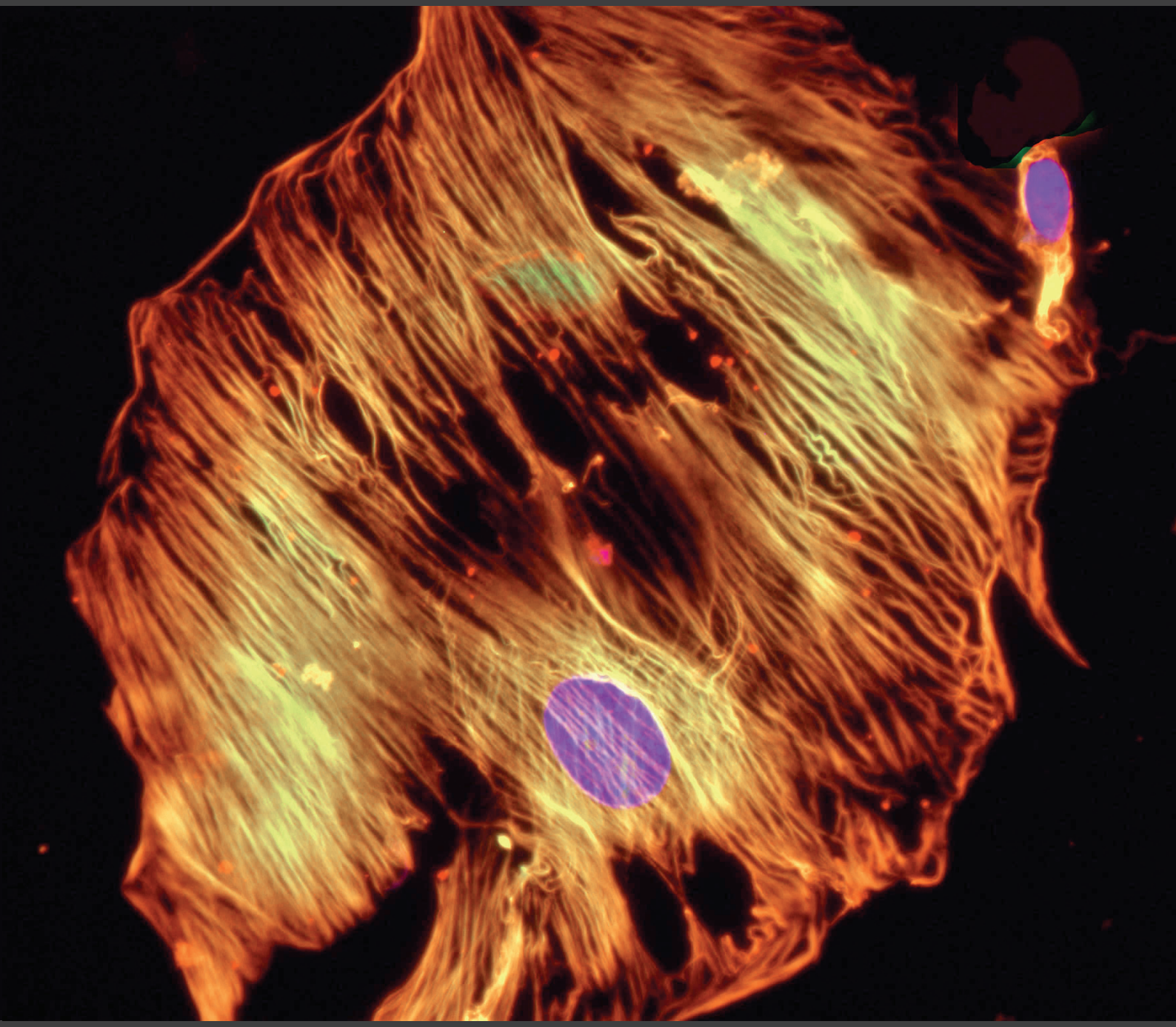
NATIONAL INSTITUTE OF IMMUNOLOGY



ANNUAL REPORT
2012-2013

NATIONAL INSTITUTE

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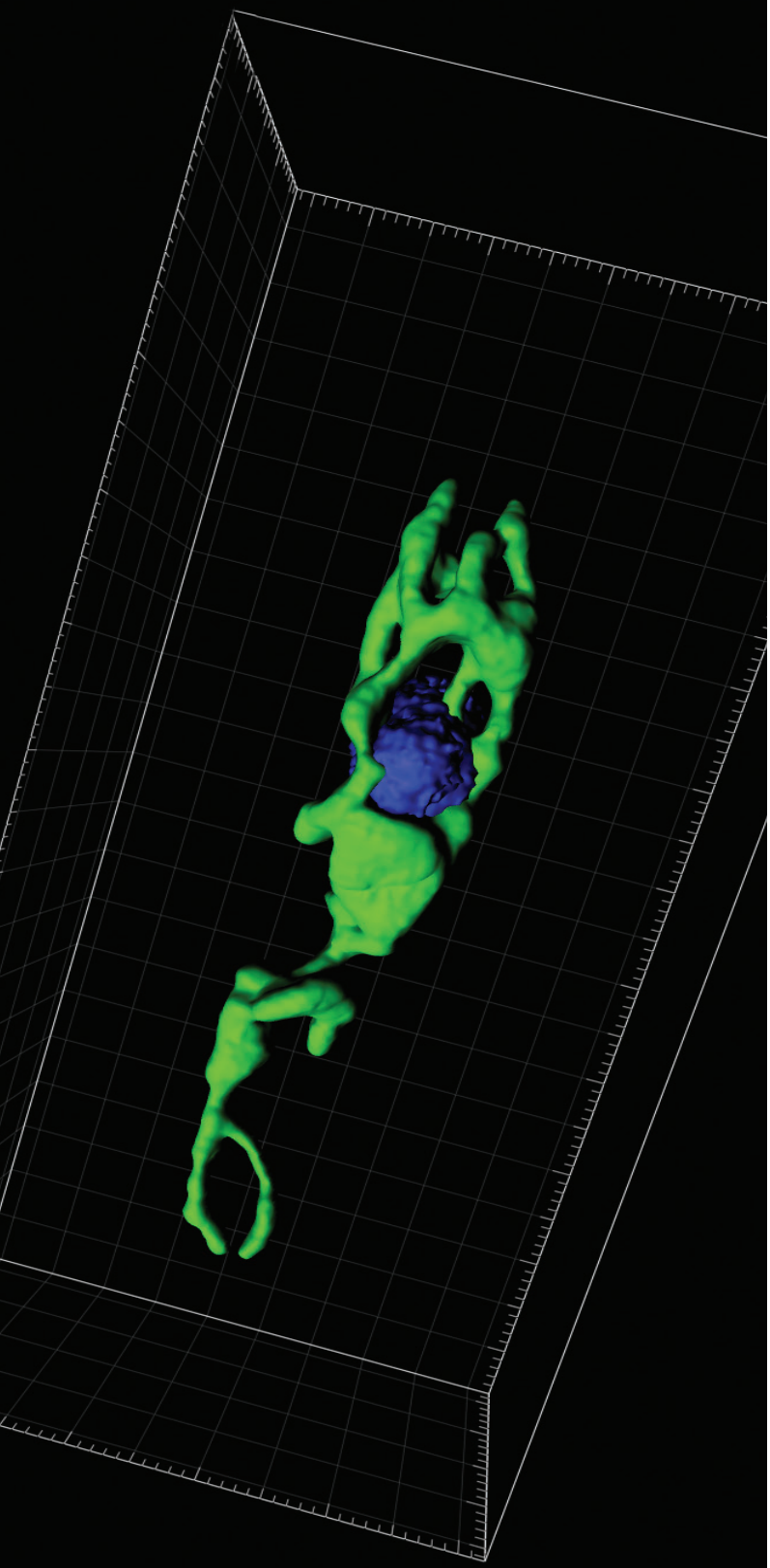


Left:
Mitochondria of *Leishmania donovani* overexpressing GFP

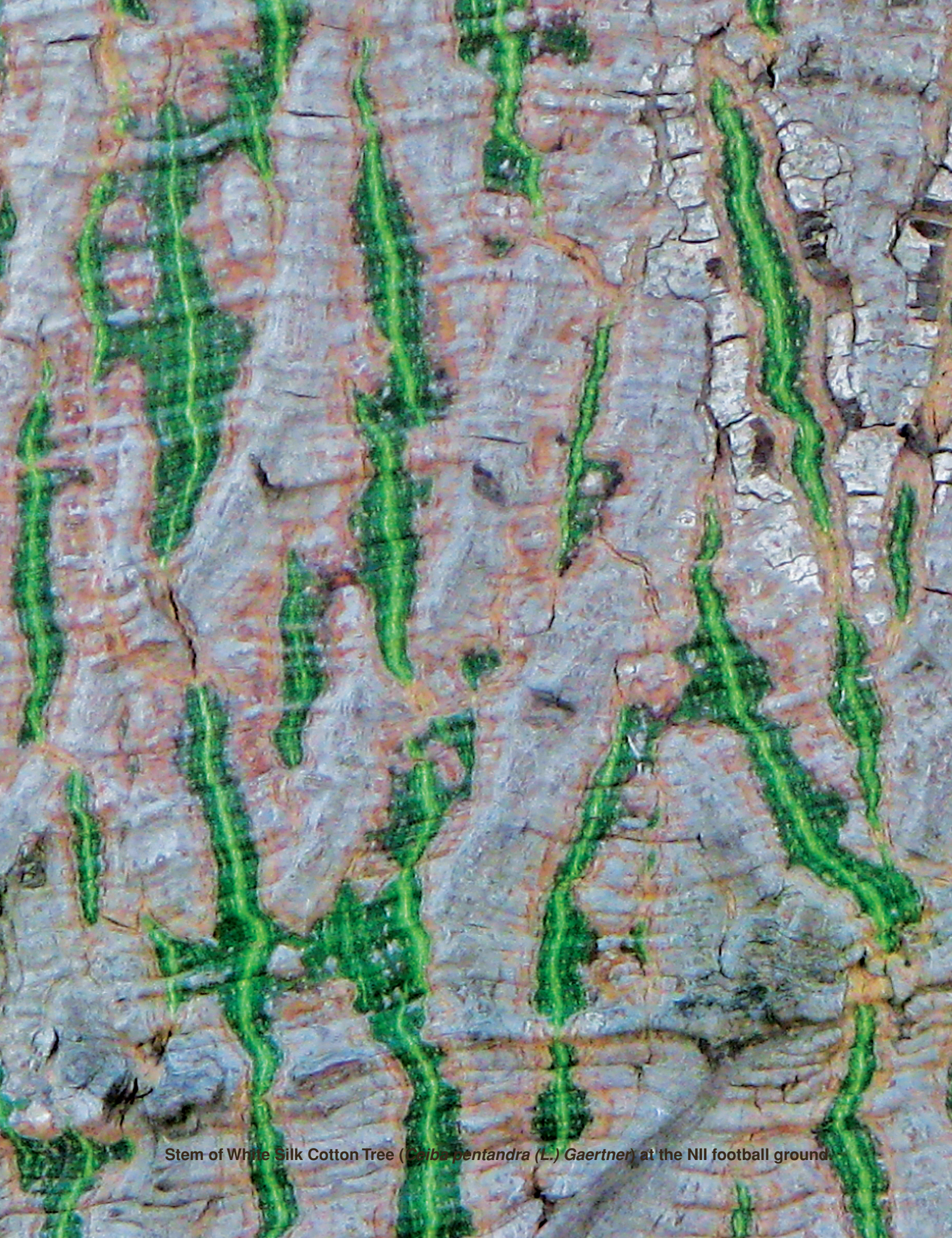
Cover page:
Mouse hepatic stellate cells expressing alpha-smooth muscle actin (green) protein

CONTENTS

Rock Garden, NII campus

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 organize postgraduate courses, workshops, seminars, symposia and training programmes of a specialized nature in the field of immunology, vaccine development and related areas.
- To organize 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 organizations in fields relevant to the objectives mentioned above.



Stem of White Silk Cotton Tree (*Ceiba pentandra (L.) Gaertner*) at the NII football ground.

FOREWORD

Over the past 30 years, the institute has nurtured a community of scientists who are making a mark in their respective turfs contributing to the understanding of disease processes. Collegial interactions amongst the faculty within the institute and the spirit of collaborations, nationally and internationally, have helped us to foray into various areas of life sciences where expertise of our scientists has contributed to the growth of science at large. Our doctoral students, post-doctoral fellows, project trainees, technical, administrative and other support staff have inspired and propelled the research programs to succeed. The variety of institute's advanced research in modern biology encompasses four major themes, namely, infection and immunity, molecular design, gene regulation and reproduction and development. Under these four rather broad areas, the institute continues to pursue cutting edge science in contemporary biology with a view to generate innovation leads. Some of the interesting observations made in the past one year and outlook for the future is detailed below.

Development and functioning of the immune system critically depends on intercellular communication. Inflammatory responses appear to have an important role in this. Interestingly, some of the molecules important in signaling pathways and inflammation are shared with those required for cellular differentiation. This leads to synergistic as well as antagonistic interactions between signaling pathways. In a study exploring such cross-talks in the context of signaling via toll like receptors (TLR), it was shown that the expressions of several NF-κB dependent inflammatory genes are augmented in TLR4/LTβR crosstalk regime as compared to cell activation through individual TLR4 and LTβR. Further, duration of signaling via LTβR and TLR was found to be an important factor in cellular differentiation and inflammation.

Cross-talk between TLR and TCR engagement induced signalling was also demonstrated in the context of T cell activation for regulation of CXCL8, a neutrophil chemo-attractant produced by T cells. Stimulation with TLR agonists, leading to CXCL8 down regulation, was reduced in presence of a serum borne lipid, S1P. However, when TLR and TCR were stimulated simultaneously, such a reduction was not seen. The study demonstrated hitherto unknown role for S1P in regulating TLR-induced CXCL8 secretion from human T cells. Also, inflammatory signals, based on TLR4 stimulation, appeared to be necessary for the entry of memory B cells to enter lymph nodes to elicit secondary response. Consequently, long-lived memory B cells, most of which are spleen resident, were demonstrated to require inflammatory signals for mounting recall responses at distal challenge sites.

Mis-regulation of the immune system at various levels can be problematic. Aberrations in

antigen presentation are well known to lead to autoimmune disorders like type 1 diabetes and rheumatoid arthritis. Analysis of association between Idiopathic hypoparathyroidism (IH) and alleles of the human leukocyte antigen (HLA) class I and II loci suggested an important role of MHC class I-mediated presentation of autoantigenic peptides to $CD8^+$ cytotoxic T cells in the pathogenesis of IH also.

Several studies have dissected the structure-function relationships of proteins specific to pathogens with an overarching view to develop therapeutics. The structure of a bifunctional enzyme, GlmU, of *Mycobacterium tuberculosis* (Mtb) was investigated. It was found to be unique in possessing a 30-residue extension at the C terminus. A detailed analysis provided an insight into substrate recognition and catalytic mechanism of the enzyme which may be exploited for the development of therapeutic inhibitors.

Analysis of calcium-dependent protein kinases (CDPKs) from *Plasmodium falciparum* (PfCDPK1) showed the propensity of several residues to be autophosphorylated and responsible for kinase activation. Also, an ATP binding site was identified in the N-terminal end of PfCDPK1. Delineation of these and other novel features of PfCDPK1 regulation might be shared by other members of CDPKs in parasites and aid in the designing of inhibitors against these targets.

While all arginases have conserved features, *Helicobacter pylori* arginase has a unique ESEEKAWQKLCSL motif whose role was unknown. That this motif is crucial for stability and catalytic function of the enzyme was established through a variety of biochemical and biophysical approaches. The studies revealed that the motif was located near the active site and formed a loop-cum-small helix structure which was crucial for the activity of the arginase. These findings can possibly be utilized for engineering it in human arginases for therapeutics against a number of carcinomas.

Beyond understanding pathogen dependent diseases and their management, several scientists at NII are also investigating the fundamentals underlying nuclear and cell biology. Cell cycle-related neuronal apoptosis has implications for neuronal degeneration. In this context, β-amyloid peptide was observed to cause aberrant activation of mitogen-activated kinase (MEK)-extracellular signal-regulated kinase (ERK) signalling. This promoted the entry of neurons into the cell cycle leading to apoptosis and highlighted the neurotoxic signal's ability to alter the signalling machinery and lead to neuronal death.

Ubiquitin-mediated degradation is essential to maintain cellular homeostasis. UV-irradiation leads to stalling of DNA replication by promoting proteolysis of Mcm10. Mcm10 degradation was shown to be a process mediated by E3 ubiquitin ligase, comprising VprBP, Roc1 and Cul4. Interestingly, ubiquitin interacts with numerous domains and motifs in its lifetime that vary in structure but bind the same hydrophobic patch. To understand its ability to interact with a variety of proteins, an NMR based study was undertaken. This revealed a role of the inherent backbone flexibility of ubiquitin in its interactions with a large array of binding partners thus providing an insight into the molecular dynamics associated with ligand recognition by ubiquitin.

Spermatogenesis is regulated by FSH and testosterone. Sertoli cells, where germ cell maturation occurs, are bestowed with FSH and testosterone receptors. Interestingly, maturation of the germ cells does not occur even though both hormones are found to be sufficiently

high in circulation in postnatal animals. The observations show that in pre-pubertal animals, the interaction of FSH to its receptor and production of stem cell factor are much higher as compared to the neonates. The switch from FSH refractory to FSH sensitive mode is the switch which determines the onset of spermatogenesis and defects in this switch would lead to infertility.

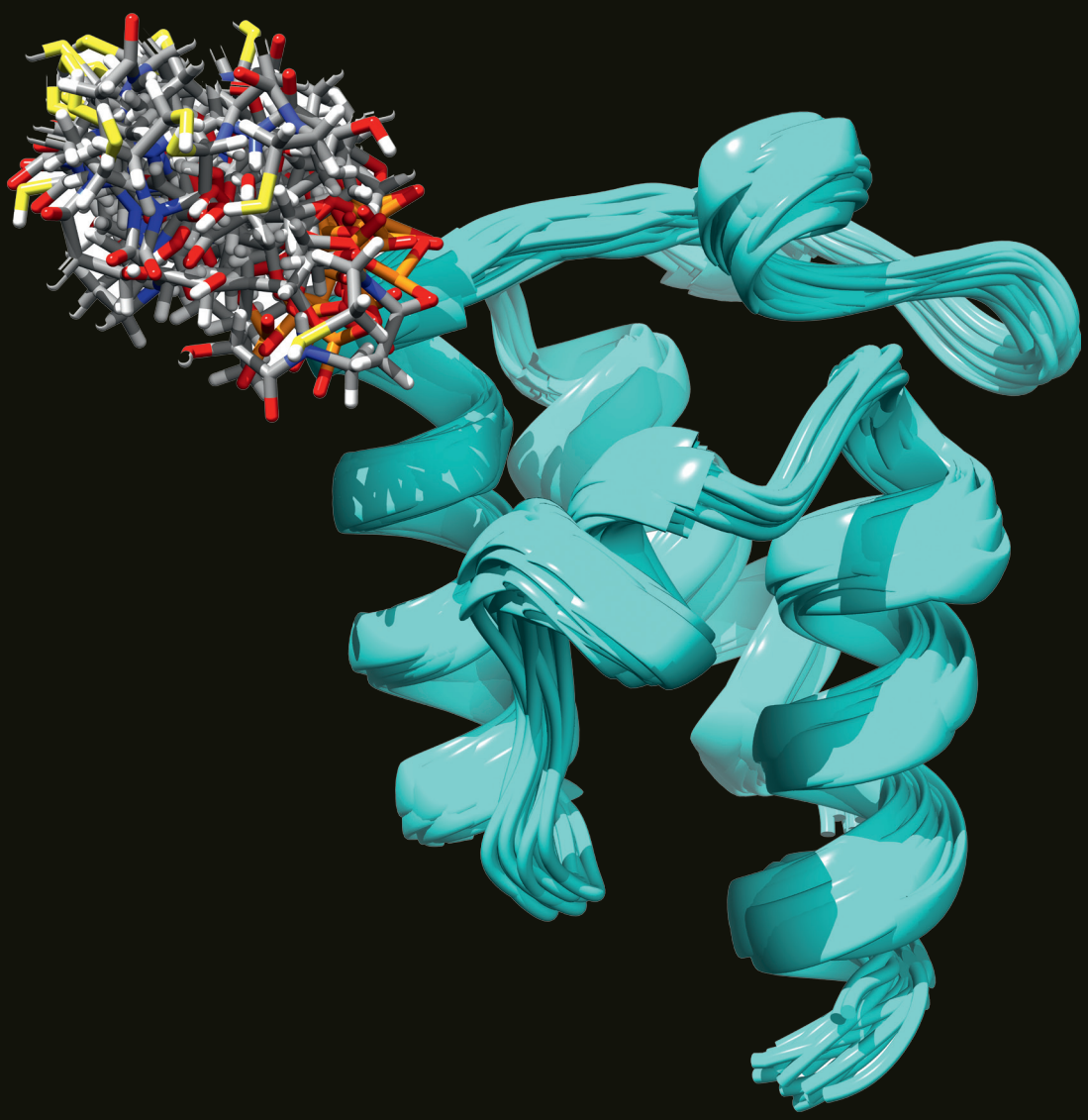
The above summary marks the strong basic science contributions made by the institute over the past one year. In the past, in addition to doing basic research, the institute has made efforts to contribute to the translational space through research on vaccines and identifying drug targets based on a variety of approaches. Over the past year the institute continued to use novel tools and advances to generate knowledge for designing new inhibitors for pathogens important from the perspective of public health.

For much of the scientific efforts of future, it is essential to develop large collaborative programmes to forge different expertise for providing solutions to questions of complex nature. While the institute nurtures many collaborative efforts all across the world, it is also a founder partner in a programme to develop multi-institutional collaborative effort which is coming up as a Biotech Science Cluster at the National Capital Region. Fostering collaborations between the biotech industry and the institute for the promotion of joint R&D programmes and for technology transfer is an ongoing activity that will be driven forward by new initiatives.

As NII strives to meet the challenges in the coming year, I would like to congratulate the entire scientific and allied staff of the institute for their enthusiastic and inspiring participation. I am sure they will propel the institute forward to sustain its excellence and contribute to the growing needs of human health care.

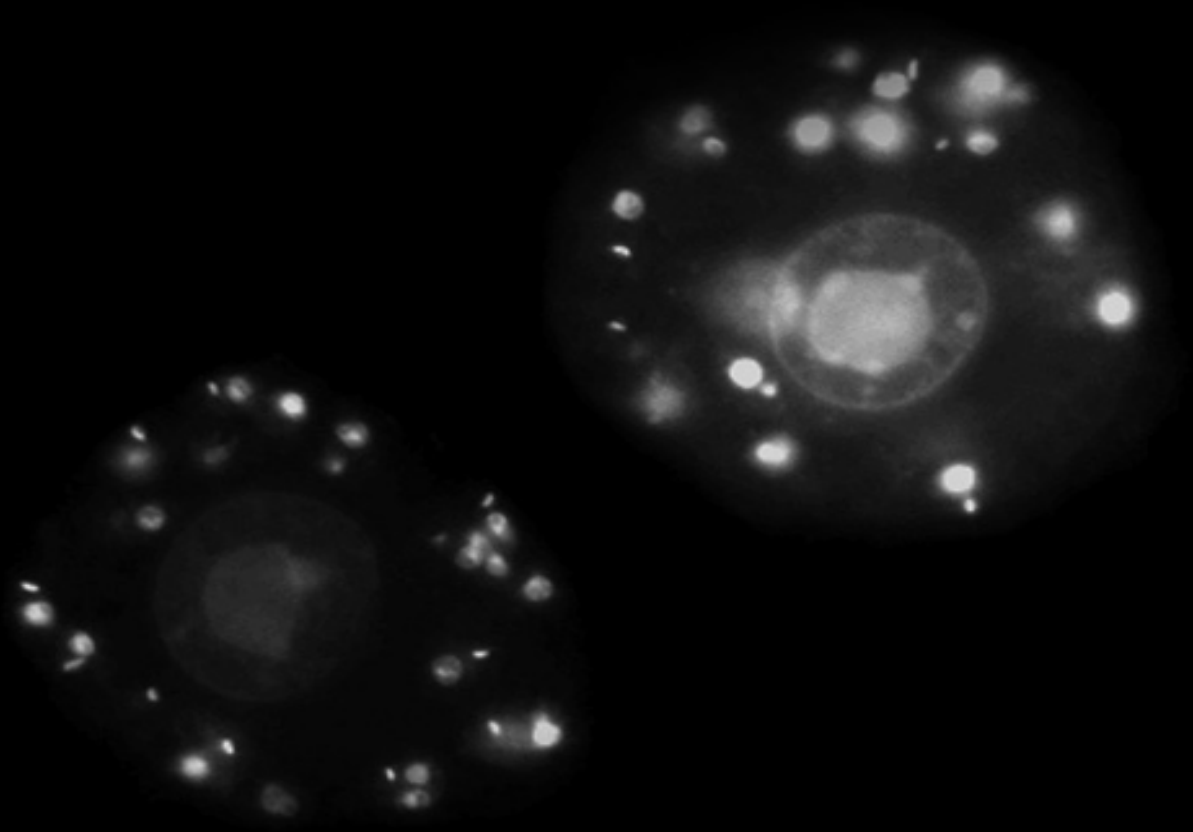
Chandrima Shaha
Director

Date : January 1, 2014



Lowest twenty NMR structures of *Leishmania major* holo-Acyl Carrier Protein (bound phosphopantetheine moiety shown in sticks)

RESEARCH REPORTS



Macrophages (J774A1) infected with *Leishmania donovani* showing amastigotes within parasitophorous vacuoles

IMMUNITY AND INFECTION

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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 ctivation 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.

A. Cellular aging of CD8 T cells

With accumulating evidence that thymic function survives well into late age in both elderly mice and humans, it is increasingly clear that naïve T cells in aged animals are likely to be a mixture with some being of relatively young post-thymic age while others would be of greater cellular age. To extend these *in vitro* studies further, we have developed an *in vivo* aging model for naïve CD8 T cells where MHC class I (MHCI)-restricted T cell receptor (TCR)-OT-I-transgenic T cells are adoptively transferred and 'parked' for varying time durations into young congenic recipients. Using this strategy, we observe that, as naive CD8 T cells are parked for increasing time, they show progressive decline in survival rates, activation, and proliferation capacity with reduced mitochondrial health status as well as a very significant decrease in CD8 co-receptor levels. Since naïve T cells require tonic signals from self-peptide MHC (pMHC) for their peripheral maintenance and survival, CD8 T

cells transferred for longer time periods would be expected to receive self-pMHCI tonic signals for extended durations. So, to assess the role of tonic signaling in altering CD8 T cell phenotype and function, we transferred OT-I CD8 T cells into wild-type (WT) C57BL/6 or TAP-1-null recipient mice. Since TAP-1-null cells (and mice) have very low MHCI surface expression, donor CD8 T cells would be expected to receive weaker self-pMHCI tonic signals in TAP-1-null recipients than in WT recipients during the period of parking. Signaling through self-pMHCI is known to be important for peripheral survival of CD8 T cells, we restricted the analysis of parked cells to early time points, namely, within the first week of parking. We find that, one week after transfer, OT-I CD8 T cells parked in TAP-I-null recipients showed higher cell-surface CD8 levels as compared to cells parked in WT recipients. OT-I CD8 T cells parked in TAP-1-null recipients were of smaller sizes and showed reduced surface levels of the negative regulatory molecule CD5. Decrease in CD5 intensity has been commonly thought to represent lack of signaling through self-pMHC. In fact, cell-surface CD8 levels on parked OT-I CD8 T cells showed differences between cells from TAP-1-null and WT recipients within 24 h, while differences in CD5 levels became visible by 48 h. Thus, these data on modulation of the cell-surface phenotype indicate that naïve CD8 T cells may begin to accumulate age-related alterations due to signaling via self-pMHCI ligands in the periphery.

A 'young' naïve CD8 T cell compartment is composed of cells that have either recently emigrated from the thymus and/or have been recirculating for variable numbers of days in the peripheral lymphoid organs without encountering a cognate antigen. Depending upon the time of egress from the thymus and dwell time in the periphery, individual naïve CD8 T cells will have relatively different age and based our data suggest that aged CD8 T cells may show reduced cell-surface CD8 expression. Expression levels of CD8 in naïve T cell populations are normally distributed, and this is thought to be due to stochastic variation in gene expression. We hypothesized that naïve CD8 T cells that lie on the two extremes of the normal distribution of CD8 levels may be differentially enriched in cells of different post-thymic ages such that CD8-low naïve CD8 T cells may show over-representation of cells of long post-thymic age. We therefore sorted naïve polyclonal CD8 T cells from young C57BL/6 mice for the brightest 10% and the dimmest 10% cells from the distribution of CD8 levels. These cell subsets were subjected to anti-CD3 and anti-CD28 stimulation. We find that, like CD8 T cells from aged animals, fewer CD8-low naïve CD8 T cells survive upon polyclonal stimulation.

Together, these data suggest that the duration of tonic signals received by naïve CD8 T cells might lead to major progressive alterations and refractoriness to TCR-mediated stimulation such as is seen in CD8 T cells from aged individuals. They also suggest that reduction of CD8 levels may be a means to identify these relatively aged cells, allowing us to distinguish the cellular aging process in the immune system from organismal aging.

B. Bruton's tyrosine kinase (Btk) in B cell development

Btk plays a crucial signaling role in B cell development, such that a lack of its functioning in patients of XLA (X-linked agammaglobulinemia) results in greatly reduced numbers of mature circulating B cells, severe agammaglobulinemia and bone marrow B cell development arrest. However, the defect manifested in the murine XID or even the Btk-deficient *Btk*-null mice is subtle, with a reduction in circulating mature B

cells to half the normal numbers, and relatively normal bone marrow B cell development. However, introducing the XID defect into a FOXN1-null *nude* phenotype has been reported to lead to an XLA-like phenotype, indicating a possible role for T cells in mediating B cell development in the absence of Btk. Neither this possibility nor possible mechanisms have been definitively explored as yet. In this context, we had previously reported generation of XID-*nu*, XID-TCRβ-null and XID-wild-type (WT) mice and compared their B cell differentiation status. There was great heterogeneity in the data with XID-*nu* mice, possibly due to the presence of mature T cells in the periphery subsequent to escaping the leaky *nude* T cell developmental block. Consistent with this, data from the XID-TCRβ-null mice were far more consistent, and showed substantial reduction of levels of serum IgM and IgG as compared to the CBA/CaJ WT or TCRβ-null mice, and at least 10-fold lower than in CBA/N or littermate XID-TCRβ$^{+/-}$ mice. Both spleen and lymph nodes from the XID-TCRβ-null mice had few B cells. Initial data had suggested that the frequency of B220^{+} B lineage cells were extremely low in the bone marrow of XID-TCRβ-null mice. However, more recent subsequent data show that, while this phenotype is indeed seen is some mice, a more common phenotype is that the most striking deficiency in the XID-TCRβ-null bone marrow is a lack of re-circulating mature B cells. This suggests that the major defect contributed by a combined lack of Btk and of TCRβ-expressing T cells is in the post-bone marrow peripheral maturation of B cells.

We have now characterised the peripheral maturation stages of B cells in these mice. We observe that, firstly, XID mice show normal numbers of splenic T1 and T2 stage transitional B cells, but show radical reductions in subsequent stages such as T3 or follicular B cells. In XID-TCRβ-null mice, this peripheral maturation block is moved upstream; while they have near-normal numbers of cells at the T1 stage, they show substantial reduction in cell numbers at the T2 stage itself, unlike in XID mice. Further, we have observed that the levels of reactive oxygen species ROS), most likely peroxide as detected by DC-FDA staining, are regulated during peripheral maturation of the B cell lineage, with T1 and T2 cells showing high levels compared to later stage such as follicular B cells. Notably, in XID mice, ROS levels are higher in all stages of peripheral B cell maturation, and in XID-TCRβ-null mice, levels of ROS are even higher. Thus, in the absence of functional Btk, T cells provide a part-redundant signal to the T1-stage B cells in the periphery to regulate their developmental transition.

Publications

Original peer-reviewed articles

1. Panda SK, Kumar S, Tupperwar NC, Vaidya T, George A, Rath S, Bal V, Ravindran B* (2012). Chitohexaose activates macrophages by alternate pathway through TLR4 and blocks endotoxemia. *PLoS Pathogens* **8**: e1002717.
2. Shenoy GN, Chatterjee P, Kaw S, Mukherjee S, Rathore DK, Bal V*, Rath S*, George A* (2012). Recruitment of memory B cells to lymph nodes remote from the site of immunization requires an inflammatory stimulus. *J Immunol* **189**: 521-528.
3. Banerjee H, Das A, Srivastava S, Mattoo HR, Thyagarajan K, Khalsa JK, Tanwar S, Das DS, Majumdar SS, George A*, Bal V*, Durdik JM*, Rath S* (2012). A role for apoptosis-inducing factor in T cell development. *J Exp Med* **209**: 1641-1653.

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Prafullakumar Tailor

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) play important role in DC subset development and their respective functions. Main area of research of the laboratory is to understand the significance of signaling pathways and contribution of IRFs and other critical transcription factors in DC subset development and functions.

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

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

Our earlier observation suggested that besides induction of *Id2* and *Batf3* genes; *Irf8* plays an essential role in development of $CD8\alpha^+$

DC development. *Id2* expression in the haematopoietic precursor cells abrogates pDC development and hence we examined the effect of *Id2* and *Batf3* expression on the pDC specific gene transcription. Co-expression of *Id2* and *Batf3* led to the decrease in the surface expression of CD11c and SiglecH. Though, *Id2* and *Batf3* when co-expressed with *Irf8* led to synergistic increase in the CD8α^+ DC specific transcripts; such synergistic effect was not extended to the pDC specific gene expression. We noticed the decrease in the expression of *Siglech* gene when *Id2* and *Batf3* expressed together and also when co-expressed with *Irf8*. Co-expression of *Id2* and *Batf3* in the mouse bone marrow DC cultures lead to very low SiglecH expression and decrease in B220$^+$MacI^{low} pDC population. A recent study of *Nfil3* (nuclear factor, IL-3 regulated; also called E4BP4) gene knock out mice suggested that it indirectly controls the CD8α^+ DC development by regulating *Batf3* gene expression. We noticed the modest yet reproducible two fold increase in the *Nfil3* transcript levels in *Irf8* expressing population. *Nfil3* expression in DC9 cells showed a modest increase in the MHC II induction upon CpG treatment, though CD8α was not detected. We further examined the CD8α^+ DC specific gene induction pattern by *Nfil3* in comparison to *Irf8* expression. CD8α^+ DC specific genes like *Necl2*, *Xcr1* and Tlr3 were induced at comparable levels by *Nfil3* yet *Irf8* expression led to comparably higher transcript levels of key transcription factors *Batf3* and *Id2*. CD8α^+ DC specific Clec9a gene remained undetected in *Nfil3* expressing cells and appeared only upon *Irf8* expression. Though *Nfil3* induced some of the CD8α^+ DC specific gene transcripts, *Irf8* could induce these transcripts at comparably higher levels and *Nfil3* could not activate transcription of Clec9a gene. Expression of *Nfil3* in DC9 line led to expression of some of the CD8α^+ DC specific gene expression and only *Irf8* expression could induce complete profile of CD8α^+ DC phenotype. Thus, taken together our data suggests that *Irf8* is a master regulator of CD8α^+ DC development. As *Batf3*, *Id2* and *Irf8* are essential for CD8α^+ DC development; we would like to conduct the detailed analysis of DC subtype development by these transcription factors. We are in a process of making the appropriate hybrid clones to understand interactions (if any) between *Batf3*, *Id2* and *Irf8* by suitable reporter assay. By understanding the interactions among these transcription factors we may be able to address questions relative to their putative roles in specific DC subtype commitment.

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

Transforming growth factor-β (TGF-β) signaling is shown to have important roles in DC development. In DCs, TGF-β signalling induces *Id2* expression. ID (ID1-4) proteins are members of helix-loop-helix (HLH) group of transcription factors and are termed as antagonists of another activator class of HLH members. *Id2* expression is essential for CD8α^+ DCs and Langerhans cells. A recent study identified *Irf8* as a direct target of TGF-β signaling in DCs; and as per our studies, *Irf8* expression in DC9 cell line (*Irf8*$^{-/-}$ DC line) led to induction of *Id2* gene. Our preliminary results suggest that *IRF8* may regulate CD8α^+ DC development by controlling *Id2* gene expression.

In the first step, we initiated the study towards understanding the role of TGF-β signalling in the DC development. Experimental treatment of the murine BMDCs with the TGF-β led to the

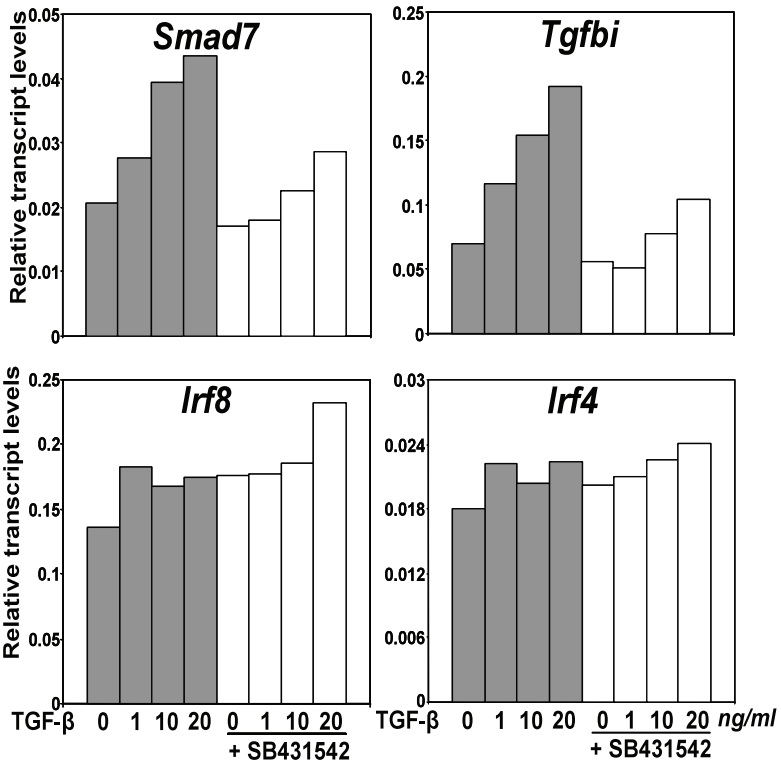

Figure 1: Effect of TGF-β signaling on the Irf8 gene transcription. Bone marrow dendritic cells were treated with different concentration of *TGF-β* for 8 hr in absence and in presence of *TGF-β* signaling inhibitor SB431542. Data suggests that treatment with *TGF-β* leads to increase in transcript levels of *TGF-β* inducible genes *Smad7* and *Tgfbi* but there was no significant change in *Irf8* or *Irf4* gene transcript levels. Transcript levels were normalized to *Gapdh* gene.

increase in the *Smad7* and *Tgfbi* genes and this increase in the transcript level was inhibited by TGF-β signalling chemical inhibitors SB431542 and LY364945. We observed that addition of TGF-β to the DCs led to SMAD2 phosphorylation as early as 30 min and addition of inhibitors completely blocked this activation, though IRF8 protein levels did not change over time period till 24 hr. Results of BMDC experiments suggest that *Irf4* and *Irf8* transcript levels are not sensitive to chemical inhibition of the TGF-β signalling (Figure 1). By flow cytometry analysis, we confirmed that treatment with the TGF-β signalling inhibitors did not affect the appearance of CD8α marker on the DCs. We further investigated this observations using molecular biology approach by blocking the TGF-β signalling. We expressed the *Smad7* and dominant negative TGF-β receptor II (dnTGFbRII) in the murine DC cultures and examined the CD8α DC differentiation. Expression of *Smad7* and dnTGFbRII led to decrease in the transcript levels of *Id2* and *Sis* but the *Irf8* and *Irf4* transcript levels remained comparable. In our experiment *Smad7* and dnTGFbRII were expressed in the ongoing BMDC cultures and there is a possibility that commitment towards CD8α$^+$ DC may be an early event. Hence, we procured the transgenic mice expressing dnTGFbRII. Transgenic mice study confirmed our finding from the *in vitro* BMDC cultures. *Irf8* gene expression remained unaffected by blocking TGF-β signalling in DCs and thus we show that TGF-β signalling is redundant in pDC or CD8α$^+$ DC development. In our further experiments we are conducting in depth study to analyse the role of *Irf8* in regulating *Id2* gene as well as its effect on the TGF-β signalling on the subtype specific gene transcription.

Publications

Original peer-reviewed articles

1. Chang T, Xu S, Tailor P, Kanno T, Ozato K* (2012) The Small Ubiquitin-like Modifier-Deconjugating Enzyme Sentrin-Specific Peptidase 1 Switches IFN Regulatory Factor 8 from a Repressor to an Activator during Macrophage Activation. *J Immunol* **189**: 3548-3556.

2. Goel P, Tailor P, Chande A, Basu A, Mukhopadhyaya R* (2013) An infectious HHV-6B isolate from a healthy adult with chromosomally integrated virus and a reporter based relative viral titer assay. *Virus Res* **173**:280-285.

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Biology of T lymphocytes

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Ongoing work on T cell fate decisions encompassing differentiation, proliferation, survival and death is reported. In addition, work on the mechanistic linkages between renal dysfunction and proteinuria are explored using mouse models.

The investigations have following objectives:

1. To understand the regulatory mechanisms involved in cell intrinsic differentiation of CD4 cells in Th1 and Th2.
2. To characterise the effects of *in vivo* aging on CD4 T cell function and phenotypic features.
3. To study mechanisms associated with renal dysfunction and proteinuria.

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

We have been studying relative contributions of CD4 T cell intrinsic and extrinsic factors in the differentiation to Th1 or Th2 phenotypes. So far our studies have focussed on intrinsic factors with a comparison between NCD4 cells from C57Bl.6 (B6) and BALB.b mice. We showed that BALB.b NCD4 cells differentiate to produce both IFNγ and IL-4/IL-13, whereas B6 only produce IFNγ during recall of memory generated *in vitro*. We compared *in vitro* generated memory with memory generated *in vivo*. For this memory CD4 cells were sorted from the 2 strains of mice and recalled *in vitro* to evaluate their Th1/Th2 commitment status. It was observed that *ex vivo* memory cells from BALB.b mice continued to produce both IFNγ and IL-4/IL-13 similar to *in vitro* memory. However, unlike memory generated *in vitro* from NCD4 cells of B6 background, *ex vivo* memory cells were producing both IFNγ and

IL-4/IL-13. These data suggest two possibilities. Firstly, contribution of T cell intrisic factors in the generation of memory in B6 mice may be poorer than T cell extrinsic factors in contrast to BALB.b mice. This could be genetically and/or epigenetically regulated. Secondly, and not necessarily exclusively, the duration of survival of once generated memory cells might be differentially regulated in the two mouse strains studied. There are multiple factors which can potentially regulate survival of T cells *in vivo* such as reactive oxygen and nitrogen species, transcription factors such as STAT family, suppressors of cytokine signalling (SOCS) etc. We will begin investigating some of these in future.

Another aspect of *in vitro* generated memory cells is interesting and novel. We observe that *in vitro* generated memory cells from both B6 and BALB.b mice co-express T-bet and Gata-3 transcription factors in large proportion of cells, unlike the cells activated in polarising conditions. Preliminary experiments to quantitate T-bet and Gata-3 from *in vitro* generated memory cells by qRT-PCR confirm upregulation of both the transcription factors in cells from B6 and BALB.b mice. Further, only a small proportion of T-bet^{+}/Gata-3^{+} cells show presence of intracellular IFNγ or IL-13. There is hardly any dual positivity in cytokine producing cells. These data are very much in contrast to *in vitro* generated memory cells from polarised cultures and hence interesting for further investigation into regulatory differences – possibly at epigenetic level.

We analysed blood samples from children of Nephrotic Syndrome (NS) for Th1/Th2 differentiation tendency. These studies were undertaken in collaboration with AIIMS. NCD4 cells from NS patients were differentiated *in vitro* in memory cells, as described above for mouse cells, and supernatants were collected for cytokine analysis. Unfortunately even with bead array, the number of samples which gave detectable and reliable signal of secreted cytokines was minimal. These data by themselves were hard to interprete because of this limitation and also for making comparisons with published literature. So we modified the approach. *Ex vivo* CD4 memory cells and naïve cells were sorted using flowcytometer instead of magnetic sorting which was used earlier. We estimated the relative Th1/Th2 ratios of secreted cytokines from *in vitro* generated memory versus *ex vivo* memory. These cytokine patterns will be correlated with the clinical outcome i.e. steroid resistance versus sensitivity in the patients of NS.

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

In our effort to study cellular aging in NCD4 cells, we have reported that NCD4 cells with low CD4 intensity isolated *ex vivo* from young mice show phenotypic and functional features comparable with NCD4 cells from aged mice, including accumulation of ROS. It has been reported that for naïve T cells to survive *in vivo* continuous presence of tonic signals provided by self peptide-MHC complex is necessary as demonstrated by loss of naïve cells in MHC-deficient mice. We hypothesised that prolonged tonic signalling may also be deleterious to naïve cells. Thus, individually old naïve T cells are likely to receive tonic signals for prolonged period as compared to individually younger naïve cells. Prolonged tonic signalling may actually result in poor ability to respond to an activating signal through TCR and CD4 down-regulation may be a marker of it. We tested this by transferring NCD4 OT-II cells in Ii$^{-/-}$ and MHC class II$^{-/-}$ mice with WT mice as control recipients. As compared to WT

mice Ii$^{-/-}$ mice have lower levels of MHC class II on APC surfaces whereas they are completely absent in MHC II$^{-/-}$. We observed differences in the CD4 staining intensity on OT-II cells in 3 days post transfer. Mean fluorescence intensity (MFI) of CD4 on OT-II cells transferred in Ii$^{-/-}$ and MHC II$^{-/-}$ was higher than that in WT suggesting that efficient tonic signalling is indeed downregulating CD4 intensity. These cells were stimulated with Ova-II peptide and it was observed that OT-II cells transferred in WT mice responded poorly as compared to OT-II cells transferred in MHC II$^{-/-}$ mice. When ROS levels in these transferred OT-II cells were examined by DCFDA staining, OT-II cells from WT recipients showed more accumulation of ROS as compared to those from MHC II$^{-/-}$ mice. OT-II cells transferred in Ii$^{-/-}$ mice showed intermediate profile, suggesting that the relative abundance of MHC class II is likely to determine frequency of tonic signalling leading to cellular ageing. These data clearly show that persistence of tonic signals can bring about senescent status to the individual naïve CD4 T cells.

C. To study mechanisms associated with renal dysfunction and proteinuria

We have undertaken studies to understand molecular partners involved in signalling resulting in proteinuria because of our interest in clinical renal diseases in collaboration with AIIMS and PBC/THSTI. Proteinuria is also a common feature in sepsis and expression of many TLRs on renal podocytes is likely to contribute to the pathology observed. We have been looking at proteinuria 24 h after exposing the mouse to an insult such as lipopolysaccharide (LPS) injection as a simple model to understand contribution of many immunologically relevant molecules. While it has been reported that CD80 is secreted in urine during NS the exact role of CD80 expression in kidney and/or bone-marrow (BM) dervied cells in proteinuria is unknown. We confirmed that CD80$^{-/-}$ mice do not develop proteinuria in response to LPS, a TLR-4 ligand, whereas WT mice show proteinuria (detected as albumin levels/ml urine). In order to see whether CD80 expression on renal podocytes or on BM-derived cells is necessary to cause proteinuria in response to LPS injection, BM-chimeras were made. BM cells from CD80$^{-/-}$ and WT mice were transferred to irradiated WT and CD80$^{-/-}$ cells. Early experiments show that LPS challenge to chimeric mice where CD80$^{-/-}$ BM is transferred to irradiated WT mice show proteinuria whereas WT → CD80$^{-/-}$ mice do not. Thus it appears that expression of CD80 on podocytes is a major determinant in proteinuria induced by LPS injection. We are also exploring outcome after use of other TLR ligands in proteinuria.

Publications

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Sudhanshu Vrati

Biology of Japanese Encephalitis virus

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Japanese Encephalitis virus (JEV) is a member of the *Flaviviridae* family of animal viruses that contains several other medically important viruses such as Dengue and Yellow fever. JEV is a major cause of human encephalitis and is responsible for considerable mortality and morbidity in India. Frequent epidemics of Japanese encephalitis (JE) are being reported from various parts of India and JEV has become endemic in several parts of the country. We are studying the biology of JEV replication with a view to develop novel anti-virals.

JEV genome is a plus-sense single-stranded RNA of ~11 kilobases. A minus-sense RNA template is generated during virus replication, which is then copied to produce a large number of plus-sense genomic RNA molecules. Based on the amino acid sequence homologies with other replicases, NS3 and NS5 viral non-structural proteins have been speculated to be involved in replication of the JEV genome. However, we do not know if any of the cellular proteins also are needed for viral replication. We are, therefore, studying cellular proteins that interact with JEV genome sequences, which are likely to be involved in viral replication. Besides, viral non-structural proteins may interact with viral genome or host proteins during replicaion. We wish to study these interactions.

The site of PTB interaction wih JEV 5'-NCR RNA was further confirmed by mutagenesis study to be a polypyrimidine tract present in the first stem of viral RNA. We also validated and measured this interaction *in vivo* upon natural infection by co-immunoprecipitation RT-PCR. Interestingly, we observed phosphorylation coupled relocalization of nuclear PTB to cytoplasmic foci that co-localized with JEV RNA as soon as JEV infection manifests. Furthermore we assessed the inhibitory role of PTB during viral replication by measuring viral titers in PTB knockdown and overexpression systems. Using competition assay we show that PTB competitively inhibit association of JEV 3'-SL(-) RNA and viral NS5 protein, an event preceding initiation of viral replication early during infection. Treatment of cells with cAMP analogue to promote PKA mediated PTB phosphorylation also leads to diminished viral replication upon JEV infection. In summary we have identified PTB as a cellular partner of JEV NCRs and its intriguing consequences on regulating fate of nascent JEV RNA during JEV infection.

We have also studied interaction of JEV non-structural proteins with host proteins. The

proteins that are under study are: NS5 which has the RNA-dependent RNA polymerase activity, and NS2A whose function is yet unknown. Yeast two hybrid system has been used to fish out potential interacting partners with these two proteins. These interactions are under validation using conforcal microscopy as well as pull-down assays.

Publications

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Agam Prasad Singh

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. Basic theme is to identify new parasite molecules that affect the host cellular processes and possible intervention strategies.

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. 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. 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.

A. Host parasite interactions

In the previous report we described generation of the knockout, here we give details of phenotypic characterization.

Phenotypic characterization of Pb-871 (SLTRIP) knockout clones

SLTRIP is a tryptophan-rich malaria parasite

protein of unknown function. SLTRIP-KO parasites show following phenotypes:

I) Localization: Immunofluorescence assay (IFA) using anti-SLTRiP polyclonal antibody confirmed that SLTRiP protein has cytosolic expression in plasmodium sporozoites. Immuno-localization of SLTRiP protein in sporozoites using transmission electron microscopy (TEM) also confirmed cytosolic expression. IFA showed expression during liverstage from 12- 42 hours of LS and protein was detected in hepatocyte cytoplasm as well. The expression of SLTRiP protein increased from 12 to 42 hr, and completely abrogated in 48hr. of *ex vivo* growing LS. SLTRiP expression was absent during blood stage of malaria parasite.

II) Development in mosquito: We found there were no significant differences in the development of oocyst, number of sporozoites in oocyst or salivary glands of SLTRiP-KO and WT infected mosquitoes. In the case of SLTRiP-KO, the average number of sporozoites in mosquito salivary glands was 14000 ± 2000 compared to 16000 ± 2000 in WT parasites. SLTRiP deficient sporozoites have normal gliding motility as compared to WT sporozoites. (III- growth in hepatocyte and RBCs) To find the effect of SLTRiP gene disruption during liver stage of malaria parasite, we quantified the SLTRiPKO and WT parasite load, in the infected liver, by measuring parasite 18S-rRNA using quantitative real time PCR. Real time PCR result shows that there was around 21 folds difference in 18S-rRNA copy number of WT and SLTRiP-KO parasite during liver stage. Using IFA we noticed, SLTRiP-KO parasites were smaller in size. SLTRiP-KO parasites average area was 4.67 fold less compared to WT parasite. To check the effect of SLTRiP-KO on blood stage parasite growth, 10000 SLTRiP-KO or WT blood stage parasites were intravenously injected in five mice each and results show there was no significant difference.

Phenotypic characterization of SLTRIP pexel mutant knockout clones

To delineate the role of exported SLTRIP in host, this year we prepared a SLTRIP pexel motif mutant parasite line using the standard transfection method. Mutation was confirmed in the clones by sequencing genomic DNA spanning the mutation region. The phenotypic characterization is in progress. In one experiment, we observed that SLTRIP pexel mutant parasites show approximately 10 folds less parasite load in liver.

Immunization with SLTRiP recombinant protein results in significant level of protection against P. bergehi sporozoite challenge

In control and SLTRiP immunized group followed by sporozoite challenge, blood stage parasites were detected on 3rd and 7th days respectively. Result demonstrated that immunization with SLTRiP gives strong (a four day delay in pre patient period) but partial protection against sporozoite challenge.

Pb-268 (PB 122500) knockout and phenotypic characterization of clones

PB 122500 a putative kinase domain containing protein has an active pexel motif. To explore the roles played by this protein in liver stage parasite development we generated 122500 knock out parasite strain using standard method. Clonal populations were obtained and verified before they were used in characterization. 122500 KO were passaged through mosquito three times before documenting the phenotype. 122500-KO had four fold less salivary gland sporozoite numbers, compared to wild type parasites, although morphologically they were

similar. Invasion of salivary gland by sporozoite was normal and no defect detected. Reduced number of sporozoite was due to less number of sporozoites developing in the oocyst stage. When equal number of 122500-KO or WT sporozoites were inoculated in mouse and subsequently measured for liver load, there was two log (100 fold) less liver burden in case of 122500-KO parasite compared to WT.

Expression of 122500 during parasite life stages

In an IFA using polyclonal antibody against a c-terminal peptide (b3), we confirmed expression in oocyst (from day 3-14 post blood meal), in salivary gland sporozoites and liver stages (12-48 h). In the blood stage parasites, PB 122500 expression was not detectable by IFA. Immuno-EM studies demonstrated that protein is distributed in cytosol.

B. Novel drug targets

The remnant plastid of *Plasmodium* has lost its primary function of photosynthesis during the course of evolution. Nevertheless it has retained several other important functions. Apicoplast has emerged as a significant drug target because of its bacterial origin. The C3, C5, C6 type sugar phosphate transporters bring sugars inside apicoplast, thus providing energy, reducing power and elements like carbon to apicoplast. *Plasmodium berghei* has two C3 type sugar phosphate transporters in the membrane of apicoplast: triose phosphate transporter (TPT) and phosphoenolpyruvate transporter (PPT). We found that P. berghei TPT knockout parasites failed to survive.

However, PPT knockout parasite behaved similar to the wild type in the blood stages. The absence of PPT in other life stages, leads to defects in the development of parasite and was required at both mosquito as well as liver stages. This study underlines the essentiality of triose transporters for apicoplast and its downstream pathways. Since TPT is essential for parasite survival it could be a novel target for drug development.

Publications

Original peer-reviewed articles

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Akhil C. Banerjea

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 in India is mainly due to the genetic subtype C (>90%), followed by B and then B/C & A/C recombinants and it is therefore important to understand how our epidemic is different than subtype B dominated regions like UK and USA. This involves constant genetic and functional analysis of circulating HIV-1 genes from India.

Host generates its own anti-viral response and the viruses have evolved to overcome restriction factors. Understanding the mechanistic details about the host-virus interaction is crucial for understanding biology of HIV-1. Micro-RNAs are involved in various disease conditions and our broad aim is to explore their role in HIV biology and pathogenesis.

HIV-1 has evolved several strategies to ensure its survival by systematically overcoming host restriction factors. The various small HIV-1 ORFs like Nef, Vpu, Vif and Vpr are known to play a major role in pathogenesis. We wish to understand the role of accessory genes in modulating cellular functions important for causing pathogenesis. Equally important is to understand how cell is using various counter measures to reduce the virus burden. It is important to study how key regulatory and accessory proteins of HIV-1 are degraded inside the mammalian cell; how they directly or indirectly modulate the key regulators of cell cycle/apoptosis etc. How micro-RNAs (viral and cellular) influence these functions and contribute to pathogenesis.

Genetic characterization of natural variants of Vpu and their impact on virus release and cell death

Based on phylogenetic analyses of natural

Vpu variants from North India, we were able to categorize them broadly into B and C-subtypes. Group B variants always showed greater virus release activity but exhibited moderate levels of cell death potential. Group C variants displayed lower virus release activity but exhibited greater apoptosis. The Vpu variants with a natural S61A mutation displayed greater intracellular stability; were hypo-ubiquitinated but caused greater virus release. We came across a natural Vpu mutant (S61A). This mutation resulted in remarkably increased intracellular stability. We came across a novel group C variant that possessed a non-functional B-TrcP motif (S52 & S56 being substituted with isoleucine). This mutant was compromised with respect to virus release and cell death. Interestingly, this individual, had the highest levels of CD4 count (>1000) out of 13 that we analysed. The Indian Vpu B variants showed close relationship with subtype B reported from America, Japan and Brazil but the Vpu C showed resemblance with Zambia, Botswana & South Africa. To determine the nature and extent of changes due to evolutionary selection, the average DN/DS values (the ratio of substitution rates between non-synonymous and synonymous sites) were determined for all samples. Majority of the samples showed values greater than one which is suggestive of a positive selection. Taken together this study clearly suggests that Vpu locus in the viral genome is highly polymorphic and that some of these mutations have a significant functional implications. These observations have obvious implications for HIV subtype-specific pathogenesis.

Tat and Rev have RNAi suppressor activity and role of miRNA34a

The two regulatory proteins, Tat and Rev were earlier shown to possess the suppressor activity using the suppressor assay described by us (J Virol 84, 10395-401, 2010). Both of these proteins possess a 12 – 13 long arginine rich motif (ARM) and we show that this ARM motif is important for this function. This was achieved by making specific deletion mutants of Tat and Rev. We also found that ARM alone is able to interact specifically with some of the components of RNA-induced silencing complex (RISC). Interestingly, when the ARM was fused to a reporter gene, it now possessed suppressor activity. This is the first report of its kind where a suppressor function is assigned to ARM. The role of p53-sensitive miRNA34a was studied in viral infection. This was clearly up-regulated in HIV-1 infected T-cells.

HIV-1 protein degradation mechanisms

We have earlier reported that E6AP ligase is involved in carrying out Nef-mediated degradation of p53. We have preliminary evidence that Vif is also degraded by a novel ligase called CHIP. Similary, Tat was found to be largely degraded by 20-S mediated -ubiquitin-independent mechanism. Tat was found to stabilize MdM2 by specific post-translational mechanisms which are being worked out.

HIV-1 Vpr possess de-ubiquitinase activity

A balance of ubiquitination & de-ubiquitination has been described for many viruses Influenza and specific viral gene products have been assigned with such functions in few cases. We report, for the first time that Vpr possesses global de-ubiquitinase activity and that integrity of all 3 helices are important for this remarkable activity. Functional relevance of this observation with HIV-1 pahto-physiology is being worked out.

Publications

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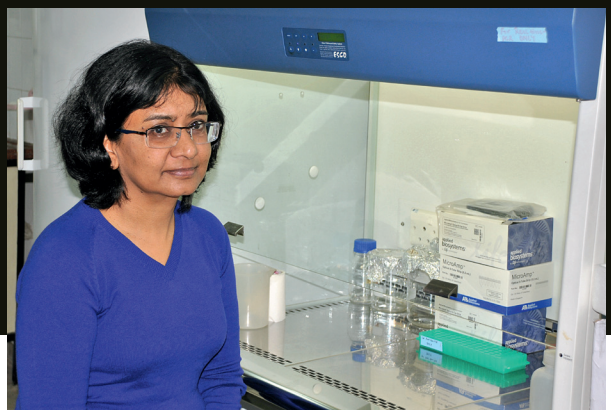

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Study of mucosal immune responses

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This laboratory is currently engaged in exploring signals that influence the activation, differentiation and death of lymphoid cells, in evaluating the role of endosome biogenesis in antigen presentation by B cells, and in dissecting the role of gut microbes in modulating the mucosal-systemic immune interface.

Current ongoing research efforts in the lab revolve, to varying degrees, around the following objectives: a) studying the role of CD27 and CD40 in B cell differentiation, b) identifying factors that influence plasma cell survival, c) evaluating at the relative roles of B cell antigen presentation versus BCR-mediated signal transduction in determining the final outcome of antigen recognition by B cells d) attempting to generate an IgA response to systemic immunization and e) evaluating the contribution of intestinal flora in regulating immune homeostasis.

A. Studies on plasma cell survival: This is the main area of research on which progress is being reported this year.

We have previously reported preliminary data indicating that when iNOS$^{-/-}$ B cells are stimulated with LPS, the frequency of live plasma cells (PCs) observed on day 3 or day 4 was about two-fold lower than that seen following stimulation of wild-type B cells and that the difference was not due to poor B cell activation or differentiation. Over the current reporting year we have cultured sort-purified PCs from the two strains and scored their survival over time as viable counts and their function as antibody secreting cells (ASCs). We report that iNOS$^{-/-}$ PCs show poor survival in both assays. To look at lifespan *in vivo*, we transferred purified PCs from the two strains into Igh congenic recipients and scored for donor IgM allotype in the recipients over time. We find that less donor IgM is present in recipients receiving KO PCs as early as 24h after transfer, and that while donor Ig levels decrease in both sets of recipients, the decay is more acute in recipients of KO PCs. Further, addition of two independent iNOS inhibitors enhances the death of purified iNOS-sufficient PCs *in vitro* and addition of an NO donor reduces the death

of iNOS$^{-/-}$ PCs. Physiological relevance for the findings comes from the further observation that iNOS-null mice mount a poor antibody response to immunization with NP-Ficoll as compared to WT mice, a T-independent antigen being used to avoid possible confounding effects of iNOS-deficiency on T cells. Together, the data provide compelling evidence that iNOS has a protective role during terminal differentiation of B cells.

We next tested whether pro-survival signals from known effectors like IL-5, IL-6, APRIL or CD44 ligation that contribute to PC survival (especially of long-lived ones in the bone marrow) could compensate for the lack of iNOS. Anti-CD44 supplementation enhanced the survival of PCs from both strains, and while KO PCs did not respond to IL-5, WT PCs did not respond particularly well either. Surprisingly, while IL-6 and APRIL enhanced the viability of WT PCs significantly, they had absolutely no effect on the survival of KO PCs. IL-6R levels were similar on PCs from the two strains, ruling out low receptor density as a cause for non-responsiveness in KO PCs. No differences were seen in levels of CD44, CXCR4, Blimp-1 and Bax, either, but Bak levels were consistently higher in KO PCs. Notably, addition of iNOS inhibitors to WT cultures negated the protective effect of these cytokines, indicating that signaling downstream of the cytokine receptors intersects with iNOS signaling. The simplest explanation was that these factors might induce iNOS expression, and we found that IL-6 did, indeed, induce iNOS transcripts in WT PCs. Our data provide a mechanism for the known protective effect of IL-6 on PC survival. They also show that both iNOS-dependent and -independent pathways contribute to PC viability.

NO can activate soluble guanylyl cyclase, leading to the activation of PKG via cGMP and to the activation of PKA via cAMP, both of which can regulate components of the ER stress pathway and inhibit death. To determine whether these pathways were involved in PC survival, we assessed PC generation and survival in the presence of the soluble guanylyl cyclase inhibitor ODQ, the PKG inhibitor KT5823 or the PKA inhibitor Rp-8-Br-cAMPs. Fewer live PCs were detectable in WT cultures in the presence of ODQ or KT5823, but Rp-8-Br-cAMPs had only minimal effect, indicating the involvement of PKG and not PKA downstream of NO. Further, WT PCs were unable to respond to IL-6 if PKG was inhibited and addition of the PKG mimic 8-pCPT-cGMP promoted the survival of KO PCs. Together, our data indicate that the iNOS-NO-PKG pathway is involved in PC survival.

The metabolic stress associated with antibody secretion can lead to ROS accumulation, ER stress and to mitochondrial dysfunction. We found that intracellular ROS levels were similar in PCs from the two strains and that ROS inhibition had no effect on PC viability. Mitochondrial potential was also unaffected. However, transcripts for sXBP-1, GADD34, EDEM1 and GRP94 (molecules involved in coping with ER stress) were significantly lower in iNOS-null PCs. Thus, iNOS affects components of the unfolded protein response. Since NO has been shown to inhibit the activation of various caspases by nitrosylation of the cysteine thiol group in the catalytic site and since cGMP analogs prevent caspase activation, we looked at PC viability in the presence of individual caspase inhibitors. Inhibition of caspases 3, 6, 9 or 12 enhanced the survival of iNOS-null PCs while inhibition of caspase-8 or -10 afforded no protection. Further, WT PCs showed enhanced activation of caspases 3, 6, 9 and 12 when they were cultured for 4h with the PKG inhibitor KT5823. Together, the results indicate that iNOS deficiency in PCs leads to an enhanced activation of initiator caspases 9 and 12 and executioner caspases 3 and 6.

Taken together, our data show that iNOS is a significant signaling intermediate in PC survival pathways. Endogenous iNOS supports the survival of newly generated PCs via the soluble guanylyl cyclase-cGMP-PKG pathway that leads to modulation of components of the UPR that allow PCs to cope with ER stress, as well as to inhibition of caspase activation. iNOS is also an intermediate in survival signals provided to PCs by BM stromal cells and thus also influences the lifespan of the long-lived PC pool.

B. Other projects: Some progress is also being reported on other research efforts in the laboratory. In our analysis of the role of CD40 in B cell differentiation, we have previously reported that CD40 ligation inhibits terminal differentiation by a pathway that includes activation of JNK and induction of Blimp-1. These results are strengthened now by our observation that many genes downstream of JNK are modulated in our B cell microarrays ($LPS^{+/-}$ CD40 ligation; 24 h, 48 hr. and 72 h comparisons) as early as 24 h These include Rbp4, JunB, ATF2, Elk-1, NFATC2, Foxo3a, STAT3, Pax2, Bcl-2, RxRalpha, Mcl-1 (and a number of related genes). The microarray data have been validated by real-time RT-PCR and the rapid kinetics suggest that cell fate decisions can be made even before cell division has been initiated. We also found that CD40 ligation leads to a substantial reduction in Blimp-1 transcript levels as early as 12 h in LPS-stimulated B cells, suggesting that CD40 signaling may have a dominant role in suppressing plasma cell generation.

In our attempts to induce mucosal priming following systemic immunization, we report that treatment of mice with vitamin D prior to or at the time of immunization leads to slightly higher serum antibodies, but to significantly higher levels of IgA in serum, saliva and fecal pellets. It has been reported that one effect of vitamin D is to allow dendritic cells to leave the draining lymph node and traffic to distal sites. We tested this and report that in addition to draining lymph nodes, the spleen and MLN of mice immunized subcutaneously with ovalbumin under cover of vitamin D can serve as APCs to stimulate ovalbumin-specific transgenic T cells. Further, when spleens from bone marrow chimeras (VDR knockout and CD45 congenic wild-type mice), are sorted for the two cell types in similar experiments, only wild-type cells serve as APCs.

Publications

Original peer-reviewed articles

1. Panda SK, Kumar S, Tupperwar NC, Vaidya T, George A, Rath S, Bal V, Ravindran B* (2012) Chitohexaose actiates macrophages by alternate pathway through TLR4 and blocks endotoxemia. *PloS Pathogens* **8**: e1002717.
2. Shenoy GN, Chatterjee P, Kaw S, Mukherjee S, Rathore DK, Bal V*, Rath S*, George A* (2012). Recruitment of memory B cells to lymph nodes remote from the site of immunization requires an inflammatory stimulus. *J Immunol* **189**: 521-528.
3. Banerjee H, Das A, Srivastava S, Mattoo HR, Thyagarajan K, Khalsa JK, Tanwar S, Das DS, Majumdar SS, George A*, Bal V*, Durdik JM*, Rath S* (2012) A role for apoptosis-inducing factor in T cell development. *J Exp Med* **209**: 1641-1653.

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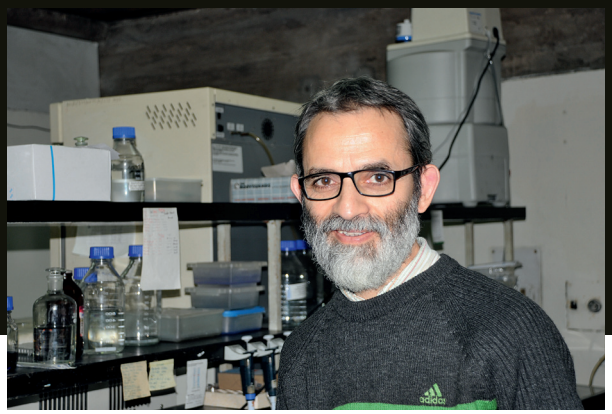

Ayub Qadri

Analysis of *Salmonella Typhi*-host cell interaction

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Pathogenic *Salmonella* species produce different clinical manifestations depending upon *Salmonella* serovar and the type of host. *Salmonella Typhi* infection in humans causes systemic disease, typhoid, during which bacteria disseminate into different organs including spleen, liver, bone marrow and gall bladder. On the other hand, infection with non-typhoidal *Salmonella* species such as *Salmonella Typhimurium* produces self-limiting localized gastroenteritis in which infection is restricted to the gut. In mice, however, *S. Typhimurium* infection leads to a systemic disease that is analogous to human typhoid, therefore it has been used as a model to understand *S. Typhi* pathogenesis; *S. Typhi* does not establish successful infection in wild type mice. The reasons for different clinical outcomes produced by these two closely related *Salmonella* serovars are not understood. Further, the mechanisms by which pathogenic *Salmonella* modulates host immune defenses in order to establish systemic infection are also not well worked out. Work in our laboratory addresses these two aspects of *Salmonella*-host cell interaction.

Broadly, studies in our laboratory are aimed at i) identifying differences in host-pathogen interactions which ensue during infection with *S. typhi* versus *S. Typhimurium* and ii) understanding modulation of immune responses during infection with pathogenic *Salmonella*.

TLR agonistic activity in Vi facilitates antibody switching to IgG

Vi capsular polysaccharide is currently in use in humans as a vaccine against typhoid caused by *Salmonella Typhi*. The efficacy of this vaccine has been shown to correlate with IgG anti-Vi antibodies. Vi is a T-independent antigen and the mechanism by which antibody switching to IgG occurs during immunization with this vaccine is not understood. To investigate this mechanism, we analyzed possible role of T cells and of TLR agonistic proinflammatory activity of Vi, which we reported recently, in isotype switching of anti-Vi antibodies. The role of T cells was examined by studying anti-Vi antibody responses in wild type (WT), TCR $\beta^{-/-}$ and TCR $\delta^{-/-}$ mice. WT and TCR $\beta^{-/-}$ mice elicited comparable levels of IgM anti-Vi antibody responses, whereas TCR $\delta^{-/-}$ mice showed slightly reduced levels. Interestingly, IgG anti-Vi antibodies were considerably higher in TCR $\beta^{-/-}$ as compared to wild-type and TCR $\delta^{-/-}$ mice. We believe that these heightened IgG responses might be due to the presence

of increased number of $CD11b^+$ inflammatory cells which are known to accumulate in these mice as they get older. The IgG antibodies in our analysis were mainly of IgG3 type. These results suggested that T cells may not be directly involved in bringing about switching of anti-Vi antibodies to IgG.

To study possible role of proinflammatory capability of Vi in the induction of IgG antibodies, we analyzed anti-Vi antibody responses in mice lacking the TLR adaptor MyD88. Consistent with our previous findings, Vi triggered secretion of KC and IL-6 from peritoneal adherent cells derived from WT mice but not from MyD88 knock out mice. Immunization of MyD88 deficient mice with the Vi vaccine produced IgM anti-Vi antibody levels which were comparable with those seen in WT mice but unlike WT mice, MyD88 deficient mice showed very poor IgG anti-Vi antibody responses. Our findings suggest that TLR agonistic MyD88 dependent proinflammatory activity in Vi might be crucial for generation of IgG anti-Vi antibodies.

Prohibitin regulates inflammatory responses from epithelial cells

Our previous work based on engagement of membrane prohibitin complex with Vi suggested that this protein complex might play an important role in regulating cellular responses following activation of cells with proinflammatory stimuli such as microbial TLR ligands. Our findings also indicated that prohibitin might communicate with src-kinases during signaling through immune receptors. To get a better understanding of the role of prohibitin in inflammatory responses, we over-expressed this molecule in model epithelial cell line HeLa. The expression of prohibitin in stable HeLa line was established by Western blotting and flow cytometry with prohibitin – specific antibodies. To assess the role of prohibitin in the regulation of inflammatory responses, cells were infected with pathogenic *S.Typhimurium* and secretion of neutrophil chemoattractant CXCL8 was analyzed. Interestingly, infection with this pathogen resulted in much reduced CXCL8 secretion from prohibitin over-expressing HeLa (HeLa-Phb) as compared to HeLa cells tranfected with only the vector (HeLa-pcDNA). This difference was not due to reduced bacterial invasion as the number of intracellular bacteria was higher in HeLa-Phb as compared to HeLa-pcDNA. Moreover, even with comparable number of intracellular *Salmonella*, secretion of CXCL8 was significantly compromised in prohibitin over-expressing cells. This difference was also not due to cell death in HeLa-Phb. Analysis of intracellular signaling following infection with *S. Typhimurium* showed that activation of NF-κB pathway and JNK were if at all better in cells expressing higher levels of prohibitin. Prohibitin overexpresion also did not dramatically change activation of p38 MAPK and ERK. Therefore, the differences in CXCL8 secretion between HeLa-Phb and HeLa-pcDNA following infection with *S.Typhimurium* do not seem to result from differential activation of NF-κB and MAK-kinase pathways of intracellular signaling.

Prohibitin is enriched in lipid rafts in the membrane and these membrane microdomains have been previously shown to regulate invasion and induction of inflammatory responses with *Salmonella*. Moreover, prohibitin is also known to regulate lipid biogenesis in mitochondria. Therefore we analyzed expression of GM1 on HeLa cells by flow cytometry. This analysis showed increased binding of CTB, which specifically interacts with GM1 enriched in the raft, to HeLa-Phb. Treatment of cells with raft-disruting agent methyl-β-cycodextrin did not however change the inability of HeLa-Phb to produce CXCL8 in response to infection with *S. Typhimurium*. Preliminary data suggests that

cells over-expressing prohibitin might also be inefficient at generating CXCL8 in response to stimulation with TNF-α. We are currently exploring the mechanism of this unresponsiveness and also investigating the possibility that TNF-α might constitute a feed forward stimulus in generating CXCL8 secretion during infection of HeLa with *Salmonella*.

T cells secrete IFN-γ and IL-17 during Salmonella infection in antigen non-specific fashion

T cells play a vital role in long term immunity against infection with pathogenic *Salmonella*. Both CD4 and CD8 T cell responses have been shown to participate in clearance of this pathogen from infected animals. IFN-γ is believed to play a major role in CD4 T cell mediated immunity against *Salmonella*. We show here that during infection of mice with *S. Typhimurium*, the ability of T cells to secrete IL-2 in response to stimulation with anti-CD3 antibody is significantly reduced with the progression of infection. There is also some reduction in the number of CD4 T cells at later stages of infection. However, in spite of this reduction, splenocytes from *S.Typhimurium* infected mice secrete considerably higher amounts of IFN-γ and IL-17 upon stimulation with anti-CD3 antibody *ex vivo*. This phenomenon is also observed when splenocytes are stimulated with antigenic extract prepared from *S. Typhimurium*. *Salmonella* antigens do not activate secretion of IL-2 from splenocytes isolated from infected mice but readily trigger secretion of IFN-γ from these cells. This response does not require presentation of *Salmonella* antigens on MHC class II as secretion of IFN-γ is also observed when T cells from infected mice are incubated with *Salmonella* antigens in presence of accessory cells from MHC class II deficient mice. The defect in IL-2 secretion is also seen in IL-10 knock out mice infected with *S.Typhimurium* suggesting that increased IL-10 secretion during infection with *Salmonella* may not contribute to IL-2^{lo} IFN-γ^{hi} phenotype. These findings have implications for inflammation and immunity during infection with pathogenic *Salmonella*. The mechanism responsible for giving rise to the IL-2^{lo} IFN-γ^{hi} phenotype is currently under investigation.

Publication

Original peer-reviewed article

1. Sharma N, Akhade AS, Qadri A* (2013) Sphingosine-1-phosphate suppresses TLR-induced CXCL8 secretion from human T cells. *J Leukoc Biol* **93**:521-528.

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Devinder Sehgal

Molecular basis of B cell responses

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

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

Functional characterization of monoclonal antibodies generated against Pneumococcal surface protein A

Pneumococcal surface protein A (PspA) is a polymorphic, cell surface, choline-binding protein. PspA interferes with fixation of C3 on the pneumococcal surface, and its lactoferrin-binding activity is believed to protect pneumococci from bactericidal activity of apolactoferrin. PspA, a virulence factor, is present in all strains of pneumococci and is serologically variable, cross-reactive, and cross protective. PspA proteins from different strains have a similar basic molecular structure. PspA consists of four distinct structural domains: an N-terminal α-helical domain that is surface exposed, a proline-rich region, a stretch of highly conserved 20-amino acid repeats (choline-binding domain), and a slightly hydrophobic sequence at the C terminus. Based on the amino acid sequence of C-terminal 100 amino acid of the surface exposed domain, PspAs have been classified into six

clades and three cross-reacting families (>55% identity). Family 1 is comprised of clade 1 and 2; family 2 covers clade 3, 4 and 5; and family 3 has only clade 6. Families 1 and 2 account for the majority (94-99%) of the pneumococcal isolates. Anti-PspA monoclonal antibodies (mAbs) have been shown to be protective. Immunization of human volunteers with a single dose of PspA stimulates broad cross-reactive antibodies to heterologous PspA molecules which when transferred protects mice from fatal infection with *S. pneumoniae.* The protective epitopes have been mapped to the α-helical domain of PspA. Previously, we reported the molecular characterization of 36 mouse monoclonal antibodies (mAbs) generated against the extracellular domain of PspA ($PspA^{3-286}$) from strain R36A (PspA family 1 / clade 2). Antibodies to $PspA^{3-286}$ were encoded by diverse V_H and V_κ families/genes. Unexpectedly, 7 hybridomas expressed heavy chains that lack DH gene-derived amino acids. 'N' addition(s) were observed in the heavy chain expressed in 6 of these 7 hybridomas; 'P' addition(s) were absent. Absence of D_H gene-derived amino acids did not prevent anti-$PspA^{3-286}$ mAbs from attaining average relative avidity. Avidity maturation occurred during primary IgG anti-$PspA^{3-286}$ polyclonal antibody response in $PspA^{3-286}$ and R36A immunized mice. Compared to $PspA^{3-286}$ immunized mice, the relative avidity of the primary polyclonal IgG antibodies was higher in R36A immunized mice at day 72, 86 and 100. Analysis of replacement/ silent mutation ratio in the CDR and FR regions provided evidence for antigen-driven selection in 11 mAbs. Based on epitope localization and competition experiments, the mAbs were classified into 12 independent groups. ELISA additivity assay indicated that members within a group recognized topographically related epitopes.

As a next step we wanted to test whether the mAbs raised against PspA from R36A cross-react with PspA belonging to other 5 clades. Towards this end, the subfragment of PspA open reading frame encoding the N-terminal half was amplified by PCR using genomic DNA from strain TIGR4 (family 2 / clade 3; serotype 4), and ATCC 6303 (family 2 / clade 5) as template. The PCR products were cloned in the expression vector pQE-30Xa with histidine affinity tag at the N-terminus. The constructs were confirmed by restriction digestion and nucleotide sequencing. The corresponding PspA constructs for family 1 / clade 1 (strain L82016; serotype 6B), family 2 / clade 4 (strain JCP#56; serotype 8) and family 3 / clade 6 (strain BG9300; serotype 37) were obtained from Prof. Susan Hollingshead, USA. All the PspA constructs were expressed in *E. coli*. Recombinant PspAs were purified to homogeneity using Ni-NTA affinity chromatography and were confirmed by western blotting by using antibodies that recognize the histidine affinity tag. The reactivity patterns of the culture supernatant from the 36 hybridomas with the 6 clades of PspA were analyzed by ELISA. Out of the 36 hybridomas, only three mAbs (all 3 are IgM) exhibited some degree of cross-reactivity with all 6 clades of PspA. Thirty anti-PspA mAbs cross-reacted with clade 1 and 2 PspA but not with the other clades. The results indicated that majority of the mAbs are family 1 specific. For subsequent analysis we restricted ourselves to the mAbs that were raised against PspA using heat-killed R36A as an immunogen and were of the IgG isotype. The cross-reactivity of the culture supernatants from these 21 IgG secreting hybridomas were tested with 3 strains that express PspA of clade 1 (BG8838, AC-094 and BG6692) and 4 strains that express PspA of clade 2 (WU2, D39, R36A and A66.1) using flow cytometry. The analysis indicated that only 8 of the 21 mAbs showed surface binding with the 7 strains that express PspA of family 1. These mAbs are B3D12, B3H8, L5C8, L5F10, M4F4, P2A4,

P2B5 and P1E11. These mAbs were analyzed for bactericidal activity and their ability to confer protection *in vivo*. Pneumococcal strain BG8838 (family 1 / clade 1) or D39 (family 1 / clade 2) (~500 cfu) were incubated in freshly collected blood (containing anticoagulant) and 5 μg of purified anti-PspA mAb or the corresponding isotype control. The contents were incubated at 37°C for 3 hr. with rotation. The surviving bacteria were enumerated by plating serial dilutions. All 8 mAbs exhibited bactericidal activity against BG8838 while B3H8, P1E11 and P2A4 showed bactericidal activity against strain D39. Passive mouse protection experiments were performed with 8 short-listed mAbs. CBA/N mice were administered purified mAbs (5 mg per kg body weight) intraperitoneally. One hour later mice were challenged with ~10^3 cfu of pneumococcal strain WU2. Survival of mice was monitored for 21 days. Mouse survival data suggested that M4F4, P1E11 and P2A4 provided 100% protection while P2B5 provided 87% protection. B3H8 and L5F10 conferred 50% or less protection. B3D12 and L5C8 failed to protect. Neither isotype controls conferred any protection.

Publication

Original peer-reviewed article

1. Gupta K, Singh S, Gupta K, Khan N, Sehgal D, Haridas V, Roy RP* (2012) A bioorthogonal chemoenzymatic strategy for defined protein dendrimer assembly. *Chembiochem* **13**: 2489-2494.

Patents

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Amulya K. Panda

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

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

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

1. Analysis of immune response from antigen loaded polymer particles and evaluation of adjuvant properties associated with polymeric particle formulation. Evaluation of memory antibody response using polymer particle based immunization.
2. Development of polymeric membrane as a scaffold for three dimensional growths of animal cells and its application as an artificial skin equivalent.
3. Solubilization and refolding of inclusion body

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

A. Immune response from polymeric particle formulations entrapping antigens

Improved immunogenicity of antigens using PLA particles was studied by entrapping Pneumococcal polysaccharide in polymer particles in combination with protein antigens. Preparation of polymer particle entrapping protein antigen was also standardized using spray drying method. Cellular interaction studies of particles formulations with macrophages and DCs were carried out to understand the presentation and processing of particulate antigens. The details work carried out in these areas are as follows:

Memory antibody response from pneumococcal surface polysaccharide by entrapping them in polymer particles

The objective was to elicit memory antibody response from single point immunization and to understand how size of polymer particles influences immune response. Pneumococcal capsular polysaccharide was entrapped in different sized PLA particle and immunogenicity was evaluated in mice. Polylactide nanoparticles entrapping pneumococcal capsular polysaccharide from serotype-1 (PCP-1) elicited significantly higher anti-PCP-1 IgG responses as compared to that observed with soluble PCP-1 and PCP-1 entrapped in microparticles. Immunization with particles co-entrapping both polysaccharide and protein antigen of *S. pneumoniae* origin did not improve the antibody titer in comparison to that achieved with particles entrapping carbohydrate alone. Immunization with polymer particle formulation entrapping PCP-1 also promoted antibody isotype switching and elicited protective memory antibody responses from a single intramuscular injection. Antibody responses generated using particle based immunizations were functionally competent in opsonizing and promoting the phagocytosis of pneumococci by macrophages. Mice immunized with polylactide particles entrapping PCP-1 were protected when challenged intraperitoneally with lethal dose of virulent pneumococci. The observed protection was both antigen and serotype dependent, suggesting the specificity of antibody responses elicited by PCP-1 entrapped nanoparticles. Other than geometric size, antigen surface density and antigen loading were the critical parameters in determining the antibody responses. Antibody responses correlated with the enhanced uptake of nanoparticles by macrophages in comparison to that observed with microparticles. This approach provides a unique mode of improving the immunogenicity of T cell independent polysaccharide antigen using biodegradable polymeric particles.

Formulation and evaluation of polymer particles prepared using spray drying

To facilitate the production of polymer particle using GMP guidelines, spray drying method was used. The objective was to produce free flowing powder without using lyophilization. Spray drying process was optimized for the formulation of polymer particle entrapping recombinant Pneumococcal surface protein (PspA) as a candidate antigen. Spray drying method produced microparticles entrapping PspA with uniform size distribution and good re-dispersibility. The spray dried particles showed excellent flow properties with reproducible entrapment efficiency and good antigen load. The feed rate, percentage of dissolved solids,

viscosity of feed and type of emulsion employed played a key role in determining the particle size distribution. The antigen entrapped in particles was observed to be stable and on immunization produced comparable immune responses. Both spray dried particles as well as particles prepared using double emulsion solvent evaporation method elicited comparable antibody responses. When compared to standard batch processes which yielded particles in mg scale, spray drying process resulted in particles in gram scale. These findings would help in designing and developing optimized processes for manufacturing of polymeric particles entrapping antigen. The process engineering parameters analyzed during particle formulation will be useful for the entrapment of water soluble drugs in polymer particles.

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

Our study aims at developing a novel method of scaffold fabrication using polymeric particles fusing into different type of structures. This method of scaffold fabrication takes place at room temperature. Drugs, growth factors, antibiotics can be incorporated into scaffold to suit for a particular biomedical application. Evaluation of this polymeric membrane towards transplantation and wound healing is also being studied. The efficacy of this scaffold towards development of *in vitro* model for tumor microenvironment is being evaluated currently. A well-characterized 3-D model can particularly be useful for rapid screening of a large number of therapeutics for their efficacy during the drug discovery phase. Cancer cell lines were grown on these polymer scaffolds and evaluated for drug sensitivity. It was observed that these *in vitro* three D cell culture models have different drug sensitivity behavior. This was supported by identification of markers of tissue type structure such as collagen, secretion of metal proteases from the cells grown on polymer scaffold. Attempts are being made to grow cells on scaffold in spinner/bioreactor culture. Growth in controlled condition will provide details of metabolic activities of cells when grown in 3-D configuration on polymer scaffold.

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. Previously we have reported that different sized inclusion body aggregates are formed during expression of recombinant protein in *E. coli*. We also reported that many more recombinants proteins could be refolded from inclusion bodies after solubilizing them in propanol containing buffer. Currently we are applying this solubilization process for refolding of multimeric and membrane proteins from inclusion bodies. The detail mechanism of inclusion body solubilization using propanol is also being studied using biophysical techniques. We are also studding how different growth conditions during fermentation can give rise to different types of aggregates.

Publications

Original peer-reviewed articles

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Patents

1. Anish CK, Khan N, Sehgal D, Panda AK (2012) Design and developmemsnt of nanoparticles based carbohydrate vaccine against *Streptococcus pneumonia* (2793/DEL/2012, Indian patent filed on 07/09/2012).
2. Gupta SK, Gupta N, Shreshtha A, Panda AK (2012) A process for production of tag-free recombinant fusion protein encompassing promiscuous T cell epitope of tetanus toxoid and dog zona pellucida glycoprotein-3 and its use as contraceptive vaccine (1269/DEL/2012, Indian patent filed on 24/04/2012).

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Disorders of proliferation: Analysis of novel pathways and targets

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The lab focuses on two disorders characterized by proliferative aberrance, systemic autoimmune disease and tumorigenesis. In particular, the work seeks to investigate the consequences of aberrant cell death in systemic autoimmune disease as well as to delineate mechanisms and pathways by which human chorionic gonadotropin (hCG) can impact upon systemic autoimmunity and cancer.

The pathological consequence of autoreactive immune responses are the subject of intense investigation. Systemic Lupus Erythematosus (SLE) is a prototypical non-organ specific autoimmune disease. Animals genetically modified to impair the uptake of apoptotic cells exhibit lupus-like pathology. Autoimmune cascades initiated by autoantibody responses specifically directed towards apoptotic cells are being investigated, since apoptotic debris appears to constitute the original antigenic insult. The immunological and physiological sequelae arising as a result of the release of sequestered hemoglobin in animals prone to autoimmunity also form a focus of current investigations.

Human chorionic gonadotropin (hCG) is critical for the sustenance of pregnancy, but has also been shown to be secreted by a variety of cancers; its presence has been associated with poor patient prognosis. Understanding the molecular pathways by which hCG impacts on tumor progression as well as the development of novel immunotherapeutic anti-hCG vaccination strategies form a focus of the laboratory. Given reports of pregnancy-associated lupus flares in humans and the ameliorating influence of hCG in murine models of organ-specific (Th1-mediated) autoimmune disease, the effects of the hormone in animals genetically prone to systemic autoimmunity (a state which can notionally be considered to constitute a Th2 skew) are also being investigated.

A. hCG in tumorigenesis

Studies in βhCG transgenic (TG) mice

TG mice expressing βhCG are being employed to study the influence of hCG on tumorigenesis and infertility. These mice exhibit accelerated

weight gain, as well as ovarian, pituitary and mammary gland tumors. Previous work indicated that active anti-hCG immunization of transgenic mice prevented increases in body-weight; serum prolactin levels and gross ovarian and pituitary morphology remained normal. Immunization also led to the resumption of estrous cyclicity and the restoration of fertility. Significantly, pituitary transcript levels of the hormone-responsive and tumor-associated genes *ccnd1, hmga2, e2f1, galanin and prolactin*, which are greatly enhanced in transgenic mice, were comparable to levels in non-transgenic litter-mates. The study was then extended to additional genes implicated in human and murine pituitary tumorigenesis; anti-hCG immunization also prevented increase in transcript levels of *growth hormone, pttg1, fgf2, bmp4* and induced up-modulation (or prevented down-modulation) of inhibitors of cyclin-dependent kinases such as CDK1b (p27), CDK 2a (p16) and CDK 2c (p18), to achieve levels found in non-transgenic mice. Serum derived from βhCG transgenic mice enhanced the viability of tumor cells and also led to heightened transcription and expression of VEGF, IL-8 and MMP-9, effects not seen when sera derived from hCG-immunized transgenic mice and transgenic sera incubated with anti-hCG serum were employed. Using a stringent *in vivo* system in which βhCG acts both a ''self'' antigen and a tumor-promoting moiety, the data builds a case for anti-gonadotropin vaccination strategies in the treatment of gonadotropin-dependent or secreting malignancies that frequently acquire resistance to conventional therapy.

The effect of the transgenic expression of βhCG on the growth of implanted tumors was also studied. Heterozygous male FVB βhCG transgenic mice were first mated with wild-type female C57BL/6 mice. Male and female F1 transgenic mice (as well as non-transgenic littermates) were subcutaneously implanted with Lewis lung cancer (LLC) cells; higher tumor incidences as well as larger tumor volumes were recorded in transgenic mice of both sexes. Previous work using *in vitro* culture systems had established the ability of hCG to induce the transcription and expression of genes associated with tumorigenesis. Whether the findings held true for tumors developing in an βhCG/CG-containing environment *in vivo* was assessed. Markedly higher transcript levels of genes associated with inflammation (IL-6, IL-8 and TNFα) were observed in LLC tumors isolated from βhCG transgenic mice. Similar increases were also observed in transcript levels of the vascular endothelial growth factor and matrix metalloproteases MMP-2 and MMP-9. Transcript levels of Bcl-2 and Bcl-xl as well as of survivin were similarly enhanced. These results demonstrate that endogenous βhCG/CG can have pro-tumorigenic effects on implanted tumor cells as well.

hCG and chemo-resistance

An increasing number of cancers have been found to express hCG or its subunits, and its presence has been linked with poor patient prognosis in several instances. In ongoing work, hCG was found to increase cellular viability as well as proliferation of tumor cells. Additionally, hCG provided protection against nutrient deprivation-induced cell death. The growth-promoting effects of hCG could be reversed by anti-hCG antibodies.

Published literature has drawn associations between the presence of hCG and chemo-resistance. Last year, working with both human and murine tumor cells, it was determined that pre-incubation with hCG reduced the loss of cellular viability induced by 5-flourouracil, curcumin, cisplatin, tamoxifen and etoposide; enhanced viability in the presence of hCG

was attributable to a significant reduction in drug-induced apoptosis. In an extension of this work, hCG was shown to reduce the extent of proliferative inhibition induced by these anti-cancer agents. Quantification of cytokines provided an independent indicator of hCG-mediated chemo-resistance; when independently employed, while hCG induced increases in the tumor-associated factors IL-6, IL-8 and EGF and the chemotherapeutic drugs reduced basal concentrations, co-incubation with hCG along with the drugs restored (and sometime exceeded) levels observed with hCG alone.

Studies assessing the influence of hCG on the mRNA levels of molecules known to be associated with chemo-resistance (HIF-1α, survivin, livin, BCL-2, PARP, c-FLIP, KLK10, KLK11, TLR1, TLR3, TLR6, TLR8, TLR9, TLR10), initiated last year, were extended. Western blots and flow cytometric analysis confirmed effects on protein levels. The data prompted studies into the combined effects of hCG and various TLR ligands on tumor cells; hCG and some TLR ligands (such as for TLR1, TLR2, TLR3, TLR5, TLR6, TLR8 and TLR9) acted in synergy to provide enhanced chemo-resistance (in terms of both viability and proliferation) against curcumin and tamoxifen. Levels of released IL-6, IL-10, TGF-β, IFN-γ and TNF-α were in consonance with these observations.

While studying tumor chemo-resistance, it becomes important to study the contributions of non-transformed cells in the tumor micro-environment, such as macrophages and fibroblasts. IL-6 has been traditionally associated with chemo-resistance, a finding that was verified and extended by our studies in data reported last year. Macrophages incubated with supernatants of tumor cells previously incubated with hCG were found to produce significant amounts of IL6, which in turn mediated resistance of tumor cells to curcumin. These results indicate that macrophage-derived IL-6 may be a significant contributor to hCG-induced chemo-resistance.

B. Systemic autoimmunity

Previous work had established that immunization with apoptotic cell-specific antibodies in syngeneic autoimmune-prone animals (NZB/W F1) leads to hyper-gammaglobulinemia and expansion of the autoantibody repertoire. Antibodies non-reactive to double-stranded DNA (dsDNA) could elicit antibodies that bind dsDNA upon immunization. This is a significant observation vis-à-vis the etiology of immune pathology, since anti-dsDNA antibodies are associated with nephritis. Whether apoptotic blebs differentially affect innate immune components of autoimmune-prone and non-autoimmune prone animals was investigated. Purified blebs potently inhibited the generation of bone marrow-derived dendritic cells (BMDCs), an effect more severe in autoimmune-prone animals as opposed to non-autoimmune prone animals. Blebs were also more effective than healthy cell lysate in the induction of BMDC maturation, with effects once again being more pronounced in autoimmune-prone animals.

Our lab has been investigating autoreactivity towards hemoglobin (Hb) in auto-immune prone mice and in humans. In addition to documenting endogenous serum and organ-associated anti-Hb autoantibodies in autoimmune-prone mice, previous work had also described the effects of immunization of NZM 2410 mice with hemoglobin (Hb). Serum reactivity towards Hb and anti-double stranded (ds) DNA was heightened and antibodies to Hb, dsDNA, Ro52, Ro60, RNP A, La and Sm sequestered in the kidneys. Hb-immunized NZM 2410 mice demonstrated moderate thickening of basement membrane,

along with severe increase in mesangial matrix, associated with pronounced glomerulosclerosis. Additionally, the tubular epithelium showed degenerative changes and evidence of hyaline casts in the lumen. Glomeruli demonstrating normal morphology and architecture were significantly decreased, with about half showing severe sclerosis. Whether immunization of non-autoimmune prone animals (FVB) with Hb or its dominant epitope Hbβ (110-119) led to similar down-stream effects was then investigated; though some increases in serum antibody reactivity to Hb and Sm were observed, reactivity of antibodies in serum and organ eluates to moieties in murine cellular lysate (as determined by Western blot) and towards the autoantigens La, RNP A, RNP68k, Ro52, Ro60 was minimal. Glomerular size and basement membrane thickness in immunized FVB mice were within normal limits. Tubules were normal and no interstitial inflammation was observed. A great majority of the glomeruli appeared normal, with minimal evidence of sclerosis. In conjunction with previous data indicating higher anti-Hb B cell precursor frequencies in autoimmune prone mice, the findings indicate that anti-Hb humoral responses exist at higher titre, can be further enhanced upon immunization, and are specifically associated with end-organ damage in these animals.

Publications

Original peer-reviewed articles

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Rajni Rani

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

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The project aims to decipher the Immunogenetic and autoimmune factors involved in the destruction of pancreatic beta cells and melanocytes in Type 1 diabetes (T1D) and vitiligo respectively. We aim to device ways to inhibit autoimmune responses in T1D. Vitiligo, is a multifactorial disease etiology of which is not precisely understood. While several hypotheses have been proposed including autoimmunity, it is not clear how the pigment producing melanocytes are destroyed by the autoimmune responses. So, the theme of this project is to understand the aetiopathogenesis of vitiligo with an aim to develop therapeutic approaches for the disease.

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 which 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.

A. Type 1 diabetes

Genetic basis of Type 1 diabetes

After studying the HLA, cytokine genes and Vitamin D receptor genes, we ventured to study the role of antigen processing genes in T1D. For this purpose about 3 SNPs in LMP2 and LPM7 genes were studied in 226 T1D patients and about 700 healthy controls. LMP-2 and LMP-7 molecules are the subunits of large cytosolic proteasomes complex which are believed to be involved in generation of endogenous immunogeneic peptides by proteolysis for presentation on MHC class-I molecules. The gens for these are localized in the MHC class-II region. Three SNPs, one in LMP2 exon 3, one in Intron 6 of LMP7 and one in LMP7 exon 2 were studied. For LMP2, G/A substitution that leads to a nonsynonymous amino-acid change in exon 3 was studied using PCR-RFLP using restriction endonucleases *Hha I*, which cleaves the G allele, but not the A allele. For LMP7-intron 6 also *Hha I* was used for genotyping which cleaves the G allele but not the T allele. For LMP7 exon 2 A/C substitution was studied using restriction enzyme *Pst-I* which cleaves the C allele whereas the A allele remains uncut. The data is being analysed.

Differentiation of mesenchymal stem cells into insulin producing cells

To understand the factors involved in differentiation of MSCs into insulin producing cells, we tried to correlate the stemness markers with the ability of the MSCs to differentiate into insulin producing cells at different passages starting from passage 4 till 12. Stemness markers were assessed using real time PCR at the beginning of induction and after induction of differentiation into insulin producing cells. However, the number of samples studied are not enough to draw any conclusions at this time. We need to add more samples before any meaningful conclusions can be drawn.

Immunosuppressive properties of Mesenchymal stem cells

Since we wanted to use MSCs to inhibit autoimmune responses, we treated MSCs with different cytokines as their immunosuppressive properties have been reported to be enhanced by these treatments. A dose response was done to check for the expression of different immunosuppressive markers like Foxp3, indoleamine 2,3 dioxygenase (IDO) and inducible nitric oxide synthase (iNOS). We observed that TNF-α and IFN-γ could induce the expression of IDO and iNOS. However, Foxp3 was not inducible by individual treatments of different cytokines. Combination treatments of cytokines could induce Foxp3 in some cases, the experiments are being standardized to get reproducible Foxp3 expression on MSCs

B. Vitiligo

Effect of cytokines increased in vitiligo on epidermal cells

To evaluate the role of cytokines that were increased in the lesional skin compared to non-lesional skin, on epidermal cells, individual and a combination of cytokines were used and alteration in transcripts of the target genes were studied. The RNAs were isolated by trizol method, real-time assays were performed for known regulated target mRNA, to assess the efficacy of the cytokine treatments and this was followed by microarray analysis. The dose responses for each cytokine have been determined by studying the expressions of known response markers/ genes regulated by these cytokines.

Effect of cytokines on Normal Human Keratinocytes

For IFN-γ we used ICAM-1 as the indicator gene. It was found that 200 U/ml IFN-γ was ideal for keratinocyte treatments (Figure 1a). For IL-17A we used CCL20 as the indicator gene. 25 ng/ml of IL-17A was considered to be ideal concentration (Figure1b). For TGF-β1 we used 3 genes as the response of keratinocytes to TGF-β1 had not been robustly determined before, namely, Intergrin α V (ITGαV), Integrin β 1 (ITGβ1) and Urokinase-type plasminogen activator (PLAU). From the data it appears that 20 ng/ml is the ideal concentration for further treatments (Figure 1c). For IL-1β we used IL-6, IL-8, and ITGβ1 as the response markers to determine that 4ng/ml was the ideal treatment concentration (Figure1d).

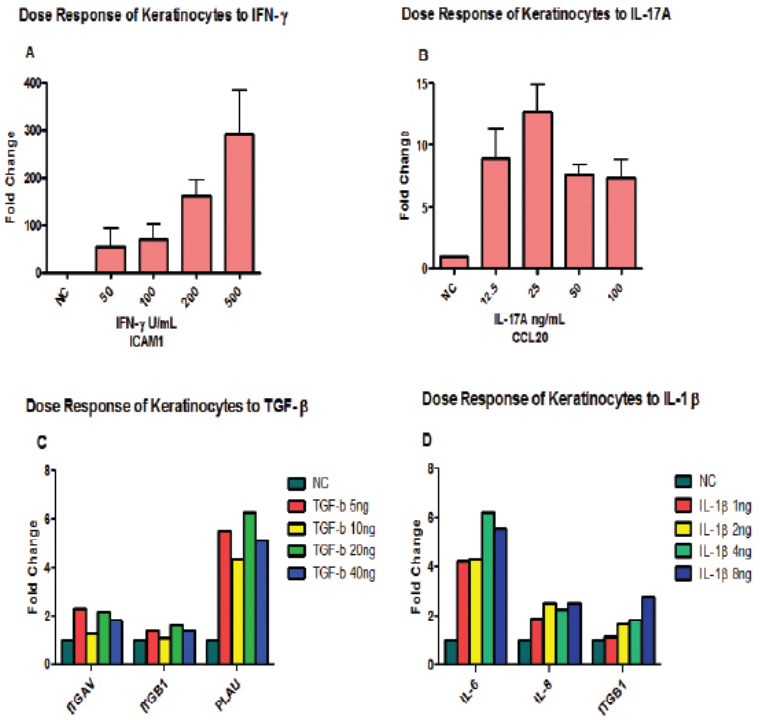

Figure 1: a. ICAM-1 expression levels in Keratinocytes in response to different IFN-γ doses. b. CCL20 expression levels in Keratinocytes in response to IL-17A doses. c. Dose response of Keratinocytes to *TGF*-β1. Intergrin-αV (*ITGAV*), Integrin-β1 (*ITGB1*) and Urokinase-type plasminogen activator (PLAU) are shown to be regulated by *TGF*-β1. d. IL-6, IL-8 and *ITG*β1 expression in keratinocytes in response to different doses of IL-1β.

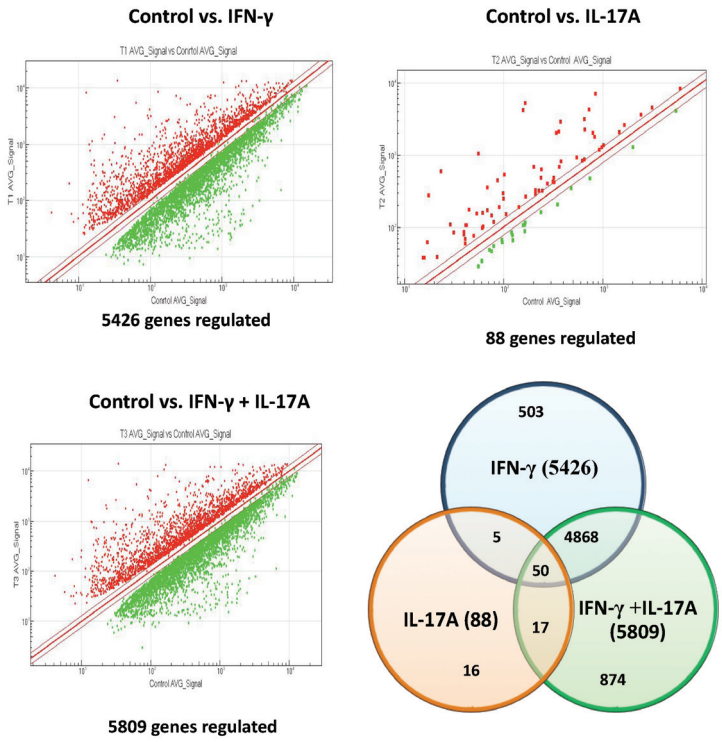

Figure 2: a. Scatter plot for genes significantly regulated in NHKs by IFN-γ treatment, red dots show the up-regulated genes and the green dots show the down-regulated gene b. Scatter plot for genes significantly regulated in NHKs by IL-17A treatment. c. Scatter plot for genes significantly regulated in NHKs by combined treatment with IFN-γ and IL-17A. d. Venn diagram showing the intersection between the genes regulated by IFN-γ, IL-17A, and the combination of the 2 cytokines. 874 genes are regulated only by the combination and not by either cytokine independently.

After finalizing the doses, primary cultures of Normal Human Epidermal Keratinocytes (NHEK) were treated with various cytokines and their combinations for 48 hours in keratinocyte serum-free medium. Total RNA was isolated from these cells and microarray analysis was performed on Illumina HT-12 gene expression chips. The resulting fluorescence intensity data was analysed on Beadstudio software with average normalization and Illumina custom model to determine the differentially expressed genes. 5426 genes were regulated by IFN-γ alone (Figure 2a,d), 88 by IL-17A alone (Figure 2b, d), and 5809 genes were regulated by a combination of both (Figure 2c, d). Similarly 2759 genes were regulated by TGF-β1 and 38 genes were regulated by IL-1β and 5314 genes were regulated by a combination of IL-1β and IFN- γ. The differentially expressed gene lists were then uploaded and analyzed on DAVID. It was observed that pathways such as cell proliferation, nucleotide synthesis etc. were downregulated in IFN-γ treated keratinocytes. However, with a combination treatment of IL-17A and IFN-γ, epidermal differentiation genes were also strongly affected. Analysis of the other data sets is currently under progress, as is the validation of the enriched pathways by real time PCR and western blots.

Effect of cytokines on Normal human Melanocytes

Human Normal Primary melanocytes were cultured and treated with different doses of cytokines (IL-6, IL-1β, TGF-β and IL-8) for 48 hrs in T25 flasks and RNA was isolated and the expressions of different genes known to be

regulated by these cytokines were checked. Finally the minimum concentrations giving good response were selected for the final treatment.

After finalization of the doses, five cultures of melanocytes were treated with the final doses of cytokines, expression of informative genes was carried out and three of the five cultures giving reproducible results were sent for micro-array analysis for global gene expression profiling. The data is being analyzed at present.

Publications

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Sangeeta Bhaskar

Study of immunotherapeutic potential of *Mycobacterium indicus pranii* (MIP) and the underlying mechanisms in animal models of tuberculosis and tumor model

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

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

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

Another objective is to evaluate immunoprophylactic and immunotherapeutic activity of MIP in mouse syngeneic tumor model. Study of MIP as an adjunct to chemotherapy in combination with commercial anti-cancer drug formulation in tumor bearing mice. Simultaneous

study of mechanism of MIP mediated host immune activation.

Protective efficacy of MIP against tuberculosis and the mechanistic insights as compared to BCG

Since MIP has shown promising immunomodulatory activity in tuberculosis as well as in tumor models, we decided to investigate the differential immunostimulatory activity of its cellular fractions viz. cell wall, cytosolic fraction and DNA. We first prepared the different cellular fractions of MIP and BCG harvested at log growth phase and evaluated their relative immunostimulatory potency in terms of macrophage, dendritic cell and splenocyte activation. Among all the cellular fractions, immune stimulating property of the cell wall was found to be the highest. Compared to the cytosolic fraction and DNA, treatment with the cell wall emulsion induced very high response. Hence, we further investigated the cell wall fraction. It was further fractionated into aqueous soluble and lipid soluble parts and determined their immune stimulatory activity in terms of macrophage and T cell activation. Aqueous extract of MIP cell wall was found to be very potent immune stimulator. Based on these findings we are further characterizing the aqueous fraction of the MIP cell wall in terms of its differential TLR agonistic activity.

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

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

Macrophages constitute the major proportion of tumor infiltrating immune cells and play a crucial role in tumor prognosis. Hence, the mechanism by which macrophages recognize and respond to stimulation with live or killed MIP was investigated and compared with BCG stimulation. Treatment of macrophages with live MIP induced significantly higher level of proinflammatory cytokines, TNF-α, IL12, IL6 as compared to treatment with BCG. Higher activation of macrophages also reflected in the upregulation of costimulatory molecules. We also investigated the role of different toll-like receptors (TLRs) in the recognition of MIP and the pathway through which it mediates immunostimulation. To understand the role of TLRs, we analysed the immune response in the presence of specific pharmacological inhibitors of different TLRs. It was observed that MIP mediates its effect through TLR2, TLR4 as well as through intracellular TLRs. Multiple TLRs contribute differentially to produce a compounded response, but signaling through TLR2 was found to be primarily responsible for the immune response. We are presently confirming the role of different TLRs in knockout mice.

We further investigated the role of MyD88, the common adaptor molecule for most TLRs, by isolating the macrophages from WT or MyD88$^{-/-}$ mice. These macophages were stimulated with MIP. The induction of major pro-inflammatory cytokines was almost completely abolished in MyD88$^{-/-}$ macrophages as compared to WT macrophages. Hence, MyD88 was found to have

the central role in the innate immune response to MIP. Signaling through MyD88 culminates in the translocation of NF-κB/AP-1 and subsequent transcription of genes for proinflammatory cytokine. Using the RAW-Blue assay system, activation of NF-κB/AP-1 was analysed in response to stimulation with MIP/BCG. RAW-Blue cells stimulated with MIP produced SEAP in dose dependent manner. MIP induced macrophage activation was shown to result in NF-κB/AP-1 activation and was drastically abrogated by MyD88 deficiency suggesting its signaling via MyD88 dependent pathway.

Quantitatively all the above immune responses were found to be significantly high with live MIP as compared to killed MIP and thus possibly explain the higher protective efficacy observed with live MIP as compared to its killed form.

Efficacy of MIP as a booster to BCG: Immunogenicity, protection and safety study when given by aerosol route in animal models

BCG is the only TB vaccine presently available and its efficacy remains controversial, particularly against pulmonary TB in young adults. BCG is effective against severe form of childhood tuberculosis however, effective protection from TB in adults is still a challenge which indicates that there is a need to boost the protective immune response against *M.tb.* Hence, it is rational to provide a booster following BCG-immunization. However, paradoxically, multiple doses of BCG have resulted in reduced protection and poor survival in the susceptible animal model.

Initial studies showed that vaccination with MIP gives protection against TB in both BCG responder & non-responder strains of mice. In large scale human trials (28,948 people) in 272 villages in Ghatampur, Kanpur, India its protective efficacy against TB was obseved. This population was administered two doses of MIP, originally to investigate its protective effect against leprosy. The vaccine/placebo was given to healthy contacts of leprosy patients who had no evidence of tuberculosis. This area is endemic for tuberculosis. After 13 years, a significant difference was observed in the occurrence of TB cases in the vaccinated and placebo groups. In the persons who had earlier received BCG in their childhood, further higher protective efficacy of MIP was observed.

MIP has also shown higher protective efficacy as compared to BCG in animal models through parentral and aerosol routes. Hence, it is proposed that a booster with MIP may enhance the protective immunity in animals primed with BCG. The intranasal route is proposed to induce both local and systemic immunity and provides a non-invasive delivery system intended to target lung as the desired site of pharmacological effect.

Hence, it is aimed to evaluate the efficacy of MIP administered through aerosol as booster to BCG vaccine in animal model. We are presently doing the dose – response study of MIP given as booster by aerosol route in BCG primed animals and have also initiated the analysis of *M.tb* specific immune response in these animals.

Publications

Original peer-reviewed articles

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Fine tunings of NF-κB signaling

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In physiological settings, cells receive multiple signals from its microenvironment those within a cell-network impinge upon pathogen responsive circuit. Indeed, such cell autonomous crosstalks are thought to engage "apparently" distinct signlaing pathways in a synergistic or antagonistic relationship involving a variety of mechanisms to regulate immune response. Here, we have focused on cell-differentiating LTβR for its ability to augment TLR induced inflammatory response through signaling crosstalk. In a multidisciplinary research program that combines biochemistry, genetics and mathematical biology, we have considered the NF-κB system for its ability to mediate signaling crosstalk to fine-tune TLR induced inflammatory response.

A. Exploring crosstalk between LTβR and TLRs: Using biochemical experiments those involve both a panel of mutant cells as well as recombinant proteins, we will parameter fit a newer version of mathematical model to describe stimulus responsive activation inflammatory NF-κB dimers. In a combinatorial approach, we will use mathematical modelling, mouse genetics and biochemistry to address stimulus and cell-type specificity of signaling crosstalk between LTβR and TLRs. We will further explore molecular mechanisms underlying crosstalk control to generate a genetically engineered "crosstalk defective" mutant cell. Through gene-expression analysis, we will identify important TLR4 induced inflammatory genes those are under crosstalk control. Finally, we will attempt to understand the physiological significance of the proposed crosstalk mechanism *in vivo* in a murine infection model.

B. Exploring cross-regulations of TLR response through PTMs: Using bioinformatics tools, we will predict novel post-translational modification (PTM) sites on NF-κB/RelA subunit. By expressing NF-κB/RelA proteins mutated at these newly identified sites, in both over-expression as well as stable cell-line based systems, we will examine the potential role of the predicted sites in inflammatory gene-expression downstream of various TLRs. Next, we will address if PTMs modulate inflammatory response at the level of NF-κB activation in crosstalk settings.

For the past one-year (2011-2012), in a coherent group effort, we have effectively implemented a multidisciplinary research program at NII, as detailed below. In a circular loop, quantitative biochemical data was used to parameter fit NF-κB mathematical model, which was used to generate a conceptual framework that led to study design, experimentation as well as further model refinements to capture emergent crosstalk behaviour of inflammatory signalling.

A. Exploring crosstalk between LTβR and TLRs

In this aim, we have carried out various biochemical measurements those utilize a panel of knockout mouse embryonic fibroblast cells (MEFs) and recombinant NF-κB/IκB proteins to stringently parameter fit the model. By iterative model simulations, using a combination of individual members from a theoretical library of 349 kinase activity profiles as inputs for NEMO/IKK2 or NIK/IKK1 arms, we identified that a duration filter discriminates between short and long duration NEMO/IKK2 or NIK/IKK1 signals and preferentially engages long duration NEMO and NIK signals into signaling crosstalk.

To experimentally verify our mathematical predictions, we analyzed NF-κB signaling induced through NEMO/IKK2 by inflammatory IL-1R or various TLRs in a crosstalk regime that involves concomitant NIK activation through developmental LTβR using an agonistic monoclonal αLTβR antibody. First, we compared NEMO/IKK2 activation profile in response to IL-1, Pam3Cys4 (activates TLR2) and LPS (activates TLR4) in MEFs using IP-Kinase assay. Consistent to the reported role of TRIF in controlling late phase signaling activity, we noted that TLR4, which engages both Myd88 and TRIF, elicits a rather long duration NEMO/IKK2 activity as compared to IL-1R or TLR2 those solely engage Myd88 adapter to elicit only a transient early activity (Covert, Leung et al. 2005). Indeed, our EMSA revealed that TLR4 induced late phase NF-κB activity was further augmented in synergy with LTβR induced signal. However, IL-1R signaling was found to be insulated from crosstalk with LTβR with respect to NF-κB activation. To further validate our duration filter hypothesis, we utilized *trif*$^{-/-}$ MEF that induces transient NEMO/IKK2 activity in response to TLR4 activation. In our crosstalk regime, we could reveal a complete lack of signaling crosstalk between TLR4 and LTβR, in spite of functional LTβR pathway in this knockout MEFs. Finally, we were able to show that the expressions of several NF-κB dependent inflammatory genes are augmented in TLR4/LTβR crosstalk regime as compared to cell activation through individual TLR4 and LTβR.

Our mathematical model also predicted requirement of a persistent NIK/IKK1 activity for the developmental cues to participate in signaling crosstalk with inflammatory pathways. Here, we have developed a pulse treatment protocol, which involves LTβR stimulation using recombinant LTα1β2 (activates LTβR) for 5h followed by stimulus withdrawal, to reveal short-duration NIK activity downstream of LTβR upon pulse as compared to chronic LTα1β2 treatment. Currently, we are distinguishing between short and long duration LTβR signals for their ability to engage into signaling crosstalk.

To identify the molecules and mechanisms those underlie crosstalk synergy between TLR4 and developmental pathways, we carried out a local parameter sensitivity analysis of our mathematical model. First, we mathematically defined synergy as the ratio of induced NF-κB activity in NEMO-NIK co-treatment regime to that of sum of the individual activities induced upon NEMO or NIK activation. Next, each of the parameters (rate constants for various biochemical reactions) was

individually increased 10% and the resultant changes in the synergy were compared to that of the wild type module. In our analysis, top five crosstalk sensitive reactions were found to be connected with two distinct NF-κB/IκB molecules implying their critical role in crosstalk regulation.

In our biochemical analysis, now we are using primary MEFs derived from various NF-κB/IκB knockout mice from a colony established at NII in biochemical and gene-expression analysis to mechanistically explore signaling crosstalk between cell-differentiating cues and inflammatory signaling pathways.

mutant of RelA in our κB-luciferase assay, as documented last year, we sought to further investigate the role of T54 phosphorylation of RelA in NF-κB driven inflammatory activity. To this end we have generated retroviral constructs those express wild-type (WT), $RelA_{T54A}$ or $RelA_{T54D}$. We have transduced $RelA^{-/-}$ fibroblasts with individual retroviral constructs to generate three different RelA reconstituted stable cell-lines those express $RelA_{WT}$ or $RelAT_{54A}$ or $RelA_{T54D}$. By Immunoblot, we ascertained that the expression level of RelA or its variants are similar in these three different cell-lines (Figure 1A).

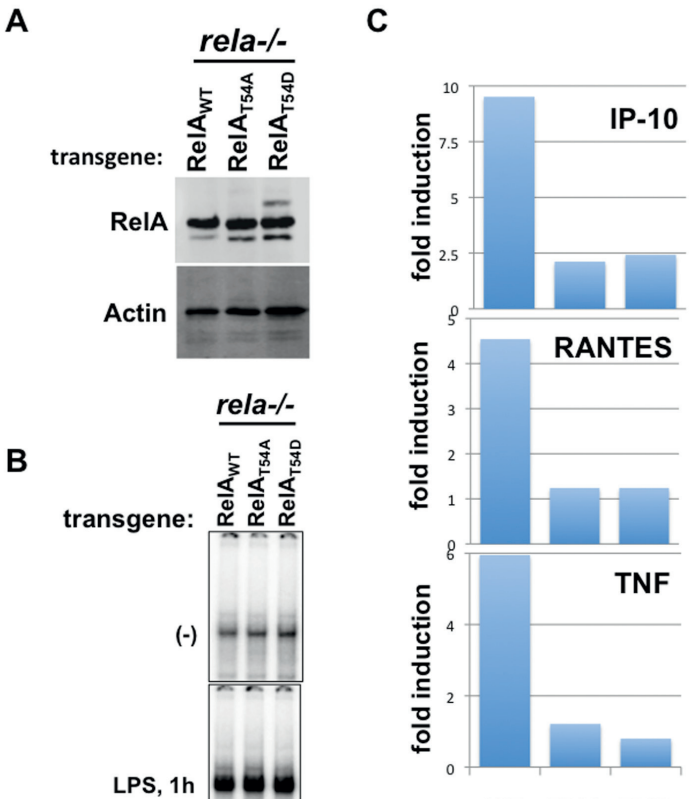

Figure 1: Biochemical characterization of $RelA_{WT}$ or $RelA_{T54A}$ or $RelA_{T54D}$ fibroblast cell-lines for RelA protein expression (A), LPS induced NF-κB DNA binding activity (B) and cytokine/chemokine gene expression (C).

B. Exploring cross-regulations of TLR response through PTMs

Given promising results with T54D/T55D

Next, we stimulated $RelA_{WT}$ or $RelA_{T54A}$ or $RelA_{T54D}$ fibroblast cell-lines with LPS to engage TLR4 and scored NF-κB activation using EMSA in a time-course. In our DNA binding assay, we

could reveal similar level of nuclear translocation and kappaB DNA binding by NF-κB/RelA dimers upon TNF treatment in the mutant cells as compared to wild-type RelA reconstituted cells (Figure 1B).

Finally, we examined TLR4 induced gene-expression in $RelA_{WT}$ or $RelA_{T54A}$ or $RelA_{T54D}$ fibroblast cell-lines. To this end, cells were stimulated with LPS, RNA was isolated in a time-course and gene-expression was measured using real-time PCR. Interestingly, LPS induced NF-κB dependent expression of several cytokines and chemokines were found to be defective in $RelA_{T54A}$ cell lines (Figure 1C), in spite of similar LPS induced NF-κB activity in these cells as compared to wild-type RelA reconstituted cells. To somewhat surprisingly, we also noticed an abrogated LPS induced NF-κB dependent gene expression of in $RelA_{T54D}$ cell lines. It is thought to be unusual to have similar negative phenotype in both phospho-mimicking and phospho-defective mutants. Currently, we are generating $RelA_{T54S}$ and $RelA_{T54E}$ cell line to further investigate this issue.

Publication

Review

1. Basak S, Behar M, Hoffmann A* (2012) Lessons from mathematically modeling the NF-κB pathway. *Immunol Rev* **246**: 221-238.

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Amitabha Mukhopadhyay

Understanding the regulation of intracellular transport : Role of GTPases

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

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

1. Modulaton of phagosome maturation by intracellular pathogens.
2. 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.
3. Mechanism of intracellular trafficking of hemoglobin in *Leishmania*.

Mechanism of survival of *Salmonella* in macrophages

It is now well evident that several intracellular pathogens modulate host cellular machinery for their benefit through some of their own proteins, collectively known as effectors. Previously, we have identified and determine the role two such effectors namely SopE and SipC from *Salmonella* which modulate host cell trafficking molecules. We have shown earlier that SopE is selectively acts as a Rab5 exchange factor and recruits host Rab5 on *Salmonella*-containing phagosomes (SCP) and thereby promote constitutive fusion with early endosomes; so that their transport to the lysosome is inhibited. Subsequently, we have shown that SipC acts as cognate SNARE and specifically binds with host Syntaxin 6 and thereby promote fusion with LAMP1-containing Golgi-derived vesicles with SCP to acquire LAMP1 which stabilize their niche in macrophages.

In the reviewing period, we have found that live-SCP specifically recruits significantly higher amounts of Syntaxin8 in comparison to dead-SCP indicating possible role of some effector in the recruitment of Syntaxin8 on SCP. Therefore, we have tried to analyze the mechanism of recruitment of Syntaxin8 by live *Salmonella*-containing phagosome. In order to detect the Syntaxin8 binding protein from *Salmonella*, *Salmonella* were grown in high salt concentration to induce the type III secretion and spent medium was collected and concentrated. Subsequently, immobilized GST-Syntaxin8, or GST alone was incubated in the presence of concentrated spent medium for 10 hr. at 4^{o}C. Under these conditions, we have found that Syntaxin8 binds with a ~74KDa secretory protein form *Salmonella*. *Salmonella* proteins bound to respective beads were detected by Western blot analysis using antibodies against different secretary proteins of *Salmonella*. Our results have shown that Syntaxin8 specifically binds with SipA but not with SopB and SopE. These results are further confirmed by mass-spectrometric analysis of the pull out protein. Consequently, we have cloned and expressed SipA from *Salmonella* and determined its ability to bind Syntaxin8. Our results indicate that SipA specifically binds with Syntaxin8, however, relatively low binding is also observed with Syntaxin6 and Syntaxin7. These results suggest that *Salmonella* possibly recruit Syntaxin8 on their phagosomes through SipA. Subsequently, we have tried to map the interacting regions of these two proteins. Accordingly, we have cloned and expressed several truncated forms of SipA with His6 tag and determined their ability to bind full length Syntaxin8. Our results have shown that N-terminal end of SipA (SipA$^{1-242}$) specifically binds with GST-Syntaxin8 and no binding is observed with C-terminal end of SipA (SipA$^{436-685}$) or with free GST. Currently, we are also trying to map the region of Syntaxin8 important for the interaction with SipA. Accordingly, we have made several truncated mutants of Syntaxin8 namely Syntaxin8$^{1-144}$, Syntaxin8$^{1-209}$ and Syntaxin8$^{145-209}$; considering that residues spanning from 145-209 of Syntaxin8 is a SNARE motif.

To determine the role of SipA on the modulation of intracellular trafficking of *Salmonella* in macrophages, we have made a SipA knock out *Salmonella*. Briefly, upstream and downstream flanking region of the SipA was PCR amplified and cloned sequentially into a suicide vector pRE112 to generate pRE112ΔSipC. In the first step of recombination, the E.coli donor strain (SM10λpir) containing the suicide vector pRE112ΔSipA was mated with *Salmonella* SL1344 strain. The transconjugants containing integrated suicide vector into the *Salmonella* genome were selected by antibiotic selection. The transconjugants, were sensitive to sucrose

due to the synthesis of levans (toxic compounds), catalysed by levansucrase, the product of *sacB* (pRE112 contains the SacB gene of *Bacillus* sp.). In the next step, a single colony from the first step was grown in enrich medium deprived of antibiotic, allowing the occurrence of a second crossing-over to replace the wild-type allele with the mutant one, and then excised the plasmid-borne *sacB* from the chromosome. Such mutants were selected on their ability to grow on medium containing 5% sucrose. The deletion of SipA gene from the mutant was confirmed by PCR, sequencing and western blot analysis. Studies are in progress to determine the role of SipA in *Salmonella* trafficking in macrophages using WT and SipA knock our bacteria.

Mechanism of hemoglobin trafficking in *Leishmania*

Previously, we have shown that hemoglobin endocytosis in *Leishmania* is mediated through a specific receptor (HbR) present in the flagellar pocket which is characterized to be a surface localized hexokinase. Subsequently, we have shown that endocytosed hemoglobin is transported to the lysosome through Rab5 and Rab7 dependent process where it is degraded to generate intracellular heme which is used by the parasite for their growth. Moreover, we have found that blocking of hemoglobin endocytosis or its intracellular degradation is detrimental to parasites. Therefore, we also initiated studies to evaluate the hemoglobin receptor as a novel target for *Leishmania*.

Previous year, we have shown that LdHbR traffic from ER to the flagellar pocket is Rab1 independent process in *Leishmania* and possibly follow unconventional secretory pathway. Recent reports in mammalian cells have shown that unconventional protein secretory pathway is possibly regulated by GRASP65 or its homologue. Therefore, in the reporting period, we have tried to clone and overexpress GRASP65 homologue from *Leishmania* to understand the mechanism of HbR trafficking ER to the flagellar pocket via GRASP65 dependent unconventional pathway. In order to clone LdGRASP65 from *Leishmania*, we have designed appropriate forward and reverse primers based on *Leishmania* major GRASP65 sequence (NCBI Reference Sequence: XP_001685999.1) which has amplified 1.6 kb fragment from L. donovani cDNA by RT-PCR. The PCR product was cloned and sequenced and our results have shown that cloned protein has 88 percent identity with the GRASP65 from *Leishmania* major. Subsequently, LdGRASP65 was subcloned into pNUS-mRFP-nD expression vector and overexpressed in *Leishmania* as RFP fusion protein. Confocal microscopy analysis of the LdGRASP65 overexperessing cells shows that this protein is localized in Golgi as a single punctate structure. In an attempt to find out whether LdHbR trafficks through an unconventional GRASP65 dependent pathway in *Leishmania* promastigotes, cells overexpressing LdGRASP65-RFP were cotransfected with LdHbR-GFP. Subsequently, positive clones were selected in the presence of G418 (30 μg / ml) and Blasticidin (15 μg/ml). Co-expression of LdGRASP65-RFP with LdHbR was confirmed by confocal microscopy. Our preliminary results have shown that GFP-LdHbR is partially colocalized with LdGRASP65-RFP in the Golgi and no GFP-LdHbR is found in the glycosomes as compared to untransfected control cells. Interestingly, some fraction of the LdHbR is also found in the apical region near flagellar pocket of *Leishmania* expressing LdGRASP65-RFP. Further studies are in progress to characterize the role of GRASP65 in HbR trafficking in *Leishmania*.

In the reporting period, we have also evaluated whether haemoglobin endocytosis in *Leishmania* is a clathrin dependent process or not. Therefore,

we have clone and express clathrin heavy chain from *Leishmania* (Ld-CHC) and shown that Ld-CHC is localized in flagellar pocket and regulates Hb-endocytosis in *Leishmania*. Kinetic analysis of Hb trafficking in GFP-Ld-CHC overexpressed *Leishmania* reveal that Hb is internalized through Ld-CHC coated region and remains associated with Ld-CHC containing vesicles at early time points of internalization and subsequently starts dissociating from Hb-containing vesicles at later time points indicating that clathrin-coating and uncoating regulates Hb trafficking in *Leishmania*. Interestingly, we have found that overexpression of dominant negative mutant of clathrin heavy chain of *Leishmania* (GFP-Ld-CHC-Hub) blocks the Hb internalization and causes severe growth defect in parasite. Moreover, we have shown that chlorpromazine (CPZ), a pharmacological agent, blocks Hb internalization in *Leishmania* by depolymerizing Ld-CHC and thereby inhibits the growth of the parasites. Taken together, our results have shown that Hb endocytosis in *Leishmania* is a clathrin dependent process and is essential for the survival of the parasites. As hemoglobin endocytosis is important for both promastigote and amastigote stages of *Leishmania*, therefore, CPZ mediated inhibition of parasite growth by blocking hemoglobin endocytosis in parasite could be useful for development of new anti-*leishmania*l agent which we are currently exploring.

Publication

Original peer-reviewed article

1. Agarwal S, Rastogi R, Gupta D, Patel N, Raje M, Mukhopadhyay A* (2013) Clathrin-mediated hemoglobin endocytosis is essential for survival of *Leishmania*. *Biochim. Biophys Acta* **1833**: 1065–1077.

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Eggs of red vented Bulbul in a tree at NII campus

REPRODUCTION AND DEVELOPMENT

Subeer S. Majumdar

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 undertake gene expression studies of rat, mice and monkey Sertoli cells to identify factors important for induction of spermatogonial stem cell division and differentiation in the testis.
3. To study biology of spermatogonial stem cells and to use germ cell transplantation technique for restoration of fertility following chemotherapy.
4. To study paracrine and endocrine modulation of signal transduction in target cells of the endocrine system.

A. Functional genomic studies of genes selected from studies of differential genomics by DNA microarray using mRNA from rhesus monkey Sertoli cells

Sperm quality has been declining globally over last 50 years (from 165 million to 60 million), which could lead to an increased prevalence of infertility in the world in coming 20-30 years. Testicular Sertoli cells (Sc) in males produce several factors generating functional niche for germ cell (Gc) division and differentiation at

puberty, failure of which may cause infertility. Congenital and/or environmental perturbations in expression of such Sc factors potentially underlie several cases of idiopathic infertility. Knowledge of such post-hormonal transcriptional and signal transductional events in Sc would help in diagnosis and treatment of hormone independent idiopathic male infertility. Present study was designed to compare genes expressed by infant (spermatogenically inactive) and pubertal (spermatogenetically active) monkey Sc treated with intermittent pulses of FSH and T, using DNA microarray. Important genes are being evaluated one by one.

Wnt3

Wnt3, a morphogen of Wnt/β-catenin signalling pathway is crucial for cellular differentiation, was found to be highly expressed in pubertal Sc as compared to infant Sc. The expression level of Wnt3 was also found to be higher in pubertal Sc of mouse origin. To evaluate the role of Wnt3 as a paracrine signalling factor in the Sc-Gc niche, transgenic mouse models were generated. Wnt3 was over expressed in Sc of infant mice using infant Sc specific promoter of Mullerian Inhibiting Substance (MIS) and to knock down (KD) natural Wnt3 expression, *Rhox5* promoter element was used for pubertal Sc specific expression of shRNA of Wnt3. Over expression of Wnt3 during infancy resulted in Gc overcrowding and precocious initiation of lumen formation within seminiferous tubules. Conversely, knocking down of Wnt3 during puberty resulted in decreased sperm count and testicular weight, along with Gc sloughing and disrupted Sc-Gc association. Wnt3 overexpression led to activation of classical β catenin mediated signal transduction whereas knockdown of Wnt3 lead to increased destabilisation of β catenin and consequently inhibited downstream transcriptional targets. Wnt3 overexpression augmented expression of GDNF, an essential factor for maintenance of Gc stemness, and associated downstream targets like cFos, Lhx1 and Erm which are known to be crucial for Gc proliferation and self renewal. Wnt3 overexpression also led to increased levels of Cx43, a gap junction molecule essential for Sc-Gc communication and spermatogenesis. Interestingly, knockdown of Wnt3 led to decreased Cx43 level which is known to be associated with disrupted spermatogenesis.

CD30

CD30, a member of the TNF receptor super family involved in cytokine mediated processes within the body, was overexpressed by Sc during puberty in monkey as well as mice. Cytokines of the TNF family are known to play a crucial role in Sertoli cell-germ cell cross talk and we believed CD30 to be a key member to such cytokine mediated cross talk. In order to evaluate the functional significance of CD30 expression on Sc mediated Gc division and differentiation during puberty, we used the Sc specific Rhox5 promoter (active onwards 14 days post birth) to knock down CD30 in Sc of transgenic mice. Sc specific shRNA mediated knockdown decreased CD30 levels by ~80%. CD30 knockdown lead to inactivation of NFκB signal transduction, as evidenced by lower expression of Iκbα, RelA and p100, which elevated the expression of the Sc immaturity marker AMH and diminished AR and Sf1 expression, both events being associated with impaired spermatogenesis (and infertility). Lower expression levels of Sf1, a transcriptional co activator essential for AR expression as well as AR transcription factor activity, could explain diminished expression levels of AR. TNFα mediated NFκB signal transduction is known to lower Gc apoptosis by Sc, and CD30 knock down was found to repress the NFκB pathway so we checked the effect of CD30 knockdown on apoptosis rate within testis of transgenic mice.

The expression level of pro apoptotic genes like FAS, FAS-L and Noxa were increased and expression level of anti apoptotic genes Bcl2 and Bcl-xl were decreased in CD30 knockdown animals, indicating a greater tendency for apoptosis in testis of CD30 knockdown mice. CD30 knock down animals exhibited delayed lumen formation and lower sperm count in comparison to control animals. The evidence from knockdown of CD30 in Sc of mice during puberty suggested CD30 as an important factor in processes leading to gain of spermatogenic function by Sc at puberty. Abrogation or aberration in CD30 expression may underlie several cases of male factor idiopathic infertility and CD30 and should be included for diagnostic and therapeutic consideration of non-hormonal idiopathic male infertility.

C. Spermatogonial Stem cell cultures and germ cell transplantation in mice

Transgenic Spermatogonial stem cells transplanted in busulfan treated recipient mice resulted into production of sperm in about 3 months. Sperm were found to carry the ransgene as revealed by PCR.

Endocrine signaling (Sertoli cells)

We have recently demonstrated that androgen binding ability of cultured infant monkey Sertoli cell (Sc) is significantly low compared to that of the pubertal Sc. However, downstream status of FSH-R signaling in primate Sc before and during puberty is not known. Sertoli cell FSH-R is a G protein-coupled receptor (GPCR), which upon binding to FSH gets activated and stimulates adenylyl cyclase (AC) mediated production of cAMP. This second messenger induces, via several intermediate steps, the transcription of several genes like Transferrin, ABP, Inhibin-β_B, SCF , GDNF etc which have a key role in the regulation of male germ cell (Gc) differentiation. Although FSH is not indispensable for murine male fertility, role of FSH in the regulation of primate spermatogenesis including human remains controversial. Men with an inactivating mutation in the FSH-R remain fertile despite having smaller testes. However, in contrast, infertility occurs in men lacking normal circulating FSH. Therefore, FSH is considered to be crucial for human spermatogenesis. In nonhuman primates, FSH is known to stimulate testicular Sertoli cell proliferation during infancy that determines the maximal spermatogenic output throughout the male reproductive life and provide the survival and differentiation signal for the expansion of the developing Gc in the testes during and after puberty. Unlike rodents, as found by us and others), the knowledge about the mode of FSH-R signaling in primate Sc before and upon testicular maturation is extremely limited. Moreover, it is also not clear whether the acquisition of appropriate FSH-R signaling leading to augmentation in gene transcription to initiate Gc differentiation is associated with the pubertal maturation of these cells. To address these issues, we for the first time, have compared the FSH-R signaling in terms of cAMP response and gene expression in infant and pubertal male monkeys exposed to similar gonadotropin stimulus using primary culture of Sc. Additionally, we have evaluated the maturational status of infant and pubertal Sc used in this study. In this study we have evaluated the signaling events in Sc following rise in intracellular cAMP known to be the major second messenger in FSH mediated pathway leading to spermatogenesis. Our results suggested that unlike pubertal Sc, increasing dose of FSH treatment to infant Sc did not show a dose responsive cAMP production. However, forskolin (which directly activates adenylyl cyclase) induced a comparable rise in cAMP production in both the age groups. Forskolin or 8Br-cAMP, but not FSH treatment to infant Sc

significantly augmented the mRNA expression of Sc specific genes which are necessary for spermatogenesis like Transferrin, ABP, Inhibin-βB, SCF and GDNF. However, the expression levels of these genes were similar to those expressed by pubertal Sc upon stimulation with FSH. Taken together, our results suggested that intracellular post FSHR downstream signaling cascades were operational in infant Sc, the restricted FSH responsiveness primarily stemming from the limited ability of cAMP production by these cells at this phase of development. This study also reveals that like the pubertal Sc, infant Sc are also transcriptionally competent provided sufficient amount of cAMP is being produced inside the cell. Dissection of the FSH signaling pathway would help identification of important steps which may be used for intervention to augment fertility in men suffering from idiopathic infertility.

Endocrine signaling (Adipose tissue)

Free fatty acids (FFAs) augment adipose tissue inflammation through the TLR4 pathway, causing insulin resistance. Interestingly, FFAs do not directly bind to TLR4. In a study led by Professor Samir Bhattacharya, where we were collaborators, it was shown that fetuin-A (FetA) seems to be the endogenous ligand that regulates insulin sensitivity via TLR4signaling in mice. *FetA* knockdown in mice with insulin resistance caused by a high-fat diet (HFD) resulted in downregulation of TLR4 mediated inflammatory signaling in adipose tissue, whereas selective administration of *FetA* induced inflammatory signaling and insulin resistance. FFA-induced proinflammatory cytokine expression in adipocytes occurred only in the presence of both *FetA* and TLR4; removing any of them limited FFA-induced insulin resistance. Modulation of *FetA* may help in managing insulin resistance.

Publications

Original peer-reviewed articles

1. Majumdar SS*, Sarda K, Bhattacharya I, Plant TM* (2012) Insufficient androgen and FSH signaling may be responsible for the azoospermia of the infantile primate testes that is exposed to an adult-like hormonal milieu. *Human Repro* **27**: 2515–2525.
2. Pal D, Dasgupta S, Kundu R, Maitra S, Das G, Mukhopadhyay S, Ray S, Majumdar SS, Bhattacharya S* (2012) Fetuin-A acts as an endogenous ligand of TLR4 to promote lipid-induced insulin resistance. *Nature Med* **18**: 1279-1285.
3. Bhattacharya I, Pradhan B. Sr, Sarda K, Gautam M, Basu S, Majumdar SS* (2012) A switch in Sertoli cell responsiveness to FSH may be responsible for robust onset of germ cell differentiation during prepubartal testicular maturation in rats. *Am J Physiol (Endocrinol and Metabol)* **303**: E886-E8898.
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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. 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.

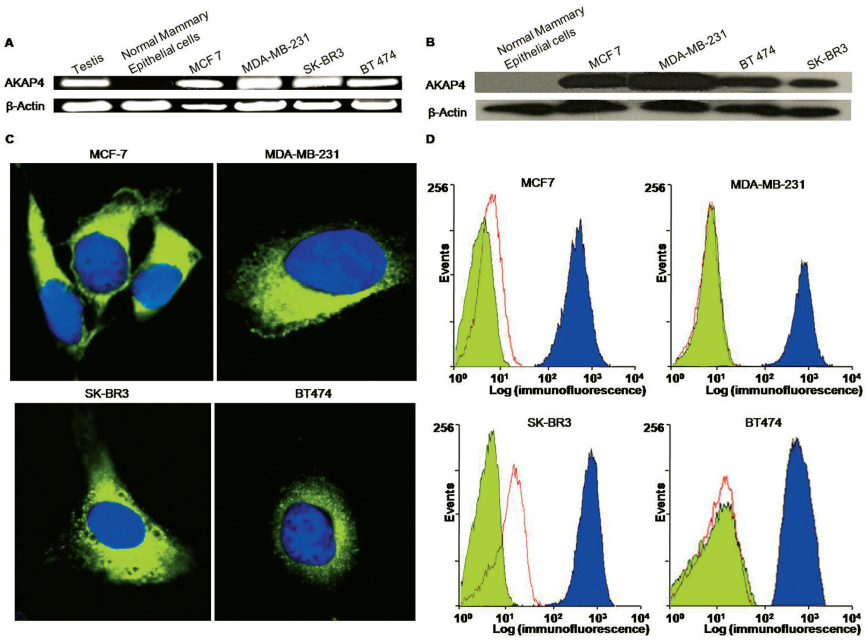

FIgure 1: AKAP4 expression and localization in breast cancer cell lines.

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

Breast cancer is the second leading cause of cancer related deaths in women worldwide. Reports about the early diagnosis of breast cancer are suggestive of an improved clinical outcome and overall survival rate in cancer patients. Therefore, cancer screening biomarker for early detection and diagnosis is urgently required for timely treatment and better cancer management. In this context, we investigated an association of cancer testis antigen, A-Kinase anchor protein 4 (*AKAP4*) with breast carcinoma.

We first investigated *AKAP4* gene expression in human normal mammary epithelial cells and breast cancer cells of two different origins namely adenocarcinoma (MCF7, MDA-MB-231 and SK-BR3), and ductal carcinoma (BT474). Our results revealed *AKAP4* gene expression in all four breast cancer cell lines (MCF7, MDA-

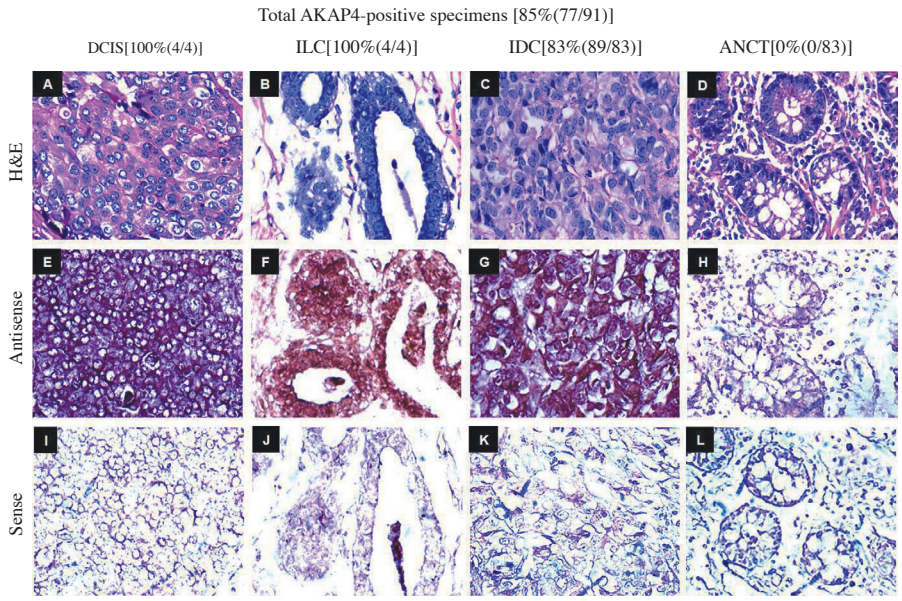

Figure 2: Gene expression analysis of AKAP4 in breast cancer tissue specimens of different histotypes (DCIS, IDC, ILC) and matched associated non-cancerous tissues by in situ RNA hybridization.

MB-231, SK-BR3 and BT474), but not in normal mammary epithelial cells (Figure 1A).

AKAP4 gene expression was validated for endogenous *AKAP4* protein expression in all breast cancer cells by using Western blotting. Polyclonal anti-*AKAP4* antibody raised in rats was used to probe the *AKAP4* protein in breast cancer cell lysates. As shown in Figure 1B, *AKAP4* protein expression was detected in all the breast cancer cell lines but not in normal breast epithelial cells. β-actin was used as an internal loading control for Western blotting experiments. We further examined the localization of *AKAP4* protein in breast cancer cells by indirect immunofluorescence assay and flow cytometric analysis. All breast cancer cell lines revealed cytoplasmic localization of *AKAP4* protein (Figure 1C). Subsequently, we also investigated the *AKAP4* surface expression in live breast cancer cells by flow cytometry which revealed a distinct shift of fluorescence on X-axis (blue histogram) indicating *AKAP4* protein localization on the surface of the cells as shown in Figure 1D. In contrast, cells that were probed with control IgG (Figure 1D, red histogram) showed no displacement with respect to unstained cells (Figure 1D, green histogram). The surface localization of *AKAP4* protein suggests that it may be a potential target candidate for therapeutic use in breast cancer patients.

Further, *AKAP4* gene expression was investigated in breast cancer patient's tissue specimens by employing in *situ* RNA hybridization. Our results revealed hybridization of anti-sense AKAP4 riboprobe depicted by chocolate brown color in cells expressing AKAP4 gene (Figure 2). As expected, sense riboprobe, having the same sequence as that of endogenous AKAP4 mRNA failed to show hybridization and resulted in no reactivity. AKAP4 gene expression was detected in 85% (77/91) of breast cancer patients. Among the various histotypes, AKAP4 gene expression was detected in 100 % DCIS (4/4), 83% IDC (69/83) and 100 ILC (4/4) specimens (Figure 2).

Validation of AKAP4 protein expression was

carried on serial sections of breast tissue specimens used for in situ RNA hybridization by employing immunohistochemistry (IHC). Our results distinctly revealed cytoplasmic localization of AKAP4 in 85% (77/91) of breast cancer patients. It is noteworthy that we did not observe discrepancy between AKAP4 gene and protein expression in breast cancer specimens under investigation. We analyzed AKAP4 protein expression in all histotypes using anti-AKAP4 antibodies which revealed AKAP4 protein localization in 100% DCIS (4/4), 83% IDC (69/83) and 100% ILC (4/4) patient's specimens suggesting its importance for developing as a biomarker. Serial tissue sections probed with control IgG showed no immunoreactivity. Similarly, no AKAP4 protein expression was detected in ANCT specimens indicating that AKAP4 expression was associated with cancerous tissues ($P < 0.001$, Pearson's Chi-square test). AKAP4 protein expression in majority of cancer patients irrespective of their clinical stages and histopathological grades indicates that AKAP4 may have a potential role in disease progression. Therefore, further studies are warranted in large number of patients to validate our findings.

We further investigated humoral response in breast cancer patients by Enzyme Linked Immuno-Sorbent Assay (ELISA) after validating AKAP4 protein expression in tissue specimens. The absorbance value of mean + 2SD of healthy normal female's sera was used as cut-off value (absorbance = 0.308) above which patient's sera were considered positive for anti-AKAP4 antibodies. The intra-assay and inter-assay coefficients of variation were 3.6% and 5.4% respectively. Our data indicated the presence of circulating anti-AKAP4 antibodies in 79% (79/91) of the breast cancer patients. However, none of the healthy normal males showed detectable circulating anti-AKAP4 antibodies. It is important to mention that 77 patients found positive for AKAP4 protein expression, 72 patients (94%) generated humoral response against AKAP4 protein. Circulating antibodies against AKAP4 were found in majority of patients irrespective of their clinical stages and histological grades. Moreover, detection of humoral response against AKAP4 represents a better and minimal invasive method of diagnosis from the sera of cancer patients. ELISA findings were subsequently confirmed by subjecting purified AKAP4 recombinant protein to sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting experiments. All the cancer patients found positive for ELISA revealed a strong reactivity in patient's sera of all histotypes, grades and stages.

It is important to mention that all sera samples were preincubated with *E.coli* BL21 (DE3) cell lysate in order to remove any non-specific binding. Probing purified recombinant AKAP4 protein with polyclonal anti-AKAP4 antibody resulted in specific AKAP4 immunoreactive band. Interestingly, in neutralization experiment sera pre-incubated with 15μg/ml of recombinant AKAP4 protein, resulted in complete loss of reactivity with AKAP4 protein. Additionally, no immunoreactivity was observed by probing *E.coli* BL21 (DE3) whole cell lysate which ensured that polyclonal anti-AKAP4 antibody was specific against AKAP4 protein. Further, to confirm the reactivity of circulating antibodies in patient's sera against recombinant AKAP4 protein in western blotting experiment, neutralization experiments were carried out by pre-incubating diluted patient's sera with 15 μg/ml of recombinant

AKAP4 protein which resulted in complete loss of reactivity in immunoblotting. Similarly, to validate the specific immunoreactivity of anti-AKAP4 antibody with endogenous AKAP4 protein in cancer specimens, polyclonal anti-AKAP4 antibody was pre-incubated with 15μg/ml of recombinant AKAP4 protein and used on serial tissue sections of IDC which resulted in complete loss of immunoreactivity.

Given consistencies in AKAP4 gene and protein expression in various histotypes, different stages and grades of breast cancer illustrates its potential diagnostic role as a biomarker in clinical settings. To the best of our knowledge, AKAP4 is the first X-linked CT antigen showing expression and humoral response in majority of breast cancer patients. Furthermore, anti-AKAP4 antibodies, present in the patient's sera provides a basis for better, affordable and routine method of detection. AKAP4 expression in breast cancer in all clinicopathological stages and grades indicates its possible role in tumorigenesis and disease progression. Future large scale studies are warranted to explore its utility as an early diagnostic biomarker and immunotherapeutic target in breast cancer.

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Asok Mukhopadhyay

Study of expansion and plasticity in bone marrow stem cells

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Bone marrow (BM) niche controls self-renewal and differentiation of HSCs. To understand the regulation of hematopoiesis and the effect of aging on stem cells, it is necessary to decode the niche microenvironment in mammals of various age groups. A comprehensive knowledge on hematopoietic niche will help to mimic an *ex vivo* system to restore stem-ness and engraftability of cells. It is now known that hematopoietic cells are involved in regeneration of many non-hematopoietic organs. *Ex vivo* cultured cells seems to facilitate transplantation required for hematological reconstitutions as well as therapeutic usage to heal diseased organs. The overall theme of our research involves the study of stem cell niches, plasticity of BM-derived stem cells, role of hematopoietic cells in the progression of cancer, and reprogramming of adult cells.

We intend to dissect HSCs niche to elucidate its function in marrow regeneration, as well as their role in various pathological conditions of certain organs, and plasticity of adult stem cells. The objectives are as follows:

1. Molecular control of self-renewal and engraftability of HSCs in mice.
2. To understand liver regeneration by BM-derived cells and to elucidate the mechanism of hepatic differentiation.
3. Role of BM cells in stem-ness and cancer progression.

4. Mechanistic insight into regeneration of neurons by MSC-derived precursor cells.
5. Study of molecular interplay during fibrosis and normal regeneration of tissue.

A. Hematopoietic stem cells niche and marrow regeneration

In irradiated host, the syngenic donor LSK cells are found to proliferate at higher rate than the host LSK cells, during marrow regeneration. The cell cycle status of these cells suggests the presence of a positive and negative feed-back regulatory loop in BM, which induces quiescent HSCs into actively proliferation and then returns them to quiescence. In addition to their greater role in marrow regeneration, the donor cells secrete mitotic and anti-apoptotic factors for the early recovery of host cells from radiation induced damage. To establish the functional roles of Areg and Clec11a genes in hematopoiesis, respective gene-silenced stromal cells were co-cultured with LSK cells. Preliminary results suggest that LSK cells undergo apoptosis in the absence of these genes when compared to the vector control cells. These results implicate that A*reg* and C*lec11*a genes and their signaling pathways protect LSK cells from undergoing apoptosis.

B. Plasticity in BM cells

Transdifferentiation of MSC into DA neurons

In continuation to the earlier work, we conducted stage specific neuronal gene expression analyses by real-time PCR during differentiation of MSCs. These results suggest that fetal liver MSC are differentiated into dopaminergic neurons within 25 days of culture, however neuronal commitment has been observed by 10 days. Further, co-localization studies of cells confirm the expression of neuron specific proteins like tyrosine hydroxylase, dopamine transporter, microtubule associated protein 2, and β-tubulin III. We have also conducted studies on the propensity of differentiation, to determine the efficiency of the differentiation programme. These differentiated cells were subjected to electrophysiological studies, and the results show large extent of K^+ currents as compare to the Na^+ currents. MSCs primed for neuronal differentiation were transplanted in the Parkinson's diseased (PD) mouse model. At present the recipient mice are undergoing different behavioral tests to evaluate the therapeutic benefit of these cells.

Therapeutic effect of allogeneic BM Lin^- cells in hemophilia A mouse

The recipient mice of the allogenic Lin^- cells were examined for the establishment of peripheral tolerance. For that, immune modulation by co-transplanted Treg cells has been examined at different time intervals of the transplantation, by monitoring effectors $CD4^+$ T cells and $CD4^+CD25^+Foxp3^+$ Treg cells in the spleen. The results suggest that allo-antigen specific Treg cells are able to check the immune response by suppressing $CD4^+$ T cells, which can be correlated with the pathology of the liver tissue. We have also evaluated various cellular sources of FVIII to find out a potential candidate for treatment of hemophilia A. Our results suggest that donor-derived hepatocytes, sinusoidal endothelial cells and Kupffer cells can synthesize FVIII protein. We have also generated induced hepatocytes (iHep) by over-expressing HNF-4α in fibroblastic cells. The iHep are now undergoing rigorous functional tests.

Studies on liver fibrosis and therapeutic benefit of BM cells

Next, we have studied the role of hematopoietic cells in the resolution of fibrosis in CCl_4-induced liver fibrosis model. Fibrotic lesions on the liver are found to be resolved much faster by transferring $CD45^+$ BM cells into the liver. The resolution of fibrosis has been confirmed by adopting METAVIR scale, determining collagen equivalent proportionate area in the liver section, and estimating hydroxyl-proline content of the tissue. Parallel with the decreasing collagen content, the activation of hepatic stellate cells has been moderated with time. It has been hypothesized that hematopoietic cells/macrophages within the graft secrete MMPs and cytokines to bring about such pathological changes. In Addition, liver steotosis (accumulation of fat) has been controlled with the resolution of fibrosis. The donor cells are detected in the liver section as hepatic phenotype, suggesting that some BM-derived cells have been involved in liver regeneration process. Liver injury by CCl4 leads to improvement in hematopoietic chimerism with respect to the donor cells, possibly through the activation of cell cycle. Further, in chronic liver injury a small number of BM resident endothelial progenitor cells (EPCs) are found to mobilize in the peripheral blood.

Bone tissue engineering

In tissue engineering project, we have established a calvarial bone defect model in rats to study bone regeneration using a silk-based scaffold in the presence and absence of bone forming cells. It has been observed that implantation of empty scaffold (without cells) on the defect bone results in its mineralization, as depicted by calcium deposition. This has been analyzed by X-ray and Von Kossa staining. As a part of cellular plasticity research, we have also worked on rat adipose-derived stem cells (ADSC) for differentiation into osteogeneic cells. In future, these cells will be grown on this scaffold for implantation in defective bone, for early recovery.

C. Ovarian cancer stem cells

Orthotropic transplantation of ovarian surface epithelial tumour cells (ID8) into mouse ovarian bursa results in the formation of highly metastatic tumour. Upon isolating tumour cells, it has been observed that the GFP^+ID8 cells gain certain hematopoietic markers, the expression of which is otherwise absent in the cultured cells. Further analyses of the results suggest that host bone marrow cells play an important role in tumour progression. Not only HSCs are recruited to the primary tumour stroma and ascitic fluid, stem cells markers are also found to be expressed in the tumour cells. Quantitative real-time PCR and fluorescence *in situ* hybridization (FISH) reveal that the expression of the hematopoietic phenotype in epithelial ovarian cancer cells is the result of cell-cell fusion, which probably occurs because of the close interaction between the two cells types. Apart from the hematopoietic phenotype, fused cells also express chemokine receptor (CXCR4), which renders a superior migratory property when compared to the crude tumour cells. The existence of a stem cell compartment within this fused population could implicate fusion as one of the mechanisms responsible for the origin of cancer stem cells. To check if this phenomenon is also seen in human ovarian cancer, cells from the ascitic fluid of four different patients have been analyzed for the co-expression of cancer biomarker

(EpCAM) with pan hematopoietic marker (CD45). All the four samples show the presence of a significant fraction of CD45-expressing cancer cells. These fused cells are found to contribute to the expression of cancer stem cell markers like CD133, CD34 and CD44/c-Kit along with the pro-migratory marker CXCR4. These findings indicate that the presence of hemato-epithelial compartment of cancer cells is not restricted to mouse ovarian carcinoma. The fusion phenomenon with tumour and hematopoietic cells has been confirmed in Lewis lung cancer (LLC) model too, by using a dual reporter system.

D. Fibrosis and molecular interplay

In chronic injuries there is abnormal muscle repair and its end stage is fibrosis, the common pathway taken virtually by all chronic degenerative muscular diseases. There have been various studies conducted on the factors that contribute to fibrosis as an individual, but the enigmatic interactions of the satellite cells with the myofibroblasts that lead to fibrosis remains unclear. It has been hypothesized that the contributors of fibrosis up-regulate certain gene(s), which are responsible for the negative regulation of cell cycle. The expressions of these genes influence the activated satellite cells to return back to quiescence, thus leading the path to aberrant regeneration. In past two years we have identified the expression profile of few such genes, spanning from cell cycle regulators (e.g. *Rgs2, cullin1*, etc.) to negative regulators of myogenic process (e.g. *MyoR, HeyL, and Gli2, etc.*), along with extracellular matrix secretion regulators (e.g. *SPARC* and *SMOC2*, etc.). The trends of these genes' expressions are shown to correspond with the results obtained in histopathology, where there is a reduction of satellite cell population during the early phase, later their number seems to increase. These results explain that even on activation of parenchyma cells, normal process to regeneration is inhibited due to early entry of cells into quiescence.

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Regulation of cell death

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The overall theme of the research program is to elucidate the processes that influence cell death programs under varying physiological conditions in diverse model systems.

Broadly, our research programme explores the underlying mechanisms of cell survival and death in diverse intracellular and extracellular conditions. Using different experimental approaches we explore the precise mechanisms by which cells die, how these processes are regulated by diverse signaling pathways, inter-relationship between the various death processes and the evolutionary significance of cell death.

A. Cell death in protozoan parasites

One of our experimental model systems is a protozoan parasite that branched early during eukaryotic evolution and survives in disparate biological environments during its life cycle. It provides an interesting system for studying cellular pathways leading to death in contrasting environments. Some of these pathways may be universal features of eukaryotic cells and would help us understand how more complex higher-eukaryotic regulatory systems evolved.

Defensive mechanisms in Leishmania donovani

Consequent to data reported last year, we sought to investigate the function of the mTXNPx in terms of cellular response when the protein was incorrectly targeted and the mechanism of the transport. The translocation of mitochondrial proteins is relatively unexplored in lower eukaryotes. Many mitochondrial proteins especially the inner-membrane and matrix ones normally encode a transient cleavable N-terminal extension of about 20-50 amino acids that functions as a MTS. To validate if this region actually serves as a MTS *in vivo*, a construct expressing the mTXNPx with a 30 amino acid deletion from the N-terminus and with a GFP tag at the c-terminus was episomally overexpressed in *Leishmania* promastigotes. Another construct

with the full-length native protein without any deletion and with a c-terminal GFP was also expressed. The native mTXNPx-GFP transfectants showed mitochondrial localization of the episomally expressed GFP-fusion protein. The truncated recombinant protein demonstrated predominant localization within the cytoplasm demonstrating that absence of the MTS failed to translocate the protein to the mitochondria. Localization of the protein was confirmed through sub-cellular fractionation studies. Complete cytosolic localization of the truncated protein clearly indicated the functional importance of the signal sequence. However, this incomplete translocation did not interfere with cell survival which could be due to the presence of the endogenous enzyme within the mitochondria or the cytosolic presence of the overexpressed enzymatically active protein was sufficient to protect the cells. It is known that MTS is cleaved upon mitochondrial translocation in many organisms but it is not known if in the kinetoplastid parasites the signal is cleaved or not. Our studies show that upon translocation of the mTXNPx enzyme, the MTS is cleaved. The MTS contained a CaM binding site that generated a hypothesis that CaM may be essential for mitochondrial translocation. Constructs containing mutations in the CaM binding site on the MTS were episomally overexpressed in promastigotes of *L. donovani* to generate parasites with mutations in the CaM binding site of the MTS of mTXNPx. The various strains generated contained either 2,3 or 5 mutations in the CaM binding site such that the mutations do not interfere with the amino acids required for mitochondrial transport. Native protein tagged to GFP co-localized with Mitotracker red confirming mitochondrial localization. This co-localization pattern decreased in transfectants expressing the mutant proteins, the maximum decrease occurring in transfectants with 5 mutations. This clearly indicated that substitution of 5 amino acids in the CaM binding site did interfere with transport to the mitochondria while substitution of 2-3 amino acids resulted in translocation with decreased efficiency. Thus, we propose that mTXNPx requires CaM for translocation.

CYP450s of Leishmania donovani

We have earlier generated parasites expressing CYP710, a sterol desaturase. These parasites express much less sterols than their normal counterparts and are susceptible to reactive oxygen species (ROS). Interestingly, the normal counterparts with normal sterol levels showed less sensitivity to ROS. Drug treatment resulted in an increase in levels of a number of sterols in *Leishmania*, both during a shorter regimen as well as a longer regimen of treatment. Drug generated ROS or directly added ROS increased sterol levels. Presence of sterol biosynthesis inhibitors during drug treatment increased cell death. The presence of antioxidants prevented sterol increase and decreased cell death. These studies propose that sterols can function as anti-oxidants.

B. Mechanisms underlying cell death in cancer

The primary purpose of this programme was to investigate how chemotherapeutic stress influenced both the processes of apoptosis and autophagy in EC cells and if chemotherapeutic efficacy could be improved through manipulation of either of the processes. EC cells are malignant surrogates for the normal stem cells of the early embryo and express characteristics of both stem cells and cancer cells. Our earlier studies established that EC cells (NT2D1) are sensitive to cisplatin and undergo death by apoptosis at certain doses. Microarray studies identify both decrease and increase in expression of autophagy and apoptosis related genes at doses that do

not induce apoptosis but induces autophagy. Unlike many malignant cells, these cells express wild-type p53, a major modulator of apoptosis and autophagy. In cells expressing the wild-type p53, apoptosis was preceded by increased autophagy after cisplatin exposure, however, this autophagy was not required for the apoptosis because inhibition of autophagy did not stop apoptosis. Lowering of p53 levels through shRNA mediated interference resulted in resistance to apoptosis and increase in Beclin-1. Beclin-1, known as the autophagy protein interacts with several cofactors and regulates the lipid kinase Vps34 protein. It promotes the formation of Beclin-1-Vps34-Vps15 core complexes, responsible for inducing autophagy. The high Beclin-1 expression in shp53 cells correlated with the higher basal autophagy observed in these cells. Expression of Beclin-1 increased in wtp53 cells with increase in cisplatin dose. At higher cisplatin dose, Beclin-1 expression decreased in wtp53 cells with concomitant PARP cleavage suggesting apoptotic activity instead of autophagic responses. Cisplatin treatment did not significantly influence either Beclin-1 expression or autophagic vacuole formation in p53 down-regulated conditions where autophagy and Beclin-1 were constitutively higher. As reported earlier, inhibition of autophagy through pharmacological inhibitors or through shRNA mediated down-regulation of ATG-5 or Beclin-1 increased apoptotic death. Xenografts were induced *in vivo* with the EC cells in nude mice where the sh53 cells with higher basal autophagy showed the growth of much larger tumors in 60 days' time as compared to cells with normal complement of p53. To expand the *in vitro* work to gain an insight on how autophagy inhibition would alter the outcome of cisplatin induced cell death *in vivo*, we used 30 day old tumors developed in nude mice to see the effects of autophagy inhibition after cisplatin treatment. While drug treatment reduced wtp53 tumor development in terms of size and weight, the presence of wortmannin, the autophagy inhibitor during cisplatin treatment, the reduction in tumor size was significant. Similar observations were also made with the shp53 cell induced tumors. The observations were commensurate with *in vitro* observations of partial resistance of sh53 cells to cisplatin induced effects on cell death.

Therefore, in the above studies, we demonstrate a complex interplay between apoptosis and autophagy where apoptosis was preceded by autophagy but autophagy was not required for apoptotic death. Autophagy inhibition pushed the cells towards apoptosis that was measurable in reduced tumor sizes *in vivo* and increased apoptosis of cells *in vitro*. Therefore, autophagy inhibition during drug treatment can potentially improve efficacy of the treatment. These data can help unravel the underlying molecular mechanism of autophagy in DNA-damaged embryonal carcinoma cells and also provide a rationale for clinical evaluation of autophagy inhibitors in combination with DNA-damaging chemotherapy in human embryonal carcinoma.

Satish Kumar Gupta

Cellular and molecular aspects of reproduction and viral infections

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Keeping in view of the critical role of zona pellucida (ZP) glycoproteins during fertilization, our group is evaluating their potential as candidate for developing contraceptive vaccines for the management of wildlife population including street dogs. These efforts are being complemented by additional use of spermatozoa specific recombinant proteins. Other areas of study include evaluation of the role of cytokines and hormones in regulating the trophoblast cell invasion and differentiation; identification and characterization of novel anti-HIV compounds from medicinal plants or from synthetic sources; and development of influenza virus neutralizing monoclonal antibodies.

1. To develop contraceptive vaccine for the management of wildlife population.
2. To understand the molecular mechanisms associated with invasion and differentiation of trophoblast or trophoblast derived cancer cells.
3. To discover molecules with anti-HIV activity for their application as potential microbicide.
4. To develop influenza virus neutralizing monoclonal antibodies.

A. Development of contraceptive vaccine for street dog population management

Last year we had reported in mice, immunogenicity and contraceptive efficacy of recombinant protein encompassing

promiscuous T cell epitope of tetanus toxoid [TT; amino acid (aa) residues 830-844] followed by di-lysine linker, dog zona pellucida 3 (dZP3; aa residues 23-348) without His6-tag cloned in pQE-60 vector and expressed in M15[pREP4] strain of *E. coli* (TT-dZP3). Further, sera from TT-dZP3 immunized mice recognized zona matrix of mouse and dog oocytes as visualized by indirect immunofluorescence. However, we did not get the desired results during upscaling the production of this protein in fermenter. Therefore, this year we cloned the same construct in pET-22b(+) vector and expressed TT-dZP3 in BL21[DE3]pLysS strain of *E. coli* as host. The protein was successfully produced at large scale in fermentor. Subsequently, the procedure to purify and refold the fermentor produced TT-dZP3 was optimized with a yield of 50-72 mg purified protein from 5 gm pellet of bacteria. The fluorescent spectroscopy revealed the wavelength for emission maxima (λmax) of refolded protein as 338 nm and for denatured sampled was 362 nm, suggesting the presence of defined secondary structure in refolded protein. CD spectrum of refolded protein showed negative peaks at ~218 nm and 208 nm, which is the hallmark for the presence of beta sheets and alpha helices present in the protein, respectively. Active immunization studies in female mice are in progress.

Additionally, immunization of female FvB/J mice with *E. coli*-expressed chimeric recombinant protein encompasing promiscuous T cell epitope of TT followed by di-lysine linker, dog ZP3 (aa residues 165-346), tri-glycine spacer and sperm specific protein, Izumo (aa residues 165-250) (TT-dZP3-dIz) led to a significant curtailment in fertility as compared to adjuvant control group. Antibodies generated against fusion protein reacted with anterior head of the acrosome-reacted mouse spermatozoa as well as mouse and dog ZP in an indirect immunofluorescence assay.

B. Molecular mechanisms associated with trophoblast invasion and differentiation

Comparative analysis of the invasion-associated genes expression pattern in first trimester trophoblastic (HTR-8/SVneo) and JEG-3 choriocarcinoma cells

Previous year we had reported that IL-11 mediated change in the expression of MMP23B as well as PIAS3 mediated sequestration of activated STAT3 was responsible for the IL-11 mediated increase and decrease in invasiveness of JEG-3 and HTR-8/SVneo cells, respectively. Further, to investigate whether IL-11 associated differential responsiveness was an attribute of the cellular model to study trophoblast invasion, we compared the expression of invasion-associated molecules in these two cell lines by performing cDNA microarray followed by quantitative RT-PCR. We have observed that HTR-8/SVneo cells have significantly higher invasiveness than JEG-3 cells, which might be due to higher expression of proteases and signaling intermediates of JAK/STAT and MAPK signaling pathways. Like extravillous trophoblasts (EVTs), a higher expression of functionally significant proteases like MMP1, MMP2, MMP9, MMP23B, PLAU etc in HTR-8/SVneo cells, project them as a close mimic of EVTs under *in vitro* conditions.

Role of Pappalysin 1 in Leukemia Inhibitory Factor (LIF) mediated invasion of HTR-8/SVneo cells

Previously we had reported that LIF increases the invasiveness of HTR-8/SVneo cells through activation of STAT1, STAT3 and ERK1/2 (thr202/tyr204) dependent signaling pathways. This was associated with increase in the expression of several invasion- associated genes. Taking that information further, we have validated the expression of invasion associated genes at

transcript and protein level as well as silenced the expression of pappalysin 1 to decipher its role in invasion. LIF mediated increase in the invasion of HTR-8/SVneo cells was associated with the upregulation of novel regulatory molecules like pappalysin 1, Podoplanin, SERPINB3, ICAM1, ID1, integrin B3 (which are also expressed by human placenta) and downregulation of the expression of TIMP1, TIMP2 and TIMP3. Silencing of pappalysin 1 expression by siRNA led to abrogation of LIF-mediated invasion of HTR-8/SVneo cells (Figure 1).

were silenced by shRNA and its impact on the forskolin mediated cellular fusion as well as gene expression of key fusion associated molecules like syncytin-1, syndecan-1 and CD98 were analysed. It was observed that with a decrease in hCG levels (confirmed by ELISA as well as qRT-PCR), BeWo cells showed decreased fusion as well as a significant decrease in the transcript for syncytin-1 and syndecan-1.

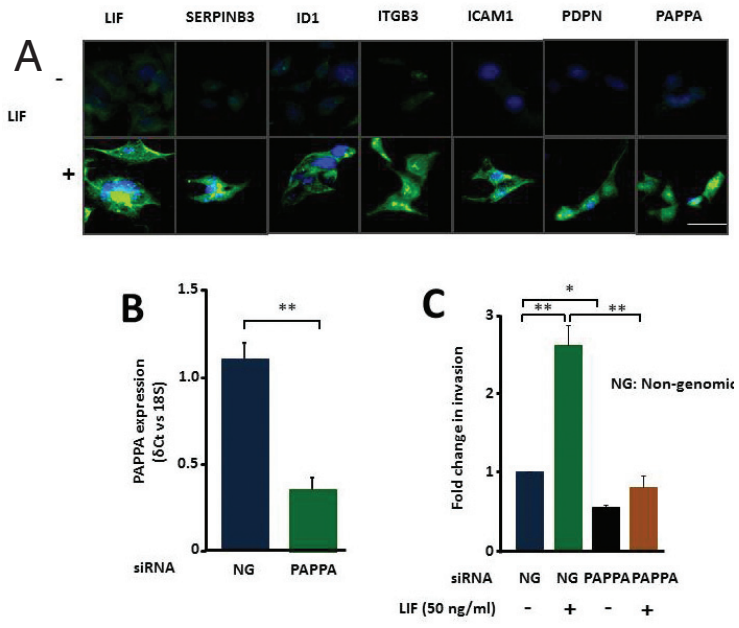

Figure 1: Effect of LIF on gene expression and role of pappalysin1 in LIF mediated increase in invasiveness of HTR-8/SVneo cells. Panel A: HTR-8/SVneo cells were treated with LIF for 24 h and probed for immunofluorescence using specific antibodies for PAPPA, podoplanin, ICAM1, ITGB3, ID1, SPB3 and LIF. Panel B: HTR-8/SVneo cells were transfected with either PAPPA siRNA or non-genomic siRNA for 72 h and level of silencing was checked by qRT-PCR. The transfected cells were used to study their invasive behavior in the presence or absence of LIF (Panel C). The results are expressed as mean ± SEM of fold change in invasion as compared to non-genomic siRNA transfected cells, observed in 3 independent experiments. *p<0.05; **p<0.001

Molecular mechanisms underlying the trophoblastic BeWo cell fusion

A significant proportion of unsuccessful pregnancies are due to implantation failure, which in turn could be driven by impaired syncytialization. Treatment of BeWo cells (used as model) with forskolin led to an increase in the transcript of α as well as β subunits of hCG concomitant with increased in cell fusion. Subsequently, expression of α and β subunits

C. Evaluation of anti-HIV activity and pre-clinical safety of medicinal plants and semi-synthetic compounds

To prepare herbal formulation for prevention of human immunodeficiency virus (HIV)-1 infection, extracts prepared from various plants have been evaluated for anti-HIV activity. Both aqueous (IC_{50} = 15 μg/ml) and 50% ethanolic (IC_{50} = 26 μg/ml) extracts prepared from leaves of NBRH-16 showed anti-HIV activity in TZM-bl cells-

based assay wherein the virus was treated with the extracts prior to infection. Further these extracts also inhibited virus load in HIV-1 infected CEM-GFP cells and human peripheral blood lymphocytes (PBLs). The anti-HIV activity was found to be mediated through inhibition of HIV-1 protease activity. Both the extracts exhibited no deleterious effect on the integrity of monolayer formed by epithelial Caco-2 and HEC-1A cells. The extracts were also found to be safe upto 100 μg/ml when tested for their effect on viability of *L. plantarum, L. fermentum, L. rhamnosus and L. casei*. The extracts (100 μg/ml) did not reveal any cytotoxic effect on vaginal keratinocytes (Vk2/E6E7). Levels of pro-inflammatory cytokines secreted by Vk2/E6E7 cells treated with aqueous and 50% ethanolic plant extracts were within the non-inflammatory range. The extracts from NBRH-16 may be useful in formulating herbal formulation for topical application to prevent sexual transmission of HIV.

Further, in collaboration with Dr. Sujata V. Bhat, Labdane analogues have been synthesized. Three compounds showed potent anti-HIV activity, which was further supported by inhibition of integrase activity and computational studies.

D. Neutralizing monoclonal antibodies (MAbs) against influenza virus

Humanization strategy of MA2077 (IC_{50} of 0.08 μg/ml, KD = 2.1±0.4 pM) against pandemic H1N1 by grafting of CDR (2077Hu1) or CDR and other important framework residues (2077Hu2) was reported last year. Full length expression of these antibodies in HEK-293T cells followed by ELISA against pandemic H1N1 showed that 2077Hu2 was able to bind to pandemic H1N1 significantly with minimal difference as compared to parental antibody; whereas 2077Hu1 failed to show any reactivity (Figure 2). In addition, we have been able to isolate one more MAb neutralizing pandemic H1N1 and 5 additional antibodies neutralizing seasonal H1N1. Two of these MAbs also broadly cross-react with the H2 as well as H5 group influenza HA proteins. Characterization and humanization of these antibodies is being carried out.

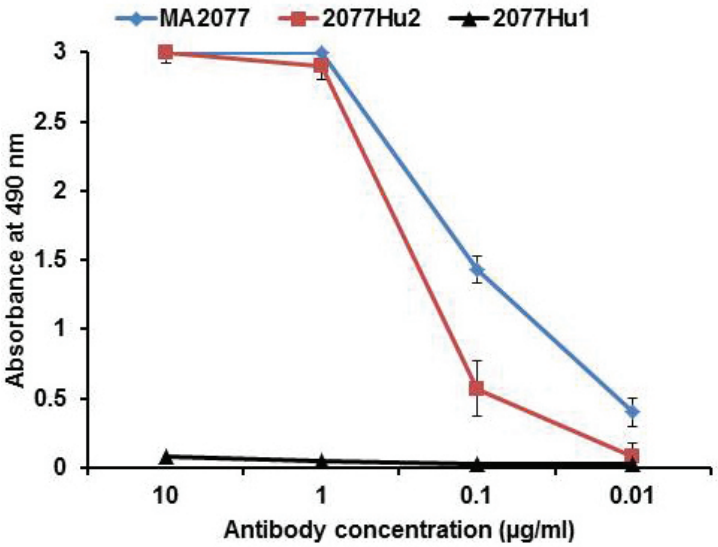

Figure 2: ELISA cross-reactivity of purified MAbs; MA2077, 2077Hu2 and 2077Hu1; against pandemic H1N1 virus.

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7. Gupta N, Shrestha A, Panda AK, Gupta SK* (2013) Production of tag-free recombinant fusion protein encompassing promiscuous T cell epitope of tetanus toxoid and dog zona pellucida glycoprotein-3 for contraceptive vaccine development. *Mol Biotech* **34**: 853-862.
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1. Gupta SK, Gupta N, Shrestha A, Panda AK (2012) A process for production of tag-free recombinant fusion protein encompassing promiscuous T cell epitope of tetanus toxoid and dog zona pellucida glycoprotein-3 and its use as contraceptive vaccine (1269/DEL/2012, Indian Patent filed on 24/04/2012).
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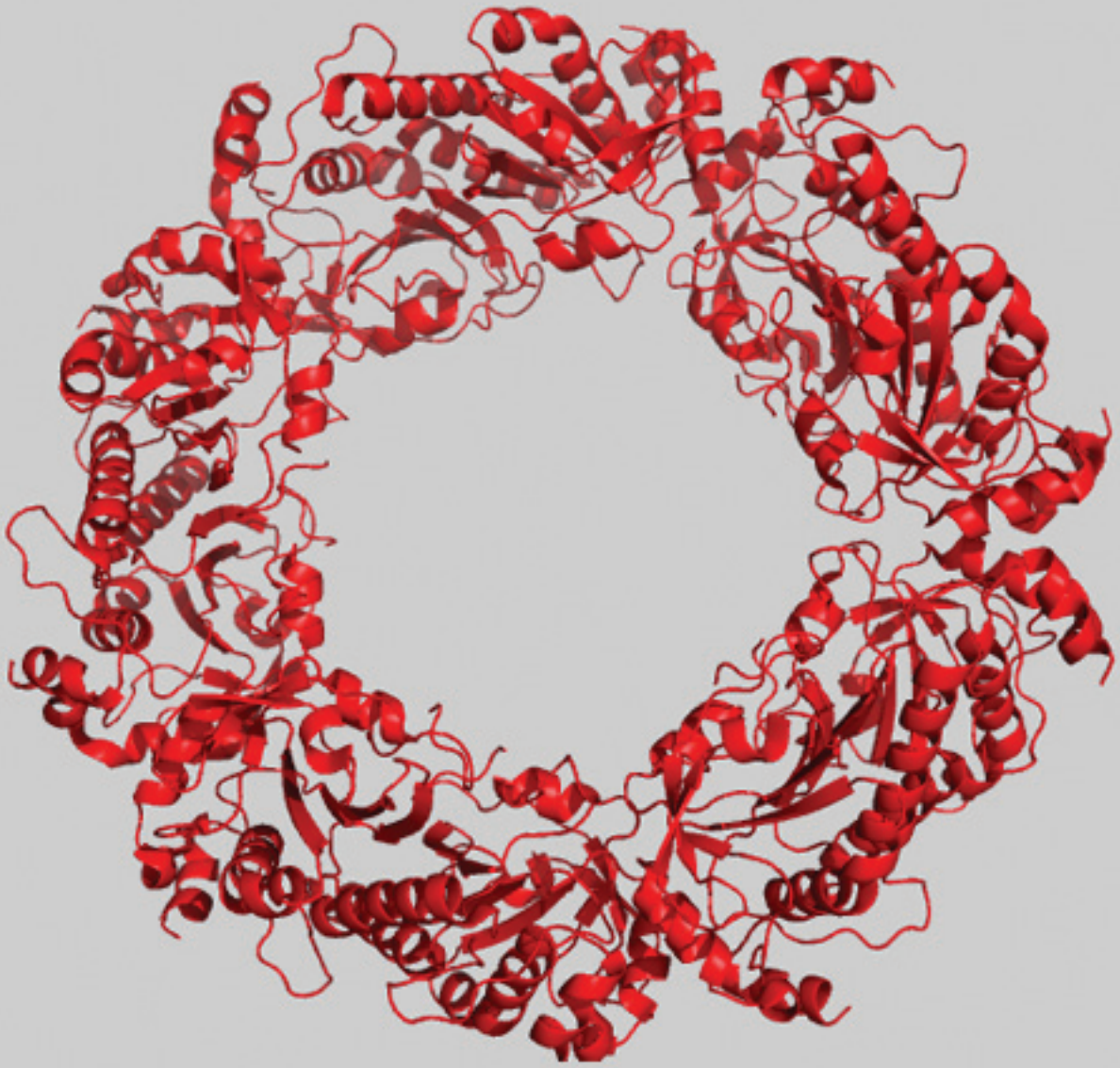
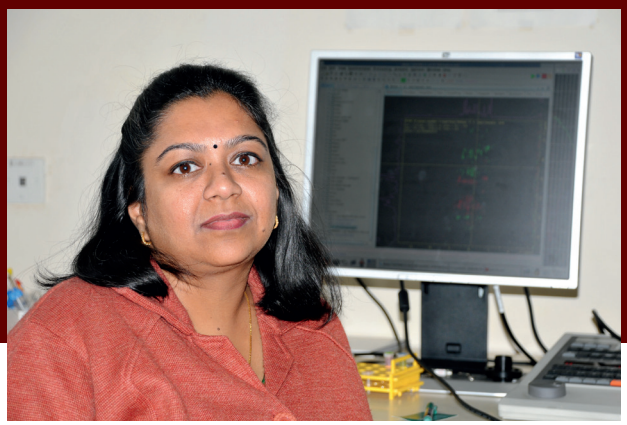


Model of *Leishmania donovani* cytosolic tryparedoxin as a decamer visualized through PyMOL

MOLECULAR DESIGN



Monica Sundd

Structural studies on proteins, dynamics and ligand interactions using NMR

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The theme of our research is to understand the structure and dynamics of proteins using NMR, and other biophysical techniques to understand their function. Presently, we are working on several proteins, viz. ubiquitin, acyl carrier protein, acyl CoA binding protein etc., all involved in protein-protein interactions.

The primary objectives of the project involve cloning, expression, purification and structural characterization of various proteins using NMR and study their interaction with the naturally occurring partners with a goal to better understand their biological function.

We are interested in understanding the structural and functional aspects of type I and type II fatty acid biosynthesis pathways, in order to exploit their disparities for drug targeting. With regard to type I fatty acid pathway, we are using human system as the representative model, while the type II pathway is being studied in *P. falciparum* and *Leishmania*. Interestingly, both type I and type II pathways share very similar proteins, viz. the acyl carrier protein (ACP) that plays a central role by protecting the acyl chain from the hydrophobic environment. How this protein differs with regard to its functions in the two pathways remains to be fully understood. Moreover, the structural differences that contribute to the difference in function remains unclear. Over the past one year, we have completed the structure solution of *Leishmania major* holo-acyl carrier protein, a novel protein using NMR and also characterized its acyl-ACP intermediates (Figure 1).The holo-

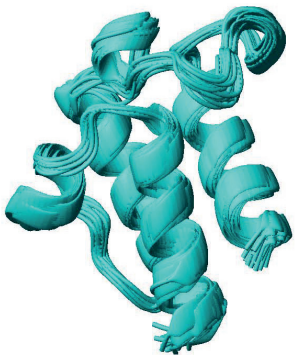

Figure 1: Lowest twenty NMR structures of Leishmania holo-ACP.

ACP of *Leishmania* is a four helix bundle protein, with a hydrophobic cavity. An important question we are trying to address in this study is: Does the type II pathway of *Leishmania major* at all contribute to fatty acid biosynthesis. If yes, then what is the function of this pathway? Very

little if any information is available with regard to this pathway in *Leishmania*. Given the fact, that the thiolactomycin analogs that target fatty acid enzymes like acyl carrier protein acetyl transferase and 3-oxo-acyl-ACP synthase kill *Leishmania donovanii* cultures completely offer indirect proof of an important role of this pathway in its survival. Our structural studies too support the latter findings. The acyl ACP of *Leishmania* forms acyl intermediates, and upto –C8-ACP, the intermediates are fairly stable. The acyl-ACP intermediates longer than C8 were observed to be relatively unstable at room temperature in comparison to *P. falciparum* acyl-intermediates. We are trying to extrapolate how this difference in stability would effect the function of the protein as compared to other type II ACPs. We have also carried out some *in vivo* studies on the type II pathway of *Leishmania* and our data suggests that the ACP along with other enzymes like ENR, Phosphopantetheine transferase are localized to the mitochondria. Microarray data from GEO NCBI and http://tritrypdb.org suggests that there is a marked increase in the expression of ACP and ENR based on RNA analaysis in the amastigote stage as compared to premastigotes.

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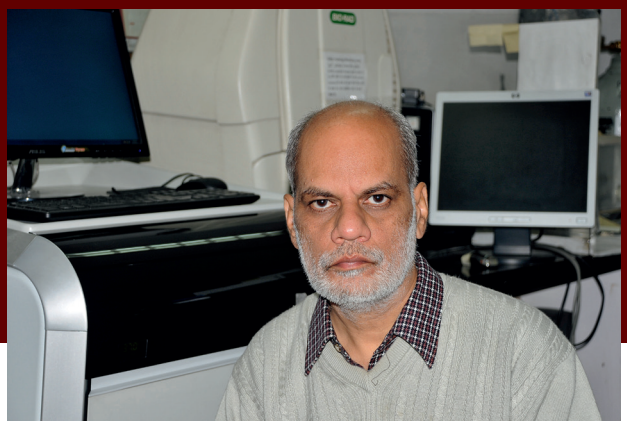

Pramod K. Upadhyay

To develop strategies for making sensors and actuators for biological processes

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Aim of research

1. To develop systems for monitoring biological processes.
2. To develop tools for needle free immunization.
3. To study the biological processes like differentiation, hybridization etc. and to develop devices and sensors based on such studies.

***Ex-vivo* 3D liver culture**

The successful generation of hepatocyte like cells *in vitro* will have clinical applications as it may give a cell based therapeutic option for patients who are suffering from liver cirrhosis.

Hematopoietic stem cells (HSCs) are known to differentiate to hepatocytes *in vitro* but it is difficult to get significant number of HSCs from adults. Earlier we have shown how to reprogram peripheral blood mononuclear cells (PBMCs) to 'hematopoietic stem like cells'.

The hepatocytes like cells from the monocytes of HBV infected (HBsAg positive) patient has been generated. A brief description of steps involved is as follows.

Isolation of $CD14^+$ monocyte from PBMCs

Blood from HBsAg positive patient was mixed with equal volume of RPMI 1640. Density gradient centrifuge was done using Ficol Paque density gradient solution to isolate PBMCs from blood. From the suspension of PBMCs, $CD14^+$ monocytes were isolated using CD14-MicroBeads. The isolated $CD14^+$ monocyte cells ($1.3 \times 10^7/cm^2$) were allowed to adhere to tissue culture plastics plates coated with Matrigel for 1–2 hours in RPMI 1640 medium containing 10% FCS, 2 mmol/L glutamine, 100U/mL penicillin, and 100 μg/mL streptomycin.

Reprogramming $CD14^+$ monocytes

After 2 hr. of incubation, supernatant was

discarded and fresh RPMI 1640 media supplemented with 10% FCS, 140 μM of β-mercaptoethanol, 5 ng/ml of MCSF, 0.4 ng/ml of IL-3 added to the culture plate. The culture continued for 6 days.

Differentiation of reprogrammed CD14⁺ monocytes

After the reprogramming process, differentiation of the cells was done by replacing the supernatant with fresh RPMI 1640 media supplemented with 10% FCS solution, 10 ng/ml EGF, 20 ng/ml HGF and 3 ng/ml FGF-4. The culture was continued for 21 days.

Characterization of differentiated cells

After 21 days of differentiation, the cells were examined for the presence of cytosolic albumin and Connexin 32. A representative result is given below, which shows cytoplasmic albumin (Figure 1A) and Connexin 32 (Figure 1B) produced by hepatocyte like cells derived from the monocytes of HBsAg positive blood sample.

qPCR based characterization differentiated cells at transcript level

After 21 days of culture, relative expression of transcripts in differentiated hepatocytes like cells derived from the monocyte of HBsAg positive blood sample were determined with respect to HepG2 (positive control) and hepatocytes like cells derived from the monocyte of healthy blood sample. It was found that hepatocyte like cells have expression of Albumin and HNF-4. The expression of HNF-4 in hepatocyte like cells from healthy blood sample is as high as that of HepG2. The expression of HNF-4 in hepatocyte like cells derived from HBsAg positive blood sample was 100 fold lesser than that of hepatocyte like cells from healthy blood sample. The expression of Albumin in hepatocyte like cells derived from healthy blood sample was 10 fold higher than that of hepatocyte like cells from HBsAg positive blood sample. The CK18 expression in hepatocyte like cells derived from HBsAg positive blood sample was higher than that of HepG2, hepatocyte like cells from healthy blood sample and PBMCs at Day 0.

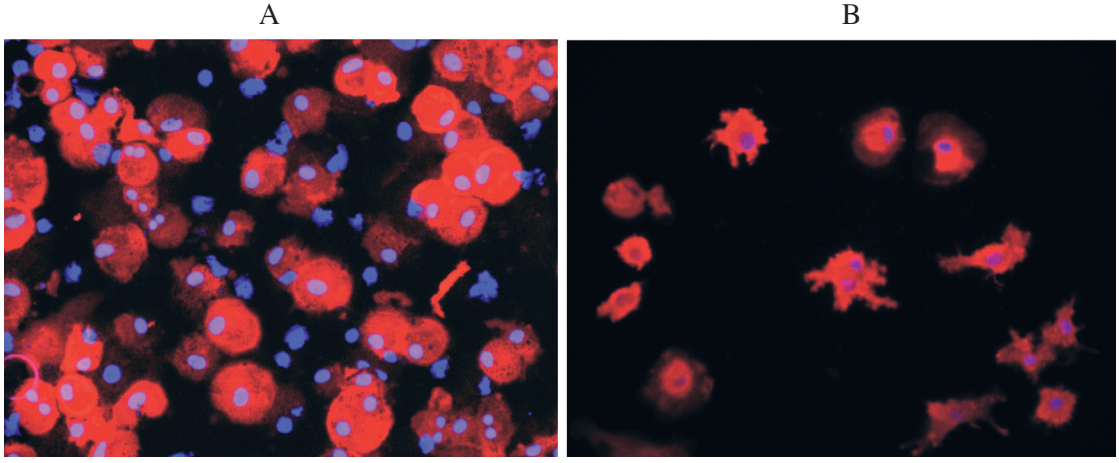

Figure 1: Albumin (A) and Connexin 32 (B) in the hepatocyte like cells differentiated from reprogrammed monocytes. Blue (DAPI) represents nucleus, Red (Alexa Fluor 588nm) represents cytoplasmic albumin (A) and Red (Alexa Fluor 594) represents Connexin 32(B).

Drug metabolism assay

The functional viability of the hepatocyte like cells derived from PBMCs of HBsAg positive blood sample was determined. The P-450 enzyme of hepatocytes detoxifies 7-pentoxy resorufin to resorufin which has a fluorescent emission at 585nm. In a typical experiment, HepG2 (positive control), HBV1 and HBV2 (hepatocytes like cells derived from HBsAg positive blood samples and PBMCs (Day 0, healthy blood) were incubated with 7-pentoxy resorufin for 24 hr. After incubation, the fluorescent spectrum was recorded. It was found that HepG2, HBV1 and HBV2 were able to detoxify 7-pentoxy resorufin and PBMCs had no such activity.

Trans-differentiation of PBMCs to endothelial-like cells

Endothelial cells occupy the core position in the successful treatment of vascular diseases. PBMCs can be trans-differentiated to endothelial like cell by culturing them in an appropriate angiogenic medium. Being abundant in number and easily harvestable, this method is very promising source of autologous endothelial cells for use in vascular treatment.

A composite culture of PBMCs obtained by means of a density gradient centrifugation was cultured in EGM-2 medium, supplemented with a cocktail of angiogenic growth factors including VEGF, EGF, FGF and IGF and fortified with serum and the culture continued for 30 days, with one half of media change every 3 days.

After 30 days in culture, the PBMCs differentiated into endothelial-like cells as shown by the expression of various endothelial markers verified by immunostaining, western bloting and RNA expression profiling using qPCR. The differentiated endothelial-like cell also showed good proliferation as well as functional properties similar to the endothelial cells as verified by the wound healing assay.

Publications

Original peer-reviewed articles

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Protease-catalyzed splicing of peptide bond

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We study the principles underlying peptide ligation reactions catalyzed by proteases and transpeptidases with a view to apply them to the semisynthesis of proteins, assembly of well defined bioconjugates and protein dendrimers that may be useful in a variety of biotechnological applications. Transpeptidase sortase that catalyzes covalent anchoring of surface proteins to the cell wall in gram-positive bacteria has turned out to be a wonderful enzyme in this endeavor. The propensity of sortase to ligate LPXTG proteins to an aminoglycine-derivatized moiety offers unprecedented opportunities in synthetic protein chemistry.

Objectives

1. Sortase-mediated protein labeling and conjugation
2. Studies on structure, dynamics and function of sortases.

Objective 1

In our previous work, we had utilized lysine-based multiple antigenic peptide (MAP) scaffold for creation of multivalent proteins. However, assembly of multivalent constructs can be achieved using a variety of chemically disparate scaffolds endowed with special and unique attributes viz., shape (linear, cyclic or dendrimeric), spacing, flexibility and hydrophilicity. β-cyclodextrin (β-CD), a naturally occurring carbohydrate scaffold appeared to be important in this regard. We adapted the Sortase-Click strategy for display of multiple copies of proteins on β-CD. For this β-CD was converted to per-6-deoxy-6-azido-β-CD and subjected to click reaction with PspA-alkyne obtained by sortase-mediated ligation. SDS-PAGE and size exclusion chromatography of the reaction mixture indicated a 50-60% conversion of monomeric protein into high molecular weight species thus demonstrating the versatility of Sortase-Click strategy for macromolecular assemblage on a variety of dendrimeric scaffolds.

Objective 2

During the current period of review catalytic residues of pSrtA were delineated through a combination of mutagenesis and kinetic

experiments. The crystal structure is continued to be refined and analyzed with a view to delineate the mechanistic imperatives of catalysis. Contemporaneously, attempts were also focused towards elucidating the substrate recognition mechanism in archetypal sortase A (SaSrtA) from *Staphylococcus aureus*. SaSrtA is considered a *bona fide* target of therapeutic intervention besides serving as a versatile tool in protein labeling/engineering and bioconjugation. The question as to how SaSrtA recognizes the LPXTG peptide substrate is central to the design of inhibitors as well as to expand its synthetic utility.

SrtA displays an eight strand β-barrel fold in which β-strands are connected by multiple loops and two helices. The loop regions, especially those connecting the β6 and β7 strands, located in the vicinity of the active site, are observed to be highly disordered and mobile. Substrate bound structures of SaSrtA are reported by both NMR and X-ray crystallography. While the LPETG peptide substrate is seen in an extended conformation in the crystal, LPATG peptide covalently linked to the active site Cys184 assumes an "L-shaped" conformation due to a kink in Ala-Pro peptide bond. NMR structure also reveals severe restriction of β6-β7 loop movement in the substrate bound structure. Recent molecular dynamics (MD) simulation studies have indicated that both poses may be genuine and can be reconciled with a conformational selection cum induced fit model of substrate recognition. Based on this, we have investigated if the "kinked" conformation generated as a consequence of Pro residue in LPXTG substrate connects dynamics, recognition and catalysis in SaSrtA. The results gleaned from MD simulations of sortase-substrate complex using an experimentally validated minimal SaSrtA recognition motif, experiments with SaSrtA mutants and designer peptide substrates endowed with kinked conformation suggest the following: (a) The kinked conformation is essential for productive binding but does not per se control the loop dynamics, (b) Leu of the substrate plays an important role in immobilizing the β6-β7 loop, (c) The β6-β7 loop engages Leu and Pro residues of LPXTG substrate through hydrophobic interactions mediated by residues Leu169 and Ile182, (d) The residue at "X" position provides optimum backbone separation between binding site anchor residues (Leu-Pro) and scissile peptide bond (Thr-Gly). The overall results appear to be consistent with a conformational selection model of substrate recognition.

Publication

Original peer-reviewed article

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Patents

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Sarika Gupta

Therapeutic interventions in chronic diseases: Investigations on the effects of homocysteine on bone remodelling

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My group is a multi-disciplinary group adopting an integrated approach in drug discovery that combines medicinal chemistry, basic biology and biochemistry principles for efficient drug design process. Interests of the group lie in identifying underlying principles in a disease pathogenesis to discover new targets, designing molecular intervention strategies and confirming the biological/therapeutic activities of the designed compounds. The small molecule regulators contribute to both drug development and understanding biological systems in human body.

Hyperhomocysteinemia, a condition marked by elevated levels of homocysteine in circulation is a causative factor for altered bone remodeling and osteoporosis. However the rationale for this is ambiguous. A focal point of research in our laboratory is assessing reasons for such altered bone remodeling so that modulation of signaling mechanisms can serve as a therapeutic modality for treatment of bone loss during hyperhomocysteinemia.

To dissect molecular signaling pathways that participates in OPG and RANK ligand synthesis in the osteoblast and to investigate cellular mechanisms that contribute to altered RANK ligand and OPG synthesis during hyperhomocysteinemia.

Preliminary data showed that the oxidant nature of homocysteine facilitates abnormal sRANK ligand synthesis in osteoblast cultures and inhibits the synthesis of OPG in homocysteine treated osteoblast cultures. An interesting observation was the ability of radical scavenger N-acetyl cysteine to revert OPG:RANKL ratio towards normalcy in homocysteine treated osteoblast culture (Figure 1A-C). In view of the ability of an antioxidant to reinstate OPG:RANKL ratio in homocysteine treated osteoblast culture, we presumed the role of anti-oxidative signaling molecules in OPG and RANK ligand synthesis in

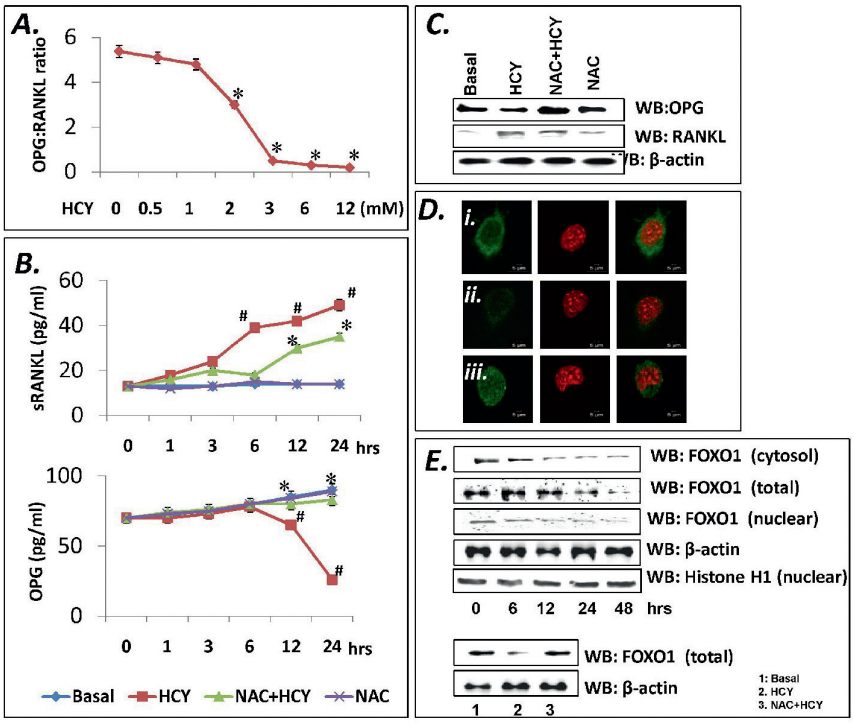

Figure 1: Effect of homocysteine on RANKL, OPG synthesis and FOXO1 expression in MC3T3E1 osteoblasts. Osteoblasts were grown in osteogenic medium containing β-glycerophosphate (10 mM) and ascorbic acid (100 μg/ml) for 5 days. (A) OPG/RANKL ratio measured by ELISA in osteoblast culture treated with increasing concentration of homocysteine (0.5-12 mM). (B) sRANK ligand and OPG levels in culture supernatants of osteoblast cultures exposed to homocysteine (3 mM) and N-acetyl cysteine (5 mM); alone and in combination for 24 hours as measured by ELISA. Results are mean ±SEM (n=3; #P<0.05 vs. Basal; *P<0.05 vs. HCY). (C) Expression status of OPG (top) and RANK ligand (middle) by Western blot. sRANK ligand and OPG expressions in homocysteine treated total cell lysates were measured at 3rd and 12th hours respectively. (D) Confocal images showing changes in the expression of FOXO1 in osteoblasts after 24 hours of treatment with homocysteine (3 mM). Cells were immunostained with antibodies against FOXO1. The secondary antibody was goat anti-rabbit F(ab')2 fragment conjugated with Alexa 488 (for green color). Representative images are in split view showing channel green for FOXO1 and red for DAPI (nuclear counter staining). Photomicrographs were acquired on Axio imager Z1 (Carl-Zeiss). Scale bar, 5 μm. (E) Changes in the expression of FOXO1 in nuclear and cytosolic fractions of homocysteine (3 mM) treated osteoblasts at different time-periods by Western blot. Changes in the expression of total FOXO1 after N-acetyl cysteine pre-treatment is also shown. Osteoblasts were pre-treated with N-acetyl cysteine (5 mM) for 15 mins prior to homocysteine treatment. One of the three experiments with similar result is shown. HCY=*homocysteine; NAC=N-acetyl cysteine.*

the osteoblast. The role of FOXO1 transcription factor was therefore evaluated.

Our *in vitro* studies using MC3T3E1 osteoblast cultures showed that total FOXO1 expression in homocysteine treated cell cultures declined in a time dependent manner (Figure 1D-E) and this was attributed to phosphorylation of protein phosphatase 2A (PP2A) (S566), a physiological regulator of insulin-sensitive FOXO1 signaling pathway (Figure 2A-C). Downregulation of homocysteine induced PP2A phosphorylation improved FOXO1 levels and also reinstated phosphorylated p38 and OPG synthesis in osteoblast culture (Figure 2D-F). Experiments employing siRNA mediated knockdown of

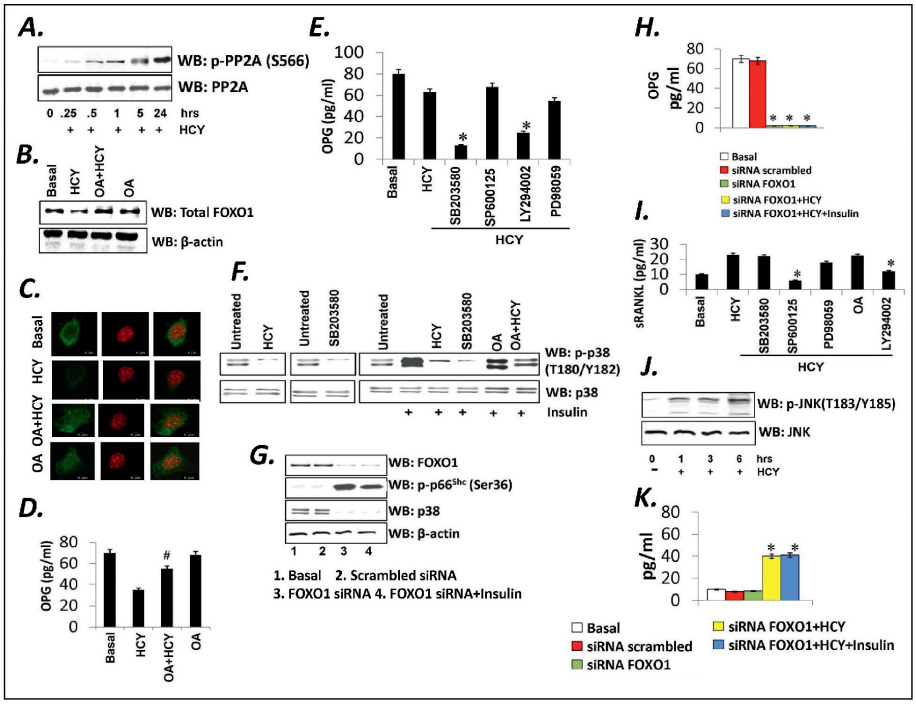

Figure 2: Homocysteine inhibits OPG synthesis involving FOXO1/p38 signaling and activates RANK ligand synthesis by c-Jun/JNK signaling in MC3T3E1 osteoblasts. (A) Changes in the expression of total and phosphorylated form of PP2A in osteoblasts by Western blot at specified time points after homocysteine (3 mM) treatment. (B-C) Changes in the expression of total and phosphorylated form of FOXO1 in osteoblasts after treatments with okadaic acid (40 ng/ml) and homocysteine (3 mM), alone or in combination as evidenced by Western blot and immunostaining. (D) OPG level measured by ELISA in osteoblast cultures exposed to okadaic acid (40 ng/ml; 15 min) and homocysteine (3 mM; 15 mins). Results are mean ±SEM (n=3; #P<0.05 vs. HCY). (E) OPG level measured by ELISA in osteoblast cultures exposed to SB203580 (25 μM; 20 min), SP600125 (10 μM; 20 min), LY294002 (10 μM; 20 min), PD98059 (10 μM; 20 min), and homocysteine (3 mM; 15 mins). Osteoblasts were pre-treated with the pharmacological inhibitors for specific time-periods before exposing to homocysteine. Results are mean ±SEM (n=3; *P<0.05 vs. Basal). (F) Western blot showing total and phosphorylated forms of p38 in homocysteine, SB203580 (25 μM; 20 min), okadaic acid (40 ng/ml; 15 min) and insulin (100 nM) treated cells; alone or in combination. (G) Western blot showing change in expression of FOXO1, p38 and $p66^{shc}$ in FOXO1 siRNA knockdown osteoblasts treated with and without insulin (100 nM). (H) Effect of FOXO1 siRNA interference on OPG synthesis in osteoblast culture. Scrambled siRNA was used as negative control. siRNA knockdown efficiency was 89% as evidenced by Western blot. Results are mean ±SEM (n=3; *P<0.05 vs. Basal and scrambled siRNA). (I) sRANK ligand level measured by ELISA in osteoblast cultures exposed to SB203580 (25 μM; 20 min), SP600125 (10 μM; 20 min), LY294002 (10 μM; 20 min), PD98059 (10 μM; 20 min), okadaic acid (40 ng/ml; 15 min) and homocysteine (3 mM; 15 mins). Results are mean ±SEM (n=3; *P<0.05 vs. HCY). (J) Western blot showing changes in the expression of total and phosphorylated JNK at specified time-points. Effect of FOXO1 siRNA interference on sRANK ligand synthesis in osteoblast culture. Results are mean ±SEM (n=3; *P<0.05 vs. Basal and scrambled siRNA).

FOXO1 showed that FOXO1 is integral to p38 and OPG synthesis (Figure 2G-H). Nevertheless sRANK ligand synthesis in osteoblast culture was not dependent on FOXO1 and the rationale for the augmentation in sRANK ligand synthesis was mainly JNK phosphorylation (T183/Y185) by homocysteine (Figure 2I-K).

In summary an alteration in the redox regulatory mechanism in the osteoblast due to decline in

FOXO1 and derangement in MAPK signaling cascades is a majpor contributory factor for the shift in OPG:RANKL ratio towards increased osteoclast activity and decreased bone quality during hyperhomocysteinemia.

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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.

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

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

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

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

Temperature dependent studies reveal existence of reversible intermediates during the dissociation of GDP-bound enzyme dimer

To understand how temperature regulates the product formation in hGBP1, we carried out temperature dependent GTPase assays using radiolabeled (α-^{32}P) GTP. Unlike GMP, GDP decreases with increase in the temperature. To get a further insight, steady-state kinetics assay was carried out in the temperature range

10-42°C and the k_{cat} for GDP and GMP was determined. The Arrhenius plot for GMP shows a nonlinear curve. The nonlinear dependence of k_{cat} for GMP with temperature clearly indicates that the conversion of GTP-bound enzyme complex to GMP must be associated with at least more than one step. The Arrhenius plot for GDP shows a nonlinear curve with positive slope in the temperature range 30-42°C, indicating that GDP decreases with increase in the temperature and the activation energy at this range is associated with a negative value. This implies that there should be an intermediate during the conversion of GDP-bound enzyme dimer to free GDP and the enzyme, which is shown in Eq. 1.

However, the Arrhenius plot for GDP shows a straight line with positive slope (i.e. negative activation energy) within the temperature range 30-42°C. Hence, the mechanism described above does not provide a complete description of the reaction. Alternatively, if the mechanism is considered as described in eq. 2, where, $k_{cat}^{GDP} = k_3.k_4/(k_3 + k_{-3} + k_4)$, it would give a linear Arrhenius plot with positive slope, if k_{-3} is much larger than the sum of k_3 and k_4. Under this condition, the equation will become, $k_{cat}^{GDP} \approx (k_3/k_{-3}).k_4 \approx K \times k_4$ (where K is the equilibrium constant for the first step of Eq. 2). The activation energy in this case would be equal to the sum of the enthalpy change for the first step and the activation energy for the second. Thus, the observed activation energy in the higher temperature range could be negative, if the first step becomes an exothermic reaction and the heat released associated with this is more than the activation energy of the next step. This possibility is in good agreement with our experimental data and hence the mechanism suggested in Eq. 2 appears to be valid. Additionally, it suggests that there is reversibility between GDP-bound enzyme dimer and GDP-bound enzyme monomer at higher temperature. The GDP-bound enzyme monomer further reversibly dissociates into the free enzyme and GDP.

At low temperature the Arrhenius plot for GDP is linear, suggesting that at lower temperature the activation energy associated with GDP is zero. This is possible, if the first step of the reaction described in Eq. 2 becomes exothermic and the heat change associated with this step is equal to the activation energy for the next step. This is in good agreement with our experimental data and therefore suggests that the decrease in temperature will favor the dissociation of GDP-bound enzyme dimer to GDP-bound enzyme monomer. This also clearly indicates that GDP formation from GDP-bound enzyme dimer is tightly regulated with temperature.

$$(\text{hGBP1.GDP})2 \xrightarrow{k_1} 2\ \text{hGBP1.GDP} \xrightarrow{k_2} 2\ \text{hGBP1} + 2\text{GDP} \qquad (1)$$

$$(\text{hGBP1.GDP})2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} 2\ \text{hGBP1.GDP} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} 2\ \text{hGBP1} + 2\text{GDP} \qquad (2)$$

Biochemical studies for the existence of a reversible intermediate

To verify the reversibility at higher temperature, we carried out GTPase assays with external GDP. GMP formation was marginally affected with increasing concentrations of external GDP. However, GDP was significantly reduced,

suggesting that external GDP reduces the dissociation of GDP-bound enzyme monomer to free GDP and the enzyme. This clearly indicates the reversibility between GDP-bound enzyme monomer, and free GDP and the enzyme. This also agreed well with GMP being a predominant product at higher temperature, where GDP-bound enzyme dimer preferably undergoes subsequent phosphate cleavage yielding GMP-bound enzyme dimer, which ultimately produces GMP.

Thermodynamic insight into the difference in product formation at different temperature

To get a thermodynamic insight into difference in the product formation with temperature, we carried out urea-induced unfolding studies to determine the stability of hGBP-1 in the presence of various nucleotides. This was carried out by measuring the intrinsic tryptophan fluorescence of hGBP1 alone or in the presence of GppNHp, GDP and GMP, separately at 37°C. $\Delta G_D°$ was determined from the plot of the fluorescence vs concentrations of urea. The values of ΔG_D at 37 °C for the free, GppNHp-bound, GDP-bound and GMP-bound proteins are 5.4, 7.1, 4.3 and 5.2 kcal/mol, respectively. This implies that the binding of the nucleotides alters the stability of the protein. Interestingly, the GDP-bound protein is found to be less stable than the free protein, but the GMP-bound complex shows similar stability to the free protein.

To determine the ΔG_D at lower temperature, we first estimated ΔC_p, T_m and ΔH_m of the protein with various nucleotides, separately. These were done by heat-induced unfolding of wild type protein by measuring the tryptophan fluorescence with varying concentrations of urea, and ΔH_m and T_m were determined. ΔC_p was evaluated from the plot of ΔH_m versus Tm and was determined to be 5.9 kcal.mol^{-1}.K^{-1} for the free protein. Using the thermodynamic parameters, the ΔG_D at 10°C for these complexes were determined. The values of ΔG_D at 10°C for the free, GppNHp-bound, GDP-bound and GMP-bound proteins are 5.2, 5.9, 0.5 and 5.6 kcal/mol, respectively. The stability of the free and GppNHp-bound proteins at 10°C is similar to at 37°C. But for GMP-bound protein it is slightly more stable at 10°C compared to at 37°C. In contrast, GDP-bound hGBP-1 exhibits significant lower stability at 10°C compared to at 37°C (0.5 versus 3.4 kcal/mole at 10 versus 37°C respectively). Interestingly, at 37°C the stability of the GppNHp-bound protein increases compared to the free protein. But the ΔG_D of the GDP-bound hGBP-1 is found to be lower than the GppNHp-bound hGBP1 at both these temperatures, suggesting that after the first hydrolysis GDP-bound protein becomes less stable compared to GppNHp-bound and the free protein at both temperatures. After the first hydrolysis, the difference in the stability of GDP-bound hGBP1 complex at these two temperatures determines whether the formation of GDP could be higher or lower. The data show that after the first hydrolysis as well as release of the first phosphate, the stability of the GDP-bound protein complex plays a crucial role in the product formation.

Role of SSEHA motif in the stability of *H. pylori* arginase

To examine the role of this motif in the stability, thermal denaturation studies were carried out using CD measurements on the mutant proteins with and without metal ions. The Tm was calculated using two states transitions. Similar analysis was done for the apo-proteins. The apo-proteins also showed similar unfolding curves and the Tms were calculated except for His91Ala, as it did not show proper transition.

Comparison of the Tm values between the mutant and wild type proteins showed a significant decrease in the thermostability of Ser88Gly and His91Ala by ~10-11 degree compared to the wild type. Glu90Ala showed a decrease in the Tm by ~5-6 degree both in the apo and holo proteins. Ser89Ala and Ala92Ser showed the T_m values similar to the wild type suggesting that these residues did not have major impact on the stability of the protein. It is evident that all mutant proteins except His91Ala in the apo form showed significant decrease in the thermostability (~10-12 degree lower in the T_m) compared to their holos suggesting that the metal ions play important role in providing stability to the mutant proteins.

Publications

Original peer-reviewed articles

1. Rani A, Pandita E, Rahman S, Deep S, Sau AK* (2012) Insight into Temperature Dependence of GTPase Activity in Human Guanylate Binding Protein-1. *PLoS One* **7**: e40487.
2. Srivastava A, Meena SK, Alam M, Nayeem SM, Deep S, Sau AK* (2013) Structural and Functional Insights into the Regulation of *Helicobacter pylori* Arginase Activity by an Evolutionary Nonconserved Motif. Biochemistry **52**: 508–519.

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Janendra K. Batra

Ribonucleases and heat shock proteins: Involvement in host defense

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The work is focused on two major themes:

1. Investigation of the role of human ribonucleases, particularly eosinophil ribonucleases, eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) in host defense. Human ribonucleases, and natural protein toxins targeting RNA or ribosomes are being analyzed for structure-function relationships to understand their molecular mechanism of action, to explore them to design knowledge-based recombinant toxins.

2. Investigation of crucial housekeeping proteins of *M. tuberculosis.* We are studying the functioning of Caseinolytic protease (Clp) machinery, and RNase P mediated tRNA maturation in *M. tuberculosis* as these two protein families could be promising drug targets. Clp proteases regulate the expression of virulence genes, and also help bacterial pathogens in countering stress in the host. RNase P is a key housekeeping enzyme involved in tRNA maturation, and is structurally completely different in bacteria and human.

The work revolves around the following broad objectives:

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

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

Stress regulation and persistence mechanisms in Mycobacteria

To investigate the involvement of heat shock

proteins of Clp family in the survival and virulence of *M. tuberculosis*, the regulation of stress response in the pathogen is being studied. The heat shock response in *M. tuberculosis* appears to be regulated both positively and negatively. There are two heat shock protein repressors, HspR and HrcA annotated in the genome of *M. tuberculosis*. His-tagged HrcA of *M. smegmatis* and *M. tuberculosis* were expressed in *E. coli* and recombinant proteins purified from inclusion bodies. Upstream region of groES and hrcA contains the probable CIRCE DNA binding element, which is the putative binding site for HrcA. A 200bp of this region has been amplified, cloned into pGEMT easy vector and sequenced. The binding properties of mycobacterial HrcA are being analyzed using radioactive DNA template.

To identify the target DNA regions of HspR of *M. smegmatis* and *M. tuberculosis*, three probable HAIR motifs upstream of dnaK, and one upstream of clpB gene were amplified as four different DNA fragments, cloned into pGEMT easy vector and sequenced. The three fragments upstream of dnaK included a 202bp region comprising of two HAIR elements, and two regions respectively of 134bp and 78bp from the 202bp region comprising of one HAIR element each. The fragment upstream of clpB gene was of 200bp comprising of one HAIR element. HspR of both, *M. smegmatis* and *M. tuberculosis* bound all four DNA fragments containing the putative HAIR motifs. Further, the DNA binding ability of HspR of *M. smegmatis* was checked at 4°C, 25°C, 37°C, 42°C, 50°C, 60°C and 70°C. The protein showed good binding from 4°C till 50°C, the binding was slightly reduced at 60°C, however it was significantly reduced at 70°C, indicating that HspR of *M. smegmatis* is quite stable up to 60°C. The study has identified regions in dnaK and clpB genes that bind HspR, and indicates that transcription of these genes is regulated by HspR.

Structure-function analysis of Clp proteins of M. tuberculosis

The bacterial ClpP has been shown to be autocleaved at its amino-terminus. The DNAs encoding the N-terminal processed versions of the ClpP1 and ClpP2 of *M. tuberculosis* with C-terminal 6xHis tail were amplified by PCR, and cloned in a *M. smegmatis* expression vector. Both proteins were partially purified. The proteolytic activity of ClpP1 and ClpP2 of *M. tuberculosis* were analysed on FITC-casein and GFP-Ssra in the presence of di-peptides, Z-Leu-Leucinal and Z-Leu-Leu, reported to be working as activators. These proteins demonstrated poor proteolytic activity. Currently, we are in the process of optimizing the activity of ClpP1 and ClpP2 of *M. tuberculosis*.

M. tuberculosis ClpX is a 428 amino acid long protein containing a zinc binding domain at the N-terminus and a single ATPase (AAA+) domain divided into a large ATPase domain, containing the Walker motif, and a small C-terminal ATPase domain. We investigated the role of the small AAA+ domain of ClpX of *M. tuberculosis* in its function. To study the role of conserved as well as unconserved regions, ClpX mutants, having internal deletions at C-terminus were consructed. The mutants include five C-terminal internal deletion mutants, ClpX-316-399 (Δ316-399 aa), ClpX-316-336 (Δ316-336 aa), ClpX-336-356 (Δ336-356 aa), ClpX-356-376 (Δ356-376aa), ClpX-376-399 (Δ376-399 aa), and one C-terminal deletion mutant ClpX-399 (Δ399-428 aa). The wild type ClpX and mutants were expressed in *E. coli,* and the proteins were purified to homogeneity. The CD spectra of ClpX-WT and its deletion mutants were found to be very similar indicating that the deletions did not affect the overall conformation of the protein. *M. tuberculosis* ClpX was found to contain significant ATPase activity. All the mutants

showed ATPase activity similar to that of the wild type protein. The ClpX-WT showed enhanced ATPase activity in the presence of its model substrates, GFP-SsrA, λO, casein and poly-L-lysine. All ClpX C-terminal mutants showed the ATPase activity in presence of substrates similar to that of ClpX-WT, except for ClpX-376-399 which showed less enchancement in the ATPase activity in the presence of λO as compared to ClpX-WT. ClpX-WT, and mutants ClpX-336-356, ClpX-376-399 and ClpX-399 formed hexamer in the presence of ATP. However, the mutants ClpX-316-336, ClpX-356-376 and ClpX-316-399 failed to form hexamer, and remained monomeric. ClpX has been shown to unfold its substrate proteins and translocate them to the associated protease, ClpP. The *M. tuberculosis* ClpX was also able to trap the unfolded model substrate, GFP-SsrA in an unfolded state in a dose dependent manner. ClpX-336-356, ClpX-376-399 and ClpX-399 mutants showed trapping activity similar to that of the ClpX-WT. However, ClpX-316-336, ClpX-356-376 and ClpX-316-399 mutants did not any show trapping activity. ClpX and its mutants that were showing trapping activity were further analyzed for their ability to trap GFP-SsrA in the presence of competitive substrates. The ClpX-336-356 and ClpX-399 mutants showed trapping activity in the presence of competitive substrates, similar to that of ClpX-WT, however ClpX-376-399 mutant showed reduced trapping activity in the presence of poly-L-Lysine and λO suggesting this region to be important in the interaction of ClpX with its substrates. The study identifies regions in *M. tuberculosis* ClpX, involved in its oligomerization, and in turn its chaperonic activity.

The ClpX hexamer has a translocation channel, and three different pore loops called “GYVG,” “pore 2,” and “RKH” which play crucial role in binding the SsrA tag in its substrates. To establish the role of these loops in *M. tuberculosis* ClpX in SsrA-tag recognition, in each of these loops, a conserved amino acid residue was mutated to generate three ClpX mutants, namely R230A (RKH), Y155A (GYVG) and R202A (pore-2). In addition, a triple mutant termed as ClpX-YRR, having all the three mutations was also constructed. The mutants were expressed in *E. coli*, and the proteins were purified to homogeneity. CD spectral analysis of the mutants in the far-UV range revealed that the overall structure of the mutants R230A, Y155A, R202A and ClpX-YRR was similar to that of ClpX-WT. All the mutants showed ATPase activity similar to that of the wild type protein. Among the pore loop mutants ClpX R202A and ClpX R230A showed trapping activity similar to that of ClpX-WT. However, ClpX Y155A and ClpX YRR showed less binding of the substrate suggesting that the Y155A mutation specifically impairs recognition and initial binding of SsrA-tagged proteins with ClpX.

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

To investigate the role of *M. tuberculosis* RNase P *in vivo*, we are making conditional knockouts of rnpA gene, encoding the RNase P protein in *M. tuberculosis*, H37Rv. As the rnpA gene appears to an essential gene, we decided to integrate a copy of rnpA gene under inducible tetracycline promoter in the genome, before attempting to disrupt the endogenous copy. The vector pTC-mcs, containing teracycline promoter and integrase gene from mycobacteriophage L5 was used. The pTC vector integrates in the genome of mycobacterium at a neutral L5 attP locus. This vector also incorporates tetracycline repressor for reduced expression of the gene in the uninduced medium, consequently allowing for tight regulation of the gene under tetracycline promoter. The *M. tuberculosis* rnpA gene was independently cloned in pTC vector in sense and

anti-sense orientation. Through pTC vector the genomic integration of rnpA-sense orientation has been achieved. In addition, one strain of *M. tuberculosis* that contains an integrated copy of rnpA gene in the reverse, antisense orientation has been constructed. The endogenous rnpA locus will be shut to make the conditional knockout. As rnpA gene is transcribed from a promoter that controls the transcription of eight other genes, many of which are involved essential functions, we intend to minimally manipulate the rnpA region. Accordingly, we aim to silence the endogenous rnpA locus by introducing stop codon through point mutations, using a recombineering plasmid pJV62. Currently, the mutation of endogenous rnpA gene is being carried out.

Publications

Original peer-reviewed articles

1. Bajaj D, Batra JK* (2012) The C-terminus of ClpC1 of *Mycobacterium tuberculosis* is crucial for its oligomerization and function. *Plos One* **7**: e51261.
2. Rath P, Singh PK, Batra JK* (2012) Functional and structural characterization of *Helicobacter* pylori ClpX, a molecular chaperone of Hsp100 family. *Prot Pep Lett* **19**: 1263-1271.
3. Sikriwal D, Seth D, Parveen S, Malik A, Broor S, Batra JK* (2012) An insertion in loop L7 of human eosinophil derived neurotoxin is crucial for its antiviral activity. *J Cell Biochem* **113**: 3104-3112.

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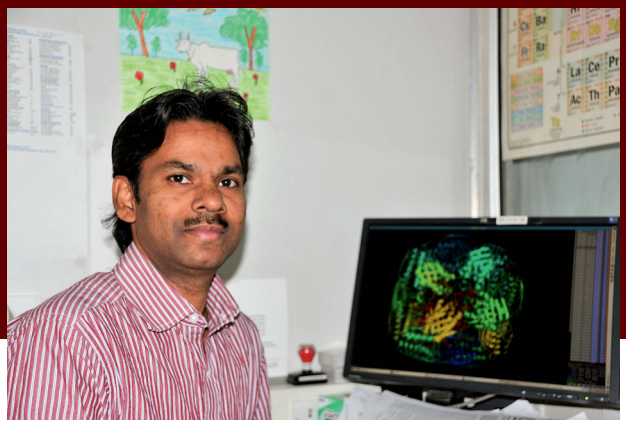

Bichitra K Biswal

Structural and functional studies of *Mycobacterial* proteins

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We have been pursuing two major projects. In the first project, we aim at understanding the molecular mechanisms underlying the actions of the enzymes involved in biosynthesis of Histidine in *Mycobacterium tuberculosis (Mtb).* The second project deals with the structural and functional characterization of membrane associated proteases (MAPs) from *Mtb*.

Objectives

1. Elucidate the structures of the native and substrate-bound forms of these enzymes primarily using X-ray crystallographic technique, deduce the mechanisms underlying their action and site-directed mutagenesis study.
2. Identify the exact physiological substrates of MAPs through a proteomics approach.
3. Examine whether these enzymes are involved in Mtb pathogenesis through *in vivo* studies.
4. Design inhibitors against these targets through a structure based inhibitor design approach, examine the potency of these inhibitors in *Mtb* infect macrophages and determine the 3D structures of enzyme/inhibitor complexes.

A. Structural and kinetics studies of HisB in complex with its substrate and an inhibitor

In the previous year, we have reported the crystal structures of HisB and HisC2. In the past one year, we have carried out detailed structural and kinetics studies of HisB, which catalyses the conversion of imidazole glycerol phosphate (IGP) to imidazole acetol phosphate (IAP), with its substrate IGP and an inhibitor 3-Amino-1,2,4-triazole (ATZ). To establish that HisB indeed possesses enzymatic activity as a dehydratase, we performed enzymatic assay with IGP. The enzyme obeys the Michaelis–Menten kinetics and exhibits specific dehydratase activity with a K_m value of about 120 μM. Inhibition study shows that ATZ inhibits HisB competitively with a K_i value of 300 μM. HisB is a 24-mer in crystal as well as in solution.

To map the interactions between HisB/IGP and HisB/ATZ, the structures of these complexes were determined. The enzyme/substrate structure clearly showed the binding of the IGP in a cleft comprised of mainly charged residues such as histidine, lysine, arginine, aspartate and glutamate protruding from three different subunits (Figure

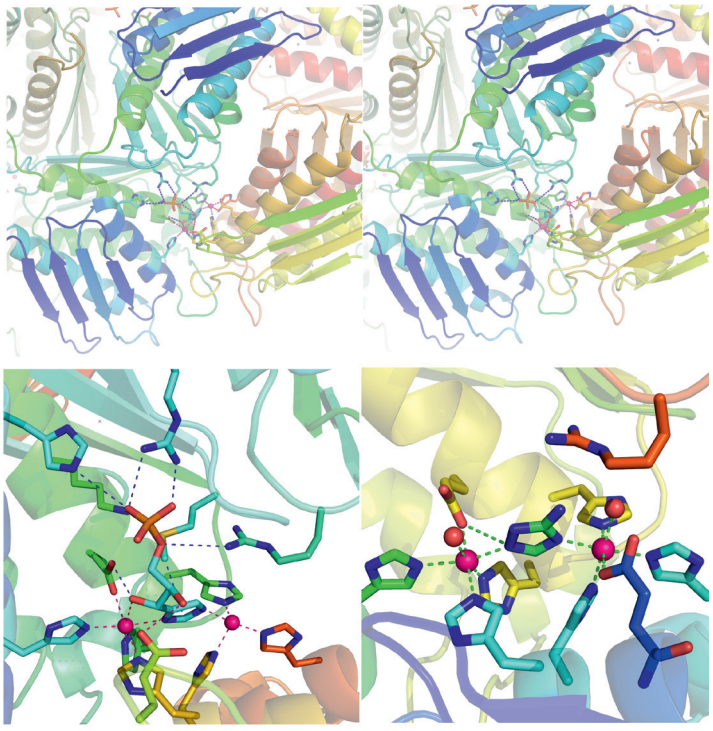

Figure 1: (A) Stereoview of the HisB active site. The three subunits that form a complete active site are labeled A, B and C. The substrate IGP and interacting residues are shown in stick representation. In panels (B) and (C), the interactions (shown by dotted lines) between the enzyme and the substrate and between the enzyme and inhibitor are shown. Four manganese atoms are two water molecules are shown by pink and red spheres, respectively.

1A). Briefly, the imidazole ring of the IGP is positioned between the two active site manganese ions (Mn1 and Mn2) located about 6 Å apart; each nitrogen atom of the imidazole ring interacts with a manganese atom (Figure 1B). Examination of the Fourier electron density maps in the active site region of HisB/ATZ complex reveals that 1,2,4-triazole ring of the inhibitor binds in the same place as the imidazole moiety of the IGP does (Figure 1C). The N1 and N4 atoms of the ATZ are coordinated to Mn1 and Mn2 ions, respectively.

Superimposition of the native and substrate complex structures showed that the side chain of Arg121, apart from few minor changes, undergoes approximately 160° rotation about C_β-C_γ bond and this brings the guanidyl moiety to make interaction with the substrate. Histidine biosynthesis pathway has been shown to be essential for the optimal growth of *Mtb*. In this context, these structural studies undoubtedly will be helpful to design new inhibitors for HisB with enhanced affinity. In addition, during the last one year, we have solved the structure of another enzyme HisC which catalyses the conversion of IAP to histidinol phosphate. Model building and refinement are currently in progress.

B. Understanding the localization and function of MAPs

Of the three MAPs (Rv2223c, Rv2224c and Rv2672) whose over-expression profiles were checked in small scale culture in *M. smegmatis* expression host, Rv2224c was over-expressed in large scale (8 litres) and the membrane fractions were harvested using ultra centrifugation. Subsequently, cell wall and cell membrane fractions were homogenized separately in a dozen of different detergents. The samples were examined on SDS-PAGE. The protein was purified to homogeneity using affinity and gel filtration chromatography. In addition, various truncated versions of Rv2224c were over-expressed in *M. smegmatis*. One of

the constructs, a 30 amino acid truncation from the N-terminus, was purified and enzymatic assay was performed. The preliminary results suggest that Rv2224c possesses an esterase activity. We also have set up crystallization trials for Rv2224c at room temperature using commercially available crystallization screens. Importantly, couple of conditions containing PEG 4000 as the precipitant yielded needle-like clusters. Optimization of these conditions by varying salt concentration, pH, additives, etc. is in progress. Large scale over-expression of Rv2223c is currently being carried out.

Publication

Original Peer reviewed article

1. Nasir N, Vyas R, Biswal BK* (2013) Sample preparation, crystallization and structure solution of HisC from Mycobacterium tuberculosis. A*cta Cryst. Section F* **69**: 445-448.

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Debasisa Mohanty

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 (PRMs) like MHCs, kinases, PTB, PDZ and WW domains etc, (3) structure and dynamics of microRNA-protein complexes.

A. *In silico* analysis of enzymes associated with novel post-translational modifications and biosynthesis of lantipeptides

We have carried out a comprehensive bioinformatics analysis of enzymes associated with five different types of novel post-translational modifications (PTMs), namely, AMPylation, Eliminylation, hydroxylation, sulfation and deamidation. The sequence and structural features of the experimentally characterized enzymes involved in catalyzing these PTMs have been analyzed in details. Based on extensive manual curation of published literature, information about three dimensional structures, experimentally verified active site residues, known substrates for enzymes associated

with these PTMs have been compiled. Based on a variety of analysis of these sequence and structural information, whole sequences as well as active site residue profiles have been built for enzymes involved in each of these PTMs. These profiles can be utilized for identification of various types of PTM catalyzing enzymes from among un-annotated proteins in various genomes. Our benchmarking studies on completely independent dataset indicate that, these profiles can predict the presence of these 5 PTM catalyzing domains with sensitivity in the range of 0.8 to 1.0 and specificity of 1.0. For some of the PTMs like AMPylation and Eliminylation, we have also carried out detailed analysis of genomic neighborhoods of the enzymes associated with these PTMs. Based on these results, we have developed specific search tools using HHSearch for identifying inhibitory domains which are known to regulate AMPylation activity (Engel P *et al*., Nature 2012, 482(7383), because of their presence on the same polypeptide chain or in the genomic neighborhood. Similarly, in case of Eliminylation, analysis of the genomic neighborhood of putative eliminylation domains has helped in discovery of "eukaryotic BLES03"-like proteins in bacterial gene clusters associated with biosynthesis of lanthipeptides. This observation provides further support to our earlier prediction of phosphothreonine lyase activity for BLES03.

We have also carried out a comprehensive analysis of LanM like enzymes involved in class II pathways for biosynthesis of lantipeptides. The C-terminal of LanM contains a putative cyclase domain, while N-terminal of LanM is known to catalyze dehydration of Ser/Thr to Dha/Dhb in an ATP dependent manner. However, unlike class III and class IV lanthipeptide synthetase enzymes, N-terminal of LanM does not show sequence similarity to kinase or phosphothreonine lyase domains. We have carried out fold prediction as well as profile to profile comparison for identifying possible presence of kinase or phosphothreonine lyase domains in LanM. Interestingly, a stretch of 300 amino acids in the N-terminal of LanM showed similarity to phosphatidylinositol 3-kinase (PI 3-kinase) in profile-to-profile matches. Catalytic residues of LanM reported by mutational studies also fall in this stretch. Threading programs like GenThreader and Muster also show presence of kinase domains in LanM. Thus our analysis revealed possible presence of kinase domain in LanM.

B. Analysis of interaction networks involving kinases and other PRMs

Development of computational method for prediction of protein phosphorylation networks

Currently available computational approaches for prediction of substrates of protein kinases are mainly phosphosite predictors. They identify phosphorylated residues in a given substrate protein using sequence motif or structure based scoring and this limits their applicability at genome wide identification of cognate kinase-substrate pairs due to high computational costs and potential increase in number of false positives. We have utilized the results from our earlier analysis of the conserved features of protein phosphorylation networks to develop an alternate computational approach which will be more appropriate for genome wide identification of protein phosphorylation networks. Our earlier analysis involving classification of protein kinases based on the PFAM domains they phosphorylate had revealed distinct correlation between kinase families and PFAM domains of their substrate proteins. This novel correlation was successfully utilized to develop an automated computational tool called PhosNetConstruct

for distinguishing cognate kinase-substrate pairs from all other non-cognate combinations. Benchmarking on a completely independent test dataset indicates that, our approach for prediction of phosphorylation networks can identify cognate kinase-substrate pairs with sensitivity of 68% and specificity of 78%. We have compared its performance with other methods namely, GPS, NetPhosK and iGPS. The results of benchmarking indicate that, PhosNetConstruct performs better than GPS as well as NetPhosK at the task of prediction of target kinases for a given protein. However, iGPS which incorporates protein-protein interaction information is the only approach which attains a trade-off between sensitivity and specificity comparable to our approach. Incorporation of co-occurring domains along with those phosphorylated, further improves the sensitivity of PhosNetConstruct to 89% with a specificity of 58%. Interestingly, the fraction of true positive kinase-substrate pairs identified by PhosNetConstruct is highest among all the methods, while phosphosite predictors like GPS, NetPhosK etc. have comparatively larger large false positive hits. PhosNetConstruct will be a useful computational tool for generating potential list of cognate kinase-substrate pairs by filtering out large number of non-cognate kinase-substrate pairs and subsequently exact phosphosites can be identified using phosphosite predictors like GPS and NetPhosK.

In silico identification of small molecule modulators of PDZ-peptide interactions

Inhibitors of interactions involving PDZ domains have important implications for treatment of diseases like metastatic breast cancer, prostate cancer and Parkinson's disease. Therefore, in this study an attempt was also made to benchmark a computational protocol for identification of small molecule or peptidomimetic modulators of PRMs using PDZ domain as test case. Based on literature search, known small molecule inhibitors of PDZ domain mediated interactions and their experimentally determined Ki/Kd values were compiled. In order to identify the common chemical scaffold in these inhibitors of PDZ-peptide complexes, clustering based on similarity in chemical structure was carried out. A set of 38 small molecules with known Ki/Kd values for PDZ2 and PDZ3 domain of PSD-95 protein were selected for the benchmarking study. Each of these small molecule ligands were docked on the peptide binding sites of PDZ2 (PDBID 1QLC) and PDZ3 (PDBID 1BE9) domains using AUTODOCK VINA. The binding pocket residues of the receptor were made flexible during the docking. In order to incorporate ligand as well as receptor flexibilities, the final docking poses for each of these compounds on PDZ2 and PDZ3 domains of PSD-95 were further refined by multiple explicit solvent MD simulations of 5 ns length. Binding energy values for each of these 38 compounds were calculated using MM-PB/SA module of AMBER. The affinity scores predicted by AUTODOCK VINA and the MM-PB/SA binding energy values calculated after MD simulations were compared with the experimental binding energy values. Surprisingly, binding energies obtained from AUTODOCK VINA showed better correlation with the experimental inhibition constants compared to MM-PB/SA binding free energy values calculated from dynamics trajectories.

C. Structure and dynamics of microRNA-protein complexes

Most of the currently available computational methods for analysis of miRNAs and their targets focus primarily on sequence complementarity based on secondary structure prediction, rather than the role of tertiary structure of miRNA/mRNA and their interactions with key proteins

in the RNA induced silencing complex (RISC) or miRNA biogenesis pathways. Recently available crystal structures of miRNAs in complex with Argonaute and Lin28 have provided opportunities for understanding the structural basis of target recognition by miRNAs and inhibition of miRNA biogenesis by other regulatory proteins. We have carried out explicit solvent molecular dynamics (MD) simulations on the crystal structures of miRNAs in complex with Argonaute and Lin28. The MD trajectories have been analyzed to identify the crucial tertiary structural features of miRNA and key specificity determining residues of the interacting proteins, which govern the specificity of recognition. To better understand the role of Argonaute protein and the structural features involved in miRNA target recognition, we have modelled Argonaute-miRNA-mRNA ternary complex and refined it by MD simulations.

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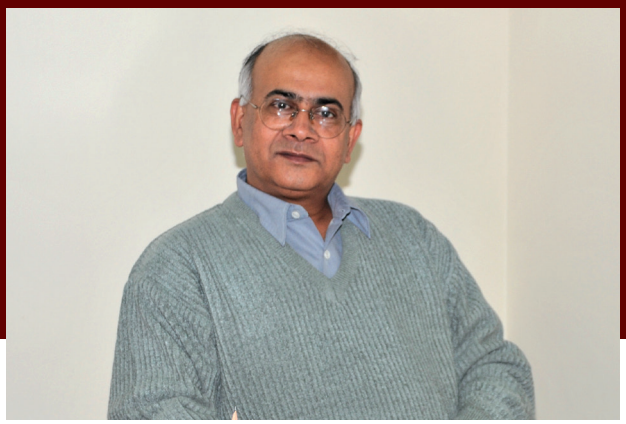

Dinakar M. Salunke

Structure, interaction and design studies involving regulatory peptides and proteins

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

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

The hallmark of acquired immune system is the remarkable specificity in its recognition repertoire that not only counters the invading pathogen but also ensures self-non self discrimination. Although immune system has evolved to discriminate finer differences between the molecules, degeneracy in immune response is often been observed. In order to understand the issue of specificity/degeneracy against an invading pathogen we had earlier taken up the HIV, a common immune evading virus. We have further extended these studies to another immune evading virus, Influenza A Virus. Its genome is made up of 8 strands of ssRNA encoding 10 viral proteins. Envelope of the virus is made up of 3 viral proteins: hemagglutinin or HA (16 subtypes), neuraminidase or NA (9 subtypes) and M2 ion transporter. Neutralizing antibody responses are only known to be generated against hemagglutinin but because of high intersubtype and intrasubtype variations in the antigenic regions it is challenging to develop a broadly neutralizing antibody. However this effect of genetic drift can be overcome if the immune system can generate antibodies with broader recognition specificities. Therefore it would be interesting to investigate the issues of specificity/degeneracy against neutralizing epitopes on influenza virus hemagglutinin.

The influenza hemagglutinin's antigenic regions were previously been mapped. A seven amino acid (WTGVTQN) epitope from an unusual protruding loop near the receptor binding region which has been previously shown to

have neutralizing effect was selected for further studies. All the hemagglutinin sequences from influenza research database (flu database) were extracted and aligned in order to get the analogues of the epitope. Out of 46 analogues found, 9 were selected for further studies (based on the maximum variation present). Peptide epitope and its analogues were synthesized on an automated peptide synthesizer using solid phase Fmoc (9-fluorenylmethyloxycarbonyl) chemistry on HMP (p-Hydroxymethyl phenoxymethyl) polystyrene resin, purified using C18 column on HPLC and were characterized by ESI-MS. Epitope was conjugated to 4 carrier protein and of which 3 conjugates were used to study physiological response in terms of degeneracy against the analogues. Polyclonal humoral response has shown differential degenerate response against different analogues and also overall differential degenerate response in the case of the three different conjugates. When the booster was given with lowest responding peptide conjugate we have seen an increase in the overall crossreactivity as well as against the lowest responding peptide. The degenerate profile was remained to be the same when the peptide-conjugate was injected in 4 strains of mice (Balbc, Black6, fvbj and carter). Two monoclonal antibodies 1A5 (KWTGVTQN-TT) and 4B1 (KWTGVTQN-KLH) were generated and characterized by DNA sequencing. Hybridoma cells were injected in the peritoneal cavity of mice to generate antibody in ascites. Both the antibodies were then purified from ascetic fluid by various chromatographic techniques (protein G purification and DEAE ion exchange chromatography). These two antibodies have shown differential crossreactivity profile as was indicated in the polyclonal responses against the two conjugates. Purified antibodies were digested with papain to generate Fab molecules. Fab was further purified by DEAE ion exchange chromatography and characterized by western blot. Purified Fabs were concentrated to 10 mg/ml concentrations and were further used in crystallization trials.

The seeds of a medicinally important plant, *Mucuna Pruniens*, have been known for pharmacological properties such as anti-Parkinson, antineoplasty, antioxidant, antidiabetic and antivenom activity. A 21 kDa protein, MP-4, was earlier purified and subjected to crystallographic studies, which were stagnated due to non-availability of suitable model for molecular replacement. The protein was repurified and subjected to further sequencing and mass spectroscopic analysis showing significant sequence homology (63%) with the serine protease inhibitor from *Delonix regia*. Protein was subjected to re-crystallization and were collected at higher resolution (2.1Å resolution) in a new crystal form, primitive orthorhombic space group $P2_12_12$, with unit-cell parameters a = 51.0 Å, b = 69.2 Å and c = 45.3 Å and single protomer per asymmetric unit. The structure is being refined starting by gradually increasing resolution. The current refinement statistics, Rcryst and Rfree are 30.14 % and 30.62 % respectively at 3.1 Å resolution.

Towards functional characterization of MP-4, the protein was subjected to antivenom activity. Different sets of balb/c mice were immunized with MP-4 protein and non-lethal dose of *Echis carinatus* whole venom protein and sera collected. The polyclonal antibody raised in mice against MP-4 protein react with one of *Echis carinus* venom proteins, phospholipase A2 (PLA2). Also polyclonal antibody raised for whole venom reacts with MP-4. We have further confirmed this cross-reactivity by immunizing balb/c mice with commercially available purified PLA2 from *Naja mossambica mossambica*. These studies indicate that MP-4 protein and PLA2 share some common epitopes. Further, the observed sequence homology of MP-4 with

serine protease inhibitor from *Delonix regia*, led to screening the inhibitory activity and binding efficiency with trypsin and chymotrypsin. Competition ELISA and binding experiments using surface plasmon resonance (SPR) were carried out. MP-4 showed IC_{50} values of 1.96uM and 1.03uM for trypsin and chymotrypsin, respectively. Thus, Inhibitory efficiency of MP-4 was found to be less as compared to commercially available soybean trypsin inhibitor (STI) and chymotrypsin inhibitor (chymostatin). Binding efficiency of MP-4 with trypsin and chymotrypsin was analysed indicating that MP-4 binds with the K_D values 2.65e-6 and 3.39e-6, respectively, which comparatively poor affinity as compared to other known Kunitz type inhibitors.

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Chemical Glycobiology: Glycoproteomics and carbohydrate-based drug design

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Mammalian cells display a wide variety of molecules on their surfaces that are known as clusters of differentiation (CD) antigens, majority of which are glycoproteins. Chemical structures of glycans attached to CD antigens exhibit microheterogeneity and are subject to dynamic modifications. The theme of research of our laboratory of chemical glycobiology (CGB) is to design and develop chemical tools to address challenges of functional glycomics and to advance understanding of importance of glycosylation in regulating fundamental biological processes. We design and synthesize carbohydrate-based small molecules, and study their ability to intercept enzymes of glycosylation pathways for metabolic incorporation and inhibition.

Glycosylation is the most abundant and complex post-translational modification (PTM) found on proteins. Our goal is to harness the power of synthetic chemistry and small molecules to probe biological systems with questions specific to protein glycosylation, the importance of which is highlighted by its day-to-day relevance in blood group antigens, transplantation, and biopharmaceuticals (e.g. erythropoietin, therapeutic antibodies). Our current objectives include four distinct areas of research:

1. Design, synthesis, and characterization of non-natural monosaccharide analogs for interception of mammalian glycosylation pathways.
2. Application of metabolic glycan engineering (MGE) methodology for modulation of glycoforms of cell surface antigens, and its consequence in immunological processes.
3. Design, synthesis, and development of carbohydrate-neuroactive (CH-NA) hybrid molecules for delivery of non-natural monosaccharides to brain across blood-brain-barrier (BBB).
4. Design, synthesis, and evaluation of

glycopeptidomimetic based small molecules as potential inhibitors of matrix metalloproteinases (MMPi) and anti-metastatic agents. We employ an extensive range of techniques from nuclear magnetic resonance (NMR), ultra-high pressure liquid chromatography (UPLC), nano-liquid chromatography – high resolution mass spectrometry (LC-HRMS) to flow cytometry and confocal microscopy. Our studies will provide new insights on the intricate mechanisms that govern spatiotemporal changes associated with glycans and its effect on cellular responses.

A. Non-natural analogs for metabolic glycan engineering (MGE)

Using MGE, chemical functional groups with unique reactivities (e.g. alkyl, hydroxyl, azidoacetyl, fluoro, thioacetyl, and others) have been successfully expressed on cell surface glycans enabling bio-orthogonal ligations. MGE has provided new avenues for imaging of glycans in living animals, tagging of cancer cells *in vivo*, and study of dynamic roles of glycosylation in biological processes. Non-natural analogs of ManNAc, GalNAc, and GlcNAc have been applied, respectively, for processing through sialic acid biosynthetic pathway, mucin-type O-glycosylation, and O-GlcNAc modifications. A unique functionality in biological systems is the presence of unsaturated double bond containing structures, commonly found in cholesterol, membrane lipids, and vitamin A, but not in glycans. In order to expand the repertoire of non-natural groups new analogs of ManNAc and GalNAc carrying unsaturated double bonds at the N-acyl side chain (vinyl, crotonoyl, but-2-enyl, and but-3-enyl) have been synthesized, purified, and characterized thoroughly using NMR (^{1}H and ^{13}C), HPLC, and mass spectrometry.

B. Modulation of glycoforms of cell surface molecules

There is an ever growing appreciation of importance of glycoforms in modulating biological processes. For example, resting and activated T-cells display different glycan structures, known as the 'glyco-code', on their surface. Depending on the structure of glycans present on antibodies either pro- or anti- inflammatory responses could be elicited. Glycoforms of cell surface antigens are remodeled in a dynamic manner by various glycosyl transferases and glycosidases.

MGE provides a powerful chemical tool to modulate glycans through metabolic incorporation and inhibition. In this context, we have studied structure dependent effects of non-natural GalNAc analogs on modulation of glycoforms of CD43 using a panel of glycoform-dependent and glycoform-independent antibodies. Ac_5GalNTGc **(1)** caused thiol-specific inhibition of elaboration of O-glycans on CD43 with minimal toxicity, as revealed by western blotting, lectin blotting, flow cytometry, and confocal microscopy. A comparison of *N-thioacetyl* with *N-methylthioacetyl* analog confirmed the requirement of free sulfhydryl moiety for observed changes to CD43 glycosylation. Ac_5GlcNTGc – a C-4 epimer of **1** which does not get processed directly through mucin-type *O*-glycosylation pathway – did not affect CD43 glycoforms thus indicating specificity of **1** towards mucin-type *O*-glycans. Lectin blots of immunoprecipitated CD43-myc/FLAG revealed abrogation of MAL-II (*Maackia amurensis* lectin-II which binds to NeuAc-a(2→3)-Gal moiety) and PNA (peanut agglutinin which binds to Gal-b(1→3)-GalNAc moiety known as Thomson-Freidenreich (TF) antigen and T-antigen) epitopes induced specifically by **1** but not control analogs. Abrogation of sialoglycoforms and TF-antigen, reduction in apparent molecular weight, and

thiol-selective biotinylation of GalNTGc of engineered CD43 taken together suggested that **1** might induce reduced site occupancy of initiating *O*-GalNAc as a consequence of its metabolic incorporation. A parallell study in K562 (human chronic myeloid leukemia) cells showed similar reduction in sialoglycoforms of CD43, but with no drastic changes in mobility in western blots, indicating that although effects of **1** are general its threshold for activity might vary between cell lines. A comparative qPCR study of isoforms of UDP-GalNAc: polypeptide transferase (E. C. 2.4.1.41) (ppGalNAcT; T1- T14) revealed unique expression patterns between Jurkat and K562. ppGalNAc-T3 and T14 were expressed exclusively in Jurkat but not in K562. Such differential expression of ppGalNAcT and their differential permissivity to MGE might govern the outcome of glycoform modulation. Further studies to (a) understand isoform-specific mechanisms, (b) effect of MGE on T-cell activation, (c) site mapping of *O*-glycans in CD43, and (d) glycomics study of permethylated *O*-glycans from various cell lines using MALDI-TOF mass spectrometry are currently underway.

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

Sialic acids, also known as neuraminic acids (NeuAc), are a unique class of nine-carbon containing acidic monosaccharides found at the termini of glycans in glycoconjugates. Sialoglycoconjugates, particularly, polysialic acid carrying neural cell adhesion molecules (PSA-NCAM) play important roles in neuronal development and brain tumors. Having shown proof-of-concept studies that ManNAc analogs when conjugated to cleavable neuroactive molecules results in efficient expression in brain across BBB, we further expanded the repertoire of CH-NA hybrid molecules. Hybrids of peracetylated N-azidoacetyl-D-mannosamine (ManNAz) with additional neuroactive molecules such as valproic acid, nicotinic acid, theophylline acetic acid, choline, tyrosine, glutamate, and benzyl succinate have been synthesized using selective protection / deprotection strategies and characterized. In order to identify glycans carrying an azido group, a water soluble biotin-linker-alkyne derivative for copper (I) catalyzed bio-orthogonal ligation was synthesized and optimized for avidin-blotting using CH-NA treated Jurkat and SH-SY5Y (human neuroblastoma) cells. Peracetylated ManNAz-NA hybrids were administered to mice (both C57BL/6j and Balb/c, up to 300 mg/kg) intravenously. Non-hybrids were used as negative controls. Heart, brain and other tissues were harvested post-treatment, *N*-azidoacetyl-neuraminic acid (NeuAz) containing glycoproteins were derivatized using a bio-orthogonal click reaction using biotin-linker-alkyne and analyzed by western blotting, flow cytometry, and confocal microscopy. Expression of azide groups was found to increase in a dose-dependent manner for nicotinic and valproic conjugates. To enable characterization of metabolically engineered sialic acids from brain tissues, conditions for derivatization with 1,2-diamino-4,5-methylenedioxy-benzene (DMB) followed by UPLC detection have been established.

Having found efficient carriers for ManNAz and consequent engineering of brain sialic acids, we focused our attention for modulation of PSA biosynthesis *in vivo*. *N*-butanoyl-D-mannosamine (ManNBut) has been shown to reduce PSA levels *in vitro*. ManNBut is thought to inhibit PSA biosynthesis resulting in truncated PSA chains. In order to achieve modulation of PSA-NCAM across BBB, we synthesized a panel of ManNBut-NA hybrids. ManNBut-NA hybrids were studied *in vitro* in SH-SY5Y and HL-60 (human myeloid leukemia) cells for effects on PSA-NCAM expression, using anti-PSA

(clone 12F8) and anti-NCAM (clone OB11, PSA-independent) antibodies by western blotting. mRNA levels of NCAM and two polysialyl transferases – PST and STX – measured by RT-PCR revealed that while both PST and STX were expressed in SH-SY5Y cells, STX was expression was absent in HL-60; NCAM expression was positive in both cell lines. ManNBut-NA hybrids abrogated PSA-NCAM expression while NCAM levels remained unaltered, in both cell lines. Intravenous administration of ManNBut-NA hybrids to mice resulted in significant (~30-40%) reduction in brain PSA-NCAM levels. Corresponding non-hybrid molecules did not show any effect on brain PSA-NCAM levels. Notably, the wild-type ManNAc-NA conjugates enhanced PSA-NCAM levels by 10-20% of controls indicating that depending on the choice of hybrid molecules PSA levels could be modulated in both directions. Studies on the effect of modulation of PSA levels by ManNR-NA hybrid molecules in neuronal behavior, memory, and brain tumor growth are currently underway.

D. Glycopeptidomimetics (GPM) as potential anti-metastatic agents

Towards development of carbohydrate-based small molecules as potential MMPi, a panel of monosaccharide derivatives with a zinc binding group (ZBG) at C-6 position was synthesized through multi-step multi-gram scale synthesis. α- and β- Allyl D-glucopyranoside derivatives locked with benzyl or methyl groups at C-2, C-3, and C-4 carrying an acyl-hydroxamic acid moiety at C-6 were synthesized using selective protection / de-protection strategies. Similarly, allyl D-galactopyranoside derivative were synthesized to exploit stereochemical variations for differential binding to MMPs. Monosaccharide-ZBG compounds were screened for toxicity in Jurkat and LL/2 (mouse Lewis lung carcinoma) cells using cell counting and MTT assays. 2,3,4-Tri-*O*-Benzyl derivatives were found to be toxic mainly due to their hydrophobicity and limited solubility in cell culture medium. On the other hand, 2,3,4-tri-*O*-methyl derivatives of gluco- and galacto-compounds were found to be non-toxic up to 500 μM. Additionally, a known chromogenic peptide substrate for thermolysin – N-[(3-furyl)acryloyl]-Gly-Leu-Amide (FA-GL-NH_2) has been synthesized in four steps in good yields, which will be used for study of kinetics of metalloproteinases and its inhibition using above-mentioned compounds, along with known MMP inhibitors.

Kanwaljeet Kaur

Role of carbohydrates in modulating the structure and function of glycopeptides

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

1. Synthesis and structural characterization of glycosylated amino acids
2. Structure-function analysis of the synthetic glycoconjugates

Continuing the studies for understanding the role of glycosylation in the antibacterial activity, an example of disaccharide containing antibacterial peptide, drosocin, was undertaken. Drosocin is found to contain monosaccharide (2-acetamido-2-deoxy-D-galactopyranosyl; GalNAc) or a disaccharide (Galβ(1→3)GalNAcα1-) attached to threonine in its sequence. The synthesis of disaccharide building block, N^{α}-Fmoc-Thr(Ac_4-β-D-Gal-(1→3)-Ac_2-α-D-GalNAc)-OH, was tried following different synthetic schemes and finally it was standardized by the methodology which involves sixteen steps from free galactose. Firstly, the monoglycosylated, N^{α}-Fmoc-Thr(Ac_3-α-D-$GalN_3$)-OBzl, was synthesized as reported earlier, in which the formation of the α-glycosidic linkage between threonine and GalNAc saccharide was obtained in a glycosylation reaction with a nonparticipating substitutent at C-2 of the glycosyl donor. The azido group was found to be efficient for such stereoselective α–glycosylation and subsequently it can be converted into the naturally occurring acetamido group. The acetate groups of this monoglycosylated amino acid were subsequently removed using sodium methoxide, which was followed by treatment with α,α-dimethoxytoluene and a catalytic amount of *p*-TsOH in acetonitrile. This yielded the benzylidene compound with selectively protecting the C4 and C6 positions while the C3 hydroxyl group remained unprotected. This compound was glycosylated with the peracetylated galactose trichoroacetimidate in the presence of catalytic amounts of trimethylsilylmethanesulfonate affording exclusively the β-(1→3)linked disaccharide in good yield. The benzylidene group was removed by treatment with 80% acetic acid followed by acetylation of the free hydroxyl groups. The azide moiety was reduced and acetylated in one step using thioacetic acid and pyridine to give GalNAc derivative. Excess thioacetic acid was only seperable by column chromatography. Subsequently, the carboxylic

group was deprotected by hydrogenolysis yielded the final compound, N^{α}-Fmoc-Thr(Ac_3-β-D-Gal-(1→3)-Ac2-α-D-GalNAc)-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 disaccharide containing drosocin (di-drosocin) was synthesized by using synthetically prepared N^{α}-Fmoc-Thr(Ac_3-β-D-Gal-(1→3)-Ac_2-α-D-GalNAc)-OH. This peptide was assayed for its comparative spectrum of antimicrobial activity against different bacterial strains. It was observed that though the di-drosocin exhibited higher level of antibacterial activity than nonglysoylated drosocin but it showed lower level of activity than monoglycosylated drosocin against all the tested bacterial strains. The results indicated that the conformation of the peptide is effected by sugars and different sugars alter the conformation of same peptide differently.

Bacterial DnaK has been reported as one of the possible targets for the proline rich class of antibacterial peptides. In order to understand bioactive conformation of Drosocin, we need to have its structure in presence of its plausible receptor. Hence we cloned and expressed the substrate binding domain of *E.coli* DnaK, truncating its flexible C terminus part which hinders crystallization. Its binding with monoglycosylated as well as nonglycosylated analogues of Drosocin was confirmed using SPR. The co-crystallization trials were done with DnaK and drosocin analogues. The crystals were grown at room temperature using hanging drop vapour diffusion method. The crystals were diffracted without a cryoprotectant upto 4°A. But the data could not be processed probably because of faulty crystal packing. The trials are still ongoing to improve the crystals quality.

With an aim to provide a structure based understanding of functionality in differentially glycosylated peptides, we have also undertaken a model system of thrombin inhibitory glycopeptides because the receptor for this class of peptides, thrombin, is structurally well characterized. Expanding our repertoire of the differentially glycosylated variegin analogues, α-GalNAc and α-Mannosyl bearing variegin analogues were synthesized. Besides variegin, peptide analogues belonging to the Hirudin class of proteins were also synthesized. Hirudin variants, Hirudin P6 and Hirudin P18 isolated from *Hirudinaria manillensis* are characterized to be strong inhibitors of thrombin and are known to undergo differential O-glycosylation on one of its C-terminal threonines. Hence, the C-terminal peptides of these two inhibitors were synthesized along with the α-GalNAc-Hirudin P6.

Functional characterization of the above synthesized peptides along with few previously synthesized analogues was carried out by chromogenic assays. While performing the chromogenic assays, human thrombin was incubated either with various concentrations of the peptide or without peptide for 30mins at room temperature and the rate of release of *p*-nitroaniline (pNA) from substrate, s-2238, was monitored at a wavelength of 405 nm for 10 mins. From this, the percentage of inhibition in the activity of thrombin was calculated and thus their IC_{50} values were obtained. The nonglycosylated and mannosylated variegins showed the comparable IC_{50} values.

Surface plasmon resonance studies were also performed to obtain the kinetic parameters of the peptide – thrombin interactions. For this, pure thrombin was covalently immobilized onto CM5 chip and different concentrations of synthesized peptides were passed. Porcine trypsin immobilized on flow cell served as a negative control. The sensograms obtained with

each peptide were fitted into a 1:1 (Langmuir) binding model and the kinetic parameters were obtained. Hirudin P6 and glycosylated Hirudin P6 exhibited the comparable affinity constants.

To understand the structural effects of glycosylation on their conformation and its correlation with the function, co-crystallisation trials were carried out with recombinant human thrombin at 10 mg/ml concentration and various, above mentioned peptide analogues at a molar ratio of 1:10 (protein to peptide). These trials resulted in some initial hits where either small clusters of needles or planar rhomboidal crystals were obtained. These conditions are to be further optimized.

Vidya Raghunathan

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

It is known that *Leishmania* sp. unlike mammalian counterparts uses multiple isoforms for many enzymes of the energy pathway, one of which is phosphoglycerate kinse or PGK. *Leishmania* PGK isoforms 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. Drug development efforts can be targeted, either at the glycosome itself or at the enzymes present within them for which, targeting unique structural features is critical.

We also would like to use nucleur magnetic resonance spectroscopy to map the metabolic profile of *Leishmania* spp cultures and correlate this to the enzymological studies with purified proteins.

1. Expression, purification and determination of specific activities of PGKB-Lmex and PGKC-Lmex and steady state kinetics study.
2. Comparison between PGKB-Lmex and PGKC-Lmex of, pH optimum of activity and enzyme inhibition by salt and suramin.
3. ^{31}P NMR studies using substrate / enzyme (PGKB-Lmex or PGKC-Lmex) 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.
4. Peptide based studies of glycosomal membrane association of PGKC-Lmex. The peptides used in these studies will be evaluated as useful models to understand the structural basis of the biochemical differences between PGKC-Lmex and PGKB-Lmex.
5. Structure determination of PGKB-Lmex and PGKC-Lmex by x-ray crystallography and conformational studies by NMR using site non-radioactive isotope labeling.
6. Using promastigote and amasitoge cultures of *Leishmania* spp for metabolome mapping. The concentration of specific metabolites in the cell at a particular time can be monitored at the micromillimolar level by ^{31}P, ^{1}H and ^{13}C NMR spectroscopy. The metabolites that can be detected are alanine, lactate, acetate, pyruvate, succinate, glycerol, urea, CO_2, oxalate, valine, glutamine and arginine.

In lieu of the structure of the peptide as determined by NMR in solution, we have launched into looking at the structure of the entire C-terminal domain extension of PGKC-Lmex by cloning in *E. coli*. The 62 residue domain of the C-terminus of PGKC has been cloned in *E. coli* BL21 (RP) strain. The protein

expression is good and western blotting shows the presence of a strong clean band after purification by metal affinity chromatography. The protein is pure enough to be used at this stage. A sample of ^{15}N labeled protein was made as an NMR sample. For this the *E.coli* was grown in M9 minimal media in the presence of $^{15}N\ NH_4Cl$.

Publication

Original peer-reviewed article

1. Kaushik S, Krishnarjuna B, Raghothama S, Aggarwal S, Raghunathan V*, Ganjiwale A (2012) Theoretical and *in vitro* studies of a C-terminal peptide from PGKC of *Leishmania* mexicana mexicana. *Mol Biochem Parasitol* **185**: 27-35.

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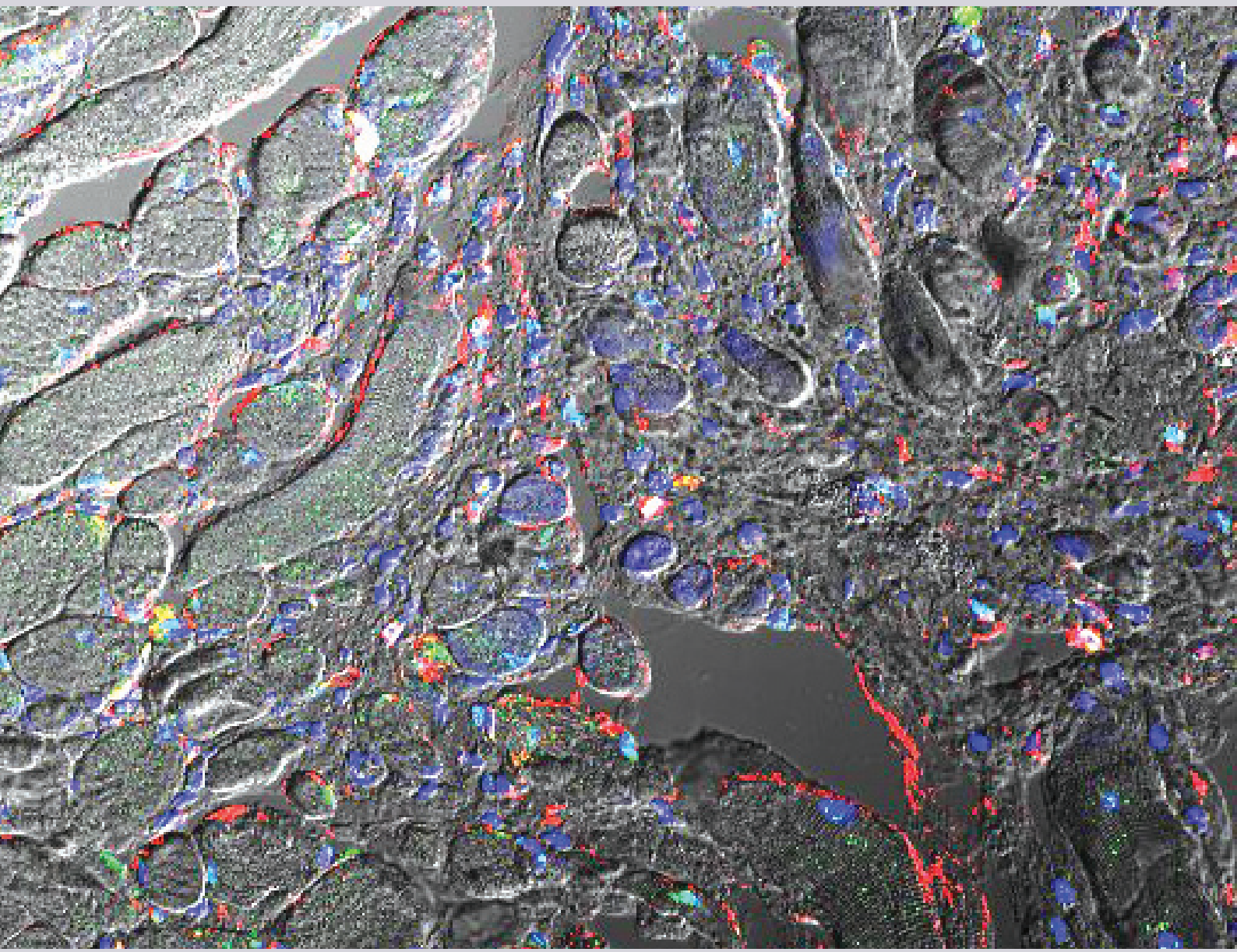

Mouse skeletal muscle tissue expressing Pax7 (red) and P57 (green) proteins

GENE REGULATION

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Molecular analyses of the human and animal genomes

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Our lab focus has been on genome analysis of animal and human systems including cancer. More than 50 different types of brain tumors have been reported of which Meningiomas and Gliomas each contribute to about 30%. However, number and kind of genes involved are still not fully understood. Despite gender bias in these tumors, Y chromosome linked genes and loci were not assessed earlier. In the present study, we analyzed *MN1* and *KIT* genes both in Gliomas and Meningiomas for their mRNA transcription, copy number variation and protein expression. In addition, we included Y chromosome linked genes and loci in our study.

Objectives

1. Expression analysis of *MN1* and *KIT* genes in the biopsied meningioma and glioma tumor tissues.
2. Mutational Status of the affected gene/s.
3. Analysis of the Y-Chromosome linked genes and loci encompassing *SRY*, *PABY*, *DYZ1*, *DYS1*, and *AZFa*, *AZFb*, & *AZFc* regions.

A. Genomics of Brain Tumors Meningiomas and Gliomas in Human

Primary brain tumors (PBTs) originate from the cranial nerves, meninges and associated tissues, unlike their secondary counterparts arising from the metastatic invasion. Of the 50 different types of PBTs catalogued by WHO, meningiomas and gliomas are the most common ones. Together with Y chromosome, we analyzed *MN1* and *KIT* genes for their mRNA transcription, copy number variation and protein expression.

A subset of meningiomas show KIT immunoexpression that correlates with transcript over-expression without gene alteration

Contrary to earlier reports on the absence of *KIT* expression in meningiomas, we detected its expression in 20.6% cases (n=7/34). IHC staining and western blots showing *KIT* expression in representative cases is shown in Figure 1.

not correlated with copy number variation (p>0.05, Figure 2C).

None of the *KIT* immunopositive cases showed loss or gain of functions as reported in other pathological conditions. Though 1/7 meningioma *KIT* positive cases showed a transversion (A→C) in exon 10, leading to a mis-sense substitution, (Met→Leu) at codon 541 (M541L). This SNP with minor allele frequency has been reported by

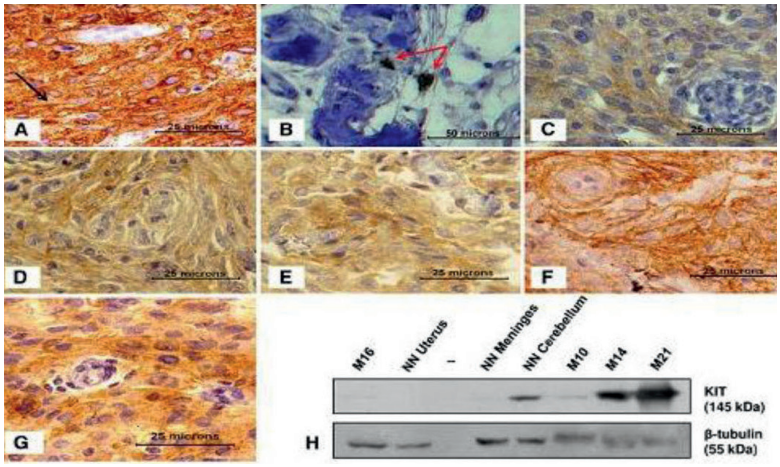

Figure 1: Immunoexpression of KIT in meningioma and control samples. (A) GIST (positive control) showing strong immunopositivity (B) an otherwise *KIT* negative meningioma showing interspersed positive mast cells (red arrows) control (C) meningothelial meningioma (M10) showing weak staining, (D) transitional meningioma (M14) displaying moderate staining, (E) meningothelial meningioma (M15) with weak to moderate staining, (F) fibroblastic meningioma (M21) showing strong staining of the cytoplasm, (G) an atypical meningioma (M29) with strong *KIT* expression. (H) Immunoblots of neoplastic and non-neoplastic (NN) tissue lysates probed with antibodies to *KIT* and β-Tubulin.

Expression of *KIT* receptor and its ligand *KITLG* transcripts was assayed using RT-qPCR. *KIT* immunopositivity was found to be associated with up-regulation of its transcript in meningiomas (p<0.001). Most of the *KIT* immunopositive cases showed co-expression of *KIT* and *KITLG* transcripts.

KIT copy number variation was found to be in accordance with fluorescence in situ hybridization (FISH) on the neoplastic tissues. Of the 7 *KIT* positive meningiomas, none showed copy number gain of this gene. Notably, M29 tumor tissue showed loss of *KIT* allele detected by FISH (red signal) and substantiated by qPCR (Figures 2A & B). The loss of *KIT* allele in M29 represented neoplastic event as the matched blood sample was found to have 2 copies of the gene (Figure 2B). *KIT* expression was

other groups. However, its clinical significance is unknown.

B. Over-expression of *KIT* without its amplification in a subset of Gliomas

In gliomas *KIT* over-expression and amplification of the gene have been reported. It was suggested that increased transcription and subsequent translation were due to amplification of the gene. However, none of the earlier studies validated this premise. In our study, 32.5% (n=13/40) gliomas showed *KIT* positivity validated by both IHC and western blotting (Figure 3).

KIT expression was found to be associated with transcriptional up-regulation (p<0.001). However, correlation between the level of *KIT* transcription

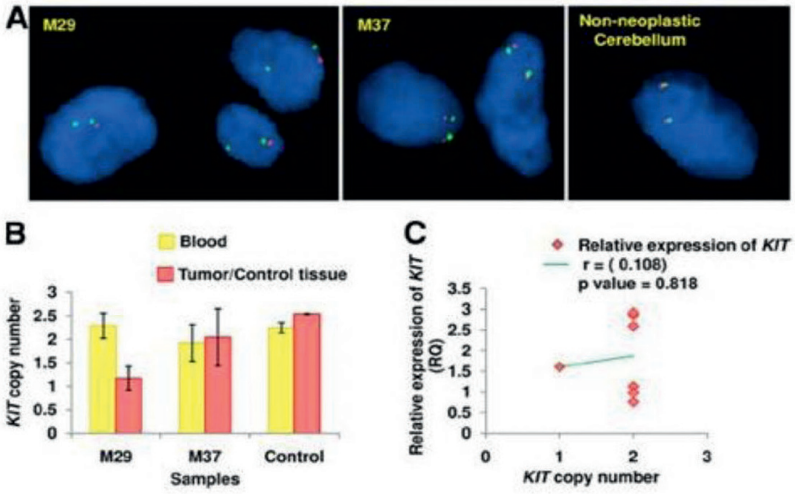

Figure 2: Copy number status of KIT and its correlation with expression (A) dual-colour FISH showing *KIT* signal (red) and centromeric sequences of chromosome 4 (green) on tissue sections (100x). M37 and non-neoplastic cerebellum (control) showed normal, M29 showed decreased *KIT* copy numbers. (B) qPCR based *KIT* copy number results of meningioma cases. (C) Scatter plot showing relative expression of *KIT* Vs its copy number in immunopositive meningioma cases.

and translation was not always proportionate. KIT immunopositive gliomas were found to co-express receptor and ligand (KITLG) transcripts.

We assessed the gene's copy number by qPCR and validated the findings by FISH on the neoplastic tissue (not shown). Contrary to literature report, no correlation was found between the altered copy number of the gene and expression ($p>0.05$).

In order to determine whether mutations in *KIT* contributed to its altered expression, we sequenced seven key exons and observed nucleotide substitutions in 6 cases though no activating mutations were identified. The clinical significance of these substitutions remained unclear.

C. *MN1* shows altered expression and copy number variations in both meningiomas and gliomas

Owing to its identification from a meningioma patient, having structural rearrangements

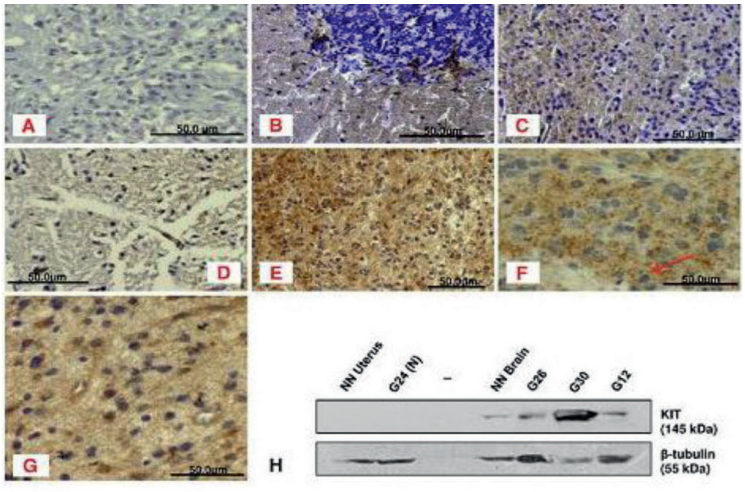

Figure 3: Immunoassays for KIT in glioma and control samples (A) Non-neoplastic uterine tissue (negative control) lacking *KIT* expression. (B) Non-neoplastic cerebellar tissue shows immunopositivity (C and D) represent an anaplastic astrocytoma (G15) and GBM (G26) showing moderate and faint *KIT* staining, respectively, (E) corresponds to a GBM (G30) showing strong *KIT* expression, (F) an anaplastic oligodendroglioma (G19) showing moderate granular and occasional subtle membranous staining (red arrow), (G) diffuse astrocytoma (G12) showing moderate *KIT* staining. (H) Western blots of tissue lysates with antibodies to *KIT* and β-Tubulin. NN signifies non-neoplastic tissue, N denotes negative for *KIT* IHC staining.

involving the chromosome 22, *MN1* (22q12.1) was proposed to be involved in this neoplasia. However, it was not clear if *MN1* expression is altered in meningiomas as compared to the normal meninges. In view of this, we undertook quantitative estimation of *MN1* transcripts in meningiomas and gliomas in comparison to the non-neoplastic meninges and brain tissue.

MN1 expression was quantified by RT-qPCR in 38 meningiomas. 21% (n=8/38) cases showed over-expression whereas ~53% (n=20/38) showed down-regulated expression as compared to the non-neoplastic meninges. Transcriptional up-regulation of *MN1* was more common in gliomas (~53) than meningiomas. Also, over-expression of the gene was correlated ($p = 0.013$) only with low grade gliomas (grades I and II).

Evaluation of the copy number status of *MN1* showed no loss of the gene in any of tumors. As compared to gliomas, meningiomas were found to be more prone to copy number variations. Moreover, copy number losses of *MN1* were more prevalent than its gain in meningiomas, while the scenario was completely reverse in gliomas. High grade gliomas evinced correlation ($p = 0.030$) with copy number variations (mostly gains), but did not show transcriptional up-regulation as often as LGGs. Conversely, the LGGs showing frequent over-expression rarely displayed copy number alterations (n=1/14, showing loss of an *MN1* copy). Thus, copy number changes in *MN1* did not strictly correspond to alterations in its level of expression. It was intriguing that some meningioma and glioma cases despite loss of an *MN1* copy demonstrated normal or up-regulated transcriptional status. Significantly, in meningiomas and gliomas both, higher levels of transcription did not always show proportionately enhanced protein expression.

Our work on the Y –linked genes and loci showed that there was no alteration. However, DYZ1 copy number variation was detected in about 60% cases.

According to the global scenario and reports from CBTRUS (Central Brain Tumor Registry of the United States) meningiomas (~34%) and gliomas (~31%) account for the most commonly occurring brain tumors. These tumors are graded based on clinical and histopathological criteria laid down by WHO. However, genotypic evaluation reveals several sub-grades conferring uniqueness to every single case. Present work is envisaged to augment accurate diagnosis and prognosis of brain tumors.

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1. Sher Ali* (2012) Animal DNA Fingerprinting- Beyond Dolly. *Biotech News* **7**: 5-7.
2. Samad R, Pathak D, Ali F, Rawal L, Sher Ali* (2012) Uniqueness of the Extracellular Domain of Protooncogene c-kit Receptor across the Species: Implications in Conservation of Biodiversity. *Proc Indian Natl Sci Acad* **78**: 651-670.
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Deciphering the role of cell signalling in *M. tuberculosis* biology and in the function and dynamics of nucleoporins

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A. Elucidating serine/threonine kinase mediated signalling pathways in *M. tuberculosis* and their role in the survival of pathogen in the host

Protein phosphorylation in prokaryotes plays a regulatory role in events as diverse as chemotaxis, bacteriophage infection, nutrient uptake and gene transcription. Analysis of the *M. tuberculosis* genome sequence suggested the presence of 11 eukaryotic- like serine/threonine protein kinases, and one serine/threonine protein phosphatase. Recent reports have established the role of these kinases in modulating cell shape and morphology, glucose transport, glutamine transport, glutamate metabolism and regulation of the expression/activity of transcription factors. Our aim is to decipher the signaling pathways in *M. tuberculosis* and investigate their role in modulating the host signalling network and the survival of pathogen in the host. Towards this goal, following objectives are being pursued:

1. To identify novel downstream targets and determine the role of kinase mediated phosphorylations of these targets.
2. Generate phospho-specific antibodies and gene replacement mutants and utilize them to investigate their activation status under various growth conditions and upon infection.
3. Investigate modulation of host signalling

pathways upon infection with *M. tuberculosis* H37Rv wild type or kinase gene replacement mutants.

B. Deciphering the role of signaling in modulating the function of nucleoporins

Nucleocytoplasmic transport between the nucleus and the cytoplasm occurs through nuclear pore complexes (NPC), ~ 60 MDa macromolecular structures that span from cytoplasm to nucleus across the lipid bilayers of the nuclear envelope. Nucleoporin Tpr is a component of the nuclear pore complex (NPC) that localizes exclusively to intranuclear filaments. Our aim is to decipher the role of phosphorylation on the functions of nucleoporin Tpr. In this context, following objectives are being pursued:

1. Identification and validation of *in vivo* phosphoryaltion sites on Tpr.
2. Determining the functional significance of identified phosphorylation sites.

A. Elucidating serine/threonine kinase mediated signalling pathways in M. tuberculosis and their role in the survival of pathogen in the host

Protein Kinase A and B (PknA and B)

Despite the fact that many of the substrates were initially identified to be substrates for one specific kinase or other, most are actually phosphorylated by multiple STPKs. Thus, a key feature amongst all these kinase-substrate interactions is the redundancy of signalling, suggesting a complicated temporal and spatial control over key mycobacterial processes. Since PknA and PknB are essential genes, it was necessary to provide an inducible integrated copy of these genes. Recently, we reported generation of new series of vectors that allow for the constitutive and regulatable expression of proteins, appended with peptide tag sequences at their N- and C- termini, respectively. With the help of developed shuttle vectors, we have created integrating shuttle vector where *pknA* or *pknB* genes have been cloned under a tetracycline inducible promoter.

These integration proficient plasmids containing inducible *pknA* or *pknB* genes were transformed into *M. smegmatis* and *M. tuberculosis* to create pknA or pknB merodiploid strains. Conditional gene replacement mutants of PknA and PknB in *M. smegmatis* (mc^2ΔpknA and mc^2ΔpknB) were created from the merodiploids following two step recombination protocol. The authenticities of the mutants were confirmed by PCR and western blot analysis. We have procured *M. tuberculosis* H37Rv ΔpknB (RvΔ*pknB*) conditional gene replacement mutant from Dr. Franscisca Forti, in which the PknB gene expression was regulated by pristinamycin. Importantly, we have also successfully generated *M. smegmatis* and *M. tuberculosis* Δ*pknA*Δ*pknB* (mc2Δ*pkn*AΔ*pknB* & RvΔ*pkn*AΔ*pknB*) double mutants.

Growth analysis of the conditional mutants in the presence and absence of inducer (ATc or pristinamycin) demonstrated that both PknA and PknB are individually essential for the survival of mycobacteria. Complementation of the mutants with ectopically expressing PknA or PknB showed that both their kinase activities are necessary for functionality *in vivo*. Using these conditional mutants, we showed that extracelluar PASTA domain of PknB plays a critical role in PknB mediated signaling and its

absence results in cell death. In contrast, in case of PknA the localization to cell membrane seems to be critical, however, the extracellular domain does not play any role. The above tools would be used to decipher PknA and PknB signaling networks in *M. tuberculosis*. Further, work is also underway to develop novel inhibitors of PknB.

Protein Kinase G (PknG)

Protein kinase G (PknG) is closely related to the mammalian protein kinase Cα. PknG has been shown to be secreted into the cytosol of host macrophages during M. bovis BCG infection. Though PknG lacks an identifiable N- terminal signal sequence for secretion, it is known that mycobacterium species have alternate secretory pathways that may aid in secretion of such molecules. Work is in progress to investigate the secretion mechanism of PknG. The N-terminal region of PknG harbours two Trx fold motifs ^{106}C-x-x-^{109}C and ^{128}C-x-x-^{131}C. In order to investigate their role in regulating PknG activity, we have generated single, double and quadruple mutants of cysteine to alanine and serine residues. We are in the process of investigating the precise role-played by Trx motifs in survival of pathogen in the host.

N-acetylglucosamine-1-phosphate Uridyltransferase (GlmU)

N-acetyl-glucosamine-1-phosphate-uridyl-transferase (GlmU), a bifunctional enzyme involved in bacterial cell wall synthesis is exclusive to prokaryotes. Several studies now recognize GlmU as a promising target to develop new anti-bacterial drugs. GlmU catalyzes two key reactions; acetyl transfer and uridyl transfer reactions, at two independent domains. Previously, we identified GlmU to be a substrate for PknB and determined the biochemical significance. Recently we showed GlmU from *M. tuberculosis* ($GlmU^{Mtb}$) to be unique in possessing a 30 amino acid extension at the C-terminus. In collaboration with Dr. Balaji Prakash, we solved the crystal structures of $GlmU^{Mtb}$ in complex with substrates/products bound at the acetyltransferase active site. Analysis of these and mutational data, allowed us to infer a catalytic mechanism operative in $GlmU^{Mtb}$. In this SN2 reaction, H374 and N397 act as catalytic residues by enhancing the nucleophilicity of the attacking amino group of glucosamine-1-phosphate. A short helix at the C-terminal extension is uniquely found in $GlmU^{Mtb}$, which provides the highly conserved W460 for substrate binding. Furthermore, the structures revealed an uncommon mode, termed the U conformation, for acetyl-CoA binding. It is distinct from the L conformation seen thus far in the crystal structures of GlmU from non-mycobacterial species. With the help of mass spectrometry and *in vitro* kinase assays, we identified PknB mediated phosphorylation site on GlmU to be T418, which is present in a region close to the acetyltransferase active site. The importance of this region to structural integrity and therefore the observed decrease in activity upon phosphorylation was rationalized based on the structures.

Transposon mutagenesis experiments revealed $GlmU^{Mtb}$ to be an essential gene. In line with this previous work demonstrated the inability of *M. smegmatis* to grow in the absence of $GlmU^{Mtb}$. However, which among the two activities of the protein are essential has not been addressed. To address these questions we wanted to generate conditional gene replacement mutant

in *M. semgmatis* and *M. tuberculosis*. Towards this we have generated merodiploid strains wherein, a regulatable copy was integrated in to the attB site of mycobacterial genome. In this background we have successfully generated *M. smegmatisΔglmU* mutant, in which the gene at its native locus is replaced with hygromycin resistance gene cassette. Efforts are underway to generate *M. tuberculosisΔglmU* conditional mutant. Our preliminary results confirm that GlmU is indeed essential for growth and survival of *M. smegmatis*. Since, GlmU is essential and is responsible for generating UDP-GlcNAc, a very important metabolic intermediate, we are also focusing on development of novel inhibitors against both uridyl and acetyl transferase activities.

B. Deciphering the role of signaling in modulating the function of nucleoporins

Previously, we demonstrated that Tpr is phosphorylated *in vivo* in MAP kinase ERK2 dependent and independent fashion. We have now identified ERK2 independent phosphorylation sites. We raised phospho-specific antibodies that can recognize phosphorylation on these residues. Extensive characterization of antibodies clearly demonstrated that antibodies specifically recognize phosphorylated Tpr phosphorylated both in western blots as well as immunofluorescence experiments. Based on the sequence analysis, *in vitro* kinase assays and *in vivo* siRNA experiment, Protein kinase A was identified to be one of the kinase that phosphorylates Tpr. Work is in progress to determine the biological significance of ERK independent phosphorylation of Tpr on various functions of Tpr.

Publications

Original peer-reviewed articles

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Arnab Mukhopadhyay

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

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Our laboratory uses a combination of genetics, molecular biology, genomics coupled to Next Generation sequencing (NGS) to decipher the underlying mechanisms of aging. As a model system, we use *C. elegans*, a nematode that has been instrumental in understanding various important biological processes including aging and innate immunity. We are trying to understand the complex interplay of transcription factors and co-regulators downstream of the Insulin-IGF-1 signalling (IIS) pathway in regulating gene expression required for longevity, metabolism, stress and pathogen resistance. Additionally, we are also interested in understanding the molecular events that take place during dietary restriction (DR), the only intervention that can prolong longevity in almost all organisms tested. We have also developed a chemical genetic screen to identify compounds that can prolong life span.

We would like to map the genome-wide recruitment of two transcription factors that function downstream of IIS pathway, namely HSF-1 and DAF-16/FOXO, to their target gene promoters following different physiological inputs. NGS-based applications like ChIP-seq and transcriptomics will be used for this purpose. In the second objective, we will try to understand how DR works at the molecular level by characterizing several novel genes and miRNAs that work in the DR pathway. Thirdly, we would perform screens to identify novel compounds or reposition existing drugs to increase life span in the model system and decipher their mechanism of action.

A. Deciphering coordinate regulation of genes downstream of IIS pathway

In *C. elegans*, lowering the IIS leads to increased life span as well as increased resistance to various biotic and abiotic stresses. We are using a multi-pronged approach to understand why these worms are resistant. We are interested in understanding the complex regulation by transcription factors downstream of the IIS pathway to co-ordinately control their direct targets that may be involved in increased resistance. In this direction, we used RNAi to knock down different transcription factors, that are known to function downstream of IIS, in an IIS-deficient worm strain. This was followed by RNA sequencing at our GAIIx NGS facility. We have been able to delineate specific transcription factor-regulated genes downstream of IIS. For example, currently we have generated data for transcriptional targets of FOXO/DAF-16, SKN-1/NRF2, SMK-1/SMEK1, HSF-1/HSF and BAR-1/B-catenin that are controlled by the IIS pathway. We are in the process of generating data for several other transcription factors including the ELT and GATA family proteins. Preliminary observation points to coordinate as well as independent regulation of the IIS downstream genes by different transcription factors (Figure 1D).

In order to understand how two of the most important transcription factors downstream of IIS, FOXO/DAF-16 and HSF-1, co-ordinately regulate gene expression, we have raised polyclonal antibodies against these proteins using NII animal facility. We have standardized chromatin immunoprecipitation using the DAF-16 antibody. First, we confirmed the ChIP procedure by confirming the recruitment of FOXO/DAF-16 to *sod-3* promoter in a IIS knockdown worm strain using real-time PCR (Figure 1A). Following this, we prepared sequencing libraries using Illumina procedures and validated enrichment in them by real-time PCR analysis (Figure 1B).

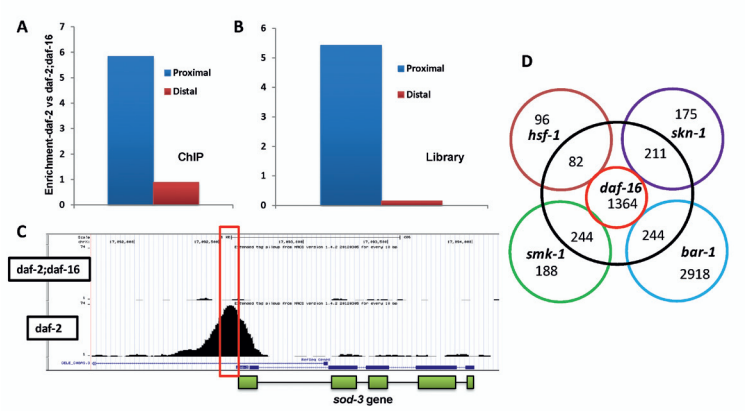

Figure 1: Standardization of ChIP-sequencing and transcriptomics. A) increased recruitment of DAF-16 to proximal promoter of sod-3 compared to distal. B) Same enrichment retained in the ChIP-seq library. C). Snapshot of reads aligned to the sod-3 promoter. D) Transcriptomics analysis shows overlap of transcription factor-dependent genes in a daf-2 insulin receptor mutant.

Preliminary analysis of deep sequencing data confirmed recruitment of DAF-16 to the DAF-16-binding element proximal to the sod-3 promoter (Figure 1C).

B. Involvement of miRNA-Transcription factor networks in dietary restriction

MicroRNAs have emerged as important regulators of many biological processes

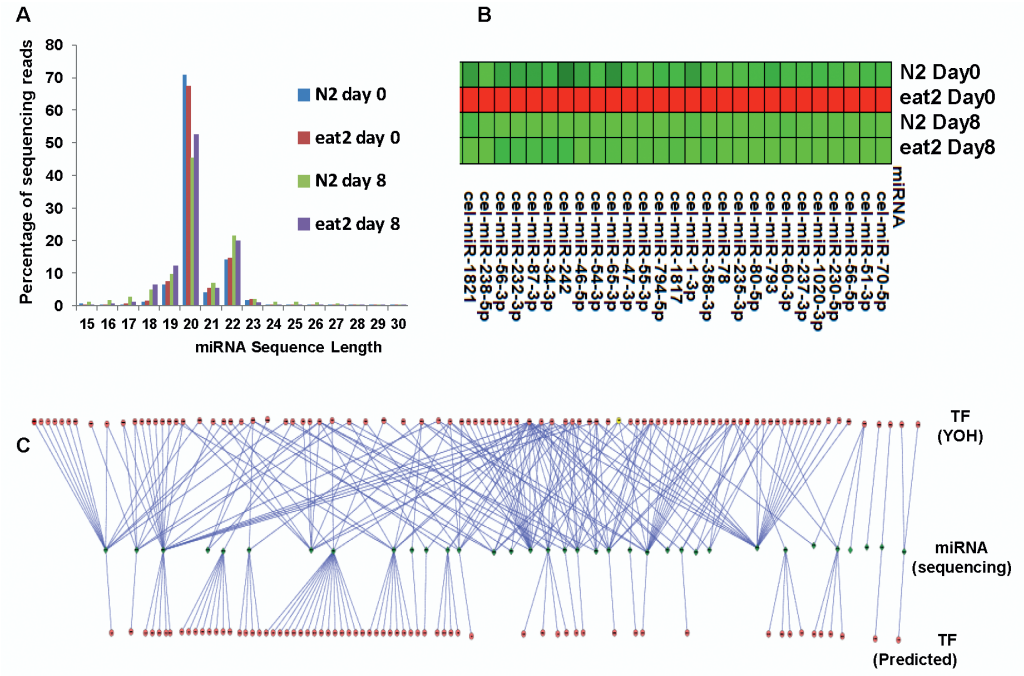

Figure 2 : miRNA profile during dietary restriction. A) miRNA length following sequencing and adapter trimming. B) Snapshot of miRNA profile between wild type N2 and dietary restricted eat-2 mutant. C) Trancription factor-miRNA regulatory network.

including aging. Since we are interested in the mechanisms of dietary restriction, we initiated a study to understand the role of miRNAs in this process. We performed a NGS-based profiling of miRNA in wild-type and dietary restricted worms during early and late life. (Figure 2A) We find a general upregulation of miRNA by dietary restriction (Figure 2B) which we also validated by real-time PCR. Due to the depth of sequencing, we identified and validated several novel miRNA.

Extensive prediction of miRNA targets using three different softwares showed that these miRNAs mostly target genes involved in early embryogenesis and larval development. This is important as resource allocation during dietary restriction needs repression of egg production and reduced reproductive energy expenditure. We integrated our data with a published yeast one-hybrid screen using *C. elegans* miRNA promoters and identified several transcription factors that target the miRNAs upregulated during dietary restriction (Figure 2C). We also used motif search softwares to find common elements of the miRNA promoters and found enrichment for helix-loop-helix transcription factors. We are currently in the process of determining the physiological role of these factors during dietary restriction.

C. Involvement of novel kinases in dietary restriction

We have identified a kinase gene *eddf-1* (which we currently call *idr-1*) involved in dietary restriction. We have extensively characterized this gene by molecular genetic analysis. We showed that *idr-1* knockdown initiates a process

of internal dietary restriction without changes in food intake. Initiation of dietary restriction leads to shift in metabolism towards beta-oxidation. This is achieved through the transcriptional activator HNF4/NHR-49, an important regulator of beta-oxidation genes (Figure 3A). We also found that peroxisomal beta-oxidation is indispensable for *idr-1*-mediated longevity, possibly due to the break down of long-chain fatty acids for energy generation. Change in metabolism to beta-oxidation leads to low ROS production; this is independent of SOD activity as none of the *sod* genes or their activity is upregulated. The shift in metabolism also leads to increased expression of xenobiotic detoxification genes. These genes are targeted through transcription factors/ nuclear hormone receptors NHR-8 and ARH-1 (Figure 3B). Finally we show that increased beta-oxidation is the reason for increased xenobiotic detoxification gene expression, leading to long life (Figure 3C).

D. A drug that can extend *C. elegans* life span

Glycation is a non-enzymatic reaction leading to a covalent bond between an amino acid or lipid and a sugar molecule. Advanced glycation end-products (AGEs) are formed in all glucose-utilizing systems, leading to aberrant protein functions that form an important basis for debilitating diseases and aging. Thus we hypothesized that preventing glycation may lead to increased life span. We identified a FDA-approved drug that can prevent glycation *in vitro* and *in vivo*. We developed several assays using liquid culture in *C. elegans* to evaluated the efficacy of the drug. Treatment with this drug even late in adulthood increased life span by ~50-80% (Figure 3A) and delayed A-beta as well as polyglutamine aggregation-related proteotoxicity. The drug was also able to prevent glucose toxicity in a nematode model of diabetes (Figure 3B). We showed that the drug works by activating the

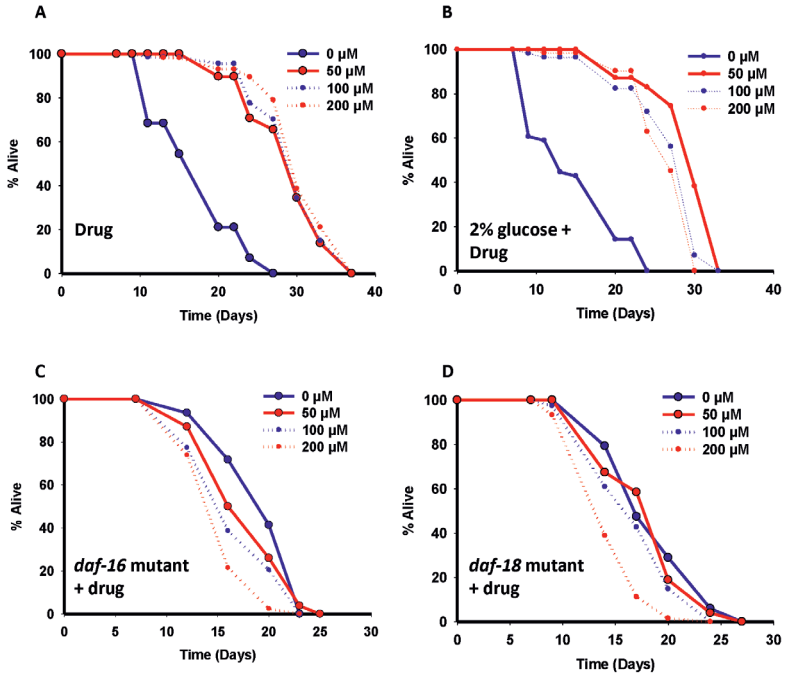

Figure 3: (A) Treating C. elegans with the new glycation inhibitor increases life span. (B) Treatment with the drug ameliorated glucose toxicity. The drug requires DAF-16/FOXO (C) as well as DAF-18/PTEN to function as no life spanextension was seen it the mutants.

FOXO transcription factor DAF-16 (Figure 3C) leading to its nuclear localization and subsequent activation of several downstream genes, including two SODs. However, we found that the subset of genes targeted by the drug was significantly different than the FOXO dependent targets of IIS pathway. Bioinformatic analysis of promoters of genes upregulated by drug treatment showed that majority of these genes have FOXO binding sites although they are not regulated by IIS. Thus the drug may be targeting a different set of FOXO targets. Interestingly, we showed that the drug requires PTEN/DAF-16 for its action (Figure 4D). However, it is independent of the IIS downstream target PDK-1. Our findings suggest that the drug may target an alternate pathway parallel to the IIS to regulate FOXO. Preliminary results suggest the involvement of JNK.

Publications

Original peer-reviewed articles

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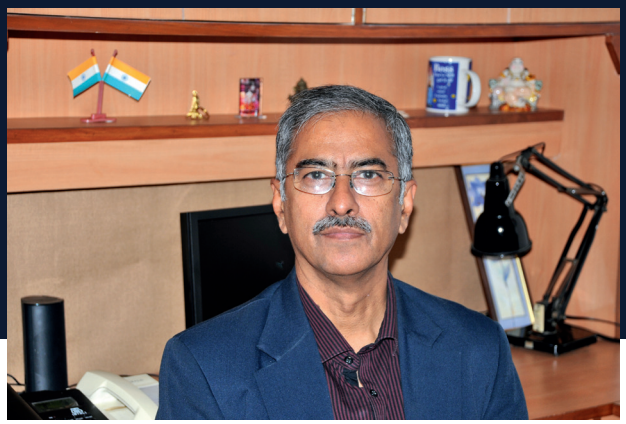

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Molecular biology of infectious diseases

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

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

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

B. Studies on functional characterization of PE_PGRS and PE_PPE proteins of *Mycobacterium tuberculosis* H37Rv

Sequence analysis of the *Mtb* H37Rv genome resulted in identification of novel multigene families- the PE (proline-glutamic acid) and the PPE (proline-proline-glutamic acid). These families account for much of the genomic difference between *Mtb* and nonpathogenic

mycobacterial genomes. Therefore, they may play role in *Mtb*'s virulence and host specificity. However, their exact role in *Mtb* biology is not clearly understood. We aim to explore the possible role these proteins may play in the biology of *Mtb*.

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

In order to evaluate the efficacy of intranasal immunization of recombinant epsilon toxin (Etx), BALB/c mice were immunized intra-nasally with heat inactivated Etx followed by one booster after 2 weeks of primary immunization. Serum samples were collected at different time intervals of immunization. The specificity of the antibody against Etx was confirmed by Western blot analysis and immune response at the level of antigen-specific antibodies was assayed by indirect ELISA. Intranasal immunization of the recombinant Etx generated a very good immune response. After intranasal immunization, specific immunoglobulin isotypes was analyzed by isotyping ELISA and *IgG1* was found to be dominant. The capacity of intranasal immunization of Etx to induce cell mediated immunity was analyzed by *in vitro* T cell proliferation assay by inducing the splenocytes of immunized mice with Etx. Specific T cell response was determined by cytokine analysis.

Neutralization capacity of the anti-sera was analyzed by incubating sera and Etx prior to the addition to MDCK cells and a significant protection of cells was observed. *In vivo* protection potential of sera from intra-nasally immunized mice was evaluated by incubating sera and the lethal dose of toxin before injecting the naïve mice and a high level of protection was observed. To check the vaccine potential of intra-nasal immunization of recombinant Etx, *in vivo* protection studies were carried out in the immunized mice followed by challenge with 50 LD_{50} dose of Etx. One hundred percent protection was observed.

The immunized mice were challenged with 50 LD_{50} dose of Etx and mice survived after challenge, these mice were kept and observed for an additional two weeks to ensure that they survived the infection.

Using bioinformatics tools, five potential surface exposed regions of the ε-toxin were predicted. In order to develop subunit vaccine against Etx, we have earlier reported the cloning of these five putative epitopes of Etx in translational fusion with LTB at the C-terminus of LTB. Secretory expression of one of these fusion proteins in *Vibrio cholerae* cells has also been reported. During the current year, studies on two more fusion proteins have been carried out. Both the Etx epitope-LTB fusions were subcloned in pMMB vector for secretory expression in *E. coli* DH5α cells. The constructs were then conjugally transferred to Vibrio cholerae cells. Expression of the fusion genes in *E. coli* and *V. cholerae* under the control of the tacP promoter was carried out by inducing the cells with IPTG. Periplasmic and extracellular expression of the fusion proteins was obtained in *E. coli* and *V. cholerae* cells, respectively. Purification of the fusion proteins from the Vibrio culture supernatant was obtained by ammonium sulphate precipitation followed by cation exchange chromatography. BALB/c mice immunized with the fusion proteins emulsified in alum followed by a single booster dose generated a very good immune response against each fusion protein. Western blot analysis and ELISA using the anti-sera against the fusion proteins indicated that both the fusion proteins retained

the antigenicity of the fusion partners and was able to detect the Etx as well as LTB. The anti-sera generated against the fusion proteins were able to confer protection against epsilon toxin toxicity in MDCK cells.

B. Studies on the functional characterization of PE_PGRS and PE_PPE proteins of *Mycobacterium tuberculosis* H37Rv

In order to address the question of differential effect of over expression of PE_PGRS30 and PE_PGRS35 in *M. smegmatis* sequence comparison of these proteins was performed. Sequence comparison revealed that PE_PGRS30 possesses a much larger PGRS domain than PE_PGRS35 and both the proteins contain an additional, highly similar C-terminal domain. This suggested that PGRS domain may be responsible for change in colony morphology of PE_PGRS30-recombinant *M. smegmatis* while delayed lag phase may be caused by the C-terminal domain. To test this hypothesis, 3 other PE_PGRS proteins (PE_PGRS11, PE_PGRS16 and PE_PGRS62) possessing the C-terminal domain, were selected and expressed in *M. smegmatis* but none affected the growth of recombinant mycobacteria, indicating that the change in growth profile is not solely due to C-terminal domain.

Proteomic analysis of recombinant *M. smegmatis* expressing PE_PGRS30 showed differential expression of 8 proteins in the cytosol, of which, 3 up-regulated proteins (encoded by Msmeg_2516, Msmeg_1874 and Msmeg_1634) could be identified using MS/MS analysis. Twelve proteins were differentially expressed in the cell wall of PE_PGRS30 recombinant *M. smegmatis*, of which 2 up-regulated proteins (encoded by Msmeg_4011 and Msmeg_6725) were identified. Msmeg_1874 encodes for DNA-binding response regulator, MtrA, which has been shown to be essential in *M. tuberculosis* and suggested to regulate growth of *Mtb in vivo*. Msmeg_2516 and Msmeg_1634 encode for putative F420-dependent oxidoreductase and probable forkhead associated protein, respectively. A putative pyrimidine permease and an ABC transporter, ATP binding protein are encoded by Msmeg_4011 and Msmeg_6725, respectively.

Further, to dissect the functional domains of PE_PGRS30, the 3 mutants (PE only, PE+PGRS and C-term only) were expressed in *M. smegmatis*. All the 3 mutants localized in the cell wall suggesting that not only PE domain but C-terminal domain also directs the protein to the cell wall. Fluorescence microscopy data illustrated that the PGRS domain of PE_PGRS30 is responsible for association of the protein with cell poles since only PE+PGRS mutant showed polar localization. Further, proteinase K sensitivity assay revealed that PE_PGRS30 is exposed on mycobacterial surface through its PGRS domain. The change in colony morphology caused by PE_PGRS30 was found to be due to the presence of PGRS domain but none of the domains could individually cause delay in the lag phase of growth of *M. smegmatis* in broth culture. In fact, PE+PGRS mutant rendered a more pronounced effect on growth of *M. smegmatis* on solid media as compared to the complete protein as colonies of PE_PGRS30-recombinant *M. smegmatis* appeared after 4 days of electroporation while those of *M. smegmatis* expressing PE+PGRS mutant appeared only after 5 days.

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Original peer-reviewed article

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6. Deshmukh SK, Kaushik H, Sharma N, Tiwari A, Trivedi P, Garg LC (2012) Development of membrane bound expression and heterologous booster based DNA vaccine against ε-toxin of Clostridium perfringens (1462/DEL/2012, Indian patent filed on 11/05/2012).
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Madhulika Srivastava

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

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

To explore the mechanisms underlying insulator activity, antigen receptor loci like IgH, TCRα/δ, TCRβ etc, are particularly interesting. Transcription as well as RAG mediated recombination is exquisitely regulated during development at these loci and underscores the importance of appropriate enhancer-promoter interactions. Further, recombination requires physical interaction between RSS elements associated with the V, D and J segments. These segments are located at large distances from each other on the chromosome and higher order chromatin reorganization is necessary to bring them together prior to recombination. By exhibiting long range interactions between different types of elements, the antigen receptor loci present a useful framework to explore the role of CTCF in defining independently regulated chromatin domains. Taking advantage of this, we are currently investigating the chromatin structure and organization of the wild type and genetically manipulated TCRβ loci to understand various aspects of insulator function as well as VDJ recombination.

Our functional analysis of TCR-ins mutants in the previous years clearly showed that normal chromatin interactions for ordered VDJ recombination can be disrupted simply by the ectopic insertion of non-locus specific CTCF binding sites. The inserted *H19-ICR* interfered in promoter-enhancer interactions providing a useful system to explore enhancer-promoter-insulator interactions. Also, the ectopic CTCF binding at the inserted insulator reorganized the chromatin landscape and influenced Vβ-to-DβJβ interactions. To understand these observations, analysis of several aspects pertinent to chromatin organization has been initiated. Some observations of ongoing investigations are summarised below:

1. The enhancer mediated accessibility generation is prevented by the ectopic insulator and influences Rag binding

The region known to be modulated by enhancer Eβ, showed an enormous reduction in activating histone modifications H3K4-me3 and H3K9-Ac as shown in the previous year. This reduction was seen only for PDβ1 and associated DJCβ1 segments and not for PDβ2 and DJCβ2 segments correlating well with the position dependence of the insulator activity detected functionally. H3K4-me3 has been reported to act as a signal for recruitment of Rag2, an important cofactor of Rag1. Hence the presence of Rag2 on the wild type and mutant alleles was analysed by allele specific ChIP. The Jβ segments of DJCβ1 cluster did not exhibit any Rag binding while Jβ segments of DJCβ2 cluster showed high enrichment of Rag2 protein by ChIP and correlated well with the presence of H3K4-me3. By an entirely independent assay relying on restriction enzyme accessibility, we confirmed that the Eβ mediated accessibility is altered in a position dependent manner. Thus, it forms the molecular basis of the altered transcription and recombination profiles observed earlier at the DJCβ1 cluster.

2. The insulator leads to complete repression of the PDβ1 promoter

The functional Eβ-PDβ1 interaction as well as deposition of activating histone modifications markedly reduced at the promoter. We next investigated the requirement of Eβ in removing repressive histone modifications. We analysed the presence of H3K9-me2 and DNA methylation in the PDβ1 promoter region. Clearly, the inactive promoter exhibited association with H3K9-me2 as well as methyled CpG residues. Both these features are known to be developmentally regulated by Eβ. Additionally, we analysed H3K27-me3 association on this promoter as it known to be associated with repressed chromatin but has not been investigated so far in context of TCRβ. Our analysis of the TCR-ins allele showed an enhanced associated of H3K27-me3. However, the levels of the modification as assessed by ChIP, were much lower than of the same region in non-lymphoid cells (NIH3T3). This suggests that Eβ is essential for removal of repressive marks as well as recruitment of activating marks. Notably, Eβ is shown to activate the 25 kb region in a PDβ1 dependent as well as independent manner, and the inserted insulator prevented both these activities in a CTCF dependent manner.

3. Altered Vβ-to-DβJβ recombination does not correlate with an altered TCRβ locus contraction

Antigen receptor loci IgH, Igβ as well as TCRβ have been shown to undergo 'locus contraction' prior to VDJ recombination which brings distally located V segments in spatial proximity of DJ

segments. We speculated, therefore, that the basis of the observed alterations in Vβ-to-DβJβ recombination in the TCR-ins mutant alleles could be an inability to undergo appropriate degree of locus contraction. Using DNA-FISH analysis, we have investigated this aspect. Our preliminary findings suggest that the extent of contraction in mutant alleles is not different form the wild type alleles. Considering that the TCRβlocus has several endogenous CTCF binding sites, it is not surprising that overall contraction of the locus was not dramatically altered by insertion of four ectopic CTCF binding sites as *H19-ICR*. However, this aspect needs further analysis. It seems likely that alterations in specific interactions between the CTCF binding sites might be more relevant.

4. Long distance interactions between endogenous CTCF binding site of TCRβ locus are observed

We have earlier validated CTCF and cohesin binding at several sites on the TCRβ locus by chromatin immunoprecipitation in thymocytes derived from Rag deficient mice i.e. prior to initiation of VDJ recombination. The interaction between these sites is being investigated using chromosome conformation capture analysis. Our preliminary analysis suggests interactions across large distances as would be required for effective VDJ recombination. It will be interesting to investigate the alterations in the interactions under the influence of the ectopic CTCF binding sites in TCR-ins allele. This investigation will rely on allele specific 3C analysis.

Publication

Original peer-reviewed article

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Pushkar Sharma

Role of cell signaling in eukaryotic development

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

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

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

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

While apoptosis of neurons is critical for proper brain development, it also leads to neuronal loss in neurological disorders. A subset of neurons upon exposure to neurotoxic stimuli 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). We are interested in molecular mechanisms that regulate Cell Cycle Related Neuronal Apoptosis (CRNA), which are poorly understood.

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

Cross talk between cAMP and calcium in the blood stage development of malaria parasite

During the course of above described studies on the regulation of glideosome associated protein 45 (PfGAP45) by calcium effectors like PfCDPK1 and PfPKB, we noted that PfGAP45 may also be modulated by calcium independent pathways. Interestingly, the modulators of cAMP

like cAMP phospodiesterase inhibitor IBMX and 8BrcAMP caused a decrease in phosphorylation of GAP45 at PfCDPK1/PfPKB target sites. These observations suggested that cAMP may either modulate calcium levels in the parasite or regulate the down stream effector cAMP-dependent protein kinase (PfPKA), which in turn may control the activity of PfCDPK1 and PfPKB. Subsequent studies to test these possibilities are in progress. The modulators of cAMP inhibited PfCDPK1 activity suggesting that cAMP needs to be optimal for PfCDPK1 activation. The role of cAMP dependent protein kinase, PfPKA, was also investigated. PfPKA may prevent the activation of PfCDPK1 *in vitro*. We also observed that the alteration in cAMP affects RBC invasion by the parasite. Studies investigating the contribution of calcium and its effectors in this process are being pursued.

Role of phosphoinositides in parasite signaling and trafficking

We are interested in dissecting the role of phosphoinositides (PIPs) in the parasite life cycle. In this context, we have previously reported the role and regulation of a PI3K kinase PfPI3K, a PI3P binding protein FCP and PfPLC. *In silico* studies lead to the identification of several proteins that contain PIP binding domains and motifs in *Plasmodium falciparum*. Several of these proteins may be involved in trafficking events that regulate processes like endocytosis and autophagy. One of them (PLASMODB ID: PF11_0242) is a protein kinase, which has a C-terminal kinase domain (KD) preceded by a pleckstrin homology (PH) domain, therefore, was named as a PH-domain containing kinase (PfPHDK). In addition to the PH and the kinase domains, PfPHDK also has an EF hand motif making it a putative target of calcium. IFAs performed to localize PfPHDK suggested that it may be expressed in the ER at early stages of development and in ER derived vesicles as the parasite matures; immuno-EM studies also supported these results. PIP binding assays indicated that PfPHDK interacts with PI(4,5)P2 via its PH domain. In order to explore if the PH domain of the kinase is responsible for its targeting to the vesicular compartments, parasite lines expressing PH-GFP, PH+KD-GFP and KD-GFP were generated. While the PH domain constructs exhibited vesicular localization as observed for PfPHDK, the KD-GFP was present in the parasite cytoplasm suggesting that PH domain, via its interaction with PIP2, is responsible for cellular targeting of *PfPHDK*. Efforts were made to disrupt PfPHDK in *P. falcparium* by using a single cross-over homologous recombination strategy previously used to successfully knockout other *P. falciparum* kinases. Briefly, a portion corresponding to the central region of the kinase domain was cloned in pCAM-BSD vector for this purpose. The blastidicine resistant parasites contained a population in which the integration had occurred at the expected locus. Several attempts to clone the knock-out (KO) parasites by limited-dilution resulted in the identification of a clone in which *PfPHDK* was disrupted. In comparison to wild type, the growth of PfPHDK-KO asexual blood stage parasites was significantly compromised. A close examination of the parasite life cycle suggested that the transition of ring to trophozoites was significantly hampered in PfPHDK-KO parasites. In addition, the number of nuclei per schizonts was also lesser than the wild type parasites. These observations indicated that PfPHDK is crucial for proper development of asexual blood stage malaria parasites. Efforts to elucidate the mechanisms via which PfPHDK contributes to the parasite development are being made.

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

As mentioned above, we identified a novel mechanism via which beta amyloid peptide $A\beta_{42}$ causes Cell Cycle Related Neuronal apoptosis (CRNA) by elevating the expression of cyclin D1. The role of CDK inhibitor (CKI) p27 and cdk5 in this process was further explored. The inhibition of cdk5 or its knockdown lead to CRNA via hyperactivation of the MEK-ERK pathway, which suggested that one of the roles of cdk5 is to keep the neuronal cell cycle in check. Furthermore, the treatment of neuronal cells with $A\beta_{42}$ or $A\beta_{42}$ leads to the formation of inactive cyclin D1-cdk5 complex. p27 further enhanced the formation of this complex by interacting with cdk5 and cyclin D1. In contrast, p27 did not interact with p35 and cdk5.

Several miRNA are directly or indirectly involved in the cell cycle progression as several cell cycle related proteins are miRNA targets. Interestingly, the levels of some of these miRNA are modulated in Alzheimer's disease brains and other models of neuronal apoptosis. However, their contribution to neuronal cell death is poorly understood. We are investigating if miRNA contribute to the process of CRNA by targeting cell cycle related genes. Ongoing studies on miR34a indicate its possible involvement in CRNA. The overexpression of miR34a in cortical neurons attenuated the expression of cyclin D1 induced by Ab_{42} and also blocked the S-phase entry and cell death. *In silico* analysis suggested that miR34a may interact with the 3'UTR of cyclin D1 and thereby may control its expression, which was supported by preliminary studies. These findings suggested that miR34a may regulate the CRNA possibly by modulating cyclin D1 levels. Efforts will be made to dissect the underlying mechanisms.

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Original peer-reviewed articles

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Rajesh S. Gokhale

Reconstructing the chemico-cellular trestle to decipher biology of Tuberculosis and Vitiligo

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Our group is interested to elucidate mechanistic and spatiotemporal coherence of cellular processes that result in two distinct pathological diseases – Tuberculosis (TB) and Vitiligo. Although unrelated, both disorders involve decisive role of unusual metabolites- complex lipids with very-long branched acyl chains produced by the TB pathogen *Mycobacterium tuberculosis* (Mtb) and heteropolymeric structurally uncharacterized melanins produced by melanocytes. Both these diseases are also characterized by unpredictable disease progression profiles. TB manifests in active, latent, reactivated and dissemination phases. Vitiligo is a chronic unstable depigmenting disorder that often shows symmetry in its manifestation. While 1/6th of the Mtb genome encodes genes involved in lipid metabolism; the only well-characterized function of melanocytes is to produce melanins. Our endeavor is to understand how small molecule metabolic networks are elaborately tuned in nature and how these pathways provide distinct advantages to the specific biological system and finally their underlying implications in disease outcome.

To summarize, the objectives of the studies proposed are:

1. Decoding enzymes and pathways involved in the biosynthesis and/or degradation of lipidic metaboilites
2. Identify factors that can trip coordination between melanocyte-keratinocyte biology
3. Demarcate differences between lesional and non-lesional skin to understand homeostasis

A. Evolutionary conserved spatiotemporal expression of phenolic lipids in biofilm development

Spatio-temporal regulation of metabolic processes in response to various environmental cues is an important paradigm in successful survival. One such important dynamic biological process is the cell surface remodeling of various bacilli.

Our previous studies with *Mycobacterium tuberculosis* type III polyketide synthases (PKSs) had revealed novel role to catalyze

formation of long chain acyl phenolic lipids. Recently, a three-gene operon consisting of *type III pks*, *methyltransferase* and *hydroxylase* in *Streptomyces griseus* was shown to produce acyl benzoquinones. In *Bacillus subtilis* the *hydroxylase* gene was missing and the two-gene operon was found to encode for alkyl pyrone methyl ethers. Surprisingly, these metabolites could not be isolated under normal culture conditions and were characterized after heterologous overexpression. The type *III pks* knockout of *S. griseus* was shown to be sensitive to few cell wall disrupting antibiotics, however, this property could not be replicated in *B. subtilis*. We decided to examine this capability to produce phenolic lipids in Mycobacteria. A phylogenetic analysis revealed that while the three-gene operon is restricted to *Actinobacteria*, the two-gene operon is predominantly present in *Firmicutes* and *Proteobacteria* (Fig.1). Remarkably, the three-gene operon is conserved across nine different mycobacterial species and therefore we studied this operon in *Mycobacterium smegmatis* (Ms). Biochemical characterization of the recombinant Ms type III PKS revealed its ability to accept C14-C22 long-chain acyl CoA and form products with both malonyl-CoA (MCoA) and methyl malonyl-CoA (MMCoA) as extender units. Tandem mass spectrometry characterization of the products revealed the ability of the protein to form triketide pyrones, tetraketide pyrone and resorcinols using MCoA as extender and triketide pyrone using MMCoA as extender. When both MCoA and MMCoA were used together tetraketide pyrone and tetraketide resorcinol formed by ordered combination of both MCoA and MMCoA were obtained as additional products.

To understand the importance of the operon in mycobacteria we generated a knockout (KO) for msmeg_0808 and performed comparative metabolite analysis. No differential metabolite was observed between the WT and KO extracts and most of the metabolites corresponded to glycopeptidolipids (GPLs). Since abundant GPLs could hinder the detection of lowly expressed metabolites, we decided to perform studies in a GPL-null strain of Ms RB-WT (Rough Background). However it was only after feeding of long chain fatty acids to the Ms RB-WT strain engineered to over-express the three genes of the operon that we succeeded in isolating long chain acyl resorcinols and pyrones from the shake flask cultures. Surprisingly, none of the metabolites isolated were modified by the other two enzymes methyltransferase or hydroxylase. The remarkable conservation of this cluster across several microbes led us to argue that these molecules must be relevant to bacteria under alternate bacterial niches. Biofilm represents a physiologically relevant model for studying the assemblage of microorganism in a sedentary habitat. RT-PCR studies revealed all three genes of the operon to be significantly upregulated in the Ms biofilm cultures compared to stationary phase planktonic cultures. Moreover the biofilm formed by the KO strain was found to be fragile and lacked the reticulation pattern that could be observed for the WT biofilm. On analysis of the lipid contents of the WT and the KO biofilm, a differential peak in WT extract was observed in HPLC studies. TOF-MS analysis of this metabolite in the positive ion mode showed two major molecular ion peaks of m/z 391.3 and 405.3. Tandem MS for both the peaks produced product ions of m/z 167.1, 151.09, 139.09 and 121.08. Similar fragmentation pattern has been reported for acyl benzoquinone metabolites from *S. griseus*. The products were thus identified to be 5-methoxy-2-methyl-3-(octadecyl)-1,4-benzoquinone and 5-methoxy-2-methyl-3-(nonadecyl)-1,4-benzoquinone. Further studies with *B. subtilis* NCIB3610 biofilm also revealed presence of triketide pyrones and their methylated analogs formed by condensation of

C14 to C16 acyl CoA with MCoA, suggesting this to be a general mechanism. Together, our studies demonstrate that modified phenolic lipids are spatio-temporally expressed during biofilm formation and we propose this to be an evolutionary conserved mechanism in microbial world.

B. Discerning the dilemma of discoloration

The depigmenting disorder, vitiligo, is characterized by localized loss of melanin and melanocytes from epidermis. While melanocytes synthesize melanin inside specialized organelles called melanosomes, keratinocytes are the cells that harbour them in the epidermis. Vitiligo is proposed to have different etiologies. However, the current treatment regimen emphasizes more on autoimmune disorder and is largely symptomatic with minimal success. A tempting hypothesis therefore, is that vitiligo could be a manifestation of the breakdown of melanocyte-keratinocyte cellular homeostasis, which primarily dictates epidermal pigmentation. The challenge however is to identify the various fuses that trip the homeostatic circuit and brings about white vitiligo patches on the skin. We have exploited the inherent periodicity feature of the biological systems to delineate operative mechanisms that are important during the process of pigmentation. In order to recognize the negative feedback regulatory network involved in pigmentation, we designed an oscillatory model of pigmentation-depigmentation. The logic behind this design is based on the observation that all biochemical oscillators require a negative feedback loop for their functionality. Such common regulatory circuits are known to control various aspects of cell physiology. We decided to exploit the known unstable melanotic behavior of B16 cells. By effectively generating reversible cell autonomous model systems, we have identified new functional modules whose perturbations could be the cause of disease manifestation (Figure 1). Such modules could then be investigated by studying lesional and nonlesional skin of patients or by developing a suitable animal model system.

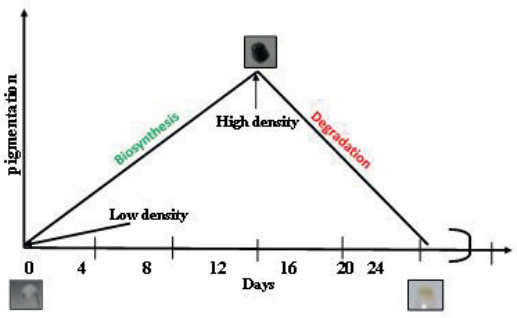

Figure 1: Pigmentation oscillator model illustrating facile pigmentation-depigmentation cycle.

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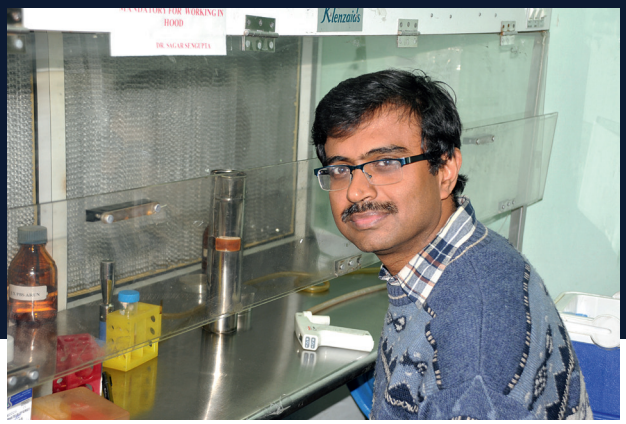

Sagar Sengupta

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

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

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

1. Decipher the role of BLM in regulating c-Myc functions
2. Determine how post-translational modifications on BLM affect its function during homologous recombination

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

BLM inhibited c-Myc driven tumor initiation in mouse xenograft model

Based on earlier reported results, it was possible that c-Myc functions on proliferative pathways were altered by the presence or absence of BLM. To test our hypothesis we had to generate a set of isogenic stable cell lines having genotypes

BLM^+ $c\text{-}Myc^+$, BLM^+ $c\text{-}Myc^-$, BLM^- $c\text{-}Myc^+$, BLM^- $c\text{-}Myc^-$. We could not use the A-15/BS celllines for this purpose was these fibroblast derivatives did not have tumorigenic potential. Hence two new cell lines HCT116 shc-Myc (Clone #9 and #7) and HCT116 $BLM^{-/-}$ (Clone #5 and #13) were generated by stably co-expressing Tet repressor (pcDNA6TR) and shRNA against c-Myc (pTER c-myc) in HCT116 and HCT116 $BLM^{-/-}$ cells. These cells, expressing tetracycline regulated shc-Myc, showed complete shutdown of c-Myc expression on addition of tetracycline. While 1μg/ml of tetracycline was sufficient to shutdown c-Myc expression upto 24 hr., 0.5 μg/ml of tetracycline led to almost complete shutdown of c-Myc protein expression within 12 hr.

Since the ability of the cells to penetrate through a barrier of reconstituted basement membrane correlates with their invasive potential, we carried out *in vitro* matrigel invasion assays with HCT116 shc-Myc and HCT116 $BLM^{-/-}$ shc-Myc cells in presence and absence of tetracycline. As expected absence of c-Myc (i.e. BLM^+ $c\text{-}Myc^-$ genotype) decreased the invasive potential of the cells by almost half (compare BLM^+ c-Myc- genotype to the normal BLM^+ $c\text{-}Myc^+$ genotype) thereby indicating that the invasive percentage of the HCT116 cells was largely due to c-Myc expression. However compared to the normal condition (BLM^+ $c\text{-}Myc^+$ genotype), absence of BLM in presence of c-Myc (i.e. BLM^- $c\text{-}Myc^+$ genotype) statistically enhanced invasive capability of the cells by approximately 1.6 fold, indicating that BLM is a critical component regulating the invasive potential of c-Myc.

To conclusively link the increased invasive potential of cells expressing BLM but lacking c-Myc to the action of BLM on Fbw7, *in vitro* matrigel invasion assays were carried out in parallel in HCT116 (wildtype) or HCT116 $Fbw7^{-/-}$ cells in which the expression of BLM was shut down by transfecting its cognate siRNA. As expected due to their respective tumor suppressive functions, ablation of BLM or Fbw7 alone mildly enhanced the invasion potential of the cells. However lack of both Fbw7 and BLM i.e. BLM^- $Fbw7^-$ genotype (which should also indicate the BLM^- $c\text{-}Myc^+$ genotype) statistically enhanced the invasive potential by 5-6 fold. The invasive potential of BLM^- $Fbw7^-$ cells was more compared to BLM^- $c\text{-}Myc^+$ cells. The effect of BLM on the stability of other $SCF^{Fbw7\gamma}$ substrates like Cyclin E and c-Jun may also contribute during the invasion process.

The above studies were next extended to a xenograft model in nude mice. The generated stable cell lines were subcutaneously injected into nude mice. Feeding of tetracycline to 50% of the animals led to the generation of four different genotypes within the microenvironment of the injected cells. The absence of BLM staining in HCT116 $BLM^{-/-}$ derivatives and ablation of c-Myc staining after tetracycline treatment were confirmed in the respective tumor sections. In accordance with the role of BLM as a tumor suppressor, its absence alone mildly accelerated tumor initiation [compare BLM^+ $c\text{-}Myc^+$ (9.1 days) with BLM^- $c\text{-}Myc^+$ (5.4 days)]. Lack of c-Myc delayed tumor initiation whether in presence of BLM [compare BLM^+ $c\text{-}Myc^+$ (9.1 days) with BLM^+ $c\text{-}Myc^-$ (16.6 days)] or in absence of BLM [compare BLM^- $c\text{-}Myc^+$ (5.4 days) with BLM^- $c\text{-}Myc^-$ (9.2 days)]. The delayed initiation of tumor formation due to BLM expression (by approximately 1.7 fold) was statistically significant. Significantly the initiation of the tumor formation was most delayed when BLM expression was present in concurrent absence of c-Myc [compare BLM^+ $c\text{-}Myc^-$ (16.6 days) with any other genotype], thereby indicating that apart from its intrinsic function as

a tumor suppressor, BLM can also act on c-Myc, enhance its degradation, thereby delay tumor initiation to the maximal extent.

The role of c-Myc in diverse functions led us to determine the levels of proliferation and apoptosis in the tumor sections derived from nude mice by Ki67 staining and TUNEL assay. The net proliferative capacity of the tumors (measured by the ratio of proliferation versus apoptosis) was decreased by the loss of c-Myc (compare BLM^+ $c\text{-}Myc^+$ with BLM^+ $c\text{-}Myc^-$) and increased by the loss of BLM (compare BLM^+ $c\text{-}Myc^+$ with BLM^- $c\text{-}Myc^+$). Since proliferation is less in $BLM^+c\text{-}Myc^-$, the tumor initiates later for these cells in the mouse xenograft model compared to $BLM^+c\text{-}Myc^+$. Loss of BLM coupled with the presence of c-Myc increased the net proliferative capacity of the tumors (compare BLM^- $c\text{-}Myc^+$ with any other genotypes). Expression of CD31 (a marker for the extent of angiogenesis) was also highest in tumor sections from BLM^- $c\text{-}Myc^+$ genotype. Altogether these results indicate that the negative regulation of c-Myc stability by BLM led to a delay in tumor initiation in nude mice.

2. Determine how post-translational modifications on BLM affect its function during homologous recombination

BLM is recruited to sites of replication stress in a ubiquitin-dependent manner

It has been demonstrated that BLM helicase and 53BP1 together function to limit HR by suppressing RAD51 response. Given the biochemical association between BLM, 53BP1 and the Ub-DDR, we hypothesized that the Ub-DDR may regulate the accumulation of BLM in response to replication stress. Hence cells stably expressing GFP-tagged BLM were either mock treated or exposed to HU, fixed, stained with antibodies directed against PML or poly-ubiquitin chains (FK2). BLM is known to primarily reside in PML-containing nuclear bodies (PML-NB) in undamaged cells. Following treatment with HU, BLM relocalises from PML-NBs to sites of stalled replication forks. Consistent with the potential for the Ub-DDR to regulate the nuclear recruitment of BLM, HU-treated cells exhibited a significant co-localisation of BLM with sites of ubiquitylation.

To investigate whether the RNF8/RNF168-dependent E3 ubiquitin ligase cascade functioned during replication stress to promote the focal relocalisation of BLM, cells expressing GFP-BLM were treated with either a control siRNA or siRNA directed against UbcH5a, Ubc13, RNF8 or RNF168. Following exposure to HU, immunofluorescence was used to determine the localization of BLM. Depletion of RNF8 or RNF168 resulted in a failure of cells to properly recruit BLM to sites of DNA damage. These observations were also recapitulated in cells derived from a RIDDLE syndrome patient. Taken together this indicates that BLM functions downstream of RNF8 and RNF168 during the DDR invoked by exposure to HU. Infact we observed both RNF8/RNF168 relocate to sites of replication damage that contained BLM. Interestingly, BLM was predominantly localized to the nucleoli of cells lacking RNF8 suggesting that a basal level of RNF8- but not RNF168-dependent ubiquitylation is required for the normal localization of BLM within the nucleus even in undamaged cells. This is supported by the observation that BLM co-localised at sites of ubiquitylation in asynchronously growing cells. Interestingly, depletion of either Ubc13 or UbcH5a alone did not affect BLM relocalisation after HU treatment. However, the combined loss of Ubc13 and UbcH5a completely abrogated the formation of BLM foci induced by replication

stress comparable to a lack of RNF168, possibly indicating functional redundancy between these two E2 enzymes.

BLM is targeted by RNF8 and RNF168 for ubiquitylation in vitro and in vivo

Based on the above observations it is conceivable that the requirement for RNF8/RNF168 to facilitate BLM relocalisation after HU treatment is mediated through their ability to target it for ubiquitylation. To investigate whether BLM is a substrate for RNF8/RNF168, *in vitro* ubiquitylation assays were carried out using recombinant BLM in combination with either bacterially purified RNF8/RNF168 in the presence of Ubc13/UbcH5a as the E2 conjugating enzyme. Both E3 ligases strongly stimulated the K63-dependent poly-ubiquitylation of BLM *in vitro* when in the presence of Ubc13. Moreover, we demonstrated that this reaction was dependent on the active RING domain of the two E3 ligases. Though UbcH5a could also be utilized as an E2 to catalyse BLM poly-ubiquitylation, BLM ubiquitylation by Ubc13 was more robust, indicating that the RNF8/RNF168 preferentially utilize Ubc13 to poly-ubiquitylate BLM *in vitro*.

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1. De S, Kumari J, Mudgal R, Modi P, Gupta S, Futami K, Goto H, Lindor NM, Furuichi Y, Mohanty D, Sengupta S* (2012) RECQL4 is essential for the transport of p53 to mitochondria in normal human cells in the absence of exogenous stress. *J Cell Sci* **125**: 2509-2522.
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Understanding the regulation of DNA replication

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DNA replication is a vital process of life and must be completed precisely during each cell cycle. When mammalian cell experiences DNA damage, it activates checkpoint mechanisms to stall the progression of cell cycle and DNA replication. Our laboratory is working towards understanding the mechanisms by which microRNA and checkpoint proteins stall the cell cycle preventing genomic instability and cancer.

We are studying the regulation of replication machinery during stress in order to identify underlying mechanisms responsible for inhibition of essential replication factors during stress. We are trying to understand the role of ubiquitination machinery in regulating replication proteins under normal and stressed conditions. Further, we are investigating the cellular response to aberrations in replication complexes. The objective is to identify yet unknown checkpoint pathways that monitor the replication apparatus. Emerging evidences suggest that microRNA target genes that regulate DNA replication and cell cycle progression and we aim to determine the role of microRNA in regulating the DNA replication machinery as cell progresses from one phase to the next. This would provide an insight to understand the mechanisms by which microRNAs regulate mammalian cell cycle and DNA replication during normal and pathological conditions. Summing up, we are attempting to unravel the protective regulatory control of mammalian cells, failure of which is likely to lead to genomic instability.

A. Role of non-coding RNAs in regulation of cell-cycle and DNA replication

We recently reported that mammalian cells specifically down-regulate essential replication proteins during stress to block DNA replication and we wanted to ascertain if microRNAs down-regulate the expression of replication genes during stress. Although DNA Damage response

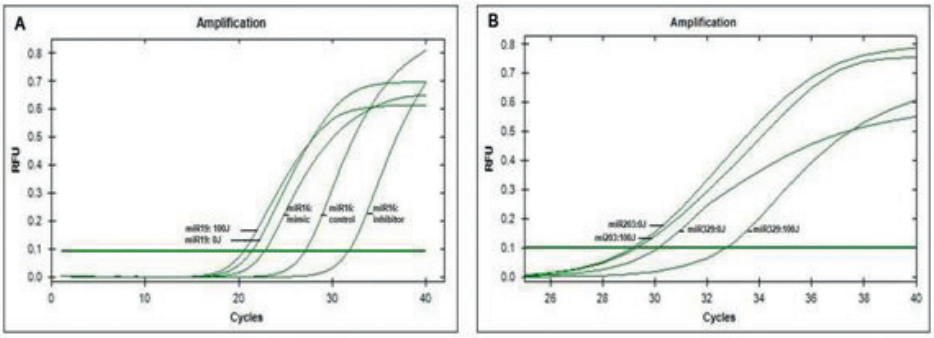

Figure 1: (A) Real-time PCR amplification of miR16 and miR19 from non-irradiated and UV irradiated U2OS cells. Note that mimics and inhibitors of miR16 respectively decrease or increase its Ct values. (B) Real-time PCR amplification of miR203 and miR329 from non-irradiated and UV irradiated U2OS cells. Note that Ct values of miR329 increase significantly following UV irradiation.

is well studied at the transcriptional and post-translational level, much less is known about post-transcriptional regulation by microRNAs. Some studies have evaluated the levels of microRNAs after UV irradiation and reported up-regulation or down-regulation of multiple human microRNAs. On the basis of target prediction algorithms, we postulate that many of the stress-regulated microRNAs may be involved in targeting DNA replication genes during stress. In our laboratory we have determined the UV-irradiation triggered changes in the levels of individual microRNA by Real-time PCR amplification: For example, we have observed that miR19 is induced after UV irradiation, and on the basis of target prediction, we postulate it may be targeting replication factor Mcm8 after UV irradiation (Figure 1). We have evaluated the effect of individual microRNAs on the activity of firefly luciferase expressed in fusion with 3' UTR of DNA replication factors. We have observed that tumor suppressor miR16 inhibits the expression from Cdc25a UTR (Figure 2). We have also observed that miR329 is down-regulated after UV irradiation, and this may lead

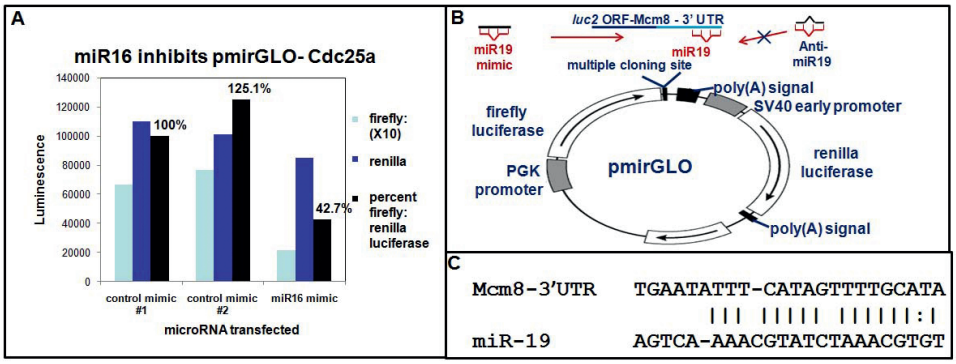

Figure 2: (A) MicroRNA miR16 inhibits firefly luciferase activity when expressed in fusion with Cdc25a-3'UTR: Normalized luciferase activity using the pmirGLO vector. (B) Silencing of firefly luciferase expressed in fusion with Mcm8-3'UTR: Mcm8 UTR is predicted to be targeted by miR19 which is enhanced by miR19 mimic and inhibited by miR19 inhibitor. (C) Base pairing between Mcm8 3'UTR with miR19.

to a cell cycle block due to up-regulation of its target, p21 (Figure 1). Our results demonstrate that miR16 inhibitors and mimics increase or decrease the cycle threshold value (Ct), implying significant change in the levels of the microRNA (Figure 1). In summation, our study will expand our understanding of the regulatory mechanism that mediates the stress-triggered proteolysis of the replication machinery to prevent aberrant replication.

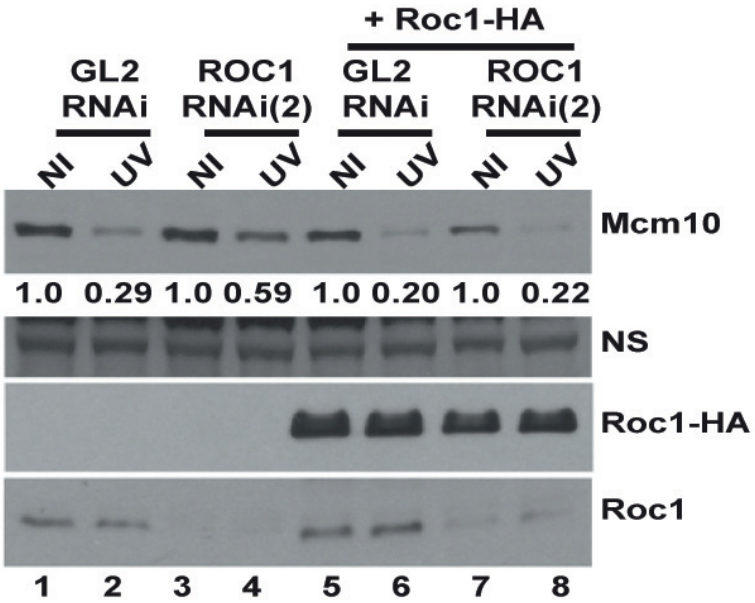

Figure 3: Overexpression of siRNA resistant Roc1 reverses the suppression of UV-triggered Mcm10 degradation following Roc1 depletion. HeLa cells were transfected on three consecutive days with either control GL2 or ROC1 siRNA(2) along with plasmid expressing HA-tagged Roc1 as indicated.

B. Regulation of replication machinery during stress

We previously reported that Mcm10, an essential human replication factor, is selectively proteolyzed after UV-irradiation to inactivate the replication machinery. We attempted to identify the ubiquitin ligase that mediates stress-induced Mcm10 proteolysis. We observed that Mcm10 downregulation is independent of the cell cycle regulatory E3 ligase, SCF^{Skp2}, and Cdt2, the substrate recognition subunit essential for Cdt1 ubiquitination. We have observed that Mcm10 associates with E3 ubiquitin ligase comprising of DNA damage-binding protein, DDB1, cullin, Cul4 and ring finger protein, Roc1. Depletion of DDB1, Roc1 or Cul4 abrogates the UV-triggered Mcm10 proteolysis, implying that Cul4-Roc1-DDB1 ubiquitin ligase mediates Mcm10 downregulation. Complementation of Roc1 protein after UV-irradiation reversed the suppression of UV-triggered Mcm10 degradation due to Roc1 depletion, strongly implicating Roc1 in the Mcm10 turnover (Figure 3). Purified Cul4-Roc1-DDB1 complex ubiquitinates Mcm10 *in vitro*, proving that Mcm10 is its substrate. By screening the known DDB1 interacting proteins, we discovered that VprBP is the substrate recognition subunit that targets Mcm10 for degradation. We evaluated its effect by expressing the UV-resistant NTD+ID domain of Mcm10, which does not interact with VprBP, on the rate of DNA replication following UV irradiation. We observed that the NTD+ID domain of Mcm10 cannot attenuate the inhibition of DNA replication post-UV irradiation. In conclusion, we establish that E3 ubiquitin ligase composed of

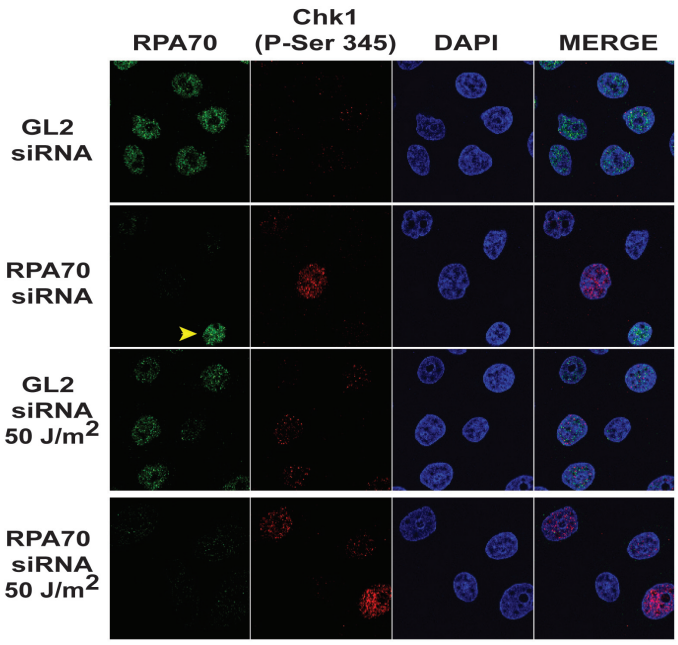

Figure 4: RPA1 depletion leads to Chk1 phosphorylation. HeLa cells transfected with either control GL2 or RPA1 siRNA with or without UV irradiation were assayed for thephosphorylation of Chk1.

Cul4, Roc1, DDB1 and VprBP imparts a vital role in regulating replication by mediating the stress-induced turnover of replication factor, Mcm10.

C. RPA independent ATR phosphorylation of Chk1

Single-strand DNA generated at stalled replication forks and during DNA repair serves as an intermediate for activating the Ataxia telangiectasia and Rad3-related protein (ATR) checkpoint kinase which phosphorylates Chk1, initiating a signal transduction cascade. It is believed that binding of Replication protein A to the single strand DNA is essential for recruitment of ATR-ATRIP complex to the sites of DNA damage facilitating the initiation of checkpoint response. We have observed that ATR can phosphorylate Chk1 independent of RPA (Figure 4). The function of single stranded DNA binding and ATR activation is provided by a recently identified protein called human single-stranded binding protein 1 (hSSB1) and its partner protein called INTS3. In the absence of RPA, hSSB1 and INTS3 form DNA damage foci and recruit the ATR-ATRIP complex to the sites of damage. Depletion of either hSSB1 or INTS3 prevents the ATR mediated Chk1 phosphorylation. hSSB1 mediated activation of ATR requires partner protein ATRIP and coactivators, TopBP1 and 911 complex. In summation, we have observed that replication stress resulting from acute depletion of replication proteins trigger RPA independent hSSB1 mediated ATR activation

Publication

Original peer-reviewed article

1. Kaur M, Khan MM, Kar A, Sharma A, Saxena S* (2012) CRL4-DDB1-VPRBP ubiquitin ligase mediates the stress triggered proteolysis of Mcm10. *Nucleic Acids Res* **40**: 7332-7346.

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Sanjeev Das

Role of tumor suppressor p53 in stress response: understanding the regulatory milieu of p53 family members

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Tumor suppressor p53 mainly functions as a sequence-specific DNA-binding transcription factor to regulate a large number of target genes. These genes mediate diverse cellular processes including cell-cycle arrest, apoptosis, senescence, differentiation, DNA repair, inhibition of angiogenesis and metastasis. Temporal regulation of diverse sets of target genes is thought to be achieved by post-translational modifications and through its interaction with other cellular proteins. To understand the regulatory milieu of tumor suppressor p53, we screened for p53 interacting proteins and have now identified HDAC5 as a bonafide p53 interacting protein.

p73 is one of the tumor suppressors of the p53 family of nuclear transcription factors. p73 exhibits many p53-like properties: it can bind to p53 DNA target sites, transactivate p53-responsive genes and induce cell cycle arrest or apoptosis. However the molecular mechanisms underlying p73 regulation remain unanswered. To address the lacunae in the understanding of p73 stability and function, we carried out a proteomics screen to identify p73 interacting proteins under normal and genotoxic stress conditions. We have now identified an ubiquitin ligase TRIM28 as potential p73 interacting protein.

1. Characterization of HDAC5 as a key modulator of p53-mediated transactivation: Our previous studies have led to characterization of HDAC5 as a bonafide p53 interacting protein. We plan to investigate the functional significance of this interaction.

2. Identification and characterization of novel p73 interacting proteins involved in regulation of p73 stability and function : We plan to use a proteomics based approach to identify novel p73 interacting proteins. For this we intend to generate a recombinant adenovirus expressing HA and Flag tagged p73.

A. Characterization of HDAC5 as a key modulator of p53-mediated transactivation

Regulation of transcription involves dynamic

acetylation and deacetylation of histones and various components of transcription machinery. The latter process is mediated by histone deacetylases (HDACs). HDAC5 belongs to the class IIa HDAC subfamily. It has a nuclear localization signal (NLS) towards the N-terminus and a catalytic domain on the C-terminal half of the protein. The cellular localization of HDAC5 is a carefully regulated process. HDAC5 resides in the nucleus during the proliferation phase (pre-differentiation) and is triggered to relocalize from the nucleus to the cytoplasm during differentiation. HDAC5 is phosphorylated by several kinases, including calmodulin-dependent protein kinases, protein kinase D, salt-inducible kinase, and protein kinase A. Not much is known about the biological functions of HDAC5. It is thought to regulate muscle differentiation as it interacts with and represses myocyte enhancer factor-2 (MEF2). MEF2 plays an essential role, as a DNA binding transcription factor, in muscle differentiation. When MEF2 is bound to HDAC5, the function of MEF2 as a transcription factor is inhibited, thus blocking muscle cell differentiation.

To study the p53-HDAC5 interaction under physiological conditions, we first checked whether genotoxic stress had any effect on HDAC5 protein levels. Upon etoposide treatment p53 protein levels increased with time but there was no change in the HDAC5 protein levels. Since HDAC5 is known to shuttle between nucleus and cytoplasm, we checked for its cellular localization upon DNA damage. HDAC5 is primarily nuclear during early phases of DNA damage but it becomes cytoplasmic upon prolonged DNA damage. p53 remained nuclear throughout the course of genotoxic stress. We next investigated the interaction between endogenous p53 and HDAC5 under genotoxic stress conditions using nuclear extracts. We found that during early phase of genotoxic stress when both p53 and HDAC5 are nuclear, HDAC5 coimmunoprecipitated with p53. However upon prolonged genotoxic stress when HDAC5 goes out of the nucleus this interaction was lost. We also mapped the domains involved in p53-HDAC5 interaction. We found that C-terminal deacetylase domain of HDAC5 binds to the DNA binding domain of p53.

The molecular mechanism underlying the nuclear-cytoplasmic shuttling of HDAC5 upon genotoxic stress is not known. Previous studies report that phosphorylation of HDAC5 at Ser259 and Ser498 is essential for its nuclear export. Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII) has been reported to phosphorylate HDAC5, leading to its nuclear export. Moreover, CAMKII acts as a sensor for cellular ROS (reactive oxygen species) levels and triggers apoptosis. As DNA damage leads to ROS accumulation, we checked if genotoxic stress leads to CAMKII activation and subsequent HDAC5 phosphorylation and nuclear export. Indeed, prolonged DNA damage leads to high ROS levels. We found that with increased accumulation of ROS at extended periods of genotoxic stress, there is a concomitant activation of CAMKII, which phosphorylates HDAC5 at Ser259 and Ser498. The HDAC5 phosphorylation is CAMKII dependent, as a specific CAMKII inhibitor KN-93 abolished HDAC5 phosphorylation. We also examined the cellular localization of HDAC5 with respect to its phosphorylation status upon DNA damage. We found that at early time points of genotoxic stress when HDAC5 is not phosphorylated, it is nuclear but at extended periods of genotoxic stress concomitant to HDAC5 phosphorylation, it becomes cytoplasmic. CAMKII inhibitor KN-93 which inhibits HDAC5 phosphorylation, prevents HDAC5 nuclear export. Phospho-dead double mutant of HDAC5 (S259/498A HDAC5) incapable of undergoing CAMKII-mediated

phosphorylation, remained nuclear upon DNA damage, emphasizing the role of phosphorylation in nuclear export. Taken together, these results demonstrate a unique mechanism of phosphorylation-dependent HDAC5 nuclear export upon genotoxic stress.

Since HDAC5 is a member of class IIa HDAC subfamily, we examined the effect of HDAC5 on p53 acetylation upon genotoxic stress including the C-terminal lysine residues of p53 (K320, K373, K381, K382) and K120 located in DNA binding domain. Our results suggest that nuclear HDAC5 prevents p53 acetylation at K120 residue during early periods of genotoxic stress but at late phases of genotoxic stress, concomitant to nuclear export of HDAC5, p53 gets acetylated at K120 residue. To further investigate the effect of HDAC5 on p53 acetylation status, we performed an *in vitro* deacetylase assay using peptides containing different acetylated lysine residues of p53 (K120, K320, K373, K381and K382) as substrates. Mass spectrometry analysis of the peptides revealed that HDAC5 exhibits specific deacetylase activity towards p53 peptide containing acetylated K120 but showed no activity towards other lysine acetylated p53 peptides tested. Taken together, these results indicate that HDAC5 is a deacetylase with specificity for K120 site of p53.

B. Identification and characterization of novel p73 interacting proteins involved in regulation of p73 stability and function

p73 is one of the tumor suppressor of the p53 family of nuclear transcription factors. p73 contains the characteristic features of the p53 protein- an acidic, amino terminal transactivation domain, a proline-rich domain, a central core DNA-binding and a carboxy-terminal oligomerization domain. Consistently, p73 exhibits many p53-like properties: it can bind to p53 DNA target sites, transactivate p53-responsive genes and induce cell cycle arrest or apoptosis. DNA damage-mediated induction and activation of p73 is regulated by post-translational modifications such as phosphorylation and acetylation, and protein-protein interactions. Like p53, multiple phosphorylations following DNA damage regulate the stability as well as activity of p73. It has been shown that DNA damage activates non-receptor tyrosine kinase c-abl through ATM-dependent phosphorylation. c-abl interacts with p73 and phosphorylates p73 at Tyr99 in response to genotoxic stress. c-abl-mediated phosphorylation of p73 at Tyr99 increases its stability and enhances its transcriptional as well as proapoptotic activity. Chk1 and Chk2 have been shown to be the downstream effector kinases of ATM and ATR, which play a critical role in the regulation of DNA damage response. Chk1 interacts with p73 and phosphorylates p73 at Ser47 in response to DNA damage. Chk1-mediated phosphorylation enhances p73 transactivation as well as proapoptotic function. However the molecular mechanisms underlying these observations remain unanswered. Why certain post-translational modification should result in stabilization of p73 protein and why others should result in increased transactivation function is not clearly understood. To address these lacunae in the understanding of p73 stability and function, we carried out a proteomics screen to identify p73 interacting proteins under normal and genotoxic stress conditions. For this purpose a recombinant adenovirus expressing HA and Flag tagged p73 was generated. This virus would be used to infect p53 null cell lines followed by tandem immunoprecipitations using HA and FLAG antibodies and mass spectroscopy to identify the p73 interacting proteins. We have now identified an ubiquitin ligase TRIM28 and a transcriptional coactivator Med15 as potential p73 interacting proteins. As part of the MAGE-RING Complex (MAGE-C2-TRIM28) TRIM28 has

been shown to exhibit E3 ubiquitin ligase activity while MED15 is a component of the activator-recruited cofactor (ARC) complex or the Mediator complex.

Publication

Original peer reviewed article

1. Satija YK, Bhardwaj A, Das S* (2013) A portrayal of E3 ubiquitin ligases and deubiquitylases in cancer. *Int J Cancer* **133**: 2759-2768.

Review

1. Sen N, Satija YK, Das S* (2012) p53 and metabolism: Old Player in a New Game. *Transcription* **3**: 119-123.

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ANCILLARY RESEARCH

Subeer S. Majumdar

Production of transgenic animals and development of new transgenic technologies

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Theme of the research is to produce transgenic animals for using them as a system for the study of functional genomics and other gene expression related studies.

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 new techniques for making transgenic mice expressing gene(s) with an objective to reduce time and efforts required for generating transgenic animals.

A. Studies with SG2NA

There was a high rate of mortality in mice overexpressing SG2NA (a cell cycle regulated WD-40 repeat protein with potential scaffolding function) under CMV promoter. Therefore, a new construct is made with SG2NA cloned under brain specific promoter and mice are being generated.

B. Aif mediated circumvention of T cell defects

Transgenic mice over-expressing Aif generated by us helped in establishing that Aif rescues T cell developmental defects in harlequin (Hq) strain of mice where naive T cell numbers are low in circulation. This work of Satayjit Rath has resulted into publication of a paper (see reference).

C. SMAR1 mediated susceptibility to infection by *M. tuberculosis*

Studies with Mycobacterium tuberculosis infected SMAR1 transgenic mice revealed that over expression of SMAR1 decreases resistance towards the pathogen. This might have occurred due to reduced production of IFNγ compared to wild type mice in response to infection. SMAR1, hence causes defective Th1 induction. This work is being communicated.

D. Attempts to generate transgenic buffalo expressing therapeutic protein in the milk

After the characterization of the buffalo β-Casein promoter *in vitro* and *in vivo*, we tried to generate construct expressing therapeutic proteins under this promoter.
For generation of transgene construct which can express human interferon gama (hIFNγ) in the milk of buffalo, cDNA coding for hIFNγ was PCR amplified from its native carrier construct using primer specific for hIFNγ coding region and subcloned in pBucsn2-IRES2-EGFP construct (EGFP under β-casein promoter) to generates pBucsn2-hIFNg-IRES2-EGFP construct. This construct was validated through restriction digestion and sequencing. Finally, this construct was digested with PstI and SfoI to generate the desired linear fragments which were used for electroporation in buffalo testis.

Generation of another transgene construct which can express human growth hormone (hGH) in the milk of buffalo, cDNA coding for hGH was PCR amplified from its native carrier construct using primer specific for hGH coding region and subcloned in pBucsn2-IRES2-EGFP construct (EGFP under Beta casein promoter) to generates pBucsn2-hGH-IRES2-EGFP construct. This construct was also validated through restriction digestion and sequencing. The construct was digested with PstI and SfoI to generate the desired fragments for electroporation in buffalo testis.
In addition to above gene preparation and electroporation experiment, analysis of previously electroporated buffalo with pCX-EGFP construct are underway.

E. Transgenic mice to study dendritic cell development

This study is initiated from the laboratory of Prafulla Tailor to generate transgenic animals for intervention of different signal transduction pathways involved in dendritic cell subtype development. This will allow to asses role of these molecules in dendritic cell subtype development and function. Transgenic mice are being made with various constructs for this study.

F. Functional genetics to decipher the molecular basis of vision loss in inherited retinal dystrophies through transgenic mice (Indo-Spain)

Retinal dystrophies (retinitis pigmentosas and macular degenerations) are a group of genetically heterogeneous disorders that involve the degeneration of photoreceptors, resulting in partial or complete blindness, that affect 1 in 4000 individuals in Western countries (15000 affected individuals in Spain according to FAARPEE) and have a considerably higher prevalence in India (up to 1 in 372 individuals reported for rural areas of South India. There is no current cure for these diseases. The goal is to use testicular transgenesis methodology to generate transgenic mice to do rapid and affordable gene function studies in the mouse retina. This is an Indo-Spain collaborative work. We have electroporated the gene construct MOP-bovineGCAP2-Mp1, having the mouse opsin promoter. The electroporated mice are cohabitated for breeding.

G. Transgenic mice expressing xenoreceptors

Transgenic mice are being generated for Dr. Rakesh Tyagi using mouse PXR gene (with flag tag) which acts as a xenoreceptor and has a major role in drug metabolism.

H. Transgenic mice over expressing BLM helicase

BLM is a multi-functional protein, acting not only during the resolution of the DNA damage and in fact working as an upstream sensor of the DNA damage signal. Dr. Sagar Sengupta's laboratory hypothesized that the function of BLM during its role as a sensor to DNA damage could be regulated by post-translational modifications, especially phosphorylation. To address this and other issues constructs are being designed to generate transgenic animals.

I. Establishment of a new technique for generation of transgenic mice without any major invasive procedures or surgery

In contrast to time consuming fertilized oocyte mediated technique, we had established a surgical, *in vivo* testicular gene electroporation method for generation of transgenic animals based on the fact that germ cells are amenable to integration of foreign genes. Since, in this procedure surgical steps are involved, including hemi-castration, the chances of post-surgical infection and impotency of male mice is high. In addition, such surgical procedure in scrotal area may not be suitable for use in farm animals where conditions are conducive to infection.

Overcoming above caveats, we have established an easier, ethically superior, non-surgical, incision-free alternative procedure for generating transgenic

animals at faster pace by exploiting the male germ cell of the testis. The delivery of the transgene was mediated through the hypotonic solution into the testis.

Standardization of the procedure

Standardization of the procedure was done with the help of *pCX-EGFP* linearized plasmid. This plasmid consisted of chicken beta actin promoter (known for constitutive expression) which drove the expression of EGFP reporter gene. Various DNA injection parameters such as amount (10μg-30μg), volume of DNA (20-30μl) injected and number of injections (1-4) in the testis and the concentration of hypotonic solution (under process of patent) were varied to determine the most suitable condition for achieving a successful *in vivo* testicular transfection.

After standardization of the procedure in FVB/J mouse, transgenic mice overexpressing different kind of genes construct with tissue and time specific gene expression *(GFAP-hIGFBP6, MIS-IRES2-EGFP, BuCSN2-IRES2-EGFP)* were successfully generated using this technique.

Validation of the procedure

In *GFAP-hIGFBP6* transgenic line, the hIGFBP6 transgene was successfully expressed only in brain. This caused >75% decrease in GFAP expression in brain of transgenic mice as compared to that of wild type mice.

In another transgenic line carrying *MIS-IRES2-EGFP* construct developed by this procedure, EGFP transgene was specifically expressed in 7 days old Sertoli cells of the testes of the transgenic mice as compared to age matched wild type mice. MIS is known to be expressed at very high levels in immature Sertoli cells of the testis from 12.5 dpc in the mouse up to 2 weeks post partum.

In *BuCSN2-IRES2-EGFP* transgenic line, transgenic mothers carrying *BuCSN2-IRES2-EGFP* transgene displayed very intense native GFP fluorescence, specifically in their mammary gland post delivery when observed under fluorescence stereozoom microscope. Western blot analysis of various tissues of mice showed the presence of EGFP protein only in extracts from mammary glands tissue as compared to other tissues of same transgenic female generated by this procedure. Beta casein promoter is known to be specific to mammary gland. Thus this construct was chosen to get EGFP expression specifically in mammary gland of lactating transgenic female mice.

Publications

Original peer reviewed articles

1. Roy S, Javed S, Jain SK, Majumdar SS, Mukhopadhyay Asok* (2012) Donor hematopoietic stem cells confer long-term marrow reconstitution by self-renewal divisions exceeding to that of host cells. *PLoS One* **7**: e50693.
2. Banerjee H, Das A, Srivastava S, Mattoo HR, Thyagarajan JK, Tanwar S, Das DS, Majumdar SS, George A*, Bal V*, Durdik JM*, Rath S* (2012) A role for apoptosis-inducing factor in T cell development. *J Exp Med* **209**: 1641-1653.

Patents

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2. Mazumdar SS, Ganguli N, Usmani A (2012) Isolation, cloning, sequencing and functional analysis of buffalo (Bubulus bubalis) β- Caesin Promoter along with regions of Exon1, Intron1 and Exon2 using mammary gland derived cell line (PCT/IN2012/000267, International PCT filed on 13/04/2012).

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CANCER RESEARCH
ADVANCES IN CARBOHYDRATE CHEMISTRY AND BIOCHEMISTRY
ADVANCES IN IMMUNOLOGY
ADVANCES IN PHARMACOLOGY
GABA

PUBLICATIONS, PATENTS AND TECHNOLOGY TRANSFER

A. ORIGINAL PEER-REVIEWED ARTICLES

1. Agarwal S, Rastogi R, Gupta D, Patel N, Raje M, Mukhopadhyay Amitabha (2013) Clathrin-mediated hemoglobin endocytosis is essential for survival of *Leishmania*. *Biochim Biophys Acta* **1833:** 1065-1077.

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Flowers of *Portulaca sp.* at NII campus

AWARDS AND DISTINCTIONS

Dr. Sher Ali availed Indo-Australian Senior Scientist S&T visiting Fellowship and visited Australian Phenomic Facility Canberra, Australia on 20-25, February 2013. He was also conferred distinguished Fellowship of the Sudan Academy of Sciences, Khartoum, Sudan.

Dr. Akhil C. Banerjea was awarded the Indian Immunology Society Senior Scientist Oration Award for the year 2012, for his recognition in the area of HIV at the national level.

Dr. Satish K. Gupta was a recipient of Ranbaxy Research Award 2011 in Medical Sciences-Medical Research, Ranbaxy Science Foundation (2012).

Dr. Anil Suri was a recipient of Prof. L.S. Ramaswami Memorial Oration Adward, conferred by Indian Society for the Study of Reproduction and Fertility (ISSRF) in recognition of research contribution in Reproductive tract cancers in February 2013.

Ph.D Degrees Awarded to NII Scholars

Seventeen scholars of the Institute were awarded the degree of Doctor of Philosophy by Jawaharlal Nehru University on completion of their work. The details are as follows:

S. No.	*Student's Name*	*Title of Thesis*	*Guide*
1.	Ms. Swadha Anand	In silico analysis of protein interaction regulatory networks in polyketide biosynthetic pathways	Dr. Debasisa Mohanty
2.	Ms. Masum Saini	Molecular characterization of genetic lesions in primary brain tumors in human	Dr. Sher Ali
3.	Ms. Divya Catherine Thomas	Characterization of novel signaling pathways in *Plasmodium falciparum*	Dr. Pushkar Sharma
4.	Ms. Shweta Tikoo	The role of ubiquitination in mediating BLM function in response to stalled replication	Dr. Sagar Sengupta
5.	Ms. Priyanka Verma	Investigation of lipid metabolic network in *Mycobacteria*	Dr. Rajesh S. Gokhale
6.	Mr. Sachin Verma	Genetic and functional studies on HIV-1 VPU gene	Dr Akhil C. Banerjea
7.	Ms. Sheetal Kaw	Molecular analysis of the role of CD4 and CD27 in B cell differentiation	Dr. Anna George
8.	Ms. R. Mallika	Role of the hematopoietic population in progression of solid ovarian carcinoma	Dr. Asok Mukhopadhyay
9.	Ms. Debjani Dutta	Role of membrane prohibitin in T cell receptor signalling	Dr. Ayub Qadri
10.	Ms. Nutan	Identification and characterization of novel plant based compounds for anti-human immunodeficiency virus activity	Dr. Satish K. Gupta

S. No.	Student's Name	Title of Thesis	Guide
11.	Mr. Sayon Basu	A study to identify some genes which are differentially expressed by sertoli cells of spermatogenically active and inactive testis	Dr. Subeer S. Majumdar
12.	Mr. Raghava Sharma	Analysis of functional structure and interactions of antibacterial peptides of innate immune origin	Dr. Dinakar M. Salunke
13.	Ms. Esha Pandita	Understanding the structure-function relationship of Interferon- γ inducible GTPases	Dr. Apurba K. Sau
14.	Ms. Tandrika Chattopadhyay	Supramolecular insulin assembly-II (SIA-II) as a therapy for diabetes mellitus and maintenance of glucose homeostasis by bone morphogenetic proteins	Dr. Sarika Gupta
15.	Ms. Manpreet Kaur	Study of proteins and their interactions that regulate DNA replication.	Dr. Sandeep Saxena
16.	Ms. Alpana Satsangi	Effect of gonadotropin and pregnancy on systemic autoimmune responses	Dr. Rahul Pal
17.	Ms. Deepika Bhullar	Studies on the host cell proteins interaction with the Japanese encephalitis virus genome	Dr. Sudhanshu Vrati

LECTURES AND SEMINARS

COLLOQUIA

1. **Prof. Lalita Ramakrishnan,** Department of Microbiology and Immunology, University of Washington (USA) delivered a Colloquium lecture on **"Tuberculosis as an Inflammatory Disease"** on 9th April, 2012.

2. **Prof. Raghavendra Gadagkar,** IISc and JNCASR (Bangalore) delivered a Colloquium lecture entitled **"The Functional Organization of a Tropical Insect Society"** on 21st June, 2012.

3. **Prof. Mriganka Sur,** Director, Simons Center for the Social Brain, Massachusetts Institute of Technology (USA) delivered a Colloquium Lecture on **"Brain Circuits and Brain Disorders"** on 22nd August, 2012.

4. **Prof. Jerry Workman,** Stowers Institute of Biomedical Research (USA) delivered a Colloquium Lecture entitled **"Histone Modification and Exchange During Gene Transcriptions"** on 3rd December, 2012.

RAMALINGASWAMI MEMORIAL LECTURE

Prof. Ramalingaswamy, a pioneer and visionary who laid the foundations of Biomedical research in India, was one of the founding members of NII. In his honour, RAMALINGASWAMY MEMORIAL LECTURE was organized on 27th April, 2012.

On this occasion, **Prof. Guido Kroemer,** University of Paris Descartes, Director of Apoptosis, Cancer and Immunity Research Unit, French Medical Research Council (INSERM) and Director, Metabolomics Platform, Institut Gustave Roussy, Villejuif (France) was invited to deliver the Lecture entitled **"Cancer Immunosurveillance Determines the Efficacy of Anticancer Therapies"**.

Prof. Guido Kroemer and Dr. Chandrima Shaha after the Ramalingaswami Memorial Lecture.

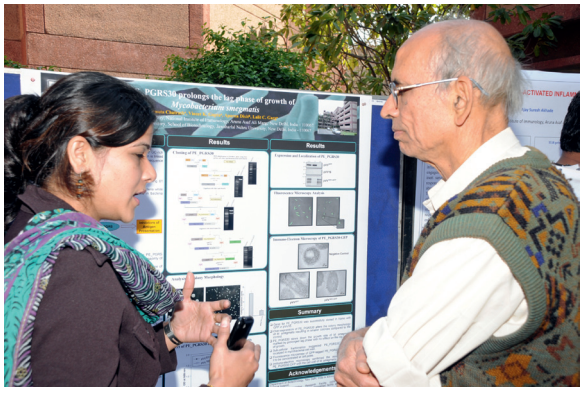
FOUNDATION DAY LECTURE

On 6th October, 2012, the 26th Foundation Day of NII was celebrated. **Prof. Rohini Godbole**, a noted Physicist at Indian Institute of Science (Bangalore), was invited as the Guest of Honour. She delivered a lecture **"Theoretical Significance of the Discovery of a Boson at the Large Hadron Collider"**.

Prof. Rohini Godbole delivering the Foundation Day Lecture.

SCIENCE DAY LECTURE

National Science Day was celebrated on 28th February, 2013. **Prof. Madhav Gadgil,** Emeritus Scientist, National Centre for Cell Science (Pune), was invited to deliver a Lecture on **"Science and the Art of Engaging People in a Scientific Enterprise."** Also, a scientific poster session was organized during which the NII students presented their research work.



Prof. Madhav Gadgil interacting with a student during the poster session on National Science Day.

NII SILVER JUBILEE LECTURES

1. **Prof. P. Balaram,** Director, Indian Institute of Science (Bangalore) delivered a lecture on **"Chemical Analysis in the Age of Biology"** on 19th October, 2012 as a part of NII Sliver Jubilee Lecture Series.

Prof. P. Balaram delivering the Sliver Jubilee Lecture.

2. **Prof. Robert Clarke,** Director, Lombardi Comprehensive Cancer Center, Georgetown University (USA), delivered a Lecture on **"Systems Biology Approaches to Explore the Complex Biology of Endocrine Resistant Breast Cancer"** on 19th November, 2012 as a part of NII Sliver Jubilee Lecture Series.

Prof. Robert Clarke delivering Silver Jubilee Lecture.

SEMINARS

S. No.	Title	Speakers	Date
1.	Widening the spectrum of therapeutic approaches for cancer	**Prof. Ashok Venkitaraman** Director, MRC Cancer Cell Unit Cambridge Cancer Centre, UK	17th April, 2012
2.	Loops within loops generate the chromatin configuration of the immunoglobulin heavy chain gene locus	**Dr. Ranjan Sen** National Institutes of Health, Baltimore, USA	23rd April, 2012
3.	Nucleocytoplasmic transport and its significance to cell differentiation	**Prof. Kiyoshi Takeda** Department of Microbiology and Immunology, Graduate School of Medicine, WPI Immunology Frontier Research Center, Osaka University, Japan	24th April, 2012
4.	Interplay between mitochondria, microRNAs and NAD-dependent Sirtuins: implications in aging and age related diseases	**Dr. Ullas Kolthur Seetharam** Department of Biological Sciences Tata Institute of Fundamental Research Homi Bhabha Road, Colaba, Mumbai, India	18th May, 2012
5.	Novel strategies in cancer immunotherapy	**Dr. Rahul Purwar** Harward Medical School, Boston, USA	22nd May, 2012
6.	The role of hSSB1 in genome stability pathways	**Dr. Derek Richard** Genome Stability Laboratory ARC Future Fellow, Institute of Health and Biomedical nnovation, Queensland University of Technology, Australia	15th June, 2012
7.	Linking causal relationships between genes and phenotypes of the immune system through large scale ENU mutagenesis of the mammalian genome	**Dr. Edward Bertam** Australian National University, Canberra, Australia	3rd July, 2012
8.	Early life experience shapes lifelong changes in behaviour	**Dr. Vidita Vaidya** Department of Biological Sciences, Tata Institute of Fundamental Research, Mumbai, India	3rd July, 2012
9.	Differential mechanical stability of nucleosomes with histone post-translational modifications assayed at the single molecule level.	**Dr. Abhijit Sarkar** Department of Physics, Catholic University of America, Washington DC, USA	7th August, 2012
10.	Taking Cilia apart looking for signal transduction events	**Dr Andrew Peterson** Director, Genentech Inc., USA	10th August, 2012
11.	Mechanism of IκB Kinase (IKK) activation	**Dr. Gouri Ghosh** Department of Chemistry and Biochemistry University of California, San Diego, USA	13th August, 2012
12.	Apoptosis and apoptotic mimicry in leishmanial infection: studies in the mouse model and inpatients with diffuse cutaneous leishmaniosis	**Prof. Marcello A. Barcinski** Instituto Oswaldo Cruz, Rio De Janerio, Brazil	12th September, 2012

S. No.	Title	Speakers	Date
13.	Beyond two-state model of protein folding	**Prof. G. Krishnamoorthy** Department of Chemical Sciences Tata Institute of Fundamental Research, Mumbai, India	14th September, 2012
14.	Epigenetic modifications in transcription regulation and differentiation: Implication in therapeutics	**Dr. Tapas K. Kundu** Molecular Biology and Genetic Unit, JNCASR, Bangalore, India	19th September 2012
15.	FcRn as a therapeutic target: from subcellular behaviour to *in vivo* studies in mice	**Prof. Elizabeth Sally Ward** Paul and Betty Meek - FINA Professor in Molecular Immunology, Department of Immunology, University of Texas, Southwestern Medical Center, Dallas, Texas, USA	1st October 2012
16.	Leveraging temporal regulation to prevent chronic diseases	**Dr. Satchidananda Panda** Regulatory Biology Laboratory, Salk Institute, La Jolla, USA	8th October 2012
17.	Light, melatonin and cancer: current results and future perspectives	**Dr. Christian Bartsch** Center for Research in Medical & Natural Sciences, University of Tubingen, Germany	9th October 2012
18.	Whole genome predictions of protein sub-cellular localisation in apicomplexan parasites	**Dr. Stuart Ralph** Bio21 Institute, University of Melbourne, Australia	5th November 2012
19.	Host remodelling in malaria disease and immunity"	**Dr. Kasturi Haldar** Julius Nieuwland Chair of Biological Sciences, Parsons-Quinn Director, Center for Rare and Neglected Diseases, University of Notre Dame, USA	30th January, 2013
20.	DNA replication stress- The knowns and unknowns"	**Dr. Arnab Roy Choudhury** Institute of Molecular Cancer Research, University of Zurich, Switzerland	4th February 2013
21.	Fourth fluorescence in proteins with	**Prof. Purnananda Guptasarma** IISER, Mohali, India	15th February 2013
22.	Nuclear power: Motivations and problems"	**Dr. M.V. Ramana** Princeton University, Princeton, USA	19th February 2013
23.	Drug Discovery and development challenges in tuberculosis: Our learning in last decade"	**Dr. Anil Koul** Infectious Diseases Area, Janseen Research and Development, Johnson and Johnson Pharmaceuticals, Beerse, Belgium	1st March 2013
24.	DNA Breaks to Repair: New Tales from an Old Story"	**Dr. S. Raghavan** Department of Biochemistry IISc, Bangalore, India	26th March 2013

OTHER NOTABLE ACTIVITIES

ACADEMIC COURSES, TRAINING PROGRAMMES AND INTERACTION WITH OTHER ACADEMIC INSTITUTES

The Institute imparts long term residential training leading to Ph.D Degree of the Jawaharlal Nehru University, New Delhi. Every year 35-40 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.

The Ph.D Programme of the Institute was launched in the academic year 1986-87. Since then the Institute has admitted a total of 548 students in 27 batches. So far 245 students have been awarded the Ph.D degree including 17 that have obtained the degree in academic year 2012-13. Many others are at various stages of their research work for the degree.

In addition, 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 their Post Graduation course.

IMPLEMENTATION OF OFFICIAL LANGUAGE POLICY

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

The National Institute of Immunology was adjudged best Institute for implementation of Govt. of India Official Language Policy in its official work for the year 2011-12 amongst all the DBT Institutes, for which the Secretary, Department of Biotechnology awarded Shield and Commendation certificate to the Institute during the Rajabhasa Conference organized by the Department of Biotechnology, Ministry of Science and Technology on 27/11/2012 at Scope Building, CGO Complex, Lodhi Road, New Delhi. The Official Language team, Department of Biotechnology also inspected this Institute on 18/3/2013 and was satisfied with the efforts made by the Institute on implementation of Official Language policy in official work.

Dr. Chandrima Shaha, NII Director giving prize to one of the competitors (Mrs. Vinod Kumar) for Hindi Vaad-Vivad Pratiyogita (Debate Competition)

To promote Official Language Hindi in official work, the Hindi Pakhwara (Hindi Fortnight) was celebrated in the Institute with great zeal from 1st to 14th September, 2012. During this period, various Hindi competitions via Hindi Sulekh (Hindi Writing), Hindi Shrutlek (Hindi dictation), Hindi Nibandh (Hindi Essay), Hindi Anuvad (Hindi Translation), Hindi Vaad-vivad (Hindi Debate) and Hindi Kavita pathan (Hindi Poetry recitation) were organized in the Institute, in which a large numbers of faculty members, staff members and students participated. Hindi Diwas (Hindi Day) was celebrated on 14th September, 2012 on the culmination of Hindi Pakhwara. To sort out the difficulties and to remove the hesitation of the staff while doing their official work in Hindi on Computer, Hindi workshop on use of Unicode at Computer has also been organized in the Institute on 26/10/2012.

CULTURAL ACTIVITIES

During the last week of February, 2013 several cultural activities including in-house competitions like Quiz, Antakshri, Treasure hunt, Mixed bag,

Dance performance by the children of NII Staff during the Cultural Night.

Rangoli was celebrated amongst scholars and staff members 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.

NII staff presenting a classical song during the Cultural Night.

FAREWELL TO EMPLOYEES

Three members of NII staff retired during the reporting year. They are Mrs. Vimlesh, Mr. S. Kannan and Mr. K. Rajan.

Mr. Kanan and his wife receiving flowers at his farewell function.

INDEPENDENCE DAY CELEBRATION

Independence Day was celebrated in the Institute on 15th August, 2012. 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.

Independence Day celebration during flag hoisting by Dr. Chandrima Shaha.

ANTI-TERRORISM DAY, SADHBHAVNA DIWAS AND COMMUNAL HARMONY WEEK

Anti-Terrorism Day was observed by all employees of the Institute on 21st May, 2012 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 pledge 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.

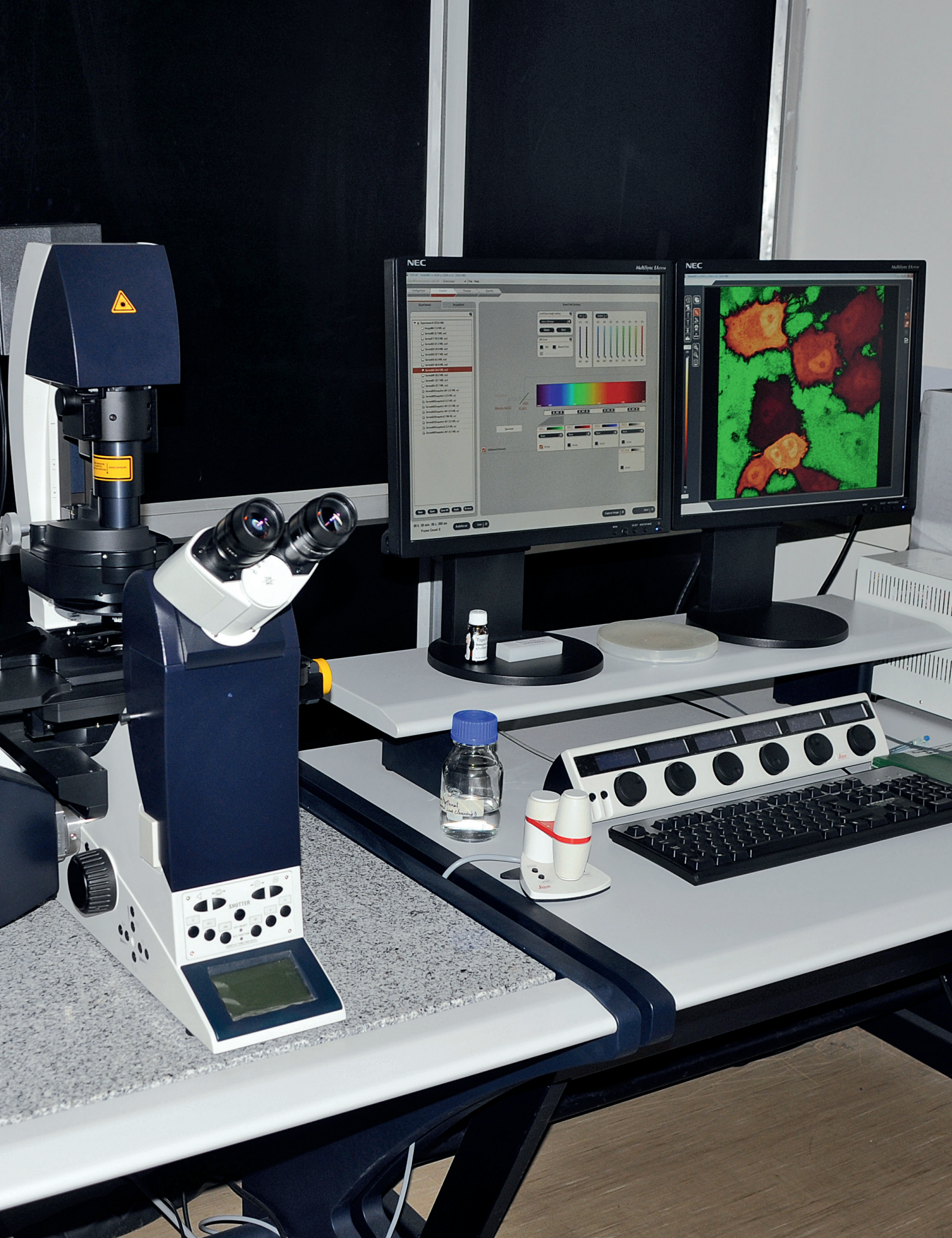
With the theme to promote national integration and communal harmony among people of all religions, languages and regions, 'Sadhbhavna Diwas' was observed in the Institute on the birth anniversary of late Shri Rajiv Gandhi on 20th August, 2012. All staff members of NII took the pledge 'I take this solemn 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 20th August to 3rd September, 2012 was observed as Communal Harmony Week. A Communal Harmony Campaign and Fund Raising Week was observed in the Institute from 19th to 25th November, 2012 and Flag Day on 25th November, 2012, 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 AND 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 challenged 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.

FAREWELL TO PhD STUDENTS

Tree Plantation by 2007 batch of Ph.D students as the Institute bids them farewell.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SHUTTER

INFRASTRUCTURE

RESEARCH FACILITIES

EQUIPMENT

While most of the routine equipment is available in various laboratories of NII, some high-end instrumentation facilities are shared by various research groups and their collaborators. The equipment in these facilities includes Mass Spectrometers, NMR Spectrometers, Confocal Microscopes, Atomic Force Microscope, Scanning and Transmission Electron Microscopes, High Throughput DNA Sequencer, Flow Cytometers, Dual wavelength X-ray Generator and X-ray device for in-vivo imaging.

BSL-III FACILITY

There are three Biosafety Level III facilities at NII – one each for handling *Mycobacterium tuberculosis, Streptococcus pneumoniae* and HIV.

SMALL ANIMAL FACILITY

The Small Animal Facility of the Institute is committed to ensure the human care of animals used in approved research and provides defined strains of mice and rats to the scientific community of the Institute. Animals are issued to the respective investigators on the basis of the approval from NII Institutional Animal Ethics Committee.

At present the small animal facility holds 85 mouse strains (wild types, transgenic and knockout), 6 rat strains and 1 strain of rabbit. Majority of the breeding colonies of mice are maintained in IVC systems of international standards especially since many of these are immunocopmpromized. Defined breeding protocols are followed to minimize genetic drift. Health monitoring procedures are strictly followed and animals are routinely screened for pathogens such as Sendai virus, Rodent corona virus, Mycoplasma pulmonis, PVM, MHV, MVM, TMEV and MPV. The facility is attended by full-time veterinary and other support staffs that are experienced in providing high quality care to the laboratory animals.

PRIMATE RESEARCH CENTER

Rhesus monkeys (*Macaca mulatta*) are bred and maintained in the Primate Research Centre for generation of animals of known ages for approved basic, pre-clinical and toxicological research. Primates are issued to the investigators on the basis of approval given by NII Institutional Animal Ethics Committee and clearance of the research proposals by CPCSEA. The macaques at this Centre are used for research related to infectious diseases, reproduction, endocrinology, immunology and contraception. The staff of Centre makes sure that all the procedures involved in animal handling are pain-free and involve minimum stress to the animal. The center also extends technical expertise for surgery, immunization, bleeding, biopsy, electro-ejaculation and fertility studies to investigators in addition to maintaining and providing primates free of microbial pathogens. The center has a well-equipped operation theatre where major/ minor surgeries can be performed on the primates.

OTHER SUPPORTING UNITS

Establishment, Personnel and General Administration

The Division has been providing effective administrative support in terms of manpower employment, liaison, secretarial assistance and infrastructure to meet its goal of coordination of human and material resources. The activities include service matters, policy implementation, preparation and submission of periodic reports to nodal Ministry, foreign visits of scientists for training, conferences, exchange visits etc. The Institute also conducts periodical trainings for its Administrative and Technical Cadre on subjects of relevance to them. In addition to the routine jobs, the staff of Administration has also been involved in other activities like maintenance of the NII campus, organizing various seminars, workshops, training courses, lectures etc.

Finance and Accounts Department

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

Stores and Purchase Department

The Stores and Purchase Department of the Institute is responsible for all purchases of indigenous and imported items such as chemicals, and other consumables as well as research equipment. The important function of purchase is overseen by various purchase committees comprising of three or more NII Scientists, Finance and Accounts Officers and Stores and Purchase Officers.

Engineering, Maintenance and Instrumentation Services

The Engineering department of the Institute has been entrusted with all the engineering activities (including running and maintenance of 1000 KVA generating set) and ensures timely maintenance and necessary up-gradation of facilities that influence research at NII. Major activities under taken during 2012-2013 include renovation of air handling units in various laboratories and offices, New Electrical Control Panel for Pump House, SITC of Automatic Fire Detection and alarm System, Setup of new Common Flow Cytometry facility including electrical and air conditioning system and up-gradation of humidification system of the animal house.

Instrumentation workshop provides service and maintenance of the minor laboratory equipments as well as maintenance and operation of audio-visual systems of the institute. Other important responsibilities of the Cell are to co-ordinate services to the instruments and negotiate animal maintenance contracts with service providers/ instrument vendors.

Library and Documentation Services

Library and Documentation Department acts as a Scientific Information Management Centre and provides information support to the scientific staff of the Institute using both archival and contemporary digital resources. All the housekeeping activities of the library are computerized. Online Public Access Catalogue (OPAC) is available for searching databases. The library subscribes to several E-journals directly or through DeLCON consortium project. Besides, the unit is supposed to be responsible to co-ordinate and produce and different kinds of reports of the institute.

Academic and Training Department

The activities of the Academic and Training Department can be grouped under three major groups viz. Students Affairs, Outside Training, and In-House training. The unit has been involved in admission of students to the Ph.D. programme of NII, conducting the Pre-PhD courses, and facilitating Doctoral Committee and Academic Committee meetings. In addition to the Ph.D. students, The academic and training Department also coordinates the training of Post-doctoral researchers who come to NII through various fellowship programmes like Department of Bio-technology-Post Doctoral Fellowship (DBT-PDF), Indian Council of Medical Research-Research Associates (ICMR- RAs), Wellcome Trust Fellowship and Department of Science and Technology Women Scientist fellowship (DST-WOS). The unit also co-ordinates MSc/M. Tech project and summer training programme of the students who come to NII for short term training from various Institutions/Universities of the country. The unit has been involved in arranging the participation of scientific, technical and administrative officials of the Institute in the training courses, workshops and seminars organized by outside organizations in different parts of the country.

Vigilance Cell

The Institute has a Vigilance Cell headed by a Scientist nominated as part–time Chief Vigilance Officer (CVO) by the Chief Vigilance Commissioner (CVC). The CVO and the support staff perform vigilance functions as adjunct duties in addition to their responsibilities. The Cell has effectively followed various instructions issued by the CVC from time to time to ensure effective implementation of the measures outlined in the instructions and strengthening vigilance and anti-corruption work. Emphasis has been laid primarily on preventive vigilance. The Institute has been constantly reviewing procedures to identify corruption prone areas, making policies more transparent to avoid ambiguity and streamlining procedures to achieve a corruption free environment. Plans for rotation of staff employed in sensitive areas prone to corruption have been implemented. Purchase of chemicals, consumables and instruments are handled by various purchase committees of the Institute. The Institute has thus far been able to maintain a clean slate in corruption matters.

ORGANIZATION

NII SOCIETY

Prof. G. Padmanaban
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Molecular Biophysics Unit
IISc, Bangalore

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Department of Biotechnology
New Delhi

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Director General Health Services
Ministry of Health and Family Welfare
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Prof. R. C. Deka
Director, AIIMS
New Delhi

Dr. V. M. Katoch
Secretary, DHS and
Director General ICMR,
New Delhi

Dr. S. Ayyappan
Director General ICAR,
New Delhi

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Chairman, UGC
New Delhi

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New Delhi

Prof. Ramakrishna Ramaswamy
Vice Chancellor
University of Hyderabad
Hyderabad

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Department of Biotechnology
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Ministry of Health and Family Welfare
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Director, NII
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(Non-member Secretary)
Senior Manager, NII
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Registrar, JNU
New Delhi
(Nominee of Vice Chancellor, JNU)

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Dr. Asok Mukhopadhyay, PhD, Staff Scientist VII

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** On Lien/ Deputation*

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Dr. Archana Saini

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Ms. K. Suchitra
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Dr. Anjali
Dr. Anuj Tripathi
Mr. Ashok Saini
Dr. Avishek Anant
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Ms. Deva Sukanya Parthipati
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Ms. Garima Tiwari
Ms. Himani
Ms. Himanshu Singh
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Mohammad Kashif
Ms. Monika Garg
Mr. Mukesh Gautam
Md Muntaz Khan
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Ms. Saborni Chattopadhyay
Mr. Sachin K. Deshmukh
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8	Kolkata	Phyto Biotech Pvt. Limited, IICB
9	Lucknow	NBRI, SGPI, IITR
10	Mohali	IISER
11	Mumbai	NIRRH, Mumbai University
12	New Delhi	JNU, RMLH, ICGEB, AIIMS, IIT, DU, IOP, VMMC
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13	Pune	NCCS, Serum Institute, CSIR-NCL, BAIF
14	Sagar	DHG Vishwavidyalaya
15	Shantiniketan	Vishva-Bharati
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17	Varanasi	BHU Medical College
18	Vadodara	MS University of Baroda
19	Vellore	CMC

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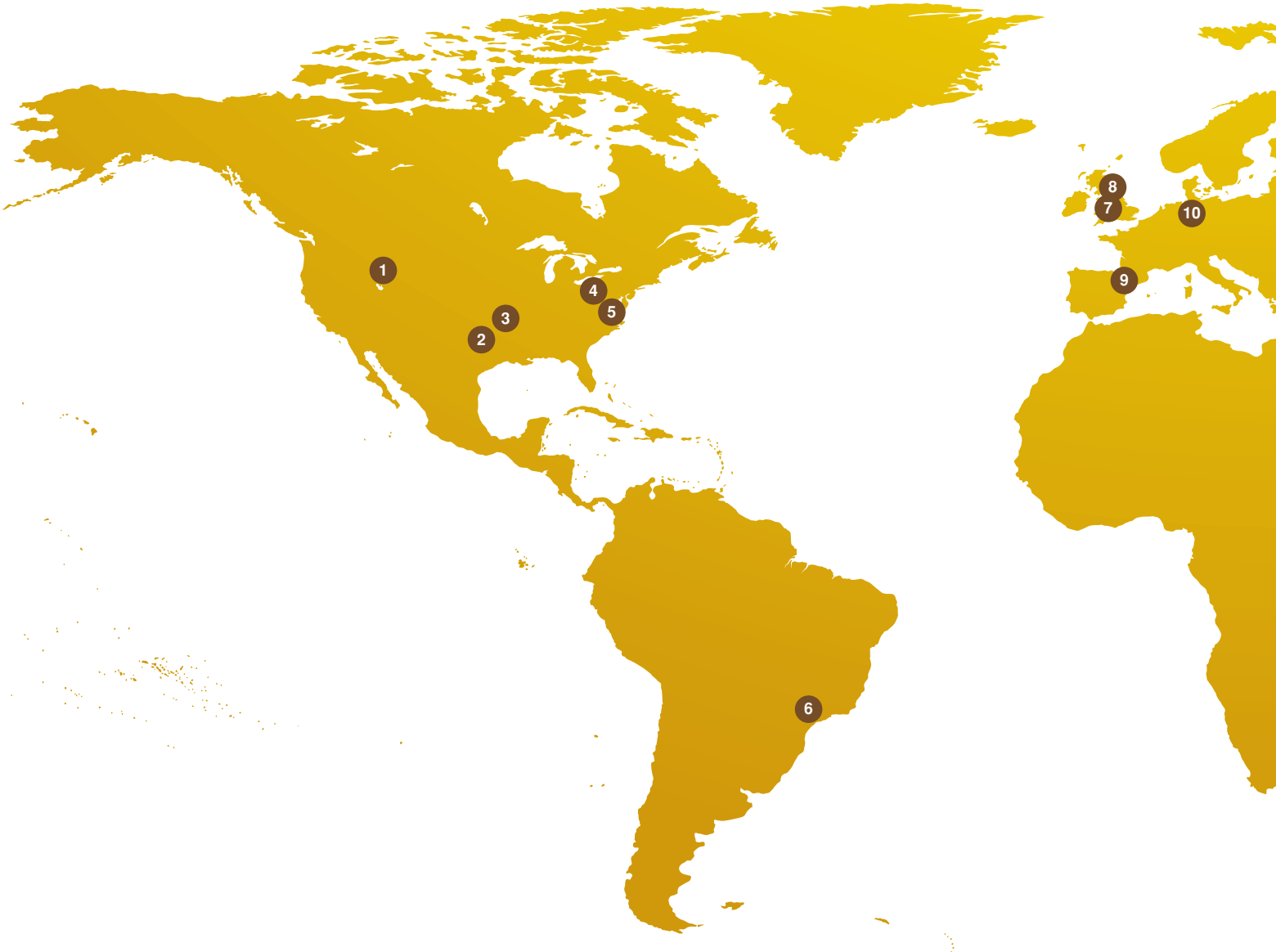

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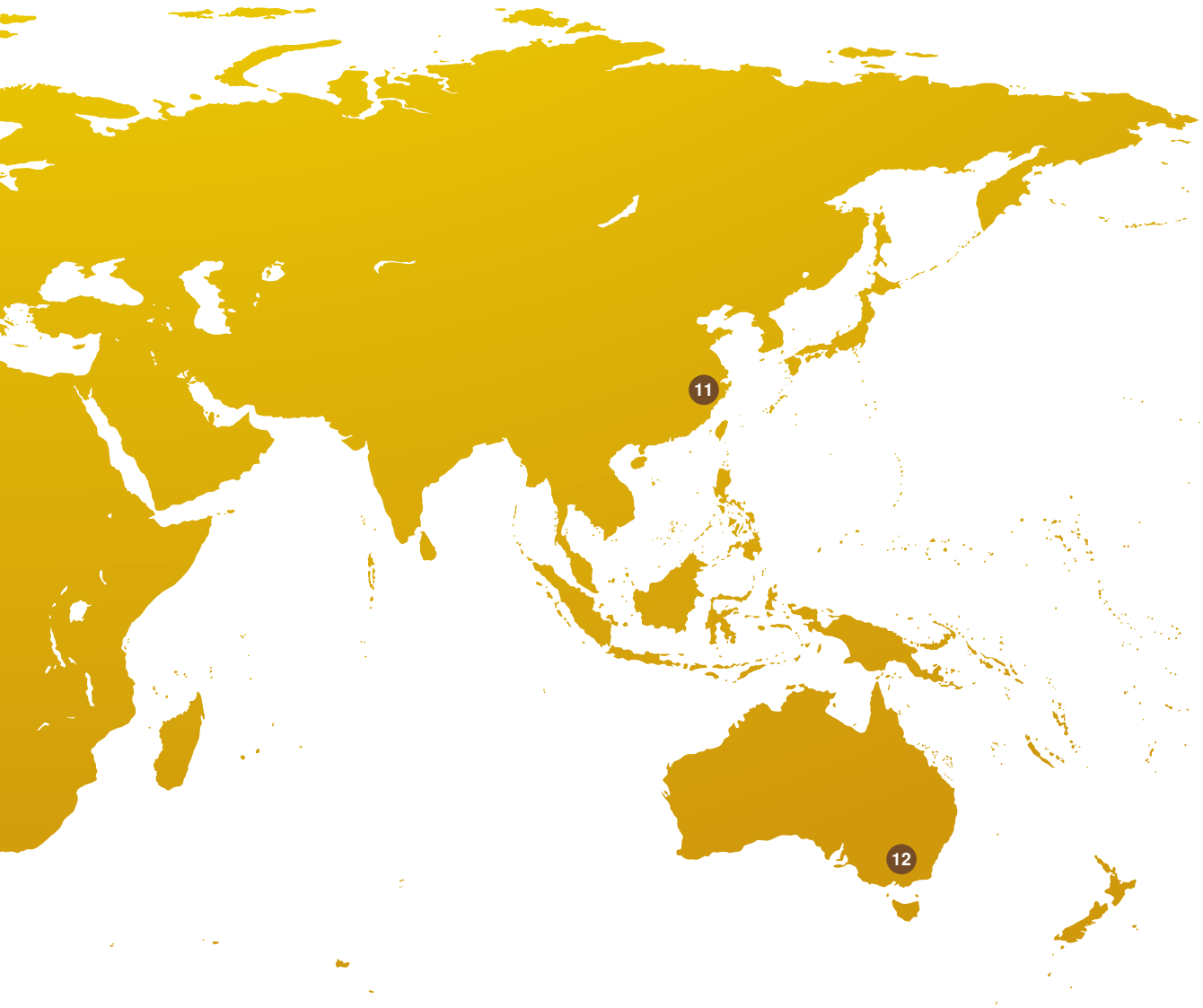

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