Kaur M, Ranade SM, Sankaran A, Misra S, Rawat K. and Saxena S (2010) Specific replication factors are targeted by different genotoxic agents to inhibit replication. **IUBMB Life 62**: 764-775.

2. Kaur M, Sharma A, Khan M, Kar A and Saxena S (2010) Mcm10 proteolysis initiates before the onset of M-phase. **BMC Cell Biology 11**: 84.

Role of tumor suppressor p53 in metabolic stress response

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Most cancers show metabolic changes that result in upregulation of glycolysis and glucose consumption. How this glycolytic switch happens, and whether it is a cause or a consequence of the transformation process, has remained a matter of debate. p53 is one of the most important tumor suppressors in the cell. 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 serves as an inducible "booster" to equip the organism to meet the energy demands of diverse physiological and dietary conditions. The amino-terminal region of PGC-1 coactivator interacts with proteins containing histone acetyltransferase (HAT) activity, including CREB-binding protein/p300. 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 transactivation function of p53 especially with respect to p53-mediated metabolic stress response. We intend to look for physical interaction between p53 and PGC-1 and map the domains involved in this interaction. We also plan to investigate the effect of this interaction on p53 function i.e. its ability to induce the expression of its diverse target genes to regulate different cellular processes like cell cycle arrest, ROS clearance, apoptosis etc. 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.

Progress of work during the current reporting year

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

Since our results established that PGC-1 can serve as p53 coactivator by binding to it, we next determined its effect on p53-mediated transactivation of its target genes. We stably transfected HepG2 cells with control (luciferase) or PGC-1 shRNA. These cells were then subjected to glucose starvation for increasing time periods and the mRNA and protein levels of different p53 target genes including pro-arrest, pro-apoptotic and metabolic targets were analyzed. In HepG2 control cells different classes of p53 target genes were induced with different kinetics. The pro-arrest and metabolic

target genes such as *p21*, *GADD45*, *TIGAR*, *SCO2* and *sestrin 2* were rapidly induced at early time points (24 and 36 hours) but at extended periods of starvation (48 and 72 hours) their levels reduced drastically. On the other hand p53 pro-apoptotic target genes such as *Bax*, *Noxa* and *Puma* were induced only at extended periods of starvation (48 and 72 hours). In HepG2 PGC-1 knockdown cells no induction of the pro-arrest and metabolic target genes was observed at any of the time points whereas the pro-apoptotic target genes were rapidly induced from early time points itself. In HepG2 control cells *PGC-1* transcript levels were induced at 24 hours and remained steady throughout the rest of time course but the protein levels reduced drastically at extended periods of starvation concomitant to the induction of pro-apoptotic target genes. Taken together these results indicate that PGC-1 is required for the selective induction of p53 pro-arrest and metabolic target genes at early periods of starvation while at later time points PGC-1 protein gets degraded resulting in downregulation of p53 pro-arrest and metabolic target genes and induction of pro-apoptotic target genes. To further analyze the effect of PGC-1 on the transactivation function of p53, we measured DNA binding activity of p53 induced in response to glucose starvation on the promoters of p53 targets including *p21*, *TIGAR*, *Bax* and *Puma* in HepG2 control and PGC-1 knockdown cells by carrying out quantitative chromatin immunoprecipitation assay (ChIP-qPCR). At early time point of starvation (36 hours) p53 preferentially bound to the promoters of its pro-arrest and metabolic target genes such as *p21* and *TIGAR* but at extended time point (72 hours) it preferentially bound to the promoters of its pro-apoptotic targets such as *Bax* and *Puma*. However, in HepG2 PGC-1 knockdown cells, no significant amount of p53 could be detected at *p21* and *TIGAR* promoter at any of the time points of starvation but p53 was selectively recruited to the promoters of pro-apoptotic targets from early time point itself. To look for presence of PGC-1 in the transcription complex, we performed "ReChIP" experiments using PGC-1 antibody. PGC-1 was detected only at the promoters of p21 and TIGAR at early time point (36 hours) but not at the promoters of Bax and Puma at any time point. Taken together, these data demonstrate that PGC-1 selectively enhances p53-mediated transactivation of its pro-arrest and metabolic target genes at early time points of starvation but at extended periods of starvation PGC-1 protein is down regulated and p53 is preferentially recruited to the promoter of its pro-apoptotic targets. We next examined the effects of PGC-1 on p53-mediated cell cycle arrest, apoptosis and ROS clearance. Our results indicate that PGC-1 modulates the p53-mediated stress response promoting cell cycle arrest and ROS clearance over apoptosis. However at extended periods of starvation concomitant to PGC-1 degradation p53-dependent apoptosis is induced.

Since ubiquitin-mediated proteasome degradation pathway is a frequently engaged mechanism for protein downregulation, we investigated the potential role of the proteasome in stress-induced PGC-1 protein degradation. Loss of PGC-1 triggered by metabolic stress at 72 hours time point was significantly attenuated by the proteasome inhibitor MG132. We next determined whether prolonged stress in cells triggered the polyubiquitination of PGC-1 prior to degradation. Glucose starvation of HepG2 cells in the presence of MG132 revealed that endogenous PGC-1 undergoes extensive ubiquitination at extended periods of starvation (72 hours), while no such ubiquitinated forms were observed at early time point (36 hours). Thus, the appearance of ubiquitinated forms of PGC-1 in starved cells treated with MG132 supports the idea that the PGC-1 protein is targeted for degradation during extended exposure to metabolic stress due to activation of an ubiquitin-proteasome pathway.

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

HepG2 control and HepG2 PGC-1 knockdown cells were subjected to glucose starvation for 24 and 48 hours and microarray analysis of gene expression was carried out using Affymetrix platform. *In silico* analysis of the sequence proximal to transcription site of the upregulated and downregulated genes revealed several p53 targets. The putative p53 upregulated targets include genes such as *Caspase 10* (involved in apoptosis), *Artemis* (DNA repair protein), *KLF1* (transcription factor), *PANK2* (regulatory enzyme in CoA biosynthesis) and *TLR3* (member of Toll-like receptor). The putative p53 repressed targets include genes such as *NAE1* (involved in Neddylation), *CDK7* (cell cycle regulator), *Cyclin D2* (cell cycle regulator). p53-dependent changes in the levels of these genes upon glucose starvation was confirmed by RT-qPCR.

ANCILLIARY ACTIVITIES

Production of transgenic animals and development of new transgenic technologies.

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Shushanta Roychowdhury (IICB, Kolkata)
Shyamal Goswami (JNU, New Delhi)
Satyajit Rath (NII, New Delhi)

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.

Transgenic mice with CGG and CTG repeats

The goal of the collaboration was to analyze the epigenetic modifications on the transgene sequence in both CGG and CTG repeat transgenic mice. We found that the nucleosome occupancy in the unstable transgene (Tg*hFMR1*) was lower than that in the endogenous *Fmr1* gene of mouse, as assessed by tiling assays and data analysis by three independent statistical tools. In this context we observed that primary sequence has a major role in dictating nucleosome positioning. Our studies demonstrated that flanking DNA sequences can influence repeat instability through modulation of nucleosome occupancy in the region.

In case of CG transgenics we observed silencing of the transgene in one line while expression in the other. We identified that this is due to position effect, implying that the transcriptionally active transgene is integrated in a contig of active region on chromosome 18, while it is within repetitive LINE 1 region in the other case. The transcriptional status correlates with the epigenetic state; DNA methylation and histone modifications that we studied.

SMAR1 mediated susceptibility to infection by M. tuberculosis

Infection with *M. tuberculosis* invokes an inflammatory host response associated with increased secretion of IFN-γ, symptomatic of a T_H1 type response. As SMAR1 expression was down regulated upon T_H1 polarization, we were inquisitive to find out its expression in T_H1 immune response induction. As mycobacteria-specific $CD4^+$ T cells are typically of the T_H1 type, which are potent IFN-γ producers and SMAR1 Tg mice demonstrated reduced secretion of IFN-γ, we were interested to study *M. tuberculosis* mediated pathogenesis in these mice. For this, both SMAR1 Tg and normal mice (LM) were infected with H37Rv at dose of 1×10^6 bacilli per ml through aerosol nebulizer. This part of work was done at collaborative centre at JALMA, Agra. The target organs lung and spleen were excised aseptically after indicated period of time, homogenized and plated on 7H11 agar plates. A significant difference in CFU (colony forming units) was observed in both set of animals. SMAR1 Tg infected mice demonstrated increase in the CFUs in lungs and spleen respectively compared to normal mice suggesting the susceptibility of these mice towards *M. tuberculosis* infection

Gene knock down studies

A novel technique for generating knock down mice, by *in vivo* electroporation of shRNA vector into the testis, in a short span of time was established. With this technique, one can generate a variety of knock down mice in one go because differential integration of constructs in various spermatogonial stem cells of a testis produce variety of sperm that generate mice bearing different levels of gene specific shRNA. Hence, choice of the extent of reduction (a range)in gene expression also becomes available in one go. This is an advantage over null mutants produced by presently existing knock out technique.

shRNA knock down mice of several Sertoli cell specific genes were generated on the basis of differential display of mRNA obtained from Sertoli cells cultured from spermatogenically active (fertile) and inactive (infertile) testis. Role of these genes in spermatogenesis was evaluated. shRNA knock down mice generated using this procedure served as models for study of function of these genes. Inhibition in mRNA was evaluated using Real time PCR. We have generated 8 transgenic lines of Knock down mice and found that spermatogenesis was modulated upon specific inhibition of mRNA in all of them. Dickoppf homolog 3 (Dkk3) was one of the genes which was over expressed by pubertal Sc. We generated transgenic mice expressing shRNA against Dkk3 by testicular electroporation technique developed by us. Testicular weights of adult transgenic mice were reduced as compared to age matched controls. Histological analysis of atrophic testis revealed a compromised differentiation of germ cells along with multinucleated giant cells and vacuoles. Inhibition of the Dkk3 expression had lead to sloughing off of germ cells. TUNEL assay showed a higher incidence of Gc apoptosis which led to tubular degeneration. Severe reduction in sperm counts was also observed. Litter size of such mice were significantly ($p<0.5$) reduced. Lack of Sc maturity due to inhibition of Dkk3 may underlie defective testicular spermatogenesis and Gc apoptosis in these transgenic mice during adulthood. This may have relevance to idiopathic infertility in men.

Transgenesis in large animals

This work has progressed well in monkeys and males electroporated with growth hormone , Mamu A01 and other genes are put for mating with the females. During April-June, 2011, babies are expected to be born and we can then evaluate propagation of transgene to offspring. Similarly, in an attempt to generate transgenic buffalo, we have isolated beta caesin promoter from buffalo genomic DNA and have successfully cloned EGFP under this promoter. Further work is in progress.

Generation of transgenic mice as a central facility

The collaborative work with Dr. Shyamal Goswami of JNU and Dr. Stayajt Rath Of NII has just begun and we have made transgenic mice for them. Results are awaited.

Publication

Original peer-reviewed article

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PUBLICATIONS

A. ORIGINAL PEER-REVIEWED ARTICLES

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spermatozoa is potentiated by glycodelin-A via down-regulation of extracellular signal-regulated kinases and up-regulation of zona pellucida-induced calcium influx. *Hum Reprod* **25**: 2721-2733.

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B. REVIEWS /PROCEEDINGS

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PATENTS

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DISTINCTIONS/HONOURS/FELLOWSHIPS

Dr Sher Ali is the fellow of the Indian Academy of Sciences,Banglore,the Indian National Science Academy, New Delhi,The National Academy of Sciences, Allahabad,Alexander Von Humboldt Foundation, Bonn, Germany and Fullbright Foundation,USA. He is member of the Editorial Board of International Journal of Human Genetics, Indian Journal of Biotechnology and International Journal of Biosciences and Technology (IJBST). He is member of Research Advisory Committee NBFGR, Lucknow, Fast Track Committee for Young Scientist, DST and Technology Information Forecasting and Assessment Council (TIFAC) DST, Delhi.

Dr Akhil C Banerjea is the fellow of the National Academy of Sciences, Allahabad, UP, India. He is the member of the Editorial Boards of several AIDS journals. He is the recipient of the National Bioscience Award for career Development of DBT (2001-2002). DBT-National Overseas Fellowship; several International Awards/ Full Scholarships to attend and present work in Conferences in USA, UK, Canada, Japan, South Africa etc. Thomson Reuters has recently recognised Dr Banerjea as a distinguished expert in his field and was included in the Thompson Pharma KOLexperts database.

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

Dr Satish K Gupta is the fellow of the Indian National Academy, Delhi, the Indian Academy of Sciences, Banglore, 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 fellow of Indian National Academy of Sciences, Delhi, National Academy of Sciences, Allahabad. Dr Majumdar is the member of the Endocrine Society, Society for Experimental Biology and Medicine, and Society for the Study of Reproduction, USA. He is the member of Guha Research Conference, Society of Biological Chemists, and Indian Society of Reproduction and Fertility, India.

Dr Arnab Mukhopadhyay received the Ramalingaswami Fellowship from the Department of Biotechnology, Government of India for the year 2010-2011.

Dr Vinay K Nandicoori is the recipient of National Biosciences Career Development Award.

Dr Sagar Sengupta is member of Indian Association of Cancer Research.

Dr Chandrima Shaha is recipient of J.C. Bose National Fellowship, fellow of the Indian National Science Academy, Delhi, Indian Academy of the Sciences, Banglore and the National Academy of Sciences, Allahabad. She is recipient of Dr. Darshan Ranganathan Memorial Lecture Award of INSA for the year 2010. Dr Shaha is the member of Editorial Boards of 'Molecular and Cellular Endocrinology" (Elsevier) and 'Spermatogenesis', Landes Bioscience, TX, Austin. She is a sectional Committee Member of Biochemistry-Biophysics, INSA, New Delhi and General Biology, Indian Academy of Sciences, Banglore. She is member of the Science Education Panel, Indian Academy of Sciences, Banglore and the Summer Fellows Selection Committee of the Indian Academy of Sciences.

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

Dr Anil K Suri is the recipient of Drs Anand and Lata Labhsetwar Cheritable Trust, Labshetwar Foundation USA in recognition of outstanding contribution in the field of Reproductive Health.

Prof Avadhesha Surolia is the recipient of Thomson Reuters Innovation Award 2010, Themis Chemicals UICT Diamond Jubilee Distinguished fellow in Pharmaceuticals Science Award 2010-2011, and 11th Smt. Pushpa Sriramachari Foundation Day Oration Award 2011, Institute of Pathology, ICMR, New Delhi.

Dr Sudhanshu Vrati is the fellow Indian Academy of Sciences, Banglore, National Academy of Sciences, India and Tata Innovation fellow of the DBT, Govt of India. He is the recipient of Australian Alumni Excellence Award 2010, Australian High Commission , New Delhi.

Ph.D. DEGREES AWARDED TO NII SCHOLARS

Fourteen scholars of the Institute were awarded the degree of Doctor of Philosophy by the Jawaharal 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. Anasua Ganguly	Delineation of structural and functional attributes of human zona pellucida glycoprotein-1	Dr. Satish K Gupta
2	Mr. Pankaj Bansal	Structural and Functional Characterization of Human Zona Pellucid Glycoprotein-3, the Primary Sperm Receptor.	Dr. Satish K Gupta
3	Ms. Namrata Ojha	Regulation of expression of different Isoforms of Rab5.	Dr. Amitabha Mukhopadhyay
4	Ms. Priyadarshini Chatterjee	Study of antigen presentation in B cells.	Dr. Anna George
5	Mr. Narendra Kumar	Modelling protein-peptide complexes using rotamer library approach: application to prediction of substrates for MHC and Kinases.	Dr. Debasisa Mohanty
6	Mr. Manu Anantpadma	Studies on Japanese Encephalitis Virus Replication	Dr. Sudhanshu Vrati
7	Mr. Ravikant Ranjan	Dissection of signaling pathways in *Plasmodium Falciparum*	Dr. Pushkar Sharma
8	Ms. Uttra Marathe	Studies on sortases from *Streptococcus pneumoniae and Staphylococcus aureus*	Dr. Rajendra P Roy
9	Mr. Vineet Gaur	Comparative Structural proteomics of Novel Seed proteins with allergenic Potential	Dr. Dinakar M Salunke
10	Ms. Anubha Singh	Human ribonuclease eosinophil cationic protein: understanding the mechanism of its biological actions.	Dr. J K Batra

Sl	Student's Name	Topic of Research	Guide
11	Ms. Rohini Garg	Deciphering role of *Salmonella typhi* virulence polysaccharide in the modulation of immune cell functions	Dr. Ayub Qadri
12	Ms. Sarabpreet Kaur	Studies on the Chk1 mediated phosphorylation of BLM Helicase	Dr. Sagar Sengupta
13	Mr. Tarun Chopra	Investigation of novel mechanisms involved in generation of metabolic diversity	Dr. Rajesh S Gokhale
14	Ms. Aalia Shahr Bano	Funtional Analysis of HIV-1 co-receptor CXCR4 and vpr Gene of HIV-1 Subtype	Dr. A C Banerjea

CONFERENCES/SYMPOSIA/WORKSHOPS ORGANIZED

An International Symposium on "French-Indian Inter-Academic Symposium on Infectious Disease" was organized from 30th November to 3rd December 2010 at the National Institute of Immunology (NII), New Delhi. This symposium was part of 25th Anniversary celebrations of National Institute of Immunology. Both France and India have a strong tradition and excellent scientists are working in this important area of research. Fifteen eminent scientists from France, including Dr. Luc Montagnier, 2008 Nobel Laureate in Physiology and Medicine and seventeen distinguished scientist from all over India participated and delivered lectures. The conference had more than 150 registrants including faculty, students and post-doctoral fellows. Symposium program included two poster sessions to encourage young students. In addition to providing wider platform for students and postdocs, these activities were essential for developing closer scientific interactions and collaborations between Indian and French Scientists. The scientific deliberations of the Symposium were focused on infectious diseases such as HIV, tuberculosis, zoonoses & vector-borne diseases, enteric infections, man & microbes and chronic infections, infections and cancer were covered in this symposium.

Participants of the French-Indian Inter-Academic Symposium on Infectious Disease held in NII between 30th Nov- 3rd Dec 2010.

Noble Laureate Dr. Luc Montagnier with NII Director Prof Avadhesha Surolia and NII Scientists, Dr Vinay K Nandicoori and Dr Akhil C Banerjea.

An "Indo-US bilateral symposium on Aging and Age-related Diseases" was organized in the Institute on 3rd-4th March 2011. The symposium was aimed at developing collaborative research projects between US and Indian scientists in the field of molecular aging and age-associated diseases as well as expose young Indian scientists and students to this relatively new field of research. Seven US and 17 Indian Principal Investigators presented their work and deliberated on topics ranging from basic research on the biology of aging to development of pharmaceutical interventions to cure age-related diseases. More than 75 registered delegates participated from all over India. Poster sessions were organized on both days; best two posters were invited for oral presentation

Professor Avadhesha Surolia (Chairman Scientific Committee), Dr Arnab Mukhopadhyay (Convernor), Dr Sagar Sengupta and Dr Sanjeev Das (Co-convenors) with the US delegates at the Indo-US Bilateral Symposium on Aging and Age-Related Disease, 3rd – 4th Mar 2011.

VISITORS TO THE INSTITUTE

The Hon'ble Prime Minister of Niedersachsen, Germany, Mr David McAllister visited the Institute on 30th September 2010. During this visit he held discussions with the Chairman, Governing Body and Director of the Institute. Prof V M Katoch, Secretary, Department of Health, Ministry of Health and Family Welfare, Government of India, welcomed the delegation.

The Hon'ble Prime Minister of Niedersachsen, Germany, Mr David McAllister during his visit to the Institute.

Noble Laureate Prof Torsten Wiesal of Rockfeller University NY, USA visited the Institute on 1st Dec. 2010. During his visit he held discussions with Director and scientists of the Institute.

Noble Laureate Prof Torsten Wiesal with NII Director Prof Avadhesha Surolia .

A delegation of Eminent Scientist of EMBO comprising of Prof Maria Leptin, Director, European Molecular Biology Organisation, Heidelberg, Germany, Prof Thomas Lecuit, Director, Institute of Developmental Biology of Marseille, France, Nobel Laureate Prof Tim Hunt, Principal Scientist, Cancer Research, UK Clare Hall Laboratories, South Mimms, Hertfordshire, UK and Prof Anne-Marie Glynn, Programme Manager of the EMBO Courses & Workshops EMBO Global Exchange Programmes visited the Institute on 4th January 2011 and held discussions with the scientists of the Institute.

Eminent Scientists of EMBO delegation Prof Thomas Lecuit, Prof Maria Leptin, NII Director Prof Avadhesha Surolia , Noble Laureate Prof Tim Hunt and Prof Marie Glynn during their visit to the Institute on 4th January 2011.

SUPPORTING UNITS

SMALL ANIMAL FACILITY

The Small Animal Facility of the Institute is committed to supply defined strains of mice and rats to the scientific community of the institute. Apart from the breeding and maintenance of defined strains of mice and rat undefined stocks of other laboratory animals are also raised.

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.

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. In many strains occasional mice show minor physical abnormalities that are characteristics of that strain and are considered to be normal.

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 are monitored for presence of ectoparasites. Fecal samples are randomly selected for the presence of endoparasites by sedimentation method. In addition to sedimentation method specifically for checking pinworm infestation, we undertake Tape test method on randomly selected animals. The health quality procedures are implemented to prevent the transmission of infection between use of sterilized bedding, autoclaved cages, feed and zero bacteria/ 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 is taken by the veterinarian concerning the necropsy/ autopsy of the suspected animals. The recommended

schedule of medication is strictly followed to prevent the infection/s. The sick animals are euthanized immediately.

PRIMATE RESEARCH CENTER

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

Group mating is done under the breeding program for the production of healthy animals, This helps in providing animals of known age and parentage. The centre has large open pens which are used for group mating under semi-natural conditions where food and water is provided adlibitum. 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. At puberty they are housed in independent cages. To prevent cross-cage contamination strict procedures are followed. All cages are washed routinely by scrubbing with soap and are painted once a year. Routine deworming of the colony is done at least once a year. A stock of medicine is maintained at the Centre, to treat the minor injuries, gastrointestinal disorders and to revive animals during acute cardio-pulmonary crisis. To check outbreak, the routine TB tests are performed because non-human primates are susceptible to this infection. 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.

Commercial 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. Steel nozzles with teflon interior are fitted at the tip of these hose pipes for continuous access (of animals) to water for drinking . 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 anti rabies vaccine when required. TB test and chest x-ray of staff and the security personnel are performed periodically. 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 properly.

The Centre has technical expertise for surgery, immunization, bleeding, biopsy, electroejaculation and fertility studies which is extended in addition to maintaining and providing primates free of microbial pathogens The Centre also provides services involving modern techniques like laparoscopy, endoscopy, etc. 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 surgicals 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. This facility is the first of its kind in India.

The macaques at this Centre are used for research related to infectious diseases, reproduction, endocrinology, immunology and contraception. 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 staff of Centre makes sure that all the procedures involved in animal handling are pain-free and involve minimum stress to the animal. Experimental animals are provided with special feed, whenever needed. There are thirteen open enclosures with swings and shelters, some of these are used for rotation of monkeys and some for rehabilitation. A constant effort is made to keep the animals in comfortable and stress free environment as per the available guidelines.

KEYNOTE LECTURES

FOUNDATION DAY LECTURE

The 24th Foundation Day Lecture of the Institute was delivered by Prof Suresh C Jhanwar, Director, Pathology Laboratory and Member, Memorial Sloan Kettering Cancer Centre, Professor of Genetics, Weill Cornell Medical Centre, New York on "Cancer Genetics and Translational Research" on 6 Oct 2010. The function was presided over by Prof H Sharat Chandra, Director and Professor Emeritus, Centre for Human Genetics, Indian Institute of Science, Bangalore.

Prof Suresh Jhanwar, Prof H Sharat Chandra and Director NII Prof Avadhesha Surolia after Foundation Day lecture.

After the Foundation Day Lecture awards of Prof GP Talwar Medals for the Best PhD Thesis and Best Guide/Supervisor were distributed.

Recipient of award for the best PhD thesis (father of Dr Neha Subramanium) and best Guide/ Supervisor, Dr Ayub Qadri, on the NII Foundation Day with NII Director Prof Avadhesha Surolia and Founder Director Prof G P Talwar.

RAMALINGASWAMI MEMORIAL LECTURE

The Ramalingaswami Memorial Lecture 2010 was delivered by Noble Laureate Prof Ada Yonath, Director, Kimmelman Centre for Biomolecular Structure and Assembly, Department of Structural Biology, Weizmann Institute of Science, Isreal on an interesting topic of "The Amazing Ribsome, Its Tiny Enemies and Hints About Its Origin" on 2 Feb 2011. Prof Avadhesha Surolia, Director, National Institute of Immunology presided over the function.

Noble Laureate Prof Ada Yonath and NII Director Prof Avadhesha Surolia after the Ramalingaswami Memorial Lecture.

NII SILVER JUBILEE LECTURE

Dr R Chidambaram, Principal Scientific Adviser to the Govt of India delivered the NII Silver Jubilee Lecture on "Research and Innovation: Nuclear to Rural" on 24 March 2011. The function was presided over by Prof Avadhesha Surolia, Director, National Institute of Immunology, New Delhi.

Dr R Chidambaram , Principal Scientific Advisor to Govt of India, during NII Silver Jubilee Lecture with NII Director Prof Avadhesha Surolia and Dr Dinakar K Salunke.

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 2010-2011, 2688 candidates applied for admission, out of which 2355 candidates took the National Level Test at New Delhi, Ahemdabad, Kolkata & Bangalore centres. On selection 35 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 472 students in 25 batches. Out of these 216 students have successfully completed the course and have been awarded Ph.D. degree by the Jawaharlal Nehru University, New Delhi. Out of the remaining 256 students, 79 have left the course in between and the rest 177 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 four 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 twelve 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.

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 eight applications and six patents were granted.

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. They also attended many other such events as delegates.

SEMINARS BY VISITING SCIENTISTS/GUEST INVESTIGATORS

The Institute continued to receive visiting scientists and guest investigators from all over the world. Thirty three seminars from thirty two investigators 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 five seminars were delivered by investigators from India and the rest twenty seven 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 2010

The twenty fourth Foundation Day of the Institute was celebrated on 6th Oct 2010. 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.

The dance performance by the children of NII Staff on the Foundation Day.

An inter-institutional drama competition of teams from several institutes and colleges from University of Delhi was organized at the Institute as part of Foundation Day celebrations. The celebrations ended with the Cultural Programme.

HINDI PAKHWARA

The Hindi Pakhwara (Hindi Fortnight) was celebrated in the Institute from 1st to 14th September 2010. To promote the use of Hindi in the official work of the Institute competition such as Hindi Sulekh (Hindi writing), Hindi shrutlek(Hindi dictation), Hindi nibandh (Hindi essay writing), Hindi anuvaad (Hindi translation),Hindi vaad-vivad (Hindi debate) and Hindi Kavita Pathan (Hindi poetry recitation)were organized for the faculty, students and staff of the Institute in which the participation has been overwhelming. Prizes and certificates of merit were awarded to the winners adjudged first,second and third in various competitions. Hindi Diwas (Hindi Day) was celebrated on 14 September 2010 on the culmination of Hindi Pakhwara.Kavi Sammelen was also organized in the Institute on 23 April 2010. To help the difficulties of staff while doing their official work in Hindi, a Hindi workshop had also been organized in this Institute on 23 Dec 2010.

Prof Avadhesha Surolia giving away the prize to one of the competitors (Mr Madan Mohan) for Hindi Poetry Competition.

INDEPENDENCE DAY CELEBRATION

Independence day was celebrated in the Institute on 15th Aug 2010. 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 by the students during the Independence Day celebration at the Institute. 15th August 2010.

FAREWELL TO PhD STUDENTS

At the Farewell function of the 2005 batch of PhD students which was marked by planting of the tree at the Institute 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 2010 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 2010 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 2010 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 2010 and Flag Day on 25 Nov 2010, 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 Government of India, while making 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 complied with.

COMMITTEES OF THE INSTITUTE

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Hony Professor (IISc)
Emeritus Scientist (CSIR)
& Distinguished Biotechnologist, DBT
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Director General Health Services
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Indian Council of Medical Research
New Delhi

Dr S Ayyappan
Director General
Indian Council of Agricultural Research
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Prof R C Deka
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All India Institute of Medical Sciences
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