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KARIN GRUNEBAUM
cancer research foundation

December 2021 ♦ Volume 18

From the Chair

Dear Friends of the Karin Grunebaum Cancer Research Foundation:

Just as I started writing this letter on August 23, 2021, my iPhone lit up with the breaking news that the FDA has now given full approval for the Pfizer-BioNTech Covid-19 vaccine! That, of course, is great news for the world and especially for our Foundation. Why is that, you may ask? Isn't the Foundation dedicated to cancer research rather than viral immunology? The answer is "yes," but in true science there is always an overlap between the various fields – and sometimes a great story, too.

In this case, the untold story behind the successful Covid-19 vaccine approval began in 1984 when the Karin Grunebaum Cancer Research Foundation agreed to help sponsor the research work of a promising Boston University School of Medicine (BUSM) medical/Ph.D. student named Drew Weissman. For two years (1984-1986) Drew was awarded a Karin Grunebaum Fellowship, and his research into cancer-related immunology was funded in part by the Foundation.

After graduating as an M.D./Ph.D. from BUSM in 1987 and completing his residency at Beth Israel Deaconess Medical Center, Dr. Weissman did a fellowship at the National Institute of Health (NIH) under the supervision of one Anthony Fauci! Yes, the same Anthony Fauci who is currently Director of the National Institute of Allergy and Infectious Diseases, and the Chief Medical Advisor to President Biden.

Fast forward to 2005: Dr. Drew Weissman is now a Professor of Medicine at the University of Pennsylvania's Perelman School of Medicine – and his specialty is RNA biology and immunology. He has been working for many years with a colleague, Katalin Karikó, trying to develop a method whereby modified RNA (mRNA) can be successfully inserted into the human body without triggering an immune response which results in the body degrading the inserted mRNA.

In 2005 they were finally successful, and a patent was obtained. In 2020, when Covid-19 vaccine makers Pfizer and Moderna needed a viable method for inserting mRNA into people without fear of having the vaccine rejected or degraded, they obtained licenses based on the work done by Dr. Weismann and his colleagues.

So, the Foundation's sponsorship of Drew Weissman's two years of research long ago as a medical student turns out to be one of the best investments ever made for the world.

Please, please give another of our Fellows the opportunity to again achieve a world-changing milestone by contributing generously to the Karin Grunebaum Cancer Research Foundation.

You never know when or how such miracles will occur.

Thank you.

Steven Wallach

Chairperson

From Boston University Medical School

Julie Palmer, M.P.H., Sc.D
Karin Grunebaum Professor in Cancer Research, Boston University School of Medicine; Director, Slone Epidemiology Center; Co-Director, Boston University-Boston Medical Center Cancer Center

Boston University Researchers Develop Breast Cancer Prediction Tool for Black Women

Our esteemed member of the board, Julie R. Palmer Sc.D has had a paper published in the *Journal of Clinical Oncology.* Professor Palmer comments that "developing and testing a risk prediction model for breast cancer in Black women has been foremost on my agenda for the last year and it was gratifying to see it published in this journal."

Black women have the highest breast cancer mortality rate in the United States but Boston researchers have developed a new tool to help close that gap and save more lives.

"My sister died of breast cancer two years ago. My mother had breast cancer. Her sister had it," said Dr. Julie Palmer, of Boston University's Slone Epidemiology Center.

Palmer has spent more than two decades focusing on the health of Black women and studied why that part of the population is hit so hard by breast cancer.

"Barriers to the best treatment, also higher incident of the tumor type that's more aggressive, but another barrier is the delayed diagnosis," she said.

Diagnosis is where her research team was focused. They developed a breast cancer prediction tool focused on risk factors specific to Black women.

"Young, Black women are dying too often from breast cancer," she said. "I especially wanted something that could be used for women who otherwise wouldn't be screened because nobody is at that age until someone realizes that person is at really high risk."

For example, Palmer's tool includes a question about having a family member with prostate cancer — since that disease is a lot more common among Black men and may indicate a higher risk for breast cancer among related women.

The prediction tool asks women to answer a series of questions and responds with a risk factor for developing breast cancer in the next five years.

Palmer said the easy process is designed to help, but not replace, a professional diagnosis.

"It wouldn't be valuable for someone to look at this and do this calculation herself," she said. "We really believe in shared decision making and this we hope will make the women themselves have a part of that decision-making."

Drew Weissman, MD PhD, Grunebaum Fellow 1984-1986, Nominated for a BU Distinguished Alumni Award

Dr. Weissman has been instrumental in the development of several of the COVID-19 vaccines. He is best known for his contributions to RNA biology. His work helped enable development of effective mRNA vaccines, the best known of which are those for COVID-19 produced by BioNTech/Pfizer and Moderna. Weissman is a professor of medicine at the Perelman School of Medicine at the University of Pennsylvania (Penn). He and his research colleague Katalin Karikó have received numerous awards including the presigious Lasker-DeBakey Clinical Medical Research Award.

Weissman grew up in Lexington, Massachusetts. He received his B.A. and M.A. from Brandeis University in 1981, where he majored in biochemistry and enzymology and he worked in the lab of Gerald Fasman. He performed his graduate work at Boston University in immunology and microbiology where he received his M.D. and Ph.D in 1987. Afterward, Weissman did a residency at Beth Israel Deaconess Medical Center, followed by a fellowship at the National Institutes of Health (NIH), under the supervision of Anthony Fauci, the current director of the National Institute of Allergy and Infectious Diseases.

COMPETITION PARTICIPANTS

Beatrice Awasthi | G3
Biological and Biomedical Sciences
Kevin Haigis lab
Interrogating how tissue type influences hyperactive KRAS-induced MAPK signaling
The oncogene KRAS is frequently mutated in two of the deadliest human cancers, pancreatic and colorectal cancer. KRAS activation by mitogenic signals initiates the MAPK phosphorylation cascade that consists of the proteins RAF, MEK, and ERK, the latter of which has hundreds of known substrates, including many transcription factors. In some tissues, like the liver, KRAS-mutant cancers are notably rare. The basis for this is unknown. Understanding differences between tissues with distinct profiles of oncogenic KRAS mutations could aid in the novel identification of targetable vulnerabilities in KRAS-mutant cancers. Phospho-proteomic data from our lab suggests that MAPK-induced signaling networks are tissue-specific. We hypothesize that the basal interactome and epigenome together dictate substrate phosphorylation by ERK and subsequent gene expression changes in tissues downstream of KRAS oncoproteins. Here, I will employ a mutant of ERK2 that can label its substrates to study how the oncogenic allele KRAS-G12D affects ERK substrates in the pancreas, colon, and liver of mice. Simultaneously, I will interrogate the chromatin landscape and identify ERK binding partners in each tissue. My ultimate goal is to understand how basal tissue state affects ERK signaling in response to hyperactive KRAS. This could reveal new therapeutic targets in KRAS-mutant tumors.

Francisco Fernandez | G2
Biological and Biomedical Sciences
James Gusella and James Walker Lab
Protein glycosylation in NF1-related tumors: therapeutic targets and biomarkers
Neurofibromatosis type 1 (NF1) is an autosomal dominant disorder affecting 1/3000, caused by mutations in the NF1 gene encoding neurofibromin, a negative regulator of RAS. Patients are predisposed to developing benign peripheral nerve associated tumors including plexiform neurofibromas (PNFs). In about 10% of NF1 patients, PNFs, which are derived from Schwann cells (SCs), transform into malignant peripheral nerve sheath tumors (MPNSTs), which are invariably fatal. Protein glycosylation is a highly regulated post-translational modification playing a critical role in protein structure, function and stability. Changes in glycosylation are a hallmark of cancer with specific glycans driving tumor development and progression. We propose to use mass spectrometry (MS)-based glycoproteomics to identify global glycosylation changes in patient-derived NF1-deficient SCs from PNFs and MPNST cell lines. We will also determine the glycosylation profile of neurofibromin using CRISPR-engineered SC lines in which a FLAG tag has been introduced in-frame with NF1. Modified residues will be compared with our extensive database of pathogenic missense mutations in NF1 to determine if altered glycosylation of neurofibromin itself may play a role in the disease. Profiling and characterization of glycoprotein variations could lead to identification of disease-associated glycan alterations which could be exploited for therapeutic and biomarker potential.

Alissandra Hillis | G3
Biological and Biomedical Sciences
Alex Toker lab
Identifying syn
thetic lethal combination therapies with PI3K/AKT inhibitors in TNBC
The phosphoinositide-3-kinase (PI3K) pathway is hyperactivated in almost all human cancer types, promoting cell growth and survival. Triple negative breast cancer (TNBC) is a highly heterogeneous disease with poor prognosis and limited targeted therapies. Among the few common genetic features in TNBC is PI3K pathway hyperactivation, which occurs in greater than 50% of TNBC cases. PI3K/AKT inhibitors have been developed to treat PI3K pathway-mutant cancers, but they have been generally ineffective as monotherapies due to the presence of other oncogenic mutations in heterogeneous tumors, the development of acquired resistance, or on-target toxicities. However, in 2019, the PI3Ka-selective inhibitor, BYL719, was approved, in combination with hormone therapy, for the treatment of estrogen receptor-positive, PIK3CA-mutant breast cancer. This motivates developing additional combination therapies with PI3K/AKT inhibitors in other cancer types. I hypothesize that PI3K/AKT inhibitors can effectively treat PI3K pathway-mutant TNBC, if synthetic lethal drug combinations are identified. With the myriad of potential anti-cancer drug combinations available, it is difficult to predict which combinations will be effective. To identify effective drug combinations, I performed a genome-wide negative selection CRISPR screen with PI3K/AKT inhibitors in TNBC. This work aims to advance the treatment of TNBC by identifying targetable vulnerabilities in this heterogeneous disease.

Sophia Liu | G4
Biophysics
Fei Chen Lab
Spatially mapping T cell receptors and transcriptomes for understanding immune response to cancer
T cells mediate antigen-specific immune responses to disease through the specificity and diversity of their T

cell receptors (TCRs). Although determining the spatial distributions of T cell clonotypes in tissues is essential to understanding T cell maturation and behavior, spatial sequencing methods remain unable to profile the TCR repertoire. Here, we develop Slide-TCR-seq, a method to sequence whole transcriptomes and TCRs within intact tissues. We confirmed the ability of Slide-TCR-seq to map the characteristic architecture of T cells and their receptors in mouse spleen. We then spatially profiled T cell clonotypes and their infiltration in renal cell carcinoma specimens before and following immune checkpoint blockade, which identified uniquely distributed T cell clonotypes and transcriptional states. Our method is anticipated to facilitate dissection of the immune microenvironment, yielding insights into the complex spatial relationships between T cell clonotypes, neighboring cell types, and gene expression that drive T cell responses across diseases.

Francesca Nardi | G2
Biological and Biomedical Sciences
Karen Cichowski Lab
Targeting the eIF4F translation initiation complex as a novel therapeutic strategy for KRAS-mutant lung cancer
Although KRAS is the most frequently mutated oncogene in non-small cell lung cancers (NSCLCs), there are currently no effective treatments for KRAS-mutant tumors. We have recently discovered that inhibition of eIF4A, the helicase of the eIF4F translation initiation complex, synergizes with either MEK or KRAS inhibitors and triggers apoptosis of NSCLCs. We further identified several eIF4A-regulated mRNAs in NSCLC including c-Myc, cyclin D1, Bcl-XL, Bcl-2, and Mcl-1. However, using functional genomic studies we found that the therapeutic effects of these drug combinations where mediated by the suppression of the anti-apoptotic bcl-2 family proteins rather than c-Myc or cyclin D1. Importantly, because eIF4A inhibitors suppress the excessive translation of these components in tumors, rather than their biochemical function, this strategy should selectively affect tumor cells while sparing normal tissues. Nevertheless, to complement these studies and gain more insight into the broader functional role of the eIF4F complex in NSCLC, we will perform polysome profiling as an unbiased approach to identify additional translational targets. Together, our studies highlight two promising therapeutic strategies for KRAS-mutant NSCLCs, define the bcl-2 family proteins as critical contributors to the therapeutic response, and will reveal important insight into mechanisms that are required for NSCLC survival and development.

Shikha Sheth | G6
Biological and Biomedical Sciences
Kevin Haigis Lab
Understanding resistance to Kras-G12C inhibitors in colorectal cancer (CRC)
Although mutations in the Kras oncogene are frequent across cancers, they have historically been difficult to target directly. Recent studies have led to the development of inhibitors targeting Kras-G12C mutant cancers (G12Ci), and though these inhibitors are effective against lung tumors, patients with colon tumors do not respond as well. It is not entirely clear what accounts for the differential response in lung and colon tumors, and we believe that the underlying biological processes inherent to colon tumors-but not lung-can contribute to the intrinsic resistance seen in colon tumors. Specifically, loss of Apc and consequent activation of Wnt signaling is very common in colorectal cancer (CRC), and these mutations are rare in lung cancer. We hypothesize that activation of Wnt signaling contributes to intrinsic resistance to G12Ci in colon tumors. Using mouse colon organoids, we evaluate the effect of Apc loss in a Kras-G12C mutant background on response to G12Ci. Our preliminary findings indicate that loss of Apc increases resistance to G12Ci, and that co-treatment of Apc-mutant organoids with a Wnt inhibitor increases sensitivity to G12Ci. Altogether, these studies underscore the importance of understanding tissue context when targeting the same pathway in different tumors, which will hopefully aid in the development of improved drug treatments and combinations.

Georgia Stirtz | G4
Biological and Biomedical Sciences
Leonard Zon Lab
Tumor Microenvironmental Regulation of T cell Infiltration
The advent of immune checkpoint blockade (ICB) has greatly improved survival rates for melanoma patients, yet 40% of patients are unresponsive. Research has demonstrated that tumor-infiltrating lymphocytes are a predictor of response to ICB and increased survival. Therefore, developing strategies to increase T cell infiltration has strong therapeutic potential. Our lab developed a zebrafish reporter that labels CD8+ T cells and used this reporter to visualize tumor-immune interactions in endogenous, nonpigmented melanomas. Using an approach of longitudinal, intravital microscopy to visualize T cell infiltration within the same tumor across development, I identified progressive stages of T cell infiltration and found that CD8+ T cells preferentially infiltrate within clefts in the surface of the tumor. These clefts are commonly adjacent to cxcl12a+ vessels and stromal cells. Single-cell RNA-sequencing of cxcl12a+ stromal cells revealed a cancer-activated stromal population that expresses genes found to correlate with T cell infiltration in human melanomas. I am functionally evaluating these candidate genes in vivo by knockout and overexpression for an effect on T cell infiltration. These experiments will advance understanding of the mechanisms underlying the anti-tumor response to allow for the development of therapeutics to induce T-cell infiltration and expand the population of responders to ICB.

Karin Grunebaum Poster Competition at the Cancer Biology Spring Symposium

Since 2017, the Cancer Biology Spring Symposium has included the Karin Grunebaum Poster competition funded by the Karin Grunebaum Cancer Research Foundation. Due to the ongoing pandemic, we held an online research competition via Zoom webinar. The competition was held on Monday, June 28, 2021 at 2:30 pm.

Students at all levels of their training were invited to showcase their research in a different format than previous years – one slide and 3 minutes in total, showcasing big picture questions and the significance of their research.

Seven graduate students participated across two different graduate programs at Harvard (Biological and Biomedical Sciences and Biophysics). *The complete program is attached.*

We awarded three monetary prizes aimed at professional development:

1. **First place ($750) – Georgia Stirtz, G4**
   PhD candidate in the lab of Leonard Zon
   Title: "Tumor Microenvironmental Regulation of T cell Infiltration"

2. **Second place ($500) – Shikha Sheth, G6**
   PhD candidate in the lab of Kevin Haigis
   Title: "Understanding resistance to Kras-G12C inhibitors in colorectal cancer (CRC)"

3. **Third prize ($250) – Alissandra Hillis, G3**
   PhD candidate in the lab of Alex Toker
   Title: "Identifying synthetic lethal combination therapies with PI3K/AKT inhibitors in TNBC"

The students were judged by four faculty members during the competition – Pratiti (Mimi) Bandopadhayay (DFCI), Christian Dibble (BIDMC), Andrea McClatchey (MGH), and Mohammad Rashidian (DFCI). The prizes were awarded at the end of the event.

More than 30 people were in attendance, including three members of the Foundation Board (David Golan, Shelby Schultz, and Steven Wallach).

In addition to the research competition, we heard short talks from last year's winners:

Kristin Qian, G3
PhD candidate in the lab of Cigall Kadoch
"Elucidating the dynamics and organization of SWI/SNF chromatin remodeling complexes in transcriptional regulation"

Bing Shui, G5
PhD candidate in the lab of Kevin Haigis
"Hyper-activated KRas suppresses global microRNA function in colon"

Incoming Fellow

Gareth J Morgan, PhD
Karin Grunebaum Faculty Research Fellow,
Research Assistant Professor, Section of Hematology and Oncology, Boston University School of Medicine

The roles of antibody stability in plasma cell cancers

We study the antibodies secreted by cancerous plasma cells, aiming to understand why some antibodies and their fragments can form pathological aggregates including amyloid fibrils, and how the secretion of different antibodies modulates the cells' metabolism and proliferation.

All cells need to maintain a functional proteome, comprising the thousands of proteins that carry out most biological processes. This maintenance is carried out by an array of quality control systems, known as the proteostasis network, which sends misfolded or defective proteins for repair or degradation. Cancer cells accumulate mutations that disrupt normal proteins, leading to greater dependence on these quality control systems. Although this dependence represents a vulnerability that could be targeted for therapy, attempts to do so have not yet been broadly successful.

One notable exception is the success of proteasome inhibitors such as Velcade (bortezomib) in treating multiple myeloma and related cancers of antibody-secreting plasma cells. Myeloma affects an estimated 140,000 Americans and is considered incurable, but targeted therapies including proteasome inhibitors have improved survival rates since the turn of the century. The proteasome is one of the cell's main garbage-disposal systems, a key part of the proteostasis network that degrades unwanted proteins and recycles their component amino acids. It also plays a key role in signaling by removing inhibitory components of protein complexes. In myeloma, proteasome inhibition suppresses activation of the NF-κB signaling pathway and also leads to accumulation of misfolded proteins, causing cellular stress that eventually leads to cell death.

Plasma cells are long-lived, terminally-differentiated B lymphocytes, which are specialized to secrete vast amounts of a single antibody, providing long-term immune memory. Each plasma cell secretes a unique antibody heavy chain and light chain protein, and clonally expanded or cancerous plasma cells all express the same antibody. Myeloma cells continue to secrete antibodies, requiring substantial cellular resources, even though antibody production does not directly contribute to proliferation of the cells. Indeed, antibody production may be an important limit on myeloma progression. The secreted, "monoclonal" antibody, especially the light chain protein, is an important biomarker for the status of a patient's disease.

Importantly, overproduction of a single antibody can lead to downstream pathology, including formation of protein aggregates such as renal tubular cast nephropathy, known as "myeloma kidney". In some cases, the secreted antibody can form ordered aggregates known as amyloid fibrils, which can accumulate in multiple tissues and cause severe organ damage. Amyloid formation can occur without overt myeloma, if the antibody light chain made by otherwise slow-growing clonal cells is particularly prone to aggregation. The resulting disease, light chain amyloidosis or AL amyloidosis, is often rapidly fatal if untreated, as cardiac amyloid deposition leads to heart failure.

Myeloma and AL amyloidosis therefore highlight a potential tradeoff for cancer cells. We aim to ask whether production of an unstable, aggregation-prone antibody limits cellular proliferation. Conversely, is production of a stable antibody associated with more aggressively proliferating myeloma? Identifying the mechanisms and pathways that couple protein production and quality control to survival and proliferation could highlight potential targets for drugs, and allow more effective use of existing therapies.

To address these questions, we will measure gene

expression in primary plasma cells from individuals with AL amyloidosis and multiple myeloma. Boston Medical Center is a major referral clinic for amyloidosis, and we have a unique opportunity to study bone marrow and peripheral blood samples from patients with this rare disease. Using flow cytometry and RNA sequencing, we will ask how plasma cells from individuals with AL, which are known to secrete an unstable antibody, differ from cells from individuals with myeloma. Gene expression has been extensively studies in myeloma, and we are fortunate to be able to draw on large published datasets in order to benchmark our data.

A challenge with this work is the low population of cancerous plasma cells in bone marrow from AL amyloidosis patients. Our initial flow cytometry experiments observed populations of cells expressing both κ and λ antibody light chains, as shown in the figure, rather than a dominant population expressing a single light chain type, as would be expected in myeloma. Therefore, we turned to single-cell RNA sequencing in order to differentiate between clonal, cancerous plasma cells and the healthy plasma cells within the same sample.

In parallel, we have developed a bioinformatics pipeline to extract antibody sequences from RNA sequencing datasets. This will allow us to substantially increase the number of antibody protein sequences known to be associated with myeloma. In combination with our existing database of amyloid-associated antibody sequences, AL-Base, these data will allow us to identify sequence features associated with stable or unstable antibodies. We can test the effect of mutations by measuring the stability of recombinant proteins.

This work is at an early stage, and the funds from the Karin Grunebaum Cancer Research Foundation will be critical to establishing our experimental systems and acquiring early data. I am very grateful to the Foundation for the opportunity to pursue this research, and honored to join such a distinguished group of Fellows.

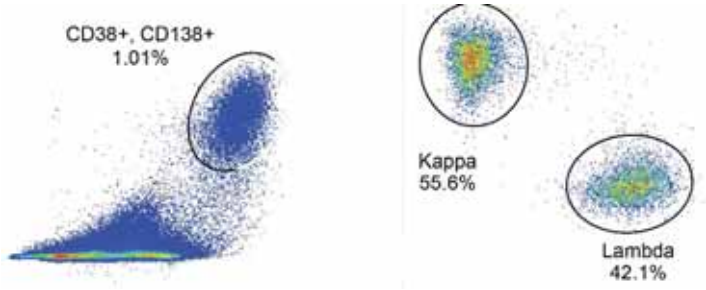
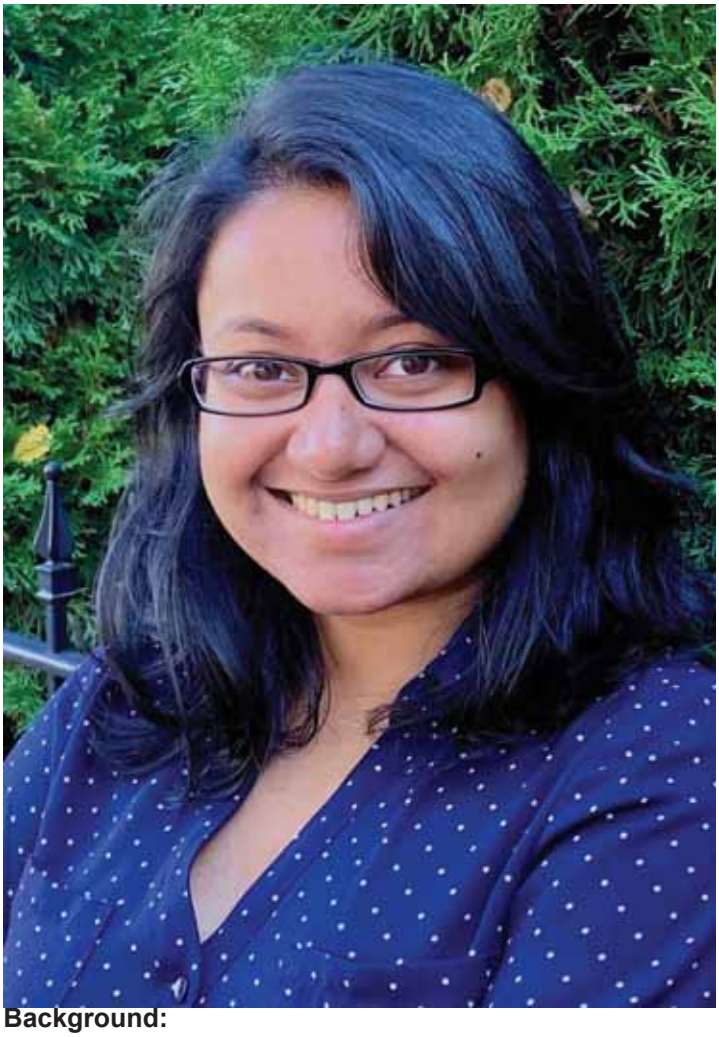


Figure: Flow cytometry identifies plasma cells in a bone marrow aspirate from a patient with AL amyloidosis. Two million Ficoll-fractionated cells were stained with antibodies to surface markers including CD38 and CD138, then fixed, permeablized and stained with antibodies for λ and κ light chains (LC). Flow cytometry was carried out on a Cytek Aurora instrument at the BUSM Flow Cytometry Core Facility and data were analyzed in FlowJo. Each point represents a single cell and false color indicate multiple cells. In amyloidosis, a small fraction of plasma cells secretes a toxic antibody so there is no dominant population of λ- or κ-expressing cells, as would be the case in myeloma.

Incoming Fellow

Debattama Sen, PhD
Karin Grunebaum Faculty Research Fellow,
***Member of the Faculty*, Harvard Medical School; Massachusetts General Hospital**



Background:
Over the last decade, cancer immunotherapy has rapidly revolutionized the treatment of many types of tumors. However, there is still great unmet need. Effective immunotherapy responses have been limited in part due to the development of T cell exhaustion wherein $CD8^+$ T cells become dysfunctional and fail to control tumor growth. Current strategies to alleviate T cell exhaustion have largely focused on modulating inhibitory T cell signaling through receptors such as PD-1. However, to date there has been no comprehensive analysis dissecting the fundamental mechanisms by which PD-1 is regulated specifically in dysfunctional tumor-infiltrating T cells.

This knowledge is needed to develop more targeted approaches to reduce inhibitory signaling and reprogram exhausted cells in the tumor microenvironment (TME). **The goal of this project is to investigate the epigenetic regulation and functional consequences of chronic PD-1 expression in anti-tumor immunity.** We hypothesize that modulating exhaustion-specific regulation of molecules like PD-1 in dysfunctional T cells could prevent pathological expression of inhibitory genes while otherwise preserving their physiological role in T cell activation.

Current work:
By comparing across several contexts of T cell dysfunction, we have identified a core epigenetic signature of exhaustion that is shared across all subsets of exhausted T cells, suggesting that T cell exhaustion is a fundamental immune adaptation to settings of chronic stimulation (Figure 1). **We discovered a novel candidate enhancer near the PD-1 gene that is unique to dysfunctional $CD8^+$ T cells and a component of the core epigenetic program of exhaustion (Figure 1).** In collaboration with the Sharpe lab (Harvard Medical School), we used Cas9-mediated genome editing to create a novel mouse strain with germ-line deletion of this region.

Our preliminary data suggested that enhancer loss reduces but does not abrogate PD-1 upregulation in vivo, consistent with our hypothesis that this enhancer is specifically responsible for chronically high PD-1 levels in the TME (Figure 2A). Furthermore, we observed 2-3-fold enrichment of PD-1 enhancer-null cells over control cells in the tumor, suggesting that $CD8^+$ T cells in these mice might be less prone to exhaustion (Figure 2B). PD-1 enhancer-null mice also exhibited slower tumor growth and increased survival when challenged with either B16-ova melanoma or LLC-ova lung carcinoma (Figure 2C). Taken together, these data suggest that deletion of a state-specific enhancer in exhausted T cells can enhance anti-tumor immunity.

Moving forward:
<u>Aim 1: Identify key transcription factors regulating pathologically high PD-1 expression in vivo.</u>

Our data suggest that the -23kb enhancer specifically mediates sustained high PD-1 expression within exhausted CD8+ T cells. However, the mechanisms by which this enhancer regulates PD-1 levels remain unknown. We previously conducted an in vitro CRISPR-based saturation mutagenesis screen across this enhancer in the EL4 T cell line to finely map functional “hotspots”, which identified binding sites for several key exhaustion-associated transcription factor (TFs). We will now translate this in vivo, leveraging our long-standing collaboration with Arlene Sharpe’s lab at HMS and their recently published CRISPR screening platform in immune cells.

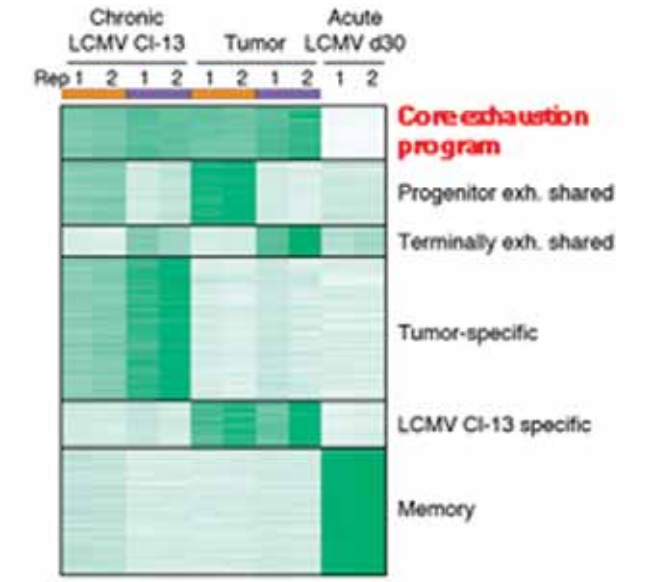


Figure 1. Clustering of chromatin accessibility in exhausted CD8⁺ T cell subsets from chronic infection and tumors, compared to memory T cells identified a core exhaustion program.

We propose a multi-scale screening approach to profile at progressively finer resolution to identify the most impactful regulatory elements. First, we will leverage dCas9 inhibition through steric hindrance paired with sgRNAs tiling across the entire 1.2 kb enhancer to broadly map active subregions. Second, we will screen only within the functional "hotspots" defined from the dCas9 screen, using nuclease Cas9 to introduce indels that will pinpoint the important TF motifs.

Aim 2:
Interrogate the functional impact of disrupting this PD-1 enhancer in tumor-infiltrating CD8⁺ T cells.

Downregulating PD-1 signaling (by antibody blockade or gene knock-out) has profound effects on multiple facets of CD8⁺ T cell biology, including proliferation, persistence, cytotoxicity, cytokine production, and population heterogeneity. By tuning down PD-1 expression in exhausted settings, we hypothesize that enhancer deletion could recapitulate the benefits of PD-1 gene knock-out (enhanced proliferation) while retaining the benefits of wildtype PD-1 expression (relative maintenance of function, improved long-term persistence) in the tumor microenvironment.

Therefore, we will examine the effect of PD-1 modulation through enhancer editing deletion in settings of CD8⁺ T cell

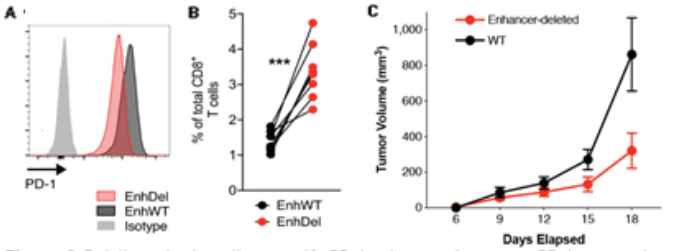


Figure 2. Deletion of exhaustion-specific PD-1 enhancer decreases PD-1 expression (A), increases CD8⁺ T cell persistence in tumors (B) and leads to better control of B16-ova melanoma cells (C).

exhaustion and compare to both WT and full PD-1 gene knockout T cells. To normalize potential differences from the microenvironment, we will co-transfer antigen-specific enhancer-deleted and WT or PD-1 KO CD8⁺ T cells into mice that will be implanted with B16-OVA tumors. We will then profile transferred cells at an early timepoint (day 8) and a late timepoint (day 21+ in tumor) to capture the impact of the enhancer on the differentiation of exhausted T cells.

If successful, these studies will enable unprecedented context-specific engineering of PD-1 expression in CD8⁺ T cells to generate "exhaustion-proof" CAR-T therapies against cancer.

Outgoing Fellow

Nilay Sethi, MD, PhD
Karin Grunebaum Faculty Research Fellow,
Principal Investigator at Dana-Farber Cancer Institute

Progress Report

BACKGROUND

Colorectal cancer (CRC) is the third most common and second most deadly cancer worldwide. The rising rates of early onset CRC is also undeniable, and a concrete reason for this unfavorable trend remains unclear. Current first-line therapeutics consistent of empiric chemotherapy with modest efficacy and considerable toxicity. We recently learned that SRY-box transcription factor 9 (SOX9) suppression induces intestinal differentiation and cancer cell death by interrupting an essential stem cell program in CRC1. These results inspire a new treatment approach: discovering and developing chemical compounds that overcome the genetically wired differentiation block in CRC. To this end, we implemented a novel screening platform to identify genetic perturbations and chemical inhibitors that interrupt stem cell signaling, promote intestinal differentiation, and lead to CRC cell death.

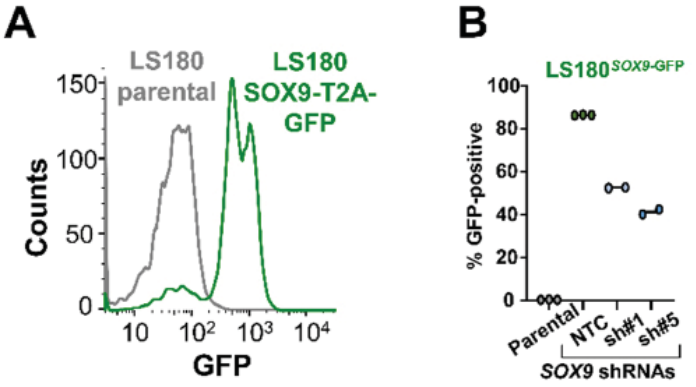


Figure 1. Endogenous stem cell reporter. A, *GFP positivity in engineered stem cell reporter CRC line compared to parental control by flow cytometry.* ***B,*** *GFP positivity in stem cell reporter cell line with SOX9 knockdown by flow cytometry.*

The overall objective of our proposal was to (1) define the molecular mechanisms of hyperactive stem cell programs and (2) determine the therapeutic potential of blocking stem cell signaling in colorectal cancer. In order to accomplish this, we proposed to design a new discovery platform that will enable drug screens to identify compounds that block hyperactive stem cell signaling and promote differentiation/death of cancer cells.

SUMMARY OF PROGRESS:

To identify drug compounds that block stem cell activity and induce intestinal differentiation, we successfully developed a dual endogenous reporter system with the help of the Karen Grunebaum Foundation funding, which was the primary objective of the grant. After validation and optimization, we utilized the reporter platform to perform a small chemical library screen, generating preliminary proof-of-concept data that blocking aberrant stem cell activity in CRC is a promising therapeutic approach.

DETAILED RESULTS:

Development of endogenous stem cell reporter cell line:

To establish an endogenous stem cell activity reporter, we knocked-in GFP in-frame at the end of the *SOX9* coding region using the combination of CRISPR/Cas9 and template-based homologous recombination (Figure 1A). Compared the parental CRC cell line, the engineered GFP

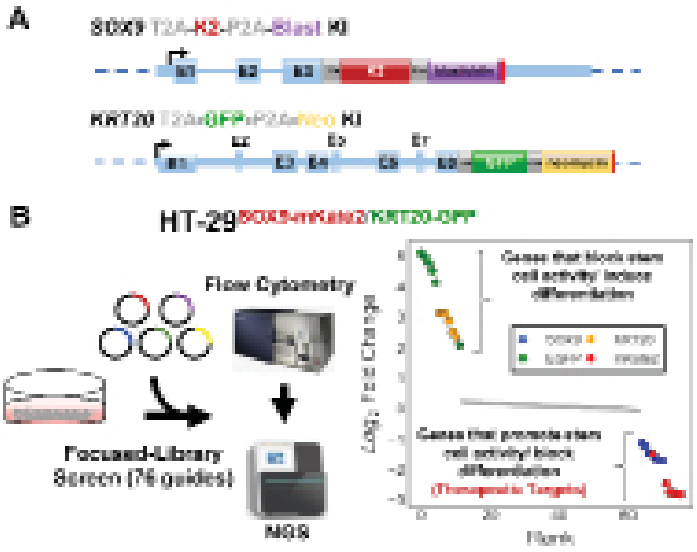


Figure 2. Genome engineered dual reporter system measures stem cell and differentiation activity in human CRC cell lines. *A, Diagram of genome-edited SOX9 and KRT20 genomic loci with mKate2 and GFP reporter knock-in, respectively. B, Flow cytometry-based CRISPR genetic screen validates endogenous stem cell and differentiation dual reporter system.*

knock-in stem cell reporter line demonstrated high GFP levels due to the elevated SOX9 expression in CRC (Figure 1B). Since SOX9 drives a stem cell transcriptional program, GFP levels in the engineered reporter line will reflect stem cell activity. To validate that GFP levels faithfully reflect SOX9, we stably expressed two different SOX9 shRNAs in the stem cell reporter line, which led to a greater than 50% reduction in GFP (Figure 1C).

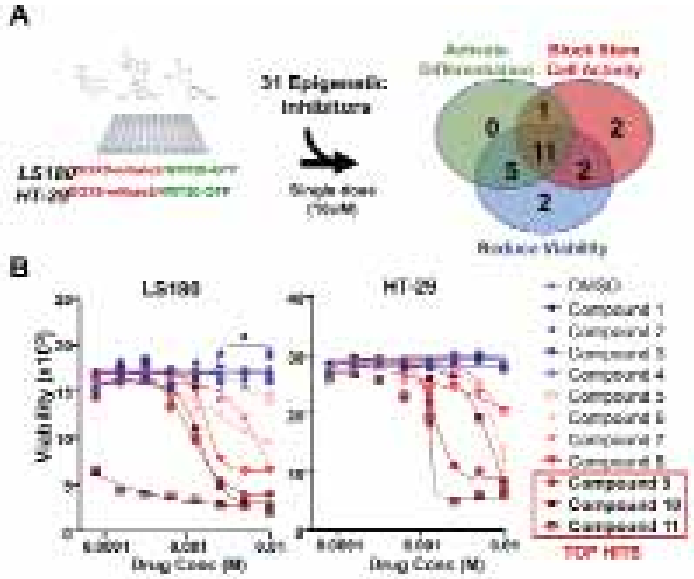


Figure 3. Epigenetic drug screen using dual reporter system. *A, 31 epigenetic drugs were screened using our reporter system at a single dose (10uM). 11 candidates reduced viability, decreased stem cell activity and activated differentiation. B, A secondary viability screen of 11 candidates at 8 different doses.*

Development of dual stem cell and differentiation reporter cell line: We knocked-in mKate2 (mK2, a next-generation RFP probe) and GFP fluorescent markers in-frame at the end of the SOX9 and KRT20 coding regions using the combination of CRISPR/Cas9 and template-based homologous recombination to establish an endogenous dual stem cell and differentiation activity reporter (Figure 2A). We next performed a CRISPR/Cas9 focused-library screen to validate the platform. In a pooled format, 6 sgRNAs targeting GFP, 6 sgRNAs targeting SOX9, 6 sgRNAs targeting mKate2, 6 sgRNAs targeting KRT20 and 52 sgRNAs targeting 6 different negative control genes were introduced into the dual reporter system. After seven days, cells were sorted by flow cytometry, capturing fractions with $GFP^{high}/mK2^{low}$ expression and vice versa (Figure 2B). Genomic DNA was extracted and sequenced to determine sgRNA representation. If the stem cell reporter is functional, we would expect that mK2 and SOX9 targeting sgRNAs to be enriched in the $GFP^{high}/mK2^{low}$ sorted populations. By contrast, sgRNAs targeting KRT20 and GFP would be recovered in the $GFP^{low}/mK2^{high}$ fractions if the differentiation reporter is functional. Indeed, this pattern of guide enrichment was recovered from the sorted fractions (Figure 2B), validating the dual stem cell and differentiation reporter line.

We have now used this system to perform three genetic screens. The most mature results stem from an epigenetic regulator genetic screen that corresponds to annotated drug targets of the chemical compound library screen described below. We identified epigenetic regulators that once genetically inactivated promote intestinal differentiation. One of these epigenetic regulators corresponds to a chemical compound that also scored in our drug screen. The other regulator is involved in mitochondrial metabolism and is the focus of further investigation.

Epigenetic drug screen using our fluorescent reporter cell lines: We utilized our dual reporter CRC cell lines to screen 31 well-annotated small molecules that inhibit specific epigenetic regulators (Figure 3A). We obtained this drug library through our collaborator Dr. Jun Qi (DFCI), an expert chemist focused on drug discovery. The library consists of synthesized compounds that either degrade or disrupt specific enzyme activity of ATP-dependent (e.g. SNI/SWF family members) and covalent modifying (e.g. histone deacetylases (HDAC)) chromatin remodelers among other epigenetic regulators. The initial screen was performed at a relatively high concentration of 10μM of each compound and resulted 11 candidates which reduced viability, blocked the stem cell program and activated differentiation (Figure 3A). We next performed a viability secondary screen involving these 11 compounds at 8 different drug concentrations to establish dose-response curves, which yielded 3 promising candidates (Figure 3B). Reassuringly, 5 of the top 6 compounds fall in two classes of epigenetic inhibitors (i.e. 2 compounds in one class and 3 compounds in another). One of these compounds was tested in an in vivo mouse model and showed promising results. We now aim to understand its mechanism of action using RNA-seq and drug-protein pulldown experiments.the top 6 compounds fall in two classes of epigenetic inhibitors (i.e. 2 compounds in one class and 3 compounds in another). One of these compounds was tested in an in vivo mouse model and showed promising results. We now aim to understand its mechanism of action using RNA-seq and drug-protein pulldown experiments.

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KARIN GRUNEBAUM
cancer research foundation