

FARE2019 WINNERS

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Ling Zhang

Postdoctoral Fellow

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Radiology/Imaging/PET and Neuroimaging

Convolutional Invasion and Expansion Networks for Tumor Growth Prediction

This study investigates the possibility of using deep learning to model and predict tumor growth, which has long been formulated by mathematical modeling. Tumor growth is associated with two fundamental processes: cell invasion and mass-effect. Medical imaging data provides non-invasive and in vivo-measurements of these underlying tumor physiological parameters over time. We propose a two-stream end-to-end convolutional neural network (ConvNet) to directly represent and learn the cell invasion and mass-effect from longitudinal medical images, and to predict the subsequent involvement regions of a tumor. The network architecture can easily be trained on population data and personalized to a target patient, unlike most previous mathematical modeling methods that fail to incorporate population data. Experiments are taken on a longitudinal pancreatic tumor data set with comparison with a state-of-the-art mathematical model-based approach. To the best of our knowledge, this is the first time to use learnable ConvNet models for explicitly capturing the two fundamental processes of tumor growth. The invasion network can make its prediction based on the metabolic rate, cell density and tumor boundary, all derived from the multi-model imaging data. Mass-effect $\hat{=}$ the mechanical force exerted by the growing tumor $\hat{=}$ can be approximated by the expansion/shrink motion (magnitude and orientation) of the tumor mass. This expansion/shrink cue is captured by optical flow computing, based on which the expansion network is trained to infer tumor growth. To exploit the inherent correlations among the invasion and expansion information, we study and evaluate three different network architectures, named: early-fusion, late-fusion, and end-to-end fusion. Our proposed ConvNet architectures can be both trained using population data and personalized to a target patient. The proposed ConvNets are tested on a NVIDIA TITAN X Pascal GPU of 12 GB of memory. Quantitative experiments on a pancreatic tumor data set show that the proposed method has a Dice coefficient of 86.8%, and RVD (relative volume difference) of 6.6%, substantially outperforms a state-of-the-art mathematical model-based approach (Dice=84.4%, RVD=13.9%). The proposed ConvNets requires \sim 5 mins for training and personalization, and 15 s for prediction per patient - significantly faster than the state-of-the-art model-based approach (\sim 24 hrs $\hat{=}$ model personalization; 21 s $\hat{=}$ simulation).

Laetitia Gorisse

Visiting Fellow

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Biochemistry - General, Proteins, and Lipids

IQGAP1 Binds The Axl Receptor Tyrosine Kinase And Inhibits Its Signaling

IQGAP1 is a scaffold protein that integrates cell signaling pathways by binding several growth factor

receptors and intracellular signaling molecules (Trends Cell Biol 2015; 25:171-184). The Axl tyrosine kinase receptor is important in hematopoiesis, the innate immune response, platelet aggregation, engulfment of apoptotic cells and cell survival (Cold Spring Harb Perspect Biol 2013; 5:a009076). Binding of growth arrest-specific protein 6 (Gas6) to immunoglobulin-like (Ig-like) domains of Axl activates intracellular signaling. However, the mechanism of inactivation of the Axl receptor is poorly understood. Here, we investigate the interaction between IQGAP1 and Axl. Using GST pulldown assays and immunoprecipitation from mammalian cell lysates, we found that IQGAP1 associates with Axl in cells. Analysis with multiple discrete fragments of each protein revealed that the binding is direct and mediated by the IQ domain of IQGAP1 and the Ig-like domains of Axl. We observed by proximity ligation assays that Axl:IQGAP1 complexes are located in the cytoplasm, and this interaction is reduced by 64% when cells are incubated with Gas6. Consistent with these findings, reducing IQGAP1 levels in cells by CRISPR/Cas9 enhanced the ability of Gas6 to stimulate both Axl phosphorylation and activation of Akt. Moreover, reduction of IQGAP1 in cells promoted Gas6-stimulated transcription of matrix metalloproteinase (MMP)-2, MMP-3, MMP-9 and urokinase-type plasminogen activator genes. Finally, we observed by confocal microscopy that IQGAP1 downregulates the heterodimerization of Axl with the epidermal growth factor receptor. In summary, we identify IQGAP1 as a previously undescribed suppressor of Axl. These findings differ from the previously reported functions for IQGAP1 as a scaffold that promotes signaling by growth factor receptors (J Biol Chem 2007; 292:3273-3289; J Biol Chem 2011; 286:29734-29747; J Biol Chem 2011; 286:15010-15021). Our data expand the repertoire of binding partners for both Axl and IQGAP1, and provide additional insight into the regulation of Axl function.

Youbao Tang

Postdoctoral Fellow

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Radiology/Imaging/PET and Neuroimaging

Semi-Automatic RECIST Labeling on CT Scans with Cascaded Convolutional Neural Networks

Response evaluation criteria in solid tumors (RECIST) is the standard measurement for tumor extent to evaluate treatment responses in cancer patients. The quality of RECIST annotations will affect the assessment result. To perform RECIST annotations, a radiologist first selects an axial image slice and then he or she measures the diameters of the longest axis and the orthogonal short axis. Using RECIST annotation faces two main challenges: 1) measuring tumor diameters requires a lot of professional knowledge and is time-consuming. 2) RECIST marks are often subjective and prone to inconsistency among different observers. However, consistency is critical in assessing actual lesion growth rates, which directly impacts patient treatment options. To overcome these problems, we propose a RECIST estimation method based on a cascaded convolutional neural network. Specifically, the stacked hourglass networks (SHN) is employed for RECIST estimation, where a relationship constraint loss is introduced to improve the estimation accuracy. Regardless of class, the lesion regions may have large variability in sizes, locations and orientations in different images. To make our method robust to these variations, the lesion region first needs to be normalized before feeding into SHN. The spatial transformer network is used for lesion region normalization, where a localization network is designed for lesion region and transformation parameter prediction. Thus, given a region of interest cropped using a bounding box roughly drawn by a radiologist, our method directly outputs RECIST annotations. As a result, our method is semi-automatic, drastically reducing annotation time while keeping the

human in the loop. In addition, our method can be readily made fully automatic as it can be trivially connected with any lesion localization framework. We train our system on a large scale lesion dataset where RECIST annotations are performed by multiple radiologists over a multi-year period. Experimental results compare our method to the multi-rater annotations in that dataset, plus annotations from two additional radiologists. The mean and standard deviation of inter-reader variation of long diameter are 3.40mm and 5.24mm, while those for the variation between our system and the manual annotations are 2.67mm and 3.95mm, which demonstrate that our system performs more stably and with less variability, suggesting that RECIST annotations can be reliably obtained with reduced labor and time.

Kirill Gorshkov

Postdoctoral Fellow

NCATS

Clinical and Translational Research, and Gene Therapy

A novel high-throughput assay for Niemann-Pick disease type A small molecule therapeutics discovery

Niemann-Pick disease type A (NPA) is a rare and fatal lysosomal storage disease caused by a deficiency of acid sphingomyelinase (ASM) that results in lysosomal sphingomyelin (SM) accumulation. There is no treatment available for NPA. In this study, we have developed a washless, 384-well plate, quantitative high-throughput screening (qHTS) fluorescence assay to identify compounds that alleviate the SM accumulation phenotype by restoring ASM function or other mechanisms. We first loaded NPA patient fibroblasts with green-fluorescent BODIPY-FL-labeled sphingomyelin, followed by treatment with drug compounds at 37 degrees C for 72 hours. The red-fluorescent LysoTracker dye and DRAQ5 nuclear dye were added at the end of the experiment one hour prior to fixation with Mirsky's fixative, enabling the detection of individual cells using the Mirrorball fluorescence cytometer, which uses fluorometric microvolume assay technology to exclude background signal. The cellular imaging data consisting of cell count and fluorescent intensity measurements were analyzed using the Cellista software. Our primary and follow-up compound screen used wild-type fibroblasts as a control cell line. DMSO and delta tocopherol were used as negative and positive controls, respectively. We first screened the LOPAC drug library consisting of 1280 bioactive compounds producing 19 positive hits. The hit selection criteria consisted of greater than 30% reduction green fluorescence (decreased SM accumulation) without a decrease in cell counts. Seven hits clustered into two distinct classes targeting Receptor A or B, suggesting a common mechanism of action. The assay parameters from control plate statistics were robust with a signal-to-basal ratio of 2.45, Z-factor of 0.54, coefficient of variation of 6.58 percent in wild type cells and 7.22 percent in patient cells. From the follow-up screen, the EC50s for lead compounds A and B were 1.39 nM and 10.1 nM with efficacies of 62.5 percent and 50.0 percent, respectively. All hits were previously FDA-approved for other indications. In summary, our assay advantages include (1) no wash, (2) high throughput, and (3) live or fixed cells. In contrast, traditional imaging assays require multiple plate washes to remove the background signal, long scan and analysis times, and large data files. Therefore, our Mirrorball-based qHTS method advances high-throughput screening by increasing sensitivity, throughput, and efficiency while reducing assay artifacts.

Xue Liu

Postdoctoral Fellow

NCATS

Vascular Disease and Biology

Human vascularized Bioprinted skin tissues as tissue-in-a-dish models for drug screening

In vitro cell assays and animal models are extensively used in early stage drug development for efficacy, risk and toxicity tests. However, the lack of physiologically relevant human native skin models results in limited predictive value of human response. Three-dimensional (3D) human skin equivalents are employed as an alternative method for drug discovery. Despite the improvements in culturing 3D human skin equivalents, very few models have vasculature with proper epidermal barrier function. We sought to develop a physiologically accurate 3D bioprinted full-thickness human skin model with associated vasculature to enable preclinical drug development studies. Primary neonatal fibroblasts, pericytes, and induced pluripotent stem cell (iPSC)-derived endothelial cells, were suspended in a fibrinogen based bio-ink and bioprinted on top of a membrane of a 12-well transwell insert. Bioprinted dermal constructs cultured in submerged media conditions for 7 days produced a microvascular network. Primary neonatal keratinocytes were then seeded on the surface of the dermal tissue. After 3 days of submerged condition, the epidermal layer was exposed to the air-liquid interface for 14 days to allow the differentiation and stratification of the tissue. In the dermis, confocal images of intact tissues stained with collagen IV and CD31, an endothelial cell marker, showed the 3D microvascular network and basement membrane of microvessels. Strong expression of integrin β 1 and laminin5 indicated well-formed dermal-epidermal junctions. Late epidermal differentiation markers, keratin10, loricrin and filaggrin further illustrated the epidermal layer was fully differentiated. Transepithelial electrical resistance (TEER), which measures the integrity of the tight junction and the barrier function of the tissue, showed values of >10000 ohm, indicating a well formed skin barrier. The accumulation of Texas Red-conjugated 10k MW dextran within the endothelial network structures demonstrated both structural and mechanical integrity of the vascular system. These combined data demonstrate we have developed a rapid and robust 3D bioprinting platform faithfully recapitulating human native skin in microwell plate format. This first 3D bioprinted skin tissue-in-a-dish assay platform is a major advance for studies in which vasculature is used to mimic systemic delivery of drugs, biological reagents like antibodies, immune cells, and viral vehicles for genomic editing, into the skin tissue

Joe Matarlo

Postdoctoral Fellow

NCI-CCR

Oncology, Tumor Immunology/Immuno-oncology, and Therapy

Inhibition of Pre-miRNA Processing by bPGN Leads to Cell Cycle Arrest in Colorectal Cancer Cells

Colorectal cancer (CRC) is the second most common cancer worldwide. Elevated levels of oncomiRs such as miRNA-21 play pivotal roles in regulating tumorigenesis. Controlling aberrant RNA expression in cancer cells and restoring miRNA homeostasis can provide a new therapeutic strategy in the treatment of cancer. In this study, we developed a novel thermofluor-based high-throughput screening method to identify small-molecule inhibitors of premiR-21 processing. A small-scale screen of 5000 pure natural product compounds afforded 35 selective modulators of premiR-21 stability. These compounds were then tested for tumor cytotoxicity against HCT-116 colorectal cancer cell line. One compound, bPGN,

inhibited cell viability at 33 nM and exhibited an apparent binding constant (Kd) of 1.6 μ M to premiR-21 in vitro. Our results also revealed that bPGN can inhibit Dicer activity in a dose and time-dependent manner in vitro. qRT-PCR analysis of treated cells showed an 80% decrease in miR-21 level within 2 hrs post-treatment without affecting miR-1246 level, another overexpressed miRNA in CRC. Furthermore, transcripts of miR-21 target genes such as PDCD4 and PTEN, increased 3-5 fold in 4-8 hrs post-treatment, suggesting that inhibition of bPGN is on-target. To gain insight into the global effects of bPGN, we performed a pan-cancer pathway-transcriptomics analysis of cells treated from 0-72 hours. Principle component and heatmap analyses depict clustering of samples in a time-dependent manner. Gene expression analysis showed highly differentiated genes from 4 signaling pathways; JAK-STAT, PI3K-AKT, and FOXO signaling are upregulated, while the Cell Cycle is highly downregulated. Although PI3K and other upstream genes are hyperactive, upregulation of PTEN prevents phosphorylation (activation) of AKT. Our data also suggest that bPGN can upregulate negative regulators of the cell cycle such as p27, p15, and p21, through inhibition of SKP2 (also a miR-21 target). Thus, although the JAK-STAT, PI3K-AKT and FOXO pathways are hyperactivated, downstream effectors of the cell cycle are downregulated via premiR-21 inhibition, resulting in cell cycle arrest. Finally, mini-chromosome maintenance 2-7 (MCM2-7) transcripts are all highly downregulated by bPGN up to 80%, consequently inhibiting DNA replication in S-phase. Taken together, bPGN can inhibit premiR-21, upregulating miR-21 target genes, and inducing cell cycle arrest. Furthermore, bPGN may function as a novel MCM2-7 inhibitor.

Snehal Gaikwad

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Oncology, Tumor Immunology/Immuno-oncology, and Therapy

A model of proteasome inhibitor resistance driven by metabolic reprogramming

Introduction: Multiple myeloma (MM) is both a rare disease and the second-most common hematological malignancy. Despite treatment regimens with proteasome inhibitors (PI) and immunomodulatory drugs, MM patients relapse. PI-resistant MM cells were found to harbor high levels of MCL1, a pro-survival protein of the BCL2 family. When these cells were treated with a small molecule capable of targeting MCL1, the mitochondrial apoptotic pathway was induced via metabolic deregulation. Methods: We established a clinically relevant Oprozomib-resistant MM subline (KMS28BM-R) from parental KMS28BM-P. Microarray, exome sequencing and druggable-genome RNAi sensitizer screening approaches were employed to characterize molecular and genetic differences between parental and resistant cells. Host cell bioenergetics were assayed using a Seahorse XFe analyzer. Immunoblotting and Q-PCRs were used to validate protein and gene expression levels. Results: KMS28BM-R cells were pan-resistant to multiple PIs, with a 10-fold increase in IC50 for oprozomib compared to parental cells. No differences were seen between KMS28BM-P and -R cells with respect to expression of proteasomal subunits, and exome sequencing confirmed that all subunits were intact. As expected, knock-down of proteasomal subunits sensitized the drug-resistant cells to apoptosis. To identify genes involved in modulating PI resistance, gene expression profiles and high throughput druggable-genome RNAi sensitizer screen data were analyzed using a Foundry data integration platform. Mcl1 was one of the top hits upregulated in resistant cells, and RNAi against Mcl1 in the resistant cell line showed reduced viability. Drug resistant cells had higher bioenergetics, as measured by oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) indicative of higher glycolytic

activity. Treatment with an MCL1 inhibitor reduced cell-viability in the resistant cells, with concomitant reductions in the gene expression of glycolytic enzymes LDHA and PKM. Ongoing: High expression of MCL1 has been confirmed in additional PI-resistant cell lines, and the in vivo efficacy of MCL1 inhibitors are being evaluated preclinically. Thus, a systematic screening approach has identified MCL1 as one of the molecular modulators of PI resistance and suggests metabolic reprogramming can be used to combat PI resistance in MM.

Nivedita Ratnam

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Oncology, Tumor Immunology/Immuno-oncology, and Therapy

Tapping into the choroid plexus gateway to improve T cell trafficking to CNS tumors

Background: Glioblastoma (GBM) has a dismal five-year survival of 5.1% due, in part, to anatomical and physiological barriers surrounding the brain that limit delivery of drugs or other potential therapeutic compounds. In contrast, effector immune cells can penetrate some of these barriers, offering hope for immunotherapy strategies for intracranial cancers; yet GBMs are recognized as immunologically cold. Therefore, approaches are needed to enhance lymphocyte trafficking into GBM tumors. One gateway for immune cell entry into the brain is the choroid plexus (CP), which enhances immune migration from the blood in response to IFN γ signaling. Thus, we hypothesized that targeting the IFN γ pathway would increase lymphocyte migration to GBM tumors through the CP, resulting in enhanced immunotherapeutic response. Methods: We interrogated surgically resected GBM patient samples, primary non-human primate CP, as well as human and murine GBM cell lines. Flow cytometry was used to identify cell populations and quantify cytokine production. Gene expression analyses were performed using qRT-PCR alongside mining the TCGA database. Cell migration was evaluated using transwell assays. Results: Although rare, GBM patient tumor infiltrating lymphocytes (TILs) were enriched for IFN γ -expressing effector T cells. IFN γ exposure resulted in increased secretion of T cell chemoattractants CXCL9 and 10 in human and murine GBM, offering a potential means to enhance immune trafficking to the tumor site. Conditioned media from IFN γ -exposed murine GBM cell lines revealed new players including IFN β , IL23, IL27 and MCP1. Finally, T cell migration was enhanced toward GBM cells exposed to IFN γ . Conclusions: While IFN β is known to negatively counteract IFN γ function, IL23 and IL27 promote T cell differentiation and dendritic cell antigen presentation, and MCP1 is a known chemoattractant. Further, in the presence of IFN γ , GBM cell lines up regulated MHCII, facilitating antigen presentation to T cells. Together, these data support IFN γ signaling as a promising approach to enhance lymphocyte trafficking across the CP and into the "immune desert" of GBM. By understanding and enhancing immune entry through the CP, we aim to develop novel and critically needed immunotherapy strategies for patients with GBM.

Hejiao Bian

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Oncology, Tumor Immunology/Immuno-oncology, and Therapy

Construction of phage-displayed shark single domain antibody libraries

Background: The shark VNAR (variable domain of new antigen receptor) antibody is one of the smallest antibodies with sizes ranging from 12-15 kDa. With great stability, solubility, and ability to penetrate tissues and bind with hidden functional sites in target proteins, shark VNAR single domain antibodies possess distinct advantages as novel therapeutic and diagnostic tools in cancer and other diseases.

Methods: With a highly efficient method developed in this study based on PCR- Extension Assembly and Self-Ligation (named EASEL), we constructed a phage-displayed VNAR antibody library with a size of 1.2×10^{10} from six naïve adult nurse sharks (*Ginglymostoma cirratum*). The plasmid DNA of the naïve shark phage library was extracted from phagemid library for Next Generation Sequencing (NGS) analysis and over 1.19 million full length unique VNAR sequences were analyzed. To expand the diversity of the naïve shark library with type IV VNARs that possess longer CDR3s bind to buried sites in antigens, we constructed 3 semi-synthetic Type IV VNAR phage libraries with 18, 22, 24AAs in CDR3. Phage display technology was used to isolate VNAR binders from both naïve and engineered libraries that target cancer and viral antigens.

Results: This naïve shark library is highly diversified because it covers not only all four known VNAR types (types I-IV) but also VNAR sequences that have not been categorized in any known types. The amino acid variability analysis in each VNAR sequence position showed the sequence diversity is mostly in the CDR3 for naïve nurse sharks. CDR3 lengths, full length cysteine numbers and locations, and CDR3 cysteine numbers based on NGS analysis showed unique patterns as compared to conventional IgG antibody sequences. To validate the use of the shark VNAR library for therapeutic antibody discovery, we isolated a panel of VNAR phage binders to cancer therapy related antigens and viral antigens, including glypican-3, HER2, PD1, MERS and SARS spike proteins. Several cross-species shark VNAR binders that bind to both human and mouse antigens were also isolated from the 3 engineered long CDR3 shark libraries.

Conclusions: The 4 nurse shark libraries including 1 naïve and 3 engineered libraries are the largest diversity nurse shark antibody selection platform constructed so far. This platform can be used for selecting cross-species therapeutic single domain antibodies target challenging antigens.

Romina Araya

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Oncology, Tumor Immunology/Immuno-oncology, and Therapy

Neutrophils are required for optimal response to chemotherapy and their function is regulated by the microbiota

Neutrophils represent the first line of defense and are rapidly recruited to sites of acute inflammation and infection. Although their function in host defense is clear, the role of neutrophils in the tumor microenvironment remains controversial and their presence often associates with bad prognosis. Both pro- and anti-tumorigenic functions have been described and depending on environmental cues, tumor associated neutrophils (TANs) can be polarized towards either phenotype. However, their role in response to cancer therapy and the signals that control their polarization remain unclear. We tackled these questions with two approaches: using high-dimensional flow cytometry, functional assays and transcriptional profiling to characterize TANs from mouse tumor models with different susceptibility to oxaliplatin (oxa), or from oxa-sensitive tumors in animals with different microbiota status. We found that the number of reactive oxygen species (ROS)-producing TANs positively correlate with therapy efficacy, and tumors lacking TANs did not produce the ROS needed for response to oxa. However, no

differences were observed in circulating neutrophils indicating a TAN-specific requirement for successful therapy. Oxa-susceptible tumors injected in microbiota-deficient (GF or Abx) versus -competent (SPF) mice showed significant reduction in neutrophil function, recruitment and activation pathways, significantly lower number of TANs, ROS production, and impaired response. The same tumors injected in SPF animals with different microbiota composition differed in their susceptibility to therapy with higher numbers of TANs correlating with better response. Microbiome analysis identified 17 differentially abundant bacteria that correlate with TAN numbers. Administration of a NOD2 ligand restored ROS production in TANs from Abx-treated mice, providing a mechanistic pathway by which microbiota can modulate neutrophil function and anti-tumor response. Phenotypic characterization revealed high levels of ICAM1 expression in TANs from both oxa-resistant tumors and GF tumors, identifying a possible biomarker for tumors likely or not to respond to this therapy. These data indicate that TANs are required for optimal response to oxa, and that microbiota-derived signals regulate their function. Our study underscores the importance of having a clear understanding of the beneficial role of neutrophils and the necessary environmental factors when designing successful therapeutic strategies.

Fardokht Abulwerdi

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Chemistry

Discovery and Characterization of Small Molecules of Long Noncoding MALAT1 Triple Helix with Anti-Cancer Therapeutic Potential

Long noncoding RNAs (lncRNAs) have diverse biological functions and are associated with various disease states, including cancer. Human metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) features a highly conserved triple helix at its 3'-sequence which contributes to its stability and is highly associated with its increased oncogenic activity. MALAT1 plays a crucial role in breast cancer tumor progression and metastasis, making it an attractive target for cancer therapy. Small molecules offer an opportunity to target highly structured RNA motifs such as a triple helix. Therefore, as a first step in developing a therapeutic strategy, we elected to perform a high throughput small molecule microarray screening of ~26,000 small molecules to identify compounds binding to a 5'-fluorescently-labeled MALAT1 triple helix motif. After statistical analysis and visual inspection, 188 hit molecules were identified. From the hit list, 28 compounds representing the most selective binders were re-purchased from commercial vendors. We next elected to evaluate the compounds in a mouse mammary cancer organoids derived from MMTV (mouse mammary tumor virus)-PyMT luminal B tumors and grown in 3D matrigel. MMTV-PyMT tumors are undifferentiated, aggressive mammary carcinomas that are prone to metastasizing to the lungs. All 28 compounds were evaluated in this model and our results showed that when treated with 1 μ M of hit candidates 5 and 16, MALAT1 expression in organoids reduced by 54% and 41% respectively. We also observed a 38% and 27% decrease in organoid branching, a property of organoids which correlates with metastasis, with 5 and 16 respectively. A recently developed four-dimensional fluorescence resonance energy transfer (4D FRET) assay provided a detailed conformational landscape of MALAT1 triple helix interactions with 5 and 16 and determined their dissociation constants (K_ds) to be in the low micromolar range. Computational modeling predicted their basis of binding to MALAT1 triple helix. The superior compound 5 further modulated downstream MALAT1 target genes in a dose-dependent manner while sparing the expression of multiple endocrine neoplasia beta (MENbeta)

lncRNA which shares a similar triple helix to MALAT1. These results demonstrate the feasibility of selective targeting of an RNA triple helix with small molecules and the potential to leverage these molecules in treatment and understanding of MALAT1-driven breast cancer.

Chad Brocker

Research Fellow

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DNA-binding Proteins/Receptors and DNA Repair

Long non-coding RNA Gm15441 attenuates hepatic inflammasome activation in response to metabolic stress

There is growing support for a strong link between metabolism and inflammation. Peroxisome proliferator-activated receptor alpha (PPARA) is a ligand-activated transcription factor that is a key regulator of the fasting response. PPARA facilitates metabolic remodeling that promotes lipid oxidation and its dysregulation contributes to metabolic disorders and liver disease. Interestingly, studies have revealed that synthetic PPARA agonists also act as potent anti-inflammatory agents. The underlying mechanisms of how PPARA prevents inflammation are not well understood. To elucidate the impact of PPARA on metabolic and inflammatory pathways, we performed RNA-seq on livers from mice treated with the PPARA agonist WY14643. Comparing differentially expressed coding and non-coding genes, we identified a potential regulatory axis between thioredoxin-interacting protein (TXNIP) and its antisense long non-coding RNA (lncRNA) Gm15441. TXNIP was recently shown to act as a critical relay that links oxidative and ER stress to inflammation through NLRP3 inflammasome activation. Gm15441 was upregulated by over 400-fold in response to PPARA agonist. Conversely, TXNIP mRNA was reduced by 50%. ChIP-seq datasets were analyzed for PPARA binding sites within the Gm15441 promoter. Gm15441 regulatory elements were confirmed using reporter gene assays and PPARA ChIP studies. Gm15441 transgene expression in Hepa-1 cells downregulated TXNIP supporting lncRNA-mediated gene suppression in vitro. CRISPR/Cas9-mediated gene editing was used to develop a Gm15441 knockout mouse. Basal TXNIP levels in Gm15441-null mice were elevated five-fold over wild-type supporting Gm15441 as a negative regulator of TXNIP expression in vivo. Mice were treated with WY14643 to assess how loss of Gm15441 effects hepatic inflammasome activation in response to metabolic stress. TXNIP protein levels were unchanged in agonist-treated wild-type mice but increased by ten-fold in Gm15441-null mice. Caspase 1 and IL1B cleavage was elevated in Gm15441-null mice and further enhanced by PPARA activation indicating that lncRNA expression plays a major role in attenuating inflammasome activation. Herein, we demonstrate that hepatic PPARA directly regulates the lncRNA Gm15441. Gm15441 in turn suppresses TXNIP expression attenuating NLRP3 inflammasome activation during periods of metabolic stress. These studies reveal a novel regulatory mechanism supporting the beneficial effects of fasting, namely reduced inflammation.

Daniel Arango

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Molecular Biology - Prokaryotic, and Eukaryotic

Acetylation of cytidine in messenger RNA promotes translation efficiency

Nucleoside modifications expand the metabolic and regulatory functions of nucleic acids. In contrast to a handful of well-studied modified bases that constitute the DNA epigenome, over a hundred modified ribonucleosides are present in RNA. Mainly studied in non-coding RNAs, ribonucleoside modifications have more recently been described in protein-coding messenger RNAs (mRNAs), where they form the basis of the epitranscriptome. These epitranscriptome modifications have the capacity to regulate all steps of posttranscriptional mRNA metabolism including processing, structure, stability and translation. While most ribonucleoside modifications are methylation events, only a single acetylated ribonucleoside has been described in eukaryotes, occurring at the N4-position of cytidine (N4-acetylcytidine or ac4C). Initially discovered in transfer RNA (tRNA) and ribosomal RNA (rRNA), ac4C prevalence and locations transcriptome-wide has not been studied. The aim of this study was to investigate the distribution and function of ac4C in the human transcriptome. Using a combination of mass-spectrometry and antibody-based techniques in a human cell line, I identified ac4C as a novel mRNA modification that is catalyzed by the N-acetyltransferase enzyme NAT10. Enrichment of acetylated RNAs followed by sequencing identified discretely acetylated regions that displayed a specific localization bias near start codons within the coding sequence (CDS) of target mRNAs. Depletion of ac4C through CRISPR/Cas9 ablation of NAT10 followed by RNA-seq, revealed a relationship to mRNA levels, wherein a reduction in ac4C within the CDS was globally correlated with transcript down-regulation. Analysis of mRNA half-lives and translation transcriptome-wide demonstrated increased RNA stability and enhanced translation in the cohort of acetylated targets compared to non-acetylated mRNAs. Accordingly, NAT10 depletion led to decreased mRNA half-life and translation of specific acetylated targets indicating a role for NAT10-catalyzed cytidine acetylation in both these processes. Remarkably, examination of sequence biases and codons content within ac4C peaks uncovered a strong enrichment of cytidine within wobble sites, suggesting a direct role for ac4C in decoding efficiency. Altogether, these results expand the epitranscriptome to include an acetylated residue highlighting a role for cytidine acetylation in regulating translation and stability, potentially at the level of decoding efficiency.

Michael Boylan

Visiting Fellow

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Developmental Biology

Keeping it together: the role of the Fgf8 subfamily in murine embryonic ventral wall closure

Fibroblast growth factor (FGF) signaling is integral to processes such as cell survival, motility and differentiation and is studied in fields such developmental biology, cancer biology and stem cell biology. We generated mice in which the genes coding for the Fgf8 subfamily (Fgf8, Fgf17 and Fgf18) have been conditionally inactivated in the primitive streak and emerging mesoderm using Cre-loxP technology. We found that such mutants frequently display the ventral wall (VW) defect omphalocele, a herniation of the liver and bowel through the umbilical ring. By examining the contribution of each of the three genes to the penetrance of the phenotype, we were able to determine a genetic hierarchy (Fgf18>Fgf8>Fgf17). In humans, omphalocele is associated with increased morbidity and mortality, but the causes of this condition are poorly understood. It is thought that omphalocele is a result of a failure in VW closure due to an arrest in myoblast migration from the somites to the ventral midline. Histological examinations of Fgf8 subfamily mutant embryos show that muscle migration is impaired, suggesting the defect observed is analogous to omphalocele in humans. Experiments using different Cre lines show that the Fgf8

subfamily is dispensable in the muscles themselves and may be required in the presomitic mesoderm (PSM) or somites. The PSM is a reservoir of undifferentiated tissue that will form the somites and contribute to other tissues. The role of Fgf8 in the PSM has been well studied, but the roles of Fgf17 and Fgf18 are completely unknown. We have observed ectopic cell death in the somites of Fgf8 subfamily mutants, which could explain the defects in the ventral muscles. In order to test whether the cell death observed contributes to the VW defect, we have additionally knocked out the pro-apoptotic genes, Bak and Bax. We found that the incidence of omphalocele dropped dramatically when apoptosis is suppressed genetically, suggesting that the somitic cell death causes omphalocele. We are currently investigating whether the somite specification program is malfunctional, and we are also examining the role of FGF receptors 1 and 2 in VW closure. This project will clarify the role of FGF signaling in VW formation, which has received little attention despite its medical relevance.

Yu-Ting Su

Postdoctoral Fellow

NCI-CCR

Clinical and Translational Research, and Gene Therapy

Novel Targeting of Transcription and Metabolism in Glioblastoma

Background: Glioblastoma is an aggressive primary malignant brain tumor with a median survival of less than 15 months. The current standard treatment includes maximal surgical resection followed by radiation and/or chemotherapy with the alkylating agent, temozolomide but shows limited efficacy in patients with glioblastoma. Finding a promising drug targeting multiple survival mechanisms of glioblastoma is urgently needed. TG02 is a multi-kinase inhibitor, mainly inhibiting cyclin-dependent kinase 9 (CDK9), thus diminishing RNA polymerase II activation to suppress the expressions of anti-apoptotic proteins such as Mcl-1 and Survivin. To investigate TG02, an agent with known penetration of the blood-brain barrier, we examined its cytotoxic effects as monotherapy and in combination with temozolomide in glioblastoma. Methods: We utilized human glioblastoma cells and a syngeneic orthotopic mouse model, evaluating the survival and the pharmacodynamics of TG02. Mechanistic studies included TG02-induced transcriptional regulation, apoptosis and RNA sequencing in treated glioblastoma cells as well as the investigation of mitochondrial and glycolytic function assays. Results: We demonstrated that TG02 induced cell death in eight glioblastoma cell lines with the variable genetic background but not in normal cells such as astrocytes and endothelial cells. TG02-induced cytotoxicity was blocked by the overexpression of phosphorylated CDK9, suggesting a CDK9-dependent cell killing. TG02 suppressed the protein expressions of Mcl-1 and Survivin and induced apoptosis in glioblastoma cells. We further demonstrated that TG02 induced mitochondrial dysfunction by decreased the activity of complex I and induced Cytochrome c release to the cytoplasm. Moreover, TG02 synergizes with temozolomide to induce cell death, partially by inhibiting glycolysis and subsequently depleting energy in glioblastoma cells. A prolonged survival was observed in GBM mice receiving combined treatment of TG02 and temozolomide. TG02 decreasing in CDK9 phosphorylation was confirmed in the brain tumor tissue. Conclusions: TG02 inhibits multiple survival mechanisms and synergistically decreases energy production with temozolomide, representing a promising therapeutic strategy for glioblastoma. These preclinical findings, supporting the pharmacological efficacy of TG02 and temozolomide for glioblastoma, have led to the launching of a phase I/II clinical trial (NCT02942264).

Yeap Ng

Visiting Fellow

NCI-CCR

Physiology

Characterization of mitochondrial metabolic oscillations in live rodents

Mitochondria are specialized cellular compartments that function in energy production and calcium homeostasis. Although these organelles have been primarily investigated in cell cultures, very little is known about their morphology, function, and dynamic properties in live multicellular organisms. In order to address this issue and, specifically, to investigate mitochondrial bioenergetics in vivo, we used Intravital Subcellular Microscopy (ISMic), an imaging approach that enables the visualization of a variety of biological processes in live animals. Using the salivary glands of live rats as a model organ, we found that: 1) mitochondrial metabolic activity exhibits rapid and periodic oscillations under basal conditions, and 2) mitochondrial oscillations are synchronized throughout the salivary epithelium via the activity of gap junctions. Moreover, in the last few years several reports have linked dysfunctional metabolic oscillations to a variety of diseases which include obesity, cardiovascular disease, developmental defects and cancer. This prompted us to address new challenging questions such as: 1) what is the physiological role of mitochondrial metabolic oscillations and 2) and how oscillations are orchestrated at the whole organismal level. Here, we have extended our previous work in order to characterize mitochondrial metabolic oscillations in a variety of rat and mouse tissues under both physiological and pathological conditions. We found that under basal conditions metabolic oscillations occur in all the tissues tested (skeletal muscle, tongue muscle, kidney), and that they are not induced by surgical injuries or laser photo-toxicity. In addition, we developed a semi-automated method that enables a rapid quantitative analysis of the oscillations, and we found that each tissue exhibits metabolic oscillations with distinct periods and amplitudes, that are not significantly altered by the age of the animal. Importantly, we observed differences in oscillatory patterns between mice and rat tissues, which may be potentially linked to species-specific differences in metabolism. In conclusion, we provided for the first time a detailed quantitative analysis of the characteristics of the metabolic oscillations under basal conditions, which will serve as a baseline to study these processes during pathological states such as cancer progression.

Jacob Paiano

Doctoral Candidate

NCI-CCR

Chromatin and Chromosomes

Elucidation of mitotic chromosome condensation by genome-wide sequencing of topoisomerase II-induced DNA breaks

At risk of mutagenesis, cells employ DNA damage and repair to organize chromatin into discrete genomic architectures. Upon mitosis, chromosomes are rapidly condensed into metaphase chromatids by concerted DNA loop extrusion via condensin proteins and controlled DNA breakage by topoisomerase II (Topo2) at chromatid axes. However, precisely how these modes of organization are mechanically coordinated has yet to be demonstrated. Recent Hi-C models conclude a stochastic process of condensation, purportedly through unbiased loading and action of condensin. Yet no direct evidence against a more deterministic model of site-specific condensin and Topo2 activity has been reported. In

this study, we used END-seq to map Topo2 DNA breaks genome-wide in condensed chromosomes and found strikingly specific sites of Topo2 activity, arguing against a purely stochastic model for mitotic condensation. We recently identified preferences for Topo2 activity at interphase cohesin (a protein relative of condensin) loop extrusion. With these findings and previous literature on the requisite role for Topo2 in mitosis, we hypothesized that Topo2 has an analogous role in condensation at sites of condensin loops, possibly to overcome topological issues associated with extruding entire chromosomes in minutes time. We prepared murine sperm able to fully condense chromosomes in *Xenopus* egg protein extracts to study a simplified in vitro condensation system with no DNA replication. To ensure accurate END-seq, we treated condensed sperm with the restriction enzyme AsiSI and detected breaks at 90% of AsiSI cut sites. We next treated condensed sperm with etoposide to trap Topo2 in its native cleavage complex, occluding its break repair, and detected 50-100 kb Topo2-induced fragment sizes by gel electrophoresis, consistent with predicted condensin loop sizes. Across four END-seq replicates, we found approximately one to three thousand reproducible Topo2 breaks evenly distributed across all chromosomes, possibly reflecting novel sequence- or structure-specific hubs of condensin action. We will further test this by condensin depletion and ChIP-seq experiments. Our studies strongly indicate site preferences for Topo2 activity, and possibly condensin, arguing against a fully stochastic process. Characterization of these active Topo2 sites will further our understanding of how cells use DNA damage at specific sequences or structures to organize the genome.

Kazusa Ishii

Clinical Fellow

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Oncology, Tumor Immunology/Immuno-oncology, and Therapy

Perforin Contributes to Chimeric Antigen Receptor (CAR) T-Cell Contraction and Regulates Host Inflammatory Response

Chimeric antigen receptor T cell (CART) therapy against B-cell malignancies is highly effective, but is frequently associated with inflammatory toxicities. In severe cases, patients develop a prolonged life-threatening systemic inflammatory response mimicking hemophagocytic lymphohistiocytosis (HLH). Our understanding of CART-induced HLH-like toxicity has been limited by the lack of experimental model. Primary HLH occurs among individuals harboring genetic defects in granule-mediated cytotoxicity pathway, such as perforin gene mutations. Here, we describe the role of perforin in CART response, and demonstrate that CART-induced HLH-like toxicity can be modelled in a syngeneic murine system by using perforin-knockout (Prf^{-/-}) donors. Compared to wild-type (WT), Prf^{-/-} CART produced a higher amount of IFN γ upon CAR-mediated activation. As expected, Prf^{-/-} had inferior cytotoxicity compared to WT. The differential cytotoxic activity between WT and Prf^{-/-} became apparent in vivo when CART was given at cell dosage of 1E5 or less. However, at higher dosage, Prf^{-/-} eradicated leukemia with the same clearance kinetics as WT and achieved long-term complete remission. Following maximal in vivo CART expansion, both WT and Prf^{-/-} CART contracted by day 8 as leukemia cleared. Surprisingly, Prf^{-/-} CART had a second proliferation between day 13-20 despite no measurable leukemia or endogenous B-cells at that time. Host-derived CD8 T-cell also skewed towards activated phenotype in Prf^{-/-} CART recipients. Furthermore, Prf^{-/-} CART recipients developed splenomegaly, increased phagocytosis, and elevated serum ferritin level, recapitulating HLH phenotype seen among human CART recipients. These changes in host-derived immune-associated cells suggest that the lack of perforin not only affects CART but may

also induce a pro-inflammatory microenvironment through soluble mediators. Among the first 10 cytokines that we tested, IFN γ was the only cytokine differentially elevated in vivo, comparing Prf $^{-/-}$ to WT. IFN γ blockade is utilized in clinic to manage primary HLH. However, contrary to our expectations, IFN γ neutralization exacerbated later CART expansion. IFN γ -overproducing CART model also showed that higher IFN γ ameliorates later CART expansion. Collectively, our data suggest that perforin contributes to CART contraction and the regulation of a secondary inflammatory response. Current work aims to elucidate the main cytokine axis and therapeutic target of the HLH-like toxicity.

Tsion Minas

Postdoctoral Fellow

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Epidemiology/Biostatistics - Prognosis, Response Predictions, Etiology, Risk, and Prevention

IFNL4-deltaG Predisposes to Aggressive Prostate Cancer Among Men at Increased Risk of Sexually Transmitted Infections

Prostate cancer incidence and mortality rates are highest among men with African ancestry. Previously, we and others have described a tumor-specific and distinct immune-inflammation gene expression signature in prostate tumors of African-Americans (AA), but not in European Americans (EA). The fact that the signature included several up-regulated interferon (IFN)-related genes that are part of the antiviral signaling pathway suggested viruses may play a role in inducing this signature and in the tumorigenesis of prostate cancer in men with African ancestry. Humans are polymorphic for the dinucleotide TT/deltaG allele (rs368234815) in the interferon lambda 4 gene (IFNL4). The deltaG variant, the variant predominantly present in those with African ancestry, is associated with impaired viral clearance. Thus, we hypothesized that increased likelihood of exposure to sexually transmitted infections (STIs) may increase prostate cancer risk in IFNL4-deltaG-dependent manner. Number of sexual partners was used as a surrogate to assess likelihood of exposure to STIs. Multivariable logistic regression models were used to examine the association between the number of sexual partners and prostate cancer in the NCI-Maryland Prostate Cancer Case-Control Study. The study included 976 prostate cancer cases (489 AA and 487 EA) and 1034 population controls without disease diagnosis (486 AA and 548 EA). Sexual history was reported using a self-administered questionnaire. Overall, men who had 10 or more sexual partners in their twenties and thirties had a significantly increased risk of developing prostate cancer when compared to men with 0-1 partners. IFNL4-deltaG modified this risk. Those with 10 or more partners and at least one copy of IFNL4-deltaG had a significantly increased risk of prostate cancer while those with the same number of partners but lacking IFNL4-deltaG did not. A test for synergy showed that the number sexual partners and IFNL4-deltaG positively interacted in the development of aggressive prostate cancer. In conclusion, we discovered a gene-environment interaction between the IFNL4-deltaG allele and sexual activity during early adulthood that was associated with increased risk of prostate cancer. Our finding indicates that IFNL4-deltaG is a candidate prostate cancer risk factor in men with high likelihood of exposure to STIs, suggesting a yet unidentified relationship between an infectious agent and prostate cancer in men with IFNL4-deltaG.

Xavier Bofill De Ros

Visiting Fellow

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Molecular Biology - Prokaryotic, and Eukaryotic

Tertiary Structure Differences in Pri-miRNAs Promote Alternative Drosha Cleavage and Expand Target Repertoires

Background and Hypothesis: MicroRNAs (miRNA) biogenesis starts with Drosha cleavage. The fidelity of this initial cleavage is critical for the posterior cleavage by Dicer as well as for establishing the seed sequence that determines the miRNA target specificity. To understand how pri-miRNA sequence and structure impact on the choice of Drosha cleavage sites, we study the processing of the three mir-9 paralogs, which harbor the same sequence of miR-9 but differ in loop and surrounding sequences. **Study Design and Methods:** Here, we systematically explore how different pri-miRNA paralogs of miR-9 are processed. To this end, we have analyzed the relative abundances of canonical and alternative cleaves in pri-mir-9-1, pri-mir-9-2 and pri-mir-9-3 by in vitro cleavage assay. In parallel, the in vivo cleavage was measured by deep sequencing the products. We also generated chimeric and hypothesis-driven mutations of pri-miRNA structures to study the contribution of each structural element to Drosha cleavage fidelity. Finally, we have evaluated the differential targeting between the miR-9-can and miR-9-alt, which are products of canonical and alternative Drosha cleavages. **Results and Conclusions:** Interestingly, we reveal that pri-miR-9-1 has a unique Drosha cleavage profile due to its kinked tertiary structure which is confirmed by small-angle X-ray scattering (SAXS). Pri-miR-9-1, but not pri-miR-9-2 or pri-miR-9-3, generates abundant miR-9-alt isoform with a shifted seed sequence that expands the scope of its target genes. Further analysis in gliomas cells and patient samples indicates a distinct role of miR-9-alt in tumorigenesis. Finally, we generalize our conclusion by demonstrating that the tertiary structure is a major determinant of Drosha cleavage fidelity on all pri-miRNAs. **Relevance and Importance:** 1) Our results demonstrate for the first time that pri-miRNA tertiary structure impact Drosha processing. 2) We provide compelling evidence that isomiRs play important roles in a physiological condition. 3) More than 30% of miRNA genes are members of families. Our results indicate that individual members can exert a different function via distinct Drosha processing. 4) We provide new guidelines in shRNA design to reduce heterogeneous processing and its associated off-target effects.

Santhi Devasundaram

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HIV and AIDS Research

IL-15 treatment increases cytotoxic lymphocytes in LN follicles and reduces SHIV RNA

Background: Heterodimeric interleukin-15 (hetIL-15) is a native stable form of the cytokine that activates and expands cytotoxic T and NK cells. Based on its properties and extensive preclinical data, hetIL-15 is currently evaluated in humans for the treatment of cancer. We study the effects of hetIL-15 in infected macaques to evaluate its use in HIV infection and especially in the reduction of SIV/SHIV reservoir towards a functional cure. **Methods:** Rhesus macaques, either chronically infected by SHIV or uninfected received injections of hetIL-15 over 2 weeks using increasing doses of cytokine (step-dosing). At the end of the treatment, the animals were sacrificed and the hetIL-15 effects on different lymphocyte populations isolated from tissues collected at necropsy were monitored by multi-parametric flow cytometry and quantitative multiplexed confocal microscopy (histo-cytometry). Cell-associated viral RNA and plasma viral load was measured by quantitative PCR. **Results:** This protocol was safe in rhesus

macaques and resulted in systemic expansion of CD8+ T lymphocytes and NK cells with higher granzyme B content. These expanded cell populations were found in both effector sites, such as liver, vagina and rectum, and secondary lymphoid tissues. Importantly, a significant increase in cytotoxic effector memory CD8+ T cells was found in lymph nodes (LN) from all hetIL-15-treated macaques. CM9 tetramer staining demonstrated that the increase of CD8+ effector T cells in lymphoid organs included actively proliferating SIV-specific T cells with higher granzyme content. Imaging analysis by histo-cytometry revealed that these effector CD8+ T cells infiltrated the B cell follicles where chronically infected follicular helper CD4+ T cells are located. Following hetIL-15 treatment, cell-associated RNA was decreased in LN and plasma viral load was also decreased. Treatment of macaques under Antiretroviral Therapy (ART) with this regimen was also safe and induced cytotoxic CD8+ accumulation in LN follicles. Conclusions: Step-dose administration of hetIL-15 is a well-tolerated regimen that results in systemic activation and expansion of cytotoxic leukocytes that infiltrate areas where chronic HIV-infected cells reside. These results suggest that hetIL-15 could be useful in disrupting sanctuary sites within the B cell follicles and reducing long-term viral reservoirs in HIV-1 infected individuals, thus contributing to a functional cure of the infection.

Zhongyan Lu

Postdoctoral Fellow

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HIV and AIDS Research

Vaccine Regimen to Induce Robust and Broad Cellular Immune Responses to Both Conserved and Variable Regions of HIV Gag and Env in Macaques

Background and Hypotheses: HIV sequence diversity and the propensity of eliciting T cell responses targeting variable regions are hurdles in the development of effective immunogens. Using HIV-derived DNAs encoding only conserved elements (CE) within Gag or Env, we were able to alter the immune hierarchy and we showed that responses were redirected to otherwise subdominant viral targets. This hierarchy was preserved after boosting with DNA expressing full-length (FL) proteins. A vaccination strategy including simultaneous delivery of both CE and FL DNA in separate anatomical sites was compared to CE prime/co-delivery CE+FL DNA vaccination to examine the magnitude and breadth of epitope recognition to variable and conserved regions. Study Design and Methods: Rhesus macaques were immunized with HIV-1 CE and FL DNA delivered by IM route followed by in vivo electroporation. The vaccine comprised Gag CE and FL gag or Env CE and FL env plasmid DNAs. The vaccine components were administered in the same (CE prime/CE+FL DNA boost) or separate (CE and FL DNA administered the same time) anatomical sites. Antigen-specific cellular immune responses in blood were monitored by flow cytometry in PBMC. Results and Conclusions: The T cell responses targeting CE induced by CE prime/CE+FL boost and by CE and FL separate vaccination were similar in magnitude and function, measured by cytotoxicity (Granzyme B+). Both vaccine regimens showed similar breadth of CE recognition. Interestingly, separating CE and FL molecules resulted in increased cytotoxic T cell responses targeting variable regions. Thus, separating the immunogens allowed for development of T cell responses with overall increased breadth including conserved and variable epitopes. Relevance and importance: The CE DNA prime/CE+FL DNA booster vaccination induces immune responses targeting the CE epitopes. The vaccination strategy that separates CE and FL DNA plasmids to different anatomical sites, targeting different draining lymph nodes, induces responses to both CE and variable regions. This

vaccine regimen has the advantage of avoiding interference of the different vaccine components (CE, FL) during the priming of the adaptive immune response, redirecting immunodominance, and eliciting robust cytotoxic T cell responses targeting conserved and variable regions in the viral proteome.

Mianmian Yin

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Genetics

Mice with a "deletor" phenotype allow identification of tumor suppressor genes and oncogenic fusion genes in lymphoid malignancies.

Mice that are homozygous for a deficiency allele of the DNA replication factor minichromosome maintenance protein 2 (designated *Mcm2def*) are born viable and are healthy for the first 2 months of life. Beginning at three months, these mice develop precursor T-cell lymphoblastic leukemia/lymphoma (pre-T LBL). Copy number aberration (CNA) analysis showed that these pre-T LBL samples had 8-14 small (100-1000 kb) interstitial deletions per sample. Remarkably, all mice had two or more deletions that encompassed genes known to be relevant for human pre-T LBL, including *Pten*, *Cdkn1a*, *Tcf3*, and *Tcf12*. Mice that express a NUP98-HOXD13 (*NHD13*) transgene develop a wide array of leukemias, most commonly myeloid, less commonly T-cell, and, rarely, B-lineage. To identify myeloid tumor suppressor genes, we crossed the *NHD13* transgene onto the *Mcm2def* background. All *Mcm2def:NHD13+* mice developed CD4+CD8+ (double positive, or DP) pre-T LBL by 3 months of age, reflecting the highly penetrant nature of the *Mcm2def* phenotype. However, none of the *Mcm2def:NHD13+* mice developed myeloid leukemia. Surprisingly, approximately 30% of the *Mcm2def:NHD13+* mice developed concurrent B-cell precursor acute lymphoblastic leukemia (BCP-ALL) and pre-T LBL. The thymus was typically infiltrated with pre-T LBL cells, whereas the bone marrow and spleen were infiltrated with BCP-ALL cells, characterized by clonal *Igh* gene rearrangement and CD19 staining. Parenchymal organs (lung, kidney, liver) were variably infiltrated with pre-T LBL, BCP-ALL, or both. CNA analysis showed that the pre-T LBL were characterized by short deletions including *Pten*, *CDkn1a*, *Tcf3*, and *Tcf12* deletions, similar to the *Mcm2def* pre-T LBL, whereas the BCP-ALL were characterized by homozygous or heterozygous deletions including *Pax5* and a 400 kb region encompassing *Cebpb* and *Ptpn1*, as well as amplifications, including a recurrent CNA that encoded a NUP214-ABL1 fusion gene. There were no shared deletions present in both BCP-ALL and pre-T LBL from the same mouse, indicating that the BCP-ALL and pre-T LBL arose independently, and not from a common precursor. The *Mcm2def:NHD13+* BCP-ALL mouse model was validated by the high frequency (5/7 samples) of acquired *Pax5* deletions and generation of a NUP214-ABL1 fusion gene, both of which are commonly detected in human BCP-ALL. Furthermore, the finding of *Cebpb/Ptpn1* deletions (5/7 samples) suggests an unanticipated role of either *Cebpb* or *Ptpn1* in BCP-ALL.

Paul Zakrevsky

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Molecular Biology - Prokaryotic, and Eukaryotic

A novel tetrahedral RNA scaffold improves the efficacy of delivered Dicer substrate RNAs

RNA has become an attractive material for use in the construction of nanoparticles for biological applications. By utilizing Watson-Crick base pairing interactions, as well as defined structural motifs that make use of non-canonical interactions, RNA nanoparticles (RNA NPs) can be constructed with exquisite control over their resulting size, geometry and mode of assembly. As such, RNA NPs can be used as a core scaffold to harbor functional RNA moieties, providing spatial and stoichiometric control of the appended entities. While existing RNA NPs exhibit favorable characteristics for coalescing multiple functions and delivery of siRNA, we have aimed to increase the RNAi potency of an RNA NP by increasing its capacity for functional RNAs. By using our previously characterized hexameric RNA nanoring as a modular building block and incorporating additional structural motifs, we have constructed a novel tetrahedral RNA NP that is able to harbor twelve functional moieties. This is a two-fold increase in functional capacity over the original nanoring scaffold. Molecular dynamics simulations suggest that the tetrahedral NP is robust and resistant to large structural perturbations, despite being assembled from eighteen RNA strands. Transfection of various RNA NPs harboring eGFP-targeting Dicer substrate RNAs (DsiRNAs) into cultured breast cancer cells expressing eGFP indicate that tetrahedral NPs display greater knockdown than other RNA NPs examined. The increased silencing also translates to increased therapeutic efficacy, as tetrahedral NPs targeting polo like kinase 1, a gene involved in cell cycle regulation, induces greater loss in cell viability than equal DsiRNA quantities delivered using hexavalent scaffolds. Importantly, the tetrahedral scaffold with non-targeting DsiRNAs is not cytotoxic at therapeutically relevant concentrations. Interestingly, the increased efficacy of the tetrahedral NP does not appear to be due solely to its increased DsiRNA capacity. Cellular uptake studies using fluorescently labeled NPs reveal that tetrahedral NPs are more efficiently taken up by cells than other RNA NPs tested, suggesting that the size and/or shape of the tetrahedral core plays a role in its increased RNAi potency. This increase in uptake, taken together with an increased functional capacity that could allow for incorporation of other functional RNAs while maintaining significant RNAi potency, results in a superior RNA NP for therapeutic applications.

Carla Neckles

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Gene Expression

THE FUNCTION OF HNRNPH1 IN PROCESSING THE EWS-FLI1 PRE-mRNA IN A SUBSET OF EWING SARCOMA CELLS

The primary oncogenic event in ~85% of Ewing sarcomas involves a chromosomal translocation that generates a fusion gene containing the 5' end of EWSR1 and 3' end of FLI1 (EWS-FLI1). In a third of EWS-FLI1 driven tumors, translocations that retain exon 8 of EWSR1 generate an out-of-frame transcript unless this exon is removed. We have previously demonstrated that the heterogeneous nuclear ribonucleoprotein H1 (HNRNPH1) binds to G-rich sequences within EWSR1 exon 8 and this binding event is required to express an in-frame EWS-FLI1 mature mRNA. G-rich nucleic acids may fold into stable tertiary structures, such as RNA guanine quadruplexes (rG4s). Our current studies are focused on the tertiary RNA structure of the G-rich regions within EWSR1 exon 8 and its role in HNRNPH1-dependent RNA processing. We first employed circular dichroism (CD) spectroscopy to evaluate the tertiary RNA structure of G-rich regions within EWSR1 exon 8. CD spectra for RNA oligomers corresponding to these G-rich regions had a positive peak at 262 nm and a negative peak at 240 nm, characteristic of a parallel-

stranded rG4 structure. This RNA structure was confirmed using a probe, thioflavin T (ThT), which is known to fluoresce in the presence of rG4s. Next, to determine the relationship between rG4s and HNRNPH1 binding, RNA oligomers were compared under stabilizing and destabilizing folding conditions in the presence of HNRNPH1 protein. Using chemiluminescent EMSA and antibody-based assays, we discovered that HNRNPH1 binds preferentially to rG4s and modulation of RNA structure alter HNRNPH1 binding affinity. Small molecules that can displace HNRNPH1 binding may provide a therapeutic vulnerability in a subset of Ewing sarcoma. To evaluate if this protein-RNA interaction is amenable to small molecules, we performed displacement assays with HNRNPH1 protein and an EWSR1 exon 8 RNA oligomer at varying concentrations of a G4-binding molecule, pyridostatin (PDS). The HNRNPH1-RNA complex was disrupted by PDS (IC50 of 7 micromolar). Furthermore, treatment with PDS selectively inhibits the growth of EWS cell lines harboring EWSR1 exon 8 fusions, decreases EWS-FLI1 activity in a cell-based reporter assay, and partially restores mRNA expression of EWS-FLI1 deregulated transcriptional targets. Our studies suggest that targeting the HNRNPH1-mediated processing of the EWS-FLI1 pre-mRNA is a potential strategy for targeting the fusion oncogene expressed in a third of Ewing sarcoma.

Jonathan Rawson

Postdoctoral Fellow

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HIV and AIDS Research

Recombination is Required for Efficient HIV-1 Replication

During assembly of HIV-1 virions, two copies of the full-length RNA genome are packaged into one particle, each containing all the genetic information required for replication. Upon infecting a new cell, viral RNA is converted into DNA, but each infection event generates only a single DNA provirus, such that HIV-1 is considered pseudodiploid. Why does HIV-1 package two copies of the RNA genome but generate only one DNA provirus? One hypothesis is that packaging two RNAs allows frequent recombination, which increases genetic diversity in the viral population. Another hypothesis is that recombination is a repair mechanism: if reverse transcriptase encounters a break in the RNA genome, it can switch to the co-packaged RNA to complete DNA synthesis. We sought to determine whether recombination is strictly required for replication of HIV-1. Frequent recombination is mediated by homologous sequences. Therefore, we devised a strategy to block recombination by reducing the sequence homology in defined regions of the viral genome. If recombination is not required for replication, reduced homology in part of the genome would not affect viral replication. In contrast, if recombination is required to complete DNA synthesis, blocking recombination would cause premature termination of DNA synthesis or lead to deletions in the viral genome, thereby significantly reducing viral infectivity. For this purpose, we engineered near full-length HIV-1 genomes that, when paired with other viruses, contain 0.9-kb, 1.7-kb, or 2.6-kb non-homologous regions. We then determined the survival of the progeny generated from these pairs of viruses. We found that as the size of the non-homologous region increased, the proportion of viable progeny decreased. Importantly, ~half of the progeny generated from viruses with a 2.6-kb non-homologous region were either non-viable or contain large deletions that rendered them non-infectious. Considering that the HIV-1 genome is ~9.2 kb in length, our results imply that >93% of progeny would be non-viable if recombination was blocked in the entire genome. Therefore, HIV-1 packages two viral RNAs to facilitate recombination and maintain intact

genome structure, ultimately enabling successful viral replication. These results resolve a long-standing question in the field, provide novel insights into HIV-1 replication strategy, and reveal recombination as a potential target for antiviral development.

Delphine Lissa

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Epigenetics

Ultrasensitive Droplet Digital PCR analysis of DNA Methylation For Lung Cancer Prognosis

Early detection and timely treatment remain the most effective strategy to reduce lung cancer mortality. Annual screening with low-dose computed tomography (LDCT) is recommended in high-risk individuals, based on results from the National Lung Screening Trial (NLST), which showed great promise for detecting early-stage cancers. Surgery remains the only curative option for stage I non-small cell lung cancer (NSCLC) patients, as benefits from adjuvant chemotherapy are controversial. Despite complete resection, a third of patients suffer recurrence and die of their disease within 5 years. Thus, it is urgent to develop more accurate prognostic biomarkers to predict tumor behavior and guide medical decisions. DNA methylation has emerged as a promising biomarker for early cancer detection and prognostication. Specific patterns of altered DNA methylation are frequently observed in lung cancer, and play an important role in cancer initiation and progression. We and others reported that high methylation of Homeobox A9 (HOXA9) promoter was associated with worse clinical outcome in stage I NSCLC patients. The present study is aimed at developing a practical and cost-effective assay to facilitate the clinical evaluation of this biomarker. Given the limited availability of tumor-derived material from small early lesions, we pursued methylation-specific droplet digital PCR (ddPCR) to reliably quantify rare alleles in precious DNA samples, such as derived from formalin-fixed paraffin-embedded (FFPE) biospecimens generated during routine pathology. We first validated the analytical performance of the assay, using reference standard DNA (fully methylated and un-methylated), and DNA isolated from archival FFPE tumors. The lower limit of detection was 0.01 ng of DNA, and the assay demonstrated high reproducibility, linearity and specificity towards methylated DNA. We further confirmed the prognostic value of HOXA9 methylation in a retrospective cohort of 177 FFPE tissues of stage I NSCLC. High HOXA9 methylation was associated with worse cancer-specific survival in the Kaplan-Meier (Log-rank $P < 0.0001$) and adjusted Cox regression analyses (Hazard Ratio [HR] 3.37; $P = 0.0002$). We developed a robust and ultrasensitive ddPCR assay for quantification of HOXA9 promoter methylation, and demonstrated its prognostic value. We will evaluate its clinical performance using tissue core biopsies collected for a subset of NLST participants who developed lung cancer during the trial.

Zhenyi Hong

Visiting Fellow

NCI-CCR

Endocrinology

A novel non-neuronal role of BDNF receptor-TrkB.T1 in regulating glucose homeostasis

The escalating obesity and diabetes rates due to overnutrition are happening globally. Understanding the etiology of these metabolic diseases requires detailed dissection of regulatory elements maintaining

energy balance. Brain-derived neurotrophic factor (BDNF) and its high-affinity receptor TrkB in the central nervous system (CNS) play important roles in regulating central orexigenic pathways. Previous data showed that mice heterozygous for BDNF or mice with a TrkB hypomorphic mutation develop severe obesity and hyperphagia. More importantly, patients carrying mutations in BDNF or TrkB showed similar phenotypes. The TrkB gene encodes mainly for two isoforms: a full-length isoform containing the tyrosine kinase domain (TrkB.FL) and a truncated isoform (TrkB.T1) lacking the tyrosine kinase structure. Both isoforms are extensively expressed in the adult mouse brain. However, our recent data has showed that TrkB.T1 is the exclusive isoform in pancreas. To investigate TrkB.T1 expression in the pancreas, we generated a mouse model with TrkB.T1 endogenously tagged with a V5 epitope using the CRISPR/Cas9 technique. Immunofluorescence staining of pancreatic sections from the TrkB.T1-V5 mice showed extensive expression of TrkB.T1 in the insulin producing beta cells whereas no TrkB.T1 expression could be found in the alpha-, delta- or endothelial cells. To further test the physiological significance of TrkB.T1 in beta cells, we generated TrkB.T1 complete knockout (TrkB.T1 KO) and Tamoxifen-induced TrkB.T1 beta cell-specific knockout (Insulin-cre TrkB.T1 cko) mice for in vivo studies. Impaired glucose uptake and reduced insulin secretion ability were observed in TrkB.T1 KO mice and in Insulin-cre TrkB.T1 cko mice, strongly suggesting that this phenotype is independent of pancreatic innervation. Calcium recording showed that BDNF could trigger calcium response in beta-TC6 insulinoma cells, which can be inhibited by the PLC-gamma inhibitor, suggesting that BDNF-TrkB.T1 could be an important signaling pathway in the process of insulin secretion and blood glucose homeostasis. This study, for the first time, identifies a new unexpected function for TrkB.T1 outside of the CNS. Furthermore, its impact stretches beyond this new signaling pathway by providing novel insights into the mechanisms governing insulin secretion and glucose metabolism, and potential therapeutic targets for the treatment of obesity, diabetes and related metabolic disorders.

Haydar Bulut

Visiting Fellow

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HIV and AIDS Research

Novel protease inhibitors markedly adapting to the structural plasticity of HIV-1 protease exert extreme potency with high genetic barrier

Over the last 3 decades, HIV-1 caused a devastating pandemic with ~80 million people infected worldwide and nearly half of them died from the disease, AIDS. Antiretroviral therapy (ART) has proven to suppress virus replication and significantly elongate the survival of people with HIV-1. The inhibitors of HIV-1 protease (PIs), an essential enzyme that cleaves gag-pol polyproteins into mature functional proteins, are the key element of ART. Blocking the activity of protease (PR) leaves daughter virions replication-incompetent, enabling the immune system of the hosts restored. However, HIV-1 often acquires resistance to PIs during long-term therapy. Upon comparative analyses of X-ray structures of wild type PR and mutated PR variants such as PRs of HIVDRVRP30 and HIVDRVRP51 (in vitro selected with darunavir, DRV, over 30 or 51 weeks, respectively), we identified multiple critical residues (F33, M45, R20, and D35) that induce the structural deformation in PR structure. In order to overcome the reduced affinity as a result of structural plasticity of PR, we systematically modified the chemical moieties around the scaffold of DRV and synthesized various novel PIs. In particular, we introduced a P2^{ab} cyclopropyl-amino-benzothiazole moiety (Cp-Abt) or P2^{ip} isopropyl-amino-benzoxazole (Ip-

Abo) at the S2 sub-pocket and a benzene ring located in the S1 sub-pocket with fluorine atoms at meta or para positions, and examined their anti-HIV-1 potency and cell permeability. Although the meta- and para-positioned fluorines had similarly facilitated cell permeability, we observed ~30-fold increase in cell permeability with P1 bis-fluorobenzene in the meta positions (bis-Fbz). Overall, the new set of PIs exerted significantly greater anti-HIV-1 potency (up to ~10,000-folds) and much higher genetic barriers to the emergence of resistant variants as compared to DRV. Particularly, GRL-063 containing bis-Fbz and Ip-Abo showed extremely potent inhibition against wild-type HIV-1 with IC50s of ~25 attoM; however it turned out to be significantly less potent against resistant variants as compared to GRL-142 that contains bis-Fbz and Cp-Abt. Even upon 1-year in vitro selection with GRL-142, no HIV-1 variants acquired resistance to GRL-142. The extreme potency of GRL-142 is expected to reduce the dose to be administered, to have lesser or least side effects, and to have very high genetic barrier, making it the most promising PI for potential clinical development.

Sunmi Park

Postdoctoral Fellow

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Endocrinology

Metformin and JQ1 synergistically inhibit obesity-activated thyroid cancer in a mouse model

Thyroid cancer is the most common form of endocrine malignant tumors and its incidence has sharply rising in the past decades. Compelling epidemiologic studies have shown a strong positive correlation of obesity with thyroid cancer, but a cause-effect relationship at the molecular level has yet to be established. While well-differentiated thyroid cancer responds well to radioiodine therapy and has a favorable therapeutic outcome, the treatment of refractory thyroid carcinoma remains a major challenge. We have created a mouse model, which expresses a dominant negative thyroid hormone receptor beta (denoted as PV) and deficiency in one allele of the Pten gene (ThrbPV/PVPten+/- mice), exhibiting aggressive follicular thyroid cancer. We showed that high-fat-diet (HFD)-induced obesity promotes aggressive thyroid cancer progression by aberrantly activating leptin-JAK2-STAT3 signaling in ThrbPV/PVPten+/- mice. Using the HFD-ThrbPV/PVPten+/- mouse as a preclinical mouse model, we showed that metformin, a widely used antidiabetic drug, markedly blocked invasion and metastasis, but not thyroid tumor growth in these mice. Our results suggested that tumor growth and invasion/metastasis could be regulated by different signaling pathways. Accordingly, we searched for another potential therapeutic that could inhibit thyroid tumor growth. We chose JQ1, a potent inhibitor of the activity of bromodomain and extraterminal domain (BET), for combined treatment with metformin. We showed that this combined treatment synergistically increased overall survival rate and suppressed obesity-induced thyroid tumor growth. Pathohistological analyses showed that JQ1 together with metformin blocked occurrence of invasion and decreased lung metastasis. Additional molecular analyses showed that combined treatment synergistically suppressed thyroid tumor growth by attenuating STAT3 and ERK signaling, resulting in decreased anti-apoptotic key regulators such as Mcl-1, Bcl-2, and survivin and in increased pro-apoptotic regulators such as Bim. Furthermore, combined treatment of JQ1 and metformin reduced cMYC protein levels along with EMT markers (MMP9, vimentin, N-cadherine, and fibronectin) to suppress cancer invasion and lung metastasis. The present preclinical findings suggest that combined treatment of JQ1 with metformin can be an effective

treatment modality for obesity-activated thyroid cancer and could be tested for efficacy in future clinical trials.

Guoli Shi

Visiting Fellow

NCI-CCR

Immunology - Innate and Cell-mediated Host Defenses

mTOR inhibition relieves an intrinsic antiviral barrier imposed by IFITM3

The lysosome is an essential organelle in the cell, responsible for the digestion of intracellular components, nutrient storage, and pH regulation. The lysosomal membrane also hosts mTOR kinase, the central regulator of cell growth, proliferation, and other anabolic processes required for cellular homeostasis. Rapamycin and its derivatives are specific inhibitors of mTOR and, as a result, are considered promising anti-tumorigenic agents in the cancer setting. It was also found that this class of drug promotes cellular transduction using lentiviral vectors by facilitating vector entry into cells, revealing its potential to improve gene therapy efforts. However, the precise mechanism was unknown. Here we report that mTOR inhibitor treatment results in a transient downregulation of the interferon-induced transmembrane (IFITM) proteins. IFITM proteins, especially IFITM3, are potent inhibitors of virus-cell fusion and are broadly active against a range of human pathogenic viruses. We found that the effect of rapamycin treatment on lentivector transduction is diminished upon IFITM protein silencing or knockout in primary and transformed human cell lines, indicating that the extent to which mTOR inhibition enhances lentivector entry is dependent on basal expression of IFITM proteins, with a major contribution from IFITM3. The effect of acute rapamycin treatment on IFITM3 manifests at the level of protein, but not mRNA, and is selective, as many other endosome-associated transmembrane proteins are unaffected. Rapamycin treatment leads to IFITM3 degradation that is dependent on endosomal trafficking, ubiquitination, and lysosomal acidification. However, the degradative process occurs independently of the autophagosome and is not observed during starvation, indicating that mTOR inhibition and autophagy can be uncoupled. Since IFITM proteins inhibit the entry of diverse viruses, we also show that mTOR inhibition promotes infection by IFITM-sensitive viruses, such as Influenza A virus, but not infection by IFITM-resistant viruses, such as Sendai virus. Our results identify the molecular basis by which mTOR inhibitors enhance virus entry into human cells and reveal a previously unrecognized immunosuppressive feature of these clinically important drugs that may impact antiviral immunity in vivo. In addition, this study uncovers a novel functional relationship between mTOR and IFITM proteins at endolysosomal membranes.

Eunju Ko

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HIV and AIDS Research

DENDRITIC CELL ACTIVATION AND FUNCTIONS AFTER MUCOSAL IMMUNIZATION OF RHESUS MACAQUES WITH REPLICATING ADENOVIRUS-SIV RECOMBINANTS

Inducing strong mucosal immune responses by vaccination is important for providing protection against Human Immunodeficiency Virus (HIV) and Simian Immunodeficiency Virus (SIV). Dendritic cells (DCs) are

known to initiate and control responses of most effector immune cells such as CD4 T, CD8 T and B cells. To better understand the cellular mechanisms elicited by mucosal antigen expression, rhesus monkeys were immunized mucosally 2 times with replicating adenovirus expressing SIV Env, Gag, and Nef (n=20, vaccine) or empty adenovirus (n=10, control). To examine DC subsets and their activation, rectal biopsies and blood were collected at day 3 and 7 post-immunization, respectively. To identify antigen-specific memory T cell responses, rectal biopsies and blood at day 21 post-immunization were used. Plasmacytoid DCs (pDCs, CD123+, $p < 0.0001$) and Langerhans cells (LCs, CD11c+CD1a^{high}, $p < 0.0001$) were significantly increased in rectal mucosa after the 2nd immunization. However, in blood, pDCs showed a decreased trend and myeloid DCs (mDCs, CD11c+, $p < 0.0001$) were significantly increased after the 2nd immunization. Regardless of DC frequencies and location, all DC subsets showed up-regulated expression of activation markers (CD40, CD83 and CD86) and a lymph node migration marker (CCR7). In addition, pro-inflammatory cytokines (IL-6 and TNF- α) and B cell-activating factor (BAFF) were produced by the activated DCs. There was no significant difference in DC activation between vaccine and control groups which suggested that the DCs quickly recognized the replicating adenovirus and increased activation marker expression and cytokine production at the mucosa and blood to promote further immune responses. Antigen-specific memory T cells were increased in both rectal mucosa and blood after immunizations. Rectal DCs and autologous naïve lymphocytes were co-cultured to determine the functional role of rectal DCs in inducing adaptive immune responses. The rectal pDCs and mDCs after the 2nd immunization were capable of inducing proliferation of naïve lymphocytes, especially CD8 T cells, and the vaccine group showed higher levels of T cell proliferation than controls after in vitro Env protein stimulation. Our results highlight the rapid response and potential roles of DCs in mucosal immune activation after replicating adenovirus immunizations and identify the initial cellular mechanisms of the replicating adenovirus vaccine regimen in the rhesus macaque model.

MINSUK KONG

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Microbiology and Antimicrobials

Targeted Drug Delivery to Cancer Cells Using Synthetic Bacteria

Systemic administration of anticancer drugs can cause indiscriminate drug distribution and severe toxic side effects. To reduce these shortcomings, targeted drug delivery systems have been considered an attractive option for cancer therapy. Here we developed a novel drug delivery system using synthetic bacterial spore-like particles that our lab recently developed. These particles, termed *SSHELs*, consist of 1 μ m-diameter mesoporous silica beads covered in a lipid bilayer, and encased by two spore coat proteins from the bacterium *Bacillus subtilis* that tether to the lipid bilayer and polymerize to form a stable shell around the silica bead. The proteinaceous surface of these particles were covalently decorated with $\sim 88,000$ copies/particle of affibodies directed towards human epidermal growth factor receptor 2 (HER2), which is overproduced in $\sim 25\%$ of diagnosed breast cancers. SSHELs displaying anti-HER2 affibodies (SSHEL_aHER2) displayed specific binding to HER2-overproducing breast and ovarian cancer cells. Next, we demonstrated that the porous interior of SSHELs can be loaded with $\sim 10^7$ molecules/particle of the chemotherapeutic drug doxorubicin (DOX). SSHEL_aHER2 particles loaded with DOX effectively and specifically induced apoptosis and reduced the viability of HER2-positive cancer

cells. Confocal microscopy showed that internalized SSHELsaHER2 were trafficked to acidic organelles, and an in vitro drug release assay revealed that acidic conditions (pH 5.0) triggered rapid release of encapsulated DOX from SSHELs. Taken together, we propose that SSHELs can be modified to target specific cell types and, once internalized into an acidic compartment, can release and deliver encased cargo to the target cell. Ongoing experiments in animal models are currently testing the efficacy of SSHELs as vehicles for targeted drug delivery to tumors in vivo.

Devikala Gurusamy

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Clinical and Translational Research, and Gene Therapy

Multi-phenotype CRISPR-Cas9 screens identify p38 kinase as a target for adoptive immunotherapies

Adoptive T cell immunotherapy (ACT) using autologous tumor-infiltrating lymphocytes (TIL) has led to complete durable regression of tumors in approximately 22% of patients with advanced metastatic melanoma. While several factors can contribute to the efficacy of ACT, a major limitation is the induction of a terminally differentiated phenotype and finite proliferative capacity of TIL during current ex vivo expansion protocols. Thus, there is significant interest in identifying T-cell intrinsic and extrinsic negative regulatory pathways that limit the fitness and ex vivo expansion of TIL. Preclinical cancer models of ACT have demonstrated that less differentiated memory T cells can mediate superior anti-tumor responses with improved T cell persistence. Therefore, there is also interest in limiting effector differentiation along with reducing oxidative stress and genomic damage. To identify the T cell-intrinsic negative regulatory pathways, we developed a multi-phenotypic genetic screen to systematically target 29 major kinases in preclinical T cell expansion model. We used the CRISPR-Cas9 technology coupled with high throughput flow cytometric analysis to evaluate cell expansion, T cell differentiation, oxidative stress, and DNA damage. Results from our genetic screen identified p38 kinase as a unique multi-phenotypic regulator that limited cellular differentiation, oxidative, and genomic stress while achieving improved cellular expansion. Furthermore, pharmacological inhibition of p38 kinase in murine ex vivo T cell expansion models validated the results from our genetic screen. Pharmacological inhibition of p38 kinase in human melanoma TIL ex vivo cultures improved their memory phenotypes and preserved their TCR clonality during expansion. Cells cultured in the presence of a p38 inhibitor had increased capacity for cytokine production, specifically interferon-gamma, and demonstrated improved in vivo persistence. Additionally, cells cultured in the presence of the p38 inhibitor demonstrated enhanced in vivo cell expansion, tumor infiltration, and anti-tumor efficacy in an immunocompetent tumor mouse model. Thus, using CRISPR-Cas9 technology we have identified p38 kinase to be a major regulator of multiple desired characteristics of therapeutic TIL and results from our screen has direct implications for current ACT therapies.

Deblina banerjee

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Epigenetics

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Marina Mahynski

Postdoctoral Fellow

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Biophysics

Liquid-liquid phase separation of mitochondrial nucleoids in a premature aging disease

Accumulation of mutations and damage to mitochondrial DNA (mtDNA) contributes to normal aging. Within the mitochondrial matrix, mtDNA is coated by proteins forming nucleoids, which serve as sites for mtDNA replication and transcription. Each nucleoid contains 1-2 molecules of mtDNA with a fixed size of typically ~100 nm. However, the biophysical properties of mitochondrial nucleoid organization and their potential contributions to aging are unknown. Here, we probe the phase separation of mtDNA nucleoids in a model of premature aging. We hypothesize that mtDNA nucleoids arise from liquid-liquid phase separation of nucleic acids and proteins, and that oxidative stress promotes nucleoid condensation in the premature aging disease Hutchinson-Gilford Progeria Syndrome (HGPS). Using several light microscopy techniques, we find that HGPS patient derived cells have a population of swollen and dysfunctional mitochondria. These damaged mitochondria contain enlarged spherical nucleoids with diameters greater than 300 nm. Moreover, in response to several forms of pharmacological disruption, mitochondria swell, while the nucleoids undergo rapid liquid-like homotypic fusion events with neighboring nucleoids. Nucleoid coalescence and coarsening are consistent with the phase behavior of liquid-liquid phase separation, which has recently emerged as a novel paradigm to describe the assembly of non-membrane bound organelles. Correspondingly, sequences of nucleoid associated proteins, such as TFAM and TFB2M, contain disordered domains and multivalent binding regions. Such modular domains are well known to drive phase separation. Structured Illumination Microscopy (SIM) reveals that these nucleoid associated proteins do not intermix homogeneously with mtDNA, but rather envelope the condensed mtDNA, forming a core-shell structure. This core-shell structure is analogous to the multiphase organization seen in other non-membrane bound organelles, including nucleoli and stress granules. The liquid-liquid phase separation of mitochondrial nucleoids provides a novel framework for describing their organization and assembly, with potential implications for normal aging.

Shaoli Das

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Informatics/Computational Biology

DiscoverSL: Multi-omic data driven prediction of synthetic lethality in cancers

Targeting cancer driver genes has been successful in some cases but not all tumors have target-able driver gene alterations. Synthetic lethality is a condition when simultaneous loss of two genes is lethal to a cancer cell while loss of the individual genes is not. It offers promising therapeutic strategies for selective treatment of cancers with loss of cancer susceptibility genes. In tumors, where the driver genes or oncogenes cannot be targeted, synthetic lethal interactions can potentially serve as a drug target in

the presence of mutation in the driver gene, as the mutant tumor cells are dependent upon the synthetic lethal interactions for their survival. We developed an algorithm to predict synthetic lethality in cancers from multi-omic cancer data from The Cancer Genome Atlas (TCGA). We implemented it in form of an R package "DiscoverSL" for prediction and visualization of cancer-specific synthetic lethality. Mutation, copy number alteration and gene expression data (TCGA) were combined to develop a multi-parametric Random Forest classifier trained with known synthetic lethal gene pairs. We performed drug prediction assessing the effects of selectively targeting the predicted synthetic lethal genes. The clinical outcome of cases with mutation in the primary gene, and the effect of inhibiting the SL partner was tested using Kaplan Meier curves, shRNA and drug screening data in cancer cell lines. The method helps identify and test the clinical outcomes for targeting novel synthetic lethal partners selectively in cases with mutation in the primary cancer susceptibility genes.

Luisa Magalhaes

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Immunology - Innate and Cell-mediated Host Defenses

Sustained levels of IFN-gamma during chronic infection drive global disruption of monocyte/macrophage homeostasis

Humans are exposed to numerous pathogens in their lifetime. The immune system needs to mount an effective response tailored to control the invading microbe and return to homeostasis after pathogen clearance. Monocytes and tissue macrophages play critical roles in host defense, tissue homeostasis, and repair. In mice, monocytes are divided into Ly6Chi that contribute to inflammation and pathogen control, and Ly6Clow that patrol the vasculature and participate in the resolution of inflammation. Ly6Chi monocytes give rise to Ly6Clow in blood and bone marrow (BM), however, the signals that regulate this differentiation remain unknown. Ly6Chi monocytes are rapidly recruited to sites of acute infection where IFN-gamma (IFN γ) drives their differentiation into inflammatory macrophages. The contribution of these cells needs to be transient and resident macrophages need to restore tissue homeostasis. We hypothesized that the immune adaptation needed to cope with a chronic infection has deleterious effects on monocyte/macrophage homeostasis. To address this, we used a model of chronic infection with *T. gondii* that induces sustained levels of IFN γ throughout the length of infection. We found that chronically infected mice showed a loss of Ly6Clow monocytes in blood and BM, and replacement of resident macrophage populations by Ly6Chi monocytes in peritoneum, lung and brain. Infection of mixed BM chimeras showed a loss of Ly6Clow WT but not IFN γ receptor deficient (IFN γ RKO) monocytes, demonstrating a cell-intrinsic requirement of IFN γ to block the conversion of Ly6Chi into Ly6Clow monocytes. This was confirmed by the loss of circulating and BM Ly6Clow monocytes and the disappearance of peritoneal macrophages in naïve WT but not IFN γ RKO mice injected with IFN γ . Importantly, chronically infected mice showed impaired neutrophil recruitment following Zymosan injection, indicating a blunted innate immunity after microbial challenge in these mice. Mechanistically, IFN γ inhibited the upregulation of CSF1 receptor, a critical survival factor for Ly6Clow but not for Ly6Chi monocytes. Blockade of IFN γ in chronically infected mice restored both the monocyte and peritoneal macrophage compartments. Our data reveal a central role for IFN γ in regulating the conversion of Ly6Chi into Ly6Clow monocytes, and it indicates that sustained levels of IFN γ during chronic infection

leads to global disruption of monocyte/macrophage homeostasis reshaping the innate immune landscape of the host.

Yuhong Luo

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Systems Biology

Intestinal c-Myc deficiency protects against obesity-related non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is becoming the most common chronic liver disorder in developed countries. Persistent NAFLD triggers an increased risk of non-alcoholic steatohepatitis (NASH) and end stage liver diseases such as cirrhosis and hepatocellular carcinoma. Pharmacologic therapy that targets NAFLD remains extremely limited. c-Myc is a basic helix-loop-helix (bHLH)-leucine zipper transcription factor with broad metabolic effects on cell proliferation, energy metabolism, angiogenesis, apoptosis, adhesion, and differentiation. Accumulating reports indicate that liver c-Myc has a role in obesity and hepatic steatosis via regulation of glucose and lipid metabolism. Interestingly, c-Myc expression in the intestine was significantly higher in obese mice compared to that of lean mice. However, to date, the role of intestinal c-Myc on NAFLD has not yet been examined. To address this, villin-ERT2-cre Myc fl/fl (Myc-dIE) mice were generated to achieve temporal, intestine-specific Myc disruption in the presence of tamoxifen which activates the ERT2-cre enzyme. Wild-type mice fed a 60% high-fat and tamoxifen mixed diet (HFD/TAM) had marked obesity and hepatic steatosis, while Myc-dIE mice on a HFD/TAM showed less body weight gain and hepatic steatosis. The results from glucose tolerance test (GTT) and insulin tolerance test (ITT) suggested improved glucose tolerance and insulin sensitivity in HFD/TAM-fed Myc-dIE mice compared to wild-type mice fed HFD/TAM. Myc-dIE mice fed a 60% HFD for 8 weeks and then change to HFD/TAM for another 8 weeks showed lower body weight, less hepatic steatosis and better glucose/insulin tolerance compared to similarly-treated wild-type mice, indicating that intestinal c-Myc disruption improves metabolic disease in mice that had already developed NAFLD. Amelioration of these HFD-induced adverse metabolic phenotypes was correlated with reduced expression of lipid synthesis and transport related genes in the intestine and liver. Collectively, this study found that intestinal c-Myc deficiency protected against obesity related NAFLD, and thus may be a druggable target for the treatment of NAFLD.

Navdeep Malik

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Carcinogenesis

A novel regulatory mechanism of CBFβ in protein translation and its implications in precision medicine of breast cancer.

The success of precision medicine of oncology requires implementing new algorithms and detailed understanding of genetic alterations in each cancer type. Recent genome-wide sequencing studies in various cancers identified many under-appreciated genetic alterations. It remains challenging to prioritize and determine the functions of these genetic alterations. To increase the probability of finding functionally important cancer genes, we developed a new p53 Mutual Exclusivity Algorithm (PMEA)

which tests the mutual exclusivity of p53 mutations and the mutations of a candidate gene. Since p53 is a well-known tumor suppressor, we reasoned that if the mutations of a gene are mutually exclusive to p53 mutations, this gene may functionally connect to the p53 signaling pathway and likely act a driver gene in cancer. As a proof-of-concept, we applied PMEA to two breast cancer datasets: TCGA and METABRIC and identified several genes, such as GATA3, PIK3CA, MAP3K and CBFβ, whose mutations are significantly mutually exclusive to p53 mutations. We chose to study the roles of breast tumor-derived CBFβ mutants as a model for precision medicine because its role in breast cancer is under-studied. CBFβ is a part of transcription factor complex where it interacts with its partner RUNX1 to stabilize its interaction with DNA to regulate transcription. We observed that in breast cancer cells and human tumor samples instead of being a part of transcription factor complex CBFβ mostly localizes in cytoplasm while its partner RUNX1 is exclusively present in nucleus. CRISPR-Cas9 based knockout of CBFβ in non-tumorigenic MCF10A induced cell transformation and in vivo tumorigenesis. Interestingly, loss of CBFβ resulted in concomitant loss of RUNX1 protein indicating CBFβ is regulating RUNX1. We discovered a surprising cytoplasmic function of CBFβ, in promoting the translation of RUNX1. Mechanistically, CBFβ is recruited to the 3′ untranslated region of RUNX1 mRNA by HnRNPK (known mRNA processor). CBFβ and HnRNPK co-operatively regulate translation of RUNX1. Genomic studies reveal that the CBFβ/RUNX1 axis and p53 co-induce p73, therefore providing a rationale to the mutual exclusivity of CBFβ and p53 mutations. Further, CBFβ/RUNX1 axis represses the oncogenic NOTCH3 signaling pathway to suppress breast cancer. Our results establish an unanticipated cytoplasmic role of CBFβ and this mechanism may be therapeutically exploited as precision medicine for patients bearing CBFβ mutations.

Saghar Nourian

Postdoctoral Fellow

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Chemistry

Site-Specific Antibody Conjugation for Targeted Drug Delivery and In Vivo Imaging

Antibody targeted therapeutic and imaging approaches are rapidly progressing technologies with broad translational potential. Antibody labeling is a challenging chemical problem due to the large number of potential conjugation sites. Existing methods have used non-selective lysine labeling which results in a broad distribution of labeled products. The heterogeneous conjugation of the small molecules with antibodies can compete for antigen binding and, additionally, can lead to conjugates with suboptimal in vivo properties. Specifically, our recent studies have shown that modification of the intact therapeutics antibodies (particularly the anti-EGFR IgG panitumumab) with cyanine fluorophores and drug delivery agents can impair in vivo properties, resulting in significant uptake by the endorecticular system in the liver. We are pursuing the hypothesis that well-defined site specific conjugation can dramatically improve in vivo properties that can lead to dramatic improvement of tumor uptake; a critical parameter for both imaging and treatment approaches. Previous studies on antibody-drug conjugates have displayed this point, but homogenous conjugates with complex payloads had not been prepared prior to our studies. We have examined different site-specific antibody conjugation procedures to generate homogenous conjugated antibodies that can release the cytotoxic drugs in vivo. One efficient antibody conjugation strategy involves partial reduction of antibody interchain disulfide bonds to generate thiol groups. Subsequently, the conjugation with bis-sulfone linkers through a cysteine addition, sulfone elimination, and second cysteine addition mechanism will form a three-atom disulfide bridge that can

maintain the stability of the antibody within in vivo cancer treatment. Applying this strategy, we have conjugated different antibodies with payloads containing bis-sulfone linkers in high yields and have confirmed the excellent efficiency for in vitro studies. Also, we have developed bis-sulfone linkers containing cyanine-duocarmycin moieties and performed the homogenous conjugation of these payloads with antibodies for targeted release of duocarmycin. Ongoing studies are examining these molecules in MDA-MB-468 xenograft studies for both in vivo imaging and treatment. These studies to generate homogenous small molecule antibody conjugations will enhance tumor specificity and cellular localization with the applications in both cancer treatment and imaging.

Limin Wang

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Carcinogenesis

Clusterin Regulates a Highly Aggressive Subgroup of Pancreatic Cancer

Pancreatic cancer is one of the most lethal malignancies and is ranked as 4th leading cause of death due to cancer in the United States. Alarmingly, a consistent rise in incidence and death in pancreatic cancer is estimated to make it the second leading cause of cancer-related death by 2030. Similar to many other cancer, pancreatic ductal adenocarcinomas (PDAC) are highly heterogeneous and the underlying oncogenic mechanisms are poorly understood. We hypothesized that specific molecular signatures stratify PDAC subtype with distinct prognosis. To test this hypothesis, global gene expression profile from 142 tumor tissue samples was performed and three molecular subtypes (G1, G2 and G3) were identified by non-negative matrix factorization (NMF) analysis with consensus clustering. Class comparison between each subtype generated subtype specific gene signature (G1: 148 genes; G2: 217 genes; G3: 123 genes; FC>2, FDR<0.001), which was validated in publicly available datasets (GSE16515, n=37; GSE15471, n=39; TCGA, n=162). Kaplan Meier survival analysis showed differential overall survival (p=0.033) between high (G1, N=67) and low risk (G2, N=27) subtype in test cohort and was validated in TCGA cohort (p=0.013). Hierarchical clustering of 148 highly aggressive G1-subtype gene-signature predicted prognosis in patients in test (p=0.022, N=136) as well as validation (TCGA) cohort (p=0.008, N=162). Furthermore, pathway analysis of 148 highly aggressive gene-signature identified cellular movement, cell invasion, cell death and growth as the top molecular network associated with high-risk G1-subtype. Among the top network genes, a higher expression of MET and ANLN was associated with poor survival, while a lower expression of Clusterin (CLU) correlated with poor survival, both in the test and validation cohort. Functionally, CLU, a previously undescribed gene in PDAC, inhibited proliferation, migration, invasion and 3D growth in pancreatic cancer cell lines. Our finding showed that CLU is a novel potential negative regulator of disease progression in a highly aggressive subgroup, which may be a candidate target for designing improved treatment strategy in PDAC.

Ruhul Amin

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Tumor Biology and Metastasis

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Abstract removed at request of author

Amelia Parker

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Carcinogenesis

Defining The Functional Importance of Creatine Dysregulation in Early Stage Lung Cancer

One third of patients diagnosed with early stage non-small cell lung cancer (NSCLC) will experience disease recurrence, contributing to the dismal survival rates of this disease. The distinguishing features of this high risk population have not yet been defined. Creatine riboside is a novel metabolite that is associated with risk and prognosis in early stage NSCLC, where it is enriched within tumor tissue. However, the mechanisms underlying altered creatine metabolism and creatine riboside production in lung tumor formation and progression remain unknown. By integrating tumor gene expression data (RNAseq, n=55) with metabolomics analysis of corresponding urine and tissue samples from the NCI-MD case control study of early stage lung cancer patients we will define a gene expression signature associated with altered creatine metabolism and creatine riboside production. We will combine this with imaging mass spectrometry and immunohistochemistry for microenvironmental features (e.g HIF1a) to spatially correlate altered creatine metabolism with features of the tumor microenvironment. Together, the integration of genomic, metabolomic and microenvironmental data will enable us to define the clinical context that promotes creatine riboside production. A comparison of NSCLC cell lines with low and high endogenous levels of creatine riboside have revealed that high creatine riboside levels are associated with high rates of oxidative phosphorylation (Seahorse XFAlyser). Furthermore, cells with high endogenous creatine riboside levels have significantly higher levels of ornithine and are highly dependent on arginine for cellular growth, a metabolic vulnerability that can be ameliorated by supplementation with citrulline. This indicates that high creatine riboside levels are associated with a deficiency in the mitochondrial component of the urea cycle. Current efforts are utilizing RNAi and metabolic flux analysis with mass spectrometry to define the importance of urea cycle dysfunction in promoting the production of creatine riboside and the contribution of these metabolic pathways to lung cancer aggressiveness in cell lines and epithelial cells. These studies will provide critical insight into the importance of dysregulated creatine metabolism in promoting transformation and tumor aggressiveness in NSCLC.

Yue Qi

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Tumor Biology and Metastasis

Cross-talk of lysine acetylome and tyrosine phosphoproteome unveils abnormal cell signaling of acquired resistance to EGFR inhibitors in lung adenocarcinoma

Epidermal growth factor receptor (EGFR) mutations in non-small cell lung cancer (NSCLC) are associated with sensitivity to EGFR tyrosine kinase inhibitors (TKIs). Unfortunately, all patients eventually develop resistance. Osimertinib, a third generation EGFR TKI, has been approved to treat patients who have

developed resistance to first and second generation TKIs and harbor the EGFR T790M resistance mutation. However, the potential mechanisms of osimertinib resistance have not been fully understood. Here, we developed an osimertinib-resistant lung adenocarcinoma cell line, PC-9-AZR-NCI-1 from its EGFR mutant parental cell line, PC9, found to be sensitive to osimertinib. The osimertinib-sensitive and -resistant cell lines were labeled with stable isotope labeling with amino acids in cell culture (SILAC). The cells were digested with trypsin, the resulting peptides were first immunoprecipitated (IP) with anti-acetyl Lysine (Kac) agarose and subsequently, a second enrichment of pTyr was conducted using the supernatant from previous Kac IP. The antibody enriched Kac or pTyr peptides were acid eluted, and were subjected to high resolution tandem mass spectrometry. We identified 1364 Kac Sites in 817 proteins. 31 histone proteins that were acetylated and 15 of 31 were of the H2A family. The tumor suppressor, TP53 showed K357/K305/K120 Kac sites. Acetylated protein kinases identified include CDK1 (K6, K33) and PLK1(K9). KEGG pathway analysis indicated that 19 acetylated proteins are involved in MAPK signaling, 17 in glycolysis, and 20 in cell cycle. We identified around 1400 pTyr sites in 810 proteins of which 11 sites are on EGFR. Also, out of 110 protein kinases (230 sites) identified, more than 43% of them (48) were tyrosine kinases, such as EGFR, JAK2 and SRC, which implied a high affinity of the pTyr antibody for the tyrosine kinases. KEGG pathway analysis showed that 10 proteins are associated with NSCLC (e.g., MAPK1, GRB2, PLCG1), and 30 are involved in ErbB signaling. More importantly, 136 proteins were found to be modified by both Kac and pTyr PTM, which may synergistically or differentially regulate biological processes, such as cell-cell adhesion (34 proteins), protein folding (11) and gene expression (11). Next, we plan to perform a comprehensive PTM profile (Kac and pTyr) of human tumor biopsies to identify abnormal cell signaling that may contribute to the molecular mechanisms of acquired resistance to TKIs.

Rushikesh Sable

Visiting Fellow

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Tumor Biology and Metastasis

Precision targeting of M2-like macrophages by the innate defense regulator RP-182 in malignant and non-cancerous diseases

Tumor-associated macrophages (TAMs) are a major cell population of the tumor microenvironment and play a key role in promoting tumor progression in many solid organ cancers. During cancer progression, tumoral and microenvironmental cues educate TAMs towards a M2-phenotype which nurtures cancer stem cells, accelerates metastasis, and confers resistance to chemotherapy. Current drug development efforts targeting TAMs primarily focus on unselective systemic inhibition of macrophage recruitment to disease sites or modulation of their immuno-cytokine profile. RP-182, a 10mer striapathic peptide with immunomodulatory function which was derived from a biophysical homology screen of carbohydrate recognition domain patterns and naturally occurring host defense peptides (HDP) and innate defense regulators (IDR). We demonstrate that RP-182 specifically and effectively binds to the mannose receptor MRC1/CD206 expressed on M2-like macrophages, induces a conformational switch of the receptor, and activates in human and murine M2-like macrophages a program of phagocytosis, autophagy, and apoptosis. In genetically engineered murine models of pancreas cancer, RP-182 suppressed tumor growth, extended survival, and improved anti-tumor immunity, findings also seen in other syngeneic cancer models. RP-182 enhanced the effects of chemo and immune checkpoint therapy. In conclusion,

the RP-182 peptide depletes the immune evasive M2 population of TAMs via engagement with the M2-specific lectin receptor CD206. This is associated with improved clinical outcome in transgenic mice with pancreas cancer. Similarly, RP-182 selectively reduces immune suppressive M2-like macrophages, a finding also made in a non-cancerous murine fibrosis model. Collectively, preclinical findings show that HDPs/IDRs derived from biophysical homology studies effectively reduce innate immune cell subpopulations with therapeutic merit in cancerous and non-malignant diseases.

Ching-Wen Chang

Postdoctoral Fellow

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Tumor Biology and Metastasis

Characterization and Identification of the molecular mechanism of metabolic disorder and liver cancer in hepatocyte specific Dnaja3 knockout mice

Hepatocellular carcinoma (HCC) is sixth most common malignant cancer worldwide. The overall 5-year survival rate in patients with HCC is one of the lowest among common cancer. Therefore, identification of the rational therapeutic regimens to provide a fundamental basis for developing therapeutic candidates to treat the liver disorder and HCC is important. DNAJA3 functions as a tumor suppressor in different types of solid tumors. Consistently, we found that the expression of DNAJA3 transcript is significantly reduced in HCC compared to normal hepatocytes. We also found that DNAJA3 is hemizygotously deleted in a subset of HCC cases with poor prognosis. These findings imply that DNAJA3 may serve as a tumor suppressor during HCC tumorigenesis. To further determine the role of DNAJA3 in hepatocarcinogenesis, we generated Alb-Cre-Dnaja3f/f (Alb: albumin) mice where DNAJA3 was specifically deleted in hepatocytes. We also used Diethylnitrosamine (DEN) to induce HCC in this mouse model. We found that Alb-Dnaja3f/f mice had a retarded growth and showed ballooned hepatocytes and fatty liver during the first two months. At 13 month of age, Alb-Dnaja3f/f mice showed spontaneous tumor development in the liver. In DEN-induced HCC model, the size of the tumors was also significantly increased in the livers of Alb-Dnaja3f/f mice compared to those of control mice. Deletion of DNAJA3 resulted in the mitochondrial unfolded protein response (UPR_{mt}), which is well known to play the key role in tumorigenesis. Overall, the progression of steatosis, inflammation, fibrosis to HCC was observed in our Alb-Dnaja3f/f mice with/without chronic factors involving in HCC, which similarly reflects those observed in tumorigenesis of human HCC. Collectively, our results indicate that deletion of DNAJA3 gene can cause abnormal liver development and tumorigenesis through a mitochondria dysfunction related response. The importance of understanding the role of DNAJA3 in hepatocyte development and tumorigenesis warrants further investigation.

David Calabrese

Postdoctoral Fellow

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Biophysics

Chemical and Structural Studies Provide a Mechanistic Basis for Selective Recognition of the MYC G-Quadruplex

Developing small molecules that bind to and alter the function of regulatory nucleic acid sequences is

particularly attractive when they govern the expression of so-called “undruggable” proteins, such as MYC. The MYC gene encodes for the transcription factor MYC, which regulates a large number of genes in the human genome and has been linked to proliferation, differentiation, apoptosis, and oncogenesis. Importantly, MYC is upregulated in 70% of all cancers and is linked to ~100,000 deaths per year. An attractive alternative route is the prevention of MYC expression via small molecule-mediated stabilization of the G-quadruplex (G4), a non-B DNA structure, present within its promoter region. However, a major barrier in developing biologically active small molecules that bind to nucleic acids has been the identification of selective interactions. Understanding the chemical and structural basis for nucleic acid-small molecule interactions will greatly improve our ability to rationally design selective, high affinity small molecules and further explore nucleic acid-binding compounds as mechanistically novel therapeutics. We report the discovery of a new, drug-like compound with dramatic effects on MYC expression in multiple myeloma cells, demonstrate that it acts by a G4-dependent mechanism of action, and solve a complete structure by NMR of the compound in complex with the MYC G4. We synthesized a focused library of analogs, which were evaluated for affinity, ability to silence MYC, and limit cell growth in a MYC-driven multiple myeloma cell line. The most potent analog (DC-34) inhibits MYC at the transcriptional level only when a G4 is present in the promoter. Importantly, DC-34 does not transcriptionally downregulate several other G4-dependent genes to the same extent. We synthesized an isotopically labeled DC-34, which was used to solve the NMR structure of DC-34 in complex with the G4. DC-34 adopts a three-dimensional conformation that enables specific contacts with the G4 that govern selectivity and biological activity. Insights gained from this structure and the corresponding chemical derivatives provide a basis for the selective recognition of the MYC G4 and have implications for the development of selective nucleic acid-binding compounds with biological activity. Our work demonstrates that drug-like compounds can discriminate between different G4s by making discrete interactions.

Bhagawat Chandrasekar Subramanian

Visiting Fellow

NCI-CCR

Immunology - Innate and Cell-mediated Host Defenses

A novel role for auto/para-crine Leukotriene B4 signaling in neutrophil adhesion and extravasation in vivo

Leukotriene B4 (LTB4), an eicosanoid inflammatory mediator, promotes recruitment of immune cells to the inflammatory site in concert with the action of other induced chemoattractants such as C5a, CXCL1, and CXCL2. Recent studies have demonstrated that LTB4 produced by neutrophils relays chemotactic signals through LTB4 receptor 1 (BLT1) to drive directed migration to primary chemoattractants in vitro and in vivo. Here, we discovered a novel role for the LTB4-BLT1 axis during neutrophil adhesion and extravasation, which precede interstitial migration. We found that self-generated LTB4 is required for the sustained adhesion of human primary neutrophils (PMNs) to the Beta2-integrin substrate fibrinogen in vitro, as inhibition of BLT1 in response to stimulation with primary chemoattractants resulted in the loss of persistent adhesion of PMNs. Importantly, inhibition of BLT1 or its downstream target Myosin II, enhanced the clathrin-independent endocytosis of Beta2-integrin from the trailing edge of the PMNs, thus dampening inside-out integrin signaling in response to chemoattractant stimulation. To directly assess the contribution of self-generated LTB4 to neutrophil extravasation in live animals, we performed

intravital 2-photon microscopy to analyze intravascular neutrophil behavior in the mouse hind foot-pad treated with a heat-killed chemically-fixed E. coli K12 strain. In Alox5^{-/-} mice, that are incapable of producing LTB₄, Alox5^{-/-} neutrophils failed to adhere to the blood vessel surface and extravasate, whereas wild-type neutrophils (capable of producing LTB₄) adhered and extravasated in response to E. coli-induced inflammation. In addition, neutrophils lacking either Beta2-integrin (Itgb2^{-/-}) or BLT1 (Blt1^{-/-}), or neutrophils in which Myosin II activation was pharmacologically impaired, failed to adhere to the blood vessel surface and extravasate. In conclusion, auto/para-crine sensing of self-generated LTB₄ drives neutrophils to adhere to blood vessel surface and extravasate in a Beta2-integrin and actomyosin-dependent manner in vivo. Therefore, the self-generated LTB₄-BLT1 axis acts as a signal-relay mechanism, which we envision may play a fundamental role in neutrophil extravasation response to multiple inflammatory conditions, including cancer, where neutrophils and LTB₄ are known contributors to metastatic spread of tumors.

Tomoki Yagai

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Biochemistry - General, Proteins, and Lipids

A novel role for Let-7 microRNAs in the control of hepatic lipid metabolism

Peroxisome proliferator-activated receptor alpha (PPARA) is a ligand-dependent nuclear receptor that controls genes involved in lipid transport and metabolism. PPARA mediates the mammalian fasting response, and is the target of human hyperlipidemic fibrate drugs. To comprehensively elucidate the PPARA regulatory networks, transcriptome analyses was performed in PPARA-activated mouse liver revealing that PPARA activation markedly repressed the expression of Let-7 microRNAs (miRNA). Let-7 is a microRNA family that has nine family members including Let-7a, b, c, d, e, f, g, i and miR-98 in humans and mice. Interestingly, PPARA activation repressed all Let-7 family members. To examine the mechanism of PPARA repression of Let-7 miRNAs, transcriptional activity of the Let-7a, d and f (Let-7adf) cluster was analyzed. Genome mapping analysis of P300 and PPARA CHIP-seq data sets revealed that the Let-7adf cluster promoter/enhancer (L7adf PE) had a PPARA binding element. This region was cloned and subjected to luciferase reporter gene analysis revealing that L7adf PE had potent promoter transcriptional activity that was repressed by PPARA. Furthermore, L7adf expression was increased in PPARA-knockout mouse hepatocytes. These data indicated that PPARA represses Let-7adf cluster transcription via direct binding to the L7adf PE. To elucidate roles of Let-7 miRNAs in liver, Let-7 miRNA target mRNAs were analyzed. Target mRNAs were predicted by sequence analysis in silico and RNA-seq data in PPARA-activated mouse liver. 3'UTR regions of the predicted Let-7 target mRNAs were cloned and subjected to 3'UTR reporter assays. These assays identified ATP binding cassette subfamily B member 9 (Abcb9), adiponectin receptor 2 (Adipor2), UDP-galactose-4-epimerase (Gale) and peroxisome proliferator-activated receptor gamma coactivator 1-beta (PGC1b) as novel direct target mRNAs of Let-7. Notably, Abcb9, Adipor2 and Gale encode key proteins involved in lipid metabolism, and PGC1b is a co-activator of PPARA. To analyze physiological effects of Let-7 miRNA, Let-7c was overexpressed in mouse primary hepatocytes revealing that expression of mRNAs and proteins involved in lipid metabolism were repressed, resulting in decreased triglyceride accumulation. Collectively, these results provide evidence that PPARA interferes with transcription of Let-7a, d and f miRNA by

transrepression, and that Let-7 miRNAs influences lipid metabolism by repression of PPARA target genes and a co-activator.

James Dunleavy

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Tumor Biology and Metastasis

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Gwen Buel

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Protein Structure/Structural Biology

Defining the isoform-specific mechanism of interaction between Myosin VI and Clathrin

Myosin VI (Myo6) is a motor protein that binds actin through its N-terminus and cargo through a C-terminal domain. While other members of the Myosin family move toward the plus end of actin filaments, Myo6 uniquely processes toward the minus end, enabling Myo6 at the cell cortex to transport cargo inward from the periphery. In previous work, we found that isoforms of Myo6 that lack an insert near the C-terminus contribute to cancer cell migration, whereas those that contain this insert do not display this phenotype, and rather, have increased binding efficiency to numerous proteins in the endocytic pathway including the endocytosis mediator Clathrin. In order to elucidate the different roles of the Myo6 isoforms and to study our hypothesis that specific isoforms of Myosin VI play a role in Clathrin-mediated endocytosis, we set out to determine the mechanism of binding between Myosin VI and Clathrin. Through biochemical means, it was determined that the region of Clathrin that binds to Myo6 is a segment of Clathrin light chain alpha (CLCa). Using NMR, we measured the chemical shifts of a ¹⁵N-labeled segment of Myo6 Isoform 3 with and without a CLCa-derived peptide. Analyzing which Myo6 chemical shifts changed most upon addition of the CLCa peptide provided a prediction of the Myo6 residues involved in binding. Subsequently, we used Nuclear Overhauser Effect Spectroscopy (NOESY) NMR experiments to obtain over 2500 distance constraints that we used with other NMR-derived data to calculate the structure of Myo6 1050-1131 in complex with CLCa 46-61. We found that Myo6 in this complex forms three alpha-helices and a long unstructured linker, similar to what was observed for unbound Myo6. The most N-terminal of these helices, spanning residues 1055-1068, corresponds to the isoform specific insert that was shown previously to be necessary for strong binding to CLCa. We found that CLCa forms an alpha-helix which nestles between the helices of Myo6. The isoform-specific helix of Myo6 makes several contacts to CLCa, specifically through Pro1055, Met1058, and Met1062. These results provide an explanation as to how the isoform-specific helix of Myo6 can act in the regulation of cargo binding. While the shorter isoform of Myo6 is selectively expressed in cancer cells and is involved in a migratory phenotype, it appears that the insert found in the longer isoforms shifts Myo6 away from these functions, allowing it to function with proteins involved in endocytosis.

Lulu Yu

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Carcinogenesis

Integration of mouse papillomavirus type 1 (MmuPV1) often occurs in benign tumor

Human papillomavirus (HPV) genome integration into host DNA has been recognized as a major promoting step in HPV-related carcinogenesis. However, whether its integration in the host genome also contributes to the development of benign tumor remains unclear. Mouse papillomavirus type 1 (MmuPV1) is the first rodent papillomavirus that can infect the laboratory strain of mice and thus MmuPV1 infection provides a unique model for studying papillomavirus integration in vivo. We recently performed bioinformatics analysis of total RNA-seq from 9 MmuPV1-infected tissues bearing virus-induced tumors (warts) and 3 tumor-free infected tissues. The integration is determined by identification of chimeric sequence reads mapped to both viral and host genomes. Using this approach, we identified much higher percentage (3.5%-14.1%) of virus-host chimeric reads in warts-bearing samples than that (0.0-2.1%) in tumor-free tissues. The majority of chimeric reads with the virus half were mapped to the E2/E4 region of the MmuPV1 genome which represents a main virus breakpoint region. The disruption of HPV genome in corresponding region was associated with cervical cancer leading to subsequent up regulation of E6/E7 oncogene expression. We correspondingly mapped other half of the chimeric reads to the host genome as the integration site. Although the mapped integration sites are randomly distributed throughout the whole genome, several hot spots were identified in Rn45s, MALAT1, Rps29, Krt10, CDK8 gene loci. To overcome the limit of short sequence reads generated by RNA-seq, we performed 5' rapid amplification of cDNA ends (5' RACE) and 3' RACE on these tissue RNAs. The virus-host chimeric full-length cDNAs derived from both 5' and 3' RACEs were then identified using single-molecule real time sequencing (SMRT or Iso-seq) technology. The results from SMRT analyses confirmed majority of the hotspot genes identified by RNA-seq and additionally discovered several new hot integration sites not identified by RNA-seq. In conclusion, our study provides the first evidence of more papillomavirus genome integration in wart tissues than tumor-free, papillomavirus-infected tissues. We conclude that virus integration occurs even in the benign tumor and is a risk factor for benign tumor formation.

Mamatha Garige

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Metabolomics/Proteomics

Targeting Metabolic Vulnerabilities in Bladder Cancer using Novel Therapeutic Strategies

Chemotherapy and cystectomy have been the main-line therapy for bladder cancer for decades, and relatively little progress has been made towards better targeted bladder cancer therapies. Perturbed metabolic pathways in tumors constitute a potential target for new therapeutic strategies. Many bladder cancer cell lines exhibit the "Warburg Effect", in that they show increased dependence on glycolysis as compared to normal cells. In this study one of the altered metabolic pathways, lactate dehydrogenase A (LDHA), was targeted by a new proprietary LDHA inhibitor to understand its role in bladder cancer both in vitro and in vivo. For the first time, we bioenergetically profiled fifteen bladder cancer cell lines based on their metabolic parameters extra cellular rate (ECAR) and oxygen consumption

rate (OCR) by extracellular flux analyzer. Our analysis revealed that glycolytic bladder cancer cells were most sensitive to LDHA inhibitor; bladder cancer cell lines that depended more on oxidative phosphorylation than glycolysis were resistant to the LDHA inhibitor. We were further able to render the LDHA-resistant cell lines sensitive to LDHA inhibition under hypoxic conditions and by treating them with metformin (inhibitors of oxidative phosphorylation). Inhibition of LDHA reduced the secretion of lactate in to the growth medium and affected global metabolism in the bladder cancer cell lines. Stable isotope tracer experiments in-vitro and in-vivo by NMR and MRI respectively demonstrated a decrease in lactate generation indicating on-target effect of LDHA inhibitor. LDHA inhibitor increased apoptosis and decreased invasion and this effect was synergized with metformin. Our initial proteome profiling revealed that LDHA inhibitor alone and in combination with metformin decreased the phosphorylation of AKT-mTOR-S6K signaling pathway indicating that the molecular mechanism of LDHA inhibitor could be through modulation of this pathway. Preclinical studies of xenograft models demonstrated that inhibition of LDHA decreased progression of tumor growth in vivo. Metabolic profiling and LDHA inhibitor sensitivity may prove valuable in treating bladder cancer subtypes and identifying therapeutic susceptibilities in bladder cancer patients. Our results indicate that glycolytic tumors could be sensitive to LDHA inhibitor and a combination of LDHA inhibitor with metformin may have greater therapeutic benefit in LDHA resistant tumors.

Michael Luciano

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Chemistry

Bright, Stable, Heptamethine Cyanine Fluorophores for in vivo Cancer Imaging

Fluorescence-guided surgical (FGS) interventions enable the real-time differentiation of benign and tumor tissue. The development of near-infrared region (NIR) fluorophores with favorable properties is essential to realizing the potential of this field. The commercially available molecule IR-Dye800CW is frequently used in preclinical and clinical studies for FGS and is the NIR fluorophore of choice for a variety of other biological applications. However, some major limitations preclude its progression to FDA approval. Our recent studies have shown IR-Dye800CW is unstable to intracellular thiols and the cellular proteome - complicating its use as a contrast agent for in vivo imaging. Additionally, monoclonal antibody (mAb) conjugates of IR-Dye800CW for in vivo cancer imaging suffer from high liver uptake and diminished brightness. Finally, this molecule is challenging to prepare due to its unsymmetrical nature. We have developed a novel class of NIR fluorescent heptamethine cyanine dyes to address the limitations of IR-Dye800CW. These dyes bear a central C4-O-alkyl linker rather than the C4-O-phenyl linker in IR-Dye800CW, making them more stable to biological nucleophiles. Importantly, they can be prepared using a simple, scalable symmetric synthesis. We have prepared novel molecules that are zwitterionic in nature with an even charge distribution. These subtle changes to the substituents of the cyanine core achieve enhanced brightness of the corresponding fluorophore-mAb conjugates. Both panitumumab and trastuzumab conjugates exhibit negligible aggregation and increased emission at higher degree of labeling compared with IR-Dye800CW. Moreover, these dyes are exclusively cleared through the kidney, with negligible liver uptake in mouse, rat and pig models. These properties allow easy visualization of the ureter during abdominal FGS in a rat model. Current studies are examining additional in vivo applications of these molecules including dual-color imaging for simultaneous

visualization of the ureter and bile duct during FGS procedures using two dyes with different emission wavelengths. In addition, we are also pursuing the development of novel bioconjugatable derivatives for site-specific antibody conjugation.

Assaf Magen

Visiting Fellow

NCI-CCR

Immunology - General

Dissecting antigen-specific tumor infiltrating lymphocyte diversity by single-cell transcriptome analysis

Cancers trigger complex immune responses, which are typically neutralized and result in non-functional (exhausted) lymphocytes. Although studies have highlighted associations of specific tumor-infiltrating lymphocyte (TIL) subtypes with distinct clinical outcomes, the heterogeneity of TILs has hampered unbiased analyses of T cell responses to tumors. Furthermore, how tumor-reactive T cells are activated and directed to specific functional fates is poorly understood. Here, we use new experimental strategies to study tumor-antigen-specific CD4 T cells, combining (i) tracking of CD4 T cells specific to a defined recombinant tumor antigen, both in the tumor microenvironment and draining lymph node, (ii) genome-wide mRNA sequencing at the single-cell level (scRNAseq) and (iii) scRNAseq analyses using a novel computational approach to improve the robustness, interpretability, confounder controls, and false-positive assessment. Initial results reveal novel subpopulations of TILs expressing regulatory, memory, effector, and exhaustion programs, which we are currently characterizing. With the exception of a Treg cell subpopulation, we find the CD4 T cell populations in the lymph node to be largely different from the ones found in the tumor. High resolution single cell tracking of gene expression during immune response to tumor development opens new possibilities to tailor immunotherapy strategies to patient-specific lymphocyte composition.

Ana Dios Esponera

Visiting Fellow

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Cell Biology - Intracellular Trafficking, Cytoskeleton, and Extracellular Matrix

PAK1 kinase promotes activated CD4 T cell trafficking by regulating the shedding of CD62L

T cell trafficking is essential for the function of the adaptive immune system. Naïve T cells continually traffic from blood vessels to secondary lymphoid organs where they are activated by cognate antigens, proliferate and migrate to sites of infection. This multi-step process requires an extensive remodeling of cellular membranes, which is mediated by the constant re-arrangement of the actin cytoskeleton. Rearrangement of actin is induced rapidly by extracellular stimuli, including chemokines and via the T cell antigen receptor. The chemokine receptor CCR7, is critical in guiding the migration of naïve and memory T cells across the high endothelial venules and endothelium into lymphoid tissues. P21-activated kinases (PAKs), play a central role in cell motility by remodeling the cytoskeleton. To fully understand the role of Pak1 in T cell migration, we used a conditional Pak1 knock-out mouse, in which Pak1 is deleted at an early stage of T cell development. Our results show enhanced chemotaxis to CCR7 ligands in Pak1 KO T cells compared with WT. This enhanced chemotaxis is produced by an increase in actin dynamics, due to an increase in cofilin activity in response to CCL21 and CCL19. Moreover, Pak1 is

required for trafficking of activated T cells into lymph nodes. Deletion of Pak1 also resulted in downregulation of CD62L expression in activated T cells. CD62L is involved in lymphocyte recruitment to peripheral lymph nodes and sites of inflammation by regulating rolling during transendothelial migration. The expression of CD62L at the membrane is controlled by a balance of two activities: gene transcription and proteolytic cleavage. Levels of mRNA in WT and Pak1 KO T cells were similar, indicating that Pak1 is not implicated in CD62L transcription. However, we found an increase in CD62L in the supernatants of activated Pak1 KO T cells compared with WT, suggesting that Pak1 is regulating CD62L shedding. Constitutive binding of calmodulin to the cytoplasmic tail of CD62L prevents its shedding. The local increase in calcium concentration after T cells activation liberates calmodulin from CD62L allowing its cleavage and loss. In the absence of Pak1, CCL21 produces an increase in calcium flux in T cells. Co-immunoprecipitation experiments revealed that calmodulin binds less to CD62L in activated Pak1 KO T cells. Overall, Pak1 mediates activated T cell trafficking by promoting the rolling step of transendothelial migration in a CD62L-dependent manner.

Yasuyuki Kanke

Other

NCI-CCR

Metabolomics/Proteomics

Diagnostic and prognostic utility of urinary creatine riboside for early stage non-small cell lung cancer

Lung cancer is the leading cause of cancer related death worldwide. The recommended treatment for stage I non-small cell lung cancer (NSCLC) patients is tumor resection, which may be followed by chemotherapy in patients with pathologically high-risk tumors. Still, up to 30% surgically treated stage I patients experience recurrence leading to death. Therefore, biomarkers that molecularly categorize stage I patients after tumor resection and stratify high-risk patients who may benefit from adjuvant chemotherapy would lead to improved clinical management. We previously conducted metabolomic profiling of urines collected from 469 NSCLC patients and 536 population controls using ultraperformance liquid chromatography coupled to mass spectrometry (UPLC-MS) and found creatine riboside (CR), a novel metabolite identified by the study, is significantly elevated in stage I and II NSCLC patients compared to controls. We also found CR levels are 19-fold higher in tumor tissues compared to adjacent normal lung tissues ($P < 0.00001$) in 48 NSCLC cases. We further discerned that CR levels in tumor tissues correlate significantly with those in urine ($P < 0.0001$, $R^2 = 0.87$ by Spearman's rank order test). These data indicate CR may be a product of deregulated tumor metabolism that is detectable in urine, making urinary CR a potentially useful liquid biopsy biomarker for surveillance after surgery in early stage NSCLC patients. To further evaluate the utility of CR as a liquid biopsy biomarker, urines from 34 stage I and II NSCLC patients from Lung Cancer Biospecimen Resource Network, collected at the time of diagnosis and 6, 12, 18 and 24 months after surgery, were evaluated in the current study. The urinary levels of CR were quantitated using UPLC-MS and were analyzed for association with cancer-specific survival (CSS) and disease-free survival (DFS). As a result, in non-recurrent cases ($n=23$), CR levels showed a significant decreasing trend over 24 months after surgery ($P=0.03$). These data further support previous evidence that CR is a product of deregulated tumor metabolism that can be detected in urine. In addition, Kaplan Meier survival estimates demonstrated high CR levels at the time of diagnosis correlated significantly with worse CSS and DFS ($P=0.005$ and $P=0.003$, respectively). In summary,

urinary CR may have potential as a liquid biopsy biomarker for early stage NSCLC that aids surveillance after surgery as well as to stratify patients with worse prognosis.

Robert Boer

Postdoctoral Fellow

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Chemistry

Targeting the EWS-FLI1 Pre-mRNA in Ewing Sarcoma through Small Molecule Microarray Screening

We have developed Small Molecule Microarray (SMM) technology to screen a library of 26,000 drug-like small molecules to find compounds that selectively bind to nucleic acids. A recently published study showed that the processing of the EWS-FLI1 fusion pre-mRNA expressed in approximately a third of Ewing sarcomas (ES) requires the RNA-binding protein HNRNPH1. In addition, they demonstrated HNRNPH1 binds two G-rich sequences present in EWSR1 exon 8. To determine if the interaction of HNRNPH1 with G-rich sequences in EWSR1 exon 8 is potentially targetable using a small molecule, we have used our SMM platform to identify compounds that bind selectively to the first G-rich sequence (seq 1) of EWSR1 exon 8 in the hopes of uncovering novel therapeutic options for a subset of ES patients. We have demonstrated that RNA oligomers corresponding to the G-rich sequences in EWSR1 exon 8 form G-quadruplex (G4) structures in vitro. We employed an SMM screen using an RNA oligomer corresponding to EWSR1 exon 8 seq 1 identified an azoloquinazoline compound that bound with excellent selectivity over other RNA and DNA G4s. WaterLOGSY NMR experiments demonstrated that this compound binds directly to the RNA in solution. Next, Surface Plasmon Resonance (SPR) experiments showed that this compound has KD of 3 μ M. In addition, we developed an ALPHA Screen assay and demonstrated that this compound can inhibit the binding of recombinant HNRNPH1 to EWSR1 RNA with an IC50 of 14 μ M. We then synthesized a small library of 12 derivatives to understand the structure activity relationship of the azoloquinoline scaffold and identified an improved analog with a KD of 1.2 μ M. Future work will involve conjugating this lead compound to a pan-G4 binding compound that can target the second G-rich region of EWSR1 exon 8 (seq 2) to improve potency and specificity, allowing for evaluation of these compounds in inhibiting pre-mRNA splicing and protein function in ES cells.

Shouhui Yang

Research Fellow

NCI-CCR

Carcinogenesis

miR-331-3p is a Novel contributor of Disease Aggressiveness in Resected Pancreatic Cancer Patients

Pancreatic cancer is one of the most lethal malignancies which is mostly diagnosed at an advanced stage and is minimally responsive to currently available treatment. Even in a small number (15%-20%) of patients with early detection, and surgical resection with curative intent, disease recurrence is highly frequent. Therefore, delineating the potential molecular differences between tumors from early stage, resected patients with long and short survival may provide insights into the molecular mechanism of disease aggressiveness and may identify effective therapeutic targets. microRNA (miRNA) have been identified as potentially promising biomarkers for diagnosis, prognosis and predictors for therapeutic response. Aberrant miRNA expression is found in cancer including pancreatic cancer. We hypothesized

that distinct miRNA signature contributes to markedly different outcome in early stage resected patients with pancreatic ductal adenocarcinoma (PDAC), the most common form of pancreatic cancer. To test this hypothesis, we compared tumor miRNA expression profile in stage-matched, resected PDAC patients in short (=6 months) and long survival (=2 years) groups. We found that miR-331-3p, a previously undescribed miRNA in pancreatic cancer, is highly expressed in the patients from short survival group as compared with longer surviving patients. A higher level of miR-331-3p associated with poor survival in PDAC patients in additional test cohort (N=51, p=0.02) and validation cohorts (N=42, p=0.012). Mechanistically, miR-331-3p targeted ADAMTSL3 (A Disintegrin and Metalloproteinase with Thrombospondin motifs-like 3) and suppressed its expression. ADAMTSL3 suppressed proliferation and invasive ability of pancreatic cancer cells. Furthermore, miR-331-3p enhanced proliferation, colony formation and invasion in pancreatic cancer cells, which was mediated by the downregulation of ADAMTSL3 and attenuation of its tumor inhibitory function. Additionally, gene expression profiling of tumors in a large cohort of PDAC patients (N=136) showed negative correlation between miR-331-3p and ADAMTSL3 expression, which was validated in additional independent patient cohort. A lower ADAMTSL3 expression in tumors showed poorer survival in multiple cohorts of PDAC patients. Our study identified a novel miR-331-3p/ADAMTSL3 axis that promotes pancreatic cancer progression in patients with PDAC, which can be targeted for potentially improving patient outcome.

Gali Fichman

Visiting Fellow

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Chemistry

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Erika Palmieri

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Metabolomics/Proteomics

Nitric oxide-mediated restriction of mitochondrial keto acid dehydrogenases enables metabolite "shunting" through alternative pathways during macrophage Polarization

Pro-inflammatory or "M1" activation of macrophages results in profound intracellular metabolic changes. Increased glycolysis and high lactate production imply a "commitment" of the cell to sustain fast ATP production for activation, independently of Oxidative Phosphorylation (OXPHOS). This reprogramming leads to a "broken" Tricarboxylic acid (TCA) cycle where key metabolites important for the synthesis of critical molecules required for cellular functions, such as citrate, succinate and itaconate accumulate. We have recently shown that Nitric Oxide (NO) is necessary and sufficient for the repression of OXPHOS and results in the break in the mitochondrial TCA cycle and citrate accumulation during LPS/IFN gamma stimulation (M1) in Bone Marrow Derived Macrophages (BMDM). Contrary to previous studies which implicated Isocitrate Dehydrogenase (IDH1) as the culprit for the TCA break, our combination of experiments utilizing carbon tracing of U13C-glucose, substrate-fueled mitochondrial respiration and direct measurements of enzymatic activities has allowed us to map NO-mediated

metabolic reprogramming to mitochondrial Aconitase (ACO2), the enzyme that isomerizes citrate into isocitrate. Furthermore, we show that M1 macrophages reroute pyruvate away from Pyruvate Dehydrogenase (PDH) in an NO dependent manner that is independent of Hif1 alpha activation and its previously suggested effect on limiting acetyl-coenzyme A for the TCA. Data from high-sensitivity proteomics and native in gel activity assays interrogating the different subunits of PDH complex, show BMDM to have stalled enzymatic machinery in the presence of NO, as evidenced by the accumulation of detectable levels of PDH E2 bearing acetylated lipoate cofactor. We speculated that stalled deacetylation of lipoate facilitates nitrosylation of the distal NADH-producing portion of the enzyme (E3), dampening its activity under M1 polarizing conditions. The same holoenzyme chemistry is shared with two other key enzymes, Oxoglutarate and Branched-chain alpha-keto acid Dehydrogenases (OGDH and BCKDC). Accordingly, our preliminary data from metabolomics and U13C-glutamine tracing suggest that NO is responsible for orchestrating macrophage metabolism during inflammation not only by inhibiting OXPHOS through blockage of TCA cycle but also by limiting the activity of mitochondrial alpha keto acid dehydrogenases by interfering with their cofactor chemistry, opening the possibility for new therapeutic manipulations.

Yongli Han

Postdoctoral Fellow

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Epidemiology/Biostatistics - Prognosis, Response Predictions, Etiology, Risk, and Prevention

Accounting for random observation time in risk prediction with longitudinal biomarkers: an imputation approach

In epidemiological studies, it is important to incorporate longitudinally measured biomarkers into risk prediction of clinical endpoints, as subject-specific biomarker trajectory contains additional information on pathology and critical window. While timing of marker measurements often varies among individuals, it is not well understood how the variations affect risk prediction. Existing methods of longitudinal risk prediction, such as shared random effects model (SREM) and pattern mixture model (PMM), handle observation time variations by making strong distributional assumptions of the marker trajectory. Methods with flexible assumptions, such as sufficient dimension reduction (SDR) and logistic regression (LOGIT) ignore the observation time information, and thus render suboptimal prediction accuracy. Our work was motivated by the Scandinavian Fetal Growth Study, which predicts macrosomia (birthweight over 4000 grams) using ultrasound measurements of fetal mean abdominal diameter (MAD) at about 17, 25, 33, and 37 pregnancy weeks. Yet actual observation time has standard deviations of 0.64-1.2 weeks around the designed time. We propose a novel imputation approach to handle the random observation time without making strong model assumptions. A longitudinal regression model was estimated to impute MAD at designed time. Then the imputations, instead of the original data, were used to make risk prediction. Area under receiving operating characteristic curve (AUC) was compared for all methods by using leave-one-out cross-validation. The imputation approach improved predicting accuracy of MAD (AUC=0.844) over SREM, PMM, SDR and LOGIT (AUCs between 0.822-0.829). The imputation approach, SREM and PMM, had comparable goodness-of-fit in the risk deciles, but SDR and LOGIT without time information had slight lack-of-fit (Hosmer-Lemeshow test p-value: 0.11 and 0.05). Besides, intensive simulation studies symmetrically compared performances of those methods under scenarios of correct and misspecified model assumptions. We found that even

small variations in observation time can nontrivially deteriorate risk prediction accuracy, and that our proposed method is more robust to model misspecification than SREM and PMM. These results suggest that the imputation approach is an effective way for better longitudinal risk prediction. Future research should investigate variable selection with a large number of longitudinal biomarkers and random observation time.

Emily Vogtmann

Research Fellow

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Epidemiology/Biostatistics - Prognosis, Response Predictions, Etiology, Risk, and Prevention

Prospective cohort study of the oral microbiota and colorectal cancer risk

Background: The fecal microbiota has been observed to be associated with colorectal cancer (CRC) in case-control studies, and some common oral microbes, including the genera *Fusobacterium* and *Porphyromonas*, have been more often detected in fecal samples of CRC cases compared to controls. However, no studies have prospectively evaluated the association between oral microbiota and the risk of CRC. Methods: The Prostate, Lung, Colorectal, and Ovarian Cancer (PLCO) screening trial obtained oral wash specimens prior to cancer development. We conducted a nested case-cohort study which included 470 prospectively ascertained CRC cases and a referent subcohort of 1,218 PLCO participants. The referent subcohort was a random selection of the PLCO participants with oral wash specimens with oversampling of specific age, sex, and smoking categories. DNA was extracted from the buccal cell pellet using the QIAasympphony with microbial modifications and the V4 region of the 16S rRNA gene was PCR amplified and sequenced on the MiSeq. Microbiome analysis was performed using QIIME2, including the q2-DADA2 plugin for sequence quality control, and the q2-diversity plugin for computing alpha and beta diversity metrics and relative abundances. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated using logistic regression with adjustment for age, sex, and smoking. Results: The CRC cases and referent subcohort had similar average ages (63.7 and 63.0, respectively), but there were a larger proportion of males in the CRC cases (55.3%) compared to the referent subcohort (48.0%). Within the CRC cases, 10.9% were current smokers and 47.7% were former smokers. Alpha diversity, such as the Shannon Index, was not associated with CRC (OR 1.10; 95% CI: 0.93, 1.30) and measures of beta diversity were not associated with CRC risk. The presence of the *Fusobacterium* genus was non-significantly inversely associated with CRC (OR 0.60; 95% CI: 0.28, 1.26) while the presence of the *Porphyromonas* genus was not associated with CRC (OR 1.04; 95% CI: 0.75, 1.45). Conclusions: In this large, prospective cohort, multiple microbial metrics of the oral microbiota were not associated with CRC risk. The dysbiosis in feces at the time of CRC diagnosis may occur later in the colorectal carcinogenesis process or may only be detectable in fecal microbiota. Additional studies with prospective oral and fecal samples are needed to evaluate the temporal association between the microbiota and CRC risk.

Erikka Loftfield

Research Fellow

NCI-DCEG

Epidemiology/Biostatistics - Prognosis, Response Predictions, Etiology, Risk, and Prevention

Coffee, Mortality, and Genetic Variation in Caffeine Metabolism in the UK Biobank

Coffee is one of the most popular beverages worldwide. As indicated by meta-analyses, prospective studies have consistently observed inverse associations for drinking up to 5 cups/day with mortality, relative to non-drinking. Such evidence played a major role in the 2015 report from the United States Dietary Guidelines Advisory Committee, which concluded that moderate coffee consumption can be a part of a healthy diet. Yet, there remain concerns about heavy coffee intake, particularly among people with common polymorphisms in CYP1A2 and other genes encoding enzymes that metabolize caffeine. Several studies have suggested that coffee drinkers with polymorphisms in CYP1A2 may have increased risk of cardiovascular disease. Yet to date, prospective studies with comprehensive genotype information on all participants and a wide-range of coffee intake have been unavailable. We used the UK Biobank cohort of a half-a-million people with baseline demographic, lifestyle, and genetic data to estimate hazard ratios (HR) and 95% confidence intervals (CI) for coffee intake and mortality, using multivariable-adjusted Cox proportional hazards models. We examined overall associations and potential effect modification by caffeine metabolism, defined by a genetic score of established polymorphisms in AHR, CYP1A2, CYP2A6, and POR that functionally impact caffeine metabolism. During 10 years of follow-up, 14,225 deaths occurred. Coffee drinking was inversely associated with all-cause mortality. Relative to non-drinkers, the HRs for drinking $\leq 1, 2-3, 4-5, 6-7, \text{ and } \geq 8$ cups/day were 0.94 (95% CI=0.88-1.01), 0.92 (95% CI=0.87-0.97), 0.88 (95% CI=0.84-0.93), 0.88 (95% CI=0.83-0.93), 0.84 (95% CI=0.77-0.92), and 0.86 (95% CI=0.77-0.95), respectively. Similar associations were observed for instant, ground, and decaffeinated coffee, across common causes of death, and regardless of genetic caffeine metabolism score. For example, the HRs for 6 or more cups/day versus non-drinking ranged from 0.84-0.91, with no evidence of effect modification across levels of genetic caffeine metabolism (P-heterogeneity=0.17). Despite concerns about the potential health effects of high caffeine intake from coffee, we observed evidence for inverse associations with 8 or more cups per day and no difference across stratum of genetic caffeine metabolism score. These results suggest that even very heavy coffee drinking can be part of a healthy diet and offer reassurance to coffee drinkers.

Catherine Callahan

Postdoctoral Fellow

NCI-DCEG

Epidemiology/Biostatistics - Prognosis, Response Predictions, Etiology, Risk, and Prevention

Extended mortality follow-up in a cohort of dry cleaners

Background: The International Agency for Research on Cancer has classified occupational exposures related to dry cleaning, an industry with approximately 150,000 employees in the United States, as possibly carcinogenic to humans (Group 2B); past studies have reported excesses for bladder cancer and, inconsistently, non-Hodgkin lymphoma and cancers of the esophagus, cervix, and kidney among workers in this industry. The exposures of concern involve organic solvents; however, most occupational cohorts to date have lacked assessments of workers' solvent exposures. To address this question, we updated a mortality analysis of 5,369 dry cleaning union members previously assessed for solvent exposure. Methods: We added 21 years of follow-up, through 2014, via linkage to the National Death Index. We used Cox proportional hazards models to compute hazard ratios (HRs) relating cause-specific mortality with levels of a solvent exposure index previously developed by the study industrial hygienist based on workers' job titles from union records. The models were fit adjusting for age, sex and

decade of union enrollment, and assuming different exposure lags (0, 10, and 20 years). Results: Over the extended follow-up, the number of deaths in the cohort increased from 2,351 to 3,543. We observed exposure-response relationships with estimated solvent exposure for bladder cancer [hazard ratio (HR) = 4.1, 95% confidence interval (CI) = 0.7 to 24.5 and 9.2, 1.1 to 76.7 for medium and high exposure respectively vs. no exposure; Ptrend=0.08] and kidney cancer (HR = 4.1, 95% CI = 0.7 to 22.5 and 24.4, 2.9 to 201.6; Ptrend=0.004), assuming an exposure lag of 20 years. High exposure was also associated with mortality due to heart disease (HR = 1.6, 95% CI 1.1 to 2.2) and lymphatic/hematopoietic malignancies (4.3, 1.4 to 13.6). Information on workers' smoking habits was not available; however, results were null for other smoking-related endpoints (emphysema, cancers of the esophagus, larynx, lung and cervix), arguing against confounding from smoking. Discussion: We report, to the best of our knowledge, the first cohort evidence directly relating solvent exposure levels among dry cleaners to increases in mortality due to heart disease and cancers of the bladder, kidney and lymphatic system. Our findings offer important new evidence suggesting that previously reported excesses in these endpoints among workers in this industry are related to occupational solvent exposures.

Aman George

Postdoctoral Fellow

NEI

Genetics

Atypical cadherin FAT1 is essential for vertebrate optic fissure closure

Eye development initiates with the evagination of the optic vesicle, which eventually invaginates to form a dual-layered optic cup. The invagination is asymmetric and is accompanied by the formation of a ventral opening called optic fissure, at approximately the fifth week of human gestation. For the eye to develop normally, the two edges of the fissure must converge and fuse. Defective or faulty optic fissure closure/fusion (OFC) result in a potentially blinding congenital malformation called coloboma, affecting between 1-3 individuals per 10,000 births. We identified by whole exome and Sanger sequencing, homozygous frame shift truncating mutations in the atypical protocadherin FAT1 in ten affected individuals from five unrelated consanguineous families of Middle-Eastern, Turkish, Pakistani and North-African descent. Nine out of ten affected individuals displayed coloboma among other ophthalmic defects. In mouse, Fat1 mRNA was localized at the leading edges of the optic fissure margins. Upon detailed histological analysis, we consistently observed OFC defects in Fat1 null embryos, but not in heterozygous mutants and wild type embryos. Furthermore, morpholino-mediated knockdown of fat1a in zebrafish resulted in coloboma. In human retinal pigment epithelium (RPE) cells, the primary cell type involved in closing of the optic fissure margins, FAT1 protein was observed at the cellular processes, leading edges of filopodia and earliest cell-cell junctions using super-resolution microscopy. FAT1 colocalized with the junctional protein ZO1 at the earliest cell-cell contacts providing a potential molecular mediator of OFC in RPE cells. Short hairpin RNA (shRNA)-mediated knockdown of FAT1 in RPE resulted in loss of FAT1 and ZO1 from earliest cell-cell junctions. CRISPR Cas9 technique was used to generate frame shift truncating mutations in the last exon (to avoid non-sense mediated decay) of zebrafish fat1a which encodes a part of the cytoplasmic tail region of protein. OFC defects were observed in zebrafish embryos homozygous for frame shift truncating mutations but not in those heterozygous for the mutations. In conclusion, FAT1 plays an evolutionary conserved role in OFC in

human, mouse and zebrafish. The spatio-temporal expression of Fat1 in mouse during closure of the optic fissure and its expression in human RPE cells place it exquisitely at the exact time and cell type to suggest a role in facilitating OFC in vertebrates.

Kumarkrishna Raychaudhuri

Postdoctoral Fellow

NEI

Immunology - Autoimmune

An aberrant immune response to an ocular commensal results in disease in a mouse model of Muckle-Wells Syndrome.

PURPOSE: Recent data suggest that *Corynebacterium mastitidis* (C. mast), an ocular surface commensal, elicits production of IL-17 from conjunctival T cells that protects from infection with pathogenic organisms (PMID: 28709803). Muckle-Wells Syndrome (MWS) is 1 of 3 autoinflammatory diseases known as cryopyrin-associated periodic syndromes (CAPS), and results in arthritis, dermatitis and conjunctivitis. Pathology of CAPS is connected to a gain-of-function mutation of the NLRP3 inflammasome gene CIAS1 that leads to production of multiple proinflammatory cytokines, including, most prominently, IL-1b. We hypothesized that an aberrant immune response to commensal microbes at the ocular surface may underlie the conjunctivitis that is characteristic of MWS. METHODS: We used a mouse model of MWS, produced by a knock (KI) of the human mutated CIAS1 gene (PMID: 19501001). The mice, initially negative for C. mast, were ocularly colonized with the bacterium. In vitro and in vivo responses to the commensal were assessed by changes in transcriptome, production of IL-1b, neutrophil infiltration and clinical appearance. RESULTS: Ocular colonization with the commensal C. mast induced conjunctival inflammation in eyes of MWS mice, but not in wild type (WT) controls. This correlated with increased neutrophil infiltration into the conjunctiva of CIAS KI mice and increased production of IL-1b by MWS dendritic cells (DC) upon stimulation with C. mast lysates. Mechanistic studies showed that DCs of MWS mice were more efficient in activating both WT and MWS T cells (in particular, Vg4+ cells), and gd T cells of MWS mice responded more efficiently than WT gd T cells in presence of WT DC in co-culture experiments. Interestingly, assessment of IL-1b and caspase-1 in these cultures suggested a novel finding of intrinsic inflammasome activity in gd T cells. Finally, peripheral blood mononuclear cells from two CAPS patients responded to C mast with increased production of IL-1b and IL17A. CONCLUSION: Our results suggest that the commensal C. mast can act as a pathobiont to trigger ocular inflammation in mice with an overactive NLRP3 inflammasome. We suggest that an aberrant immune response to commensal microbes in humans with this mutation may underlie the recurrent conjunctivitis seen in patients with MWS and similar autoinflammatory diseases.

Juan Angueyra-Aristizabal

Postdoctoral Fellow

NEI

Neuroscience - Cellular, Molecular, Neurotransmission and Ion Channels, and Neurochemistry

Identification of genes involved in photoreceptor recognition and synapse formation

Vision loss caused by photoreceptor death is a leading cause of irreversible blindness worldwide, but therapeutic options remain limited. Photoreceptors have recently been derived from stem cells, raising

the promising possibility of cell-replacement therapies. A critical barrier to such therapy is the lack of successful integration of transplanted photoreceptors into existing retinal circuits, thwarting a reestablishment of function. Yet, little is known about the genes involved in recognition and synapse formation between photoreceptors and their postsynaptic partners. In the absence of this critical knowledge, the mechanistic framework necessary for developing such regenerative therapies will remain elusive. We propose a multifaceted approach to identify differentially expressed genes (DEGs) in cones that are involved in selective recognition and synapse formation. The zebrafish, a genetically accessible animal model, possesses a retina with four distinct cone subtypes (UV, S, M, and L-cones). Each subtype forms synapses with horizontal and bipolar cells in highly specific patterns. We hypothesize that DEGs in cones can be identified that are responsible for such specific patterns. We have acquired reporter lines for UV-, S-, and L-cones, allowing us to manually collect and isolate dissociated cones by subtype—we have already begun sequencing libraries generated from UV- and S-cones. Furthermore, we have generated mutant zebrafish lines using CRISPR/Cas9-guided mutations for other cone-enriched genes, providing a platform to test the consequences of knocking out identified DEGs. Finally, we have refined a screening method for evaluating cone synapse formation by combining transgenic horizontal/bipolar cell reporter lines with antibody staining against proteins both pre- and postsynaptic to photoreceptors. This combined approach will expose the genetic determinants of photoreceptor wiring, providing new therapeutic targets to promote the integration of transplanted photoreceptors in retinal degenerations.

Christie Campla

Postdoctoral Fellow

NEI

Molecular Biology - Prokaryotic, and Eukaryotic

Deletion of an Nrl-regulated alternative promoter results in retina-specific silencing of Frmpd1, a gene involved in mediating rod photoreceptor adaptation

Vision loss from retinal degeneration is largely associated with the death or dysfunction of photoreceptor cells (rods and cones). The adaptation process of rod photoreceptors following intense and/or prolonged light exposure is not yet completely understood, but is critical for optimal function and homeostasis of these cells. To gain further insight into the molecular mechanisms contributing to this process, we examined the potential role of a novel candidate gene (*Frmpd1*) in modulating rod adaptation using a retina-specific knockout mouse model. Tissue-specific transcriptional start sites of *Frmpd1* were identified by 5'-rapid amplification of cDNA ends, and its promoter activity confirmed by in vivo electroporation of mouse retina with putative regulatory regions driving GFP expression. Regulation of the *Frmpd1* promoter was further characterized using electrophoretic mobility shift (EMSA) and luciferase assays, which revealed that transcription of *Frmpd1* in the rod photoreceptors is initiated from a unique retina-specific alternative promoter, which is modulated via binding and activation by key rod transcription factors *Nrl* and *Crx*. A loss-of-function mouse model was generated by a CRISPR/Cas9-mediated genomic deletion of the retina-specific promoter of *Frmpd1*, and validated by in situ hybridization and immunoblot. Interestingly, removal of the alternative promoter resulted in a retina-specific deletion of *Frmpd1* at both the RNA and protein levels. Morphological and functional consequences of *Frmpd1* knockdown in these mice were assessed by immunohistochemistry and electroretinography. Following brief exposure to light, *Frmpd1*^{-/-} mice exhibit a delayed return of

transducin (a key molecule involved in phototransduction signaling) to their outer segments as well as a delay in the recovery of rod photoresponse. We therefore provide evidence that a novel retina-specific isoform of *Frmpd1* aids in the homeostasis and functional optimization of rod photoreceptors, and accomplishes this by mediating the recovery and adaptation of these cells following light exposure. These studies also demonstrate the potential use of CRISPR technologies to generate tissue-specific knockdown models by targeting untranslated regulatory, rather than protein-coding, regions of genes. This approach could potentially be used in various other tissues of the central nervous system, which characteristically express tissue-specific isoforms of genes.

VIJAY KALASKAR

Research Fellow

NEI

Genetics

A novel mutation in RARB DNA binding domain causes ocular coloboma

Uveal coloboma is a potentially blinding congenital ocular malformation, accounting for up to 10% of childhood blindness and caused by failure of the optic fissure to close during the fifth week of human gestation. Although mutations in several genes have been reported, they do not explain 100% of cases. In this study, we report on novel mutations in known coloboma-associated genes identified through custom capture sequencing of a large cohort of patients with uveal coloboma. We further explore the mechanisms underlying optic fissure closure defects associated with a novel mutation in *RARB* gene using cell culture and zebrafish models. Custom capture sequencing of 196 genes from 217 study subjects from 66 families was performed on Illumina HiSeq2000 platform. Data were analyzed and variants filtered using Varsifter. Potential variants in *BMP7* and *RARB* (missense), *TFAP2A* (frameshift) and *CHD7* (nonsense) were identified in four unrelated families. These variants were predicted deleterious and not reported in HGMD, ClinVar and in population databases such as 1000 genomes and EVS. Sanger sequencing segregated these mutations in the affected individuals. The *RARB* mutation was located in the conserved DNA binding domain and predicted to result in p.Arg144Gln substitution. Molecular modeling of mutant *RARB* protein suggested structural changes that are predicted to cause internal rotation of the DNA binding domain. Lower steady state amounts of *RARB* mutant than wild type protein were detected by Western blotting upon transfection of expression constructs in HEK293 cells. Immunofluorescence localized *RARB* mutant protein mostly to cytoplasm while wild type *RARB* was detected mostly in the nucleus. Differential localization of mutant and wild type proteins was confirmed by Western blotting of isolated nuclear and cytoplasmic fractions from transfected cells. The transcriptional activity of the *RARB* mutant protein was significantly reduced compared to that of wild type *RARB* in the firefly-renilla dual luciferase assay. Morpholino knockdown of *rarga* gene, which is the nearest homologue for human *RARB*, produced ocular coloboma in zebrafish embryos and was rescued with wild type human *RARB* mRNA but not with the mutant *RARB* mRNA. We conclude that the p.Arg144Gln mutation in the DNA binding domain of *RARB* causes reduced protein synthesis and/or stability, affects nuclear localization and transcriptional activity in vitro and results in ocular coloboma in zebrafish in vivo.

tao Liu

Postdoctoral Fellow

NEI

Neuroscience - General

Abstract removed at request of author

Abstract removed at request of author

Abhishek Sengupta

Visiting Fellow

NEI

Neuroscience - General

Pseudotyped rabies mediated retinal connectome in primates

We are mapping the synaptic connections between retinal ganglion cells (RGCs) and interneurons in the primate with the goal of uncovering primate specific adaptations in retinal circuits that are important for human vision. Determining the specific anatomical wiring diagrams between cells is crucial to understanding how a retinal circuit extracts features of interest to an organism. Recently, the retrograde transsynaptic transport property of rabies virus was employed to map a retinal circuit dedicated to computing object motion direction in mice. However, similar pathways in primates remain elusive, largely due to technical difficulties associated with primates. Specifically, labeling of individual circuits in primate retina has not been accomplished owing to the lack of transgenic primates, the thick inner limiting membrane restricting viral transduction of the retina *in vivo*, and live primate tissue being scarcely available. We attempted to make the retina amenable to rabies neuronal tracing *ex vivo*, with the goal of utilizing primates sacrificed in unrelated studies and gaining a unique genetic access to primate retinal interneurons. We use an EnvA-pseudotyped rabies lacking the glycoprotein (G), and encoding channelrhodopsin. This virus can only infect cells expressing its complement receptor TVA and can travel transsynaptically from cells expressing G. We sparsely transfect RGCs with gene gun bullets encoding rabies TVA and G. After a period of incubation, we infect retinæ with pseudotyped rabies that selectively infects cells expressing the TVA/G combination. It then gets trafficked retrogradely, labeling individual microcircuits in the primate retina. We have observed that the macaque retina can be robustly labeled *ex vivo* using the gene gun by TVA/G. Additionally, 2-photon imaging in live retinæ showed that the rabies labels RGCs and cells in the inner nuclear layer, where the connected interneurons reside. As a next step, we are interested in identifying and optogenetically perturbing neural circuits that include the starburst amacrine cell. The starburst, which can be recognized by its stereotypic dendritic morphology, is at the heart of a neuronal circuit for computing the direction of moving objects in several mammals. But strikingly little is known about its role in primates. This persists to be an unsolved puzzle in visual neurobiology, whose unveiling can shed light on human visual deficits like nystagmus.

Ximena Corso-Diaz

Postdoctoral Fellow

NEI

Epigenetics

Age-associated changes in the DNA methylome of rod photoreceptors

Aging is characterized by altered homeostasis and reconfiguration of the epigenetic landscape, including changes in histone and DNA chemical modifications. Although both stochastic and deterministic changes in DNA methylation have been observed in multiple mammalian tissues, the contribution of this modification to age-dependent functional decline and susceptibility to disease is poorly understood. Furthermore, most studies have used tissues containing mixed cell populations, which interferes with the identification of DNA methylation changes in cell-type specific loci. Visual function decline is evident in the aging human and mouse retina, and is accompanied by cellular and structural changes with photoreceptors being highly affected. Our aim is to identify biological pathways prone to DNA methylation changes during aging in purified rod photoreceptors. Genomic DNA from flow-sorted rod photoreceptors of 3 month (3M), 12M, 18M and 24M-old old male mice (n=3) was used for whole genome bisulfite sequencing (WGBS) to assess DNA methylation at single nucleotide resolution. Differentially methylated regions (DMRs) were identified using the R-package BSmooth. RNA-seq was performed for purified rods at corresponding ages. Open chromatin regions in 3M-old mice were assessed by ATAC-seq (Assay for Transposase-Accessible Chromatin using sequencing) and analyzed using Homer. Gene Ontology (GO) analysis was performed using GOrilla and transcription factor (TF) motif enrichment using Genomatix. Aging rods (24M) exhibited more variability in their methylation pattern compared to younger rods (3M) and harbored 1160 DMRs ($q < 0.01$). Most DMRs were hypomethylated (1037/1160), localized to coding regions (685), and were enriched in neuronal genes. DMRs harbored TFs involved in chromatin architecture and oxidative stress, and 10% of intergenic DMRs were present in open chromatin regions, indicating dysfunction of distal regulatory elements. RNA-seq revealed that mitochondrial metabolism, protein degradation and neuronal function pathways were dysregulated with age. In summary, the DNA methylation pattern of rods is dynamic during aging with a preferential loss of methylation in neuronal genes and suggests a crosstalk with stress pathways and chromatin architecture. Our study sheds light into epigenetic mechanisms contributing to functional decline in the aging retina.

So Jin Bing

Postdoctoral Fellow

NEI

Immunology - Autoimmune

Ocular autoimmunity develops in the concurrent absence of IFN-gamma and IL-17 and is driven by GM-CSF

Autoimmune uveitis is a complex group of sight-threatening CNS diseases caused by activated retina-specific Th1 or Th17 cells, but their respective contribution to disease is still unclear. Previous studies showed that IL-17A neutralization prevents and reverses experimental autoimmune uveitis (EAU), and that the intensity of Th17 response correlates with disease severity (PMID 18391061). Furthermore, it has been proposed that IL-17/IFN-gamma double-producer T cells are the actual pathogenic effectors in CNS disease (PMID 21278737). Paradoxically, however, IFN-gamma KO (GKO) mice develop exacerbated autoimmunity compared to WT controls and EAU could develop in IL-17A KO mice, suggesting that diverse pathways can lead to pathology. We hypothesized that concurrent absence of IFN-gamma and IL-17A would trigger alternative disease pathways. To test this, we immunized IL-17A and IFN-gamma double KO (DKO) mice with the retinal autoantigen IRBP to induce EAU. We found that DKO mice were

fully susceptible to EAU similarly to WT controls and displayed an eosinophil-dominant (as opposed to mononuclear) eye infiltrate as well as enhancement of IL-17F, Th2 and pro-inflammatory cytokines (IL-6, TNF-alpha and IL-1beta). To rule out a protective role of IFN-gamma that might have contributed to EAU development in DKO mice, we blocked IFN-gamma at the effector phase of disease, when its pathogenic role is presumably dominant, in WT and IL-17A/IL-17F (17AF) KO mice. While effector stage blockade of IFN-gamma ameliorated EAU in WT mice, it exacerbated EAU in 17AF KO mice. Moreover, IFN-gamma antibody treated 17AF KO mice showed enhanced production of GM-CSF and massive infiltration of eosinophils in their eyes. To test whether GM-CSF contributes to pathology of EAU in the absence of Th1 and Th17, we administered an anti-GM-CSF antibody to EAU-challenged IL-17/IFN-gamma DKO mice. Treatment with an anti-GM-CSF antibody during either induction or expression stage of EAU significantly suppressed disease severity and decreased eosinophil infiltration in DKO mice. Importantly, a single intravitreal injection of an anti-GM-CSF antibody immediately after the disease onset reduced EAU severity. These results support the interpretation that, in the concurrent absence of IFN-gamma and IL-17A, GM-CSF plays a major and nonredundant role as a pathogenic effector cytokine.

Nathan Hotaling

Research Fellow

NEI

Informatics/Computational Biology

Discovering Cell Fingerprints with Artificial Intelligence to Determine Cell Therapy Potency, Safety, and Identity

Induced pluripotent stem cells (iPSCs) have a large potential for use in regenerative medicine, tissue engineering, and cell therapies. However, evaluating the degree to which iPSCs have differentiated and their functionality is challenging. Current assays for measuring this include DNA/RNA expression, immunolabeling, blots, assessment of secreted proteins or other factors, and physiological tests. Many of these tests are invasive (require lysing/fixing cells), labor intensive (hours/days to perform), expensive (ELISAs and gene arrays), and/or increase the likelihood of contaminating the cell population (placing tools into the culture area). Thus, the ultimate clinical translation of any iPSC-based therapy is made dramatically more challenging due to the dearth of non-invasive, automated, fast, and robust assays available to scientists. The work presented here details a novel non-invasive multi-spectral imaging, machine learning, and convolutional neural network (CNN) based methodology that has none of the above pitfalls but that has similar sensitivity and specificity as traditional assays. Validity of the methodology was tested in a model system in which the identity and functionality of iPSC derived retinal pigment epithelial (iPSC-RPE) cells from three patients with dry age related macular degeneration (AMD) was assessed. Cells were differentiated using a Food and Drug Administration (FDA) compliant good manufacturing practices (GMP) level protocol to ensure that the method was translatable to the clinic. During culture live iPSC-RPE multi-spectral images were taken and levels of secreted proteins, cell physiological barrier function, and gene expression were measured. The imaging and computational algorithms predicted cell function and maturity to +/- 10 percent actual values for both physiological and molecular assays. The method also classified iPSC-RPE identity from different donors and clones with an 96.3% accuracy, and an average of 92% Sensitivity and 98% Specificity. Additionally, oncogene mutations in a single iPSC-RPE clone were detected by unsupervised clustering with 100% sensitivity and specificity. The process is fully automated, relatively inexpensive, and needs no human intervention to

perform. Additionally, while the focus of this work was on iPSC derived RPE for use in a potential cell therapy for AMD, the method is easily generalizable to any epithelium and their possible cell/tissue therapies using hyperspectral imaging.

Francisco Nadal-Nicolas

Visiting Fellow

NEI

Neuroscience - General

Hibernation with superpower of neural protection?

Neuronal death is a common outcome of neural injury and neurodegenerative diseases. Apart from the physical damage to the neural structure, e.g., axonal injury in the cases of spinal cord injury or optic nerve injury, it is becoming increasingly clear that glial cells and the neuro-inflammatory local microenvironment are critical determinants for neuronal death vs. survival. Mammalian hibernators such as the ground squirrel (GS) survive cold winters by suppressing their metabolism and reducing their body temperature to near-freezing without any cellular impairment. The molecular mechanisms underlying such an extreme form of adaptation may be harnessed for various medical applications. Specifically, investigation of global changes in the retina of hibernating GSs revealed immune suppression during hibernation. We thus speculated that hibernating animals might naturally respond differently to neural injury; Furthermore, the mechanisms of this differential response might be used to develop therapeutic strategies that promote neuronal survival following injury or during neurodegeneration. To investigate differential immune responses in hibernating vs. active GS, we assessed retinal ganglion cell (RGC) and optic nerve responses to optic nerve crush (ONC). Using immunohistochemistry and an automated image processing algorithm, we counted surviving RGCs across the entire GS retina following ONC. We found that in active GSs, ONC led to massive RGC loss (85% loss by 14d post-injury), whereas in hibernating GSs, more than 80% of RGCs survived 14d after ONC. Further investigation at the injury site and in the retina revealed that the local immune responses that were mounted by microglia in active GSs were instead largely absent in hibernating GSs. To investigate whether this lack of microglia activation contributes to RGC survival, we transiently eliminated microglia in active GSs using pharmacology, thus mimicking the local immune suppression seen in hibernating GSs. This manipulation indeed protected RGCs in non-hibernating GSs following ONC. Further experiments are being performed to determine the molecular mechanisms of microglial suppression during hibernation, which may help us discover the secrets of the neural protection with superpower possessed by hibernating GS.

Elena-Raluca Nicoli

Visiting Fellow

NHGRI

Genetics

Lysosomal storage, neurodegeneration, and albinism due to effects of a de novo CLCN7 mutation on lysosomal acidification

Lysosomes are cellular organelles containing enzymes that degrade macromolecules for salvage of small molecules. For optimal function, lysosomes rely on the maintenance of an acidic luminal pH. While

active accumulation of protons is driven primarily by V-ATPase, luminal acidification also requires a neutralizing ion movement, maintained by specific channels in the lysosomal membrane. Defects in the latter can lead to physiological disruptions, including neurodegeneration and lysosomal storage diseases. CLCN7 is a member of the CLC gene family encoding Cl⁻ channels and Cl⁻/H⁺ exchangers. ClC-7 provides the primary route for Cl⁻ passage through the lysosomal membrane, but its role in lysosomal acidification has been controversial. Inactivating mutations in CLCN7 and its beta-subunit, Ostm1, cause both autosomal dominant and recessive forms of osteopetrosis and neurodegeneration. Missense mutations of CLCN7 have led to milder forms of osteopetrosis. No gain-of-function CLCN7 mutation has been reported. Here, through next generation sequencing, we identify a novel de novo CLCN7 Y715C mutation in two children of different ethnicities. These patients presented with delayed myelination and development, lysosomal storage, and hypopigmentation. However, the patients do not present with osteopetrosis. To characterize this mutation, we have measured the outward currents in mutant *Xenopus* oocytes, which showed an increased current, and measured lysosomal pH in patients' cultured fibroblasts, which demonstrated a 0.2-unit reduction. Patient fibroblasts also exhibited greatly enlarged cytoplasmic vacuoles, a finding recapitulated by overexpression of Y715C in control fibroblasts, reflecting the dominant, gain-of-function nature of the mutation. Furthermore, we created a knock-in *Clcn7* Y713C/+ mouse (Y715C in human corresponds to Y713C in mouse) that unequivocally supports the pathogenicity of CLCN7 Y715C in the patients. This murine model has exhibited hypopigmentation, hepatosplenomegaly with storage, and enlarged vacuoles in cultured fibroblasts. Treatment with the alkalinizing drug, chloroquine, normalized the lysosomal pH, reduced the number of large cytoplasmic vacuoles in patients' fibroblasts, and showed hints of clinical improvement in one of the patients. Our results support that Y715C is a novel gain-of-function CLCN7 mutation associated with lysosomal hyperacidity and that the ClC-7 antiporter plays a critical role in maintaining lysosomal pH.

Joshi Stephen

Postdoctoral Fellow

NHGRI

Genomics

Exome sequencing and functional studies in human neurodevelopmental disorders: novel genes and phenotypes

Neurodevelopmental disorders (NDD) are genetically and phenotypically heterogeneous conditions caused due to defects in genes involved in growth, development and survival of nervous system. This study describes five families with eight affected children having mild neurodevelopmental delay, learning disabilities, speech delay, distinct facial dysmorphism and congenital heart defects (CHD) and a single family with two affected children having severe microcephaly, and progressive cerebral atrophy. In five families, whole exome sequencing (WES) identified bi-allelic truncating mutations in TMEM94, the gene that has not yet been recognized for any specific cellular function or genetic disease. TMEM94 is ubiquitously expressed, highly conserved and has over 90% protein similarity across mammals. Gene expression analysis in available cells from the affected individuals revealed reduced expression of TMEM94 compared to controls. As a mammalian model, *Tmem94*^{-/-} knockout mice generated were embryonic lethal and manifested neurodevelopmental delay, haemorrhage, facial dysmorphism and cardiac abnormalities which recapitulate the phenotype of our patients. Histopathological staining of mutant embryonic brain and heart at E15.5 and E18.5 stages revealed abnormal neuronal migration,

atrioventricular septal defects as the cause of embryonic lethality. In the family with microcephaly and seizures, WES identified bi-allelic mutations (a missense and splice site mutation) in VARS that encodes cytoplasmic valyl tRNA synthetase. Human disorders associated with VARS mutations haven't yet been clinically well characterized. cDNA analysis on patient derived fibroblasts revealed that the splice site mutant allele led to nonsense mediated decay, thus resulting in null allele. 3D modelling of VARS predicts that the missense mutation lies in a highly-conserved region and could alter side chain packing, thus affecting tRNA binding or destabilizing the interface between the catalytic and tRNA binding domains. Expression studies showed remarkably reduced level of VARS mRNA and protein in patient cells. Aminoacylation experiments on patient derived cells showed markedly reduced enzyme activity of ValRS suggesting the mutations to be loss of function. To conclude, our study reports a novel syndrome of NDD with facial dysmorphism and CHD, first *Tmem94*^{-/-} animal model and adds two novel genes (TMEM94 and VARS) associated with NDD to the medical literature.

Ramya Varadarajan

Postdoctoral Fellow

NHLBI

Developmental Biology

Cell cycle dependent regulation of Centrosome maturation by Drosophila Pericentrin

Centrosomes are the major microtubule organizing centers in most eukaryotic cells. Centrosomes consist of a pair of centrioles encircled by pericentriolar material (PCM). Centrosome maturation is a key cell cycle-dependent process that facilitates recruitment of PCM proteins to the centrioles thereby enabling centrosome-driven functions, including microtubule nucleation. Mutations in PCM proteins (Pericentrin and Centrosomin) are associated with many human genetic disorders, including cancer and ciliopathies. *Drosophila* is an excellent model system to study the centrosome and diseases that are related to their dysfunction. Here, we focus on understanding the molecular mechanisms underlying PCM recruitment and mitotic microtubules nucleation. Pericentrin like protein (Plp) is a radially organized centrosome linker protein that bridges centrioles and PCM. Plp is shown to be required for PCM recruitment, however, its cell cycle dependent molecular regulation and the crosstalk between other PCM components including Asl, Sas4, CNN and Polo kinase remain unclear. To investigate the precise role of Plp during centrosome maturation, we performed a structure-function analysis by generating Plp protein truncation and characterized its function *in vivo*. We found that the C terminal region of Plp contains a promoting signal for PCM recruitment, while the N terminal region, which is proximal to the PCM, contains an inhibitory signal. We found that the newly identified Plp regulatory domains interact with essential PCM components such as CNN and Sas4. By performing *in vivo* mass spectrometric analysis on these regulatory regions, we identified the precise phosphorylation sites that are targeted by Polo, which is a key kinase of centrosome maturation. Combinatorial analysis of PCM interactions with the phospho modification on Plp regulatory domains suggests a sophisticated autoinhibition mechanism at play. Furthermore, we noticed that the N terminus of Plp extends further towards PCM during centrosome maturation (mitotic) and this conformational modification appears to be Polo driven. Collectively, our current work suggests that Plp functions as a molecular switch to regulate PCM recruitment. That is, in interphase Plp resides in a closed/inhibited conformation that prevent PCM recruitment, and in mitosis, Polo relieves Plp from autoinhibition that in turn allows interaction with PCM components thereby activating centrosomes to nucleate microtubules.

Emily Levy

Doctoral Candidate

NHLBI

Oncology, Tumor Immunology/Immuno-oncology, and Therapy

ENHANCED BONE MARROW HOMING OF NATURAL KILLER CELLS BY MRNA TRANSFECTION WITH GAIN-OF-FUNCTION CXCR4

Natural killer (NK) cells have proven to be powerful effectors against hematological tumor targets in pre-clinical studies. However, the therapeutic potential of intravenously (IV) transferred NK cells has thus far been limited, perhaps in part the consequence of their suboptimal bone marrow (BM) homing capacity. As most hematological neoplasms arise and reside in BM compartments, developing an NK cell product that can reach those targets may lead to improved clinical responses in patients who receive adoptive NK cell-based immunotherapy for hematological cancers. Hematopoietic stem cells (HSCs) are known to engraft in BM compartments via CXCR4/SDF-1 α signaling, thus we explored the potential of NK cells genetically engineered to express a naturally occurring, gain-of-function, CXCR4 (CXCR4-R334X) receptor to enhance cell capacity for BM homing. Ex vivo expanded human NK cells were transfected with mRNA coding for the wild-type CXCR4 receptor (CXCR4-WT) and CXCR4-R334X. NK cell chemotaxis toward increasing concentrations of SDF-1 α was assessed in vitro (n=10 donors). Next, immunocompromised mice either received 1e7 unmodified control NK cells, 1e7 CXCR4-R334X NK cells, or 1e7 AMD3100-mobilized CD34+ HSCs as a BM homing control (n=9-10 animals per group). Blood, BM, liver, lung, and spleen were harvested 24 hours post IV infusion. Transfection of CXCR4 mRNAs into NK cells consistently led to an increase of surface CXCR4 with no significant impact on cell viability. CXCR4-R334X NK cells had significantly greater chemotaxis toward SDF-1 α compared to CXCR4-WT and control NK cells. Overall, surface CXCR4 expression positively correlated with NK cell migration, in vitro (p<.001). Following adoptive transfer of cells into mice, we recovered significantly less circulating CXCR4-R334X NK cells in the blood and more CXCR4-R334X NK cells in femur BM (p<.01 and p=.0001, respectively) when compared to control NK cells. As expected, HSC recovery in BM was significantly higher than the recovery of both NK cell groups (p<.0001, both). This proof-of-concept study demonstrates that low baseline chemotaxis and homing functions of NK cells can be improved by genetically modifying NK cells ex vivo. These data suggest that inducing expression of CXCR4-R334X on NK cells may enhance the therapeutic potential of adoptively transferred NK cells, and thus improve clinical outcomes for patients who receive NK cell-based therapies to target BM-residing malignancies.

Jung Mi Lim

Postdoctoral Fellow

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Biochemistry - General, Proteins, and Lipids

Lipid Transfer Protein, STARD3 is a Novel Binding Partner of Methionine Sulfoxide Reductase A.

Background : Methionine residues provide antioxidant defense by reacting with oxidizing species, with the methionine oxidized to methionine sulfoxide. Reduction of the sulfoxide back to methionine is catalyzed by the methionine sulfoxide reductases. Methionine sulfoxide reductase A (msrA) is present in mitochondria as a non-myristoylated form, while the lipid modified myristoylated form has been reported to be cytosolic. A previous study demonstrated that myristoylated msrA protected the mouse

heart against cardiac ischemia-reperfusion injury while non-myristoylated, cytosolic msrA did not. Despite the important protective role of myristoylated msrA in antioxidant defense, its binding partners and substrates have not been identified in vivo. Results : Starting with a protein microarray that has 9,483 human proteins, we identified the late endosomal protein STARD3, as binding partner of myristoylated msrA, but not of non-myristoylated msrA. STARD3 has both membrane binding and cytosolic domains that function to transport cholesterol from the endoplasmic reticulum to the endosome. The interaction between STARD3 and msrA was demonstrated by 4 independent methods: (1) Binding of msrA to STARD3 on a protein array; (2) Localization of GFP-tagged msrA to late endosomes by super-resolution confocal; (3) Coimmunoprecipitation of STARD3 and msrA from cell lysates; (4) Subcellular fractionation of mouse tissue. Thus, msrA is localized to the cytoplasmic surface of late endosome through the STARD3 cytosolic domain. With mass spectrometric analysis, we also showed that msrA can reduce oxidatively modified Met307 of STARD3. Met307 is situated near the distal end of the lipid binding site and likely binds to STARD3's lipid cargo. Met307 in STARD3 may be particularly susceptible to methionine oxidation by lipid hydroperoxides. The anchoring of msrA would greatly enhance the efficiency of its action on STARD3. Significance : The study establishes myristoylated msrA localized at the late endosome and binds to STARD3, a late endosomal protein thought to be a key transporter of lipids among subcellular domains. We demonstrated that methionine sulfoxide in oxidatively modified STARD3 can be repaired by msrA. Myristoylated msrA is a late endosomal protein that may play a role in lipid metabolism or may protect endosomal proteins from oxidative damage. Such a mechanism may explain why myristoylated msrA protected the mouse heart from ischemia-reperfusion injury.

Xing Fan

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Stem Cells - General and Cancer

Aberrant Clonal Hematopoiesis of the Erythroid and Myeloid Lineages in a Lentivirally Barcoded Rhesus Macaque

Lentiviral vectors(LV) have been used for gene(s) delivery into hematopoietic stem and progenitor cells(HSPC) in clinical trials. LV, in contrast to retroviral vectors, haven't been associated with insertion site-associated malignant clonal expansions, and been considered safer. Here, however, we present a case of markedly dysplastic clonal hematopoiesis impacting the erythroid, myeloid and megakaryocytic lineages in a rhesus macaque transplanted with LV-barcoded HSPC. Autologous CD34+HSPC from macaque ZL34 were transduced with an LV containing MSCV promoter, GFP, and a high diversity barcode library, reinfused following TBI. Transduction parameters and cell dose were similar to 14 other macaques with polyclonal/stable hematopoiesis post-transplant. ZL34 initially engrafted with normal kinetics and blood count recovery. However, beginning 4-5m post-transplant, we observed marked eosinophilia and monocytosis, neutrophil dysplasia, and severe thrombocytopenia. Most strikingly, we observed elevated numbers of nucleated red blood cells(nRBCs) in PB, persisting high levels through euthanasia at 18.5m post-transplant. BM biopsy showed hypercellularity, markedly dysplasia, micromegakaryocytes, erythroid predominance, and left-shift in all lineages but no increased blasts. The spleen contained extensive extramedullary hematopoiesis. The findings were classified as an overlap MPD/MDS. The granulocyte, monocyte and nRBC lineages became virtually 100% GFP+ as the

disorder worsened, identifying transduced HSPCs as the source of the disorder. Barcode and insertion site retrieval revealed complete dominance of a single clone with 9 LV insertions in lineages coincident with clinical abnormalities. RNA-seq of nRBCs identified genes NCAM2, PLAG1, and KITLG within 500kB of an insertion to be differentially upregulated. The PLAG1 targets IGF2 was also upregulated, and PCR proved aberrant PLAG1 splicing. Notably, PLAG1 is a downstream gene of HMGA2 which has shown its upregulation associated with clonal expansion in a LV HSPC gene therapy trial. These results suggest the PLAG1 insertion may be fully or partially responsible for ZL34's MPD/MDS phenotype. This case represents the first clear link between a LV insertion-induced clonal expansion and a clinically abnormal transformed phenotype following transplant, and suggest that strong constitutive promoters should not be included within LV, and that targeted gene correction may be preferable to pursue for HSPC gene therapies.

Yujun Hou

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Neuroscience - Neurodegeneration and Neurological disorders

Mitophagy attenuates cognitive decline via regulation of amyloid- β and p-Tau in Alzheimer's disease

Accumulation of damaged mitochondria is a hallmark of human aging and age-related neurodegenerative pathologies, including Alzheimer's disease (AD). However, the molecular mechanisms of impaired mitochondrial homeostasis and their relationship to AD are still elusive. We examined neuronal mitochondrial morphology in postmortem hippocampal tissues from AD patients and healthy individuals, and found that mitophagy, a selective cellular process mediating clearance of dysfunctional mitochondria, is abrogated in postmortem patient hippocampal tissue. We also found mitophagy is impaired in human neuronal cells and animal models of AD. We hypothesized that compromised mitophagy could contribute to AD pathophysiology. We then investigated whether pharmacological activation of neuronal mitophagy would affect AD pathogenesis. We employed two potent neuronal mitophagy-inducing agents, urolithin A (UA) and actinonin (AC) to investigate whether mitophagy modulation might ameliorate AD pathology. In *C. elegans* models of AD, by using an aversive olfactory learning paradigm-based chemotaxis assay, we found that pharmacological stimulation of mitophagy by UA or AC inhibits memory loss, through a PINK-1, PDR-1 and DCT-1-dependent mitophagy pathway. We then monitored the effects of UA and AC in the APP/PS1 transgenic mouse model of AD. The beneficial effects of mitophagy stimulation were conserved in mammals. Remarkably, UA or AC treatment greatly improved learning and memory retention in the AD mice in Morris water maze assay and Y-maze assay. Furthermore, several common features of AD pathology, such as insoluble levels of A β 1-42, A β 1-40 and extracellular A β plaque formation, were diminished upon UA and AC treatment. Gene-set enrichment analysis of the UA-treated mice display major changes in expression levels of genes, which are associated with inflammation and neuronal function. Next, we examined microglia and found that the mitophagy stimulators promote microglial phagocytosis of extracellular A β plaques and protects against neuroinflammation. Besides A β plaques, tau neurofibrillary tangles are a hallmark feature of AD pathology. Importantly, mitophagy stimulation also abolished Tau hyper-phosphorylation. In view of the atrocious performance of drug development in AD, this study provides a novel drug development strategy for AD patients.

SOO HYUK KIM

Postdoctoral Fellow

NIA

Cell Biology - Intracellular Trafficking, Cytoskeleton, and Extracellular Matrix

Aortic Tropoelastin Degradation by MFG-E8 Directed p38 mediated signaling

Arterial stiffness, a risk factor for cardiovascular disease that is independent of blood pressure, increases as we age. In addition, there is an increase in aortic wall collagen, and increased arterial stiffness which occurs in the context of elastin fiber fragmentation and elastin structural disorganization in which p38 activation has been implicated. Milk fat globule-EGF factor 8(MFG-E8), a.k.a. Lactadherin, a proinflammatory molecule secreted from VSMCs, also increases in the arterial wall with age and colocalizes with the elastin laminae in stiffened arterial walls. Whether or how MFG-E8 signaling affects age-associated elastin remodeling, however, is unknown. We tested the hypothesis that MFG-E8 signaling is central to the molecular mechanisms of elastin remodeling with age in the arterial wall. Methods: We studied both thoracic aortic tissue and cultured VSMCs from young (8mo) and old (30mo) F344XBN male rats. Immunoblotting was performed with antibodies against Tropoelastin, MFG-E8, p-p38, and MMP-2. Recombinant human MFG-E8 protein was used for MFG-E8 overexpression and transfected VSMCs with lipofectamine silencing. Statistical Analyses: Student's t-tests and one-way ANOVA. Results: Aging reduces Tropoelastin expression by 76.7% in aortic tissue and 65.9% in VSMC (both $p < 0.05$). MFG-E8 expression increased with advancing age by 34.5% in aortic tissue and 63.8% in VSMC (both $p < 0.05$). Treatment of VSMCs with MFG-E8 increased p38 activation by 88% on average of young and old VSMCs and reduced Tropoelastin expression by 60% in young, and 53% old in VSMCs (both $p < 0.05$). Further, treatment with MFG-E8 reduced the contractile SMC phenotype marker protein (SM22a) by 23% in young, and by 18.5% old. In contrast, siRNA MFG-E8 enhanced Tropoelastin expression, 61.3% in young, and 50.7% in old (both $p < 0.05$). When p38 is inhibited after MFG-E8 treatment, Tropoelastin levels and expression of SM22a remain preserved compared to control young and old VSMC. Conclusion: An age-related increase of MFG-E8 signaling via p38 activation is linked to a reduction in Tropoelastin protein levels. This molecular mechanism is involved in VSMC phenotype shifts leading to Calcification with age. Targeting MFG-E8 therefore merits consideration as a novel therapeutic approach to retard age-associated elastin remodeling, related arterial calcification, and stiffening that increase exponentially with advancing age.

Mark Hutchinson

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Vascular Disease and Biology

Aortic B cells: Identifying antibodies specific for atherosclerosis in mice

Atherosclerosis is characterized by the development of arterial plaques containing lipid deposits and immune cells, such as B cells. Once recruited to the site of inflammation, B cells become activated and form germinal centers within the adventitia of the artery adjacent to the plaque. To determine if these B cells produce antibodies specific for atherosclerosis, abdominal aortas were collected, and germinal center B cells were isolated by flow cytometry. I characterized their heavy and light chain repertoires by next generation sequencing to compare non-diseased (C57BL/6) and diseased (ApoE -/-) tissues. Furthermore, the aortic repertoires were compared to the germinal center repertoires from spleens

taken from the same mice to address whether local (aortic) and systemic (spleen) B cell responses were similar. Preliminary results suggest that all the splenic repertoires were similar, independent of diet or disease. However, aortic repertoires from ApoE^{-/-} mice indicated that some VH and Vk genes were overexpressed, suggesting selection for particular antigens. I therefore performed single cell analysis and identified over 250 VH and Vk gene pairs, with different frequencies of expression and somatic hypermutation. Antibody gene pairs were then cloned and expressed in culture using Expi293 cells. Several purified antibodies were then tested for their ability to bind a known atherosclerotic antigen without success, suggesting a different antigen may be driving disease. These purified recombinant antibodies will be used to identify antigens specific to atherosclerosis by protein array. These results may be useful in creating potential vaccines for its prevention, as well as treatment via antibody immunotherapy.

mustafa okur

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Stress, Aging, and Oxidative Stress/Free Radical Research

NAD⁺ supplementation prevents hearing loss in Cockayne Syndrome mice

Age-related hearing loss (ARHL) is the most common disorder affecting elderly populations, reaching up to 80% of individuals over the age of 85. Due to its high prevalence, ARHL is considered a hallmark of aging. Cockayne Syndrome (CS) is a premature aging disease with a very high rate of ARHL (up to 84%) by the age of 12. CS is caused by mutations in CSA and CSB genes and hearing loss is a cardinal clinical symptom of the disease. Recent research revealed that hearing loss in CS is caused by defects in cochlear hair cells. These cells are highly metabolically active, making them particularly vulnerable to mitochondrial dysfunction. Interestingly, our lab demonstrated that CS patient cells manifest mitochondrial abnormalities, which can be reversed by supplementation with the essential metabolite NAD⁺. Given that sensorial hair cells are high-energy requiring cells due to their active metabolism and that CS shows mitochondrial dysfunction, we hypothesize that NAD⁺ repletion may ameliorate the hearing loss seen in Cockayne Syndrome through enhancement of mitochondrial homeostasis. To address this hypothesis, we have examined hearing loss in CS mice by measuring electrical potential changes derived from the auditory brain stem (ABR). ABR is a novel technology to record brain wave activity and measure hearing thresholds in response to sound with different intensities (Decibel-dB) and frequencies (Hertz-Hz). We find that CSB mice have increased hearing loss at 6 weeks of age (over 20dB hearing loss at 32kHz with p= 0.01). We then treated WT and CSB mice with the NAD⁺ precursor, nicotinamide riboside (NR), in their drinking water. We observed that short-term NR treatment (appx. 10 days) significantly prevented hearing loss in CS mice and reduced the hearing threshold in response to sound from 65 dB to 40 dB intensity (p=0.009) at high frequency. NR treatment did not have significant effect on young wild type mice with normal hearing. We then tested the effect of NR on the hearing defect in the CSA mouse, and also detected improvement with short-time treatment (over 25dB hearing loss at 32kHz with p<0.001). Currently, we are performing cochlear histology analysis to examine whether NR prevents cochlear hair cell loss and improve hair cell functionality. Given that NR is a natural product with no known toxicities in mice, rats, or people, it might be an effective intervention against hearing loss in mice and possibly in humans with CS and older adults.

Andrea Di Francesco

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Stress, Aging, and Oxidative Stress/Free Radical Research

Enhanced insulin sensitivity and protection against diet-induced obesity in transgenic mice overexpressing NQO1

NAD(P)H: Quinone oxidoreductase 1 (NQO1) is a multifunctional protein widely known for its role as an antioxidant enzyme and for its ability to catalyze the 2-electron reduction of quinones. Alteration in cellular NAD⁺/NADH ratio via pharmacological stimulation of NQO1 activity has been associated with protection against metabolic stress and aging in mice, although the underlying mechanisms are largely unknown. More recently, novel functions for NQO1 have been reported, including the ability to associate with mRNAs and to regulate mRNA translation in vitro. Here, we show that NQO1 overexpressing mice (NQO1-Tg) are resistant to diet-induced obesity and fatty liver, and display superior glucose tolerance and insulin sensitivity. On a high-fat diet, NQO1-Tg mice maintained significantly lower plasma levels of glucose and free fatty acids compared with their littermate controls. These changes were independent of food intake, body composition and energy expenditure. A significant increase in glucose infusion rate during hyperinsulinaemic-euglycemic clamps demonstrated that skeletal muscle was the major site of insulin sensitivity in NQO1-Tg mice. In fasting/refeeding experiments, NQO1 overexpression attenuated activation of ribosomal protein S6 kinase 1 (S6K1), an effector of mTOR, preventing insulin receptor desensitization in the muscle. Moreover, NQO1-Tg mice displayed striking differences in S6 ribosomal protein and eukaryotic translation initiation factor 4G (eIF4G) levels, indicating a major role for NQO1 in regulation of mRNA translation initiation. Using a ribonucleoprotein immunoprecipitation (RIP)-Chip approach, we identified several mRNA targets of NQO1 that have a role in ribosome assembly and mRNA translation. NQO1 co-fractionated with monoribosomes within a sucrose gradient and alterations of NQO1 levels and activity were associated with major differences in global protein synthesis. These results shed new light on the relationship between translational control and glucose metabolism in vivo. We propose that NQO1 up-regulation may allow animals to remain healthier under metabolic stress conditions, partly through regulation of mRNA translation.

Yuki Kishimoto

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Stress, Aging, and Oxidative Stress/Free Radical Research

Evidence that amyloid β -peptide induces senescence in oligodendrocyte progenitor cells in Alzheimer's disease

Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder for which there are no disease-modifying treatments. Studies have shown that amyloid β -peptide (A β) is toxic to oligodendrocytes, causing demyelination and axonal damage. In the absence of A β , axonal damage triggers microglial activation and the subsequent recruitment of oligodendrocyte progenitor cells (OPCs), which then differentiate to myelinating oligodendrocytes to compensate for deteriorating oligodendrocytes. Here, we investigated whether OPCs can properly repair axonal damage when A β is present. Using two different AD mouse models (APP/PS1 and APP-NL-G-F) we demonstrated in the first time that OPC (Olig2/NG2)-positive cells present in A β plaques, which exhibit a senescence-like

phenotype characterized by the presence of p21, p16INK4, and senescence-associated β -galactosidase (SA- β -gal) activity. Senescent cells are resistant to stress and apoptosis and exhibit a senescence-associated secretory phenotype (SASP). We confirmed the same phenotype in brain tissue from AD patients as well. Interestingly A β plaque-associated microglia and astrocytes did not exhibit a senescence phenotype. In addition, cultured OPCs exposed to A β oligomers (A β 1-42) underwent senescence, indicating that A β itself may be sufficient to induce the conversion of OPCs to senescent OPCs (sOPCs). Gene expression array analysis of laser microdissected A β plaques from AD mouse brains indicated an up-regulation of OPC-related genes, as well as genes associated with a senescence and SASP. Next, we found that weekly treatment of AD mice with the senolytic drugs dasatinib and quercetin, which selectively destroy senescent cells by inducing apoptosis signaling pathways, resulted in decreased sOPCs in pathological tissue and cognitive improvement on the Y-maze and Morris Water Maze behavioral tests. Furthermore, the drugs treatment group showed decreased numbers of senescent cells and A β plaques in the hippocampus with a reduction in pro-inflammatory cytokines (IL-1 β /TNF- α /IFN- γ). Together, these results suggest that OPCs exposed to A β exacerbate AD pathology and induce inflammation. Next, we will investigate the mechanism by which A β converts OPCs to sOPCs and how SASP factors released from sOPCs affects disease progression. Our findings which eliminate sOPCs from AD brain using senolytic drugs may lead to new therapeutic strategies to treat AD.

Craig Myrum

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Neuroscience - Neurodegeneration and Neurological disorders

Surveying the epigenetic landscape of Arc-mediated age-related cognitive decline

Old age is accompanied by varying degrees of memory loss that threatens independent living, quality of life, and identity. Pinpointing the molecular basis of age-related cognitive decline is thus paramount for early detection, treatment, and slowing neurodegenerative disease progression. The protein Arc (activity-regulated cytoskeleton-associated protein) has been dubbed the "master regulator" of memory-related synaptic plasticity in relevant brain regions such as the hippocampus. While we know that Arc is a major mediator in age-related cognitive decline, studies are lacking that examine whether altered epigenetic control (a hallmark of aging) of Arc affects cognitive outcome. Utilizing a well-characterized rat model of normal cognitive aging, young and aged animals' spatial memory capacities were assessed in a water maze. Rats were then classified as either young (Y), aged unimpaired (AU), or aged impaired (AI). The hippocampal CA3 subfield (a key site of altered plasticity in age-related memory impairment) was then microdissected and processed for analysis. An innovative, three-pronged approach provided an in-depth survey of the Arc epigenetic landscape, including three key epigenetic mechanisms: (A) nucleosome positioning assessed via micrococcal nuclease digestion followed by Ion Torrent next-generation sequencing (NGS); (B) DNA methylation examined by bisulfite conversion and analyzed using an NGS MiSeq platform; and (C) histone modifications analyzed via chromatin immunoprecipitation (ChIP) followed by RT-qPCR. Controls for each method included undigested tissue, unconverted tissue, and IgGs, respectively. Among the most prominent results: (A) Nucleosome positioning experiments showed that Arc nucleosome occupancy, fuzziness, and positioning were strikingly stable between Y, AU, and AI rats; (B) Methylation experiments identified one intragenic and two promoter bases that were significantly more methylated in AI rats than AU or Y (p 's < 0.05), but Y

vs AU comparisons showed no difference ($p > 0.05$); and (C) Two histone markers associated with active transcription (namely H3K9AcS10p and H3K9Ac) were more enriched in AI animals than in Y or AU animals ($p < 0.05$), while Y vs AU comparisons again showed no difference ($p > 0.05$). Together these data identify important neuroepigenetic signatures that distinguish impaired and successful cognitive aging, and represent novel, potential drug targets for treating age-related memory loss.

Justine Moonen

Postdoctoral Fellow

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Radiology/Imaging/PET and Neuroimaging

Cerebrovascular reactivity and midlife cognitive decline, a longitudinal functional MRI (fMRI) sub-study of the Coronary Artery Risk Development in Young Adults (CARDIA) cohort

Cerebrovascular reactivity (CVR) represents a measure of a vascular response in the brain i.e. metabolic changes, to a vasoactive stimulus, such as increased arterial CO₂. Reduced CVR predicts stroke and has been implicated in Alzheimer's disease and cognitive decline. Typically, CVR is measured by a CO₂ challenge in fMRI that tracks changes in the blood-oxygen-level-dependent (BOLD) signal. Existing studies are mainly cross-sectional and include small samples of older persons with a clinically manifest disease. Given the increasing burden of cardiovascular disease at mid-age, it's of interest to study CVR and its cognitive consequences in a middle-aged community-dwelling population. We assess the association between change in CVR and change in cognition over a 5-year period in 454 participants of the brain MRI sub-study of the longitudinal bi-racial community-based CARDIA cohort. CVR was measured in mean % change in BOLD signal over 4 successive breath-hold challenges during fMRI. Each challenge requires normal breathing for 30s, then breath holding for 16s, and resumption of normal breathing. This method offers an easy implementable alternative to CO₂-gas inhalation techniques. Cognition was measured with the Digit Symbol Substitution Test (DSST). CVR differences by age, sex and race were examined by a t-test. Linear regression analysis examined change in DSST score in relation to change CVR in the total brain and by gray and white matter of the 4 lobes, adjusting for age, gender, race and the field center. Participants had a mean age of 50 years (SD 3.5) at baseline and 55y (SD 3.5) at follow-up. CVR in the total brain declined by 0.01% change in BOLD signal (SE 0.03), $p=0.86$, and DSST score by 1.68 points (SE 0.42), $P<0.001$, with a greater decline in blacks vs whites (mean difference - 2.3 points (SE 0.85), $P=0.007$). In the total sample, decline in DSST was not associated with change in CVR in the total brain, or by tissue class in 3 lobes, and significantly associated with increase in CVR the occipital white matter [Beta -1.83 (SE 0.76), $P=0.02$]. Change in CVR was non-significantly different by age-groups or sex and significantly different by race in the parietal lobe. This community-based study of middle-aged persons showed that regional differences in CVR in the brain may be associated with cognitive decline. To further assess the impact of CVR changes on cognitive function in the community, future studies should examine region-specific associations.

SANKET AWATE

Postdoctoral Fellow

NIA

DNA-binding Proteins/Receptors and DNA Repair

FANCI Separation of Function Mutant Distinguishes its Role in Interstrand Cross-link Repair Versus Response to Replication Stress

Fanconi Anemia (FA) is characterized by bone marrow failure, congenital abnormalities, and cancer. Of the 22 FA-linked genes, FANCI uniquely encodes a DNA helicase and mutations are also associated with breast and ovarian cancer. *fanci*^{-/-} cells are sensitive to DNA interstrand cross-linking (ICL) agents and replication fork stalling drugs. Here we delineated molecular defects of two FA patient-derived FANCI helicase domain mutations (H396D and R707C). Our biochemical data demonstrated that the R707C mutation reduced DNA unwinding and ATP hydrolysis in multi-turnover assays by approximately 65%, whereas the H396D mutation nearly abolished catalytic function. Magnetic tweezers-based single molecule experiments showed that wild-type (WT) FANCI and R707C had comparable unwinding rates but R707C processivity was half that of WT. We determined that defective dimerization by FANCI-R707C was responsible for its reduced catalytic functions. Expression of FANCI-R707C or FANCI-H396D in *fanci*^{-/-} cells failed to rescue sensitivity to the DNA ICL drugs cisplatin or mitomycin C. Live-cell imaging demonstrated a compromised recruitment of FANCI-R707C to laser-induced DNA damage, suggesting that poor localization and reduced helicase due to a dimerization defect are responsible for defective ICL repair. However, FANCI-R707C expressed in *fanci*^{-/-} cells conferred nearly complete resistance to the DNA polymerase inhibitor aphidicolin, and partial rescue to the G-quadruplex ligand telomestatin or DNA strand-breaker bleomycin; FANCI-H396D failed to complement for all these agents. Aphidicolin-treated cells expressing H396D showed ~35% reduction in fiber length compared to cells expressing WT FANCI or R707C. *fanci*^{-/-} cells expressing either R707C or H396D showed significant increase in cisplatin-induced γ -H2AX foci, whereas aphidicolin-induced DNA damage was only elevated in cells lacking FANCI or expressing helicase-dead FANCI-H396D. Thus, a minimal threshold of FANCI catalytic activity is required to overcome replication stress. In contrast, rapid recruitment and a greater level of helicase function is required for FANCI to aid in the reconstruction of broken forks imposed by ICL-induced damage. Moreover, sensitivity of FA patient cells to a DNA ICL agent is an excellent marker for the disease. These findings have implications for therapeutic strategies relying on DNA ICL sensitivity or heightened replication stress characteristic of cancer cells.

Maja Mustapic

Visiting Fellow

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Neuroscience - Neurodegeneration and Neurological disorders

Successful isolation of plasma extracellular vesicles enriched for neuronal origin

Extracellular vesicles (EVs) are released by all cell types and a portion is found in circulating blood. Their dual biogenesis (exosomes from the endosomal-lysosomal system and microvesicles budding from the plasma membrane) results in variable sizes and transmembrane and intraluminal proteins cargo, a valuable source of potential biomarkers and a window into cellular and pathogenic processes. Our laboratory has introduced a method for isolating EVs enriched for neuronal origin using a two-step isolation consisting of capturing total plasma EVs by particle precipitation (Exoquick) followed by immunoprecipitation against L1 neuronal cell adhesion molecule (L1CAM) and has used them as a source of biomarkers for neurodegenerative diseases. L1CAM is highly expressed on neurons thus L1CAM⁺ EVs may be considered enriched for neuronal origin. Here, we sought to further characterize L1CAM⁺ EVs and provide evidence of their neuronal enrichment. We performed quantification of

L1CAM by western blot in L1CAM+ EVs compared to EVs immunoprecipitated by nonspecific isotype control IgG2a antibody (Fig 1A). L1CAM+ EVs showed higher intensity of L1CAM bands when normalized to CD9 (general EV marker). To assess the size distribution and concentration of L1CAM+ EVs in comparison to total and control EVs immunoprecipitated by general EV marker CD81 we used Nanoparticle tracking analysis (NTA) and found that their majority had sizes typical for exosomes and smaller microvesicles (30-200nm). To assess the efficiency of our method for capturing EVs of known neuronal origin we spiked EV depleted plasma with EVs isolated from cultured rat cortical neurons. Based on NTA, we showed that using L1CAM immunoprecipitation we recovered 26% of these neuronal EVs. To further assess enrichment for neuronal proteins we compared L1CAM+ to non-specific IgG2a+ and total EVs using OLINK, a high-throughput, multiplex immunoassay enabling analysis of 92 neurology-related protein biomarkers. Our results showed higher concentration of neuron specific proteins in L1CAM+ EVs when compared to IgG2a+EVs or total EVs. These findings provide strong additional evidence that L1CAM immunoprecipitation successfully isolates EVs of neuronal origin which can be used as a source of diagnostic and treatment response biomarkers for neurological disorders.

Tyler Demarest

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Neuroscience - Neurodegeneration and Neurological disorders

Abstract removed at request of author

Abstract removed at request of author

POONAM PANDEY

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Cell Biology - General

circSamD4 promotes myogenesis

Circular RNAs (circRNAs) lack free 5' and 3' ends and are thus believed to be quite stable. Given that they can regulate microRNA activity and the fate of interacting mRNAs and proteins, they have been proposed to be key players in diseases including cancer and neurodegeneration. However, the impact of circRNAs on muscle differentiation is poorly understood. In this study, we compared the expression patterns of circRNAs during myogenesis using as a model mouse C2C12 myoblasts differentiating into myotubes. We identified 31 differentially expressed circRNAs with distinct subcellular localization (nuclear or cytoplasmic) in myotubes relative to undifferentiated C2C12 myoblasts and hypothesized that they may influence myogenesis. Validation by sequencing and by RT-qPCR analysis indicated that circSamD4 and circStern3 were preferentially expressed in the cytoplasm of differentiated C2C12 cultures. Silencing and overexpression interventions revealed that circSamD4 enhanced the differentiation of human and mouse myoblasts. Among possible mechanisms under investigation, we tested if the association of circSamD4 with RNA-binding proteins HuR, FUS or FMRP influenced circSamD4 function. The interaction of circSamD4 with FUS was particularly interesting, as a decline in FUS levels enhanced myogenic progression, and circSamD4 reduced FUS levels in differentiating muscle

cells. Taken together, we propose that circSamD4 is a novel regulator of myogenesis, and that it modulates muscle differentiation by controlling FUS abundance.

Seung-Jin Kim

Postdoctoral Fellow

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Physiology

Acute adipocyte death causes liver inflammation and injury via the activation of macrophages: insight from a novel model of specific adipocyte death

BACKGROUND & AIMS: Adipocyte death is concomitant with metabolic dysfunctions and nonalcoholic fatty liver disease (NAFLD). However, whether adipocyte death is a cause or consequence of metabolic dysfunctions remains obscure. The aim of this study was to evaluate the pathological role of adipocyte death in vivo by using a model of acute adipocyte death in mice. **METHODS:** To study the pathological role of adipocyte death in vivo, we developed a model of acute adipocyte injury by using the Intermedilysin (ILY)/ihCD59 system. Briefly, floxedSTOP-hCD59 knock-in mice were crossed with Adiponectin Cre (AdipoCre+) mice to generate adipocyte-specific hCD59 transgenic mice (AdipoCre+ihCD59). **RESULTS:** Injection of ILY induced specifically adipocyte death, which subsequently induced macrophage infiltration and crown like structure (CLS) formation in adipose tissues, as well elevation of serum mtDNA, free fatty acid (FFA), free glycerol and lipase levels. Interestingly, injection of ILY caused specifically liver-injury and inflammation in AdipoCre+ihCD59 mice as evidenced by elevation of serum alanine aminotransferase and positive F4/80 staining. Depletion of Kupffer cells/macrophages markedly ameliorated adipocyte death-induced liver inflammation and injury. Furthermore, injection of ILY enhanced hepatic expression of adipocyte-specific miRNAs. In addition, injection of ILY caused elevation of blood glucose levels in AdipoCre+ihCD59. **CONCLUSIONS:** We developed a unique acute and rapid adipocyte death model. By using this model, we demonstrated that adipocyte death directly causes CLS formation, systemic inflammation and liver injury.

Lucas Glover

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Psychiatry

Ambiguous threat governs generalization of anxiety-like behavior and differentially recruits cortico-amygdala regions in the mouse

Ambiguity about whether a potential threat cue will end in an aversive event can catalyze anxiety. Unraveling the neural mechanisms that govern the behavioral components of anxiety can aid in the treatment of these disorders. To elucidate the behavioral mechanisms that lead to "generalized anxiety", we first used a fear conditioning paradigm wherein one group of mice heard 3 tones co-terminating with footshock (reliable conditioning) and another group in which 6 tones were presented but only 50% co-terminated with footshock (ambiguous conditioning). When we assessed memory retrieval of the threat in a control context, the reliable group froze more than the ambiguous group. When placed back into the original context, the ambiguous and reliable groups did not differ in fear-induced freezing, suggesting context is a modulator when a threat is ambiguous. In follow-up

experiments using new cohorts of mice, mice received fear conditioning as above and each cohort received a different “anxious” generalization test in a control context and compared to tone-only controls. To test stimulus generalization, the threat-related tones were intermixed with novel tones. Mice in the reliable group discriminated the two tones, but the ambiguous group showed intermediate freezing toward the novel tones. To test “anxious” situational generalization, the second cohort showed increased defensive behavior in the new situation. Both results suggest that these mice did learn about the ambiguous tone and predictability about a threat can catalyze “generalized anxiety” behavior in two distinct tests. To elucidate neural mechanisms utilized when retrieving a reliable or ambiguous threat memory, we used immunocytochemical staining of the Fos protein as an analog of neural activity. All results are compared to a tone-only control group. We found that the amygdala showed more Fos+ cells upon retrieval of a reliable threat memory. Strikingly, the infralimbic prefrontal cortex and bed nucleus of the stria terminalis (BNST) showed more Fos+ cells upon retrieval of an ambiguous threat memory. Silencing the infralimbic cortex, which sends dense projections to the BNST, using AAVdj-hM4Di DREADD, resulted in selective increase in freezing when retrieving an ambiguous, but not reliable, threat memory. This dissociation pinpoints a locus in the prefrontal cortex that selectively mediates threat ambiguity, which governs generalized “anxiety” behavior in mice.

Patrick Piantadosi

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Neuroscience - General

Serotonergic modulation of risky decision-making

Adaptive decision-making under risk or uncertainty requires activity in a distributed meso-cortico-limbic-striatal network that is heavily interconnected with the dorsal raphe nucleus (DRN) serotonin (5-HT) system. Despite this anatomical arrangement, little is known about the specific circuits through which 5-HT may bias decision-making. Global depletion of 5-HT promotes risk-taking in humans, and local manipulation of 5-HT receptors in rodents affects impulsivity and other functions related to decision-making. To begin to interrogate these circuits, we designed a touchscreen version of a risky decision-making task (RDT) that has been shown to be sensitive to manipulation of relevant cortico-limbic nodes. Mice were first trained to discriminate between two touchscreen windows (left or right; counterbalanced across animals) that delivered milkshake reward of different magnitudes. Animals were then subjected to two test sessions, whereby selection of the large reward option was associated with an increasing probability of foot-shock delivery concomitant with receipt of the large reward option. Selection of the small reward was never associated with punishment. Neural activity (measured by Fos expression) within the DRN, nucleus accumbens (NAc), basolateral amygdala (BLA), and prefrontal cortex (PFC), was differentially affected by RDT performance (as compared to no-shock controls). Based upon this, we pharmacologically blocked 5-HT receptors within the NAc of mice performing the RDT, which increased the proportion of animals predominantly selecting the large, risky option, as compared to vehicle-infused controls. Similarly, optogenetic inhibition of 5-HT terminals within the NAc of SERT-cre mice produced a comparable increase in risk-seeking propensity (which was not observed in cre-negative control mice) when conducted during the choice period, suggesting a temporal window during which 5-HT neuron activity may temper potentially risky reward-seeking. These results provide insight into the neural basis of serotonergic control over cost/benefit decision-making, a facet of cognition that

is disturbed in numerous neuropsychiatric disorders associated with 5-HT dysregulation. Future studies will aim to identify how 5-HT within the NAc may differentially affect dopamine D1 versus D2-receptor expressing neurons, and determine whether subregions of the PFC and BLA contribute differentially to risky decision-making.

Young Eun Cho

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Stress, Aging, and Oxidative Stress/Free Radical Research

Novel mechanisms for increased gut leakiness, systemic endotoxemia and inflammatory liver injury by binge alcohol or fructose exposure

Background: Elevated endotoxemia via leaky gut is a leading cause of multi-organ damage and death in rodents and humans. However, the underlying mechanisms of leaky gut and liver disease caused by alcohol or other substances such as fructose (contained in many soft drinks) and western-style high fat diets are poorly understood. We hypothesized that ethanol-inducible cytochrome P450-2E1 (CYP2E1)-mediated oxidative stress and decreased gut tight junction (TJ) proteins with increased apoptosis of enterocytes play causal roles in binge alcohol- or fructose-mediated gut leakiness, systemic endotoxemia and inflammatory liver injury. Methods: The levels of oxidative stress markers, ileum junctional complex proteins, and apoptosis-related proteins in rodents, T84 colon cells and autopsied people who died from heavy alcohol intoxication and their respective controls were determined by immunoblot, immunoprecipitation, immunofluorescence, confocal imaging and mass-spectral analyses. Results: Binge alcohol or fructose exposure increased oxidative stress marker proteins and caused apoptosis of gut enterocytes with elevated serum endotoxin and liver inflammation. Differential mass-spectral analyses of the purified TJ-enriched fractions of gut epithelial layers showed that several TJ, adherent junction (AJ) and desmosome proteins were markedly decreased in alcohol-exposed rats. Consistently, the levels of intestinal TJ proteins (e.g., claudin-1, claudin-4, occludin, and ZO-1), AJ proteins (e.g., β -catenin and E-cadherin) and desmosome plakoglobin were very low in alcohol- or fructose-exposed rats, wild-type mice, and autopsied alcoholics compared to those of the respective controls and the alcohol-exposed Cyp2e1-null mice. Pretreatment with specific inhibitors of CYP2E1 and iNOS prevented disorganization and degradation of TJ proteins in alcohol- or fructose-exposed T84 colon cells. Immunoprecipitation followed by immunoblot confirmed that gut TJ and AJ proteins were nitrated and degraded via ubiquitin-dependent proteolysis, resulting in their decreased levels in alcohol- or fructose-exposed rats. Conclusion: These mechanistic results for the first time demonstrate the critical roles of CYP2E1 and nitration of gut TJ/AJ proteins in binge alcohol- or fructose-induced gut leakiness and endotoxemia, contributing to liver inflammation or fibrosis. These results can also explain the molecular mechanisms of gut leakiness with decreased TJ proteins in many other disease states.

Ayesha Sengupta

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Neuroscience - General

Anatomical specificity of a 5-HT pathway that controls fear learning

Fear memories are essential to survival, but when pervasive, lead to emotional dysregulation in disorders such as anxiety, post-traumatic stress, phobia, and obsessive compulsion. First-line drug treatments for emotional disorders target the neuromodulator serotonin (5-HT), with variable and sometimes unwanted effects. This is partly due to 5-HT neurons projecting to widespread targets across the brain. These different 5-HT pathways are thought to be involved in distinct behavioral functions, but the pathway-specific roles of 5-HT neurons are not well understood. To address this issue, we characterized the anatomy of the specific 5-HT pathway that projects from the midbrain dorsal raphe nucleus (DRN) to the basal amygdala (BA), a brain region strongly implicated in fear-related emotions. To verify the relevance of this 5-HT pathway in fear learning, a light-gated excitatory opsin or a control fluorophore was introduced into 5-HT neurons in the DRN. The opsin enabled selective activation of the 5-HT pathway projecting to the BA through delivery of light stimulation via an implanted opto-fluid cannula during behavior, which resulted in elevated fear learning. Importantly, local infusion of 5-HT receptor antagonists, but not a vehicle, prevented this opsin-induced fear enhancement. DRN 5-HT neurons labelled by the fluorophore projected throughout the forebrain, with particularly dense innervation of the BA, as well as the nucleus accumbens (NA), which is associated with reward-based behaviors. Next, we used the retrograde tracer cholera toxin B (CTb) to identify neurons in the DRN that project to the BA. The arrangement of BA-projecting neurons demonstrated a highly restricted topographical localization in the DRN. In contrast, DRN neurons projecting to the NA were found elsewhere in the DRN and rarely colocalized with BA-projecting neurons. Finally, we labelled BA neurons that receive inputs from DRN cells using a trans-synaptic viral strategy. These BA neurons projected to other brain regions important to the fear learning process, such as the prefrontal cortex. This study shows that the BA-projecting 5-HT pathway does not collateralize to another 5-HT target, the NA. Its topography may provide an anatomical foundation for its behavioral function, since the BA 5-HT pathway is part of a wider neural network that is involved specifically in fear learning. Thus, this particular 5-HT pathway may be more relevant than others in emotional disorders.

Rajan Guha

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Immunology - Infectious Disease

Malaria-induced epigenetic reprogramming of monocytes plays a role in immunity to malaria

In malaria-naïve individuals, *P. falciparum* infection results in high levels of parasite-infected red blood cells (iRBCs) that trigger systemic inflammation and fever. Conversely, individuals in endemic areas who are repeatedly infected are often asymptomatic and have low levels of iRBCs, even young children who have yet to acquire fully protective antibodies. The molecular mechanisms underlying these clinical observations remain unclear. We showed that PBMCs collected from healthy Malian children before the malaria season responded to iRBCs by producing pro-inflammatory mediators such as IL-1 β , IL-6 and IL-8. However, following febrile malaria there was a marked shift in the response to iRBCs with the same children's PBMCs producing lower levels of pro-inflammatory cytokines and higher levels of anti-inflammatory cytokines (IL-10, TGF- β). Moreover, we showed that genome-wide expression analysis showed that molecules involved in phagocytosis and intracellular killing were upregulated in PBMCs after malaria as compared to before. Together, these data suggested that malaria-induced epigenetic reprogramming of innate immune cells or "trained immunity" might play a role in immunity

to malaria. To further test this hypothesis we are assessing *P. falciparum*-induced changes in monocyte/macrophage function (cytokine production, phagocytosis, ROS generation), gene expression (RNA-seq) and epigenetic patterns (ChIP-seq and ATAC-seq). These assays are being applied both *ex vivo* to monocytes collected from malaria-exposed children, as well as *in vitro* to monocytes stimulated with iRBCs. Our data support the hypothesis that malaria-induced epigenetic reprogramming of monocytes dampens pathogenic inflammation while enhancing anti-parasite effector mechanisms, consistent with our observation that children are often afebrile and tend to control parasite replication in the face of repeated *P. falciparum* exposures.

David Cook

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Immunology - Infectious Disease

Use of NSAIDs for mitigation of malaria-associated symptoms at high doses of PfSPZ Challenge under chloroquine prophylaxis

Intro/Background: Symptoms associated with malaria infection are common in malaria vaccine trials that administer live malaria sporozoites given with antimalarial chemoprophylaxis, a vaccine strategy termed CVac. This approach has achieved high-level (>90%) protection in malaria-naïve volunteers in recent clinical trials. Reducing the severity of malaria-associated symptoms would significantly improve the tolerability and viability of this promising vaccine strategy. An ongoing clinical trial (NCT03083847) at the NIH Clinical Center is evaluating healthy malaria-naïve adult volunteers immunized by direct venous inoculation of aseptic, purified, cryopreserved, non-irradiated *Plasmodium falciparum* sporozoites (PfSPZ Challenge) while taking antimalarial chemoprophylaxis (PfSPZ-CVac) using either chloroquine (CQ) or pyrimethamine as the antimalarial chemoprophylaxis agent. A pilot arm received a high dose (200K) of sporozoites of PfSPZ Challenge in August and September of 2017. This group experienced several severe symptoms including fever, chills, and myalgia. In an effort to improve tolerability in the future it was decided to administer prophylactic non-steroidal anti-inflammatory drugs (NSAIDs) during the main phase of the study in January 2018 in a comparable arm. Methods: Participants in the CQ arm (n=5) of the main study of NCT03083847 were asked to take their choice of NSAID (either ibuprofen or naproxen) whether or not they felt any malaria-associated symptoms. Participants were seen twice daily for clinic visits during this time period. Their symptoms were graded by severity according to the FDA's Toxicity Grading Scale for Healthy Adult and Adolescent Volunteers Enrolled in Preventive Vaccine Clinical Trials. Results: Participants in the pilot group (n=4, no prophylactic NSAIDs) experienced 19 mild symptoms, 5 moderate symptoms, and 3 severe symptoms. For the main study group (n=5, prophylactic NSAIDs) there were 23 mild symptoms and no moderate or severe symptoms. Conclusions: There was a small (3.1%) per-person reduction in mild malaria-associated symptoms in the group using prophylactic NSAIDs. More impressively, there were no moderate or severe symptoms reported at all in the prophylactic NSAIDs group. This indicates that the use of prophylactic NSAIDs may reduce or eliminate severe malaria-associated symptoms if used during future PfSPZ-CVac trials, furthering the effort to develop an effective malaria vaccine.

Jill Fritz

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Genomics

Abstract removed at request of author

Abstract removed at request of author

Forrest Jessop

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Immunology - Infectious Disease

Virulent Francisella tularensis resists IFN-gamma dependent antimicrobial responses through manipulation of host macrophage mitochondrial function

IFN-gamma signaling is critical for host defense against intracellular pathogens. Thus, the ability to evade and modulate anti-microbial effects of IFN-gamma is an important feature of virulence. We have established that the highly pathogenic intracellular bacterium *Francisella tularensis* ssp. *tularensis* (Ftt) resists host IFN-gamma mediated responses early during infection whereas the attenuated live vaccine strain (LVS) does not. Specific mechanisms induced by IFN-gamma that control infection with attenuated strains but are impaired by Ftt are unknown. Uncovering these mechanisms are critical for design of novel therapeutics and vaccines for Ftt and potentially other intracellular pathogens. Following infection of gp91/nos2 deficient bone marrow derived macrophages and addition of IFN-gamma, we established that production of reactive oxygen and nitrogen species was dispensable for control of LVS. This suggested that non-canonical host cell responses to IFN- γ , including modulation of host cell metabolism, may contribute to host defense against this intracellular bacterium. Therefore, we next assessed the contribution of modulation of host metabolism by the bacterium and IFN-gamma to control of infection. Using extracellular flux analysis, we found IFN-gamma impaired mitochondrial function through decreased electron transport chain complex II (CII) activity, and increased glycolysis in LVS-infected macrophages. Impairment of CII activity was directly correlated with increased levels of itaconate in LVS-infected macrophages following IFN-gamma treatment as assessed by LC-MS. Bacterial replication was partially restored in aconitate decarboxylase deficient macrophages, which are unable to produce itaconate, supporting a role for itaconate in control of LVS infection. Control of LVS infection by itaconate was not due to direct activity of the metabolite on cell free LVS. Therefore, our data suggested that production of itaconate supported an antimicrobial environment via inhibition of CII activity and decreased mitochondrial function. Unlike LVS infection, Ftt-infected macrophages were resistant to IFN-gamma dependent modulation of mitochondrial function and associated itaconate production. These results present evidence for a non-canonical pathway by which IFN-gamma controls intracellular infection through altered host metabolism and suggests treatment strategies that inhibit host mitochondrial function may be effective in controlling Ftt infection.

Michael Letko

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Virology - DNA, RNA, and Retroviruses

Forced adaptation of MERS-CoV spike protein to species variation in DPP4

Middle East Respiratory Syndrome Coronavirus (MERS-CoV) was discovered in Saudi Arabia in 2012, infects hundreds of people every year and has an estimated mortality rate of 35%. The virus is thought to have originated in bats and transmitted from dromedary camels to humans. While much work has demonstrated the potential hosts for MERS-CoV, the genetic mechanisms underlying how this virus crosses species barriers remains poorly understood. In order to enter host cells, the MERS-CoV spike protein binds to host dipeptidyl peptidase 4 (DPP4). Rodent-derived DPP4 is genetically distinct from human DPP4, which blocks the interaction with MERS-CoV spike, forming a species barrier to viral replication. In contrast, MERS-CoV is capable of replicating in several bat species despite their DPP4 variation. Thus, we sought to better understand the species promiscuity of the MERS-CoV spike protein. We synthesized a taxonomically and geographically diverse set of bat-derived DPP4 sequences and functionally tested their ability to support MERS-CoV replication in non-permissive cells. Surprisingly, MERS-CoV was capable of utilizing all bat DPP4 variants tested, to varying degrees. However, DPP4 from the common vampire bat (drDPP4) rendered the cells only semi-permissive to a low level of viral replication. We passaged MERS-CoV in cell lines that stably expressed drDPP4 and observed rapidly increasing cytopathic effects with each passage, indicative of viral adaptation. Sequencing of culture supernatants from every passage in combination with structural analysis revealed the step-wise accumulation of viral mutations, which reversed the surface charge of the spike protein to complement the surface charge of drDPP4. Two independent assays demonstrated that these spike mutations enhanced viral replication by increasing viral entry into drDPP4 cell lines. Taken together, these findings demonstrate that MERS-CoV spike can utilize DPP4 from a broad range of bat species and can readily adapt to species variation in the interface with DPP4. DPP4 from completely non-permissive species, such as rodents, fails to support any viral replication, which has prevented in vitro evolution studies of MERS-CoV. The identification of a semi-permissive DPP4 allowed us to observe for the first time, and in unprecedented temporal detail, viral adaptability of the MERS-CoV spike protein. These findings shed light on the evolutionary mechanisms that allow MERS-CoV to overcome species barriers.

Ivan Vujkovic-Cvijin

Postdoctoral Fellow

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Immunology - General

Identification of human pro-inflammatory gut bacteria using the endogenous systemic immunoglobulin repertoire

Numerous studies have identified shifts in the gut microbiota associated with human inflammatory diseases of several types, including arthritis, inflammatory bowel disease, asthma, multiple sclerosis, and others. However, such association studies are limited in their capacity to discern causality in the host-microbe relationship, and tools to probe such relationships are limited. We have developed a novel technique to identify gut microbes that have specifically elicited systemic immune activation via the B cell arm of the peripheral adaptive immune system (âIgG-seqâ). We have applied this technique to identify pro-inflammatory gut bacteria in subjects infected with HIV as a prototypical inflammatory disease, wherein chronic immune activation is associated with early death and shifts in the gut microbiota are linked to this persistent inflammation. Utilizing primary human gut microbiota samples

and the endogenous, systemic immunoglobulin G repertoire via IgG-seq, we have found that a conserved signature of robust systemic immune memory responses against members of the Erysipelotrichaceae family is unique to progressive HIV disease. Such a signature is also evident in non-human primates infected with SIV, a lentivirus closely related to HIV that closely recapitulates HIV disease. Using high-throughput culture techniques in conjunction with murine models, we have identified model systems to probe whether these previously uncultured novel taxa elicit systemic immune activation and play a causal role in HIV-associated chronic inflammation and HIV disease progression. Identification of human inflammation-promoting gut microbiota members in HIV thus provides a framework from which to explore the host-microbiota relationship in a variety of chronic inflammatory diseases, and opens doors to precision microbiome editing strategies for alleviating pathologic inflammation.

Vinicius Andrade Oliveira

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Immunology - General

Long-term and paradoxical metabolic impact of acute gastrointestinal infection

Infection of barrier sites such as the skin and the gut represent a frequent occurrence worldwide. The long-term consequences of defined acute infections for host physiology remain poorly explored. Defined gastrointestinal infection can have dramatic and persistent consequences for the maintenance of tissue-specific immunity and homeostasis. For instance, following the clearance of *Yersinia pseudotuberculosis* infection, mesenteric lymphoid structures and adipose tissue are profoundly remodeled structurally and functionally in a microbiota-dependent manner. Consequently, canonical mucosal immune functions, including tolerance to oral antigens and protective immunity are compromised for the long-term. However, whether these changes in adipose tissue (AT) also have an impact on host metabolism remained unclear. Here, we demonstrated that after resolution of infection, there is a persistent low-grade inflammation characterized by increasing of pro-inflammatory cytokines in the AT and in the serum. The immune tone in the AT shifted from a constitutive type 2 towards a type 1 immune pattern. Additionally, mice that were previously infected exhibited increased weight gain compared to naïve mice at the same food consumption rate. By comparing the different adipose tissue depots, we found that following the clearance of *Y. pseudotuberculosis* infection the mesenteric and subcutaneous white adipose tissue compartments were enlarged compared to naïve age-matched mice. Paradoxically, despite weight gain and expansion of adipose tissue, mice presented improved glucose disposal and insulin sensitivity, along with increased insulin secretion. Such improved response to glucose and insulin sensitivity were recapitulated when we transferred microbiota either from uninfected or previously infected mice into mice raised in absence of microbes (Germ-Free) or WT mice, suggesting that this glucose improvement is microbiota-dependent. Together, this work proposes that a single acute infection can have a profound influence on host metabolism and provide a metabolic advantage for the host. Such adaptation post infection may play an important role in sustaining survival in part of the world in which both infectious burden and malnutrition are prevalent.

Lampouguin Yenkoidiok Douti

Doctoral Candidate

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Immunology - Infectious Disease

Characterization of Plasmodium berghei PbP47 as a Malaria Transmission Blocking Vaccine Target

Malaria is an infection disease caused by Plasmodium parasites and transmitted by mosquitoes. To be successfully transmitted, Plasmodium must survive and complete its life cycle inside the vector. Interfering with the ability of mosquitoes to support the parasite could help control and ultimately eradicate malaria. A few parasite-derived antigens have been characterized and shown to induce immune responses in the vertebrate host that effectively block malaria transmission. However, there is a need to identify new antigens to optimize vaccine efficiency and to test for potential synergistic effects of co-immunization with multiple targets. Recently, our lab found that Pfs47, a protein localized on the surface of several mosquito-stages of the parasite (gametes, zygotes and ookinetes), allows *P. falciparum* parasite to become "invisible" to the mosquito immune system. Further research revealed that *P. berghei* P47 (PbP47), from the mouse malaria parasite, plays important roles in *P. berghei* female gamete fertility in addition to allowing the parasite to evade mosquito immunity. In this study, we tested whether PbP47 is a good transmission blocking vaccine target. We expressed PbP47 as *E. coli* recombinant protein and used a combination of deletion and functional studies to map a small 58 amino acids region that is highly immunogenic. We immunized mice following a prime/boost vaccination regimen with PbP47 epitope along with CpG adjuvant. Transmission blocking vaccines rely on the quality of the antibodies, so we collected sera on days 0, 21 and 42 to assess the intensity and specificity of antibody responses by ELISA. Immunized and control mice were infected with *P. berghei* parasite on day 42, and mosquitoes were challenged on the mice to evaluate the functionality of the vaccine. Results indicate that immunization induce 45% reduction in parasite density in *Anopheles gambiae* mosquito. Interestingly, purified antibodies from mice immunized with PbP47 vaccine reduced the parasite load in the mosquito by 80% when passively transferred to immunologically naïve mice infected with *P. berghei*. These data demonstrate that antibody to PbP47 can be protective. However, fine-tuning the vaccine formulation, immunization regime and optimizing the vaccine delivery system would be required to increase the titer and affinity of the antibody response in the host and ultimately enhance the transmission blocking response.

Carly Elizabeth Starke

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HIV and AIDS Research

Signatures of Protective SIV-Specific CD8+ T Cells

Simian immunodeficiency virus (SIV)-specific CD8+ T lymphocytes can reduce SIV replication, slowing disease progression. However, SIV-specific CD8+ T cells are incapable of reducing viral replication to low levels for the lifespan of most SIV-infected rhesus macaques (RMs), leading to simian AIDS. SIV can persist in lymphoid follicles, infecting CD4+ follicular helper T (TFH) cells. SIV clearance is shielded from CD8+ T cell-mediated suppression due to the lack of CXCR5, the necessary homing receptor for lymphoid follicle entry. However, in rare elite controller RMs, SIV replication is controlled and may be attributed to the unique ability of some CD8+ T cells to penetrate into lymphoid follicles and reduce SIV infection.

To determine whether there are signatures of tissue-resident SIV-specific CD8+ T cells associated with increased virologic control, we analyzed molecular and immunological characteristics of SIV-specific CD8+ T cells in lymphoid and mucosal tissues of SIV-infected RMs with high (>10,000 copies/mL) and low (<10,000 copies/mL) viral loads (VL). MHC-I tetramers loaded with SIV epitopes were used to identify SIV-specific CD8+ T cells and flow cytometric analyses were used to unravel phenotypic and functional qualities. T cell receptor (TCR) clonotypic analyses were also used to define the clonality of the SIV-specific CD8+ T cells. While we found no differences in the magnitude of SIV-specific T cell responses, RMs with low VL exhibited increased CXCR5 expression in lymphoid tissues and frequencies of CXCR5+ SIV-specific CD8+ T cells negatively correlated with plasma RNA viral load and viral DNA within TFH cells. TCR clonotypic analysis revealed distinct clonotypes across lymphoid and mucosal tissues with few clonotypes shared between sites. The TCR repertoire of RMs with low VL differed from those with high VL, with a different TCRBV and TCRBJ gene usage profile. Our data suggest that inherent functionality and trafficking of SIV-specific CD8+ T cells are important for virologic control. SIV-specific CD8+ T cells in lymphoid and mucosal tissues have a distinct repertoire comprised of tissue-resident T cells. Increased CXCR5 expression by SIV-specific CD8+ T cells of RMs with low VL may suggest an ability of CD8+ T cells to enter the follicle and reduce viral loads. Understanding the role of SIV-specific CD8+ T cells and the mechanisms that underlie viral control in SIV pathogenesis may lead to improved vaccine and therapeutic development.

Stephanie Seifert

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Genomics

Long-range polymerase chain reaction method for sequencing viral genomes from ecological and clinical samples

Infectious diseases of zoonotic origin can lead to acute outbreaks upon spillover into human populations. Of particular interest are Ebola virus (EBOV) and Middle East Respiratory Syndrome Coronavirus (MERS-CoV) which have high case-fatality rates. Full genome sequencing of ecologically and clinically-derived samples collected during viral epidemics can inform outbreak response decisions. We sought to develop a rapidly deployable method for the amplification and sequencing of the EBOV and MERS-CoV genomes from ecologically and clinically-derived samples. To achieve this goal, we developed long-range polymerase chain reaction (LRPCR) assays for the amplification of more than 99% of the EBOV and MERS-CoV genomes in two or five semi-nested reactions, respectively, which can then be sequenced without fragmentation on the portable MinION sequencing device. Primers were designed for regions which are broadly conserved across all available genome sequences for each virus species, thus our assays are robust to known variants of each virus. We tested our LRPCR assays by amplifying and sequencing viral genomes from spleen and liver tissue samples of EBOV-infected non-human primates (NHPs) and from nasal swabs collected from camels during an MERS-CoV outbreak. We were able to amplify the EBOV genome when starting with concentrations as low as 10³ TCID₅₀/mL and we were able to amplify the MERS-CoV genome from samples with as low as 500 TCID₅₀ eq/swab yielding whole or partial MERS-CoV genomes from 24 infected camels. We successfully recovered and sequenced the EBOV genome from tissue samples of two EBOV-infected NHPs with approximately 90% of our reads mapping to the reference EBOV genome allowing us to multiplex and sequence up to 96

viral genomes on a MinION flow cell costing less than \$1000. This study demonstrates the utility of LRPCR for sequencing viral genomes from clinically and ecologically-derived samples on the MinION. With long amplicons, we were able to amplify and sequence of otherwise problematic variable genomic regions such as the EBOV glycoprotein or the MERS-CoV spike protein. As our EBOV LRPCR protocol requires only two reactions per genome at each round of PCR, we are able to increase the number of samples on a PCR machine by a factor of twelve relative to existing protocols. There have been no published methods for rapidly deployable sequencing of the MERS-CoV genome and we plan to use this method at our field sites in the Middle East.

Katherine Weissler

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Immunology - General

Peanut-specific CD4+ effector and regulatory T cells in peanut allergic and non-allergic individuals

Food allergies can dramatically affect quality of life and in severe cases result in fatal anaphylactic reactions, particularly in response to peanut. Regulatory T cells are known to be involved in establishing tolerance to ingested foods, but whether defects in regulatory T cells are a major cause of food allergy in the general population remains unclear. Recent studies have suggested that exposure to food antigens through the skin may increase the risk of sensitization and allergy. Efforts to understand the T cell response to food allergens have thus far been impeded by difficulties in identifying cells with T cell receptors that recognize these allergens. We have used a novel technique to identify peanut-specific CD4+ effector and regulatory T cells in peripheral blood based on differential upregulation of CD154 and CD137, respectively, following exposure to peanut extract. In two groups of children, one school-aged and one at one year of age, the frequencies of peanut-specific effector and regulatory T cells were similar between peanut allergic, sensitized, and tolerant children. No differences were found in peanut-specific regulatory T cell suppressive function, stability, or expression of homing receptors between allergic and non-allergic children, which suggests that disparities in regulatory T cells may not be a determining factor in the development of peanut allergy. Peanut-specific effector T cells from peanut allergic children in both age groups were more likely to produce the Th2-associated cytokine IL-13 in response to peanut antigen, while in school-aged non-allergic children, more peanut-specific T cells produced the Th1 cytokine IFN- γ . Furthermore, peanut-specific effector T cells from one-year old infants with peanut allergy or sensitivity were more likely to express the skin-homing receptor CLA and less likely to express the gut-homing receptor α 4 β 7 than peanut-specific cells from non-allergic infants. Expression of homing receptors generally reflects the site of initial activation of the T cell; thus this observation indicates that peanut-specific effector T cells from peanut allergic and sensitized infants initially encountered peanut antigen following cutaneous exposure, and supports the theory that exposure to food antigen through the skin, as opposed to oral exposure, in young children can increase the chance of sensitization. A better understanding of allergen-specific T cell responses may inform future treatments of food allergy.

Isabelle Louradour

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Developmental Biology

Role of the sand fly immune response in its vector competence for Leishmania parasites

Leishmania parasites are responsible for a spectrum of diseases in their mammalian hosts, including humans, ranging from localized cutaneous lesions to fatal, visceral disease. Their transmission is achieved by hematophagous insect vectors called sand flies. For several arthropod vectors, it has been shown that insect immune response negatively impacts the development and transmission of the pathogen to its host. As an example, the vector competence, i.e. the ability to transmit the pathogen, of *Aedes aegypti* mosquitoes to Dengue virus is reduced by the activity of the Toll signaling pathway during the infection. Together with the Immune Deficiency (IMD) pathway, the Toll pathway is one of the two key signaling cascades controlling the immune response in insects, in part by the production of Antimicrobial Peptides (AMPs), directly toxic to pathogens. In sand flies, components of both the IMD and Toll pathways, as well as some AMPs such as Defensins, were found over-expressed after exposure of cell lines or flies to either bacteria or *Leishmania*, although the function of these responses was not addressed. The aim of this project is to study the contribution of the sand fly immune response to its vector competence for *Leishmania*. To this end, we adapted the CRISPR/Cas9 technology to *Phlebotomus papatasi* sand flies, the vector for *Leishmania major* parasites, to generate null mutant for immune response genes. Given that the Toll pathway is also known to be responsible for the formation of the dorso-ventral axis, we decided to focus our efforts on IMD pathway components. We successfully established a protocol for transformation in *Phlebotomus papatasi* using the CRISPR/Cas9 technology and were able to generate null mutant alleles for Relish, the only transcription factor of IMD pathway. We are currently analyzing the phenotype of Relish mutant flies in response to Gram positive and Gram negative bacteria, as well as their ability to carry and transmit *Leishmania major* parasites. In addition, we are generating other sand fly mutant lines by CRISPR/Cas9 technology, including a constitutively active allele of the Relish transcription factor. By this approach, we hope to gain a better understanding of the complex interactions between a disease transmitting insect and the pathogen, focusing on the question of immune response.

Nicholas Collins

Postdoctoral Fellow

NIAID

Immunology - General

The bone marrow supports T cell survival during malnutrition.

Malnutrition causes immunosuppression, leaving the host susceptible to infection and preventing the generation of immunity following vaccination. Furthermore, it is common to become malnourished during pathological conditions such as cancer and AIDS. The immune system is tightly regulated by the nutritional status of the host. This is especially true for T cells, which are critical to combat pathogens and tumors, as well as to generate immunological memory. The fate of T cells under nutritional stress has not been addressed. Here, we show that T cells are greatly reduced throughout the body during malnutrition, including in secondary lymphoid organs (SLO), which are critical sites of T cell activation. This reduction is due to an increase in circulating glucocorticoids (GC), steroid hormones that promote glucose production during times of need but can have the negative effect of inducing T cell apoptosis. However, T cells were found to accumulate in bone marrow (BM) in this context, a site with

constitutively low levels of GC. Thus, the BM may be an optimal site for supporting T cell survival during malnutrition. Genetic, cellular and metabolic analysis of BM during malnutrition revealed dramatic changes. These included increased production of red blood cells (RBC), key producers of the T cell chemoattractant sphingosine-1-phosphate (S1P). Experiments that boosted RBC only in the bloodstream revealed that T cells no longer increased in BM, indicating the importance of S1P gradients in T cell accumulation. Furthermore, BM of malnourished mice was enriched for signatures associated with adipocytes and fatty-acid metabolism. An increase of adipocytes in BM was confirmed by microscopy, while lipidomics showed an increase in several free-fatty acids. This included oleic acid, which promotes T cell metabolism and survival. From these results, we propose that the circulation and SLO are pro-apoptotic environments for T cells during malnutrition, due to high levels of GC. To circumvent this, T cells accumulate in BM via an S1P gradient supplied by RBC. Once in BM, a site with low levels of GC, T cells receive adipocyte-derived factors that promote survival. Thus, the BM may promote host survival in the face of malnutrition by protecting a fundamental arm of the immune system. Further, we believe that such information could have profound implications for improving the survival and longevity of T cells during conditions such as malnutrition, infection and cancer.

Albert Sek

Doctoral Candidate

NIAID

Immunology - Innate and Cell-mediated Host Defenses

IL-5 driven tissue eosinophils may limit muscle damage in Duchenne Muscular Dystrophy (mdx) mice

Duchenne Muscular Dystrophy (DMD) is a debilitating disorder of progressive muscle degeneration caused by inactivating mutations in the gene encoding dystrophin. In DMD, eosinophils are prominent at sites of muscle inflammation and necrosis, although their specific contributions to muscle pathology are not clearly understood. In this study, we hypothesized that eosinophils are recruited in response to signals from necrotic tissue but are not necessarily tissue destructive. Furthermore, because eosinophils can display substantial plasticity, we hypothesized that eosinophil function in the muscle may depend on specific cytokines and cues derived from the muscle microenvironment. To test these hypotheses, we characterized eosinophils in muscle tissue and evaluated pathology in: (1) mdx mice, which model DMD by harboring a null mutation in the dystrophin gene; (2) wild-type controls; (3) interleukin-5 transgenic (IL5Tg) mice, which have global eosinophilia due to over-expression of IL5; and (4) mdx-IL5Tg mice, in which the impact of the dystrophin mutation can be evaluated in the setting of IL5-driven hypereosinophilia. We evaluated muscle damage both by tissue histology and by assessing levels of serum creatine kinase, a biomarker for muscle damage. We detected eosinophils in muscle by immunohistochemical staining for eosinophil major basic protein (MBP) and quantitatively by flow cytometric detection of CD45+CD11c-Gr1-MHC-II-SiglecF+ cells. We have established two key findings: 1. Eosinophils in muscle do not necessarily initiate tissue damage. While we detected eosinophils in skeletal muscles of both mdx and IL5tg mice, we did not observe muscle damage in the IL5Tg mice. 2. Eosinophils generated in response to IL5 may limit mdx-associated muscle necrosis. In comparing mdx-IL5Tg mice with mdx mice, we detected increased numbers of eosinophils yet decreased muscle damage. Moving forward, we will utilize comprehensive, unbiased techniques (e.g., RNA sequencing, Proteome Profiling) to identify critical features of eosinophils from the muscle of mdx-IL5Tg as distinct from those of mdx and IL5Tg mice. In addition, we will generate mdx-PHIL mice to examine the impact

of eosinophil deficiency on muscle pathology. These studies will enhance understanding of the mechanisms by which IL5 and/or IL5-primed eosinophils limit dystrophic muscle damage and may ultimately suggest novel therapeutic strategies for DMD.

Dustin McCraw

Postdoctoral Fellow

NIAID

Protein Structure/Structural Biology

Influenza vaccine nanoparticle design and evaluation assisted by cryo-electron microscopy

Influenza virus is a major threat to public health due to the ability of the virus to rapidly mutate antigenic sites recognized by the immune system. There are 16 different avian hemagglutinin (HA) subtypes for influenza virus A (H1-H16). Commercial vaccines target currently-circulating subtypes H1 and H3. Subtypes H5, H7, and H9 circulate in animal populations, but human infection from animal contact has also occurred. Animal viruses able to infect humans pose a zoonotic and pandemic threat, whereby introducing novel influenza subtypes into the human population. Therefore, one broad question is how to design novel influenza vaccine platforms that that can (1) improve the efficacy of seasonal influenza vaccines, (2) improve pandemic preparedness from zoonotic influenza viruses, and (3) improve the breath of protection for universal influenza vaccine development. Here, we show proof-of-concept for a novel vaccine nanoplatform strategy aimed to integrate conserved epitopes for H1-H16 HA influenza subtypes into recombinant nanoparticles. We used the methods of cryo-electron microscopy with bioinformatics, protein design and expression, biochemistry and immunology for the development of a novel vaccine nanoplatform. A library of chimeric fusion proteins was designed to have conserved influenza epitopes from H1-H16 HA fused to a protein scaffolding that forms nanoparticles. We found that the library expressed in sufficient quantities for further structural, immunogenic, and challenge studies. Cryo-EM indicated that purified proteins formed symmetrical nanoparticles and the fused epitopes were on the particle surface. We found that nanoparticles were immunogenic in animals and ELISA and western blotting assays confirmed that the nanoparticles elicited antibodies that displayed heterosubtypic binding to different HA subtypes. Interestingly, the nanoparticles were immunogenic even after heating to 90 C, suggesting that they are thermogenically stable, which could allow for long-term vaccine storage and distribution without the need for a cold-chain. Furthermore, we found that mice immunized with H1 nanoparticles were protected from lethal challenge with the 2009 H1N1 pandemic virus. In summary, our nanoplatform is on a continuum of research to develop immunogens with increase effectiveness towards seasonal and pandemic influenza viruses-and aid in the development of a universal influenza vaccine.

Derek Moormeier

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Microbiology and Antimicrobials

The stationary phase sigma factor RpoS regulates genes development of the highly-resistant small cell variant developmental form of Coxiella burnetii

The intracellular bacterium *Coxiella burnetii* (Cb) is the etiologic agent of the debilitating flu-like illness Q

fever and is considered a category B biothreat with the capability to cause disease by inhalation of a small infectious dose. Following aerosol transmission, Cb invades inside host macrophages and replicates within a phagolysosome-like compartment called the Coxiella-containing vacuole (CCV). The CCV has hallmarks of a mature phagolysosome, including acidic pH and increased oxidative stress, that Cb must resist to survive. During growth, Cb undergoes a unique biphasic developmental cycle where bacteria transition from replicating (exponential phase) large cell variant (LCV) forms to a non-replicating (stationary phase) small cell variant (SCV) forms. This cycle is morphologically well-characterized, however, the gene regulation involved in the developmental transition to the stable SCV form remains poorly defined. The alternative sigma factor RpoS is an essential regulator of stress responses and stationary phase growth in several bacterial species, including the closely-related *Legionella pneumophila*, which has a developmental cycle superficially similar to Cb. Given this, we hypothesized that RpoS also regulates Cb biphasic development and host colonization. To characterize RpoS function, we constructed a Cb *rpoS* mutant to define the effects on intracellular and axenic growth as well as gene regulation during growth and SCV development. Interestingly, the Cb *rpoS* mutant exhibited intracellular growth defects in J774 mouse macrophages, but not in Vero epithelial cells. RNA sequencing of the Cb *rpoS* mutant revealed that a substantial portion of the Cb genome is regulated by RpoS during SCV development. Genes previously shown to have increased expression during SCV generation, including the expression of the SCV-specific protein ScvA and genes involved in oxidative stress, arginine transport, and peptidoglycan remodeling pathways, were dysregulated in the *rpoS* mutant. These genes were also enriched for a predicted RpoS-binding site. Gene expression data were corroborated with independent assays demonstrating that the Cb *rpoS* mutant has increased sensitivity to hydrogen peroxide and carbenicillin. Collectively, these results demonstrate that RpoS is essential for the regulation of genes involved in SCV development and growth inside macrophages.

Gaspar Canepa

Visiting Fellow

NIAID

Microbiology and Antimicrobials

Epitope Mapping of Plasmodium falciparum Pfs47 Reveals a Novel Malaria Transmission-Blocking Target

Malaria, a life-threatening disease caused by *Plasmodium* parasites, is transmitted by anopheline mosquitoes. Although the global malaria mortality rate decreased by 48% between 2000 and 2015, there were still 212 million cases and 429,000 malaria-related deaths in 2015. Reducing the rate of disease transmission is a key step to control malaria. Mosquitoes become infected when they ingest *Plasmodium* parasites as they feed on blood from an infected host. Sexual stages of *Plasmodium* in the mosquito midgut are vulnerable targets to block malaria transmission. Transmission-blocking vaccines are based on eliciting antibody responses in the vertebrate host that will disrupt parasite development in the mosquito vector and prevent malaria transmission. *Plasmodium falciparum* Pfs47 is a protein with three domains that is expressed on the surface of gametocytes and female gametes and allows parasites to evade the mosquito immune system. We evaluated the potential of Pfs47 as a transmission blocking vaccine. Immunization with full-length Pfs47 protein elicits a strong antibody response in mice, but although antibodies recognize the parasite surface by IFA, they do not confer transmission blocking activity (TBA) when feeding to *Anopheles gambiae* mosquitoes. Analysis of the protein domains targeted by fourteen independent monoclonal antibodies (mAbs) and polyclonal sera by ELISA revealed that

immunization with the full-length Pfs47 elicits antibody responses to Domains 1 and 3, but not to Domain 2 (D2). Recombinant Pfs47-D2 protein was used to generate polyclonal and monoclonal antibodies. Performing ELISA and WB with a series of protein deletions of Pfs47-D2 and mAbs generated against it, allowed us to map a 52-amino acid (aa) linear antigen that is highly conserved (98%) among Pfs47 haplotypes and confers strong TBA (72-99%). Neither human complement nor the mosquito complement-like system mediates the observed TBA. A dramatic reduction in ookinete numbers and ookinete-specific transcripts was observed after treatment of sexual stages with antibodies against the 52-aa region, suggesting that the antibodies are interacting with female gametocytes and preventing fertilization. Optimization of adjuvants, expression systems, and delivery strategies to administer this vaccine are necessary before moving into clinical trials, but these results suggest that the Pfs47-D2 52-aa antigen may synergize with other current available transmission-blocking vaccine targets.

Apollo Stacy

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Microbiology and Antimicrobials

Antiperspirants increase the pathogenicity of the skin microbiome

The prominent skin bacterium *Staphylococcus epidermidis* can defend the skin from pathogens by locally enhancing T cell-mediated immunity. Although in this capacity *S. epidermidis* acts as a beneficial commensal, it can also develop antibiotic resistance and as such cause highly antibiotic-resistant infections. As a commensal, *S. epidermidis* not only faces intrinsic, skin-produced factors but also extrinsic factors such as soaps, sunscreens, and antiperspirants. How these extrinsic factors influence *S. epidermidis*'s interactions with the immune system is largely unknown. The active ingredient in antiperspirants is the metal aluminum. As metals have been shown to select for antibiotic resistance, we hypothesized that antiperspirants enhance the antibiotic resistance of *S. epidermidis*. To test this hypothesis, we serially exposed *S. epidermidis* to aluminum in vitro (in culture media) and in vivo (on murine skin). Supporting our hypothesis, aluminum-resistant strains of *S. epidermidis* isolated from these experiments showed enhanced resistance to multiple clinically relevant antibiotics. Surprisingly, these strains also showed a shift from a commensal to pathogenic lifestyle. When introduced as a commensal (by topical application onto murine skin), aluminum-resistant *S. epidermidis* induced a weaker T cell response compared to the non-resistant ancestor strain. In contrast, when introduced as a pathogen (by injection into murine skin), aluminum-resistant *S. epidermidis* induced a stronger inflammatory response, characterized by higher monocyte numbers. Whole-genome sequencing revealed that aluminum-resistant strains harbor mutations in enzymes that modify the bacterial cell wall. Thus, in ongoing experiments we are exploring the role of host receptors that recognize bacterial cell wall components in determining the altered interactions of aluminum-resistant *S. epidermidis* with the immune system. Together, these studies shed light on how prevalent environmental triggers can negatively affect our skin microbiome and immunity.

Siddharth Krishnamurthy

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NIAID

Immunology - General

The role of the virome in mammalian immunity

The microbiome regulates immunity including immune system development and function. Although considerable work has elucidated the role of the bacterial microbiome in shaping the immune response to pathogens, the role of the viral microbiome (virome) in immunity is largely unexplored. Over 80% of the viruses in the virome infect the bacterial microbiome (bacteriophages, or phages). These phages correlate with immune disease states, even when the bacterial microbiome is not altered. For example, the gut virome of patients with inflammatory bowel disease had an expansion in phage diversity relative to healthy controls. However, because these are correlative studies, it is unclear whether expanded phage diversity caused the dysregulated immune response. Here, we set out to identify how phages modulate the mammalian immune response. To identify a specific role of phages, we will compare pairs of mice that have identical, defined bacterial microbiomes and only differ in whether they contain or lack phages. As most bacteria contain latent phages that can reactivate, we designed a bacterial microbiome that lacked phages using whole genome sequenced cultured isolates with no phage genomic signatures. This analysis resulted in a defined 15-member bacterial community that is representative of the bacterial diversity seen within the gut microbiome. Using municipal sewage, we then discovered 10 phages that infect different members of our 15-member community. Our system is innovative as it will be the first mouse model that we can completely customize to not only remove all phages from the gut microbiome, but also add back different combinations of bacteria/phages to mechanistically interrogate how these microbes alter immunity. Notably, using our phage-free system, we can dissect whether phages are also involved in the development of the immune system. Further, we will assess 1) how phages alter homeostatic immunity in the gut, 2) whether mice with phages control gastrointestinal pathogens, such as *T. gondii* and *Y. pseudotuberculosis*, better than mice without phages, and 3) how phages contribute to tissue inflammation and immunity. With these results, we will assess for the first time whether specific phage species are responsible for modulating immunity and identify the viral sensors responsible for sensing phages to confer these phenotypes. Together, these studies will help frame how a new dimension of the microbiome, the virome, contributes to immunity.

Seth Dickey

Postdoctoral Fellow

NIAID

Microbiology and Antimicrobials

Mechanisms of Toxin Secretion by MRSA

Antivirulence, in contrast to antibiotics, attempts to disarm pathogens of virulence factors that are necessary for disease. Methicillin-resistant *Staphylococcus aureus* (MRSA) kills more people in the United States than any other antibiotic-resistant pathogen. This is in part due to the myriad virulence factors that MRSA produces, which makes MRSA an excellent model for testing antivirulence approaches. MRSA secretes PSMs, a family of virulent peptides, to kill host cells and evade the immune response. The amphipathic PSMs partition into the lipid bilayers to permeabilize membranes and compromise essential processes. Additionally, greater PSM production is associated with hyper-virulent MRSA strains. However, targeting PSMs is complicated by the seven diverse PSMs produced by MRSA. Alternatively, PSM virulence can be blunted by targeting PSM secretion. Currently, only genetic evidence implicates a suite of ABC transporter genes (*pmtABCD* and *abcA*) in PSM secretion. Thus, key

mechanistic questions remain unanswered. In fact, a paradox emerged during the identification of these transporter genes. The *pmtABCD* genes were essential for bacterial survival whereas *abcA* was dispensable. Although PSM accumulation inside MRSA was deemed cytotoxic when *pmtABCD* was genetically deleted, PSMs also accumulated when *abcA* was deleted. I aimed to clarify the PSM secretion pathways in MRSA to enable rational targeting to block PSM virulence. Through a co-purification approach, I uncovered that *pmtABCD* encodes for two distinct transporters: *PmtAB* and *PmtCD*. Using cell-based secretion assays, I profiled the specificity of PSM secretion and found that *PmtCD* and *AbcA* accounted for all PSM secretion. Moreover, I discovered a strong correlation between PSM hydrophobicity and preferential transport by *PmtCD* over *AbcA*. This suggests a substrate-access model that resolves the differential-essentiality paradox. PSMs access the substrate binding sites of *PmtCD* and *AbcA* from either the membrane or cytosol, respectively. Thus, only *pmtABCD* is essential because PSM accumulation in the bacterial membrane, not the cytosol, would be lethal. Finally, based on prior work on PSMs and MRSA disease types, my data identify *PmtCD* and *AbcA* as antivirulence targets for distinct diseases: skin infections and invasive diseases for *PmtCD* and atopic dermatitis for *AbcA*. *PmtCD* is also an antibacterial target due to its essential function in preventing toxic accumulation of PSMs in bacterial membranes.

Wakako Asada

Visiting Fellow

NIAID

Virology - DNA, RNA, and Retroviruses

Development of a VSV-based pan-influenza virus vaccine

The first avian influenza virus outbreak in 1997 demonstrated the potential of this highly pathogenic avian influenza H5N1 virus to cause severe disease in humans. Effective vaccines against H5N1 viruses are needed to prevent a potential global pandemic. Our laboratory has previously developed effective vaccines against highly pathogenic viruses, e.g. Ebola virus, using the recombinant vesicular stomatitis virus (VSV) platform. In this study, we generated VSV-based influenza virus vaccines against H5 serotype influenza viruses to demonstrate the feasibility of the platform for a pan-influenza virus vaccine. The antigen of choice was full length H5 (HAfl) or a soluble version of H5 in which the transmembrane domain has been deleted (sol HA). The VSV-HA constructs were recovered and characterized in vitro. Mice were then vaccinated following a 1-dose or 2-dose regimen and challenged with a lethal dose of H5N1. We found that a single dose vaccination with VSV-solHA resulted in partial protection in mice, however, a 2-dose vaccination resulted in 100% protection. In contrast, one dose of the rVSV-HAfl was sufficient to provide 100% protection against lethal H5N1 challenge. All VSV-HA-based vaccine candidates tested here induced H5-specific antibody responses and the most promising one is currently being evaluated for its cross-protective potential against heterologous H5 influenza viruses. In the future we want to develop additional VSV-based HA vectors covering other HA serotypes such as H1 and H3. Ultimately, a cocktail of selected potent VSV-based HA vaccines will be tested as a pan-influenza vaccine.

Oliver Harrison

Visiting Fellow

NIAID

Immunology - Lymphocyte Development and Activation

Commensal-specific T cells adapt to skin injury to promote tissue repair

Barrier tissues are colonized by diverse communities of commensal bacteria, which play a key role in the education of local immune responses. We recently demonstrated that exposure to commensal species drives local accumulation of commensal-specific T cells, which are key to both host defense and tissue repair. However, how commensal-specific T cells respond to tissue injury and promote tissue repair remains unknown. To explore these questions, we employed a model of colonization with the common skin commensal *Staphylococcus epidermidis* (*S. epidermidis*). Skin colonization with *S. epidermidis* drove recruitment of IL-17A-producing CD8+T cells, crucially in the absence of overt inflammation. However, subsequent tissue injury (insect bites or intradermal injection of chitin) revealed a striking production of type-2 cytokines by commensal-specific CD8+T cells, which was not evident in the absence of tissue injury. Type-2 cytokine production by CD8+T cells is rarely described. As such we sought to investigate the transcriptional profile of commensal-specific CD8+T cells. Strikingly, even under steady state conditions, commensal-specific CD8+T cells displayed accessible chromatin and mRNA expression for type-2 cytokines. However, *in situ* hybridization for mRNA detection by flow cytometry identified that commensal-induced CD8+T cells expressed type-2 cytokine mRNA without corresponding protein production. Thus, under steady state conditions commensal-specific CD8+T cells harbor a poised type-2 immune profile that can be rapidly deployed following tissue injury. To identify factors that may elicit type-2 cytokine production from commensal-specific T cells, we first utilized an *ex vivo* screening assay, whereby isolated commensal-specific T cells were cultured with cytokines associated with tissue injury. We identified IL-18 as a factor that could elicit both IL-5 and IL-13 protein production from commensal-specific CD8+T cells. *In vivo*, IL-18 promoted commensal-specific CD8+T cells production of type-2 cytokines that were essential for wound repair. Importantly, these data demonstrate that commensal-specific T cells act as sentinels of barrier tissues, and directly sense factors released by injured tissues to promote wound repair. Future studies investigating the genetic regulation of type-2 cytokine production by commensal-specific T cells will further our understanding of tissue-specific immunity and the role of commensal-specific T cells in tissue repair.

Waipan Chan

Postdoctoral Fellow

NIAID

Immunology - Lymphocyte Development and Activation

Multiplex Dynamic Reporters Enable Single Cell Temporal Analysis of Costimulatory and Cytokine Receptor Engagement Effects on TCR Signaling

Intracellular signal dynamics are key to unraveling the complex decision-making processes cells use to enable appropriate responses to a wide range of biological, physical and environmental stimuli. T cell antigen receptor signaling pathways have been an intense area of investigations for decades; however, very few studies had probed the dynamics of multiple downstream signaling pathways in individual live lymphocytes with high spatial-temporal resolution. Utilizing an optimized split-GFP complementation detection system (CDS) and a highly-efficient CRISPR/Cas9 nickase-mediated homology-directed repair (HDR) strategy to create endogenous GFP11-tagged RelA, we developed a novel live cell reporter system that allows for high-resolution single-cell quantification of the NF-kappaB activation dynamics via

confocal time-lapse microscopy. Upon CRE-mediated recombination, this GFP11 detection system can be transformed into a nuclear-GFP11 capture mode, which allows for the cumulative reporting of RelA nuclear translocations via flow cytometry. Combining this system with the ERK kinase translocation reporter (KTR), we probe signaling dynamics in response to anti-CD3, anti-CD28, the combination and anti-CD3 plus TNF as a model of early feedback regulation of T cell activation. In addition to revealing differences between TCR and CD28 signaling in these pathways, our studies identify a unique second-wave of RelA translocations that is specifically induced by the combination of TNF and TCR-CD3 signaling, regardless of CD28 engagement, without influencing ERK activation dynamics. Unlike the initial response to TNF or TCR-CD28 stimulation, these sustained secondary RelA translocation events are dependent on induced cytokine secretion or dynamic membrane receptor expression. These data illustrate how the cellular environment can specifically influence the process of immune cell signaling and compensate for the need of a core costimulatory signal that is commonly provided through cell-cell interaction to promote optimal cell survival and proliferation. Our split-GFP CDS is suitable for studying a broad range of GFP11-tagged proteins for quantitative imaging and flow cytometric analysis, including how inhibitory receptors like PD1 modulate downstream signal dynamics linked to TCR-CD28 engagement. With the high efficiency of genomic tagging via CRISPR-based engineering tools, we expect this versatile system to be widely applicable for multiple fields of biomedical research.

Bonnie Huang

Postdoctoral Fellow

NIAID

Immunology - Lymphocyte Development and Activation

An in vivo CRISPR-based functional genetic screen reveals disparate roles for PI3K and HIF-1a in T follicular helper cell differentiation

T follicular helper (Tfh) cells specialize in helping B cells form germinal centers (GCs), produce high affinity antibodies, and generate long-term humoral responses. They are also dysregulated in many human autoimmune diseases. To discover novel Tfh-regulating genes, we developed a CRISPR-based functional genetic system to disrupt genes in primary mouse T cells in vitro, and then evaluated Tfh cell differentiation in vivo. We generated a retroviral vector to introduce guide RNAs (sgRNA) into cultured transgenic Cas9+ CD4 T cells. Three days post-transduction, we observed up to 90% loss of targeted proteins. To evaluate Tfh differentiation, we transferred sgRNA-transduced T cells into wild-type hosts, which were then infected with LCMV. One week later, control-transduced T cells differentiated into 50:50 Tfh:Th1 cells, while T cells transduced with sgRNAs against BCL6, a master transcription factor for Tfh cells, had severely impaired Tfh differentiation. We then generated and transduced T cells with a pooled library containing ~400 sgRNAs covering ~80 druggable genes, non-targeting controls, and positive controls. Post-transfer and LCMV infection, Tfh and Th1 cells were sorted, their DNA was PCR amplified and deep sequenced to quantify the abundance of each sgRNA in the two populations. Guides targeting Tfh-essential genes such as Bcl6 were severely depleted in Tfh cells relative to Th1 cells, while guides against Tfh-blocking genes such as Prdm1 (Blimp1) were enriched, thus validating the screen. We further found that PI3K p110d is required for Tfh differentiation, while HIF-1a opposes it, which we validated using single guides. Previous studies have shown that Tfh differentiation requires PI3K de-repression of BCL6 via FoxO1. However, PI3K also activates mTOR, which induces HIF-1a, yet the loss of HIF-1a resulted in increased Tfh cells, suggesting additional signaling dynamics. Previous studies of GC B

cells suggest that the hypoxic GC microenvironment modulates the humoral immune response. Since a fraction of Tfh cells occupy the GC during close interactions with GC B cells, and HIF-1a is also a master regulator of hypoxic responses, we are investigating the effects of hypoxia on Tfh differentiation. Our data suggest that this screening approach is a powerful technique for simultaneous functional interrogation of many genes in T cells in a physiological setting, and has uncovered complex signaling of the PI3K-mTOR-HIF-1a axis in Tfh differentiation.

Sanket Ponia

Postdoctoral Fellow

NIAID

Virology - DNA, RNA, and Retroviruses

Identification of a novel mechanism of Parkin translocation to mitochondria required for mitophagy and resolution of antiviral interferon responses.

The large-scale emergence of Zika virus (ZIKV), a neurotropic flavivirus, in South America during 2014/15 was associated with severe disease in humans, including microcephaly of newborns. Anti-flavivirus innate responses are initiated by activation of cellular RNA helicases that signal through the adaptor protein MAVS on the mitochondria to upregulate type I interferon (IFN) expression. Resolution of MAVS signaling is achieved in part through loss of mitochondrial membrane potential (MMP) that initiates degradation of depolarized mitochondria through mitophagy. Canonical mitophagy is controlled by two proteins that are mutated in early onset Parkinson's disease (PD), PINK1 (a serine-threonine kinase) and Parkin (an E3 ubiquitin ligase). Following loss of MMP, PINK1 is stabilized on mitochondria and is thought to activate Parkin although key molecular details including how Parkin is recruited to mitochondria are not understood. By examining MAVS signaling in the context of ZIKV, we have identified a novel protein (termed Translocator of Parkin 1 or TOP1) critical for PINK1-Parkin-dependent mitophagy. MAVS activation by virus infection or by MAVS overexpression caused translocation of the normally cytosolic TOP1 to mitochondria. Expression of TOP1 with MAVS resulted in lysosome-dependent degradation of MAVS and suppressed IFN expression, but TOP1 did not directly interact with MAVS. Therefore, we investigated the effects of TOP1 on mitophagy in cells deficient for PINK1 or Parkin. Following loss of MMP by the drug CCCP, cytosolic TOP1 also moved to mitochondria. TOP1 translocation was dependent on Parkin, as it did not occur in Parkin-deficient cells. Consistent with this, TOP1 interacted with Parkin by reciprocal co-immunoprecipitation. Interestingly, co-expression of TOP1 with Parkin caused Parkin translocation even in the absence of MMP loss or PINK1. However, once at mitochondria, TOP1 interacted with PINK1 and resulted in Parkin-mediated degradation of TOP1 suggesting a negative feedback loop to prevent aberrant mitophagy. Importantly, MEFs from TOP1^{-/-} mice had significant defects in mitophagy induced by CCCP, increased MAVS-dependent IFN responses, and reduced virus replication, thereby demonstrating a role for TOP1 in primary cells. These results reveal TOP1 as an essential component of PINK-Parkin mitophagy that may be a therapeutic target in treatment of neurological disorders including PD and ZIKV infection.

Rachel Sparks

Clinical Fellow

NIAID

Systems Biology

Towards a general indicator of immune responsiveness: A baseline blood transcriptomic signature that correlates with vaccination response and lupus disease activity

Understanding inter-individual differences in immune system states and responsiveness is a fundamental goal of human immunology. Predictive models of human immune responses are increasingly needed given the development of immune-based therapies that can treat diseases such as cancer and autoimmunity but are effective in only a subset of patients. A previous multi-modal, systems level examination of the pre- and post-vaccination immune states in response to seasonal influenza vaccine in healthy individuals performed by the NIH Center for Human Immunology (CHI) identified frequencies of temporally-stable CD20+ B-cells expressing high levels of CD38 as predictors of antibody response (Tsang et al., Cell 2014). To assess whether this predictive signature is effective in different vaccination cohorts that did not measure our cells of interest but have blood transcriptomic data available, we developed a 10-gene signature that was temporally stable and exhibited robust correlation with the frequency of the B-cells of interest (CD20+ CD19+ CD38++). Our gene signature was able to differentiate between high and low responders with high accuracy to the seasonal influenza vaccine in several publically available influenza vaccination datasets (Furman, PNAS 2014; Thakar, Aging 2015) (AUC 0.80). Notably, results were similar when we tested our signature in a study of yellow fever vaccine (AUC > 0.80; Querec, Nat. Immunol. 2009), which is a live-attenuated vaccine and thus immunologically very different from the inactivated influenza vaccine, thus suggesting that our signature may be reflective of the immune system's general propensity to respond to an antigenic challenge. If true, our baseline signature could also be indicative of responsiveness to self-antigens, often manifested clinically as autoimmune diseases. We tested this hypothesis in a longitudinal pediatric systemic lupus erythematosus (SLE) dataset (Banchereau, Cell 2016), specifically asking whether our baseline signature evaluated during clinical quiescent periods correlate with the magnitude of flares associated with plasmablast increases. Our results suggested that for a subset of SLE patients (with disease activity associated with plasmablast changes), this was indeed the case. Taken together, our study shows the utility of comprehensive immune state characterization, both at baseline and in perturbed/disease states, and postulates that there may exist "common" immune responsiveness setpoints.

Kairui Mao

Postdoctoral Fellow

NIAID-VRC

Immunology - General

Sequential innate and adaptive immunity establishes non-inflammatory gut commensalism and physiologic host lipid metabolism

The mammalian gut is colonized by numerous microorganisms termed the microbiota, which have a mutually beneficial relationship with their host. In normal individuals, the gut microbiota matures during ontogeny to a state of balanced commensalism marked by the absence of adverse inflammation. Subsets of innate lymphoid cells (ILCs) and conventional T cells are considered to have redundant functions in containment and clearance of microbial pathogens, but how these two major lymphoid cell populations each contribute to shaping the mature commensal microbiome and help maintain tissue homeostasis has not been determined. Using advanced multiplex quantitative imaging methods, here we show an extensive and persistent pSTAT3 signature in ILC3s and intestinal epithelial cells (IECs)

induced by IL-23 and IL-22 in animals lacking CD4+ T cells. In contrast, in immune-competent mice, there is only transient pSTAT3 activation induced by microbial colonization at weaning. This early signature is extinguished by Treg and Th17 cells as CD4+ T cell immunity develops in response to the expanding commensal burden. Our data show that innate and adaptive lymphocytes adopt different strategies to establish the commensal state and have different effects on microbiota, with the activity of adaptive lymphocytes dominating over that of the innate lymphoid cells in this circumstance. Physiologically, the persistent IL-22 production from ILC3s that occurs in the absence of adaptive CD4+ T cell activities results in impaired host lipid metabolism by decreasing lipid transporter expression in the small bowel. These findings provide new insights into how innate and adaptive lymphocytes operate sequentially and in distinct ways during normal development to establish steady state commensalism and tissue metabolic homeostasis.

Aleksandra Iovic

Doctoral Candidate

NIAMS

Genomics

Next generation sequencing approaches identify pathogenic somatic mutations in rare non-malignant diseases

Somatic mutations have long been recognized as the genetic drivers of cancer pathogenesis. With the advent of next generation sequencing (NGS) technologies, our understanding of the role of somatic mutations is expanding into the realms of chronic, age-related, and rare diseases. NGS technologies have enabled deep sequencing of whole exome or targeted gene sets to unprecedented depth, facilitating the study of relatively low frequency somatic mutations in mixed populations of cells. Using a combination of whole exome sequencing (WES), targeted deep resequencing, and droplet digital PCR (ddPCR), we have identified pathogenic somatic mosaic mutations in the rare bone disease melorheostosis, a rare dysostosis characterized by excess cortical bone formation in an asymmetric pattern, which results in pain, deformity, and functional impairment. Prior to our study, the genetic cause and pathogenesis of melorheostosis were unknown. 15 unrelated patients underwent biopsies of affected lesions and unaffected contralateral bone, and DNA was subjected to WES at 100X depth. Combining several variant-calling algorithms and filtering steps, we identified missense somatic mutations in the MAP2K1 gene (encoding the MEK1 protein) in 5 of 15 affected bone samples, at variant allele frequencies (VAF) of 10%-30%. Targeted deep sequencing of MAP2K1, as well as mutation-specific ddPCR, identified an additional 3 patients with MAP2K1 mutations with VAFs down to 1.5%, which were missed by WES. All 8 mutations were localized in the negative regulatory domain of MEK1 (p.Q56P, p.K57E, p.K57N), are associated with various malignancies, and are functionally activating. Bone sections and osteoblasts cultured from patient biopsies displayed phosphorylation of the MEK1 substrate ERK1/2 and increased growth commensurate with MAP2K1 mutant allele frequency. These findings identify a cause for this rare disease, elucidate the importance of the MAP2K1 oncogene in bone homeostasis, and identify a possible target for therapy with FDA-approved MEK inhibitors. We used similar NGS approaches to study somatic mutations in patients with autoinflammatory symptoms which resemble known Mendelian diseases but do not have a germline genetic diagnosis. Using PCR-based targeted deep sequencing in a cohort of >100 of these patients, we identified predicted pathogenic somatic

mutations in the MEFV and NLRC4 genes and are undertaking functional studies to examine their phenotypic consequences.

Lingling Miao

Visiting Fellow

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Developmental Biology

Abstract removed at request of author

Abstract removed at request of author

Tara Gelb

Postdoctoral Fellow

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Pharmacology and Toxicology/Environmental Health

Intersectional screening with functionally-annotated small molecules and RNAi identifies aurora kinase B inhibitors as a novel treatment for Merkel cell carcinoma

Despite being a rare malignancy, Merkel cell carcinoma (MCC) is the second leading cause of death from skin cancer. Approximately 80% of MCCs are driven by the Merkel cell polyomavirus (virus-positive MCC: VP-MCC), and the remaining 20% have a high mutational burden caused by UV mutagenesis (virus-negative MCC: VN-MCC). Anti-PD-L1 immunotherapy is the only FDA-approved treatment for MCC, however the durable response rate is less than fifty-percent. Moreover, up to 10% of patients cannot use immunotherapy due to immune-suppression or autoimmune diseases. Therefore, we sought to identify new therapeutic targets and novel treatment options for MCC patients. Towards this goal, we screened over 4,000 clinical drugs and pre-clinical oncology compounds for their ability to reduce viability in VP-MCC, VN-MCC, and immortalized control cell lines. After grouping compounds by target and ranking compounds on a combined parameter of potency and efficacy, we found that aurora kinase inhibitors selectively and effectively reduced VP-MCC viability relative to VN-MCC and immortalized controls. In parallel, using an RNAi library targeting ~700 kinases we found that knockdown of aurora kinase B (and not other aurora kinase family members) selectively reduced VP-MCC viability, with variable effects on VN-MCC viability. A specific aurora kinase B inhibitor demonstrated on-target inhibition of Histone H3 phosphorylation, induced cell cycle arrest, and reduced viability in all VP-MCC cell lines, further validating aurora kinase B as a therapeutic target. Importantly, in a preclinical xenograft mouse model of VP-MCC, a novel aurora kinase B inhibitor nanoformulation shrank MCC tumors over a four-week treatment period and increased median survival from 64 days (vehicle) to 218 days (treated). Taken together, we have identified aurora kinase B as a novel therapeutic target for VP-MCC and identified an aurora kinase B inhibitor nanoparticle as a new treatment for VP-MCC.

Daniel Benhalevy

Visiting Fellow

NIAMS

Gene Expression

Proximity-CLIP provides a snapshot of protein-occupied RNA elements in sub-cellular compartments

Many cellular RNAs localize to specific cellular compartments but methods to systematically study subcellular RNA localization are limited and lagging behind proteomic tools. Here, we combined APEX2-mediated proximity biotinylation of proteins with photoactivatable ribonucleoside-enhanced crosslinking to simultaneously profile the proteome, as well as the transcriptome bound by RNA binding proteins in any given subcellular compartment. Our approach is fractionation-independent and enables to study the localization of RNA processing intermediates, as well as the identification of regulatory RNA cis-acting elements occupied by proteins in a cellular compartment-specific manner. We applied Proximity-CLIP to study RNA and protein in the nucleus, cytoplasm and at cell-cell interfaces. Among other insights, we observed frequent transcriptional readthrough continuing for several kilo-bases downstream of the canonical cleavage and polyadenylation site and a differential RBP occupancy pattern for mRNAs in the nucleus and cytoplasm. Surprisingly, mRNAs localized to cell-cell interfaces often encoded regulatory proteins and contained protein-occupied CUG sequence elements in their 3' untranslated region, which have previously been implicated in targeting mRNAs to the membrane and in several repeat expansion neurodegenerative diseases, including myotonic dystrophy.

Younglang Lee

Visiting Fellow

NIAMS

Immunology - Autoimmune

K63-polyubiquitinated A20 regulates NF- κ B activation through K48-linked targeting of NEMO

Activation of the NF- κ B family of transcription factors is related to diverse biological processes, such as development, immune responses, and inflammation. As a result, mutations affecting the NF- κ B signaling pathway cause syndromes characterized by immunodeficiency and autoinflammatory disease. The NF- κ B essential modulator, NEMO, is a scaffolding protein that is essential for IKK kinase activation, which activates NF- κ B. Recently, we identified the C-terminus of NEMO to be a key negative regulator of NF- κ B signaling, since a class of NEMO mutations lacking the C-terminus (that we call deltaCT-NEMO mutants) confer gain-of-function properties to the IKK complex due to impaired recruitment of A20 to the TNFR. Here, we investigated details by which A20 recruitment to the TNF receptor suppresses NF- κ B signaling. The C-terminus of NEMO has been previously identified to preferentially recognize K63-linked polyubiquitin that in its unanchored form can mediate NEMO/A20 interaction. By using in vitro pull-down assays, we found that the IKK complex containing deltaCT-NEMO forms but not other mutant forms of NEMO exhibit impaired K63 linked polyubiquitin binding ability. In addition to unanchored K63-linked polyubiquitin, we hypothesized that direct K63 linked polyubiquitination of A20 could enable a functional interaction between A20 and NEMO. Using a combination of mass spectroscopy and deletion mutagenesis, we identified 4 lysine residues on A20 that were likely sites of ubiquitination. Mutation of these lysines to arginine impaired A20's ability to suppress TNF-induced NF- κ B activation. Furthermore, A20 lysine mutants showed reduced association with NEMO by co-immunoprecipitation studies. We also explored molecular mechanisms by which A20 may work to suppress NF- κ B activation. Using a combination of biochemical and microscopy studies in which we expressed ubiquitin forms that can only form K48 linkages, and studying proteasomal dependent stability of NEMO, we found increased A20 dependent K48-linked polyubiquitination of NEMO at the TNF receptor complex following TNF stimulation. Our data suggest a mechanism of NF- κ B regulation in which A20 is directly K63 polyubiquitinated to

functionally interacts with NEMO which it targets by K48 linkages proteasomal degradation. Understanding the details of these molecular interactions may lead to novel targets to modulate inflammatory disease and immune response pathways.

Kelly Hudspeth

Postdoctoral Fellow

NIAMS

Immunology - Autoimmune

Natural Killer Cell Regulation of Humoral Immune Responses

Natural Killer (NK) cells, an important component of the innate immune system, are historically known as cells that lyse virus-infected or tumor transformed cells. Recently, it has been shown that NK cells can control adaptive immune responses by killing antigen specific T cells during virus infections. However, virus-specific T cells are normally protected from NK cell lysis through the ability of virus-induced type I interferons (IFN α / β) to induce the inhibitory NK cell ligand, Qa1b on T cells. Blocking IFN's or Qa1b interaction with its' receptor, NKG2A, results in T cells being susceptible to NK cell lysis. IFNs are substantially increased in patients with the autoimmune disease systemic lupus erythematosus (SLE), suggesting that chronic exposure to IFN may protect autoreactive lymphocytes from NK-mediated killing. Interestingly, using multiple lupus-prone mouse models which have chronic expression of IFNs, we have found that Qa1b is more strongly upregulated on B cells than T cells, compared to age and sex matched healthy control mice. This strong de novo expression of Qa1b specifically occurred on plasma cells, antibody producing cells which likely contribute to disease pathogenesis by making autoantibodies. We also found that, upon immunization with soluble protein and adjuvant, which results in the production of IFNs, Qa1b is induced on B cells, similar to lupus-prone mice. Furthermore, we show that IFNs are both necessary and sufficient to drive the high expression of Qa1b on B cells. In vitro killing assays demonstrated that, while IFN β protected B cells from NK cell cytotoxicity, IFN β failed to protect Qa1b deficient B cells from NK cell killing. Future studies will address if absence of Qa1b in vivo can modulate humoral immune responses through NK cell killing after immunization and in lupus prone mice. Comparison of patients with SLE and healthy donors revealed that the human homolog to Qa1b, HLA-E, is significantly increased on SLE B cells and specifically plasma cells, similar to lupus mouse models. These results suggest a novel regulatory mechanism that occurs in both autoimmune disease as well as normal immune responses that IFNs employ to protect B cells from NK cell mediated killing. Importantly, our data reveal a potential new therapeutic avenue with which to treat SLE.

Benjamin Voisin

Postdoctoral Fellow

NIAMS

Immunology - Innate and Cell-mediated Host Defenses

Skin macrophage to fibroblast cross-talk is essential for hypodermal integrity

The skin is a complex, multilayered, and highly immunologically active organ. Unveiling the crosstalk between immune and non-immune cells within skin is a necessary challenge to understand the molecular and cellular mechanisms leading to pathological states and their treatments. Skin macrophages (Macs) have been described as important orchestrators of wound healing processes by

regulating endothelial cell and fibroblasts biology. However, little is known about the contribution of Macs to homeostatic functions of the skin. Particularly, the biology of Macs of the deepest layer of the skin, the hypodermis, remains largely unexplored. Using flow cytometry, bone-marrow transfer, and a parabiotic mouse model, we found that dermal and hypodermal Macs are a combination of tissue-resident (TR-Macs) and monocyte-derived (Mono-Macs) macrophages. We assessed their growth factor dependency using *Ccr2*^{-/-} and *Csf1*^{op} transgenic mice and confirmed that Mono-Macs of dermis and hypodermis are bone marrow dependent while both dermal macrophage subsets were highly sensitive to changes in stromal-derived CSF1. RNA sequencing analysis of sorted macrophage subsets from dermis and hypodermis revealed significant differences in gene expression between dermal and hypodermal Macs, pointing to layer specific functions. To gain deeper insights, we took advantage of the recent advances in single-cell transcriptomic analyses, and generated cDNA libraries from wild-type, *Ccr2*^{-/-} and *Csf1*^{op}/wt dermal and hypodermal single cells. Interestingly, alterations of macrophage populations in *Ccr2*^{-/-} and *Csf1*^{op}/wt mice were associated with considerable transcriptomic changes within hypodermal fibroblasts. Particularly, expression of transcripts associated with the extracellular matrix were preferentially altered. Taking advantage of our single cell data, we identified the fibroblasts and endothelial cells as the major source of CSF1, and accordingly generated a mouse model (*Tie2*-cre x *Csf1*-Flox) to specifically deplete Macs into the hypodermis. Strikingly, by chemical and immunofluorescence staining of skin sections we uncovered that the absence of hypodermal Macs was associated with a decrease of fibrillary collagen deposits into the extracellular matrix of the hypodermis resulting on the macroscopic level in a loose skin phenotype. Thus, our work provides evidences for a macrophage-fibroblasts cross-talk essential to the hypodermal integrity under steady-state conditions.

Akihiko Uchiyama

Other

NIAMS

Gene Expression

Inherent transcriptional factors SOX2 and PITX1 in human oral epithelia determine heightened wound healing

Wound healing is a dynamic process involving re-epithelialization, angiogenesis, production of soluble mediators and extracellular matrix, and migration of various types of cells, including keratinocytes, fibroblasts, inflammatory cells. Improving wound-healing resolution is a major medical and social priority due to the drastic increase in traumatic injury, chronic wounds and scarring. Oral wound healing, along with embryonic wound healing, have long been considered ideal systems of wound resolution, due to their rapid healing without scar formation when compared to skin wound healing. However, it was unclear if there were unique genes or transcriptional networks in oral mucosa which prime oral keratinocytes to accelerated wound healing in human. The object of this study is to determine the regulation and contribution of the distinct molecular events that drive wound healing resolution in oral mucosa compared with those of the skin. Moreover, defining how these oral lesions heal more efficiently will potentially aid in translating those findings in treatment of deficient healing processes. This project contains three components: a longitudinal human clinical study, in vitro examination using human keratinocytes and in vivo skin wound healing experiments using transgenic animals. Our human clinical study showed oral wounds resolved faster compared to skin wounds. RNA-sequencing, Gene Ontology, IPA and histological analysis using paired biopsies from human oral mucosa and skin samples

revealed significantly different patterns in gene expression, molecular functions and biological processes, especially keratinization, epidermal cell differentiation, responses to biotic stimulus and inflammation. We identified a characteristic expression of the transcriptional regulators SOX2 and PITX1, which confer a specific identity to oral keratinocytes. In vitro, SOX2 and PITX1 had the potential of reprogramming skin keratinocytes to acquire increased cell migration capability and improve wound resolution. In vivo, we found that skin wound healing was promoted in SOX2 conditional overexpressing mice (K14CreERTM/LSL-SOX2) via enhancement of the re-epithelialization process and formation of granulation tissue. We present a unique combination of human clinical data, histological, gene expression analysis, and mouse wound healing data. This information has been pivotal in determining the molecular anatomy of the wound healing processes in oral and skin epithelia.

Kathleen Baysac

Postdoctoral Fellow

NIAMS

Clinical and Translational Research, and Gene Therapy

Rare missense variants of PLCG2 contribute to common variable immune deficiency susceptibility - expanding the spectrum of immune dysregulation related to PLCG2

Disorders of immune dysregulation are primary immunodeficiencies that are marked by increased susceptibility to infection, autoimmunity and atopy. In these conditions, dysfunctional lymphocyte signaling leads to ineffectual humoral responses and an improperly tolerized B cell repertoire. Common variable immune deficiency (CVID), the prototypic disorder of immune dysregulation, is characterized by antibody deficiency, recurrent infections, poor response to vaccines and other complications including autoimmunity. Although a small proportion is monogenic, CVID is predominantly polygenic and its genetic risk factors are poorly understood. Phospholipase C gamma 2 (PLCG2)-associated antibody deficiency and immune dysregulation (PLAID) is an autosomal dominant form of immune dysregulation that has substantial phenotypic overlap with CVID. PLAID is caused by regulatory deletions of PLCG2, a pivotal enzyme in B cell differentiation, maturation and antibody production. Based on the function of PLCG2 and the phenotypic overlap between PLAID and CVID, we hypothesized that PLCG2 variants influence CVID risk. Using a combination of targeted deep resequencing and Sanger sequencing, we examined PLCG2 in 184 CVID patients and 92 controls. We found 9 rare (allele frequency < 0.01) missense variants in the CVID patients, including 2 previously unreported mutations (A180G, D973N). All variant sites were evolutionarily conserved and 8 variants were predicted to be damaging by 1 or more algorithm. Rare PLCG2 variants were detected in 22 CVID patients (12%) but only 4 controls (4.3%). To more rigorously evaluate this trend, we compared the distribution of rare conserved PLCG2 variants in our CVID population with that in the 33,370 Non-Finnish European ExAc subjects. Using SKAT, a rare variant association test, we found that rare variants of PLCG2 were associated with CVID ($p=5E-5$). Moreover, upon overexpressing PLCG2 mutants in cell lines, we observed that several mutants had less phospholipase activity and/or decreased BCR-induced ERK phosphorylation than wt PLCG2. Beyond CVID, we are evaluating >40 patient referrals with ultra-rare PLCG2 variants and severe immune dysregulation. In vitro studies are underway to prioritize recruitment and analysis of these patients at the NIH Clinical Center. This is the first study to implicate PLCG2 in CVID susceptibility. Collectively, we seek to characterize and explore the relationship between PLCG2 variation and immune dysregulation.

Natasha Hill

Postdoctoral Fellow

NIAMS

Pharmacology and Toxicology/Environmental Health

A novel combination of disulfiram, copper, and etoposide is highly effective in killing Merkel cell carcinoma.

Merkel cell carcinoma (MCC) is a rare and aggressive neuroendocrine skin cancer with a relative mortality higher than malignant melanoma. The FDA recently approved the checkpoint inhibitor avelumab for advanced stage MCC, however this treatment provides benefit for less than 50% of MCC patients. To identify novel treatments for MCC, we performed high-throughput screening of over 4,300 small molecules in collaboration with the National Center for Advancing Translational Sciences. Among the agents that selectively reduced MCC viability was disulfiram, an aldehyde dehydrogenase (ALDH) inhibitor used in the treatment of alcoholism. Recent studies suggest that disulfiram complexed with copper can have anti-cancer effects. To determine if disulfiram could be repurposed to treat MCC, we measured dose responses in 7 MCC cell lines and a keratinocyte cell line. Cell viability assays showed that disulfiram potently reduced MCC cell viability compared to keratinocyte controls. The addition of copper further increased the potency of disulfiram in 5 of the MCC cell lines. Next, we investigated whether disulfiram combined copper would synergize with etoposide in reducing MCC viability. Etoposide, a topoisomerase II inhibitor, in combination with cisplatin has demonstrated high response rates in advanced MCC, however responses are not durable and there is dose-limiting toxicity. In MCC cell lines, the addition of disulfiram plus copper improved the effective dose (ED50) of etoposide from 100 nM to less than 1 nM. Taken together, our data suggest that disulfiram combined with copper could be repurposed for the safe and effective treatment of advanced MCC. Moreover, combining disulfiram plus copper with etoposide could potentially serve as a new treatment regimen for MCC. One strong advantage of this combination is that all agents can be given orally and at dosages with minimal adverse effects.

Fuwu Zhang

Visiting Fellow

NIBIB

Chemistry

Polymeric Nanoparticles with Glutathione-Sensitive Heterodimeric Multifunctional Prodrug for In Vivo Drug Monitoring and Synergistic Cancer Therapy

Polymeric micelle-based drug delivery systems have dramatically improved the delivery of small molecular drugs, yet multiple challenges remain to be overcome. Part of the challenges these micellar formulations face are low drug loading capacity, premature drug release, inability of in vivo drug monitoring, and limited drug accumulation in the tumor tissues. In this work, we engineered a polymeric nanomedicine that possesses an ultrahigh loading (59%) of a glutathione (GSH)-sensitive heterodimeric multifunctional prodrug (HDMP) to effectively co-deliver two synergistic drugs to tumor. Specifically, an HDMP comprising of chemotherapeutic camptothecin (CPT) and photosensitizer 2-(1-hexyloxyethyl)-2-devinyl pyropheophorbide-a (HPPH), was conjugated via a GSH-cleavable linkage. The intrinsic fluorogenicity and label-free radio-chelation (Cu-64) of HPPH enabled direct drug monitoring by fluorescence imaging and positron emission tomography (PET). Through quantitative PET imaging, we

demonstrated that HDMP significantly improves drug delivery to tumor. The high synergistic therapeutic efficacy of HDMP-loaded NPs highlights the rational design of HDMP, and presents exciting opportunities for polymer NP-based drug delivery.

Razvan Chereji

Research Fellow

NICHD

Chromatin and Chromosomes

Precise genome-wide mapping of single nucleosomes and linkers in vivo

In eukaryotes, nucleosomes are the basic units of DNA packaging. Nucleosome positions affect all DNA-related processes in the cell because nucleosomal DNA is generally occluded from interacting with other proteins. Micrococcal nuclease (MNase) digestion followed by sequencing (MNase-seq) is the most used method of mapping nucleosomes. Unfortunately, MNase digests different sequences with different rates, and nucleosome maps are affected by the degree of digestion. Moreover, other proteins also offer protection against MNase and can be mistaken for nucleosomes in MNase-seq experiments – so called “fragile nucleosomes”. To eliminate the biases introduced by MNase, we have developed a chemical cleavage method that allows us to precisely map both single nucleosomes and linkers with very high accuracy genome-wide in budding yeast. We confirm that *S. cerevisiae* promoters are sites of strong nucleosome depletion but attribute the putative nucleosome depletion seen at termination sites to MNase bias. Our nucleosome mapping data has the highest resolution among the currently available techniques (single-bp resolution, compared to ~10-bp resolution of MNase-seq or ~100-bp resolution of ChIP-seq), and this accuracy allows us to distinguish alternative rotational positions that nucleosomes occupy in different cells. Furthermore, we show that linker DNA has quantized lengths for individual genes. By comparing our nucleosome dyad positioning maps to existing genomic and transcriptomic data, we evaluate the contributions of sequence, transcription, histone H1 and the H2A.Z variant in defining the chromatin landscape. We show that DNA sequence has a very limited effect on establishing the nucleosome organization as observed in vivo. Moreover, we find that the degree of gene compaction, measured by the spacing between neighboring nucleosomes, correlates with the transcription level, amount of histone H1 bound to the gene, and the amount of H2A.Z variant that is incorporated in the nucleosomes closest to the gene promoter (+1 nucleosomes). Furthermore, we present a biophysical model based on simple physical principles, which shows that steric exclusion between neighboring nucleosomes suffices to explain the salient features of nucleosome positioning and the complex nucleosome phasing pattern that is observed near the gene ends.

Pnina Gershon

Postdoctoral Fellow

NICHD

Epidemiology/Biostatistics - Prognosis, Response Predictions, Etiology, Risk, and Prevention

Crash Risk and Risky Driving Behavior Among Adolescents During Learner and Independent Driving Periods

Purpose: Motor vehicle crashes are the lead cause for death and disabilities among teens 15 to 19-years old. Crash rates of teen drivers are highly elevated early in licensure, despite substantial practice driving

during the learner period. This study examined the variability in measures of driving risk during the learner (supervised) and early independent driving periods according to age, gender and time-of-day. Methods: Objective data from a naturalistic cohort study of 90 teens and 131 adults were collected over a period of 2-years during the learner and early independent driving periods. Participants' private vehicles were equipped with data acquisition system that recorded driving kinematics, mileage, and video cameras. Crash/near-crash (CNC) and kinematic risky driving (KRD) rates were calculated by driving periods, gender (female/male), and time-of-day(day/night) for teens and adults. Mixed effect Poisson regression models with random intercept were used to calculate CNC and KRD incident rates (IRs). Age groups were compared by Incident Rate Ratios (IRRs). Three-way interactions between age group, driving period and each of the following factors: gender, and time-of-day were assessed. Results: CNC and KRD rates of teens were similar to adult drivers during the learner period (CNC: IRR=1.66, CI=0.98-2.82; KRD: IRR=1.04, CI=0.78-1.40), but dramatically higher in the first year of independent driving (CNC: IRR=6.51, CI=4.03-10.51; KRD: IRR=3.95, CI=2.96-5.26), and particularly elevated the first 3 months of independent driving. Teens KRD rates were higher for males than females and invariably higher than adult rates. Conclusions: This study is the first to provide objective longitudinal CNC and KRD rates for teens over the learner period and first year of independent driving. Teens' risky driving during early independent period was significantly higher compared to the learner period and to adult drivers. Relatively safe learner driving period indicated that teens can drive safely with an adult in the vehicle; however, the transition to independent driving was typified by a dramatic and persistent increase in risk that was largely invariant by the driving conditions assessed. We conclude that during the first year of driving on their own, teens either prefer risky driving or lack the judgment required to drive safely. This highlights the possible benefits of purposefully maintaining adult presence in the vehicle during early stages of independent driving.

Neha Gupta

Postdoctoral Fellow

NICHD

Gene Expression

Reconstitution of mRNA-specific and eIF4G-dependent mechanisms of Ded1 stimulation of 48S translation pre-initiation complex assembly

The steps of eukaryotic translation initiation include assembly of the 43S pre-initiation complex (PIC), recruitment of the PIC to the mRNA, and scanning of the 5'- untranslated region (5'-UTR) for the start codon. Essential DEAD-box RNA helicases eIF4A and Ded1 are thought to facilitate this process by resolving 5'-UTR mRNA structures. We showed previously that mRNAs with long, structured 5'-UTRs have a heightened dependence on Ded1 in vivo. Here we demonstrate that Ded1 stimulates the recruitment of all mRNAs tested in vitro; however, mRNAs harboring the structured 5'-UTRs from mRNAs hyperdependent on Ded1 in vivo have a higher requirements for Ded1 for efficient 48S PIC assembly in vitro. Disrupting or strengthening a stem-loop (SL) in the 5'-UTR decreases or increases, respectively, the Ded1-requirement for recruiting mRNA to the PIC, consistent with Ded1's proposed role in resolving 5'-UTR structures. Interestingly, Ded1 changes the requirements for initiation factors eIF4G eIF4E, eIF4B and eIF4A. The effects of mutations altering eIF4G-Ded1 interaction differ substantially depending on the mRNA, suggesting that distinct mechanisms for Ded1 function are dictated by the sequence or structure (geometry) of each mRNA. Whereas interaction between the C-

terminal domain of Ded1 and the eIF4G-RNA3 domain is crucial for less structured 5'-UTRs, Ded1 interaction with the eIF4G-RNA2 is important for 5'-UTRs harboring SLs. N-terminal domain of Ded1 also seems to play a role in recruitment of SL containing 5'-UTRs. Similar effects were observed with synthetic mRNAs containing unstructured 5'-UTR versus cap-proximal or cap-distal SLs, allowing us to define 5'-UTR features that influence different Ded1 interactions with the PIC and mRNA recruitment factors. In summary, we have reconstituted the role of Ded1 in unwinding structured 5'-UTRs in vitro, uncovered distinct requirements for Ded1-eIF4G interactions for different mRNAs, and delineated 5'-UTR features that dictate these mechanistic differences.

David Gershlick

Visiting Fellow

NICHD

Cell Biology - Intracellular Trafficking, Cytoskeleton, and Extracellular Matrix

Abstract removed at request of author

Abstract removed at request of author

Tal Keren-Kaplan

Postdoctoral Fellow

NICHD

Cell Biology - Intracellular Trafficking, Cytoskeleton, and Extracellular Matrix

Abstract removed at request of author

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Deepika Shrestha

Visiting Fellow

NICHD

Epidemiology/Biostatistics - Prognosis, Response Predictions, Etiology, Risk, and Prevention

Influence of maternal and offspring genetic susceptibility to obesity on birthweight in African ancestry populations: intra-uterine vs shared genetic influence?

Background: Genetic susceptibility to adulthood obesity can influence birthweight through mechanisms involving the fetus (through shared genetic effect) and/or the mother (by modulating the intra-uterine environment). We investigated the effects of fetus and maternal genetic risk of obesity on birthweight and evaluated whether these genetic influences modify the well-known association between maternal pre-pregnancy BMI (ppBMI) and birthweight. Methods: Genotypic and phenotypic data of 950 mother-baby pairs of African ancestry were obtained from the Hyperglycemia Adverse Pregnancy Outcome study (dbGAP study accession phs000096.v4.p1). A genetic risk score for obesity was generated for mothers (mGRS) and babies (bGRS) as the weighted sum of 97 BMI-increasing alleles. The median GRS was used to categorize samples as having high-low genetic risk for obesity. Linear regression analysis was performed to calculate the association adjusting for birth weight covariates and proportion of African ancestry. Results: A one allele increase in bGRS was significantly associated with a 13.0 g lower birthweight [95% CI=-24.7, -1.4]. High bGRS was significantly associated with 70.9 g lower birthweight

(95% CI=-130.5, -15.2) compared to low bGRS. However, mGRS was associated with a modestly higher birthweight but did not reach statistical significance. The significant birthweight-increasing effect of maternal ppBMI ($\beta=6.5$, 95% CI=1.2, 11.9) was modified by mGRS (P for interaction =0.03); ppBMI had a stronger and significant association with birthweight among low mGRS pregnancies ($\beta=8.7$, 95% CI =1.1, 16.2) but not among high mGRS pregnancies ($\beta=4.5$, 95% CI=-2.9, 12.0). Conclusions: Fetal genetic risk to obesity in later life had strong birthweight-lowering effect as opposed to the weak birthweight-increasing effect of maternal genetic risk to obesity. Findings suggest that obesity genetic risk loci are important components of the life course associations between birthweight and obesity in later life.

Vinay Sharma

Visiting Fellow

NICHD

Signal Transduction - G-protein and Ion Channels

New role of serotonin receptor HTR1E in mediating neurotrophic effects of NF-a1/CPE through ERK signaling

Neurotrophic factor- α 1/Carboxypeptidase E (NF- α 1/CPE) is secreted by neurons and endocrine cells where it performs different functions. It acts as a prohormone processing enzyme intracellularly and a neurotrophic factor, extracellularly in neuroprotection and cellular differentiation. Previously we showed that application of NF- α 1/CPE can induce neural stem cells differentiation to astrocytes during neural development, and it can also protect hippocampal neurons from oxidative stress-induced cell death by increasing expression of FGF2 and pro-survival protein BCL2 through ERK and AKT pathways. Based on these findings, we hypothesize that there should be NF- α 1/CPE receptors at the cell membrane to regulate these functions. Radiolabelled ligand binding studies were performed in neuronal HT22 cell line. Using recombinant CPE labeled with ¹²⁵I, specific binding was obtained which indicates that NF- α 1/CPE work through a ligand-receptor complex. Next, a high throughput screening was performed against a library of 324 orphan GPCRs using CPE protein. More than 10 different GPCRs were found to be activated from this library. These potential GPCR targets were further validated by luciferase assay based on PRESTO-TANGO assay system in HTLA cell line. Only one of them, 5-hydroxytryptamine receptor 1-E (5-HTR1E) showed a strong positive signal. HTR1E which is a member of the serotonin receptor family is a G_i-coupled receptor which inhibits adenylate cyclase activity when activated by serotonin. When HTR1E receptor was co-transfected with CPE in the HEK-293 cells, luciferase activity was increased significantly as compared to cells which were transfected with HTR1E only. A synergistic effect was observed when HTR1E and CPE transfected cells were also treated with serotonin, suggesting that HTR1E receptor can have more than one ligand binding site and can regulate different functions based on its ligand. Binding between NF- α 1/CPE and HTR1E was supported by co-immunoprecipitation of NF- α 1/CPE with HTR1E from transfected HEK 293 cells using HTR1E antibodies. Consistent with NF- α 1/CPE's action to increase ERK/FGF2 signaling for neuroprotection, we found that the level of phosphorylated ERK 1/2 and FGF2 was significantly up-regulated when HTR1E expressing cells were treated extracellularly with CPE. This study has provided evidence for a novel role of HTR1E as a potential CPE receptor that can activate downstream ERK signaling to mediate various physiological functions.

Miranda Broadney

Clinical Fellow

NICHD

Endocrinology

Estimating Plasma Glucose with FreeStyle Libre Pro Continuous Glucose Monitor (CGM) During Oral Glucose Tolerance Testing (OGTT) in Children

Background: Timely and accurate measurement of blood glucose is vital in the care of individuals with diabetes and is frequently needed in metabolic research. Approved CGM devices for children are relatively painless and provide glucose measurements over days, but must be frequently calibrated by using glucometers, which require finger-prick blood drops for analysis. Glucometer devices cause pain and although accepted by the diabetes community (due to lack of an alternative) are not easily used in young research volunteers. Thus, less painful devices that do not require glucometer calibrations are needed. The novel FreeStyle Libre Pro CGM has been found, in adults, to have acceptable accuracy compared to lab-measured glucose without requiring finger-prick blood drop calibrations. However, no studies have assessed lab-measured glucose accuracy for this CGM in children. We therefore evaluated the accuracy and practicality of this device during OGTT in nondiabetic youth. We hypothesized that children's CGM-measured glucose will not differ from lab-measured plasma glucose during OGTT. Methods: Healthy weight and overweight volunteers without diabetes (n =8; 63% male), aged 7-11y wore the FreeStyle Libre Pro CGM during a 2-hour OGTT (1.75mg/kg, max 75g). Laboratory plasma glucose was measured at 30min intervals for 120min and compared with the simultaneously obtained 15min average CGM glucose value. Glucose at each timepoint and Area Under the Curve (AUC) for CGM and lab-measured glucose were compared using paired t-tests. A correlation coefficient was calculated for each timepoint. Potential systematic errors were examined using Bland-Altman analyses. Results: AUC for CGM and lab glucose were well correlated ($r= 0.91$, $p=0.006$) and did not differ in mean value ($p=0.84$). CGM and lab glucose readings were not significantly different at any timepoint (all p 's >0.18). There were no significant systematic or magnitude errors as assessed by Bland-Altman analyses at any timepoint. No adverse events occurred with CGM use and all participants considered the device easy to wear. Conclusions: The Freestyle Libre Pro CGM device was well tolerated and provided quantitatively similar glucose readings during OGTT for healthy weight and overweight children when compared to lab-measured glucose. If confirmed in larger studies, this CGM may provide accurate adjunctive data during evaluations of glucose excursions that may be useful for pediatric clinical care and research.

Jeremy Luk

Postdoctoral Fellow

NICHD

Cultural Social and Behavioral Sciences

Sexual orientation disparities in the development of heavy episodic drinking from late adolescence into young adulthood: A two-part latent growth model

Cross-sectional studies have shown sexual orientation disparities in adolescent substance use behaviors. However, the timing of disparities is not well understood. To advance the field, trajectory analyses using longitudinal data are urgently needed. The current research examined the association between sexual orientation and past 30 days heavy episodic drinking (HED, defined as having five or more drinks in a

row on an occasion for males and four or more drinks in a row on an occasion for females) during the transition from late adolescence into young adulthood. Data were drawn from five waves of longitudinal data from the NEXT Generation Health Study (n = 2354; 55.9% female; 5% sexual minorities defined by sexual attraction to same or both sexes). Two-part latent growth modeling (Olsen & Schafer, 2001) was used to estimate two parallel processes from 11th grade to 3 years post high school: the likelihood of any HED over time and the changes in frequency of HED among those who initiated HED. For the full sample, growth model controlling for sex, race/ethnicity, and family affluence indicated that sexual minority status was associated with greater likelihood of any HED (OR = 3.59, 95% CI: 1.08-6.11) but was not associated with more frequent HED ($\beta = 0.070$, $p = 0.169$) in 11th grade. However, sexual minority youth were less likely than heterosexual youth to report increases in both the likelihood (OR = 0.68, 95% CI: 0.53-0.84) and frequency ($\beta = -0.107$, $p = 0.001$) of HED from 11th grade to three years post high school. Analyses stratified by sex indicated that the associations between sexual orientation and HED likelihood and frequency were significant among females but not among males. These findings suggest that sexual orientation disparities in HED varies across development and by sex. Specifically, late adolescence represents a developmental stage during which sexual minority females are more likely than heterosexual females to engage in any HED and serves as an important developmental window for targeted problem drinking intervention. The absence of sexual orientation disparities in HED in young adulthood reflects more rapid increases in HED for heterosexual youth during the developmental transition from late adolescence to young adulthood. Additional research is needed to identify psychosocial factors that explain these developmental patterns and understand whether similar time-specific effects can be generalized to marijuana or other illicit drug use.

Maria Queralt Martin

Postdoctoral Fellow

NICHD

Biophysics

Role of VDAC3 ion channel activity in mitochondria homeostasis and neurodegeneration

The voltage-dependent anion channels (VDACs) are the most abundant channel-forming proteins in the mitochondrial outer membrane (MOM). VDACs constitute the main pathways across MOM for organic ions and respiratory substrates, such as ATP and ADP, thus controlling MOM permeability and function. In mammals, there are three VDAC isoforms with distinct but not understood physiological roles in the control of MOM function. VDAC1 is the most abundant and best characterized isoform which has been shown to be involved in a wide variety of mitochondria-associated pathologies, including neurodegenerative diseases. VDAC1 forms highly conductive ion channels and interacts with alpha-synuclein (α S), a disordered neuronal protein hallmark of Parkinson's disease, thus mediating α S-induced mitochondrial dysfunction. VDAC3 is the least abundant VDAC isoform and is poorly characterized. Its role in the control of MOM function remains elusive. Contrary to VDAC1 or 2, VDAC3 is unable to restore the normal growth of a yeast strain depleted of endogenous VDAC. Moreover, VDAC3 ability to form functional ion channels has been questioned due to the lack of its successful reconstitution into planar lipid membranes (PLM). In this work, using PLM, we have been able for the first time to successfully reconstitute recombinant human VDAC3 channel in the high-conducting or "open" state. We also show that the channel has a low probability to be open and most of the time it resides in low conducting or partially "closed" states. However, the low-probability open state is

similar to that of hVDAC1. A set of cysteine residues predicted to be exposed toward the intermembrane space in VDAC3 has been hypothesized to account for protein low ability to form open channels. To test this hypothesis, we study an engineered cysteine-less hVDAC3 mutant (Dcys) where all cysteines are replaced by alanines. We show that hVDAC3-Dcys forms functional channels with higher open probability compared to wt, but otherwise similar properties. A comparative analysis of aS interaction with hVDAC3 wt and Dcys and with hVDAC1 wt and Dcys provides new clues about poorly understood physiological roles of different VDAC isoforms. This work is the first step towards the in-depth functional characterization of VDAC3 channel and its involvement in mitochondrial metabolism in health and in neurodegeneration.

Jin-Sik Kim

Visiting Fellow

NICHD

Protein Structure/Structural Biology

Structural basis of interactions between Huntingtin and human DHHC17

Protein palmitoylation is the most prevalent form of posttranslational protein lipidation, that is catalyzed by 23 members of the DHHC family of eukaryotic integral membrane enzymes in humans. Palmitoylation impacts almost all areas of cellular physiology and has been linked to several diseases. Between 23 enzymes and several hundreds of substrate proteins, a complex enzyme-substrate network dictates cellular protein palmitoylation. Yet, the molecular underpinnings of these interactions are very poorly understood. Substrate-interactions have functionally been the best characterized for DHHC17, which is highly expressed in the brain. DHHC17 has a non-canonical architecture with six transmembrane helices and an N-terminal ankyrin repeat domain (ANK17) that is the site for interactions with substrates. We published the first breakthrough result (Structure, 2017) where we solved the structure of the ANK17 domain of human DHHC17 in complex with a fragment of SNAP25b, a substrate of DHHC17. However, it still left open major questions such as whether the ANK17 domain interacts with intact substrate proteins in a different way and whether the same mode of interaction holds true for other substrates. My current work is focused on preparation, crystallization and solving the structure of a complex of ANK17 and the entire N-terminal domain of Huntingtin (HTT), the causative agent of Huntington's disease. HTT undergoes many post-translational modifications, prominently palmitoylation, that modulate its function and influence toxicity of mutant HTT. HTT is the most important substrate of DHHC17 and in turn, modulates the properties of DHHC17. In order to purify the complex, I carried out several iterative rounds of expression and optimization tests and honed in on a coexpression strategy. Intriguingly, the initial preparations showed DNA non-specifically bound in my complex. In order to remove DNA and increase sample homogeneity, I introduced an anion exchange column chromatography step that finally yielded enough complex for crystallization experiments. I have initial crystallization hits that I am currently optimizing. Huntingtin is thought to have many different interacting partners in the cell; yet no structures of protein complexes of Huntingtin are known. This study will not only elucidate the mechanism of substrate-interactions of human DHHC17, but will also shed further light on structural basis of interactions of Huntingtin with cellular protein partners.

Mengying Li

Postdoctoral Fellow

NICHD

Cultural Social and Behavioral Sciences

Is taking folate associated with lower risk of diabetes in pregnancy? A prospective cohort study among 14,622 women

Background: Emerging evidence from animal models suggests that inadequate folate intake may play a role in metabolic disturbances. However, few studies have examined whether lower habitual folate intake before pregnancy may be involved in the development of gestational diabetes (GDM), a common pregnancy complication that has substantial adverse health implications for both mothers and their babies over their lifespans. In the present study, we addressed the data gap. In addition, we explored whether the association of folate intake with GDM varied by methylenetetrahydrofolate reductase (MTHFR) C677T genotypes, where the TT genotype leads to substantial reduction in blood folate levels. Methods: The study included 14,622 women in the Nurses' Health Study II who reported at least one pregnancy between 1992 and 2001 (total 20,306 pregnancies). Intakes of folate and other nutrients were assessed every 4 years, starting in 1991, by a food-frequency questionnaire. Incident GDM was ascertained from self-reports of a physician diagnosis. Relative risks (RRs) of GDM by quartiles of folate intake were estimated using log-binomial models. Among a subgroup of 1,385 pregnancies, the analyses were also performed by MTHFR C677T genotypes. Results: During the study period, 829 incident GDM cases were identified. After adjusting for major risk factors, total folate was significantly and inversely associated with GDM risk (RRs across increasing quartiles were: 1.00, 1.05, 0.83, and 0.83, p-trend = 0.01). The association was primarily driven by supplemental folate intake (RRs across increasing quartiles were: 1.00, 0.83, 0.77, and 0.69, p-trend = 0.001). The lower risk of GDM associated with higher total and supplemental folate intakes was not explained by intakes of other micronutrients in multivitamins, nor was it explained by a greater likelihood of pregnancy planning related to higher folate supplement intake. In addition, there was suggestive evidence that the folate-GDM association was modified by MTHFR C677T genotypes. Conclusions: Higher intake of supplemental folate before pregnancy was associated with lower GDM risk. Folate supplementation has been widely adopted for pregnancy planning. It may offer a novel and low-cost avenue to prevent GDM.

Andrew Williams

Postdoctoral Fellow

NICHD

Cultural Social and Behavioral Sciences

Racial Residential Segregation and Racial Disparities in Stillbirth in the United States

Background: Although stillbirth rates are declining, US black women experience two-fold higher rates of stillbirth compared to US white women. Structural racism, including black-white racial residential segregation, is a key determinant of US racial health disparities. It is unclear whether current and/or persistent racial residential segregation is associated with black-white stillbirth disparities. Methods: We examined 49,969 black and 71,785 white births from the Consortium on Safe Labor (2002-2008). We measured black-white segregation using the dissimilarity index (the differential distribution of racial/ethnic groups within a geographic area) and the isolation index (the probability that a member of one racial group will interact with a member of the same racial group), categorized into population-

based tertiles: high, moderate, low. Current segregation was based on birth year segregation. Persistent segregation was measured by comparing 1990 segregation to birth year segregation. Stillbirth was fetal death ≥ 23 weeks gestation reported in medical records. Hierarchical logistic regression models estimated race-specific associations between current and persistent segregation and stillbirth; high levels of current and persistent segregation were the reference category. We estimated number of fewer stillbirths attributable to decreasing segregation using the population attributable fraction. Results: For current segregation, low segregation was more beneficial to blacks (Dissimilarity Odds Ratio [OR]: 0.43 95% Confidence Interval [95% CI]: 0.29, 0.63; Isolation OR: 0.25 95% CI: 0.16, 0.41) than whites (Dissimilarity OR: 1.42 95% CI: 0.77, 2.59; Isolation OR: 0.33 95% CI: 0.21, 0.53). For persistent segregation, decreasing segregation was also more beneficial to blacks (Dissimilarity OR: 0.53 95% CI: 0.32, 0.89; Isolation OR: 0.19 95% CI: 0.10, 0.38) than whites (Dissimilarity OR: 0.75 95% CI: 0.41, 1.37; Isolation OR: 0.82 95% CI: 0.46, 1.48). Approximately 900 stillbirths among blacks could be prevented annually by decreasing segregation. Conclusions: Low and decreasing levels of segregation were associated with reduced odds of stillbirth, with blacks benefitting more than whites. Despite decreasing levels of segregation, the black-white disparity in stillbirth remains. These findings suggest reducing structural racism, like segregation, can improve health outcomes for blacks, and could potentially reduce persistent racial health disparities.

Lan Xiao

Postdoctoral Fellow

NICHD

Neuroscience - Neurodegeneration and Neurological disorders

Carboxypeptidase E/Neurotrophic factor- $\alpha 1$ is a novel neuroprotective protein functioning independently of its prohormone processing enzymatic activity

Carboxypeptidase E (CPE) also known as Neurotrophic factor- $\alpha 1$ (NF- $\alpha 1$), was first identified as an exopeptidase and is highly expressed in the nervous and endocrine systems. It functions intracellularly as a prohormone/proneuropeptide processing enzyme. Interestingly, recent studies show that CPE/ NF- $\alpha 1$ plays a critical role extracellularly, as a trophic factor to mediate neuroprotection, stem cell differentiation and neurite outgrowth in vitro. To show that these neurological functions are independent of its enzymatic activity, we generated a CPE/NF- $\alpha 1$ knockout (CPE-KO) and a mouse model with a CPE E342Q point mutation which obliterates the enzymatic activity. We found that both CPE-KO and E342Q mice exhibited increased body weight and glucose levels due to lack of processing of prohormones and neuropeptides to yield insulin and peptides that affect feeding. Brain morphology studies by Nissl staining revealed that the CPE-KO mice had significant degeneration of CA3 regions of hippocampus, while E342Q mice had an intact hippocampus, similar to wild-type (WT) animals. In addition, doublecortin positive cells (neuroblast marker) in CPE-KO mice were significantly decreased in dentate gyrus, but there was no difference between E342Q and WT mice. Microtubule-associated protein 2 staining showed degeneration of dendrites in the hippocampal CA3 region in CPE-KO mice, but there was no difference between E342Q and WT mice. To investigate the effect of E342Q mutation on various behaviors, open field, forced-swim test, sucrose preference and Morris water maze tests were performed on the mice. The results showed that WT and E342Q mice exhibited a gradual decrease in escape latency during the 5-day training in the Morris water maze test, while CPE-KO mice demonstrated an abnormal acquisition curve. In probe test, both WT and E342Q mice spent twice as

much time in the target quadrant than other quadrants for CPE-KO mice. These results suggest E342Q mutant mice maintain normal learning and cognitive ability despite the loss of CPE enzymatic function; in contrast learning and memory function were compromised in CPE KO mice. This study provides in vivo evidence that CPE/NF-a1 is a novel trophic factor that functions in neuroprotection, independent of its enzymatic role in prohormone processing. CPE/NF-a1 is a potential therapeutic target for treating neurodegenerative disorders such as Alzheimer's disease.

Jonathan Murphy

Postdoctoral Fellow

NICHHD

Neuroscience - Cellular, Molecular, Neurotransmission and Ion Channels, and Neurochemistry

Targeting epilepsy through a novel ion channel complex

The hippocampus is a brain structure responsible for memory and is particularly susceptible to damage from seizure. Understanding the cellular basis for memory and the molecular changes associated with epilepsy requires a knowledge of the fundamental properties of the relevant neurons. Hippocampal neurons integrate thousands of synaptic inputs to reach the threshold to fire an action potential, the primary output of a neuron. Integration of synaptic input depends on ion channels that regulate excitation. The A-type voltage-gated potassium channel (Kv4.2) is a key regulator of integration that inhibits excitability and has reduced function in epilepsy. However, Kv4.2 function is enhanced by a family of Ca²⁺-binding proteins known as K⁺ channel interacting proteins (KChIPs) that associate with Kv4.2; however, it is unknown if intracellular Ca²⁺ regulates Kv4.2. Remarkably, we uncovered a mechanism by which Ca²⁺ entry through the R-type voltage gated Ca²⁺ channel (Cav2.3) may enhance Kv4.2 function by elevating local Ca²⁺. We first identified Cav2.3 in a proteomic screen for Kv4.2 binding proteins. We confirmed the interaction by coimmunoprecipitation of the Cav2.3-Kv4.2 complex, Förster resonance energy transfer microscopy, and by dual-labeling immunogold electron microscopy in the rodent hippocampus. Using fluorescence recovery after photobleaching microscopy, we measured an increased mobile fraction of Kv4.2-GFP in neurons of Cav2.3 knockout (KO) (~68%) when compared to wild-type (WT) (~58%). After confirming the Cav2.3-Kv4.2 complex in rodent hippocampus we next asked if Cav2.3 regulates Kv4.2 channel function. Using whole-cell patch clamp recordings, we measured K⁺ currents in cells expressing Kv4.2 and KChIP2 with or without Cav2.3. In the presence of Cav2.3, K⁺ current is increased (~25%). When we replaced Cav2.3 expression by elevating intracellular Ca²⁺ (10µM) we observed a similar increase in Kv4.2 current (~30%) suggesting that Cav2.3 Ca²⁺ entry may underlie Kv4.2 current enhancement. Furthermore, patch clamp recordings in hippocampal brain slices showed a decrease in Kv4.2 currents in Cav2.3 KOs when compared to WT. Therefore, the Cav2.3-Kv4.2 interaction promotes Kv4.2 function in the hippocampus and we are actively working to understand how this ion channel complex may regulate neuronal excitability. Importantly, this work may lead to the identification of new ion channel drug targets that could aid in the treatment of intractable epilepsy.

Anika Prabhu

Visiting Fellow

NICHHD

Cell Biology - General

Development of a neuronal model of Niemann-Pick C disease using human induced pluripotent stem cells

Niemann-Pick C disease (NPC) is a lethal, progressive neurodegenerative disorder with typical onset during childhood or adolescence. It is caused by mutations in the NPC1 gene, which is responsible for cholesterol efflux from the lysosome. In the absence of functional NPC1 protein, endocytosed cholesterol accumulates in the lysosome and cannot exit into the cytosol. Many in vitro studies of NPC have been performed with peripheral cells in culture. However NPC, which is referred to as "childhood Alzheimer's", is fundamentally a neurodegenerative disease. Thus, the most useful cellular model of NPC will allow the examination of processes that contribute specifically to neuronal loss and the ability to identify therapeutics that are effective in neurons. We have generated a neuronal model of NPC disease using human induced pluripotent stem cells (iPSCs) that harbors an inducible neurogenin 2 transgene. Expression of the neurogenin 2 transcription factor is induced with doxycycline, resulting in the rapid and reproducible differentiation of iPSCs to glutamatergic cortical neurons. Using CRISPR/Cas-9 technology, we successfully knocked-out NPC1 in this isogenic iPSC line, as confirmed by Sanger sequencing and the absence of NPC1 protein. In the wild-type and NPC1 null cells, neuron-like morphology becomes clear just 7 days post-differentiation. At this time, significant accumulation of cholesterol is evident in the perinuclear region NPC1 null cells using markers of cholesterol "filipin and perfringolysin O. These striking changes in the accumulation and distribution of cholesterol can be used for primary screens. In general, high-throughput screens using neurons have been disadvantaged by high costs, low neuronal yields, heterogeneous neuronal populations, and complex differentiation processes. With our NPC model, we are able to avoid these issues and will perform two critical high-throughput screens. The first is a drug repurposing screen using a collection of 2,816 clinically approved drugs, and the second uses existing CRISPR-interference libraries to identify gene modifiers of NPC1. These unbiased screens could reveal new clues to NPC pathogenesis, and help identify viable therapies that impede neurodegeneration in NPC.

Sahar Melamed

Postdoctoral Fellow

NICHD

Microbiology and Antimicrobials

FliA-dependent small RNAs integrate metabolism and flagella biosynthesis

Bacterial flagella are important for both motility and pathogenesis. While synthesis of flagella is energy intensive and is under extensive transcriptional regulation, little is known about the post-transcriptional regulation of this process. In the past two decades, it has become clear that a major class of post-transcriptional regulators are small RNAs (sRNAs), which usually base-pair with mRNAs affecting their stability and/or translation. Therefore, to fully understand the control of flagella synthesis there is a need to characterize and model the post-transcriptional regulation of flagella synthesis by sRNAs. Here we report on four *E. coli* sRNAs whose expression is controlled by the flagella sigma factor (FliA). These four sRNAs originate from the UTR regions of genes, including three genes of the flagella regulon, and add a post-transcriptional level of control to the complex regulatory network controlling flagella synthesis. RIL-seq, a broadly applicable methodology I developed, for in-vivo transcriptome-wide identification of sRNA-target interactions, identified multiple potential targets for the FliA-dependent sRNAs. We investigated the consequences of FliA-dependent sRNAs-mediated regulation of targets

encoding transcriptional regulators of the flagella regulon, metabolic enzymes and ribosomal components. Translational reporter assays showed that two FliA-dependent sRNAs negatively regulate regulators of the flagella regulon. We also found that other known sRNAs additionally repress these targets, forming a complex network controlling the initiation of flagella synthesis. In vitro structure probing assays demonstrated that two of the FliA-dependent sRNAs directly base pair with mRNAs encoding ribosomal proteins, which are required for the mass production of the flagella proteins. Translational reporter assays further revealed that while one of the sRNAs upregulates rproteins and translation, the other sRNA downregulates the rproteins, both by altering the rprotein transcripts levels. The combined effects of this regulation integrate the flagella regulon with metabolism and ribosome levels resulting in unique phenotypes in terms of flagella length and number for each of the sRNAs as documented by electron microscopy. These phenotypes exemplify the diverse impact sRNAs can have on complex regulatory networks and demonstrate how regulatory connections between ribosome synthesis and the flagella regulon enables nuanced control of flagella synthesis.

Medha Raina

Postdoctoral Fellow

NICHD

Microbiology and Antimicrobials

Not just non coding RNAs: competition between protein-coding and regulatory functions of a small RNA

Small RNAs (sRNAs) are important players in the post-transcriptional regulation of bacterial stress responses. Usually, these sRNA regulators, which act by base-pairing, are non coding. However, a few sRNAs have been identified to encode small proteins and are thus “dual-function sRNAs”, though not much is known about the interplay between the sRNA and small protein. We have identified one such new dual functioning sRNA AzuCR in E.coli which has a base pairing function (AzuR) and also encodes a 28 amino acid protein (AzuC). The synthesis of both the sRNA and protein is highly regulated. Transcription of AzuCR is repressed in the absence of glucose. Consistent with this regulation, overexpression of the sRNA causes a growth defect in various non-glucose carbon sources suggesting that the transcript functions as a regulatory sRNA under these conditions. RNA-seq revealed putative mRNAs affected by the sRNA that are consistent with some of the carbon source phenotypes that we observed. Interestingly, translation of the small protein interferes with the observed repression of some of these targets, indicating that the sRNA and the protein function in different pathways. To gain further insight into the function of the protein, we carried out affinity co-purification using SPA-tagged AzuC. This revealed GlpD, an essential enzyme involved in glycerol catabolism and cellular respiration to be the interacting partner of AzuC. Further analysis of the role of the AzuC interaction with GlpD indicated that AzuC expression is essential during metabolic flux during switch from glycerol to glucose carbon metabolism as observed by the growth defect by azuCR deletion strain. We propose that the sRNA is involved in catabolite repression where it represses genes involved in secondary carbon source metabolism in glucose, while the protein might be involved in inhibiting glpD during the flux from glycerol to glucose. We further envision that this interplay between the riboregulation and translation of AzuCR is via the two RNA chaperones Hfq and ProQ, as we found AzuCR to be bound by both. We propose that AzuCR binds Hfq as a mRNA due to the observed regulation of AzuCR translation by another sRNA FnrS and binds ProQ as a sRNA due to its destabilization in ProQ. This study highlights the

complexities of regulation by dual function sRNAs and is providing insight into how a dual function sRNA coordinates its two different activities to carry out its role in the cell.

Elodie Mailler

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NICHHD

HIV and AIDS Research

Abstract removed at request of author

Abstract removed at request of author

Saroj Regmi

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Cell Biology - General

Analysis of Y-complex subunits within the Nuclear Pore: Nup96 is a lynchpin for nuclear pore stability

The nuclear pore complex (NPC) is a channel through the nuclear envelope that facilitates macromolecular transport between the interphase cytoplasm and nucleus. NPCs contain roughly 30 proteins called nucleoporins. Disrupted NPCs and nucleocytoplasmic transport are hallmarks of numerous neurodegenerative diseases, including Huntington's disease and amyotrophic lateral sclerosis. Further, mutations in NPCs are found in many tumors, underscoring the need to better understand the functions of NPCs and their individual components. The vertebrate Y-complex is a major structural component of the NPC that forms a scaffold on the nuclear and cytoplasmic faces of the pore. It contains nine core nucleoporins, and a tenth subunit for chromatin recruitment. The abundance and stability of Y-complex subunits makes them difficult to manipulate with standard *in vivo* methods (e.g., RNAi): The extended time required for depletion produces adverse secondary consequences and leads to instability of the entire complex. To address this problem, we have used CRISPR/Cas9 to add Auxin-Induced Degron (AID) tags at the genomic locus of Nup160, Nup133, Nup96 and Nup85 in human DLD-1 cells. These AID-tagged nucleoporins assemble into functional NPCs, and they are degraded rapidly (<4 hours) after auxin addition. Using these lines, we have assessed the roles of each nucleoporin through analysis of NPC structural stability and function. We found that NPCs remained surprisingly intact after Nup160, Nup133 or Nup85 depletion. Moreover, these depleted cells showed comparable nuclear import rates before and after auxin, suggesting that the NPCs remained largely functional. By contrast, Nup96 depletion caused rapid dispersion of other Nup107-160 subunits and of nucleoporins in other domains of the NPC. Loss of Nup96 was also associated with substantially slower rates of nuclear import. Taken together, our results demonstrate that the scaffolds formed by the Y-complex, once assembled, are remarkably robust in the face of individual subunit loss. However, Nup96 loss causes extensive disruption of NPC structure and function, suggesting that it is a lynchpin for NPC structural stability. In summary, we have found distinct functional roles of individual Y-complex subunits, providing insight into nucleocytoplasmic transport and overall nuclear pore structure. Our future studies will focus on understanding the unique structural requirement for Nup96 and its regulatory implications.

Jiangnan Luo

Postdoctoral Fellow

NICHD

Cell Biology - General

Antagonistic Regulations by Two Afferent-Derived Growth Factors Ensure Appropriate Dendrite Development

1 in 68 children has been identified with some form of Autism Spectrum Disorder (ASD), a developmental and neurological disorder that begins early in childhood and lasts throughout a person's life. One of the most consistent anatomical correlates of this disorder is dendritic abnormalities such as reduced number and length of dendritic branches and aberrant morphology and number of dendritic spines. However, the developmental origins that promote these dendrite pathologies and the subsequent defects in synaptic connectivity are not well understood. In this study, we demonstrated that transient cell-cell interactions at early developmental stages provide an important mechanism for dendrites to achieve the stereotyped morphology and branching complexity. By conducting a morphometric analysis of 1941 single Dm8 cells in the adult *Drosophila* optic lobes with 121 gene mutations, we found that mutation of insulin receptor (InR) and its downstream regulators caused the most severe dendritic and synaptic defects including >30% reduced dendritic field coverage, oversimplified branching pattern and complete loss of synaptic connections with its pre-synaptic partner R7. Using CRISPR/Cas9 to knock-in GFP tag onto the InR locus, we found for the first time the spatiotemporal pattern of InR in Dm8 dendrites: 1) high expression level at early developmental stages (P30-P50) in the central area of dendrites, and 2) significantly diminished expression at the late stages (P70-adult) when the full sizes of dendritic fields are reached. Early Innervation of the InR in Dm8 dendrites requires an insulin ligand DILP2 input from L5, a non-synaptic partner of Dm8. This transient cell-cell interaction positively regulates Dm8's dendritic field elaboration, which operates in parallel but opposite to the interactions between R7 and Dm8. The latter utilized the TGF-beta ligand Activin in R7 and its receptor Baboon in Dm8 dendrites to restrict dendritic elaboration. Simultaneous disruption of these two signaling pathways in Dm8 lead to dendritic mistargeting and increased variability of dendritic field sizes, confirming that an intricate "push and pull" mechanism employed by Dm8 dendrites and their neighbors controls the robustness of dendritic elaboration. Thus, my study reveals one of the fundamental regulatory logics underlying dendrite development, and provides potential therapeutic targets for early intervention of neurological diseases like ASD.

Heeseog Kang

Postdoctoral Fellow

NICHD

Genetics

Somatic activating mutations in MAP2K1 cause melorheostosis

Melorheostosis is a rare condition of excess bone formation for which there is no definitive diagnostic test, and the causative gene was unknown. X-rays of patients have a distinctive appearance of "dripping candle wax" on the surface of cortical bone. The bone overgrowth lesions are associated with pain, deformity, and functional impairment. Because no cases of vertical transmission in families were reported, we hypothesized that somatic mutations in the lesions would be causative. Our goal was to examine bone lesions directly for potential somatic mutations associated with development of

melorheostosis. To obtain samples, we recruited 15 unrelated melorheostosis patients, who underwent paired biopsies of affected and contralateral unaffected bone for DNA extraction and whole exome sequencing. By comparing the paired samples of each patient, we identified somatic mosaic mutations in MAP2K1, encoding Mitogen-Activated Protein Kinase Kinase 1 (MEK1), in DNA from affected, but not unaffected bone, of 8 of 15 patients. The lesions occur at a hotspot (p.Q56P, p.K57E, and p.K57N) located within the negative regulatory domain of MEK1, and were previously identified in various malignancies. Mosaicism was demonstrated by immunohistochemical analysis of ERK1/2 (phos-ERK1/2) activation in affected bone and as two populations with distinct phos-ERK1/2 levels in flow cytometry of affected osteoblasts. Histology of melorheostotic bone revealed two regions: an outer region composed of distinctive parallel layers of primary lamellar bone, and an inner region of intense bone remodeling with increased osteoblasts and osteoclasts. Functional studies of patient affected osteoblasts revealed enhanced cell proliferation with increased cyclin D expression. Paradoxically, the activating MAP2K1 mutations suppressed BMP2-mediated osteoblast differentiation and matrix mineralization in culture, explaining the significantly increased unmineralized osteoid seen in affected bone histology. The immature affected osteoblasts have a higher ratio of RANKL to OPG, which indicates increased osteoclastogenesis by mutant osteoblasts in melorheostotic bone. This study uncovered a new role for the MAP2K1 oncogene in human bone formation. This suggests that MEK1 inhibition, which is already in trials for cancer, may be a good therapeutic candidate for melorheostosis treatment.

Hyun Min Jung

Postdoctoral Fellow

NICHD

Vascular Disease and Biology

Centrosome regulation is essential for endothelial cell division and vascular development

Blood and lymphatic vessel formation play vital roles during development and in the pathology of many diseases. Precisely controlled endothelial cell division is essential to generate the stereotypic, seamless vascular networks required for efficient oxygen and nutrient supply and fluid homeostasis. However, we still have only a limited understanding of how endothelial cells regulate division and migration during development. We recently developed powerful transgenic tools enabling detailed visualization of vascular development and endothelial-specific cell division in the optically clear, experimentally accessible zebrafish model. Using transgenic zebrafish, we performed a large-scale N-ethyl-N-nitrosourea (ENU) forward-genetics mutagenesis screen. From this mendelian phenotype-based mutant screening, we identified a novel recessive mutant exhibiting severe defects in vascular development, including loss of lymphatic vessels and decreased growth of sub-intestinal blood vessels. Positional cloning and exome sequencing revealed a causative nonsense mutation in Centrosomal Protein 192 (cep192), an essential component of the centrosome required for pericentriolar material recruitment, centriole duplication, centrosome maturation, and spindle assembly during cell division. Confocal imaging of cep192 mutants suggests that in addition to defective vessel growth they also have reduced endothelial cell division and aberrant nuclear division. We confirmed this result by a second ENU allele also causing premature termination of the Cep192 gene product, which resulted in an identical phenotype. These mutant animals represent the first vertebrate model for centrosome-defective endothelial cell division, potentially leading to greater understanding of vascular anomalies such as

vascular tumors (hemangioma and lymphangioma), arteriovenous malformations, and vascular gigantism, all of which are highly associated with abnormal nuclear division and endothelial aneuploidy.

MINGAN SUN

Visiting Fellow

NICHD

Molecular Biology - Prokaryotic, and Eukaryotic

Endogenous retroviruses facilitate the evolution of human placental gene expression networks

In mammals, the placenta is an essential organ that mediates nutrient and waste exchange between the mother and fetus. Although the placenta performs the same basic function in all mammals, it is fast evolving and highly diversified in size, structure, and cellular composition. Emerging evidence suggests that endogenous retroviruses (ERVs), the proviral remains of ancient infections of the germline that are abundant in mammalian genomes, can promote genetic innovation by creating novel cis-regulatory elements. Using inter-species transcriptomic and epigenomic analysis of human, macaque, and mouse placenta at birth, we identified hundreds of human-placenta-enriched genes with increased expression relative to other tissues and species – several of which are implicated in pregnancy outcomes. Using histone modification marks associated with enhancer and promoter function (H3K4me3 and H3K27ac), we annotated thousands of human-placenta-specific cis-elements that correlate with expression in human placenta. We found that several ERV families, including MLT1A, MER21A, MER39B and MER41B, are over-represented near both human-placenta-enriched genes and human-placenta-specific cis-elements - likely mediating the evolution of placental gene expression by creating novel cis-elements. Interestingly, one of these ERV families, MER41B, has also been co-opted as a regulatory module in response to interferons in humans, supporting a known link between innate immunity and placental development. In summary, this study represents the first systematic inter-species comparison of the placental transcriptome and epigenome between human and other mammalian species. Our results indicate that ERVs likely played a pivotal role in diversifying transcription and epigenetic landscapes in the placenta of diverse mammals.

Danfeng Cai

Postdoctoral Fellow

NICHD

Biophysics

Abstract removed at request of author

Abstract removed at request of author

Ivaylo Ivanov

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Gene Expression

Feedback translational regulation of the creatine transporter SCL6A8 implicated in X-linked intellectual disability

SLC6A8 is a high-affinity sodium- and chloride-dependent creatine transporter. Mutations in SLC6A8 are the second most common cause of X-linked intellectual disability (XLID), and these mutations have been linked to creatine deficiency in the brain. In a genome-wide analysis of protein synthesis, we identified SLC6A8 in a subset of genes exhibiting high levels of translational regulation. Comparative sequence analysis of SLC6A8 among vertebrates identified an evolutionarily conserved upstream open reading frame (uORF) in the 5' leader of the SLC6A8 mRNA. According to the scanning model of eukaryotic translation initiation, the 40S ribosome is loaded on an mRNA near the 5' cap and scans in a 3' direction in search of a start codon. Typically, translation initiates at the first encountered AUG codon in optimal context (with a purine at the -3 position relative to the A of the AUG codon being the strongest context determinant). Many eukaryotic mRNAs contain uORFs, and, as a consequence of ribosome scanning, most of these uORFs have stochastic cis-acting inhibitory effects on main ORF translation. A small subset of uORFs are used for gene regulation by modulation of their inhibitory function by environmental cues, often a small molecule. Although a uORF regulated by a cellular metabolite (arginine) was first discovered nearly 30 years ago in yeast, few mammalian metabolite-regulated uORFs have been described. Using an SLC6A8-luciferase fusion, we found that creatine inhibits SLC6A8 expression. This regulation was dependent on the conserved uORF as the regulation was lost upon mutation of the uORF start codon or by introducing mutations that alter the reading frame of the uORF. Thus, the encoded uORF peptide, nearly identical from lamprey to human, is critical for creatine control of SLC6A8 expression. We propose that creatine interaction with the nascent peptide in the ribosome exit tunnel causes an elongation pause during translation of the uORF and prevents ribosomes from accessing and translating the SLC6A8 ORF. Accordingly, high levels of creatine feedback inhibit expression of the creatine transporter, providing tight homeostatic control of cellular creatine levels. We believe that our studies provide a paradigm for metabolite-sensing uORFs to regulate gene expression and in the case of SLC6A8 are linked to a human hereditary disease.

Brendan Tunstall

Visiting Fellow

NIDA

Neuroscience - General

Oxytocin Blocks Compulsive-like Alcohol Drinking

From today's global population, an estimated 440 million people will die or become disabled due to alcohol. Two main sources of motivation drive alcohol drinking: alcohol's rewarding and stress-relieving effects. We hypothesized that brain signaling by the neuropeptide oxytocin, known to be involved in both stress and reward function, contributes to compulsive alcohol drinking. To our knowledge, there are no studies comparing the effect of oxytocin on alcohol drinking in alcohol-dependent vs. nondependent rodents or humans. We used a preclinical model of alcohol dependence that reliably produces somatic and motivational signs of dependence to test our hypothesis. Wistar rats were trained to lever press for access to alcohol and then either made alcohol dependent via repeated cycles of alcohol vapor exposure or non-dependent (air exposed control group). Oxytocin (0-1 mg/kg) was administered systemically (intraperitoneal route) and intranasally, a novel route of peptide administration, to facilitate brain penetrance and avoid peripherally mediated side-effects. The results indicate that alcohol-dependent rats developed escalated alcohol consumption and alcohol seeking behavior (responding when response requirement is increased) compared to non-dependent controls.

Both systemic and intranasal oxytocin blocked compulsive-like alcohol consumption and seeking in dependent rats, at doses which did not disrupt alcohol drinking in non-dependent rats. This effect in dependent rats was replicated with central (intracerebroventricular) administration of oxytocin, but not systemic (subcutaneous) administration of an oxytocin receptor agonist that does not reach the brain. This suggests that oxytocin can selectively block the compulsive-like motivation for alcohol that emerges in alcohol dependence and that this effect is centrally mediated. Additional experiments indicated that oxytocin's effects on alcohol drinking cannot be explained by effects on locomotion, coordination, or consumption of sweet or caloric fluids in general. Collectively, these findings suggest oxytocin has potential as a safe and selective new treatment for alcohol use disorders. A clinical trial of intranasal oxytocin informed by the present data is underway in humans with alcohol use disorders.

Lifeng Zhang

Research Fellow

NIDA

Neuroscience - General

Dorsal striatum medial spiny neurons encode motor skill learning

Dorsal striatum (DS) is important for motor skill learning. Two types of medium spiny neurons (MSNs) exist in the DS: the direct pathway MSNs expressing dopamine receptor D1 (D1-MSN) and the indirect pathway MSNs expressing dopamine receptor D2 (D2-MSN). Co-activation of D1- and D2- MSNs are recently shown to be important for coordinating locomotion. However, the role of D1- and D2- MSNs in motor skill learning remains largely unknown. To address this question, motor skill learning on an accelerating rotarod was employed in this study. The rotarod accelerated from 4-40 rotations per min over 300 s, mice were trained with 10 trials per training day every other day for 8 training days. Each trial ended when mice fall from the rotarod or reached the maximum performance of 300 s. There was a 300-s resting between trials. We found that bilateral partial ablation of D1-MSNs in the DS hampered motor skill learning, whereas bilateral partial ablation of D2-MSNs in the DS facilitated motor skill learning. We next employed a custom miniature fluorescent microscope to concurrently record calcium activities from hundreds of D1- or D2- MSNs longitudinally from mice training on accelerating rotarod. We identified two subpopulations of speed-related neurons within both D1- and D2- MSNs: the speed-depressing neurons (SDN) whose activities decreased as rotarod rotation speed increased, and speed-potentiating neurons (SPN) whose activities increased as rotarod rotation speed increased. We found that SDNs, but not SPNs, were related to the motor skill learning. The numbers of both D1-SDNs and D2-SDNs increased rapidly on training day 1, and gradually reached a plateau over the subsequent days, synchronizing with the behavior learning curve, while the number of SPNs didn't change over learning. Interestingly, over the course of learning, the activities of SDN but not SPN displayed increased trial-to-trial correlation, indicating increased trial-to-trial similarity and reduced trial-to-trial variability of SDN activities towards the end of learning. Moreover, over the course of learning, the number of D1-SDN increased more significantly than that of D2-SDN. Finally, optogenetic silencing of D1-SDN hampered motor skill learning, whereas silencing of D2-SDN facilitated motor skill learning. Together, our results suggest that D1-SDN and D2-SDN differentially regulate motor skill learning: the emergence of D1-SDN facilitates and that of D2-SDN delays motor skill learning.

Tianye Zhai

Visiting Fellow

NIDA

Radiology/Imaging/PET and Neuroimaging

Efficacy in Prediction of Cocaine Relapse is Distinct across Dorsolateral Prefrontal Cortex Sub-regions

Neurobiological studies have demonstrated that the dorsolateral prefrontal cortex (DLPFC), a core component of the executive control network (ECN), is crucial in modulating top-down control.

Transcranial magnetic stimulation (TMS) targeting the left DLPFC has shown to alter inter-temporal behaviors in healthy individuals and drug-using behaviors in addiction context. However, the DLPFC is a large and relatively heterogeneous brain region, it is not clear where exactly in the DLPFC is the most effective site for preventing drug relapse, and the underlying neural mechanism remains unclear.

Therefore, we utilized resting-state fMRI (RS-fMRI) and the Cox regression model to explore the relationship between the DLPFC functional connectivity (FC) and cocaine relapse, specifically to evaluate the efficacy in prediction of cocaine relapse across DLPFC sub-regions; and to explore the underlying neural mechanism of the optimal target. We recruited 45 cocaine use disorder (CUD) patients from residential treatment programs. All patients were scanned for RS-fMRI followed for 168 days or until relapse. After data pre-processing and FC calculation seeded at the left DLPFC, voxel-wise Cox regression analyses were conducted and composite indices were generated by linear summation of the FC of voxels with significant beta values ($p < 0.001$), for the positive and the negative beta values respectively. Then we built prediction model using these indices, conducted the ROC analysis for prediction model evaluation, and subsequently conducted a transverse comparison among 7 different DLPFC sub-regions applying the abovementioned analyses. Transverse comparison among 7 DLPFC sub-regions yielded prediction models with area under the curve (AUC, a measure of accuracy) ranging from 0.411 to 0.882. For the optimal target (the one with AUC of 0.882), two circuits were identified predictive of cocaine relapse. A protective circuit, mainly consisted of bilateral DLPFC, inferior parietal lobule and inferior frontal gyrus; and a risk circuit, mainly included the ventral medial prefrontal cortex and the posterior cingulate cortex/precuneus. Our study demonstrated the heterogeneity of DLPFC in predicting cocaine relapse and identified a DLPFC sub-region that has strong prediction capability that might be an optimal target of neuromodulation treatments. We further revealed two circuits of DLPFC that may provide mechanistic understanding of this optimal target for treating cocaine addiction.

Chloe Jordan

Postdoctoral Fellow

NIDA

Psychiatry

Dopamine D3 Receptor Partial Agonism as a Novel Therapeutic Strategy for Cocaine Addiction

Cocaine addiction is a chronic relapsing disorder that affects nearly one million people in the U.S.

Despite decades of intensive research, there are currently no approved medications for the treatment of cocaine addiction. In the brain, cocaine acts by blocking dopamine (DA) transporters and increasing DA receptor activity in key reward-related regions, such as the ventral tegmental area (VTA). Of the five DA receptor subtypes, D3 receptors (D3Rs) exhibit the highest binding affinity for DA and have restricted distribution in the brain reward system, thus representing attractive therapeutic targets for cocaine addiction. Previous work by our group and others has shown that D3 antagonists, which block DA from

binding D3R, can reduce the motivation to self-administer cocaine and relapse to cocaine-seeking behaviors. However, D3R antagonists are not effective in attenuating cocaine intake under *easy* (fixed ratio [FR]2) reinforcement conditions, highlighting a need for more efficacious treatments. Based on the success of varenicline (a nicotinic receptor partial agonist) for smoking cessation, we reasoned that a D3R partial agonist strategy may be effective in the treatment of cocaine addiction by: 1) acting as an antagonist during cocaine use by blocking D3R binding, and 2) attenuating craving and relapse during abstinence from cocaine through partial D3R activation. Here, we asked whether VK4-40, a novel, highly potent and selective D3R partial agonist (Kumar et al., 2016), would attenuate cocaine use in preclinical models. We found that VK4-40 significantly and dose-dependently inhibited intravenous cocaine, but not sucrose, self-administration maintained by multiple cocaine doses under an FR2 schedule and attenuated cocaine-primed reinstatement (relapse) to drug-seeking behavior. In addition, VK4-40 dose-dependently inhibited optogenetic intracranial self-stimulation of VTA DA neurons (i.e., brain reward). Strikingly, VK4-40 alone did not have dysphoric effects as assessed by optogenetic real-time and classic conditioned place preference experiments. Taken together, these converging results indicate that VK4-40 attenuates cocaine reward and relapse without altering non-drug reinforcement or producing dysphoria. These findings indicate that D3R partial agonism is a promising therapeutic strategy for cocaine use disorders and lay the foundation for translational medication development for this underserved patient population.

Justin Siemian

Postdoctoral Fellow

NIDA

Systems Biology

Activation of hypothalamic-ventral tegmental circuits gates motivation

Motivated states such as food seeking and consumption are essential for an organism's survival. Optimal performance of these behaviors is mediated by neuronal circuits that maintain energy balance and feeding. Thus, aberrant function of these circuits may result in clinical syndromes of over- and under-eating which affect more than one-third of Americans. The lateral hypothalamus (LH) has been known for decades to play a fundamental role in regulating feeding and reward-related behaviors but contains diverse neuronal populations whose contributions to such behaviors have not been unraveled. Here, we examined how lateral hypothalamic leptin receptor-expressing (LH-LEPR) neurons, one of the most abundant GABAergic cell types in the LH, may regulate motivation to obtain food. We trained mice to lever press for food pellets on a progressive ratio schedule (PR; a paradigm used to measure motivation) in which the reinforced number of responses exponentially increases following the delivery of each pellet. We found that chemogenetic activation of LH-LEPR neurons significantly increased PR performance ($244 \pm 40\%$ of control) whereas chemogenetic inhibition of these neurons decreased PR performance ($84 \pm 5\%$ of control), indicating that LH-LEPR neurons bidirectionally regulate motivation. To determine whether these neurons function through circuits that mediate motivation and reward-related behaviors, we mapped their axonal projections using a Cre recombinase-dependent viral vector for anterograde tracing (rAAV2/9-hEfla-DIO-synaptophysin-mCherry). We found that LH-LEPR neurons project to several brain regions associated with feeding (e.g., BNST, PVT, and PVH) as well as reward-related behaviors such as the ventral tegmental area (VTA). Using optogenetics, we next found that activation of LH-LEPR axons in the VTA increased PR performance ($185 \pm 23\%$ of control) suggesting that

these neurons regulate motivation through synaptic connections in the VTA. Brain slice electrophysiology recordings using optogenetics demonstrated that LH-LEPR neurons release GABA and form functional inhibitory synapses within the VTA. Thus, our results reveal an unprecedented role for LH-LEPR neurons in gating motivation and will serve as a basis for future models of LH circuitry that regulate survival behaviors. Additionally, elucidating the mechanisms that regulate the addictive nature of food intake will allow for the identification of novel targets for therapies of eating disorders.

T. Chase Francis

Postdoctoral Fellow

NIDA

Physiology

Substance P Drives Excitatory Potentiation on Nucleus Accumbens D2-MSNs via Cholinergic Interneurons

Nucleus Accumbens (NAc), deep brain, high frequency stimulation (HFS) is an efficacious treatment for mood disorders. Understanding neurobiological mechanisms of HFS holds the potential of identifying non-invasive pharmacological targets that could replace deep brain stimulation. Previously, we discovered optogenetic (o)HFS (=50Hz), but not low frequency oHFS of NAc core dopamine 1 (D1) receptor expressing medium spiny neurons (MSNs) mimics hedonic outcomes of deep brain stimulation. We aimed to determine the electrophysiological mechanism of D1-MSN oHFS by using patch clamp physiology in acute NAc brain slices from mice expressing Cre-dependent channelrhodopsin in D1-MSNs. We found D1-MSN oHFS promotes post-synaptic, glutamatergic long-term potentiation on dopamine 2 (D2) receptor expressing MSNs, but not on D1-MSNs. These results are the first to demonstrate local D2-MSN excitation by D1-MSNs. It is thought HFS drives peptide release. Therefore, we examined effects of the D1-MSN specific excitatory peptide substance P on D2-MSN plasticity. Bath application of substance P mimicked D1-MSN oHFS and antagonizing neurokinin 1 receptor (NK1R), the primary substance P receptor, blocks potentiation, suggesting substance P drives this effect. RNAscope results to determine the post-synaptic location of NK1R signaling reveal all cholinergic interneurons express NK1Rs. Neither MSN subtype expresses the NK1R, signifying a disynaptic cholinergic mechanism may drive potentiation. D1-MSN oHFS causes lasting depolarization and spontaneous firing increases in cholinergic interneurons which are blocked by NK1R antagonists, indicating increased acetylcholine release could be responsible for the effect. Indeed, blocking all muscarinic receptors with atropine or excitatory muscarinic 1-like receptors with pirenzepine prevents potentiation and selective muscarinic 1 receptor agonism with AC-42 mimics the effects of substance P and D1-MSN HFS. Conditional CRISPR and cholinergic inhibition experiments to determine the necessity of the NK1R and acetylcholine release are currently in progress. We describe a novel multi-synaptic mechanism in which D1-MSN substance P release drives cholinergic activity and excitatory D2-MSN plasticity. This study provides potential pharmacological targets for mimicking NAc HFS. Future studies will utilize compounds targeting these receptors during stressful contexts such as pain to attempt to alleviate anhedonic effects caused by emotional distress.

Yan Zhang

Postdoctoral Fellow

NIDA

Neuroscience - Integrative, Functional, and Cognitive

Distinct encoding of food and cocaine reward seeking in the medial prefrontal cortex

The search for better therapeutic strategies for drug addiction raises the challenge to diminish motivation for the drug without decreasing that for natural rewards. While medial prefrontal cortex (mPFC) has been shown to be important for reward seeking behaviors, how prefrontal neural activities code reward seeking behavior remains unknown. Here, we employed miniScope, a custom miniature fluorescence imaging system, together with detailed computational analysis and machine-learning algorithms, to simultaneously track calcium activities from hundreds of neurons longitudinally, at the single cell resolution in the mPFC during mice food and cocaine self-administration. We found that different subgroups of neurons showed increased activity around distinct behavioral events (i.e. house light on, lever extend into behavior chamber, lever press/cue presentation, and food reward consumption). The largest neuronal subpopulations were associated with lever press/cue presentation and food consumption, but these subgroups of neurons were not completely overlapping. This observation indicated separation between neural correlates for reward seeking and reward consumption. Interestingly, by imaging the same neurons over multiple days, we observed that subgroups of neurons for lever press/cue presentation over different days showed large degree but not complete overlap, indicating a dynamic population code for reward seeking in the mPFC. Further, we observed that lever press/cue presentation related neurons for food and cocaine were largely non-overlapping, suggesting that two distinct microcircuits encode natural reward and drug reward seeking in the mPFC. Finally, by using machine learning algorithms, we were able to accurately predict the timing of lever press/cue presentation across different days based on mPFC neural activities. Altogether, our results revealed dynamic and distinct neural population codes for natural reward and drug reward seeking in the mPFC, paving the way for future efforts in targeting specific neural mechanisms for drug reward seeking as novel therapeutic strategies for drug addiction.

Loksum Wong

Visiting Fellow

NIDCD

Developmental Biology

Sonic hedgehog is required for the formation of auditory ganglion in the mouse inner ear

Neurosensory hearing loss affects over 300 million people worldwide and yet treatments for patients are derisory due to lack of understanding of the complex inner ear. The sound detection apparatus of the inner ear, the cochlea, requires proper connections between sensory hair cells within the cochlea and the auditory neurons of the spiral ganglion (SG). Identifying key factors that mediate normal formation of cochlear hair cells and SG will help future design of therapies to alleviate neuropathy due to neurotoxins or sensory hair cells loss. Previously, we have found that Sonic Hedgehog (Shh), a secreted signaling molecule, is expressed in the developing SG and it regulates cochlear hair cell differentiation and SG formation. The expression pattern of Shh in the developing SG is dynamic: first this gene is expressed broadly in a subpopulation of the SG and later on its expression is confined to only SG cells located by the apex of the cochlea. This restricted expression pattern of Shh over time has been shown to be important for regulating the timing of hair cell differentiation within the cochlea; however, the significance of this dynamic Shh expression pattern on SG formation is not known. To address this question, a combination of in situ hybridization, lineage tracing, and cell cycle labeling techniques were used to decipher the spatiotemporal origin of the Shh-positive cells in the SG. The two key findings of

this study are: 1) Shh is expressed only transiently in post-mitotic, nascent SG neurons and its expression is soon down-regulated within two days as the neurons mature, and 2) neuroblast precursors adjacent to the Shh-positive cells express the receptor of Shh, Patched1 (Ptc1), and when these precursors become post-mitotic, they start to express Shh. This dynamic expression pattern of Shh in the developing SG over time represents different cohorts of newly generated SG neurons. In the absence of Shh, Ptc1 expression is downregulated and the size of SG is much reduced. Together, these results indicate for the first time that an auto-regulatory loop of Shh signaling controls the developmental timing of SG. Currently, single cell RNA-seq transcriptome analyses are being conducted on the neuronal precursors (Ptc1-positive), nascent (Shh-positive) and mature (Shh lineage-positive only) SG neurons. Understanding how Shh signaling is being regulated in the developing SG will provide insights into how SG neurons can be restored under damage conditions.

Willy Sun

Other

NIDCD

Cell Biology - Intracellular Trafficking, Cytoskeleton, and Extracellular Matrix

Intestinal glycocalyx and enterocyte microvilli integrate into a stratified transcellular structure optimized for size-exclusion barrier function and epithelial homeostasis.

Most epithelial and endothelial surfaces are covered by a lubricative, protective, and selective glycocalyx. While the composition of the glycocalyx has been explored with multiple molecular techniques, very little information is available about its precise cell and tissue specific molecular architecture. In particular, the glycocalyx lines the length of the intestinal tract and is central to both the digestive and protective functions. Exactly how the intestinal glycocalyx is structured and integrated to the enterocyte microvilli to enable its host-defense and nutrient permeability barrier functions remain poorly understood. This is mainly due to the difficulty in staining and imaging glycoproteins by conventional electron microscopy techniques. Here we combine freeze-etch and electron tomography to describe in unprecedented detail the molecular architecture of the microvilli-glycocalyx complex on the surface of mouse small intestine. We observed that the glycocalyx layer is made of very regular, $1.0 \pm 0.08 \mu\text{m}$ long, columnar filaments that emerge from the plasma membrane at the distal ends of the microvilli. Six to eight filaments emerge from each microvillus which in turn are highly cross linked and hexagonally packed with a center to center spacing of $163 \pm 14 \text{ nm}$. The closely spaced columnar glycocalyx filaments make multiple anastomosing side-to-side interactions along their length over and across the surface of the enterocytes. The anastomosing produce filaments ranging from 3-12 nm in diameter and the mesh size of the network does not exceed 40 nm. Surprisingly, the glycocalyx filament termini come together in groups of three and four to form an ordered cover net that displayed liquid hexagonal packing with a center to center spacing of $32 \pm 8 \text{ nm}$. The microvilli, the columnar glycocalyx filaments and the terminal cover net form a stratified but highly integrated transcellular organization that maintains its regular structure across the enterocyte boundaries. Fourier analysis shows that despite the regular organization all three layers exhibit liquid packing properties. Our results provide a new structural framework for understanding how the glycocalyx-microvilli complex maintains a size-exclusion filter for nutrients and an effective continuous host-defense barrier at the same time accommodating for the developmental, functional, and homeostatic rearrangements of the intestinal epithelium.

Reut Shainer

Postdoctoral Fellow

NIDCR

Cell Biology - Intracellular Trafficking, Cytoskeleton, and Extracellular Matrix

Periosteum Cells Derived from Long Bone are Unique from those Derived from Calvaria

The periosteum, a thin membrane surrounding the bone, is the source of progenitor cells needed for bone formation, remodeling and repair. Periosteum-derived cells (PDC) have the capability to differentiate into multiple skeletal phenotypes, suggesting they have stem cell-like properties. Studies of bone healing after fracture show the periosteum has an essential role in the repair process. PDC from the long bones (bPDC) have been investigated over recent years but the differences between bPDC and PDC from craniofacial bones or specifically from the calvaria (cPDC) remain elusive. In this study, we established a protocol to isolate bPDC and cPDC of adult mice where cells were further proliferated and differentiated within a novel 3D culture RAFT system to mimic the native environment of the cells in vivo. RNAseq analysis showed that the undifferentiated bPDC expressed several genes normally found in extracellular matrix (ECM) of cartilage including cartilage intermediate layer protein (Cilp), cartilage oligomeric protein (Comp) and bone sialoprotein (Ibsp). Interestingly, these genes were not expressed in the undifferentiated cPDC. When cells were subject to osteogenic differentiation in vitro the differentiated bPDC, but not cPDC, formed characteristic cartilage structures demonstrated by both H&E staining and scanning electron microscopy. Both analysis also showed that differentiated bPDC formed chondrocytes that were incorporated into the matrix. RNAseq analysis also showed that differentiated bPDC, but not differentiated cPDC, made high levels of collagen type II, aggrecan and collagen type X which further confirming our notion that bPDC undergo chondrogenic differentiation under osteogenic conditions. When the 3D RAFT scaffold with PDC cells were transplanted into mice, both differentiated cPDC and differentiated bPDC created mineralized ossicles 4w and 8w post implantation. Using μ CT we found that the transplants containing bPDC had a greater increase in total bone volume compared to cPDC, which we suspect is calcified cartilage. In summary, our results indicate that PDC from bone but not calvaria cultivated in a 3D system create a distinct microenvironment that favors cartilage formation. Considering that long bones are formed via endochondral ossification while calvaria is not, our data suggests PDC retain a "memory" of their tissue origin a feature that could be capitalized for future applications in their use in skeletal tissue regeneration.

Blake Warner

Clinical Fellow

NIDCR

Immunology - Autoimmune

Characterization of salivary gland hypofunction associated with immune checkpoint inhibitor therapy

Immune checkpoint inhibitor (ICI; e.g., pembrolizumab, nivolumab, ipilimumab) biologic agents have significantly advanced the field of cancer therapeutics. These drugs can trigger immune-related adverse events (irAEs), including salivary hypofunction and xerostomia. We describe the clinical phenotype of the largest cohort of patients experiencing dry mouth associated with ICI. Our results illustrate a novel mechanism of salivary gland hypofunction, delineate its pathogenesis, and inform the management of this irAE. Patients with ICI-related dry mouth had comprehensive evaluations at the NIH Salivary Gland

Dysfunction Unit. Salivary flow rates were measured and minor salivary gland (MSG) biopsies were obtained for histopathology, RNA sequencing, primary culture, and for functional and immunological studies (e.g., fluorescence-activated cell sorting, FACS). Patients (N=16) were evaluated approximately 10 weeks after the onset of xerostomia. All subjects reported "dry mouth" and 15/16 (94%) subjects had salivary dysfunction by unstimulated saliva flow (mean: 0.67 ± 0.69 mL/15 min, ref range >1.5 mL/15 min). MSG histopathology showed chronic sialadenitis with fibrosis and atrophy; 5 cases (31%) exhibited lymphocytic aggregates. Functional studies on MSG ex vivo demonstrated deficits in fluid secretion and calcium flux. Scanning electron microscopy revealed disrupted contacts within salivary epithelia and secretory granules disruption. Enrichment of neuronal and immune pathways, but down-regulation of protein translation pathways, was demonstrated by RNAseq. FACS on dispersed MSG demonstrated infiltration of cytotoxic T cells exhibiting high expression of PD-1 and elevated cytokine secretion in response to phorbol myristate acetate (PMA) and ionomycin (ION). Immunohistochemistry to characterize the immune cell infiltration in the MSG was performed. ICIs can elicit profound negative effects on salivary secretion, which, like other irAEs, is expected to increase in incidence with the use of these drugs. We report a mechanism of salivary hypofunction distinct from other diseases affecting the salivary glands. We propose that ICI therapy breaks immune tolerance locally leading to activation of cytotoxic T cells and associated sequelae. Because of the exquisite sensitivity of the salivary glands to injury, we hypothesize that promptly preventing the activation or function of these infiltrating immune cells may prevent long-term ICI-induced salivary hypofunction.

Jiaoyang Lu

Doctoral Candidate

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Cell Biology - Intracellular Trafficking, Cytoskeleton, and Extracellular Matrix

Abstract removed at request of author

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Marit Aure

Research Fellow

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Gene Expression

Abstract removed at request of author

Abstract removed at request of author

Shanu Jain

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Metabolomics/Proteomics

Polymorphic role of P2Y6 receptor in insulin sensitive organs: Adipose tissue and skeletal muscle

Adipose tissue and skeletal muscle are the two largest insulin-sensitive organs in the body. They play a major role in maintaining whole body glucose and energy homeostasis. Insulin action results in glucose

disposal principally in skeletal muscle and to a lesser extent in adipose tissue. Dysregulation of this function promotes the development of obesity, insulin resistance and type 2 diabetes (T2D). Many G protein-coupled receptors (GPCRs) are expressed in insulin sensitive organs and thus have emerged as potential targets for novel anti-diabetic drugs. P2Y purinergic receptors are a class of GPCRs activated by nucleotides and nucleotide sugars. The purinergic P2Y6 receptor (P2Y6R) is activated by uridine 5'-diphosphate. The potential role of P2Y6R in adipose tissue and skeletal muscle with respect to maintaining whole body glucose homeostasis remains unexplored. To address this issue, we used Cre/loxP technology to generate mice that lack P2Y6R selectively in adipose tissue (AT-Y6-KO) or skeletal muscle (SK-Y6-KO). Interestingly, AT-Y6-KO mice consuming a high fat diet (HFD) gained less weight, while SK-Y6-KO mice gained more weight than the corresponding control mice. Body composition revealed that the difference in body weight was due to loss or gain in fat mass in AT-Y6-KO and SK-Y6-KO mice, respectively. Improved vs. impaired glucose tolerance and insulin sensitivity was observed in AT-Y6-KO and SK-Y6-KO mice, respectively. Moreover, fasting blood glucose and fed plasma insulin levels were decreased in AT-Y6-KO mice. SK-Y6-KO mice showed elevated blood glucose and plasma insulin levels under both fasting and fed conditions. Plasma levels of leptin hormone released by mature adipocytes was decreased in AT-Y6-KO under both regular diet and HFD conditions. Improved metabolism in AT-Y6-KO also prevented accumulation of lipids in liver as revealed by Oil Red O staining of liver sections. Impaired metabolism in SK-Y6-KO was due to impairment in glucose uptake by skeletal muscle, as revealed by enhanced glucose uptake in the C2C12 muscle cell line upon P2Y6R agonist stimulation, which was blocked by antagonist treatment. These results revealed opposite effects of P2Y6R in two insulin-sensitive organs required for maintaining glucose homeostasis. Unveiling the P2Y6R-mediated signaling in these tissues may lead to the development of novel drugs for T2D treatment.

Saurav Majumder

Postdoctoral Fellow

NIDDK

Biochemistry - General, Proteins, and Lipids

Aryl Hydrocarbon Receptor (AHR) modulates glycosphingolipid production by transcriptional regulation of key genes involved in the de novo biosynthetic pathway.

Sphingolipids are critical components of membranes and act as important signaling molecules.

Therefore, it is not surprising that aberrant sphingolipid homeostasis has been implicated in diverse pathologic conditions, such as in aging, neurodegenerative and metabolic diseases and inflammation.

However, a global approach to identify sphingolipid regulatory genes in human cells is lacking. Here we report development of a toxin-based genome-wide CRISPR screen to identify genes involved in sphingolipid synthesis.

We also show that Aryl Hydrocarbon Receptor (AHR), a transcription factor, is a major regulator of de novo sphingolipid synthesis. Verotoxin (VT), an exotoxin, binds specifically to glycosphingolipids (globotriaosylceramide or GB3) on the cell surface. Cells lacking GB3 on the cell surface are resistant to VT. Upon binding to the cell surface, VT is endocytosed, blocks protein translation and induces apoptosis. In mammalian cells, biosynthesis of GB3 starts from sphingolipid precursors. We hypothesized that a genome wide knockout screen for VT resistance will identify novel regulators of sphingolipid/glycosphingolipid metabolism. We generated genome-wide CRISPR mediated knockout mutant pool by lentiviral delivery of human sgRNA(guide) library in HeLa cells, stably

expressing Cas9. Resulting mutants were screened for VT resistance. Loss-of-functions of genes that confer toxin resistance were identified by deep sequencing amplicons of stably integrated sgRNA regions from the genomic DNA. Relative abundance of guides between untreated and treated groups showed a robust increase of 18% of the total guides in resistant cells. Some of the highest-ranking genes enriched in resistant cells include genes such as, A4GLT, B4GALT5 which are reported to be involved in GB3 synthesis and toxin resistance. Individual knockouts of 20 top ranking candidates were validated by toxin resistance and GB3 expression on its cell surface. Surprisingly AHR-KO, one of the top candidate, is not only resistant to the toxin but also express significantly less amount of GB3. This finding, along with the fact that sphingolipid biosynthesis plays a crucial role in toxin resistance, we hypothesized that AHR might be involved in transcriptional regulation of genes in the biosynthetic pathway. We show that AHR binds to the promoter of key genes in the pathway and transcriptionally modulate their expression.

Alberto Ceccon

Visiting Fellow

NIDDK

Biochemistry - General, Proteins, and Lipids

Interaction of Huntingtin Exon-1 peptides with lipid-based micellar nanoparticles probed by solution NMR and Q-band pulsed EPR

Huntington's disease is a fatal neurodegenerative disease arising from the presence of 36 or more CAG repeats within exon 1 of the Huntingtin (htt) gene, resulting in expansion of the polyQ domain that lies immediately downstream of the 16-residue N-terminal amphiphilic sequence (httNT) of the huntingtin protein. The presence of a long polyQ stretch results in the rapid formation of polymorphic fibrils, the rate of which is modulated by the presence of flanking regions (httNT and the proline rich domain C-terminal to the polyQ sequence). Although the length of the polyQ domain is directly related to the severity of disease, the aggregation schemes of mutant exon 1 can be further complicated by additional factors including binding to surfaces (including lipid membranes) and the presence of reactive oxygen species (ROS). Recent atomic force microscopic (AFM) studies have emphasized the role of httNT in modulating the membrane-associated oligomerization on supported lipid bilayers. Nevertheless, the molecular details of how membrane association can possibly impact polyglutamine nucleation as well as the existence of transient oligomeric membrane-associated states involved in the early-stages of amyloid fibril formation are currently missing. In this paper, we have characterized the interaction of two such peptides, httNTQ7 and httNTQ10 comprising the N-terminal amphiphilic domain of huntingtin followed by 7 and 10 glutamine repeats, respectively, with lipid micelles as membrane-mimic using NMR chemical exchange saturation transfer (CEST), circular dichroism and pulsed Q-band EPR. Exchange between free and micelle-bound httNTQ_n peptides occurs on the millisecond time scale with a K_D ~ 0.5-1 mM. Upon binding micelles, residues 1-15 adopt a helical conformation. A structure of the bound monomer unit is calculated from the backbone chemical shifts of the micelle-bound state obtained from CEST. Pulsed Q-band EPR shows that a monomer-dimer equilibrium exists on the surface of the micelles and that the two helices of the dimer adopt a parallel orientation, thereby bringing two disordered polyQ tails into close proximity which may promote aggregation upon dissociation from the micelle surface through high local concentration effects. Interestingly, oxidation of Met7 to a sulfoxide due the presence of ROS reduces the binding affinity ~3-4 fold, increases the length of the helix by a further two residues and inhibits aggregation.

Cheryl Cero

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Physiology

beta3 adrenergic receptors are key regulators of lipolysis and thermogenesis in humans

Decades of preclinical studies in rodents has shown that activating brown adipose tissue (BAT) thermogenesis and white adipose tissue (WAT) lipolysis can treat obesity and confer a range of metabolic benefits. These models show that beta3-adrenergic receptor (AR) agonists potently stimulate both tissues. The recent discovery of functional BAT in humans is encouraging, but several species differences in adipocyte cellular physiology must be addressed before true advances are possible. First, the contribution of the beta3-AR signaling in human adipocytes may be different from rodents given the low abundance of such receptor in adipose tissue and access to only partial beta3-AR agonists. Second, it is unknown how human tissue compares functionally to immortalized cell lines. Therefore, we first investigated the distribution and relative expression of beta1/2/3-ARs in (i) autopsy-derived WAT and BAT from the subcutaneous abdominal and supraclavicular neck regions, respectively, and (ii) immortalized human white (WA) and brown (BA) adipocytes. We show that in WAT and BAT, the beta1-AR and beta2-ARs are similarly expressed, whereas beta3-AR has the highest expression in BAT. Similarly, differentiated BA have a significantly higher expression of the beta3-AR than WA. To assess the contribution of each receptor toward lipolysis and thermogenesis, we differentiated WA and BA, respectively, and performed dose-response curves of agonist-stimulated lipolysis. We observed that stimulation of any one of the three beta-ARs in human adipocytes induces an increase in glycerol release, a lipolytic byproduct. However, the human beta3-AR-selective agonist mirabegron, was a more potent activator of lipolysis in both BA and WA than agonists selective for the beta1-AR and beta2-ARs. These findings were translated to a clinical trial in which mirabegron was given to healthy women, where we found that mirabegron induced an increase in BAT thermogenesis and WAT lipolysis. In summary, we identify a prominent physiological role for the beta3-AR in the regulation of human BAT thermogenesis and WAT lipolysis that is consistent in both in vitro and in vivo human models. A selective beta3-AR agonist may be the optimal choice for targeted stimulation of human WAT and BAT.

Akira Nishio

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Clinical and Translational Research, and Gene Therapy

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Yanling Ma

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Clinical and Translational Research, and Gene Therapy

Human and Murine Studies Identify RORA that Contributes to the Histological Severity of Non-Alcoholic

Fatty Liver Disease

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in developed countries and can progress to liver cirrhosis and hepatocellular carcinoma, with limited treatments options. A single nucleotide polymorphism (SNP), rs339969, located in an intron of the RAR-related Orphan Receptor Alpha (RORA) gene is associated with liver enzyme levels in the general population. Previous studies demonstrated a role for RORA in the regulation of lipid and energy metabolism. However, the role of RORA in the liver metabolism remains unexplored. The current study aims to elucidate the relationship between rs339969 and histological and biochemical features of NAFLD, and to uncover whether and how liver-specific RORA affects NAFLD progression. In a cohort of 768 subjects with NAFLD, we demonstrate that the minor allele, rs339969-C increased liver fat >33% (OR=1.25, CI 0.996-1.56, p=0.056). eSNP data showed an association of rs339969 with increased hepatic expression of RORA mRNA (p=5x10E-8). Consistent with eSNP data, we found RORA expression was elevated with the rs339969-C allele in primary hepatocytes, indicating rs339969 might affect NAFLD progression by regulating RORA gene expression. Hepatic expression of RORA is 2.7-fold increased (p=0.006) in patients with NAFLD compared with healthy controls. To assess the role of RORA in the liver, we generated liver-specific Rora knock-out mice (Rora-KO). Rora-KO mice fed 12 weeks of high-fat diet gained significantly more weight compared to litter mate controls, despite a similar food intake. Rora-KO mice had increased liver to body weight ratio, marked liver steatosis, and elevated serum liver enzymes levels. Rora-KO mice were also found to have worse insulin resistance indicated by serum insulin level and insulin tolerance test. Real time qPCR revealed that Rora-KO mice had significantly elevated expression level of liver fibrosis genes (Col1a1, Col1a2, Col3a1, and Timp1), inflammatory genes (Itgax, Cd68, Ccl2, Gli2, and TNFa), and antioxidant enzyme gene Gpx1, supporting an important role of Rora in driving liver steatosis and liver injury development. In conclusion, using genetic and expression data we demonstrate an association between RORA and human NAFLD. We show in an animal model that RORA is involved in regulating insulin sensitivity, hepatic steatosis, and fibrosis. Our data suggest that RORA plays important roles in hepatic lipid metabolism and the pathogenesis of NAFLD.

Dahong Chen

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Developmental Biology

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Ivana Grabundzija

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Molecular Biology - Prokaryotic, and Eukaryotic

Helraiser transposition intermediates unveil the mechanism of eukaryotic replicative transposition

Transposons are discrete pieces of DNA that can move within their host genome. Their activity has reshaped both prokaryotic and eukaryotic genomes leading to major evolutionary innovations, but also

to disease, oncogenesis and the emergence of antibiotic-resistant microbial strains. Harnessing their ability to introduce genomic modifications can turn transposons into useful genetic tools, while understanding their mode of action can provide valuable insights into genomic pathways and evolution. Well studied eukaryotic transposons include cut-and-paste transposons and retrotransposons. Helitrons, a large and widespread family of eukaryotic DNA transposons that have profoundly affected genome variability via capture and mobilization of host genomic sequences, was discovered only recently. Based on sequence similarities with certain prokaryotic mobile elements, Helitrons were proposed to use a "rolling-circle" transposition mechanism. Even in prokaryotes, this transposition model is poorly understood, and it is not clear to what extent it can be applied to Helitrons. Here we use novel approaches and reconstituted transposon called Helraiser to study Helitron transposition in vivo and in vitro. Transposition assays with single and double-stranded transposon donors show that the transposon donors must be double-stranded. Nevertheless, our replication and integration assays indicate transposition of only one transposon strand. In addition, our experiments reveal that during Helraiser transposition the original donor sites are reused, providing a constant source of donor DNA for multiple transposition rounds. Circular transposition intermediates are actively replicated in cells by Helraiser transposase and serve as transposon donors for genomic integration. Our results support a transposition model where DNA cleavage and strand transfer occur on a single transposon strand but depend on a framework provided by the double-stranded DNA, tightly linking Helitron transposition to DNA synthesis. Generation of circular double-stranded transposition intermediates suggest similarities with the replication of certain circular single-stranded DNA viruses. Intriguingly, such viruses encode a replication initiator protein organized similarly to Helraiser transposase. The possibility that episomal forms of Helraiser transposon can both replicate and persist in cells in the presence of transposase shows the potential of this transposon system for genomic applications.

Xiang Guo

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Epigenetics

Role of ETO2 in enhancer activity and chromatin organization during hemoglobin switching

Considerable work over decades has focused on exploration of the function of novel regulators in the switch from gamma- to beta-globin transcription and re-activation of gamma-globin expression to develop therapeutic approaches for sickle cell anemia and beta-thalassemia. Transcription factor ETO2 was reported to be a repressor of gene activity by recruiting HDACs to target genes through its dimerization domain and zinc finger motifs. Our ETO2 ChIP-seq result, together with ENCODE data for K562 cells, indicated considerable co-occupancy genome wide of ETO2 with Nucleosome Remodeling and Deacetylase Complex (HDAC1 and histone demethylase LSD1), at both proximal and distal enhancers, including at the beta-globin locus control region (LCR). To determine the function of ETO2 in the beta-globin locus, we performed RNA-seq in both wild type and Crispr/Cas9-mediated ETO2 KO K562 cells. We found that loss of ETO2 elevated embryonic (epsilon) and fetal globin gene (gamma) transcription and expression of erythroid maturation factors (ALAS2, EPB42 and HEMGN), validating the repressive functions of ETO2 in globin gene expression and erythropoiesis. In the absence of ETO2, the levels of active enhancer markers, H3K27 acetylation and H3K4 mono-methylation in the LCR were significantly increased, which was associated with reduced HDAC1 and LSD1 occupancy. Furthermore,

ETO2 mutant cells showed higher interaction frequency between the LCR and gamma-globin gene than WT cells. In agreement with increased LCR/gamma-globin loop formation, loss of ETO2 function induced LCR loading of the LDB1 complex, including erythroid master regulators TAL1 and GATA1, which mediates loop formation in the beta-globin locus. Constructs expressing WT ETO2 protein and different ETO2 variant proteins missing individual domains were examined in the background of ETO2-depleted cells to explore domain functions. Proteins missing any of the domains failed to rescue ETO2 repressive function. Loss of the TAF110 domain disrupted the interaction between ETO2 and the LDB1 complex, which is required for ETO2 recruitment to beta-globin locus. Depletion of the dimerization domain and zinc finger motifs impaired both HDAC1 and LSD1 recruitment and led to a significant increase of H3K27ac and H3K4me1. In summary, our research revealed that ETO2 is a key repressor of gamma-globin gene expression though the function of individual domains in regulating enhancer activity and chromatin organization.

Young-Kwon Park

Visiting Fellow

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Cell Biology - General

Brd4 binds to active enhancers to control cell differentiation and tissue development

The epigenomic reader Brd4 is an important drug target for cancers. To investigate its role in cell differentiation and tissue development, we used two independently developed Brd4 conditional knockout (cKO) mouse strains, Brd4 f/f (flanking exon 3) and Brd4 f/f #2 (flanking exon 5). By crossing cKO mice with Myf5-Cre mice to delete Brd4 gene in progenitor cells of brown adipose tissue and muscle, we demonstrate that Brd4 is essential for adipose tissue and muscle development. Consistently, Brd4 is essential for adipogenesis and myogenesis in culture. To map the genomic binding of Brd4, we performed chromatin immunoprecipitation assays with sequencing (ChIP-Seq). Brd4 co-localizes with lineage-determining transcription factors (LDTFs) on active enhancers during cell differentiation. LDTFs coordinate with H3K4 mono-methyltransferases MLL3/MLL4 (KMT2C/KMT2D) and H3K27 acetyltransferases CBP/p300 to recruit Brd4 to enhancers activated during differentiation. Brd4 deletion prevents the enrichment of Mediator and RNA polymerase II transcription machinery, but not that of LDTFs, MLL3/MLL4-mediated H3K4me1, and CBP/p300-mediated H3K27ac, on enhancers. Consequently, Brd4 deletion prevents enhancer RNA production, cell identity gene induction and cell differentiation. Interestingly, Brd4 is dispensable for maintaining cell identity genes in differentiated cells. Our data suggest a model of sequential actions of epigenomic regulators on enhancers: 1) pioneer TFs and LDTFs recruit MLL3/MLL4 to prime enhancer regions and label them with H3K4me1; 2) MLL3/MLL4 facilitate the binding of CBP/p300, which activate enhancers and label them with H3K27ac; 3) H3K27ac and acetylated TFs are recognized by the epigenomic reader Brd4, which recruits Mediator and RNA Polymerase II to establish enhancer-promoter interactions and activate cell type-specific gene expression.

Shuang Li

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Protein Structure/Structural Biology

Structural basis of tRNA-mediated transcription anti-termination

T-box riboswitches are bacterial cis-regulatory noncoding RNAs that sense and regulate amino acid availability through a multi-partite mRNA-tRNA interaction. T-box RNA is comprised of two conserved domains, a Stem I domain and a 3' antiterminator domain connected by a flexible linker. Stem I domain recognizes the overall shape and anticodon of a cognate tRNA with sequence and structural specificity. The antiterminator domain detects the molecular volume of the tRNA 3' end to sense aminoacylation. This readout dictates the formation of either an intrinsic transcription terminator or antiterminator. However, the architecture of a full-length T-box in complex with cognate tRNA and the detailed interactions between the tRNA 3' region and T-box 3' domain remain poorly defined. In our study, we develop a novel method to produce, assemble, and isolate stoichiometric complexes of the full-length T-box RNA bound by its cognate tRNA. Using this method, we define a minimal region of the T-box that is necessary and sufficient to selectively bind an uncharged tRNA, and solve the first co-crystal structure of this tRNA-mRNA complex at 2.7 Å by de novo phasing. The structure reveals how tRNA 3' end is buried inside the antiterminator where a conserved G ψ CU wobble pair at the base of helix A2 abuts the ribose 3'-OH of the tRNA terminal adenosine. This juxtaposition creates steric clash between the universal amino group of the esterified amino acid and the uridine nucleobase, thus providing a general mechanism to reject any aminoacyl-tRNA. We also obtain a cryo-EM structure of the full-length T-box riboswitch in complex with its cognate tRNA at 4.7 Å, an unprecedented resolution for RNA of this size. The EM structure reveals that T-box Stem I and the newly defined 3' domains simultaneously bind two faces of the cognate tRNA, orchestrated and facilitated by a surprisingly ordered inter-domain single-stranded linker. Taken together, the structures show that intermolecular stacking allows Stem I and antiterminator domains of the T-box mRNA to sandwich the uncharged tRNA to stabilize the antiterminator to transcribe downstream genes. These novel structures provide a detailed mechanistic framework of how a RNA-based steric device can achieve selectivity for a cognate, uncharged tRNA. This work informs the development of new avenues and targets to battle the global epidemic of antibiotic resistance, and strategies to modulate human microbiota to improve health.

Sai Prasad Pydi

Postdoctoral Fellow

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Endocrinology

Deletion of β -arrestin-1 in AgRP neurons leads to impaired whole body glucose homeostasis

Background: Hunger and satiety are regulated, to a major extent, by the agouti-related protein (AgRP)/neuropeptide Y (NPY) and proopiomelanocortin (POMC) neurons residing in the arcuate nucleus of the hypothalamus. During starvation, AgRP neurons are strongly activated to promote hunger. Peripheral hormones and nutrients regulate the function of AgRP neurons, thus ensuring proper glucose and energy homeostasis. Recent studies from various labs, including our group, have shown the importance of G-protein coupled receptors (GPCRs) in regulating the activity of AgRP neurons. GPCR function is modulated by a pair of proteins known as beta-arrestin-1 and -2 (barr1 and barr2, respectively), which can terminate GPCR signaling and/or mediate GPCR-independent signaling. It is well established that barr1 and barr2 are involved in various physiological functions. Objective: Understanding the potential roles of barr1 and barr2 in regulating the function of AgRP neurons.

Methods: To address this issue, we used Cre/loxP technology to generate mice that lacked either barr1 or barr2 selectively in AgRP neurons. Mutant mice and their control littermates were subjected to a series of metabolic and biochemical tests. Results: When maintained on a high-fat diet, AgRP-barr1-KO mice showed impaired glucose tolerance and insulin sensitivity, as compared to their control littermates. While body weight and food intake were similar among the two groups, blood glucose and plasma insulin levels were significantly increased in AgRP-barr1-KO mice. In contrast, AgRP-barr2-KO mice showed no obvious metabolic phenotypes. Hyperinsulinemic euglycemic clamp studies indicated that hepatic glucose production was significantly increased in AgRP-barr1-KO mice. These mutant mice also displayed increased liver triglyceride levels and elevated expression of hepatic genes promoting gluconeogenesis. All metabolic phenotypes exhibited by AgRP-barr1-KO mice were absent after dissection of the vagal branch innervating the liver. Conclusion: Our findings clearly indicate that barr1 in AgRP neurons is critical for maintaining proper whole body glucose homeostasis, most likely by regulating hepatic vagal outflow. Currently, we are focusing on the mechanisms through which barr1 regulates the activity of AgRP neurons. This line of investigation may lead to new strategies to alter the activity of this class of neurons for therapeutic purposes.

Lu Zhu

Visiting Fellow

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Endocrinology

A positive allosteric modulator that promotes insulin secretion by selectively enhancing M3 muscarinic receptor signaling in beta-cells

Given the worldwide epidemic in type 2 diabetes (T2D), there is an urgent need to develop novel antidiabetic drugs with increased efficacy and reduced side effects. A key feature of T2D is the inability of pancreatic beta-cells to release sufficient amounts of insulin to maintain normal blood glucose levels. Several studies have shown that activation of M3 muscarinic acetylcholine (ACh) receptors (M3Rs) expressed by pancreatic beta-cells can promote glucose-stimulated insulin release, thereby restoring normal blood glucose levels in mouse models of diabetes. We therefore hypothesized that agents that can promote signaling through beta-cell M3Rs might become useful in the treatment of T2D. The M3R is a prototypic class A G protein-coupled receptor that is selectively coupled to G proteins of the Gq family. At present, selective M3R agonists that bind to the orthosteric ACh binding site are not available. However, it has been reported that a small molecule drug (VU0119498) can enhance ACh-induced signaling through M3Rs by binding to an allosteric site on the M3R that is distinct from the ACh binding site. In this study, we tested the ability of this positive allosteric modulator (PAM) to stimulate insulin release in vitro and in vivo. The potential clinical use of PAMs has the great advantage that it respects the spatio-temporal control of receptor activation, i.e. the PAM is only active upon receptor occupation by an endogenous orthosteric agonist. Studies with cultured beta-cells (MIN6 cells) showed that VU0119498 enhanced ACh-induced increases in calcium levels and insulin secretion in an M3R-dependent fashion. We obtained very similar results when we carried out insulin secretion studies with isolated mouse and human pancreatic islets. In vivo studies demonstrated that VU0119498 treatment caused a significant increase in plasma insulin levels in mice, accompanied by a striking improvement in glucose tolerance. These VU0119498 effects were mediated by beta-cell M3Rs since they were absent in mutant mice selectively lacking M3Rs in beta-cells. Additional in vivo studies demonstrated that

VU0119498 doses that were able to promote insulin secretion in vivo caused no significant side effects in mice. These novel findings indicate that it should be possible to develop a novel generation of anti-diabetic drugs that act as PAMs on beta-cells M3Rs.

HYE KYUNG LEE

Postdoctoral Fellow

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Genetics

Engineering the mouse genome using Cas9-APOBEC base editing

CRISPR-Cas9 genome editing is widely used, both in basic and translational research, and it holds promise in personalized medicine. However, since the current technology is based on Cas9 and its ability to introduce double stranded DNA breaks, it faces limitations. Introducing two independent allelic mutations will invariably lead to the deletion of the entire stretch of DNA between the two sites, regardless of their distance to each other. This is the result of the double strand breaks introduced by Cas9 and the ensuing repair process. To bypass this impediment, I explored novel base editing technologies, which rely on deaminases that convert cytidines to thymidine or adenosine to guanosine. I used two classes of base editors, BE4 that converts cytidines to thymidine and ABE that converts adenosine to guanosine. In addition, I used new generations of base editors that have expanded PAM sequences and therefore the capacity to recognize extended target sequences. To test the ability of these base editors to simultaneously mutate several allelic sites, I targeted three enhancers within a super-enhancer. 477 zygotes were injected with specific guide RNAs and the base editors and a total of 48 mice were born. Out of those, 31 carried the designed mutations. Most importantly, the conversion of adenosines to guanosines, using novel ABEs, was highly accurate and no additional and unexpected mutations were detected in the targeted area. This is the first study demonstrating that allelic mutations can be introduced into the genome using base editors. It is also the first study demonstrating that Adenosine Base Editors (ABEs) are highly accurate to introduce A to G transitions into the mammalian genome.

Younghoon Jang

Postdoctoral Fellow

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Developmental Biology

H3.3K4M destabilizes enhancer epigenomic writers MLL3/4 and impairs adipose tissue development

During cell differentiation, transcriptional enhancers are bound by lineage-determining transcription factors and play a key role in regulating cell type-specific gene expression. Cell type-specific enhancers are marked by specific epigenomic features. Histone 3 lysine 4 (H3K4) mono-methylation (H3K4me1) is the predominant mark of a primed enhancer state. Histone 3 lysine 27 acetylation (H3K27ac) by H3K27 acetyltransferases CBP/p300 further follows H3K4me1 to mark an active enhancer state. MLL4 is a major mammalian H3K4me1 methyltransferase with partial functional redundancy with MLL3. MLL3 and MLL4 (MLL3/4) are required for CBP/p300-mediated enhancer activation in cell differentiation and cell fate transition. Deletion of Mll3/4 genes depletes H3K4me1 in cells and prevents the enrichment of CBP/p300-mediated H3K27ac, epigenome reader BRD4, Mediator coactivator complex, and RNA

Polymerase II on enhancers. Consequently, Mll3/4 deletion prevents enhancer RNA production, cell type-specific gene induction, and cell differentiation. However, the mechanism by which MLL3/4-mediated H3K4 methylation regulates enhancer activation has remained incompletely understood. To clarify the role of MLL3/4-mediated H3K4 methylation in enhancer activation, we turned to H3.3K4M, a lysine-4-to-methionine (K4M) mutation of histone H3.3 and an inhibitor of H3K4 methylation. We found that expression of ectopic H3.3K4M in lineage-specific precursor cells depletes H3K4 methylation and prevents adipogenesis in culture and adipose tissue development in mice. Mechanistically, H3.3K4M prevents enhancer activation in adipogenesis by destabilizing MLL3/4 proteins but not other Set1-like H3K4 methyltransferases including MLL1, Set1A, and Set1B. Notably, deletion of the enzymatic SET domain of MLL3/4 in lineage-specific precursor cells mimics H3.3K4M expression and prevents adipose tissue development by destabilizing MLL3/4 proteins. Interestingly, destabilization of MLL3/4 by H3.3K4M in adipocytes does not affect adipose tissue maintenance and function. Together, our findings indicate that H3.3K4M targets MLL3/4 to prevent enhancer activation in adipogenesis.

Sing-Wai Wong

Doctoral Candidate

NIEHS

Cell Biology - General

Unravelling the role of autophagy machinery in osteoclastogenesis

Osteoclasts (OCs) are multinucleated bone cells responsible for resorbing mineralized bone, and increased osteoclastogenesis can result in disproportionate bone resorption and OC diseases, such as osteoporosis. Studies have shown that autophagy, an evolutionarily conserved intracellular degradation pathway, is genetically linked to osteoporosis and OC function. However, the role of autophagy machinery in OC differentiation remains elusive. As we observed that autophagy is induced and expression of autophagy proteins is increased in vitro osteoclastogenesis, and therefore we hypothesized that components of the autophagy machinery are required for OC differentiation. To test this hypothesis, we used LysM-Cre mice to generate myeloid-restricted autophagy deficient mice (Becn1-cKO, Vps34-cKO, Atg14-cKO, Fip200-cKO, Uvrag-cKO and Atg5-cKO) that delete autophagic components in OC precursors. Young (2-month-old) mice lacking Becn1 (Becn1-cKO) exhibit significantly increased bone mass due to defective osteoclastogenesis, compared to littermate controls. Additionally, 12-month-old Becn1-cKO mice show reduced bone resorption, suggesting that ablation of Becn1 protects against age-induced bone loss. In order to determine if Becn1 is required for differentiation of OCs, we next generated OCs from bone marrow of WT and Becn1-cKO mice. Becn1 deficiency resulted in 2-fold reduction of OC number compared to that of WT OCs. Similarly, we found that the absence of Vps34, Atg14, or Fip200, but not Uvrag or Atg5, results in reduced osteoclastogenesis in vitro. Collectively, these data demonstrate that while Fip200 and the PI3KC3 complex (Becn1, Vps34 and Atg14) are required for OC differentiation, Uvrag and the downstream conjugation protein, Atg5, are not. Mechanistically, Becn1-deficient OCs have a decreased activation of the non-canonical NF- κ B pathway, but not MAPK, CREB, or canonical NF- κ B pathways during the differentiation. Microarray analysis further confirmed an increased expression of non-canonical NF- κ B genes and a decreased expression of non-canonical NF- κ B inhibitor genes in Becn1 deficient OCs. Finally, treatment of WT OC precursors with Spautin-1, a specific PI3KC3 inhibitor, blocks OC differentiation in a dose-dependent manner. Taken together, our data reveal that upstream complexes (Fip200 and PI3KC3) in the

autophagy pathway are essential for osteoclastogenesis and could represent novel targets for therapeutic interventions of osteoclast diseases.

Lee Langer

Postdoctoral Fellow

NIEHS

Chromatin and Chromosomes

BAF47: A New Mechanism for Tumor Suppression?

Atypical teratoid/rhabdoid tumors (AT/RTs) are among the most difficult to treat pediatric cancers, occasionally presenting before birth and having extremely poor outcomes. The defining feature of these cancers, which often present in the central nervous system, is loss of the protein BAF47, part of the SWI/SNF complex. SWI/SNF is a large, multi-subunit machine that organizes DNA into accessible and inaccessible regions and thereby regulates gene activity. However, BAF47 mutations cause aggressive cancers much more rapidly than disruption of any other SWI/SNF subunit, raising the question of how BAF47 loss alters cell behavior so dramatically. Given the early onset of AT/RTs and their frequent localization in the brain, we hypothesized that an absence of BAF47 may inhibit the differentiation of human stem cells into more mature, differentiated neural cells, causing them to maintain their proliferative and tumorigenic capacity. We therefore generated human stem cells in which BAF47 levels could be inducibly decreased and then tested how these cells responded to a treatment designed to induce neural differentiation. Indeed, BAF47-knockdown stem cells showed a remarkable, almost total resistance to neural differentiation. Moreover, genes related to pluripotency, such as OCT4 and NANOG, were maintained at high levels compared to controls. When we examined how the DNA of these cells was organized, we found that the decrease in BAF47 levels completely prevented the silencing of key stem cell identity regions, an effect that has not been described for any SWI/SNF complex subunit. These results suggest that BAF47 mutations may cause cancer by preventing cells from reorganizing the genome in a way that allows differentiation. We are strongly encouraged by this result, and we believe that BAF47 loss in vivo prevents cells from suppressing regions that normally promote self-renewal and block differentiation. We are currently generating a mouse model in which BAF47 can be deleted from neural stem cells at any point during development. We will examine these cells at different time points to determine whether they exhibit the same failure to silence stem cell identity regions. We are confident that these results will significantly alter how we think about tumor formation in early life, especially AT/RTs, and may suggest more effective, life-saving treatments for children with these conditions.

Maria Petrillo

Visiting Fellow

NIEHS

Signal Transduction - G-protein and Ion Channels

Beta-arrestin 1: a novel player in the glucocorticoid receptor activity

Glucocorticoids (GCs) are the most widely used drugs to treat many autoimmune and inflammatory diseases. Although much research has been focused on investigating the GC actions, it remains puzzling how GCs regulate distinct processes in different cells. Glucocorticoids exert their actions through the

glucocorticoid receptor (GR). Upon binding with glucocorticoids, GR undergoes activation and the complex translocates into the nucleus where it binds specific DNA responsive elements to regulate the transcription of thousands of target genes. Throughout its intracellular journey, liganded-GR interacts with regulatory proteins, thus affecting its activity and function. These protein-protein interactions are necessary for the resolution of the GC-dependent physiological and pharmacological processes. In this study we have discovered the β -arrestin-1 (ARRB1) as a novel binding partner and regulator of GR. β -arrestin-1 plays a well-established role in participating in agonist-mediated desensitization of G-protein-coupled receptors. Yet it has become more appreciated as scaffold protein, thus conferring novel signaling properties independent of GPCR activity. Also, we have shown that β -arrestin-1 is a GC-responsive gene. These findings led us to explore the prospect that β -arrestin-1 may contribute to the activity of GR. Indeed, RNA sequencing performed in control- and ARRB1 knockdown- A549 cells treated with glucocorticoids, showed β -arrestin-1 reshaped the GC transcriptome by regulating the transcription of \sim 1500 new genes. Cell cycle, cell morphology and post-translational modifications are some of the cellular functions significantly altered by knockdown of β -arrestin-1. Interestingly, among the post-translational modifications, genes involved in the ubiquitin-proteasome machinery resulted activated. Among these genes, we found the E3 ligase PELI1 was the most upregulated GC-responsive gene. In vitro data demonstrated that when β -arrestin-1 was knocked-down, it upregulates PELI1 which is responsible for reducing GR half-life by promoting its ubiquitination and proteasomal degradation. Consistent with this, knockdown of β -arrestin-1 and PELI1, protects GR from its enhanced degradation. Overall, these results demonstrate that β -arrestin-1 acts as a crucial player not only for the transcriptional activity of GR but also in its stability. The novel GR/ β -arrestin-1 partnership may help unravel new mechanisms that contribute to regulating the GC-dependent processes.

Yi Fang

Other

NIEHS

Cell Biology - General

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Prashant Rai

Visiting Fellow

NIEHS

Immunology - Autoimmune

Immunity-related GTPase Irgm1 guards against interferonopathy through mitochondrial maintenance

Irgm1 is an interferon- γ (IFN γ)-inducible immunity-related GTPase that is thought to support homeostasis in macrophages by promoting autophagic clearance of both invasive microbes (xenophagy) and defective mitochondria (mitophagy). Recently, we reported that naïve Irgm1-deficient mice have an autoimmune exocrinopathy accompanied by increased autoantibodies and spontaneous induction of IFN-stimulated genes (ISGs) in several organs. We hypothesized that the spontaneous interferon response observed due to Irgm1 deficiency is driven by cGAMP synthase (cGAS)-dependent sensing of uncleared mitochondrial DNA (mtDNA) that has soiled the cytoplasm. Here, we show that the abnormal

induction of type I IFNs and ISGs in *Irgm1*^{-/-} macrophages and murine embryonic fibroblasts is associated with defective autophagic flux, decreased mitochondrial membrane potential and relative opening of mitochondrial permeability transition pore, and increased cytoplasmic mtDNA. The aberrant cell-autonomous IFN response is normalized upon depletion of cellular mtDNA, as well as by silencing or inhibition of double strand DNA sensor cGAS, the adaptor protein stimulator of IFN genes (STING), TANK-binding kinase 1 (TBK1), or IFN regulatory factor 3 (IRF3), together suggesting that a mtDNA-cGAS-STING-IRF3 axis drives the abnormal IFN signature of *Irgm1*-deficient cells. Genetic deletion of the type I IFN receptor rescues the in vivo autoimmune tissue pathology and excess serum autoantibody levels of *Irgm1*^{-/-} animals, demonstrating the pathogenicity of type I IFN in these animals. Taken together, our findings support a model wherein *Irgm1*-mediated mitochondrial maintenance represses inappropriate mtDNA-dependent activation of autoinflammatory IFN responses. We propose that this fundamental mechanism may play a key role in suppression of type I interferonopathy syndromes.

Yufeng Qin

Postdoctoral Fellow

NIEHS

Epigenetics

An obesity-associated gut microbiome reprograms the intestinal epigenome and leads to altered colonic gene expression.

The gut microbiome, a key constituent of the colonic environment, has been implicated as an important modulator of human health. The eukaryotic epigenome is postulated to respond to environmental stimuli through alterations in chromatin features and, ultimately, gene expression. How the host mediates epigenomic responses to gut microbiota is an emerging area of interest. Here, we profile the gut microbiome and chromatin characteristics in colon epithelium from mice fed either an obesogenic or control diet, followed by an analysis of the resultant changes in gene expression. The obesogenic diet shapes the microbiome prior to the development of obesity, leading to altered bacterial metabolite production which predisposes the host to obesity. This microbiota-diet interaction leads to changes in histone modification at active enhancers that are enriched for binding sites for signal responsive transcription factors. These alterations of histone methylation and acetylation are associated with signaling pathways integral to the development of colon cancer. The transplantation of obesogenic diet-conditioned microbiota into germ free mice, combined with an obesogenic diet, recapitulates the features of the long-term diet regimen. The diet/microbiome-dependent changes are reflected in both the composition of the recipient animals' microbiome as well as in the set of transcription factor motifs identified at diet-influenced enhancers. These findings suggest that the gut microbiome, under specific dietary exposures, stimulates a reprogramming of the enhancer landscape in the colon, with downstream effects on transcription factors. These chromatin changes may be associated with those seen during colon cancer development.

Heather Vellers

Postdoctoral Fellow

NIEHS

Genetics

ASSOCIATION BETWEEN MITOCHONDRIAL DNA SEQUENCE AND DNA DAMAGE IN RESPONSE TO ENDURANCE TRAINING IN MICE

PURPOSE: Exercise endurance training ensues a multitude of health benefits towards the prevention and treatment of various chronic diseases. However, not all individuals respond well to endurance training such that some individuals have no response, while others respond poorly or even worsen. Genetic background is known to contribute to the interindividual variation with endurance training. Our current understanding of the role of genetics and exercise is limited primarily to the nuclear genome, while only a limited focus has been given to a potential role of the mitochondrial genome. The purpose of this study was to characterize elements of the mitochondrial genome, including mitochondrial DNA (mtDNA) sequence, heteroplasmy, copy number and damage, in four inbred male mouse strains previously characterized as high responders (FVB/NJ), moderate responders (SJL/J), low responders (BALB/cByJ), or non-responders (NZW/LacJ) to endurance training. **METHODS:** DNA was isolated from the Plantaris skeletal muscle of mice (n=37) and mtDNA was amplified by long range polymerase chain reaction (PCR), then tagged with Nextera libraries and sequenced on a Miseq instrument. A gene-specific quantitative PCR-based assay was used for the measurement of DNA damage and mtDNA copy number. **RESULTS:** Exercise endurance training did not change within strain mtDNA sequence or total number of heteroplasmies, however the high- and non-responders each displayed unique nonsynonymous single nucleotide polymorphisms (SNPs) on mt-Co3 (position 9348) and mt-Atp8 (position 7778) genes, respectively. There were significant differences in mtDNA copy number ($p=0.0003$) with the high responders displaying significantly higher mtDNA copy number than all other strains regardless of exercise (Sedentary vs. exercise mtDNA copy number: FVB/NJ= 104,564 \pm 5448 vs. 95216 \pm 8334; SJL/J=81,816 \pm 2693 vs. 60,431 \pm 6784; NZW/LacJ= 76,155 \pm 4847 vs. 55,219 \pm 1313; BALB/cByJ=74,474 \pm 9889 vs. 65,015 \pm 6442). Exercise training did not induce significant nuclear or mtDNA damage. **CONCLUSIONS:** Our results suggest that strain-dependent mtDNA SNPs and copy number may associate with the inherent ability to respond to endurance training. Experiments are in progress that include greater sample sizes and strains to determine the contribution of the mitochondrial genome with endurance training adaptations in mice and humans.

Cynthia Sakofsky

Postdoctoral Fellow

NIEHS

Genomics

Hypermutation Associated With Bursts Of Double-strand Breaks

Knowledge of molecular mechanisms underlying severe cases of genome instability can help in preventing and/or curing cancer and other diseases associated with genome destabilization. Our previous analyses of cancer genomes have revealed an unusual distribution of mutations resulting in tightly spaced clusters of mutations. The density of mutations in clusters was about 10,000-fold greater than in the rest of the genome, and a single cancer genome harbored hundreds of clusters. Mutation signature analyses identified that the origin of these clusters were DNA lesions from single-strand (ss) DNA specific cytidine deaminases, APOBECs, in long ssDNA regions (many kilobases), raising the question $\hat{=}$ what molecular mechanisms promote long, transient ssDNA that is vulnerable to localized hypermutation? We hypothesized that since ssDNA-intermediates form during double-strand break (DSB) repair, that bursts of DSBs in yeast repaired in the presence of ssDNA-specific APOBEC3A (A3A)

cytidine deaminase would result in the formation of multiple APOBEC-induced clusters similar to cancer. Also, since A3A leaves distinguishable marks of mutated C→T or G→A in the top (Watson) DNA strand originating from damage in ssDNA, we could further determine the magnitude and patterns of clustered mutations formed from processing multiple DSBs. To test this, G2-arrested yeast expressing A3A were exposed to gamma-irradiation, generating up to 100 DSBs per genome, and surviving yeast were collected for whole-genome sequencing. We found an abundance of A3A-induced clusters with a pattern of mutated C→T followed by a single-switch to mutated G→A, consistent with damage in ssDNA formed from 5' to 3' bi-directional resection intermediates arising during DSB repair. To test the effects of DSB resection on cluster formation, parallel experiments were done with resection-defective yeast that resulted in a significant decrease of single-switch clusters and an increase in clusters containing only mutated C→T, or only mutated G→A in the top strand, indicating that a different mode of repair became predominant in the absence of canonical resection at DSBs. Next, we analyzed more than 40 million mutation calls in cancer genomes and found that the predominant pattern of mutation clusters in APOBEC hypermutated tumors was the same as in resection-defective yeast. Our next experiments are set to evaluate a new model of long transient ssDNA in tumors formed via abnormal replication initiated by a DSB.

Yu-Hua Lo

Visiting Fellow

NIEHS

Biochemistry - General, Proteins, and Lipids

ATPase activity of the AAA-protein NVL2 regulates its communication with cofactors during ribosome biogenesis

Ribosomes are large ribonucleoprotein machines that carry out the essential role of translating mRNA into proteins. The assembly of ribosomes is an intricate process that relies on the aid of hundreds of ribosomal assembly factors. Three essential AAA-ATPases are required for pre-60S maturation, including NVL2, Midasin and Drg1. The AAA (ATPase associated with various cellular activities) family is a large and functionally diverse group of enzymes that couple ATP hydrolysis with mechanical work. NVL, the gene that encodes for NVL2, has been identified as a prognostic gene for prostate cancer and knockdown of NVL2 has been shown to inhibit cancer cell progression highlighting its potential as a therapeutic target. Previous *in vivo* studies revealed that NVL2 is responsible for catalyzing the removal of the assembly factor WDR74 from pre-60S particles, however little is known about the mechanism driving ribosome formation. Our goal is to uncover the molecular role of the AAA-ATPase NVL2 involved in ribosome biogenesis. We first asked the question as to whether or not ATP hydrolysis by NVL2 is required for efficient removal of WDR74 from ribosome particles. To determine if WDR74-containing 60S subunits were present in translating ribosomes, polysomal profiles of whole cell lysates were analyzed by sucrose gradient centrifugation. Our results indicated that WDR74 accumulates with translating ribosomes upon expression of an NVL2 mutant that is deficient in ATP hydrolysis. Moreover, Co-IP analysis showed WDR74 has a greater affinity for binding to the ATP deficient mutant of NVL2, suggesting that ATP hydrolysis drives release of WDR74 from both NVL2 and pre-60S particles. NVL2 is a type-II AAA-ATPase containing a unique N-terminus followed by tandem AAA domains known as the D1 and D2 domains respectively. To distinguish the role of the D1 and D2 domains we performed yeast complementation assays, which revealed that, *in vivo*, the ATPase activity in the D2-AAA domain is strictly required for cell

cycle progression. Recently, we have solved the first structure of yeast NVL2 trapped in the pre-ATP hydrolysis state using cryo Electron Microscopy. Our structure reveals that the D1 and D2 domains of NVL2 stack on top of one another in a double-ring hexameric conformation. Taken together, our data suggest that ATP hydrolysis of NVL2 drives conformational changes to mediate its cofactor-binding and release during ribosome assembly.

Miaofei Xu

Visiting Fellow

NIEHS

Carcinogenesis

Mitochondrial genomic alterations in spontaneous and chemical-induced hepatocellular carcinomas in B6C3F1/N mice

Mitochondria play an important role in cellular energy metabolism, free radical generation, and apoptosis. An increase in reactive oxygen species has been implicated in many carcinogenic processes. We hypothesized that chronic oxidative stress and secondary mitochondrial alterations could contribute to chemical induced carcinogenesis and tested this hypothesis by evaluating the mitochondrial genome in hepatocellular carcinomas (HCCs) arising either spontaneously or due to chronic chemical exposures in B6C3F1/N mice. We performed ultra-deep (50,000x) whole mitochondrial DNA (mtDNA) sequencing and mtDNA copy number analysis in HCCs that arose either spontaneously in vehicle controls at 2 years (n=20, 10/sex) or due to 2-year exposure to a genotoxic carcinogen, ginkgo biloba extract (GBE; n=10, male only) and a non-genotoxic carcinogen, anthraquinone (n=10, male only); age-matched non-tumor liver from vehicle controls (n=20, 10/sex) were also included. In total, 958 mtDNA mutations sites were detected. Of those, the number of non-synonymous mutations in the GBE-induced HCCs (n=790) was extremely high compared to anthraquinone-induced (n=258) or spontaneous (n=290) HCCs. Mutation signature analysis demonstrated a predominant G/C to T/A transversions in GBE-induced HCCs, suggesting 8-oxo-guanine adduct formation secondary to oxidative stress. Surprisingly, only 7 mutation sites were found in D-loop region that is a common hotspot in human HCCs. The mtDNA copy number analysis revealed a significant reduction in spontaneous (p=0.006) and anthraquinone-induced (p=0.001) HCCs but not in GBE-induced HCCs (p=0.4). GBE was positive in the Salmonella mutagenicity (Ames) assay (in vitro) and negative in the mouse peripheral blood micronucleus test (in vivo). The high mtDNA mutation load/sample indicated that GBE likely has a genotoxic mode of action and cause more mtDNA damage. Anthraquinone was equivocal in the Ames assay (in vitro) and positive in the mouse peripheral blood micronucleus test (in vivo), and the low mtDNA mutation load/sample suggested that anthraquinone likely has a non-genotoxic mode of action. To our knowledge, this is the first time distinct mtDNA alterations have been demonstrated in spontaneous and chemical-induced HCCs. Unique mtDNA mutation spectra and copy number variation could help in better understanding the mode of action in chemical carcinogenesis.

Natale Sciolino

Postdoctoral Fellow

NIEHS

Neuroscience - Integrative, Functional, and Cognitive

A locus coeruleus to lateral hypothalamus circuit for suppression of feeding

Clinical evidence suggests that altered norepinephrine (NE) signaling is implicated in overeating and excessive weight gain. Although modulators of NE signaling are currently the most effective drugs for weight loss, they result in adverse side-effects due to their broad actions throughout the nervous system. Thus, there is a critical need to identify specific NE circuits that suppress feeding without other effects. Towards this goal, I used chemogenetics to reveal that activation of NE-locus coeruleus (LC) neurons results in suppressed feeding and weight loss. This key finding, along with evidence that feeding is also suppressed by delivery of NE agonists into the lateral hypothalamus (LHA), suggests that increased NE-LC activity suppresses feeding through select inputs to the LHA. To test this hypothesis, I used optogenetics to activate the LC-LHA circuit of our newly generated knockin-mouse line expressing cre recombinase under the noradrenergic dopamine beta-hydroxylase (Dbh) promoter. The Dbhcre mice were first injected with a cre-responsive virus expressing channelrhodopsin-2 (ChR2) or eYFP control into the LC, and then implanted with optical probes over the LHA. I found that photostimulation (10 Hz) of the LC-LHA circuit rapidly suppressed feeding in ChR2 mice relative to controls. To rule out the possibility that this effect was due to changes in anxiety, mice were tested in the elevated plus maze and real-time place aversion test. In both tests, photostimulation had no effect on anxiety-like behavior in ChR2 mice versus eYFP controls, demonstrating the LC-LHA circuit regulates feeding independent of anxiety. To ascertain if NE signaling is required by LC neurons to suppress feeding, I used our Dbh conditional knockout allele in combination with En1cre (LC-Dbh mutants) to disrupt NE synthesis selectively in LC neurons. LC-Dbh mutants and littermate controls were pretreated with vehicle or the alpha-2 adrenoceptor antagonist yohimbine (3 mg/kg i.p.) for its well-known ability to evoke NE release and consequently suppress feeding. In littermate controls, I found yohimbine suppressed feeding compared to vehicle. However, yohimbine had no effect in LC-Dbh mutants. Collectively, these findings reveal a novel role of LC neurons in the suppression of feeding that is mediated by release of NE in the LHA. The findings suggest that targeting specific NE neural pathways may yield improved weight loss therapies without anxiety side-effects.

Joanne Damborsky

Postdoctoral Fellow

NIEHS

Neuroscience - Cellular, Molecular, Neurotransmission and Ion Channels, and Neurochemistry

Cholinergic-GABAergic interactions in the hippocamposeptal pathway

A subset of GABAergic neurons in the hippocampus send projections to the medial septum/diagonal band of Broca (MS/DBB) region of the basal forebrain. These hippocamposeptal (HS) projection neurons are part of a reciprocal circuit that is critical for mediating spatial and episodic memory, and is disrupted in Alzheimer's disease (AD). Previous studies have shown that HS neurons form functional connections with neurons in the MS/DBB, however, there is still little known about how HS neuronal activity is integrated into this circuit. To examine how HS synaptic transmission is modulated in the MS/DBB, we used optogenetics to selectively stimulate HS terminals while performing whole-cell patch clamp recordings from MS/DBB neurons in acute slices. Most HS neurons co-express somatostatin (SST), so to target these neurons we performed stereotaxic injections of AAV containing mCherry/channelrhodopsin-2 (ChR2) into the hippocampus of SST-Cre mice. This resulted in specific expression of mCherry and ChR2 in SST+ neurons in the hippocampus, and extensive expression of

mCherry-containing fibers in the MS/DBB. Activation of these HS terminals using 470 nm light stimulation resulted in light-induced synaptic inhibitory postsynaptic currents (IPSCs) in both cholinergic and GABAergic neurons in the MS/DBB. Bath application of the acetylcholine receptor agonist carbachol (50 μ M) significantly decreased the amplitude of these light-evoked IPSCs, suggesting HS GABA release is attenuated by cholinergic activity in the MS/DBB. We next wanted to directly record from HS neurons that synapse onto cholinergic neurons in the MS/DBB. To do this, we performed monosynaptic retrograde synaptic tracing by injecting a Cre-dependent AAV helper virus and glycoprotein-deleted rabies virus that contained ChR2 and mCherry directly into the MS/DBB of ChAT-Cre mice. We were then able to record from the mCherry/ChR2-expressing neurons in the hippocampus that form direct synaptic connections with cholinergic neurons in the MS/DBB. Using this method, we will determine how the excitability of HS neurons that synapse onto cholinergic neurons in the MS/DBB is regulated. In conclusion, we have determined that GABAergic and cholinergic activity in the HS pathway is intertwined in a bidirectional manner. Future studies will reveal the importance of these cholinergic-GABAergic interactions for memory formation, and the extent to which this circuit is disrupted in AD.

Sheng Song

Visiting Fellow

NIEHS

Neuroscience - Neurodegeneration and Neurological disorders

Dysfunction of noradrenergic system induce α -synucleinopathy and neuronal loss from gut to brain

Clinical and pathological evidence revealed that α -synuclein (α -syn) pathology starts in the gut and spreads via anatomically connected structures from the enteric nervous system to the central nervous system. However, there are no suitable animal models to recapitulate this sequential pattern of pathology. Our previous study demonstrated that depletion of brain norepinephrine (NE) disrupted immune homeostasis through over-activation of microglia, producing a spatiotemporal order of neurodegeneration in the mouse brain. Since NE is the primary neurotransmitter of sympathetic neurons that supply the intestine, the contribution of which to the gut-brain axis in Parkinson's disease (PD) pathogenesis remains unknown. For this purpose, A53T-SNCA (human mutant α -syn over-expression) mice received a single injection of a selective noradrenergic neurotoxin, DSP-4 (50 mg/kg, ip.). We found DSP-4 significantly reduced the tissue level of NE in intestines, and increased immune activities in gut, characterized by altered intestinal microbiota composition and increased proinflammatory gene expression. Furthermore, a rapid-onset of α -syn pathology was observed in enteric neurons after 2 weeks, and delayed dopaminergic neurodegeneration in substantia nigra was detected after 5 months, with the appearance of constipation and impaired motor function, respectively. The increased α -syn pathology was only observed in large intestine but not in small, suggesting that the large intestine is the dominant segment of the gastrointestinal tract implicated in PD. Mechanistic studies reveal that DSP-4-elicited increased expression of NADPH oxidase (NOX2) found initially only in immune cells during the acute intestinal inflammation period, the intense NOX2 expression was also found in enteric neurons during the period of chronic inflammation. Increase of neuronal expression of NOX2 correlated well with the degree of α -syn aggregation, and subsequent enteric neuronal loss, suggesting that NOX2-generated reactive oxygen species play a key role in α -synucleinopathy. Moreover, inhibiting NOX2 by DPI or rescuing NE dysfunction by Salmeterol can significantly block intestinal inflammation, ameliorate α -syn pathology, behavioral deficits and

subsequent neurodegeneration in both gut and brain. Taken together, our "two-hit" model of PD shows a progressive pattern of pathological changes from gut to brain, and reveals a critical role of dysfunction of noradrenergic system in the pathogenesis of PD.

Kai Kang

Research Fellow

NIEHS

Informatics/Computational Biology

CDSeq: a novel deconvolution method to dissect heterogeneity using bulk RNA-seq data

Background: The cell-type composition of many biological tissues varies widely across samples. Such heterogeneity hampers efforts to probe the role of each cell type in the tissue microenvironment. Current approaches that address this issue have drawbacks. Experimental techniques, such as fluorescence-activated cell sorting (FACS), are expensive. Computational methods, such as CIBERSORT and csSAM, are flexible and promising; but they estimate either sample-specific proportions of each cell type or cell-type-specific expression profiles, not both, by requiring the other as input. Method: We developed a deconvolution method, CDSeq, designed to estimate both sample-specific cell-type proportions and cell-type-specific expression profiles simultaneously using bulk RNA-seq data only. We modeled the observed expression data using multinomial distributions whose parameters reflect the unknown cell-type-specific expression profiles and sample-specific proportions. Also, we incorporated Poisson random variables to model the varying amounts of RNA from cell types of different sizes. Integrating these components, we built a Bayesian model that fully captures the stochastic nature of RNA-seq data. We used a Gibbs sampler for estimation and developed a strategy to automatically determine the number of cell types present. Results: We benchmarked CDSeq using mixtures of varied but known composition in two ways: in silico mixtures using RNA-seq data from six pure cell lines; experimental mixtures of mRNA isolated from four human cell lines. For the former, we generated 40 mixture samples using expression data downloaded from the UCSC genome browser. For the latter, we used equal concentrations of RNA extracted from cultured cells to create 32 mixture samples. We profiled global mRNA abundance in every sample and pure cell line by RNA-sequencing and applied CDSeq separately in the two studies. We evaluated CDSeq's performance by comparing its estimates to the specified composition of our mixtures and the measured expression profiles in homogeneous samples from pure lines. In both studies, CDSeq outperformed competitors: with, respectively, 77% and 17% lower RMSE than those of CIBERSORT (only estimates proportions) and 64% and 16% lower RMSE than those of csSAM (only estimates expression profiles). Conclusion: CDSeq holds promise for computationally deciphering complex mixtures of cell types, each with differing expression profiles, using bulk RNA-seq data.

Jonathan Busada

Postdoctoral Fellow

NIEHS

Endocrinology

Endogenous Glucocorticoids are Required to Suppress Spontaneous Gastric Inflammation and Spasmolytic Polypeptide Expressing Metaplasia in the Mouse

Gastric cancer is the third leading cause of cancer deaths world-wide. Chronic inflammation causes mucosal damage and metaplasia and creates a favorable environment for the evolution of gastric cancer. Despite the established role inflammation plays in the development of metaplasia and gastric cancer, the mechanisms that regulate gastric inflammation remain unclear. Glucocorticoids are steroid hormones that are synthesized by the adrenal glands and exert potent anti-inflammatory effects. Synthetic glucocorticoids are a clinical mainstay for treating inflammatory diseases of the gastrointestinal tract, however, almost nothing is known concerning the actions of endogenous glucocorticoids in the stomach. In this study, we tested the hypothesis that endogenous glucocorticoids are required to suppress gastric inflammation and prevent metaplasia development. Removal of endogenous glucocorticoids by adrenalectomy resulted in rapid infiltration of the gastric corpus by macrophages and eosinophils. Inflammation was followed by the development of spasmodic polypeptide expressing metaplasia (SPEM) which is a common precursor of gastric cancer in humans. RNA sequencing of the gastric corpus 3-days post-adrenalectomy, which precedes leukocyte infiltration and histological changes of the gastric mucosa, revealed massive upregulation of proinflammatory genes. To determine how discrete leukocyte populations contribute to SPEM development, we adrenalectomized Rag1 knockout mice that lack B and T cells, Gata1 mutant mice that lack eosinophils, and wild type mice treated with clodronate to deplete macrophages. SPEM development in B and T cell deficient and eosinophil deficient mice was indistinguishable from wild type mice. In contrast, macrophage depletion prevented SPEM development. Our results indicate that the stomach is highly reliant on the action of endogenous glucocorticoids to suppress the expression of proinflammatory gene products and the recruitment of macrophages. Disruption of glucocorticoid signaling results in SPEM that may increase the risk of gastric cancer. Furthermore, this study is the first to explain the age old clinical finding that gastric inflammation is associated with adrenal insufficiency.

Sreenivasa Ramaiahgari

Visiting Fellow

NIEHS

Pharmacology and Toxicology/Environmental Health

Functional Characterization of Human and Rat 3D Hepatocyte Models and Application of High-throughput Transcriptomics (S1500+) in Toxicology Screening

With ever-increasing numbers of chemicals being released into the environment, their effects on public health remains a major concern, given most of them have not been studied for effects on human health. Current cell culture based assays use two-dimensional (2D) tissue culture models for chemical screening that show poor resemblance to tissue architecture and functionality, lack integrated pathway responses to xenobiotic exposures and sufficient xenobiotic metabolism capacity. To address these limitations, we have developed three-dimensional (3D) spheroid models of human bi-potent progenitor cell line, HepaRG and primary rat hepatocytes (Sprague-Dawley) with improved physiological relevance and tissue functionality. When cultured as spheroids in 384-well plates, HepaRG cells exhibit several hallmarks of polarized hepatocytes and tissue-like functionality. Assessment of xenobiotic metabolism competence with clinical substrates of CYP1A2, CYP2B6 and CYP3A4 showed robust levels of basal and inducible liver enzymatic activities that are comparable to ranges produced in Primary human hepatocyte (PHH) suspensions and a 2-20-fold higher than median activities observed with 2D cultures of PHHs. In a similar culture configuration, primary rat hepatocytes also formed polarized spheroid-like

structures and displayed a stable phenotype for more than 60 days compared to their 2D sandwich cultures which are viable for up to 5 days. Specific activities of CYP1A2 and CYP3A enzymes were markedly higher than sandwich cultured rat hepatocytes. Repeated exposure studies on both spheroid models showed differential sensitivity in identifying compounds that cause metabolism-dependent liver injury (e.g. acetaminophen, aflatoxin B1, benzo[a]pyrene). Initial characterization of high-throughput transcriptomics (HTT) assays (panel of 3000 transcripts, S1500+) on metabolically competent cells treated with 24 drug-induced liver injury compounds effectively discriminated liver injury compounds (e.g. troglitazone, trovafloxacin) from "safer" compound analogues (e.g. rosiglitazone, levofloxacin). Incorporation of these physiologically relevant in vitro models and data-rich approaches are promising new tools in predicting chemical-biological interactions and human liver toxicity potential.

YONG-MOON PARK

Postdoctoral Fellow

NIEHS

Cultural Social and Behavioral Sciences

Nocturnal light exposure while sleeping and risk of obesity in U.S. women

There is evidence from animal studies that light exposure may have direct effects on circadian rhythm, resulting in weight gain and obesity. Evidence on the association between exposure to artificial light at night (LAN) and obesity in humans has been limited to shift workers mainly in a cross-sectional study setting in which reverse-causality cannot be ruled out. We assessed the association between LAN and obesity in 50,884 Sister Study cohort participants aged 35 to 74 years enrolled from 2003 through 2009 and observed until July 1, 2014 (mean follow-up=5.7 years). Women were excluded if they had a history of cancer, heart disease, or stroke or were shift workers or pregnant at baseline, as these factors can affect sleep pattern and body weight. Exposure to LAN was assessed at baseline using a questionnaire and categorized as "no light", "small nightlight", "light outside the room", and "light or television on in room", representing increasing LAN exposure. Objective measurements for obesity at baseline included weight, height, waist and hip circumference. Incident obesity at follow-up included self-reported obesity (body mass index=30 kg/m²). Poisson regression models with robust error variance were used to estimate multivariable-adjusted relative risks (RR) and 95% confidence intervals for prevalent and incident obesity. Prevalence ratios for obesity measured using body mass index, waist circumference, waist-hip ratio, and waist-height ratio increased with increasing LAN exposure (P trend<0.001), after adjusting for potential confounders such as age, sleep duration, socioeconomic status, lifestyle factors, energy intake, caffeine consumption, depression, and perceived stress. Compared to no light exposure at night, having any light on while sleeping was associated with incident obesity (RR=1.13, 1.01-1.27). Women who reported turning on a light when they wake up during the night had higher risk of obesity than those who never wake up or do not turn on the light (RR=1.13, 1.00-1.28). We also found that having "light or television on in room" was associated with gaining 5 kg or more as compared with having no light (RR=1.14, 1.04-1.23, P trend=0.007). The associations were persistent even after adjusting for other sleep characteristics such as bedtime pattern, waking up at night, napping, sleep medication use, and snacking at night. Our findings from a large prospective study suggest that exposure to light at night may be a risk factor for obesity.

Helen Chin

Postdoctoral Fellow

NIEHS

Informatics/Computational Biology

A longitudinal analysis of ovarian growth in girls from birth to 9 months and its association with infant feeding type

Infertility and impaired fecundity are significant public health issues. Close to 1 in 8 reproductive-aged women in the US have trouble conceiving or carrying a pregnancy to term. Childhood ovarian development may influence adult ovarian function, but there are limited descriptions of healthy ovarian growth in girls, particularly during infancy. The transient activation of the hypothalamic pituitary gonadal axis during the first few months of life may represent a sensitive period for adverse exposures. Isoflavones are estrogen-like compounds found in soy (and infant soy formula) that can bind to and activate estrogen receptors and have been shown to estrogenize infant vaginal tissue. Using data from the Infant Feeding and Early Development Study, a longitudinal cohort study of estrogen-responsive outcomes in healthy infants, we characterized the ovarian growth trajectories in infant girls from birth to 9 months and evaluated the association between infant feeding type and ovarian growth trajectory. We hypothesized that infants exclusively fed soy formula would demonstrate accelerated ovarian development compared with growth patterns for infants exclusively breastfed or exclusively fed cowâ€™s milk formula. There were 136 girls who completed the study and were included in the analysis. Ultrasounds were performed on the infants within 72 hours of birth and at 4, 16, 24, and 32 weeks. Ovarian volume was calculated as the geometric mean of the right and left ovary at each ultrasound visit. We used mixed-effects regression splines to examine the overall age trajectory of ovarian volume and differences in age trajectories by feeding type. The mean ovarian volume increased from 0.2 cm³ (SD=0.2) at birth to a maximum mean value of 1.0 cm³ (SD=0.6) at 16 weeks, which was followed by a slight shrinking and leveling off in later weeks. When we assessed growth trajectories by feeding group there was a slower decrease in volume after the 16-week peak for breastfed infants compared with soy milk fed infants ($p=0.03$), but no differences between soy and cowâ€™s milk formula fed ($p=0.77$) or cowâ€™s milk formula fed and breastfed infants ($p=0.13$). Our results show an increase in infant ovarian volume shortly after birth overall followed by a shrinking and leveling off that was more rapid in soy fed girls, counter to our hypothesis. Further research is needed to understand the stimulus for early increases in ovarian volume and the influence of estrogenic exposures.

Chunfang Gu

Research Fellow

NIEHS

Tumor Biology and Metastasis

PPIP5K regulates serine, glycine and one carbon metabolism: a new cancer therapy target?

Cancer cells rewire critical metabolic pathways to support malignant transformation, tumor survival and proliferation. A molecular understanding of this fundamental metabolic dysregulation can assist the development of new cancer therapies. Our approach has been to study control mechanisms at the interface of cell signaling and metabolic homeostasis. We demonstrate a new Achilles Heel for cancer cell survival and proliferation: the catalytic activity of a small molecule kinase, PPIP5K, that synthesizes inositol pyrophosphate signaling molecules. We used CRISPR/Cas9 to prepare PPIP5K KO variants of the

HCT116 colon cancer cell line; all of our results were confirmed with two independent KO clones. We studied the impact of the KO upon cell proliferation for 3 days in a culture medium containing 0.5 mM glucose, to mimic a tumor-like microenvironment. Wild-type cell number doubled, but the cell number of PPIP5K KO cells decreased by 60%. In pursuing a metabolic basis for the proliferative defect in the KO cells, we discovered 5-20 fold lower levels of key nucleotide precursors (IMP, FGAR and dihydroorotate). We next performed ¹³C-glucose flux analysis, so as to dynamically trace substrate utilization in intact cells. We found 20-50% less ¹³C-glucose was assimilated into nucleotides such as IMP, ATP, UMP and UTP. Significantly, this was not due to any overall glycolytic defect; on the contrary, the KO cells, exhibited increased ¹³C incorporation into pyruvate and alanine (33% and 46%, respectively). So, we next focused on glucose derived serine, which provides one carbon units for de novo nucleotide synthesis via the serine, glycine and one carbon (SGOC) pathway, which has previously been identified as a key chemotherapeutic target. We found KO cells synthesized 50% less serine from glucose. Thus, we next performed ¹³C-serine flux analysis; the KO cells exhibited a 40-50% decrease in SGOC pathway-derived synthesis of the purine nucleotides IMP and AMP. Finally, we confirmed the defect in nucleotide synthesis in KO cells by rescuing their survival and growth by supplementation with either IMP or an exogenous nucleoside mix. Our data show that, particularly in cancer cells, efficient SGOC-dependent nucleotide synthesis has a strong requirement for PPIP5K. Our discovery of this new interaction between signaling and metabolism shows PPIP5K may be a novel target for tumor therapy.

Hao Hu

Visiting Fellow

NIEHS

DNA-binding Proteins/Receptors and DNA Repair

Nuclear Receptor Communications: PXR Coordinates RORα and HNF4α to Regulate the SULT1E1 Gene

Estrogen sulfotransferase (SULT1E1), a member of the SULT family, specifically and effectively sulfates estrogens to their inactive forms-sulfated estrogens. Its expression is tightly regulated by nuclear receptors such as HNF4α, RORα and PXR. Our preliminary results indicated that SULT1E1 is suppressed in low glucose treated HepG2 cells through the conserved phosphorylation sites on these nuclear receptors. This may act as a cellular defense response to elevate cellular estrogen levels thereby compromising hypoglycemia induced inflammation. We hypothesized that PXR coordinates RORα and HNF4α to regulate SULT1E1 gene expression via the conserved phosphorylation sites. First, by utilizing human hepatoma-derived HepG2 cells and HepG2-derived ShP51 cells which stably express human PXR, it was observed that SULT1E1 was suppressed in low glucose (400 mg/L) medium in a PXR dependent manner. Phosphorylation of PXR at Serine350 in low glucose treated ShP51 cells was detected by a phosphopeptide PXR antibody. Transient transfection analysis of SULT1E1 promoter-luciferase reporter genes revealed that nuclear receptors RORα and HNF4α are required for PXR to regulate the SULT1E1 gene. Co-immunoprecipitation assays were applied to elucidate protein-protein interactions among them. Chromatin immunoprecipitation and gel shift assays showed that RORα bound to the SULT1E1 enhancer (-941 bp upstream of SULT1E1) while HNF4α dissociated from it at low glucose condition. Phosphopeptide antibodies and phosphomimetic mutants of these nuclear receptors were utilized to examine whether phosphorylation becomes a regulatory factor determining the glucose response SULT1E1 gene activation. The results demonstrated that phosphorylated PXR at Serine350 interacts with HNF4α, which mediates HNF4α phosphorylation at Serine87 leading its dissociation from

the enhancer in low glucose treated cells. In the meantime, phosphorylated PXR interacts with ROR α and recruits its binding to the enhancer thereby repressing SULT1E1 promoter activation. Collectively, PXR regulates ROR α and HNF4 α to associate with and dissociate from the SULT1E1 enhancer, thereby activating the SULT1E1 gene in response to glucose exposure.

Julia Linke

Postdoctoral Fellow

NIMH

Neuroscience - Integrative, Functional, and Cognitive

White matter microstructure in disruptive mood dysregulation disorder and bipolar disorder

Background: Longitudinal data showed that chronic pediatric irritability, which affects approx. 10 million youth in the U.S., does not confer the risk to develop bipolar disorder (BD). This led to the creation of the DSM-5 diagnosis of disruptive mood dysregulation disorder (DMDD). Albeit on a different timescale, both DMDD and BD share an elevated proneness to anger, but whereas numerous diffusion tensor imaging (DTI) studies in BD suggested altered myelin plasticity as a key mechanism in this disorder, DTI has not yet been applied in DMDD. Research on how BD and DMDD differ with regard to structural connectivity might guide the development of targeted interventions for these different, but equally impairing phenotypes. Methods: We acquired DTI data from 118 participants (BD=36, DMDD=44, HV=38). After preprocessing with TORTOISE applying robust tensor fitting, images of fractional anisotropy (FA), longitudinal diffusivity (LD) and radial diffusivity (RD) were processed with tract based spatial statistics. Next, the three measures were used in an ANCOVA each with age and medication load as nuisance variables and to train Gaussian process classifiers to predict, which group participants belong to. Results: In BD, we observed widespread reductions in FA with peak voxels being located in the genu of the corpus callosum (5206 voxels, $p_{min}=0.0002$) and a finding associated with increased radial diffusivity (5098 voxels, $p_{min}=0.0002$). In DMDD, reductions in FA were confined to the genu of the corpus callosum (1494 voxels, $p_{min}=0.007$) and related to reduced longitudinal diffusivity (1453 voxels, $p_{min}=0.0002$). Based on information about FA, the machine learning algorithm predicted whether subjects were BD or HV with an accuracy of 75% and using information about LD, it was able to predict, whether subjects were DMDD or HV with an accuracy of 68% and could even distinguish between disorders with an accuracy of 60%. Conclusions: While we replicated findings of widespread reductions in FA and increases in RD in BD supporting the hypothesis of altered myelin plasticity as a mechanism in BD, alterations in DMDD were confined to the corpus callosum and seem to be driven by altered longitudinal diffusivity, which was previously shown to be more related to the degree of axonal organization. Our results support a role of altered structural connectivity in the risk architecture of both disorder, but indicate different underlying mechanisms.

Jason Avery

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NIMH

Neuroscience - Integrative, Functional, and Cognitive

Neural correlates of taste reactivity in autism spectrum disorder

Selective or 'picky' eating habits are common among those with autism spectrum disorder (ASD). These

behaviors are often related to aberrant sensory experience in individuals with ASD, including heightened reactivity to food taste and texture. However, very little is known about the neural mechanisms that underlie taste reactivity in ASD. In the present study, food-related neural responses were evaluated in 21 young adult and adolescent males diagnosed with ASD without intellectual disability, and 21 typically-developing (TD) controls. Taste reactivity was assessed using the Adolescent/Adult Sensory Profile, a clinical self-report measure. Functional magnetic resonance imaging was used to evaluate hemodynamic responses to sweet (vs. neutral) tastants delivered during scanning. Subjects also underwent resting-state functional connectivity scans. The ASD and TD individuals did not differ in their hemodynamic response to gustatory stimuli. However, the ASD subjects, but not the controls, exhibited a positive association between self-reported taste reactivity and the response to sweet tastants within the insular cortex and multiple brain regions associated with gustatory perception and reward. There was a strong interaction between diagnostic group and taste reactivity on tastant response in brain regions associated with social cognition and ASD pathophysiology, including the bilateral anterior superior temporal sulcus (STS). This interaction of diagnosis and taste reactivity was also observed in the resting state functional connectivity between the anterior STS and dorsal mid-insula (i.e., gustatory cortex). These results suggest that self-reported heightened taste reactivity in ASD is associated with heightened brain responses to food-related stimuli and atypical functional connectivity of gustatory and social brain regions, which may predispose these individuals to maladaptive and unhealthy patterns of selective eating behavior.

Jacob Nordman

Postdoctoral Fellow

NIMH

Neuroscience - Integrative, Functional, and Cognitive

Dorsal raphe regulation of aggression via the medial orbitofrontal cortex and the medial amygdala

Violence and aggression are serious concerns for modern society. Current therapeutic strategies are limited due to a lack of understanding about the neurological mechanisms underlying aggression and the environmental triggers that cause it. A number of studies have demonstrated that the dorsal raphe (DR), a major serotonin (5HT) nucleus, is a critical regulator of social behaviors like aggression, however, the precise contribution of the DR to these behaviors has remained elusive. In this study, we show that the DR inhibits aggression via a circuit involving the medial orbital frontal cortex (MeOC), the medial amygdala (MeA), and to a lesser extent the ventromedial hypothalamus (VmH), three major loci underlying aggression. We performed circuit mapping analysis and found that DR neurons densely project onto the MeOC, MeA, and VmH. c-Fos labeling revealed that optogenetic activation of the DR increases activity in these brain regions. To determine how this putative aggression circuit regulates aggression, we optogenetically stimulated or silenced neurons of the DR and its terminal projections during an aggression test. Silencing DR-MeOC synapses strongly inhibited aggression. A weaker effect was found when silencing the DR-VmH synapses. Aggression suppression could be overridden when silencing of DR somas was simultaneously coupled with activation of DR-MeOC synapses. Interestingly, stimulating DR-MeA synapses inhibited aggression. This finding, in combination with previous studies showing direct suppression of MeA activity by the MeOC, suggest a trisynaptic circuit involving the DR, MeA, and MeOC underlying aggression. Finally, to determine the contribution of 5-HT in DR regulation of aggression, we present a novel FRET based 5-HT sensor for use in vivo. Future directions will be aimed

at exploring how 5-HT is involved in this trisynaptic aggression circuit using this sensor. This study helps elucidate the role of the DR and 5-HT in regulating aggression, and provides possible therapeutic targets in the fight against pathological aggression and violence.

Nicholas Balderston

Postdoctoral Fellow

NIMH

Neuroscience - Integrative, Functional, and Cognitive

A generalized method for conducting electric-field optimized, fMRI-guided transcranial magnetic stimulation.

Transcranial magnetic stimulation (TMS) is a noninvasive method to stimulate the cortex using powerful magnetic currents. With the advent of neuronavigation and figure-8 coils, researchers can target specific cortical regions with sub-centimeter accuracy. Capitalizing on this spatial specificity, it is possible to augment specific ongoing cognitive processes with a high-degree of functional specificity. Although there are many existing TMS targeting methods, many of these do not account for individual differences in head size/shape or neural anatomy or are not generalizable to regions of the brain without obvious behavioral outputs (e.g. prefrontal cortex). Functional magnetic resonance imaging (fMRI), a widely used method to study brain activity, can deliver subject-specific, high-resolution maps of the entire brain that reflect changes in metabolic activity due to task demands. Recent advances in electric-field (e-field) modelling have made it possible to estimate the current induced in the brain, given a coil location and orientation, and this process can be iterated across possible orientations to identify the optimal orientation. In this work, we combined these tools to create a generalized algorithm for subject-specific TMS targeting, capable of optimizing both the site of stimulation, and the coil orientation. We tested the algorithm using data from an ongoing rTMS study using working memory related BOLD activity to target the right dorsolateral prefrontal cortex, using 3 different a priori target definitions (anatomical, functional, and meta-analytical) compared to no a priori target definition. We found that constraining the fMRI targeting using any of the a priori regions of interest: 1) reduced variability in the location of the fMRI target across subjects, 2) reduced the overall scalp to cortex distance of the TMS target, 3) reduced the variability in optimal coil orientation across subjects based on iterative e-field modelling. Together these results suggest that fMRI data can reliably identify functional neural target for TMS, given an a priori target definition. Additionally, by combining this approach with iterative e-field modelling, our algorithm can determine both the optimal site and coil orientation for stimulation. Finally, because this approach relies only on an a priori target region and a task to activate the target region, it is generalizable to other cognitive neuroscience paradigms and regions of interest.

Sara Kimmich

Doctoral Candidate

NIMH

Psychiatry

Neural Effects of Metacognitive Training

Recent studies have demonstrated that metacognition, the ability to effectively self-monitor behavioral performance, can be improved by training and such improvements may transfer to new, untrained

tasks. In the current study, we aimed to quantify metacognition-related neural activity before and after training to assess how neural transfer effects may underpin behavioral transfer. We recruited 41 participants who completed 10 days of online metacognitive training with pre- and post- brain imaging using functional magnetic resonance imaging (fMRI). During each training session, participants completed a 2-alternative forced-choice perceptual discrimination task, and rated how confident they were that they were correct on each trial. Participants then received feedback on the correspondence between their confidence and their performance on the task but did not receive any feedback about their performance on the task itself. Training led to a gradual improvement of metacognitive accuracy across training sessions: individuals became more accurate at knowing when they had been correct or incorrect on a trial, even though their performance on the task itself did not change. Our results show significant changes in neural activation in the precuneus, dACC and the IPFC following metacognitive training, with similar neural changes observed on both the trained and untrained tasks. Our study is the first to demonstrate that behavioral training of metacognition induces significant changes in cortical activity after feedback is removed. Metacognition is often impaired or distorted across a spectrum of psychiatric disorders, resulting in changes in self-efficacy and insight. This work is a crucial step towards making metacognitive training a viable cognitive enhancement tool for clinical populations.

Katharina Kircanski

Research Fellow

NIMH

Psychiatry

Neural Markers of Eye Gaze to Face Emotion in Pediatric Irritability

Chronic, severe irritability is common and impairing in youth, cuts across multiple psychiatric diagnoses, and increases risk for adulthood depression and suicidality. Yet, the neural underpinnings of irritability are poorly understood. Moreover, there are no empirically-based treatments specifically for irritability, making work on brain mechanisms critical. Of the limited research on irritability to date, one replicated behavioral correlate is a broad deficit in labeling facial expressions of emotion. Therefore, to interrogate brain-behavior mechanisms of irritability in a novel way, we integrated fMRI and eye-tracking methods to examine how irritability relates to neural markers of eye gaze during face-emotion labeling. fMRI data were acquired from 56 youth varying in level of irritability (mean age=15.3 years; 41% female; n=36 with disruptive mood dysregulation disorder or attention-deficit/hyperactivity disorder and n=20 healthy volunteers). A face-emotion labeling paradigm assessed neural activity by face emotion (angry, fearful, happy) and intensity (0% [neutral], 50%, 75%, 100%). Following fMRI, all participants completed an identical eye-tracking paradigm assessing gaze (duration, fixations) to the faces' eye regions as salient emotion cues. Whole-brain linear mixed effects analyses examined neural activity during face-emotion labeling in relation to level of irritability and eye gaze behavior. Age and in-scanner motion were covariates. Higher irritability was associated with a global reduction in gaze to faces' eye regions, across all face emotions and intensities ($p=.01$). Further, level of irritability significantly moderated several important neural markers of eye gaze behavior (all p s below .005, whole-brain corrected). Specifically, irritability moderated how activity in the bilateral fusiform gyrus and cerebellum related to eye gaze as a function of face emotion. Significant higher-order interactions indicated that irritability and age jointly moderated how activity in the bilateral middle temporal gyrus related to eye gaze behavior. In sum, irritability was associated with alterations in the neural substrates of eye gaze

behavior in canonical face-emotion processing regions. In the context of everyday social interactions, such substantial alterations may contribute to irritable youth's frustration and anger toward others. These neural markers of behavior inform the development of face-emotion processing interventions for irritability.

Wilma Bainbridge

Postdoctoral Fellow

NIMH

Cultural Social and Behavioral Sciences

Visual recall memory contains highly detailed and precise object and spatial information

Our recollections are highly subjective, and the veracity of memory for rich perceptual experiences is difficult to measure. While there has been research investigating the capacity of visual recognition memory (i.e., is this image familiar or not?), little work has examined the content within visual recall memory (i.e., what exact image is in your memory?), despite evidence that these may be two neurally distinct processes with different amnesiac phenotypes. Clinical tests of recall use either verbal tasks or abstract geometric drawings, with limited ability to test the limits of meaningful visual memory. Here, we developed a new method for quantifying the content of visual memory for real-world scenes, using a drawing task and systematic evaluation of these drawings by thousands of observers. Healthy adults (N=30) studied 30 real-world scene images (10s each), and after an 11-min digit span distractor task, drew as many images as possible from memory in as much detail as possible. To serve as benchmarks, separate participants (N=27) made 1) drawings from the scene category names, reflecting their baseline representation of a scene type (e.g., "kitchen"), and 2) drawings created while looking at the image, reflecting the maximum information that could be drawn. We then leveraged online experiments with 5,778 participants to objectively score the content (e.g., object information, spatial information, errors) of these 1,782 drawings. These ratings revealed an impressive detail contained in peoples' memories; memory drawings were nearly as diagnostic of the original image as those drawn directly from the image. Memory drawings contained significant detail beyond a baseline representation of the scene category, containing on average 151.3 objects across the experiment with very few false memories (only 1.83 objects drawn not in the original image). The spatial arrangement of objects in memory drawings was highly accurate, and almost identical to the original image. Further, computer vision graph-based visual saliency maps could significantly predict which objects would be remembered by participants. Collectively, these results suggest that visual recall memory contains a previously undiscovered level of detail and precision in its representations of real-world images. Such findings have meaningful implications not only for the understanding of human memory, but also provide a new way to study fine-grained memory impairments in clinical populations.

Hetal Shah

Doctoral Candidate

NINDS

Biochemistry - General, Proteins, and Lipids

Abstract removed at request of author

Abstract removed at request of author

Richard Bradshaw

Visiting Fellow

NINDS

Biophysics

Neurotransmitter transporter conformational dynamics using HDX-MS and molecular dynamics simulation

Neurotransmitter-sodium symporters (NSS) present at mammalian synapses regulate signaling processes via the reuptake of released neurotransmitters from the extracellular space, and offer a wide range of targets for both therapeutic and illicit drugs. Despite intense clinical interest, a detailed understanding of NSS mechanisms has been hampered by multiple barriers. Crystallographic studies require non-native detergent micelle environments, making it difficult to explore structural and regulatory effects of the lipid environment. Alternative strategies involving spectroscopic techniques to observe protein dynamics require protein variants with covalently-attached labels, with associated challenges in constructing and functionally verifying these mutant systems and in interpreting the limited resolution that pairwise labels provide. Hydrogen-deuterium exchange coupled to mass spectrometry (HDX-MS) is an attractive method for investigating dynamics of fully active transporters, reconstituted into a realistic membrane lipid environment, without such limitations. By providing a complementary computational interpretation of deuteration rates in an environment directly comparable to experiment, we can enhance HDX-MS information on fluctuations in protein segments with a residue-level explanation of these dynamics. We use lengthy, fully atomistic molecular dynamics simulations to generate protein structural ensembles, and empirical models to estimate deuteration rates from the accessibility of backbone amide groups. As a model membrane protein system we initially compared the bacterial NSS homolog LeuT in two distinct conformational ensembles, representing key states in the transport cycle, and tested the ability of multiple prediction models to recreate experimental trends. Simulations showed good agreement with the experimental deuteration data (with Pearson's R up to 0.81 at given timepoints), highlighted areas of divergence between the ensembles of the two states, and identified strategies for improving our prediction models given the distinct solvent accessibility of soluble, transmembrane, and pathway-lining regions of the transporter. Our results emphasize the potential to improve the resolution of HDX-MS with atomistic interpretations of conformational dynamics, and our future studies will probe dynamics as modulated by ligands and lipids in the clinically-relevant serotonin and dopamine NSS transporters.

Xiutang Cheng

Research Fellow

NINDS

Neuroscience - Cellular, Molecular, Neurotransmission and Ion Channels, and Neurochemistry

Revisiting LAMP1 as a lysosomal marker for in vitro and in vivo nervous systems

In neuronal processes, endocytic/autophagic organelles gradually mature into lysosomes during their retrograde transport toward the soma. While LAMP1 (lysosome-associated membrane protein 1) targets to lysosomes, it is also acquired by endocytic/autophagic organelles at their early stages. However, currently in the field, LAMP1 is routinely used as a lysosomal marker and LAMP1-positive organelles are often referred to as "mature lysosomes". This over-simplification has led to misunderstanding of

lysosomal distribution, trafficking and functionality in neurons. Here, to clarify the properties of LAMP1-positive organelles, we evaluated the LAMP1 distributions across various endo-lysosomal organelles in both in vitro and in vivo nervous systems. Using super-resolution light confocal imaging, we first demonstrated that ~45% of LAMP1-labeled organelles do not contain detectable lysosomal hydrolases including cathepsins D/B and glucocerebrosidase in mouse dorsal root ganglion tissues and cultured cortical neurons. To confirm these findings, we examined mouse spinal motor neurons by immunotransmission electron microscopy and detected LAMP1 signals on various endosome/lysosome-like organelles whereas cathepsin D signals only in the luminal side of mature lysosomes and autolysosomes. Moreover, BSA-gold pulse-chase assay confirmed lack of degradative capacity within some LAMP1-labeled organelles, and gradient fractionation displayed differential distribution patterns of LAMP1/2 and cathepsins D/B in neurons. To further examine if LAMP1 alone can indicate lysosomal defects in disease conditions, we co-stained motor neurons of wild-type and fALS-linked hSOD1-G93A mice at P80. We found a dramatic reduction in cathepsin D intensity in hSOD1-G93A neurons whereas LAMP1 intensity remained the same, indicating its insensitivity to lysosomal deficits. Last, we performed a quantitative assessment and revealed a heterogeneity of LAMP1-positive organelles evidenced by their co-localization with markers for early/late endosomes and retromers. In conclusion, our study suggests that LAMP1 is neither a reliable marker to represent neuronal lysosomes, nor a sensitive indicator to reveal lysosomal response in disease conditions. One must be cautious as interpreting LAMP1-labeled organelles in neurons where LAMP1 intensity, trafficking, and distribution do not necessarily represent mature lysosomes under both normal and pathological conditions. (Invited revision submit to JCB)

Monica Manglani

Doctoral Candidate

NINDS

Immunology - Infectious Disease

Peptide-Specific Engagement of Cerebrovascular Endothelial Cells Promotes Dysfunctional Calcium Signaling During Experimental Cerebral Malaria

Cerebral malaria is a potentially fatal complication associated with *Plasmodium falciparum* infection in humans. Disease pathology includes blood brain barrier (BBB) breakdown, microvascular hemorrhage, edema, brainstem herniation, and neuronal cell death. Studies in rodents (referred to as experimental cerebral malaria or ECM) have revealed that parasite-specific CD8⁺ T cells induce this pathology via engagement of cerebrovascular endothelial cells (ECs) comprising the BBB. In this study, we sought novel insights into the mechanisms underlying alterations in BBB structure and function during the development of ECM. We developed a novel transgenic reporter system that allowed us to simultaneously monitor calcium signaling in cerebrovascular endothelial cells and parasite-specific CD8⁺ T cells. We then used intravital two-photon microscopy (TPM) to monitor BBB dynamics in real-time at the peak of disease. Within venous networks, we observed a significant increase in the magnitude and propagation of EC calcium signaling. Calcium wave propagation along lengthy stretches of cerebrovascular endothelium was associated with regions of parasite-specific CD8⁺ T cell engagement (both stable and dynamic). Recognition of cognate peptide-MHC on luminal and abluminal EC surfaces resulted in calcium fluxes within parasite-specific CD8⁺ T cells, which was followed temporally by calcium wave propagation in the associated vasculature. Importantly, this pathophysiological response was completely eliminated by depletion of T cells. We postulate that T cell-mediated induction of

calcium waves along cerebral blood vessels promotes noncytopathic disruption of the BBB, resulting in fatal cerebral edema. Therapeutic manipulation of this activity might help restore BBB homeostasis and prevent neurological complications during ECM.

Jobert Vargas

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NINDS

Molecular Biology - Prokaryotic, and Eukaryotic

Abstract removed at request of author

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Emily Petrus

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NINDS

Neuroscience - Cellular, Molecular, Neurotransmission and Ion Channels, and Neurochemistry

Unilateral Whisker Denervation Drives Synaptic Remodeling Across the Corpus Callosum

Sensory cortices are bilaterally located in the brain and rely on the corpus callosum (CC) for efficient processing of external stimuli. Cortical circuitry integrates subcortical, inter- and intracortical messages in a lamina-dependent, synapse-specific manner. Early unilateral sensory perturbations inhibit correct wiring of the CC, but the effects of unilateral manipulations in adulthood are not well characterized.

Interhemispheric plasticity can be adaptive or maladaptive, for example unilateral stroke in somatosensory-motor areas produces hyperexcitability in the unaffected contralateral hemisphere, which may inhibit patients' motor recovery. In addition, amputation or nerve damage can change cortical activity in both deprived and intact systems, which may result in phantom limb sensations.

These results have been characterized with fMRI and PET imaging and hallmarks include altered connectivity between brain regions and higher spontaneous activity in affected cortical areas. Our research seeks to characterize the synaptic and circuit level mechanisms which underlie callosal dependent plasticity. We characterized the role of the CC in whisker integration by unilateral denervation of the infraorbital (whisker) nerve in adult mice. We found that the CC recruits bilateral cortical BOLD fMRI responses to the intact whisker set. We then used whole-cell electrophysiology and optogenetics to determine that this recruitment is driven by stronger callosal synapses to layer 5 (L5) principal cells in deprived primary somatosensory barrel cortex (S1BC). These synapses have increased levels of post synaptic glutamatergic receptors, including GluA, GluN, and NR2B containing GluN receptors. Callosal potentiation to L5 principal cells reversibly occludes long term potentiation along the CC, indicating that potentiation is maximized at this synapse. L5 principal neurons in deprived S1BC are also hyperexcitable, which is consistent with human literature following limb amputation or unilateral subcortical or cortical stroke. L5 cells play a pivotal role in integrating bilateral whisker stimuli and serve as the main output cells for sensory cortical columns. We hypothesize that they are specifically targeted by the CC to mediate changes which could alter downstream sensory processing. In addition, our results may characterize the circuit changes which underlie cortical plasticity which mediate patients' observed responses to amputation, nerve injury, or stroke.

Kathryn Luderman

Postdoctoral Fellow

NINDS

Pharmacology and Toxicology/Environmental Health

Positive Allosteric Modulators of the D1 Dopamine Receptor Act at Diverse Binding Sites

The D1 dopamine receptor (D1R) and its signaling is associated with several important neurological processes and neuropsychiatric disorders. Enhancing D1R signaling in the prefrontal cortex is associated with increased cognition and therefore is an appealing treatment strategy for the cognitive decline observed in schizophrenia, Alzheimer's disease, and other disorders. Because of the clinical liability inherent with D1R agonists, positive allosteric modulators of the D1R have been proposed as an alternative, due to their potential for high selectivity and larger therapeutic windows. Currently, the location and characteristics of potential binding sites for allosteric modulators on the D1R is unknown. We have identified two structurally diverse D1R positive allosteric modulators, MLS1082 and MLS6585, via a high throughput screen of the NIH Molecular Libraries Program. The compounds potentiate dopamine-stimulated G-protein- (cAMP stimulation, 3-5 fold) and β -arrestin-mediated (6-8 fold) signaling pathways and increased the binding affinity of dopamine for the D1R (3-6 fold). Neither compound displayed agonist activity in the absence of dopamine. Taking advantage of our structurally distinct potentiators, G-protein signaling and β -arrestin recruitment experiments using maximally effective concentrations of MLS6585 and MLS1082 in combination were used to determine if the compounds act at similar or separate sites. In combination, MLS1082 + MLS6585 caused an additive potentiation of the potency of dopamine beyond that caused by either compound alone for both β -arrestin recruitment (11-20 fold) and cAMP accumulation (4-6 fold). This suggests that the two compounds are acting at separate sites on the receptor. We also observed similar results using analogs of the two compounds, with analogs of MLS6585 having additive activity with MLS1082 and vice-versa. However, analogs are not additive with their parent compound, meaning the parent compounds and their analogs act at similar sites. The combination experiments were repeated with Compound B, a known D1R positive allosteric modulator. Compound B was additive with MLS6585 but not MLS1082, further suggesting that D1R has two separate positive allosteric modulator binding sites. Point mutation studies are currently underway to identify the location of these putative sites as well as whether these sites may contribute separately to potentiation of signaling efficacy versus dopamine binding affinity.

Paul Kramer

Postdoctoral Fellow

NINDS

Neuroscience - Cellular, Molecular, Neurotransmission and Ion Channels, and Neurochemistry

Dopamine Neuron Axons: Physiology and Subthreshold Currents

The activity of dopamine neurons underlies many behaviors and diseases, from reward learning to addiction. The soma and dendrites of these neurons have been the subjects of decades of electrophysiological research, and the dopamine synapse has been intensely studied in the projection targets using methods like voltammetry, microdialysis, and calcium imaging. However, the indirect nature of these methods leaves open questions about the presence and influence of subthreshold axonal currents. The first goal of this proposal is to establish whether or not GABA-A receptors are functionally expressed on the dopamine neuron axon. On many peripheral, and some central, axons

GABA-A receptors are functionally expressed. Often these receptors are depolarizing and can inhibit synaptic release through either shunting inhibition or through depolarization-induced sodium channel inactivation. The presence of these receptors on dopamine neuron axons would be novel and lead to a new appreciation for the role of GABA in controlling dopamine release. Using direct recordings from dopamine neuron axons we have established the presence of GABA-A receptors. The activation of these receptors appears to be depolarizing on the axon (unlike the soma), and decreases action potential amplitude without affecting the firing rate. Direct axonal recordings will also allow for the interrogation of axonal channels that contribute to the axonal action potential. The properties of the axonal action potential in dopamine neurons has been modeled, but has never been directly recorded. By recording from the axon, we have been able to determine the average width and height of the axonal action potential, as well as other properties like the inter-spike slope and average inter-spike voltage. These properties are important for determining release of dopamine at the synapse, and are thought to be important in neurodegenerative diseases, especially Parkinson's Disease. In conclusion, we have used direct recordings from dopamine neuron axons to ask basic physiological questions about the axon, as well as to identify the presence of subthreshold GABA-A currents, which have been so far a point of controversy in the field. We are now building upon this work to directly examine the calcium in the axon, as well as to test the interaction between the proximal axon and the soma.

Alec Nickolls

Doctoral Candidate

NINDS

Stem Cells - General and Cancer

Derivation of human sensory neurons to model a novel disorder of mechanosensation

Our sense of touch involves transducing mechanical forces from the environment into electrical signals interpreted by the brain. Similarly, proprioception - the sensation of body position and movement often considered a "sixth sense" - requires detecting mechanical strain across muscle and tendon.

Recently, our group discovered a novel human disorder caused by null mutations in the stretch-gated ion channel PIEZO2. Patients with this condition exhibit profound loss of proprioception and touch sensation, including strikingly uncoordinated motor control and an inability to detect gentle stimuli on the skin. To uncover the cellular and molecular processes underlying PIEZO2 deficiency, we established an original model system to assess human touch sensation in vitro. We found that forced expression of two transcription factors, NGN2 and BRN3A, rapidly and efficiently converts human pluripotent stem cells into peripheral sensory neurons. Within seven days, >90% of cells adopted neuronal morphology and expressed both pan-neuronal and sensory-specific markers. We observed widespread expression of peripherin in the converted cells - a neurofilament exclusive to peripheral sensory neurons - which was undetectable in stem cell-derived forebrain neurons. Further, we detected mRNA transcripts for a variety of receptor genes that bestow specific sensory functions in peripheral neurons, including TRPM8, TRPA1, P2RX3, and notably PIEZO2. Given PIEZO2's role as a mechanotransduction channel, we used a micromanipulator probe to indent the cell membrane while measuring electrical responses.

Mechanical stimulation elicited robust excitatory currents in the converted cells, a hallmark of mechanosensory neurons in vivo. Remarkably, applying this technique to neurons derived from PIEZO2-deficient patients revealed a complete absence of mechanically activated currents. Collectively, these data confirm PIEZO2's essential role in human mechanosensation and demonstrate loss-of-function

mutations with profound impact on sensory neuron physiology. This streamlined model system may broadly serve as a tool in high-throughput screens for drug discovery and identifying molecular processes involved in peripheral nervous system disorders.

Romain Quentin

Postdoctoral Fellow

NINDS

Neuroscience - Integrative, Functional, and Cognitive

Differential brain mechanisms of selection and maintenance of information during working memory

Working memory (WM) is our ability to temporarily hold information as needed for complex cognitive operations. It is required for learning, reasoning, updating information, and performing everyday visuomotor tasks. WM has been associated with sustained brain activity in the prefrontal cortex. However, recent electrophysiological research suggests that the information in working memory are only transiently coded in neural activity. To reconcile these results, we use recent models of WM that distinguish two separate WM process: (i) a selection rule that selects the content to be maintained and (ii) the maintenance of the content. Yet, most neuroscience studies do not dissociate the neural responses to these two components, leading to partially conflicting results concerning the temporal dynamic and the localization of working memory processes. To address this limitation, we aimed to characterize the spatiotemporal neural representations underlying these two components. We developed an original working memory task in which four visual attributes were briefly presented to the participant. After a short delay, a retrospective cue indicated which visual attribute the participant had to maintain and to compare to a subsequent probe. Healthy participants (n=23) performed this visual working memory task during magnetoencephalography (MEG) recordings. Multivariate Pattern Analysis (MVPA) and source analyses of time-resolved data confirmed two differentiated spatiotemporal neural representations underlying working memory selection rule and content maintenance. The selection rule is represented by stable low-frequency ($\approx 20\text{Hz}$) neural activity within a network that includes the ventrolateral prefrontal cortex. Memory content on the other hand is transiently maintained over a distributed and more posterior network different from that encoding the sensory stimulus. These results reveal differential neural mechanisms that select and maintain information in working memory and reconcile incongruent findings in the literature.

Guijing Xiong

Postdoctoral Fellow

NINDS

Neuroscience - Neurodegeneration and Neurological disorders

Defective Presynaptic Transport Is Associated with Autism-like Social Behavior

The formation, maintenance, and remodeling of synapses require delivery of newly synthesized presynaptic cargoes from the soma to synapses. We previously identified syntabulin as a kinesin KIF5 adaptor that mediates axonal transport of active zone (AZ) precursors from the soma to presynaptic terminals, which is essential for synapses formation in developing neurons and synaptic function maintenance in matured neurons. Autism is a highly inheritable neurodevelopmental disorder characterized by impaired social interaction, reduced communication, and increased repetitive

behaviors. Mutations of genes that control synapses formation and maturation are emerging to be an important cellular basis of autism. It remains elusive whether impaired axonal transport of presynaptic proteins affects synapse formation and maturation in vivo and thus contributes to autism pathogenesis. Interestingly, a genetic study of autism patients (Children's Hospital, Columbus, OH) identified a de-novo syntabulin mutation that changes a conserved arginine into glutamine within the KIF5-binding domain. We confirmed that this mutation abolished syntabulin interaction with KIF5. Thus, it is urgent to establish whether syntabulin mutations are associated with autism-linked phenotypes. To address this, our lab first generated Nestin-Cre; syntabulinloxP/loxP conditional knock out (cKO) mice in which syntabulin is null in brain since embryonic stage. Time-lapse imaging shows that hippocampal neurons of syntabulin cKO mice display impaired axonal transport of AZ precursors from the soma to developing presynaptic boutons, and reduced mature synapse density. Electron microscopy study reveals synapse loss of mature excitatory synapses and a decrease in docking vesicles per synapse in cKO mice hippocampus CA1 neurons. Moreover, syntabulin cKO mice show reduced frequency of miniature excitatory postsynaptic currents and impaired long-term synaptic plasticity in hippocampal Schaffer collateral-CA1 synapses. Consequently, the syntabulin cKO mice exhibit core autism-like traits including defective social recognition, reduced ultrasonic vocalizations, increased repetitive behavior, and impaired spatial learning and memory. Our study establishes for the first time that defective axonal transport contributes to the pathogenesis associated with synaptic and behavioral abnormalities in mice that bear similarities to autism patients, and providing new cellular targets for therapeutic intervention.

Jennifer Lefevre

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NINDS

Clinical and Translational Research, and Gene Therapy

Imaging the spatiotemporal development of spinal cord lesions in a model of multiple sclerosis

Background 75-90% of multiple sclerosis (MS) patients show focal or diffuse abnormalities in the spinal cord (SC), which are known to have a major impact in the development and progression of motor deficits. Our previous ex-vivo MRI study demonstrated that the SC of marmosets with experimental autoimmune encephalomyelitis (EAE) show demyelinated white matter (WM) lesions distributed along the entire cord. The purpose of this study was to characterize the lesion dynamic evolution by imaging in vivo the SC of EAE marmosets. Material/Methods SC imaging of three common marmosets was performed in vivo by 7T MRI using a house-made 3D-printed cradle, with a custom-built 12-channel coil. A PD-w fast-spin-echo sequence was acquired at the cervical, thoracic and lumbar levels in axial plane (0.15x0.15x0.8 mm). These animals were then immunized with human WM homogenate, and followed weekly by MRI until they met the endpoint of the study. Focal lesions were counted, and the extend of subpial lesions was evaluated by assessing the proportion of slices affected with subpial. Finally, histopathology was performed using Luxol fast blue-periodic acid Schiff (LFB-PAS) stain to evaluate demyelination. Results Disease duration ranged from 22 to 81 days post-immunization (PI), with the first clinical signs appearing 2 to 4 weeks PI. Focal lesions appeared first in the thoracic and lumbar levels as early as the fourth week PI. The rate of new focal lesions increased by the end of the disease course, and 70% of all focal lesions were at the thoracic level. Subpial lesions were visible throughout the entire cord early in the disease course. Of these, lesions along the anterior median fissure seem to be an early feature. Subpial lesions along the cord edge expanded extensively through the parenchyma only by the

end of the disease course. These lesions identified on MRI corresponded to areas of various level of demyelination on LFB-PAS stains. Conclusion We reported for the first time the spatiotemporal development of SC lesions a non-human primate model of MS. Similar spatiotemporal features for both focal and subpial lesions were observed across EAE animals, despite having different disease courses. Histopathology confirmed that demyelination is one of the underlying pathologies detected by our serial imaging. This pilot study demonstrates that our animal model and imaging platform have the potential to unravel the mechanisms of MS lesion development in the SC.

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Physiology

Astrocytes modulate brainstem respiratory rhythm-generating circuits and determine exercise capacity

Astrocytes are implicated in modulation of neuronal excitability and synaptic function, but it remains unknown if these glial cells can directly control activities of motor circuits to influence complex behaviors in vivo. We focused on the vital respiratory rhythm-generating circuits of the preBöttinger complex (preBötC) – a group of brainstem interneurons that form respiratory rhythm-generating circuits in mammals, and determined how compromised function of local astrocytes affects breathing in conscious adult rats. SNARE-dependent vesicular release mechanisms of gliotransmitters in astrocytes were disrupted by virally driven expression of the light chain of tetanus toxin (TeLC) or the dominant-negative SNARE (dnSNARE) proteins in preBötC astrocytes. Either TeLC or dnSNARE expression was found to be sufficient to effectively block the mechanisms of vesicular release in transduced cultured preBötC astrocytes. In conscious rats, bilateral expression of either TeLC or dnSNARE in preBötC astrocytes significantly reduced the resting respiratory rate by 11% (92 vs 103 min⁻¹ in control rats expressing GFP in the preBötC astrocytes (control), n=12, p=0.01) and by 11% (94 vs 106 min⁻¹ in controls, n=5, p=0.02), respectively. Moreover, the frequency of functionally important augmented breaths (sighs), produced by increased inspiratory effort was reduced in rats transduced to express TeLC or dnSNARE by 23% (n=12, p<0.01) and 26% (n=5, p<0.01), respectively. Since exercise is associated with (and ensured by) an augmented inspiratory effort to meet increased oxygen demands, we next determined whether compromised preBötC astrocyte signaling impairs exercise capacity (by employing a forced treadmill exercise model, in which the rats are run to exhaustion). Expression of either TeLC or dnSNARE in preBötC astrocytes dramatically reduced exercise capacity (by 42%, n=12, p<0.01; and 58%, n=5, p=0.02, respectively). Cardiovascular responses during exercise were not affected by TeLC or dnSNARE expression, suggesting that reduced ability to exercise in conditions of impaired astroglial function within the preBötC is due to a respiratory deficit. These results suggest that vesicular release of signaling molecules by astrocytes provides tonic excitatory drive to the preBötC rhythm-generating circuits, contributes to generation of sighs, and is essential for an appropriate respiratory response to meet the increased metabolic demands of exercise.

Martina Absinta

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Radiology/Imaging/PET and Neuroimaging

CHRONIC ACTIVE MULTIPLE SCLEROSIS LESIONS AFFECT PATIENT DISABILITY AND LONG-TERM LONGITUDINAL LESION HISTORY

BACKGROUND. In multiple sclerosis (MS), the pathologically-defined chronic active/slowly expanding lesions can be identified on susceptibility-based MRI in vivo as chronic lesions with a paramagnetic rim. Pathologically, these lesions feature smoldering inflammatory demyelination at the lesion edge, remyelination failure, and often ongoing axonal degeneration. However, the long-term effects of rim persistence in vivo are still uncertain. **AIM.** Here, we assessed whether the presence of paramagnetic rims affects the patient disability and long-term longitudinal history of MS lesions. **METHODS.** Chronic lesions with paramagnetic rims were identified on 7T or 3T brain susceptibility-based MRI in cross-sectional study of 191 MS cases. Correlations with clinical disability measures and brain volume were assessed. A retrospective yearly evaluation of lesion volume change (54 chronic lesions with vs. without rim) was performed in 23 MS patients with archival 3T yearly NIH scans for =10 years. **RESULTS.** MS patients with more than 4 chronic lesions with paramagnetic rims reached motor (EDSS and MSSS) and cognitive (SDMT and PASAT) disability milestones at earlier age ($p < 0.05$). Normalized brain volumes were lower in patients with rims than without rims ($p = 0.02$). At the lesion level, a dichotomy of longitudinal evolution of chronic MS lesions with and without rim was detected. While lesions without rim were shrinking over time, chronic lesions with rim were steady or expanding in the surrounding tissue ($p < 0.0001$). This dichotomy was more pronounced before age 45 ($p = 0.04$). On 7T T1-map, lesions with rim had higher T1 values (suggesting more tissue destruction) than lesions without rim ($p = 0.0025$). **CONCLUSION.** MS patients with several chronic active lesions in vivo show a more aggressive disease course. In addition, persistent inflammation at the lesion edge is not only a major barrier for remyelination, but can exert ongoing damage to the surrounding tissue. These results support the use of paramagnetic rim development as an outcome measure in multicenter, MRI-based clinical trials aimed at treating perilesional chronic inflammation and its potential effects on remyelination.

Marlene Boenstrup

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Radiology/Imaging/PET and Neuroimaging

A rapid form of offline consolidation in skill learning

Background Consolidation of motor skill is defined as greater resistance to interference caused by another task (stability) and/or as performance improvements after a rest period (offline gains). Offline gains are typically reported to occur after 24 hours. An emerging view is that the brain opportunistically consolidates previously encoded memories whenever it is not otherwise occupied by encoding new memories. In typical motor skill learning tasks, short periods of active practice alternate with short periods of rest to alleviate fatigue. We here explored whether the fast and strong performance improvements during early learning occur during the short periods of active practice or rest and studied potential neural correlates. **Methods** Subjects ($n=27$) naïve to the task practiced typing a 5-item numeric sequence repetitively as fast and accurately as possible with the left non-dominant hand. In this task, learning is mostly reflected in increases in speed. Practice consisted of 36 alternating training and rest periods (10 seconds each) for 12 minutes. We measured the finger tapping speed of correct sequences (keypresses per second; kp/s). Performance improvements during (online) practice and rest (offline)

periods were measured. Simultaneous magnetoencephalography was obtained to identify spatiotemporal brain oscillatory correlates of motor learning. In a control group (n=24), learning of the target sequence was interfered by typing a random sequence for equal duration after each practice and before the subsequent rest period. Results Total learning was 2.51 ± 1.3 kp/s and occurred predominantly in the initial 3 ± 1 min of training. During this initial period, online learning was virtually nihil (-0.32 ± 3.4 kp/s, n.s.) while offline learning measured across the rest periods was 2.83 ± 3.2 kp/s. Offline improvements could not be explained by learning rate. Thus, total learning was completely accounted for by the rapid offline performance gains. Interference with learning in the control group blocked the rapid offline learning. 18-22 Hz beta (but not theta, alpha or gamma) frontoparietal brain oscillatory activity contralateral to the learning hand in between practice periods predicted the magnitude of trial-by-trial offline learning. Conclusions Our results reveal a rapid form of offline consolidation during early motor learning, possibly supported by resting desynchronization of beta rhythms contralateral to the learning hand.

Thien Nguyen

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NINDS

Neuroscience - Cellular, Molecular, Neurotransmission and Ion Channels, and Neurochemistry

Functional deficit in sex-linked Neuroligin 4Y reveals potential mechanism for the gender-bias in Autism Spectrum Disorders

Autism Spectrum Disorders (ASDs) are a diverse set of cognitive developmental disorders that result in a wide range of behavioral deficits. Interestingly, ASDs have long been reported to affect many more males than females. This gender-bias in ASDs has been a puzzle in the field. Neuroligins (NLs) are postsynaptic cell adhesion molecules involved in synapse formation and modulation. There are five NLs (NL-1, NL-2, NL-3, NL-4X, NL-4Y) encoded in the human genome. NL-4X and NL-4Y are of particular interest because they are sex-linked genes located on the X and Y chromosome, respectively. In addition, multiple mutations in both the extracellular domain (ECD) and the intracellular domain (ICD) of NL-4X have been shown to associate with ASDs. NL-4X and NL-4Y are highly conserved with only fourteen amino acid differences in the ECD and five in the ICD. Due to this similarity, NL-4X and NL-4Y are presumed to have the same functions. I have shown that NLGN4Y does not have the same functions as NL-4X. Overexpressing NLGN4Y in heterologous cells or in neurons shows that NL-4Y does not traffic efficiently to the surface, and thus, NL-4Y cannot induce synaptogenesis. The lack of surface expression is due to the NL-4Y ECD. Swapping the NL-4Y ECD with NL-4X ECD rescues NL-4Y surface expression and synapse formation. The strength of synapses can also be modified through phosphorylation. Our lab has shown that NL-4X is phosphorylated by protein kinase C (PKC) at threonine 707 (T707) with a profound impact on the synaptogenic properties of NL-4X. Surprisingly, I observed that PKC cannot robustly phosphorylate NL-4Y. Furthermore, I found that protein kinase A exclusively phosphorylates NL-4X, but not NL-4Y, at serine 712 (S712). I narrowed down the differential phosphorylation to a single amino acid difference between NL-4X (arginine) and NL-4Y (histidine) at position 710. Interestingly, a point mutation in NL-4X (arginine-to-cysteine at 704) found in an autism patient diminishes phosphorylation of NL-4X at T707 and S712. Using differentiated human neurons from induced pluripotent stem cells, I show that endogenous NL-4X is phosphorylated at T707 and S712. Interestingly, female derived neurons have a stronger signal for phosphorylation of S712 compared to male neurons. Taken together, my data

indicate that functional deficits in NL-4Y contribute to the ASD gender-bias, because NL-4X mutations will have a dominant affect in males.

Mahdieh Poostchi

Postdoctoral Fellow

NLM

Informatics/Computational Biology

Computational Image Analysis and Machine Learning for Malaria Parasite Detection and Quantification

Malaria remains a major burden on global health, with roughly 216 million cases worldwide and more than 440,000 deaths in 2016. It is caused by parasites that are transmitted through the bites of infected female Anopheles mosquitoes, which infect the red blood cells. Most deaths occur among children in Africa, where malaria is a leading cause of childhood neuro-disability. The gold standard for malaria diagnosis in the field is light microscopy of blood smears, where an expert microscopist visually inspects blood smears for parasites. Accurate parasite counts are essential not only for malaria diagnosis. They are also important for measuring drug-effectiveness and classifying disease severity. However, manual counting of parasites in hundreds of millions of blood smears worldwide every year is a laborious, costly and error-prone process that depends heavily on the experience and the skill of the microscopist. We show that modern information technology and machine learning techniques can play key roles in fighting the disease and improving diagnosis. In particular, we develop an automatic system to identify and quantify malaria parasites in real-time on blood smears. The malaria parasite detection system consists of three processing steps: (i) image acquisition of blood smear images using a standard light microscope with an attached smartphone camera, (ii) detection and segmentation of red blood cells using image analysis, and (iii) classification into infected and uninfected cells using deep machine learning techniques. The accuracy of my artificial neural network classifier in correctly identifying infected cells is 99% evaluated on 10,000 cells. I measure an average absolute error of 1.18% between the true and the automatic parasite counts. For mouse cells, our automatic counts correlate well with expert and flow cytometry counts, making this the first system that works well for both human and mouse. Compared to human counting, our system is much faster and can process 100 cells per second. The system provides a reliable and standardized interpretation of blood films and lowers diagnostic costs by reducing the workload through automation. Furthermore, the implementation of the system as a standalone smartphone app is well-suited for resource-poor malaria-prone regions. Future image analysis on blood smears could also help in discriminating parasite species and identifying parasite life stages.

Elise Smith

Postdoctoral Fellow

OD

Information Science and History of Medicine

Complexity of multidisciplinary norms in collaborative teams; epistemic diversity in authorship distribution ethics

In the contemporary system of science, authorship is a proxy for productivity. Consequently, it becomes an important factor in decisions regarding funding, job advancement, salary, and prizes. For individual

researchers, authorship represents recognition, credibility and opportunities for further research in an already competitive research environment. Since norms regarding authorship attribution and order are based on disciplinary culture, multidisciplinary teams may see increased normative diversity and even conflicting norms. Given this context, we hypothesize that authorship disagreements are more prevalent in multidisciplinary collaborative teams when compared to single-discipline teams. Using the web of science, we created a sample of over 100,000 individual researchers that published in collaborative teams between 2011 and 2015; the sample was then stratified based on different levels of multidisciplinaryity. A survey on ethics of authorship distribution was answered by 6,697 respondents. We performed a descriptive analysis and multivariate logistic regression with pertinent variables (e.g. gender, rank, discipline and multidisciplinary level). Results suggest that respondents were more likely to face disagreements regarding attribution (46.6%) than ordering (37.9%). After controlling for independent factors, we found that researchers in multidisciplinary teams were less likely than disciplinary researchers to face authorship disagreements. Conversely, researchers in the medical sciences were more likely to face authorship disagreements and observe misbehaviors (e.g. sabotage, fraud, hostility) as a results of authorship disputes than in other disciplines. Such results may suggest that "one size fits all" guidelines promoted by biomedical disciplines may be ineffective in mitigating authorship disagreements. Contrary to our hypothesis, disciplinary diversity - as seen in multidisciplinary teams - seems to decrease authorship disputes. Although complete ethical relativism would most likely be detrimental to research integrity, acceptance of some normative diversity may help to resolve authorship disputes in research.
