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CCR connections

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All Natural Chemicals

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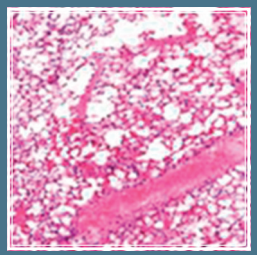

Among the Most Deadly

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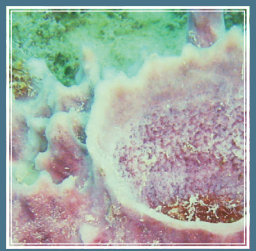

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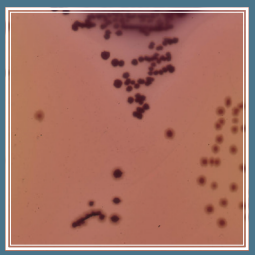

Bacterial Regulation: Past, Present, and Future

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IN THE CLINIC

Toward a Zero-Intensity Preparation (ZIP) Transplant

CENTER FOR CANCER RESEARCH

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The mission of CCR is:

To inform and empower the entire cancer research community by making breakthrough discoveries in basic and clinical cancer research and by developing them into novel therapeutic interventions for adults and children afflicted with cancer or infected with HIV.

http://home.ccr.cancer.gov/connections

Mentorship Matters

At every stage of a scientific career, the influence of good mentors can make all the difference. Although we are not a dedicated educational institution, CCR makes training the next generation of scientists a priority. Currently, CCR is home to approximately 760 postdoctoral, research, and clinical fellows, 190 pre-doctoral students, and an average of 350 summer students pursuing training in multiple areas ranging from basic research at the laboratory bench to translational research that may ultimately bring these discoveries to the bedside.

(Photo: B. Branson)

Robert Wiltrout, Ph.D.

Doctoral students find their way into CCR laboratories through several mechanisms. In "A Special Relationship," we learn that 32 graduate students have trained in CCR laboratories through a program, which originated between the NIH and Oxford and Cambridge Universities in the U.K. Each of the scientists interviewed for this story have pursued diverse career trajectories, but all look back on their experience at CCR as formative.

Chanelle Case-Borden, Ph.D., explains in this installment of our "In Conversation" series, how the NIH Graduate Partnerships Program allowed her to pursue her doctoral research under Thomas Ried, M.D., Senior Investigator, in CCR's Genetics Branch. Case-Borden continues to work as a postdoctoral fellow in CCR's Experimental Immunology Branch, under the guidance of Dinah Singer, Ph.D. Moreover, she is already "giving back" as a mentor to postbaccalaureate fellows interested in M.D./Ph.D. programs.

Mark Smyth, Ph.D., Senior Scientist in the QIMR Berghofer Medical Research Institute in Brisbane, Australia, traces a strong influence on his career to the mentors and colleagues he gained through his postdoctoral fellowship in what was then the NCI's Biological Response Modifiers Program in Frederick, Md. In "It Takes a Village," he describes the collaborative atmosphere of those days as one he has tried to replicate throughout his career as a key driver of success.

As noted in "Bacterial Regulation: Past, Present, and Future," even high school and college students have the opportunity to spend summers experiencing scientific research first hand. Indeed, it was due to these budding scientists, that Susan Gottesman, Ph.D., Co-Chief of CCR's Laboratory of Molecular Biology, was able to discover some of the first small RNAs responsible for regulating the bacterial stress response.

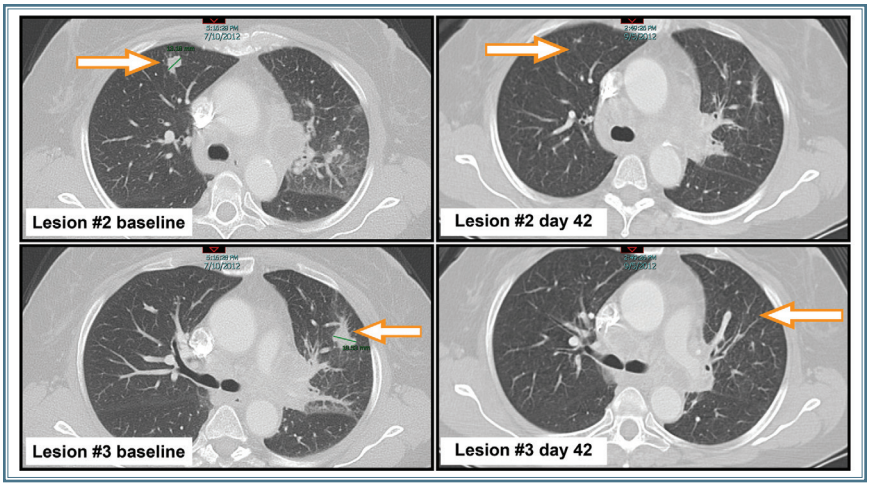
Mentoring the next generation of scientists is, thus, not only an honor and a responsibility, it is also the path to being a productive researcher. As Gottesman's postdoctoral fellow, Daniel Schu, Ph.D., remarks, "I think it is [Susan's] mentoring that has really fueled her continued success. She has two or three students, from high school, college or beyond, coming through the lab every year, either for the summer or as postbacs for a year or two. She allows them to take on their own projects, build confidence in their techniques, and really get a taste for how science is done."

While we all hope that the problem of cancer will be solved in our lifetimes, we know that new challenges will remain. Therefore, CCR is committed to laying a strong foundation for the next generation of biomedical researchers at every level, without which the future of biomedical research and solutions to emerging health threats would be imperiled.

A New Immunotherapy Makes Its Clinical Debut

A first-in-human trial of the cytokine IL-15 in metastatic cancer spurs new research.

There comes a moment in therapeutic development when the preclinical studies are compelling and the next step is a first-in-human study. For Thomas Waldmann, M.D., Co-Chief of CCR's Lymphoid Malignancies Branch, and Kevin Conlon, M.D., Staff Clinician in the Branch, and colleagues, that moment arrived four years ago in their development of interleukin-15 (IL-15) as an immunotherapy. Published earlier this year in the *Journal of Clinical Oncology*, the results of their phase 1, dose-escalation trial encourage the team to continue their efforts with further clinical studies.



(Image: T. Waldmann, CCR)

Computed tomography (CT) chest images of a melanoma patient before treatment with recombinant human interleukin-15 (rhIL-15) and 42 days after treatment. Orange arrows indicate lung metastases.

Waldmann co-discovered the cytokine in the mid-1990s (see "IL-15 Prepares for Its Clinical Debut," *CCR connections* Vol. 5, No. 2). Like IL-2, which was developed by Steven Rosenberg, M.D., Ph.D., Chief of CCR's Surgery Branch, as the first effective immunotherapy for human cancers (see "Immunotherapy's First Cure," *CCR connections* Vol. 8, No. 1), IL-15 shares an ability to stimulate the attack dogs of the immune system: natural killer (NK) and CD8+ T cells. Unlike IL-2, it does not simultaneously activate regulatory or suppressor T cells, cause activation-induced cell death in the immune system, or induce a capillary leak syndrome in animal models. This difference has made IL-15 an attractive lead for therapeutic development.

To secure enough clinical-grade IL-15 for a human trial, Waldmann, Conlon, and their team turned to NCI's Biological Resources Branch, which used the bacteria *Escherichia coli* to produce recombinant human IL-15. After preclinical toxicology testing of bolus injections in non-human primates revealed no causes for concern, the team commenced their trial and ultimately recruited 18 adult patients with metastatic malignant melanoma and metastatic renal cell cancer.

As hoped, IL-15 therapy led to increased counts of circulating NK cells and T cells, in particular, γ/δ and CD8+ memory T cells. By studying the dynamics of the cell counts, the researchers were able to develop a model of cellular activation after IL-15 infusion: immediate redistribution of cells out of circulation, followed by hyperproliferation and subsequent hypoproliferation until a baseline was achieved.

However, acute side effects from bolus administration of IL-15 led to increased levels of multiple cytokines and accompanying clinical toxicities including fever, chills, rigors, and blood pressure changes. The researchers concluded that they could not safely control the clinical toxicities produced by such intense dosing and are currently investigating alternative dosing strategies. After more preclinical testing, the researchers are beginning a new dose-escalation trial, using continuous intravenous infusions to avoid localized high concentrations. They have also joined with the Cancer Immunotherapy Trials Network to begin a phase 1 trial of IL-15 administered subcutaneously.

"Our study clearly shows that IL-15 activates NK cells, monocytes, γ/δ, and memory CD8+ T cells, which should augment the patient's own immune response to the tumors," said Conlon. "Hopefully, with new dosing strategies, we can reduce the toxicity and increase the expansion of lymphoid populations and thereby improve the antitumor effects of IL-15 in patients with metastatic malignancy."

To learn more about Dr. Waldmann's research, please visit his CCR website at https://ccr.cancer.gov/thomas-a-waldmann.

To learn more about Dr. Conlon's clinical research, please visit his CCR website at https://ccr.cancer.gov/kevin-c-conlon.

ALL's Well that Ends Well

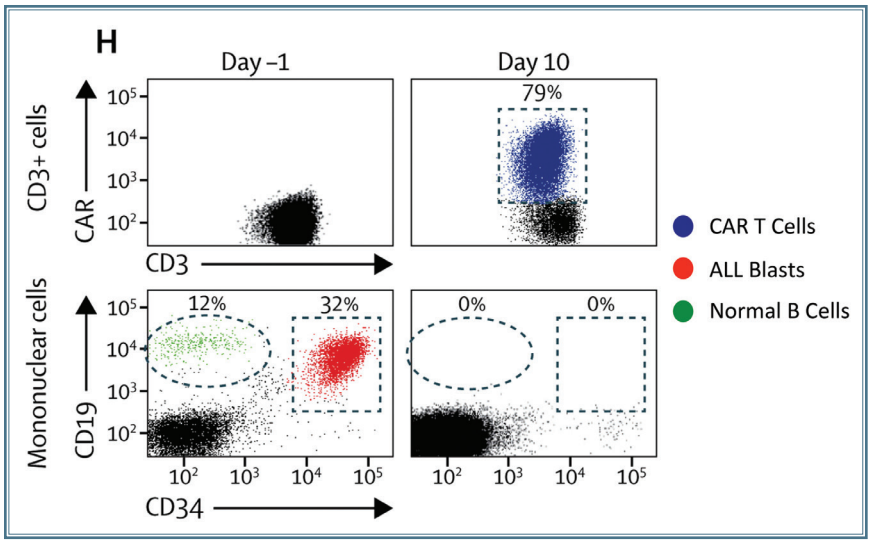
A T-cell immunotherapy attacks most acute lymphoblastic leukemia.

Recently, therapies that manipulate the immune system's response to cancer have become a new source of hope for many with otherwise intractable disease. One particularly promising strategy involves extracting and reprogramming a patient's own T cells with chimeric antigen receptors (CARs), and then reintroducing them to seek out and destroy cells bearing the specified antigen.

For the 15 percent of children with B-lineage acute lymphoblastic leukemia (B-ALL) which is refractory or relapsed after standard treatment, published case series have shown CAR T cells programmed to recognize CD19, a surface marker selectively expressed on almost all B cells, have some antitumor activity. "The anecdotal evidence is promising but does not have the rigor of a well-controlled clinical trial," said Crystal Mackall, M.D., Chief of the Pediatric Oncology Branch (POB).

So Mackall, Daniel W. Lee, M.D., Assistant Clinical Investigator in POB, and their colleagues set out to rigorously test this experimental therapy in a phase 1, dose-escalation clinical trial on consecutively enrolled patients. The results, published in the *Lancet*, showed a complete response rate of 70 percent among the 21 patients enrolled over a two-year period. By comparison, the most recent FDA-approved drug for the disease, blinatumomab, has a complete response rate of 41 percent.

After pretreatment with immune suppressants (fludarabine and cyclophosphamide), patients were infused with a single dose of CAR T cells over a 30-minute period. The maximum tolerated dose was defined at one million CD19-CAR T cells per kilogram, a dose that could be generated from the patients' cells in



(Image: D. W. Lee, CCR)

CAR T cells expand dramatically (blue) in the peripheral blood of patients and coincide with elimination of CD19+ normal B cells (green) and leukemic blasts (red).

90 percent of cases. At this dose, side effects—the most serious of which being cytokine release syndrome—were reversible. Four weeks later, the response was assessed as a percentage of blast cells in the bone marrow and in circulation.

Complete response is defined as having less than five percent marrow blasts, no circulating blasts, and no other sites of disease. Almost all responding patients (12/14) lacked even Minimal Residual Disease (MRD), meaning that blasts were not found by the most sensitive test available. Moreover, two patients were cleared of leukemia that had spread into the central nervous system.

Because most patients who achieved remission went on to have hematopoietic stem cell transplants, the extent to which CD19-CAR T-cell therapy alone could be effective in maintaining remission is not clear. The T cells themselves only persisted in patients for approximately two months, for reasons that are not well understood. What is clear, however, is that they represent a highly effective bridge to transplant therapy.

"We have a study under way that incorporates a more intensive chemotherapy regimen in patients with extensive disease, in an attempt to increase the response rate, while potentially diminishing the risk for severe cytokine release syndrome," said Mackall.

In a *Lancet* commentary earlier this year, Persis Amrolia, M.D., and Martin Pule, M.D., from University College London, summed up the impact of this study: "This approach is without question the most significant therapeutic advance in acute lymphoblastic leukaemia for a generation, and might represent the beginning of a new era of engineered T cells for cancer therapy."

To learn more about Dr. Mackall's research, please visit her CCR website at https://ccr.cancer.gov/crystal-l-mackall.

To learn more about Dr. Lee's research, please visit his CCR website at https://ccr.cancer.gov/daniel-w-lee.

A TIGER Visits Thailand

The first site visit for an international collaboration brings fresh perspectives on liver cancer.

(Photo: X. Wang, CCR)

Members of the TIGER-LC consortium at a clinical site in the Non Sang District

Of the two histological subtypes of liver cancer, hepatocellular carcinoma (HCC) is dominant throughout the world, except in Thailand, where cholangiocarcinoma (CCA) accounts for 70–80 percent of cases, particularly in the northeast province of Khon Kaen. One often proposed reason for this dubious distinction is the presence in this region of a parasite—a liver fluke, *Opisthorchis viverrini*, —whose eggs can be ingested with raw fish and develop into worms that persist in the liver for many years, causing inflammation and other complications which may eventually result in CCA.

The importance of this link is one of the many questions that are being addressed by the Thailand Initiative for Genomics and Expression Research in Liver Cancer (TIGER-LC), a collaboration between the Chulabhorn Research Institute in Thailand, CCR's Liver Cancer Research Group, and participating local institutions. The Initiative has enrolled more than 2,000 patients since 2012. In November 2014, members of the TIGER-LC consortium from the U.S. side, consisting of Anuradha Budhu, Ph.D., Staff Scientist, and Xin Wei Wang, Ph.D., Chief of the Liver Carcinogenesis Section in CCR's Laboratory of Human Carcinogenesis (LHC), along with Curtis Harris, M.D., Chief of LHC, Christopher Loffredo, Ph.D., Professor at Georgetown University Lombardi Comprehensive Cancer Center, and Robert Wiltrout, Ph.D., CCR Director, travelled for the first time to visit the local clinical sites where patients are being recruited.

"This provided us with a completely different perspective," said Wang. "We've read a lot of papers, but we couldn't have imagined a lot of the issues without seeing them first hand. For example, in Khon Kaen, we went to one of the major reservoirs located in the Non Sang District where the fish are caught. In the same locale, among 60,000 people who drink the same water and eat the same fish, one side of the village has a very high incidence of CCA; the other does not. It speaks to how detailed the analysis must be."

The visitors also learned about local campaigns to eradicate the liver fluke. Its lifecycle includes a snail that ingests the eggs from fecal matter before subsequently being ingested by fish. Local campaigns to control the snails include pesticides. "We have members of our group that can advise on these potent chemicals, some of which could be carcinogenic," said Wang. "We are including an analysis of chemical metabolites in urine to examine this link."

TIGER-LC grew out of a long-standing collaboration between the Chulabhorn Research Institute and NCI (See "Collaboration Reigns," *CCR connections* Vol. 4, No. 2). Ultimately, the initiative plans to enroll 5,000 people. The first of six proposed phases includes molecular profiling to establish biomarkers and genomic risk factors associated with HCC and CCA. The Chulabhorn Institute is responsible for coordinating patient recruitment with the five participating hospitals, and funding a national biobank and tissue repository. NCI is providing resources for data analysis and training.

"The study aims to be comprehensive," said Wang. "We want to analyze various etiologies including hepatitis, obesity, and dietary influences." As a result of the local visits, the group is also considering a dietary interventional study to see if local nutritional factors could quickly change the whole disease profile.

"I was humbled by our interactions with the local people," said Wang. "They were highly welcoming and wanted to help us in our efforts to help them."

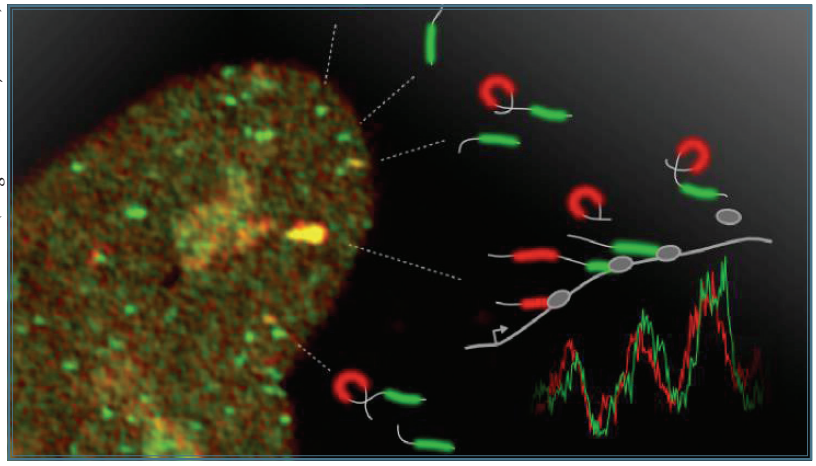
When Timing Matters

Observing mRNA transcription in real time, CCR scientists find a surprising lack of coordination between the timing of synthesis and splicing.

Cancer is a disease of dysregulation. Understanding the normal molecular process in a cell is the first step towards determining what can go wrong and how to fix it. The process of transcribing DNA into mRNA involves multiple macromolecular complexes that initiate RNA synthesis, splice disparate regions of the RNA together, and chemically modify the RNA in preparation for nuclear export. One may assume that these functions occur in a logical, stepwise fashion, amenable to tight regulation; in fact many of them overlap in time, as well as space. To what extent they occur stochastically, i.e., on a first-come-first-serve basis of random molecular interactions (kinetic competition), or are orchestrated by checkpoint molecules, has never been addressed.

Publishing in a recent issue of *eLife*, Daniel Larson, Ph.D., Investigator in CCR's Laboratory of Receptor Biology and Gene Expression, and his colleagues have studied the transcription and splicing of single RNA molecules produced from the human β-globin gene in the nucleus of engineered cell lines, in real time. The gene comprises an upstream intron that is spliced out of the final RNA product and a downstream exon. The team has taken advantage of RNA motifs from bacteriophages that form hairpin structures—PP7 and MS2—recognized by specific binding proteins. By inserting multiple PP7 hairpin-coding sequences in the intron and multiple MS2 hairpin-coding sequences downstream in the exon, the team could observe the transcribed RNA segments through the presence of corresponding fluorescently tagged PP7- (red) and MS2- (green) binding proteins.

(Image: A. Coulon, NIDDK)



Observing single RNA in living cells reveals the kinetics of RNA synthesis and processing.

Monitoring fluorescence in the two channels over time, Larson and his colleagues observed localized fluctuating signals—increasing signals reflecting RNA synthesis and decreasing signals reflecting splicing and/or release of RNA from the site of transcription—for over 1,700 transcripts. By cross-correlation analysis of the intensity signals, they showed the data best fit a model in which the transcript could either be released from the transcription site before splicing (a simultaneous extinguishing of red and green signals) or the intron could be spliced out before transcription was complete (red signal extinguished before green). The data argue for a kinetic competition model in which RNA synthesis and processing occurs at random, rather than in coordinated fashion.

Armed with this knowledge, Larson and his colleagues could ask how a cancer-associated mutation in splicing factor U2 auxiliary factor 1 (U2AF1) affected the balance of competition. U2AF1 is an essential factor involved in splicing of most human transcripts. In the presence of the mutant U2AF1, splicing before release of the transcript was completely abolished, shifting the balance of splicing activity post-transcriptionally. The researchers confirmed this result for an endogenous mRNA, *FXR1*, which is alternatively spliced in the presence of the mutant U2AF1 in cancer.

"Although variations in alternative splicing have been seen in single cells, the mechanism behind such variability has remained elusive," said Larson. "Our studies on the mutant U2AF1 suggest a role for mutations in shifting the kinetic balance to alter gene expression. The kinetic delay may allow for alternate exon pairing during transcription or reconfiguration of the mRNA after release, prior to splicing. This may be one explanation for increased levels of 'noisy splicing' which are observed in cancer."

To learn more about Dr. Larson's research, please visit his CCR website at https://ccr.cancer.gov/daniel-r-larson.

A Special Relationship

CCR and the NIH Oxford-Cambridge Scholars Program strengthen scientific training across the Atlantic.

Since Sir Winston Churchill first used the phrase in a speech in 1946, "the special relationship" between the United States and the United Kingdom has characterized the close cooperation and exchange between these two nations. In 2001, the NIH, which had only recently opened its doors to doctoral training programs, formed a partnership with Oxford and Cambridge Universities in order to create a doctoral program that capitalized on the strengths of both scientific cultures. The program would model the U.K.'s fast track to degree completion, while providing students with immersion in the collaborative, multidisciplinary, global research that characterizes modern science.

(Photo: Courtesy of F. Mussai)

Francis Mussai, M.D., D.Phil.

Fast forward 14 years, and more than 150 students have enrolled in the NIH Oxford-Cambridge Scholars Program (OxCam), 28 of whom joined CCR laboratories. An additional 30 European students, including four at CCR, have come through the Wellcome Trust, which has worked with the program to involve students from around the world and universities throughout the U.K. and Ireland.

Francis Mussai, M.D., D.Phil., an early recruit to OxCam, entered the program after a clinical fellowship at Johns Hopkins University. "I left the U.K. to gain a unique training experience, a different life experience, and broader research and clinical exposure," said Mussai.

Through OxCam, he had the opportunity to work first with Ira Pastan, M.D., Co-Chief of CCR's Laboratory of Molecular Biology, and then with Prof. Vincenzo Cerundolo, Head of the MRC Human Immunology Unit in Oxford, on two almost unrelated projects, but for their intersection in cancer and immunology. With Pastan, he worked on antibody-conjugated toxins to treat childhood cancers and with Cerundolo, he studied the immunosuppressive microenvironment in acute myeloid leukemia.

"Both projects turned out to be a real foundation for my career," said Mussai who is now Clinical Senior Lecturer in Pediatric Oncology at the University of Birmingham. Mussai is the U.K. lead investigator on a phase 2 clinical trial that builds on some of his work at NCI and is setting up clinical trials in Birmingham based on a concept developed from his D.Phil.

In contrast, Ambika Bumb, Ph.D., heard about OxCam through her (then) recently awarded Marshall Scholarship, a prestigious scholarship that brings 40 students from the U.S. to the U.K. to focus on any field of study. "I hadn't finalized which university I would attend or which area I would focus on, but when I heard about OxCam, I got very excited," said Bumb, who is now Founder and C.E.O. of Bikanta, a nanotechnology startup in the San Francisco Bay Area.

Bumb worked with four different PIs—Martin Brechbiel, Ph.D., and Peter Choyke, M.D., in CCR and Prof. Peter Dobson and Prof. Lars Fugger in Oxford—to create a nanoparticle for multimodal imaging of disease. "Now a lot more labs are engaged in interdisciplinary work," said Bumb. "But at the time, and just starting as a graduate student, it was a rare opportunity to work directly under four diverse labs and learn about all these fields. You have to be a certain kind of student who is willing to lead your project and sometimes even manage your PIs, but if you like that kind of challenge, it is incredible."

With a project involving four PIs, Bumb may have been an exception, but current Wellcome Trust student, Coralie Viollet, agrees that time-management and people skills are critical for success in the program. "You have at least two PIs, they have their own agendas and from their perspectives, you are only around for two years. You can't push them to meet your deadlines," said Viollet.

(Photo: Courtesy of A. Bumb)

Ambika Bumb, Ph.D.

"There's so much that you cannot learn any other way than taking your research across the Atlantic. I had to take my samples, ship them on dry ice and keep my fingers crossed that they would come out on the other side intact."

Viollet came to the Wellcome Trust program from an engineering degree in France. As part of a six-month internship requirement, she joined the laboratory of Jiannis Ragoussis, Ph.D., in Oxford, where she subsequently hoped she might remain for her D.Phil. "I applied for the Wellcome Trust fellowship, but Jiannis and I knew that the process was very competitive. As compared to the OxCam program application, the Wellcome Trust requires a much more detailed research proposal up front, almost a year before you start your Ph.D.!" said Viollet.

Her research, working with Ragoussis (now at the University of McGill in Canada), Robert Yarchoan, M.D., Chief of CCR's HIV and AIDS Malignancy Branch, and Prof. Francesco Pezzella at Oxford, focuses on microRNA expression in cells infected with the oncovirus, KSHV. She and her three PIs had their first co-authored paper accepted for publication in April 2015.

"I honestly feel like I've learned a lot more than the average Ph.D. student because of the exposure to so many more techniques, mindsets, people, and ways of doing research," said Viollet. She has helped others apply to the program and thinks she has a pretty good sense of who is going to make it. "Ultimately, it's how motivated you are in taking your project forward and pitching it."

A somewhat peripatetic lifestyle may be the downside to participating in a transatlantic doctoral program. "It's not always easy, especially socially. Just when you feel like you know your way around, your colleagues, and so on, soon after, you have to pack your things and go."

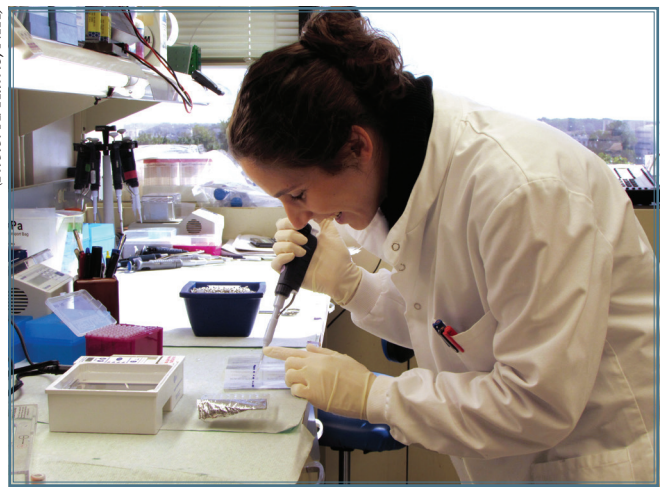
OxCam has organized several events to create closer ties among the students. "There was a lot of desire to create a community of these scholars," said Bumb. "My class was 14 people, split between Oxford, Cambridge, and the NIH. There was a yearly colloquium, during which your PIs could also meet each other. There were also outings, including an Outward Bound experience, in which we spent 3-4 days in the wilderness together."

And, Viollet lives with her fellow students on the NIH campus in buildings that formerly housed the NIH Director and the U.S. Surgeon General. "We all happen to be in the same year, and the social support has been invaluable."

"My class is very tightly knit," said Bumb. "We stay in touch and visit each other frequently. Some of us have even ended up collaborating scientifically. They are still the people I consider my best friends."

CCR's OxCam graduates are working all over the world, in research, medicine, and industry. Bumb went on to two postdoctoral fellowships at the NIH before starting her own company. Viollet has plans to ultimately work in the pharmaceutical industry after completing her degree. Mussai completed clinical training before establishing a research group in Birmingham. He is a practicing physician in addition to a scientist.

"This is no cookie-cutter program," said Bumb. "The experience, and the outcome, are highly individualized."



(Photo: A. Harris, NIH)

Coralie Viollet

Recent CCR Awards

Selman A. Waksman Award
National Academy of Sciences

For transforming our understanding of post-transcriptional regulation in bacteria through mechanisms of controlled proteolysis and small RNAs

Susan Gottesman, Ph.D.
Co-Chief, Laboratory of Molecular Biology

Harrington Prize for Innovation in Medicine
Harrington Discovery Institute and The American Society for Clinical Investigation

For key discoveries that led to development of the human papillomavirus (HPV) vaccine to prevent cancer

Douglas Lowy, M.D.
Chief, Laboratory of Cellular Oncology
Acting Director, National Cancer Institute

2015 American Cancer Society Medal of Honor

For outstanding contributions to cancer control in basic research

Steven Rosenberg, M.D., Ph.D.
Chief, Surgery Branch

John Ultmann Award for Contributions to Lymphoma Research
Weill Cornell Medical College and the SASS Foundation

Elaine Jaffe, M.D.
Senior Investigator, Laboratory of Pathology

Poland–U.S. Science Award
Foundation for Polish Science and the American Association for the Advancement of Science

For structural studies on proteins of medical relevance, which have contributed to the development of new therapies to cure human diseases, such as AIDS or leukemia in children

Alexander Wlodawer, Ph.D.
Chief, Macromolecular Crystallography Laboratory

2015 Michael J. Welch, Ph.D., Award
Society of Nuclear Medicine and Molecular Imaging

For outstanding contributions to the field of radiopharmaceutical sciences

Martin Brechbiel, Ph.D.
Senior Investigator, Radiation Oncology Branch

Rouse-Whipple Award
American Society for Investigative Pathology

For her distinguished career in research that has advanced the understanding of disease

Elaine Jaffe, M.D.
Senior Investigator, Laboratory of Pathology

2015 NPA Distinguished Service Award
National Postdoctoral Association

Jonathan Wiest, Ph.D.
Associate Director for Training and Education

Elected as Fellows of the AACR Academy
American Association of Cancer Research

Douglas Lowy, M.D.
Chief, Laboratory of Cellular Oncology
Acting Director, National Cancer Institute

Steven Rosenberg, M.D., Ph.D.
Chief, Surgery Branch

Elected to the Johns Hopkins Society of Scholars

Carole Parent, Ph.D.
Deputy Chief, Laboratory of Cellular and Molecular Biology

Elected to the American Association for the Advancement of Science

For distinguished contributions to the field of ABC transporters

Suresh Ambudkar, Ph.D.
Deputy Chief, Laboratory of Cell Biology

For distinguished contributions in the field of chemical biology in tumor biology

David Roberts, Ph.D.
Senior Investigator, Laboratory of Pathology

Newly Tenured CCR Scientists

Deborah E. Citrin, M.D.
Radiation Oncology Branch

Hisataka Kobayashi, M.D., Ph.D.
Molecular Imaging Program

Staff News at CCR

Announcement

(Photo: Courtesy of Scientific Publications, Graphics & Media)

Joel P. Schneider, Ph.D.

Joel Schneider has been named a CCR Deputy Director. He received his Ph.D. in organic chemistry from Texas A&M University. He then became a George W. Raiziss Postdoctoral Fellow at the University of Pennsylvania School of Medicine, Department of Biochemistry and Biophysics. He began his independent career at the University of Delaware as Assistant Professor of chemistry and biochemistry and was promoted to Associate and then Full Professor with a secondary appointment in materials science and engineering. He joined CCR in 2010 as Chief of the then newly established Chemical Biology Laboratory. His group's basic research entails designing soft materials, adhesives, and coatings for use in drug delivery and tissue repair. His group is especially interested in biomaterials formed via self-assembly mechanisms.

New Tenure-Track Scientists

(Photo: B .Branson)

Romina S. Goldszmid, Ph.D.

Romina Goldszmid is now an NIH Earl Stadtman Tenure-Track Investigator in CCR's Laboratory of Experimental Immunology. She received her Ph.D. from the University of Buenos Aires in Argentina. She conducted her postdoctoral research in the Laboratory of Parasitic Diseases at the National Institute of Allergy and Infectious Diseases. Goldszmid then joined the Laboratory of Experimental Immunology as a Staff Scientist. Her research focuses on linking the microbiome and mononuclear phagocyte development to cancer and infectious disease.

(Photo: Courtesy of C. Hoang)

Chuong D. Hoang, M.D.

Chuong Hoang joins CCR's Thoracic and Gastrointestinal Oncology Branch as a Tenure-Track Investigator. He received his medical degree from the University of Minnesota Medical School, where he stayed to complete clinical training in general surgery. Afterwards, he completed his cardiothoracic residency at the University of Pennsylvania. In 2008, he joined the faculty of Stanford University School of Medicine as an Assistant Professor. Choang was the Medical Director of the Stanford Cancer Center Tissue Bank. He also established an independent thoracic oncology laboratory investigating the metabolic derangements in lung cancer and microRNA interactions in mesothelioma. Currently, his research focuses on in-depth molecular interactions of microRNA and pathogenic gene networks in mesothelioma and other thoracic cancers to identify practical biomarkers or novel therapeutics targets.

(Photo: B .Branson)

Peter A. Pinto, M.D.

Peter Pinto is now a Tenure-Track Investigator in CCR's Urologic Oncology Branch. He obtained his medical degree from the State University of New York Upstate Medical School. Following a residency in urologic surgery at the Long Island Jewish Medical Center–Albert Einstein College of Medicine in New York, he was a Fellow and Clinical Instructor at the Brady Urologic Institute, Johns Hopkins Hospital. Pinto then joined the Urologic Oncology Branch as a Staff Clinician and Head of the Prostate Cancer Section. His research focuses on vaccine strategies and molecularly-targeted therapeutic approaches to modulate cancer cell growth and survival, imaging and targeted registration of genitourinary tumors to improve diagnosis and treatment, and the minimally invasive treatment of urologic cancers, including high-intensity focused ultrasound and laparoscopic and robotic surgery for prostate, kidney, bladder, and testicular cancer.

(Photo: B .Branson)

R. Taylor Ripley, M.D.

R. Taylor Ripley joins CCR's Thoracic and Gastrointestinal Oncology Branch as a Tenure-Track Investigator. He received his medical degree from Vanderbilt University. Ripley was a Surgical Oncology Fellow in CCR's Surgery Branch, and then went on to a general surgery residency at the University of Colorado. Most recently, he completed a cardiothoracic surgery fellowship at Memorial Sloan-Kettering Cancer Center with a focus in thoracic surgical oncology. Ripley is a thoracic surgeon and his research focuses on targeting the metabolism of esophageal cancer as a treatment strategy.

In Conversation:
Postdoctoral Fellow Chanelle Case-Borden, Ph.D.

CCR: Chanelle, you first came to the NIH as a graduate student. What drew you here?

Chanelle: I have always had my hands in science; I was that nerd kid playing with science kits and nesting butterflies from caterpillars. When you dream of doing science, the NIH looks like that dream, with the best research facilities, some great scientists, and multiple areas of study. I found the Graduate Partnerships Program, when I was applying to graduate school.

CCR: And what attracts you to cancer research specifically?

Chanelle: Genetically, cancer is beautiful because it adapts to survive in a hostile environment. Almost no two tumors are the same, which is an important concept. I love puzzles, and there is always a mystery or problem to solve in cancer. As a graduate student with Thomas Ried, M.D., (Senior Investigator, CCR's Genetics Branch), I worked on identifying cancer biomarkers in colorectal cancer. My project focused on characterizing the protein CKAP2. During training, I performed many trial-and-error experiments, before suddenly, the project took off. Research is full of highs and lows, but that rush when things start to take off is amazing.

CCR: And now, you are doing a fellowship with Dinah Singer, Ph.D., in CCR's Experimental Immunology Branch?

Chanelle: Yes, I am working on bromodomain-containing protein 4 (BRD4), which has been shown to play a pivotal role in several types of cancer. Our lab identified BRD4 as a kinase, regulating transcription initiation and elongation. We recently discovered that BRD4 has an additional enzymatic activity, and my project focuses on understanding its biological function.

(Photo: D. Sone)

Chanelle Case-Borden, Ph.D.

CCR: What are the clinical implications of your work?

Chanelle: BRD4 has become a popular drug target, with its major inhibitors currently in clinical trials. Some cancers heavily rely on BRD4—it's overexpressed in some and enhances the expression of BRD4-specific genes, many of which are cell cycle genes. However, what happens to the endogenous system if you block or disrupt BRD4 function remains unclear. It is a multifunctional protein, and we need to know which aspects are being disrupted by these inhibitors to determine how they are going to play out long term.

CCR: How do you see your career progressing?

Chanelle: Five years ago, I planned on running my own lab; now I'm not certain. Currently, my focus is on getting my project off the ground and publishing. Mentoring students is also a passion of mine. I've mentored four postbaccalaureate fellows and have helped several postbacs get into M.D./Ph.D. programs.

CCR: What advice do you give your students?

Chanelle: I tell them to pursue what they are passionate about. Ultimately you need to do what you love. If you go to graduate school, you have to do it with a purpose, not just as a natural progression in education. Do not take it lightly; do your research, make a plan, and don't be afraid to ask for help. Also just because you obtain a Ph.D., you don't have to stay at the bench. I am on a couple of panels for increasing diversity at NIH and could imagine working full-time on increasing diversity in science, and mentoring students to aid their retention in science.

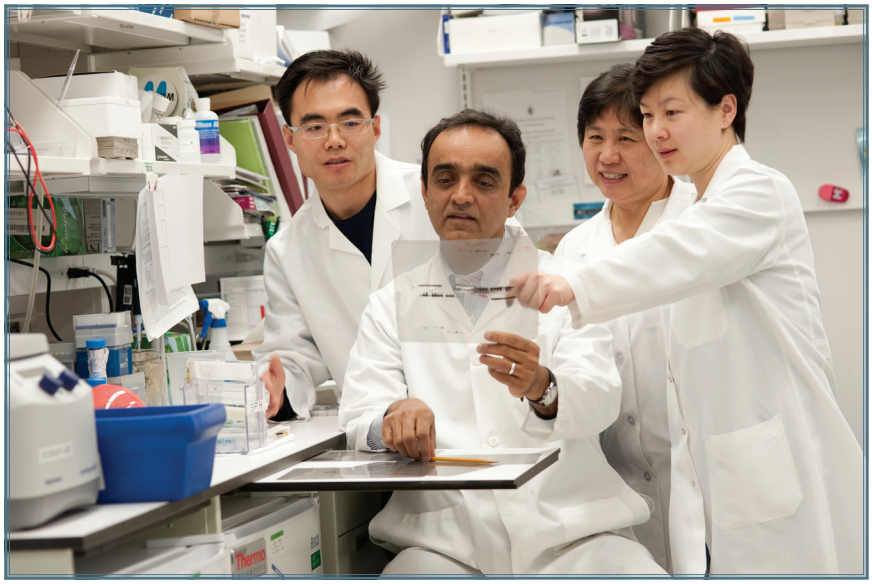
Among the Most Deadly

In 2012, Congress passed the Recalcitrant Cancer Research Act to draw attention to the deadliest cancers that afflict our society, those for which the odds of surviving for five years after diagnosis is less than 50 percent. Among the worst of the worst, pancreatic cancer—or, more specifically, pancreatic ductal adenocarcinoma (PDAC)—presents a five-year survival rate of less than five percent. With no early-detection markers, the disease is usually discovered at an advanced stage and once discovered, the response to chemotherapy is poor. Within CCR, diverse multidisciplinary efforts to understand and treat PDAC are gaining momentum.

When Perwez Hussain, Ph.D., started his group in CCR's Laboratory of Human Carcinogenesis in 2009, he decided to build a program in pancreatic cancer from scratch. "I said, let me take on this challenge. I want to understand the biology of this disease: what is the difference in this solid tumor as compared to others that makes it so aggressive? In my view, understanding the biology is the most important step because it will tell us where to strike," said Hussain.

PDAC tumors present at least two challenges: first, they are highly heterogeneous. For example, a paper published in *Science* in 2008 from Johns Hopkins researchers, found that on average, each tumor had multiple alterations affecting 12 core signaling pathways, but the particular mutations varied from tumor to tumor. In addition, unlike most solid tumors, PDACs have very few blood vessels and are surrounded by a dense tissue stroma, making therapeutic access a greater challenge.

By establishing collaborations around the world, Hussain's group started collecting patient samples for analysis and validation in multiple independent cohorts. His laboratory took both a focused, hypothesis-driven approach to the mechanisms of pancreatic cancer progression, and a global approach to defining molecular distinctions through integrative analysis of the transcriptional and metabolic profiles with a focus on inflammatory mediators.



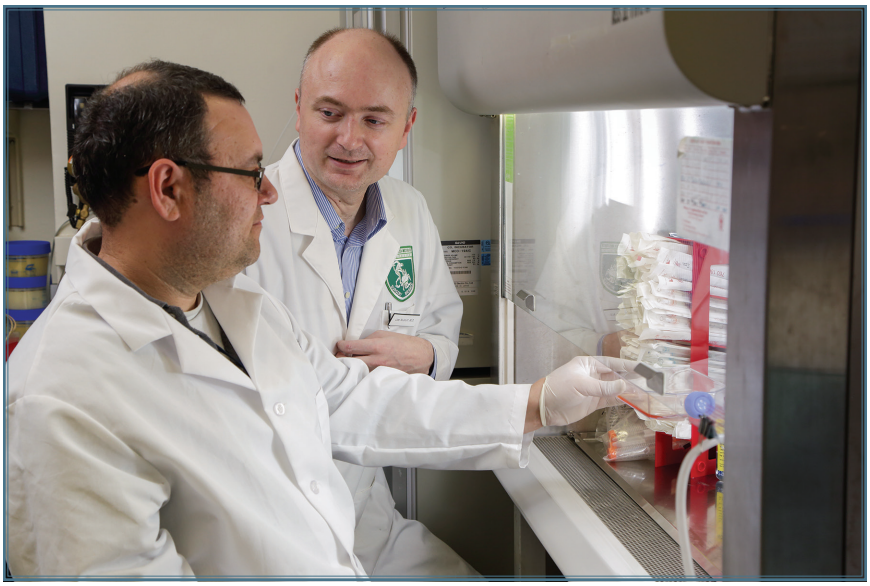
(Photo: R. Baer)

The Hussain team (left to right): Shouhui Yang, Ph.D., Perwez Hussain, Ph.D., Peijin He, B.S., and Jian Wang, Ph.D.

Inflammatory Targets

Although not well understood, many lines of evidence point to an important role for inflammation in pancreatic cancer. The most common precursors to PDAC, pancreatic intraepithelial neoplasms, are often found in association with areas of focal inflammation. Moreover, approximately 95 percent of pancreatic tumors have early mutations in the *KRAS* gene (see "RAS Takes

(Photo: B. Branson)



Udo Rudloff, M.D., Ph.D., and Yaroslav Teper, Ph.D., in the lab

Center Stage," *CCR connections* Vol. 7, No. 2). Among the many manifestations of these mutations is an increased inflammatory microenvironment.

"When you look at pancreatic tumors, you see a lot of markers of inflammation," said Hussain. "Even the tumor cells produce inflammatory mediators: chemokines, cytokines, and growth factors." Hussain's laboratory has pursued two of these mediators: macrophage migration inhibitory factor (MIF) and nitric oxide (NO).

Hussain and his colleagues found that the tumor cells in their patient samples were expressing high levels of MIF, a factor that is hypothesized to be a connecting link between inflammation and cancer. Moreover, they found that increased levels of MIF were associated with a more aggressive phenotype and a poorer prognosis in patients with PDAC.

Pursuing this observation in cell signaling studies, the Hussain group has found that altering MIF expression (either through overexpression or knockdown in cell lines and in animal models), resulted in changes to a signaling pathway that enhances the epithelial-to-mesenchymal transition, a key process in the development of metastases and disease progression to distant organs.

They now have taken this work into models. In a well-validated model of pancreatic cancer derived from pancreas specific mutations in *KRAS* and *P53* (the KPC mouse), Hussain's laboratory has found that further genetic modification to delete MIF significantly increases the survival of these mice by several months. Instead of a genetic deletion, the next step is to use small molecule inhibitors and monoclonal antibodies to target MIF in these mice. "If this ongoing preclinical study shows us that it regresses the tumor and enhances the survival of these mice, we will have very strong evidence to pursue a clinical trial," said Hussain.

A similar pattern is emerging in Hussain's studies of NO. Nitric oxide synthase 2 (NOS2) is expressed under conditions associated with inflammation, including cancer. Once expressed, it produces high levels of NO for prolonged periods. Hussain has found that increased expression of NOS2 in patient samples is also associated with poorer prognosis. Moreover, a genetic deletion of NOS2 increases survival in the KPC mouse model. His laboratory is currently working to define the cellular pathways that account for these observations.

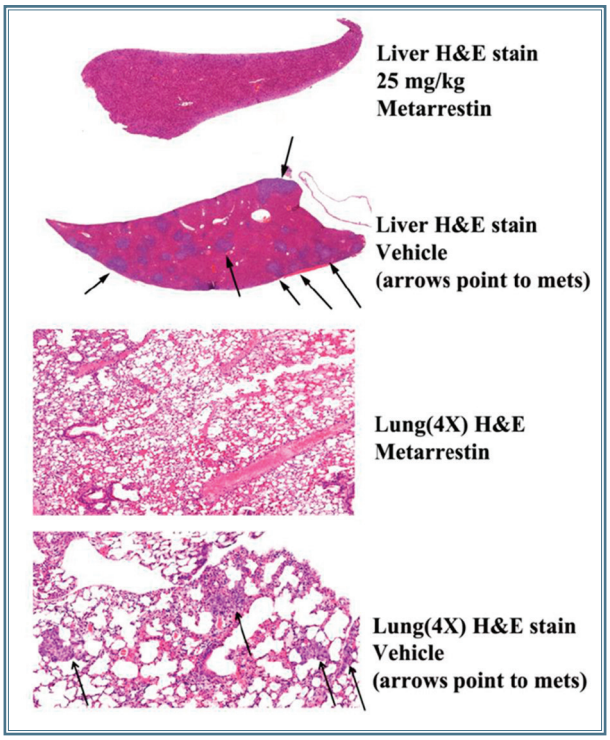
"In the KPC model, mice start dying at about three to four months of age with fully metastasized tumors. But when we deleted either MIF or NOS2, median survival significantly improved," said Hussain. "That's a pretty good start."

Increasing the Odds

"The big theme in my lab is drug development in pancreatic cancer," said Udo Rudloff, M.D., Ph.D., Investigator in CCR's Thoracic and Gastrointestinal Oncology Branch. Like Hussain, Rudloff joined CCR in 2009, after training in surgical oncology at Memorial Sloan Kettering Cancer Center. "Surgery is still associated with the best outcomes for early stages of the disease, but in nearly all patients, the cancer comes back eventually. Once metastases set in, the answer is not surgery any more. The answer to the deadliest cancers is to develop new and better drugs."

Because they grow in a very hypoxic environment, with poor vascularization, pancreatic tumors contain an unusually high number of tumor initiating (stem) cells. These cells are able to survive without a lot of oxygen and in the presence of reactive oxygen species. Rudloff and his colleagues use cellular biomarkers

> "These cancer stem cells appear to drive tumorigenesis, progression, and metastasis."



(Figure: U. Rudloff, CCR)

Metarrestin effectively suppresses pancreatic cancer progression in the liver and lungs. H&E staining of untreated and treated representative liver (top) and lung (bottom) sections.

of these stem cells that correlate well with tumor progression.

"We have isolated pancreatic stem cells from patient tissue samples and put them into immunocompromised mice; they form tumors about one hundred times more frequently than nonstem cells," said Rudloff. "These cancer stem cells appear to drive tumorigenesis, progression, and metastasis."

Often cancer cell lines, growing in monolayers at the bottom of a petri dish *in vitro*, are a first port of call for drug screening. Rudloff's team has been growing pancreatic cancer cells under conditions that promote the proliferation of cancer stem cells. The cells form spherical structures, creating their own internal microenvironments, which are more reflective of cancers *in vivo*.

"No one has used these spheres for drug screening. But our collaborators at the NIH's National Center for Advancing Translational Sciences (NCATS) optimized growth conditions for high-throughput screening and found a compound with really strong activity in the spheres containing cancer stem cell fractions," said Rudloff.

The compound, termed 10N, is a multikinase inhibitor, previously known for inhibiting the IL-2 T-cell kinase. In fact, Rudloff has conducted extensive proteomics analysis to show that the compound inhibits at least 16 kinases, four of which are important for cancer stem cell function. His team has shown that the rate of apoptosis more than doubles when you knock out two of the compound's kinase targets, suggesting the drug could create a disproportionately strong downstream effect.

"The cool thing is that this compound has intrinsic synergy; its targets cooperate, so disrupting them has an additive effect," said Rudloff. "Cancer stem cells are hugely resistant to chemotherapy, which makes an inhibitor specific for the stem cell fraction very exciting."

"Developing this drug would be a great opportunity," said Rudloff. "It would be the first pancreatic stem cell inhibitor, with a completely novel target profile."

In a parallel effort, Rudloff is working with scientists at NCATS who conducted a novel small molecule screen to identify inhibitors of metastasis. They took advantage of a poorly understood but prominent cell biological feature of metastatic cancer cells: the perinucleolar compartment (PNC). The PNC is found at the edge of the nucleolus, where it is enriched with RNA and RNA-binding proteins. It is especially prevalent in metastatic tumors, and, when found in tumor biopsies, indicative of a poor prognosis. A small molecule was discovered—metarrestin—which dramatically reduced the prevalence of this marker in metastatic cancer cell lines.

Rudloff tested metarrestin in an animal model of metastasis, which Research Fellow Yaroslav Teper, Ph.D., developed in his laboratory. Fluorescently tagged pancreatic cancer stem cell lines are injected into the pancreas of mice; the bioluminescence can be tracked not only to the pancreas, but to metastases of the liver and lungs. Metarrestin had little impact on the primary pancreatic tumor, but dramatically decreased the metastatic burden.

> "Cancer stem cells are hugely resistant to chemotherapy, which makes an inhibitor specific for the stem cell fraction very exciting."

(Photo: R. Baer)

Tim Greten, M.D., in the clinic

Encouragingly, the toxicology profile of metarrestin in rodents indicates that it is very safe, and Rudloff is currently in discussions with the Food and Drug Administration (FDA) about further testing before a first-in-human clinical trial could be approved.

"The PNC compartment disassembles after administration of the drug, but we don't know what the molecular target is," said Rudloff. "We only know that it is very well correlated with metastatic progression. It's a really good biomarker, which has been under development for several years. That's why we are so excited about it."

Requesting Immunity

Meanwhile, Tim Greten, M.D., Investigator, and Austin Duffy, M.D., Staff Clinician in CCR's Thoracic and Gastrointestinal Oncology Branch, are taking a more immediate approach to potential clinical impact, by taking existing anticancer tools into pancreatic cancer treatment.

"Tim and I built up the GI cancer program from scratch since around 2009," said Duffy, "It takes a while to set these things up, but we're now at a stage to capitalize on the investment we've made."

"There's such a huge unmet need to treat pancreatic cancer, and chemotherapy is only minimally effective," said Greten. "Immunotherapy might help, but the drugs used so far haven't shown the kind of efficacy seen with, for example, melanoma or lung cancer."

The examples Greten highlighted come from the use of so-called immune checkpoint inhibitors, inhibitors of CTLA-4 and PD-L1 signaling, which normally operate as brakes on the immune response. Lifting those brakes has resulted in remarkable, durable responses in certain intractable cancers, but has thus far not had much impact on gastrointestinal solid tumors, including pancreatic cancer, despite the fact that immune cells are found in abundance in pancreatic tumors.

Greten's laboratory has been studying tumor cell death and the effects on the immune response in preclinical models over the last decade. "Depending on how tumors die, you can have dramatic differences in the resulting antitumor activity."

Based on this line of investigation, he believes that a combination of immune checkpoint inhibition with radiation therapy might deliver a strong one-two punch to pancreatic cancer. The idea would be that initial destruction of tumor cells would cause the tissue to release antigens, which would elicit a T-cell response. Checkpoint inhibitors would then strengthen that response.

"There's strong evidence that radiation can actually stimulate

> "There's strong evidence that radiation can actually stimulate an immune response, and we are hoping to amplify that."

> "Scientifically there is a lot of opportunity here at CCR for translational research."

an immune response, and we are hoping to amplify that," said Duffy.

"Two years ago, we enrolled a similar cohort of patients with pancreatic cancer as part of a small multicenter study of two different vaccines; that study suggested that immune approaches in pancreatic cancer can be of benefit," said Duffy. "It showed me that if you use stringent inclusion criteria, you may be able to get a signal and learn from these patients."

Greten and Duffy have recently opened a study, in which they are combining the use of tremelimumab (an antibody inhibitor of CTLA-4) or MEDI4736 (an antibody inhibitor of PD-L1) with radiation treatment to the pancreas. The plan is to enroll 60 patients, who have previously had some form of standard treatment and either progressed or did not tolerate the chemotherapy.

In designing the exact treatment schedule, Greten and Duffy have worked with Deborah Citrin, M.D., Senior Investigator in CCR's Radiation Oncology Branch and her colleagues in the Branch, along with Jennifer Jones, M.D., Ph.D., Assistant Clinical Investigator in CCR's Vaccine Branch, but there is very little systematic, comprehensive evidence to guide them to the optimal dosing. "We use whatever data is out there, but it's very difficult to extrapolate from animal data to humans," said Duffy.

By taking needle biopsies before and after the treatment, Greten and Duffy will be able to study the tumor immune response. Some patients will receive one dose of focused radiation, others will receive five; and computed tomography (CT) scans will be used to monitor the size of the tumor.

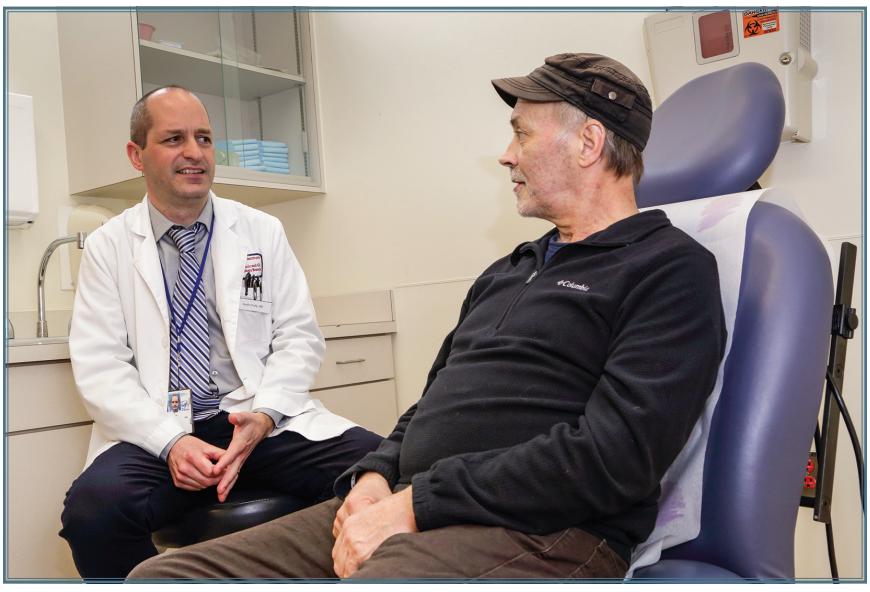
Looking Forward

"Eight to ten years ago, very few people were working on PDAC. It wasn't a priority and it was an understudied cancer. That has changed," said Hussain. "Every year, we hold a symposium on pancreatic cancer in September, bringing together experts from the U.S. and around the world. Our goal is to exchange ideas between the extramural and intramural communities working on pancreatic cancer and foster collaborations. We've been doing this for the last three years and participation is growing."

"Scientifically there is a lot of opportunity here at CCR for translational research. The proximity between lab and clinic doesn't exist to the same extent in other places," said Duffy. "Our pancreatic cancer study would be challenging to do in the community hospital setting, because of the nonstandard application of the radiation treatment. There's more freedom to break new ground."

Everyone acknowledges that there is still a lot to learn about pancreatic cancer, from its molecular subtypes and evolution, to the clinical implications of its physically constrained, hypoxic, stromal compartment. "We need completely new approaches," said Rudloff. "The vast majority are going to fail, but we need to take the risks if we're going to succeed."

(Photo: B. Branson)



Austin Duffy, M.D., in the clinic

To learn more about Dr. Duffy's clinical research, please visit his CCR website at https://ccr.cancer.gov/austin-g-duffy.

To learn more about Dr. Greten's research, please visit his CCR website at https://ccr.cancer.gov/tim-f-greten.

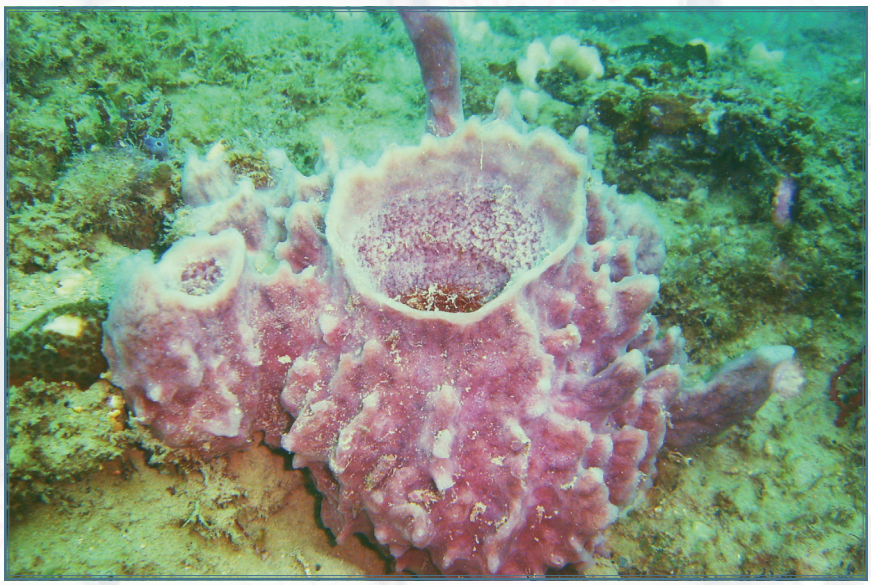
To learn more about Dr. Hussain's research, please visit his CCR website at https://ccr.cancer.gov/s-perwez-hussain.

To learn more about Dr. Rudloff's research, please visit his CCR website at https://ccr.cancer.gov/udo-rudloff.

All Natural Chemicals

The use of medicinals derived from nature is likely as old as humanity itself. Pollen evidence from caves in Kurdistan, Iraq suggests that Neanderthals may have taken advantage of the medicinal properties of plants. A Mesopotamian medical text from 2600 BC, written in cuneiform on clay tablets, lists a thousand medicinal plants. Even modern Western medicine owes a large fraction of pharmaceuticals, from aspirin to ziconotide, to natural products and their derivatives. The Molecular Targets Laboratory (MTL) works extensively with CCR investigators to allow them full advantage of nature's bounty in their fight against cancers and infectious disease. Now, CCR wants to broaden its reach.

On the submerged roots of mangroves in the warm shallow Caribbean island waters, live colonies of a tiny sea squirt, *Ecteinascidia turbinate*. In spring and summer, the colonies sexually reproduce, sending tadpoles out into the water column, but otherwise these are sedentary creatures at the mercy of their environment. Living in symbiosis with the sea squirt is a prokaryote, *Candidatus Endoecteinascidia frumentensis*, which produces a complex eight-ringed small molecule, trabectidin, for reasons not yet revealed to marine biologists. But, to oncologists, trabectidin, whose activity was first reported in a broad NCI anticancer screen of natural products, is known as Yondelis and is used for the treatment of soft tissue sarcomas and ovarian cancer.



(Photo: Natural Products Branch, DTP, DCTD, NCI)

The Natural Products Repository on the NCI campus in Frederick, Md., houses about 200,000 extracts from terrestrial and marine organisms from around the world, such as the barrel sponge *Xestospongia testudinaria*.

"When marine animals settle out of the water column onto a substrate, they're pretty much stuck there, just like plant seeds, and must fight, compete, and win in that environment," said Kirk Gustafson, Ph.D., Senior Scientist in CCR's MTL. "Thus they develop a lot of chemical strategies to deter predators, to prevent other larvae from dropping out and trying to settle on and overgrow them, to keep unwanted bacteria and fungi

from colonizing them. A compound with growth inhibitor properties in that setting just might interact with a biological target in a similar manner to, say, affect cancer cell growth."

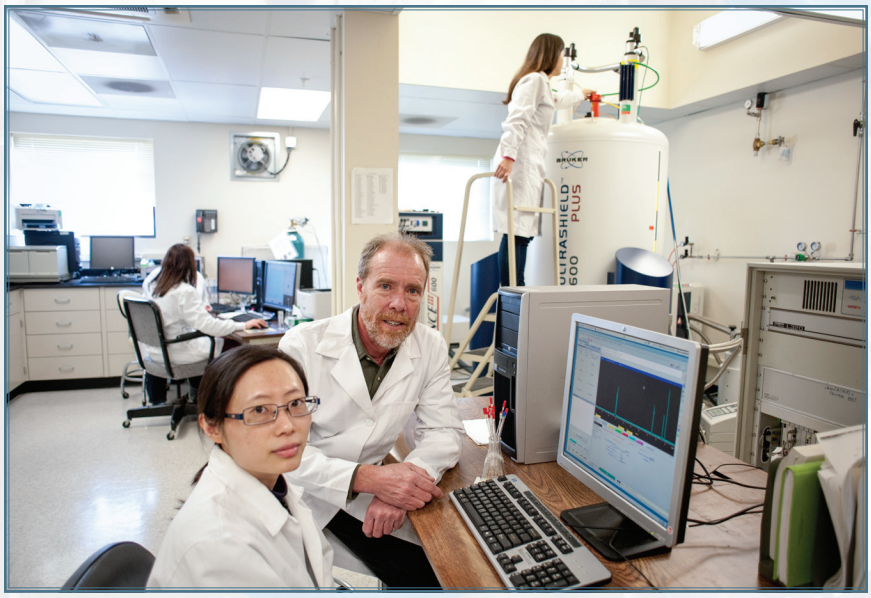
On that premise, beginning in the 1950s, NCI created a worldwide collection program, focusing on terrestrial plants and marine organisms, and developed a repository, which currently houses some 200,000 natural product extracts. This repository is the largest, most diverse, public repository of natural products in the world (See "The Natural Products Repository: A National Drug Development Resource," *CCR connections* Vol. 2, No. 2). The diversity of chemical structures springs from the diversity of organisms sampled. Among the other drugs discovered through this resource are paclitaxel, a compound found in the bark of the Pacific Yew, and noted on the World Health Organization's List of Essential Medicines for the treatment of solid tumor cancers.

"Natural products provide a pool of structural diversity that is just unparalleled with anything that humans can think up and make. Synthetic chemists are very talented, but they don't have the imagination and capabilities that Nature does," said Gustafson. "Depending on whom you talk to, anywhere from 30 to 60 percent of drugs are either natural products or derived from Nature."

Extracting and working with such a diversity of compounds requires unique expertise. Within CCR, MTL has decades of experience dedicated to turning these extracts into drugs to treat cancer and infectious disease.

Mining Small Molecules

"We're in the mining business. Our main goal is to move the research of CCR scientists forward," said James McMahon, Ph.D., MTL's Chief. "We have built up a huge database



(Photo: R. Baer)

Kirk Gustafson, Ph.D., and his team use nuclear magnetic resonance (NMR) spectroscopy to analyze compounds isolated from natural products.

and knowledge of the extracts that enables us to run high-throughput assays. The other thing we've done over the years is to build up a library of pure compounds, with a lot of interesting chemistry."

Natural products do not start out life as beautiful as the organisms that produce them. Materials are collected in the field, identified, dried, and sent to the Natural Products Branch of the Developmental Therapeutics Program (DTP) in Frederick, Md., part of NCI's Division of Cancer Treatment and Diagnosis (DCTD), where they are ground and extracted with aqueous and organic solvents. "When we get one of these extracts, we've taken everything that is soluble from an organism. These are complex mixtures; if you make an organic extract of a plant, it looks like a bottle of black tar," said Gustafson. "It's not a pretty starting material."

Gustafson is involved in the chemistry component of assay development and screening within MTL, focusing primarily on small molecules. In a typical collaboration, a CCR investigator has a target of interest and is looking for molecules that will interact in specific ways with that target. For example, Thomas Sayers, Ph.D., Senior Investigator in CCR's Laboratory of Experimental Immunology in the Cancer and Inflammation Program, is studying the mechanisms by which the immune system destroys cancer cells. His team has found that cancer cells protect themselves from apoptosis mediated by TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) with a family of anti-apoptotic proteins, known as cFLIP.

Working with assays from the Sayers laboratory, Gustafson and his colleagues were able to isolate a steroidal lactone, withanolide E, from the Cape gooseberry (*Physalis peruviana*), that enhanced apoptosis in a number of human cancer cell lines through reduction in the cFLIP proteins. A paper describing the activity of withanolide E has been published in *Cell Death and Disease.*

"Everything we do is a collaboration," said Gustafson. "My work is irrelevant without the screening results that guide me to a particular extract and through chemical separation of an extract. The biological activity is our eyes and in a

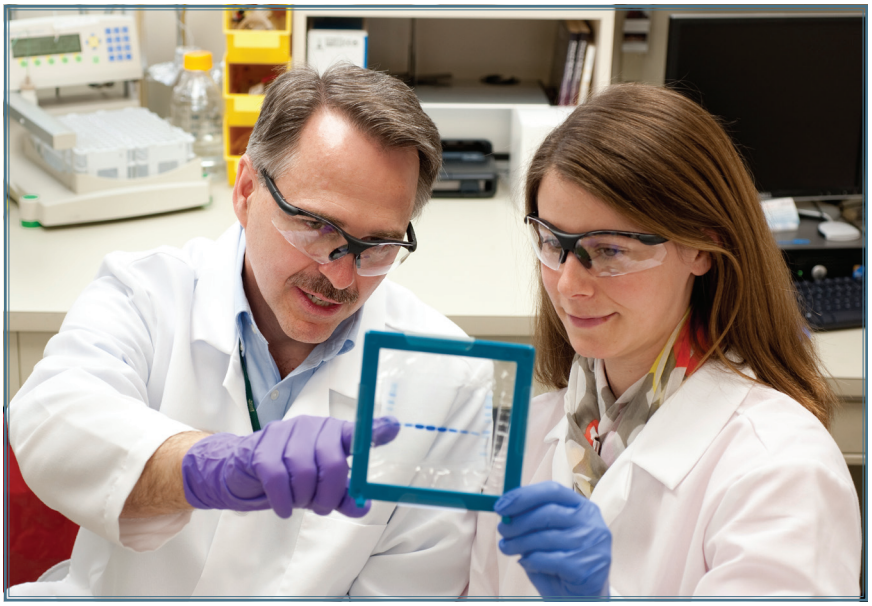
perfect world, it neatly tracks to one or a few compounds we can isolate as pure white or clear or yellow compounds. From the standpoint of the natural products chemist, the fun begins in figuring out what the entity is that we have isolated."

Gustafson and his colleagues use spectroscopic techniques: nuclear magnetic resonance (NMR) and mass spectrometry (MS). For noncrystalline compounds, NMR is the most powerful structure elucidation technique available. MS is used to identify the atomic components and define the molecular formula.

"In a really perfect world, we end up solving a new type of molecular structure and assigning it a name."

Recently, a collaboration between MTL and William Figg, Sr., Pharm.D., Deputy Chief of CCR's Genitourinary Malignancies Branch, has led to an entirely new class of alkaloids, derived from the marine sea squirt, *Eudistoma*, which they have termed eudistidines. One of these compounds is able to interfere with a protein/protein interaction critical to the ability of cancer cells to survive in the oxygen-poor milieu of tumors. Moreover, Martin Schnermann, Ph.D., Investigator in CCR's Chemical Biology Laboratory (CBL), was able to work out an efficient five-step synthesis for the new class, meaning that its availability is no longer limited by the natural product. This work recently appeared in the *Journal of the American Chemical Society*.

"Small molecules that block protein/protein interactions are quite rare and these targets are usually considered undruggable. When you screen synthetic compound libraries, you often get no hits. When you screen natural product libraries, it seems that the chance of success is higher," said Gustafson. "The difference is that these small molecules are not randomly derived molecular structures, they are the result of chemical evolution. Organisms have evolved structures that interact with biopolymers—proteins, nucleic acids, and the like—in a manner that produces a biologic effect."



(Photo: R. Baer)

Barry O'Keefe, Ph.D., and Lauren Krumpe, M.S., in the lab

Prospecting for Proteins

"Kirk isolates small molecules that interact with cancer and HIV," said Barry O'Keefe, Ph.D., Deputy Chief of MTL and Chief of DCTD's Natural Products Branch. "My group concentrates on proteins and peptides."

O'Keefe's laboratory has two main parts. One part develops cell-free systems to study the interactions of natural products with biological molecules. These can be used both to screen for compounds that affect the function of proteins or nucleic acids as well as to define the thermodynamic and kinetic interactions of extracted compounds with their targets. Recently, a collaboration with John Schneekloth, Ph.D., a CBL Investigator, led to the identification of small molecules that selectively interact with a particular RNA structure—the HIV transactivation response (TAR) RNA hairpin. One class of compounds was also able to inhibit HIV-induced death of T cells *in vivo*.

O'Keefe is also known for his work in isolating bioactive proteins from natural products. Griffithsin, a lectin isolated from the eponymous red algae *Griffithsin sp*. found off the coast of New Zealand, is a potent HIV antiviral (See "By Land or by Sea: High-Yield Production of a Marine Anti-HIV Protein in Plants," *CCR connections* Vol. 3, No. 1). Two large-scale clinical trials have now been funded to evaluate its use as a topical microbicide against HIV. The Population Council's Center for Biomedical Research is leading one trial, funded by the U.S. Agency for International Development (USAID), for vaginal administration; the University of Louisville and Intracept Biomedicine are conducting an National Institute of Allergy and Infectious Disease (NIAID)-sponsored trial for rectal administration.

Having isolated the protein, the path to synthesis is quite distinct from small molecules. "Once griffithsin was isolated and we had the amino acid sequence, we could engineer its production in *Escherichia coli*," said O'Keefe. "Then we began evaluating its mechanisms of action and

extrapolated its use to other viruses." Thus, griffithsin has shown activity against SARS, hepatitis C, Japanese encephalitis, and herpes simplex viruses. O'Keefe's team has recently shown that another lectin isolated from cyanobacteria, scytovirin, is active *in vivo* against the Ebola virus.

"When we find a protein, we play with it. We modify it and see if we can improve on its native characteristics. For example, some modifications might improve its stability, immunogenicity, bioavailability, and shelf life," said O'Keefe.

Foreign proteins have faced significant hurdles in drug development because they are automatically assumed to be immunogenic. However, there are many biotherapeutics now that are not native. The drug Exenatide, used for glucose control, was first isolated from the saliva of the Gila monster, a large venomous lizard found in Mexico and the southwestern U.S. Ziconotide, is an intrathecal pain medication, first isolated from cone snails found on tropical coral reefs. And of course the wrinkle-smoothing Botox is from the bacterium *Clostridium botulinum*.

"The hurdles for the therapeutic use of foreign proteins are much less than they were even 10 years ago," said O'Keefe.

Sharing the Bounty

"It's estimated that only one percent of existing natural products have been discovered," said Joel Schneider, Ph.D., Chief of CCR's CBL. "So, if we've got drugs that are affecting millions of people' lives by only looking at the one percent, imagine what we have yet to discover."

Screening of the Natural Products Repository has largely been limited to cancer and infectious disease. CCR is working to make this library more accessible, especially to those other disease areas.

The first step will be to make the extracts more user-friendly. Many screening centers are hesitant to use natural products because they aren't compatible with existing instrumentation. Based on studies developed by Gustafson, the process of prefractionating the crude extracts may be the answer. The goal is to create a library of pre-fractionated extracts. "The plan is a one-million fraction library, a subset of current extracts separated based on polarity in an automated fashion. This will concentrate low percentage compounds to be more readily seen in assays and sequester nuisance compounds that routinely confound assays," said O'Keefe.

The second step will be to aid external screening centers in their ability to identify the active compound. "Even when you narrow down the fraction to basically one peak on a chromatogram that is active, you still don't know what the molecule is. We can help with that," said Schneider.

A Precious Resource

"Back when Nixon declared war on cancer, there was a huge national effort in mining the natural product biome for active compounds," said Schneider. "We've seen that effort wane over the last 20 years because of the promise of newer therapies. But, to this day, small molecules derived from natural products still represent the majority of cancer treatments. With that realization, we owe it to the patients and to the science to revitalize natural product discovery at CCR."

New efforts are under way to curate a more diverse library and recognize new organisms that might provide even better molecules. "We want to build in a planned way to fill niches that we don't currently have," said O'Keefe.

To that end, McMahon and his colleague, John Beutler, Ph.D., a senior natural products chemist who serves as the MTL "librarian", have been making trips to Kazakhstan to collect new plants that have never been tested. "Kazakhstan is a huge country; there are probably a thousand indigenous plants with ethnobotanical references from the native peoples that have never been tested," said McMahon. The collaboration includes a mirror repository in Kazakhstan. "We'd like to do this with any country that's willing," urges McMahon.

"The world's diversity is going away," said McMahon. "And to me, as a scientist, that is criminal. We can get about 85 percent of our repository resupplied. The other 15 percent are just gone. The bottom line is you can only find what you have in your library. Natural products are Nature's library."

> "…small molecules derived from natural products still represent the majority of cancer treatments."

To learn more about collaborative Natural Product Research in the MTL, please visit their website at https://ccr.cancer.gov/Molecular-Targets-Laboratory.

To learn more about Dr. McMahon's research, please visit his CCR website at https://ccr.cancer.gov/james-b-mcmahon.

To learn more about Dr. Gustafson's research, please visit his CCR website at https://ccr.cancer.gov/kirk-r-gustafson.

To learn more about Dr. O'Keefe's research, please visit his CCR website at https://ccr.cancer.gov/barry-r-okeefe.

To learn more about Dr. Beutler's research, please visit his CCR website at https://ccr.cancer.gov/john-a-beutler.

Bacterial Regulation: Past, Present, and Future

Many scientists are lucky to have one fundamental insight to their name. As recently noted by the National Academy of Sciences when they awarded her the 2015 Selman A. Waksman Award in Microbiology, Susan Gottesman, Ph.D., Co-Chief of CCR's Laboratory of Molecular Biology, has made at least two seminal discoveries about post-transcriptional regulation in bacterial cells: 1) she uncovered the critical role of protein breakdown and the energy-dependent proteases that govern this process, and 2) she co-discovered small RNAs and established their role in bacterial cell regulation. Each of these discoveries has presaged their more complex counterparts in eukaryotic cells. Gottesman continues to study molecular regulation in bacteria both for the general principles to be learned and for their particular applications to host infection and immunity.

As often happens in science, Susan Gottesman took the first step toward one of her most significant discoveries through serendipity... and the persistence of a talented team.

By the early 1990s, the project to study protein turnover in *Escherichia coli (E. coli)*, which she began as a newly hired investigator at the NIH in 1976, had grown into a pioneering role for her laboratory in the field of regulation by protein degradation. One of the unstable proteins her laboratory was studying—RcsA—regulates capsule formation. Thus, it was not terribly surprising that a plasmid containing the *rcsA* gene caused overproduction of the capsule and a "mucoid" phenotype. What was surprising was that disruption of the protein-coding region of the gene did not affect the phenotype.

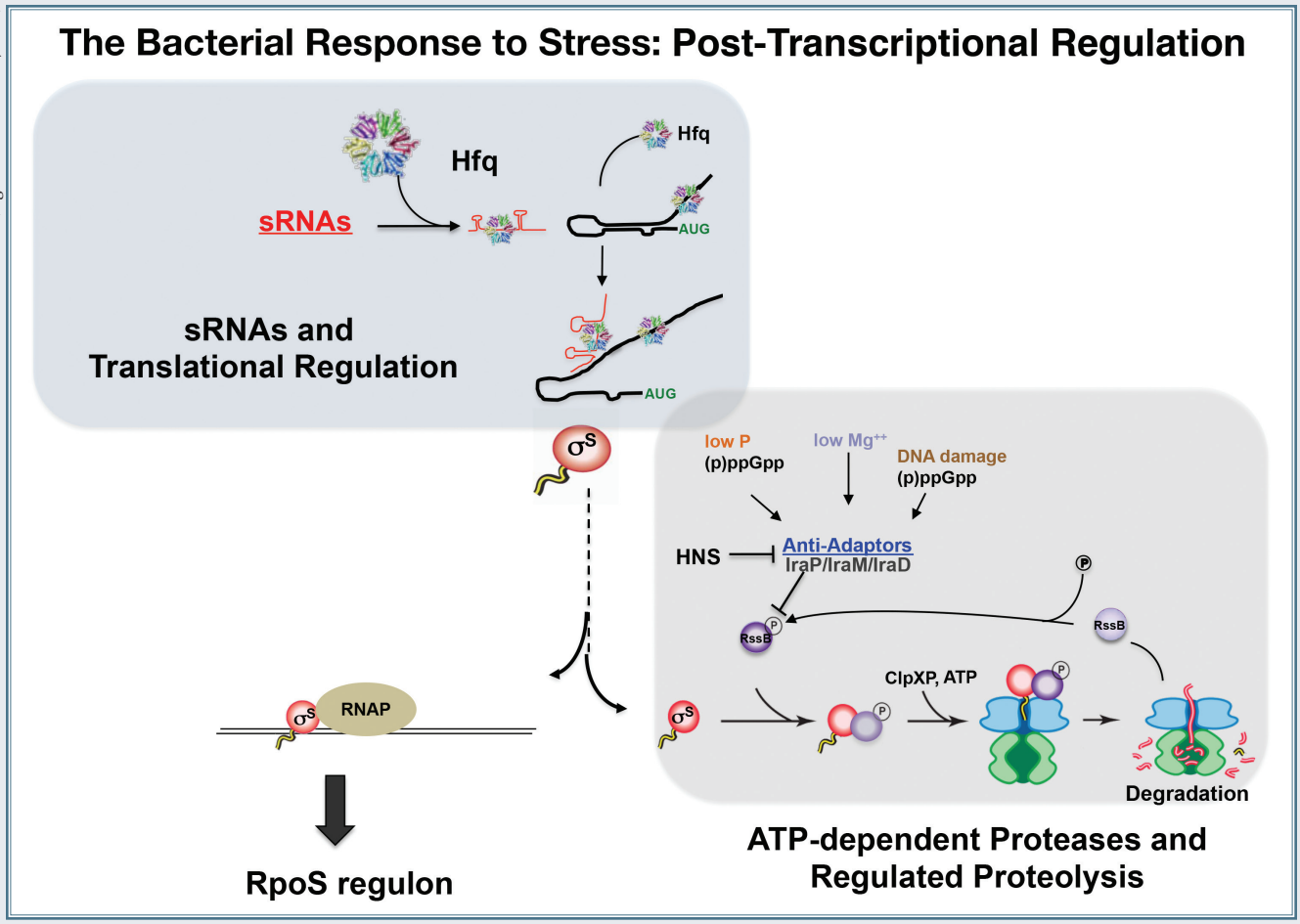
"That observation sat for a number of years. It was clear we didn't understand what was going on," said Gottesman. "Then, Darren Sledjeski, Ph.D., came to the laboratory and took another hard look. He realized that the phenotype was not due to the protein, or even the *cis*-acting regulatory elements, but was due to another gene on the plasmid. It encoded a small regulatory RNA that wasn't translated into protein." They named the element *dsrA* for "downstream of *rcsA*."

Nadim Majdalani, Ph.D., joined the group as a postdoctoral fellow in 1995. "When I came to the laboratory, there was no such thing as a small RNA," said Majdalani. He started working on the DsrA project to understand how it was creating the phenotype. Sledjeski and Majdalani's work led to the discovery that it was both inhibiting the translation of a protein, H-NS, and positively regulating the expression of a transcription factor, RpoS.

(Photo: R. Baer)

Susan Gottesman, Ph.D.

(Figure: S. Gottesman, CCR)



"Here was one small RNA that could, on the one hand, repress and, on the other hand, enhance gene activation," said Majdalani. He went on to demonstrate that DsrA was pairing with the mRNA encoding RpoS in such a way that it caused the release of an endogenous inhibitory loop within the mRNA and the resumption of translation.

The team published their findings on DsrA, a clear challenge to the existing dogma in molecular biology that RNA functioned as an intermediary between DNA and protein. But it was not clear how widespread this mechanism might be.

Large-scale, automated genomic screens were not available at the time, so Majdalani and Gottesman screened a library of genomic DNA for new small RNAs by creating summer projects for high school and college students in the laboratory. Eventually, the work of multiple students led to the discovery of a second small RNA, RprA, which also regulated RpoS, but in response to different cellular stressors.

"The next big step in the evolution of the lab began with our collaboration with Gigi Storz," said Gottesman.

Building a New Paradigm

Gisella Storz, Ph.D., Senior Investigator in the National Institute of Child Health and Human Development's Cell Biology and Metabolism Program, had come across her own small RNA, OxyS in 1985, the chance result of a flawed experiment to study the regulation of a transcription factor by oxidative stress. In 1997, she and her postdoctoral fellow, Shoshy Altuvia, Ph.D., published the mechanism of its action, namely base pairing with mRNAs.

"We were both giving talks about our small RNAs in parallel and being asked how many there are, and how general a mechanism this might be," said Gottesman. "It was relatively early in the bacterial genome sequencing era, but there was enough data to look for conserved regions that weren't encoding proteins and ask if these could be small RNAs."

By 2002, the collaboration spawned a surfeit of riches, approximately 30 small RNAs whose place in bacterial regulatory networks needed to be understood. As new members joined the lab, they could explore new regulatory territory.

A parallel story in eukaryotic

(Photo: R. Baer)

Kumari Kavita, Ph.D., Daniel Schu, Ph.D., and Nadim Majdalani, Ph.D.

cells was beginning to emerge around the same time. In the late 1990s, gene silencing by small RNA molecules—a process termed RNA interference (RNAi)—was reported, as was the extreme conservation of microRNA sequences.

Meanwhile, Aixia Zhang, Ph.D., a postdoctoral fellow in the Storz lab, discovered that at least one small RNA relied on a small protein chaperone, Hfq, to facilitate base pairing with its mRNA target. Hfq turns out to be an essential chaperone for many of the small RNAs in *E. coli*. Whereas we now know that eukaryotic cells rely on the multiprotein RNA-induced silencing complex (RISC), prokaryotes rely on the single ring-protein chaperone Hfq to mediate the regulatory interactions of small RNAs with mRNA.

"When I started working in *E. coli*, it was because that's where the genetics was; it was the model organism in which molecular biology developed and you could ask fairly sophisticated questions about mechanisms," said Gottesman. "Not by coincidence, the principles we discover often end up being applicable to other systems."

Dancing with the Chaperone

Daniel Schu, Ph.D., arrived as a postdoctoral fellow in 2009. He first met Gottesman when she came to give a lecture at Virginia Tech, where he was completing his Ph.D. Schu was working on a plant pathogen and had identified a novel small RNA involved in its virulence.

"I had two different motives to talk to her: 1) to pick her brain about what role this small RNA could be playing in our pathogen and 2) to find out what her lab was like and if there were any openings in the near future," said Schu. Six months later, he came to the NIH to join Gottesman's laboratory.

With a background in protein biochemistry, Schu became interested in the regulation of small RNAs by the protein Hfq. Gottesman suggested that he look at how this protein was facilitating pairing with mRNAs, which was the key step in regulation by small RNAs.

"Over the past decade, we and others have found an ever growing number of small RNAs and mRNAs that interact with Hfq," said Schu. "Because they are in constant competition, it didn't make sense that there would be only one pathway for binding and regulation."

"Over the past decade, we and others have found an ever growing number of small RNAs and mRNAs that interact with Hfq,"

> "For me, one of the great things about being at the NIH is the strong community of researchers working on bacteria."

Schu and Gottesman, in collaboration with Zhang and Storz, have instead identified different classes of small RNAs with different modes of interaction with the chaperone. Hfq forms a ring structure with three different faces with which RNAs can interact.

"There's a kind of gymnastics going on with the RNAs for interaction with Hfq," said Schu. "We've shown that one face always has small RNAs binding to it, but while one class of small RNAs will interact with the second face, another class interacts with the third. Small RNAs of the first class are usually transiently activated by stress; the other class seems more stable and appears to be involved in functions like metabolism."

"Depending on which class they belong to, we're finding that the small RNAs bind to this chaperone very differently and their fate is different. We think that has physiological consequences," said Gottesman. "We're hoping that understanding this will give us more insight, generally, into how cells sort small RNAs and choose which to use and maybe give us insight into how this family of chaperones is used in other organisms, certainly in other bacteria."

New Challenges

"For a while, we were the only reference for anyone doing small RNA work in bacteria. Labs were packing up their students and postdocs, and coming here to sit down with Susan and Gigi to learn how they could bring this work to their favorite organism. It was like riding the crest of a wave; it was very exhilarating," said Majdalani.

Now, Gottesman characterizes her laboratory as being in transition. "For the last few years, we've done a lot of tracking down of small RNAs and their targets. Interesting stories have emerged, but I don't think we'll be doing that for long."

Among the new explorations under way in the laboratory are attempts to work with different organisms. Clearly the molecular mechanisms of bacterial regulation they have discovered and characterized could be applied to many pathogens.

For example, in response to an outbreak of *Klebsiella* in the NIH Clinical Center three years ago, Gottesman got involved in a collaboration to look for solutions. Given the parallels between *Klebsiella* and *E. coli*, the concept is to use the tools of *E. coli* genetics to engineer a more easily manageable organism that would mimic the *Klebsiella* surface proteins to use in high-throughput screening.

In another collaboration with colleagues at the National Institute of Allergy and Infectious Diseases, Gottesman's laboratory is looking at the immune response to bacteria in mice. "We know that small RNAs regulate a lot of bacterial cell surface proteins, but we don't know why. Maybe it's to help protect the bacteria from host immunity," said Gottesman.

To test this, the antibody response of mice to bacteria with deletions of small RNAs is being compared to the response to wild-type bacteria. One problem is again engineering the mouse commensal strains to delete small RNAs. "These strains are giving us the same problems as *Klebsiella*; the normal *E. coli* engineering techniques are not working," said Majdalani.

"In *E. coli*, we can do almost anything," said Gottesman. "The difficulties start to arise when we get out of our comfort zone."

"It's never boring," said Majdalani, "In part, because of Susan's attitude towards research. She's always dynamic and never stops recruiting students."

"For me, one of the great things about being at the NIH is the strong community of researchers working on bacteria. I see that community every week at a seminar series called lambda lunch, which began before I came to the NIH as a postdoc," said Gottesman.

Anyone watching Gottesman at lambda lunch or at seminars put on by the NIH-wide RNA Biology Interest Group or the CCR RNA Initiative might notice her scribbling in the back of a little green notebook, which she carries with her to capture the "random thoughts" she has during such scientific exchanges.

"I believe it is Susan's established record of scientific achievement that attracts many talented people to her laboratory," said Schu. "But I think it is her mentoring that has really fueled her continued success. She has two or three students, from high school, college or beyond, coming through the lab every year, either for the summer or as postbacs for a year or two. She allows them to take on their own projects, build confidence in their techniques, and really get a taste for how science is done."

To learn more about Dr. Gottesman's research, please visit her CCR website at https://ccr.cancer.gov/susan-gottesman.

It Takes a Village

Professor Mark Smyth, Ph.D., Senior Scientist in the QIMR Berghofer Medical Research Institute in Brisbane, Australia, has a track record in the field of cancer immunology that is both impressive and wide-ranging. He has made fundamental discoveries in understanding how natural killer (NK) cells destroy tumors, his work has helped revitalize the concept of immune surveillance, and his preclinical studies of cancer immunotherapies have been translated into man. Undoubtedly modest, Smyth credits part of his success to an international network of close collaborators—friends, in fact—through which his science has been enriched and magnified.

(Photo: Courtesy of M. Smyth)

Mark Smyth, Ph.D.

On Becoming an Immunologist

My first exposure to immunology was through my Ph.D. with Ian McKenzie at the University of Melbourne; we were modifying antibodies—coupling drugs and toxins—and measuring their activity in mouse models of transplantation and cancer. As a next step, I wanted to learn molecular biology. I did the typical Australian thing and interviewed at laboratories across the U.S., and the U.K., but I chose NCI's Biological Response Modifiers Program in Frederick, Md., based on its collaborative feeling.

I started working with John Ortaldo, Ph.D., on a very tough project to isolate a cytotoxic factor secreted by NK cells. After six months, a couple of really good colleagues took me aside and said I ought to find a back-up project. That's how I began working with Howard Young, Ph.D. (now Deputy Chief of CCR's Laboratory of Experimental Immunology). Despite leaving John's lab formally, John remained a really good friend and mentor.

In Howard's lab, we studied the transcriptional control of pore-forming protein, a.k.a. perforin, a cytotoxic molecule in mammalian lymphocytes and a major pathway by which they kill target cells. We published our results demonstrating how interleukin-2 (IL-2) activates the killing machinery of these lymphocytes. And we discovered that TGFβ suppresses perforin activation, which has had lasting importance for tumor immunology.

I became skilled at Northern blots, which enabled me to initiate many projects with other people. Howard gave me a lot of independence, and his lab was very interactive. Up the hall, we had a new young investigator, John J. O'Shea, M.D. (now Scientific Director of the National Institute of Arthritis and Musculoskeletal and Skin Diseases), with whom I published on the pervanadate method to study phosphatase function. Through golf, I met a younger team leader, Kouji Matsushima, Ph.D. (now Professor at the University of Tokyo in Japan), with whom I collaborated on chemokine production in NK cells.

At the end of my time in Frederick, I had morphed into an immunologist.

On Building a Career

When I returned to Australia, I joined the Austin Research Institute and started working with Joe Trapani, Ph.D., on perforins. Eventually, I decided to go back to biology, which interested me more than the cellular mechanisms. I had become very interested in immune surveillance. Was the immune system continuously getting rid of cancerous cells? This idea had been around since Frank Macfarlane

> "And we discovered that TGFβ suppresses perforin activation, which has had lasting importance for tumor immunology."

Burnet, M.D., and Lewis Thomas, M.D., proposed it in the 1950s, but it's an impossible concept to demonstrate in humans precisely. Suggestive examples exist, such as patients who develop malignancies after receiving organs from a "cured" donor.

We reasoned that if perforins were important for the immune response to cancer, then mice lacking perforin must have a higher probability of developing tumors. So we engineered mice that were both p53 deficient (i.e., prone to cancer) and perforin knockouts. We saw that lymphomas developed earlier and were more prevalent in mice lacking perforin. We continued to use this approach with other molecular components of the immune system. We have accumulated over 100 genetically modified mice that now serve as a platform for investigating which host molecules are important in controlling tumor growth.

We also used an older model of cancer as a platform: the chemical carcinogen, 3-methylcholanthrene (MCA) injected into the flank, in various immune-deficient backgrounds. Some of the experiments would take a year or more to do, because the tumors were slow growing. But for studying immune surveillance, we felt it was a much more realistic model of cancer progression than, for example, tumor transplants.

On Cancer Immunoediting

In 2000, Lloyd Old, M.D., sometimes called the "Father of Modern Tumor Immunology" invited me to a meeting in New York. From that meeting sprung a fabulous collaboration with Robert Schreiber, Ph.D., at Washington University in St. Louis, Mo. Bob proposed the cancer immunoediting concept, now a cornerstone of thinking about how the immune system reacts with cancer. Because tumors are genetically unstable and the immune system is exerting selection pressures constantly, tumors eventually develop immunoresistant clones.

Immunoediting posits an equilibrium phase, during which the immune system and the tumor go into battle. We found we could describe a phase of tumor dormancy mediated by the immune system with the MCA model. When we injected low doses of carcinogen, the host formed a granuloma, a localized inflammatory response. These lesions eventually disappeared with time. But when we depleted the immune response, 60–70 percent developed fast growing sarcomas at the site of the original injection. We were able to track those tumors and show some cells within had malignant potential. We published that paper in *Nature* in 2007.

We continue to try and understand the equilibrium phase, what kind of sculpting is going on, what drives tumors to escape, whether you can bring them back to an equilibrium. If so, you might have a way of making cancer a chronic disease without necessarily curing it.

On Clinical Translation

As we've been able to understand escape mechanisms and pathways, we're increasingly interested in preclinical models for therapeutic development.

About 10 years ago, with our colleagues in Tokyo, we tested the concept that combinations of antibodies could increase therapeutic benefit. Now, it may seem obvious, but at the time, we were staggered by synergy we saw between antibodies that blocked the TRAIL receptor to stimulate apoptosis and antibodies that stimulated dendritic cell and T-cell activation. It was very satisfying to see the combination of ipilimumab (an antibody against CTLA-4) and nivolumab (an antibody against the PD-1 receptor) in a successful phase 1 trial for advanced melanoma published in the *New England Journal* two years ago.

We are currently pursuing another surface protein—CD96—which we discovered inhibits lymphocytes' ability to attack cancer cells. We were just awarded a grant to screen a series of antibodies against this target for use in a clinical trial.

It's an exciting time for immunotherapies. There's been a lot of background work that is going to come to fruition. It has been surprising people in the field how well anti-PD-1/PDL1 have worked. Approaches that have been tried and failed might have new value. We're just scratching the surface and T cells are just part of the story. There is a lot of opportunity to mobilize other cell types. Moreover, we need to recognize that patients will have their own unique antigens. We will need to stratify human tumors to match patients with the right treatment strategies.

These are really hard problems and the value of collaboration can't be underestimated. I am incredibly grateful for the postdoc period I spent in Frederick. It taught me to be a great collaborator, it really accelerated my career, and I've kept friendships with the people I met along the way.

Toward a Zero-Intensity Preparation (ZIP) Transplant

Transplanting the immune system from genetically matched donors to patients with hematologic cancers—allogenic stem cell transplants—are among the first and best examples of cures for cancer. However, the procedure is associated with potentially lethal toxicities, many of which are the direct or indirect result of the preparative regimen administered just prior to transplant. Dan Fowler, M.D., Senior Investigator in CCR's Experimental Transplantation and Immunology Branch (ETIB), has spent 25 years at NCI in a quest to convert the classical bludgeoning approach of ablating and replacing the entire immune system to a more nuanced approach that selectively transplants key mediators of the therapeutic antitumor effect: cytokine-polarized donor T cells.

Into the Minefield

Bone marrow transplantation was first evaluated in cancer treatment more than 50 years ago as a rescue strategy after high-dose chemotherapy combined with radiation therapy. Well into the 1980s, curing leukemia was thought to rely primarily upon the high-dose preparative regimens that ablated leukemic host stem cells; transplanting normal stem cells from bone marrow or peripheral blood cells was meant to reconstitute the hematopoietic system once the cancer was eradicated. The main causes of lethality with ablative transplants were due to the preparative regimen or to the transplant attack on normal tissues known as Graft versus Host Disease (GVHD, see "Treat the Cure," *CCR connections* Vol. 8, No. 2).

By 1990, experimental murine models and clinical data demonstrated that donor T cells were critical to the success of transplantation, as they mediated both detrimental effects (GVHD) and beneficial effects (prevention of graft rejection; mediation of graft-versus-tumor [GVT]). Because of this new understanding of allogeneic transplantation as an immune therapy rather than simply a rescue from high-dose preparation, the transplant field evolved to include alternative preparative regimens that were termed nonablative or reduced-intensity. The argument was: if the curative aspect of the transplant was due in part to T cells in the graft, why are we giving such toxic host preparation?

In 1990, I stepped into this minefield as a Medical Oncology Fellow and then worked in the lab of Ronald Gress, M.D., now Chief of ETIB and a CCR Deputy Director.

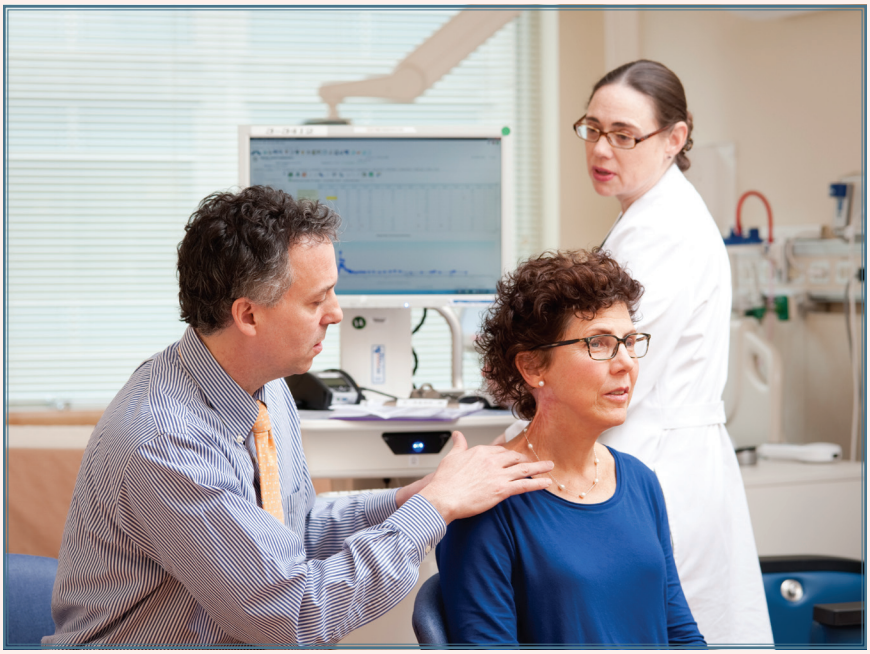
Laying the Groundwork

At this time, immunology researchers were just defining the concept of functional subsets of T cells with differential *in vivo* effects based on their pattern of cytokine secretion (initially, the TH1/TH2 subsets). My first project was to use mouse models to address the hypothesis that donor TH2 cells, which we manufactured by exposing T cells to the cytokine IL-4, would cause less GVHD. Indeed, IL-4-polarized donor T cells did not induce lethal GVHD in mice, and more importantly, they protected mice against GVHD mediated by donor TH1 cells (TH2/TH1 cross-regulation). We also studied mouse models of graft rejection and found

(Photo: R. Baer)

Dan Fowler, M.D., in the lab

(Photo: R. Baer)



Dan Fowler, M.D., patient Annette Abrams, and Daniele Avila, M.S., Nurse Practitioner

that IL-4-polarized cells prevented graft rejection by an "infectious tolerance" mechanism. That is, donor TH2 cells produced IL-4, which engaged the IL-4 receptor on host T cells; the host T cells became TH2-like and mediated less graft rejection. Meanwhile, we found that donor TH1 cells were critical for the GVT effect. So although donor TH2 cells could prevent GVHD and graft rejection, we realized early in our research that we did not want a total TH2 phenotype in the transplant; we needed some TH2/TH1 mixture.

Trials and Tribulations

We have been fortunate to translate this research at the NIH Clinical Center. My first clinical trial began in 1999, and we enrolled 49 patients. I submitted an investigational new drug application (IND) to the Food and Drug Administration (FDA) that allowed us to manufacture donor T cells *ex vivo* here at the NIH Clinical Center, Department of Transfusion Medicine, Cell Processing Section (David Stroncek, M.D., is the current Chief and a key collaborator). Before we performed the routine donor stem cell collection, we collected lymphocytes and purified the CD4+ T-cell population. We cultured these cells with IL-4 and administered them as a T-cell booster at the time of transplant. We hypothesized that the recipients of the IL-4 polarized, TH2-type cells would have less GVHD; however, relative to the control group, such recipients had similar rates of GVHD. In parallel with this initial clinical trial, our lab was evaluating new methods to optimize our approach. Usually, after a transplant, patients receive some type of immunosuppressive drug; at the time, we were using cyclosporine. I reasoned that cyclosporine might be neutralizing the effect of the infused donor TH2 cells and that it may be advantageous to use an alternative drug such as rapamycin. Indeed, manufactured T cells could not survive in media containing cyclosporine whereas some fraction of T cells always survived in rapamycin. This observation led to our now 10-year effort to characterize and harness a phenomenon known as T-cell rapamycin resistance (T-Rapa cells).

Rapamycin is an inhibitor of a protein kinase known as the Mechanistic Target of Rapamycin (mTOR). When mTOR is blocked, cells are starved and start to digest their internal organelles as a source of fuel in a survival maneuver; with their organelles downsized and cell volumes reduced, T-Rapa cells exist in a relatively quiescent state in order to survive starvation. We did not initially predict that T-Rapa cells would be powerful *in vivo* after their transplantation, but that is the somewhat paradoxical result that we obtained. In side-by-side comparisons of TH2 cells manufactured either with or without rapamycin, the T-Rapa cells were always much more powerful in preventing experimental murine GVHD and graft rejection.

So when we began a new clinical trial in 2004, in which we kept all transplant parameters the same except for the way we manufactured the TH2 cells. This time, we added rapamycin.

> "In side-by-side comparisons of TH2 cells manufactured either with or without rapamycin, the T-Rapa cells were always much more powerful in preventing experimental murine GVHD and graft rejection."

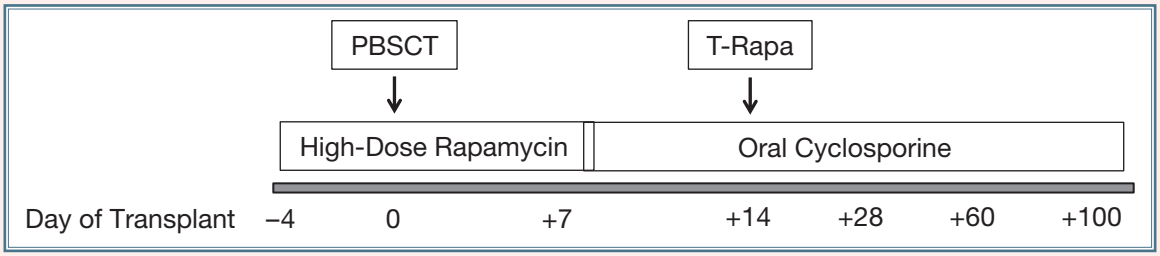
The Importance of Mixed Chimerism

On this new protocol, infusion of rapamycin-resistant T cells resulted in rapid full donor engraftment and toxic side effects due to a "cytokine storm." This observation indicated that the T-Rapa cells were indeed more powerful than the T-cell population we previously manufactured. However, the protocol needed to be amended to improve the safety of this novel cell therapy product. First, we reduced the fludarabine/cyclophosphamide preparative regimen intensity by 75 percent, which allowed the transplant to be performed on an outpatient basis. We also waited two weeks after the stem cell transplant before administering T-Rapa cells. These two changes to the transplant platform allowed for safe infusion of the T-Rapa cells. In 2013, we published the results of a multicenter, phase 2 study in 40 patients with refractory hematologic malignancies. We had no transplant-related mortality, a low rate of acute GVHD, and 18 of 40 patients remained in complete remission from their malignancy with a minimum follow-up time of 42 months (see "The Art of Living," *CCR connections* Vol. 9, No. 1).

One key element of this kind of transplant is that patients start out as a mixed chimera. At two weeks after the stem cell transplant, immune T cells and stem cell-derived myeloid cells are each typically at a 50-50 split in terms of donor and patient. The activated T-Rapa cells are thus administered in the mixed chimera state, with subsequent progression towards full donor engraftment. It is during this phase that we will observe clinical antitumor responses. We have also developed an iterative, risk-adjusted approach to allogeneic transplantation. That is, if a patient goes into complete remission, we will keep the patient as a mixed chimera, which offers a huge protection against GVHD (severe GVHD typically only occurs with full donor engraftment). On the other hand, if a complete remission is not attained, we intervene with additional infusions of donor T-cell products to achieve full donor engraftment to potentiate further GVT effects.

Most recently, we have tested the stringency of this mixed chimerism strategy by eliminating the chemotherapy preparative regimen. This represents the final step in our attempt to reduce the intensity of transplant from ablative, to reduced-intensity, to low-intensity, and now to a zero-intensity preparative regimen, or ZIP regimen. At this point, the therapeutic approach is highly focused on the donor T-cell immune therapy rather than any antitumor effect derived from chemotherapy; in the words of David Halverson, M.D., Staff Clinician in ETIB, and lead investigator on this protocol: "We have to trust the immunology."

We have now performed the zero-intensity prep transplant procedure on 12 patients (see Figure 1, treatment schema). Basically, we allow patients to recover from their prior chemotherapy and ensure that their immune system is somewhat depleted (CD4 count of less than 100). Patients are started on a high dose of rapamycin to prevent graft rejection and then they get the stem cell transplant without any further chemotherapy. Now, when we do our chimerism test at two weeks after transplant, we find a much lower contribution of the donor elements (usually only 10 percent donor T-cell engraftment, less than 1 percent donor stem cell engraftment). This exaggerated state of mixed chimerism is generally considered to be unsustainable as the host immune system can reject the outnumbered donor elements. However, infusion of donor T-Rapa cells causes a marked increase in donor elements within two weeks of cell transfer and associates with clinical antitumor effects (see Figure 2, case study). So with the zero-intensity prep transplant, we have developed a new transplant platform that should further improve the safety of the immunotherapeutic T-Rapa cells by reducing chemotherapy toxicity, reducing infection (absence of peritransplant neutropenia), and preventing GVHD (more patients may achieve remission while remaining in a mixed chimeric state).



(Figure: D. Fowler, CCR)

Figure 1. Patients with a hematologic cancer that do not respond to standard chemotherapy regimens proceed to a peripheral blood stem cell transplant. Host immunity is moderately compromised then rapamycin is administered four days prior to a peripheral blood stem cell transplant (PBSCT) with the donor being an HLA-matched sibling. Seven days after transplant, rapamycin is switched to oral cyclosporine therapy. Then at 14 days after transplant, patients receive *ex vivo* manufactured donor T-Rapa cells.

(Figure: D. Fowler, CCR)

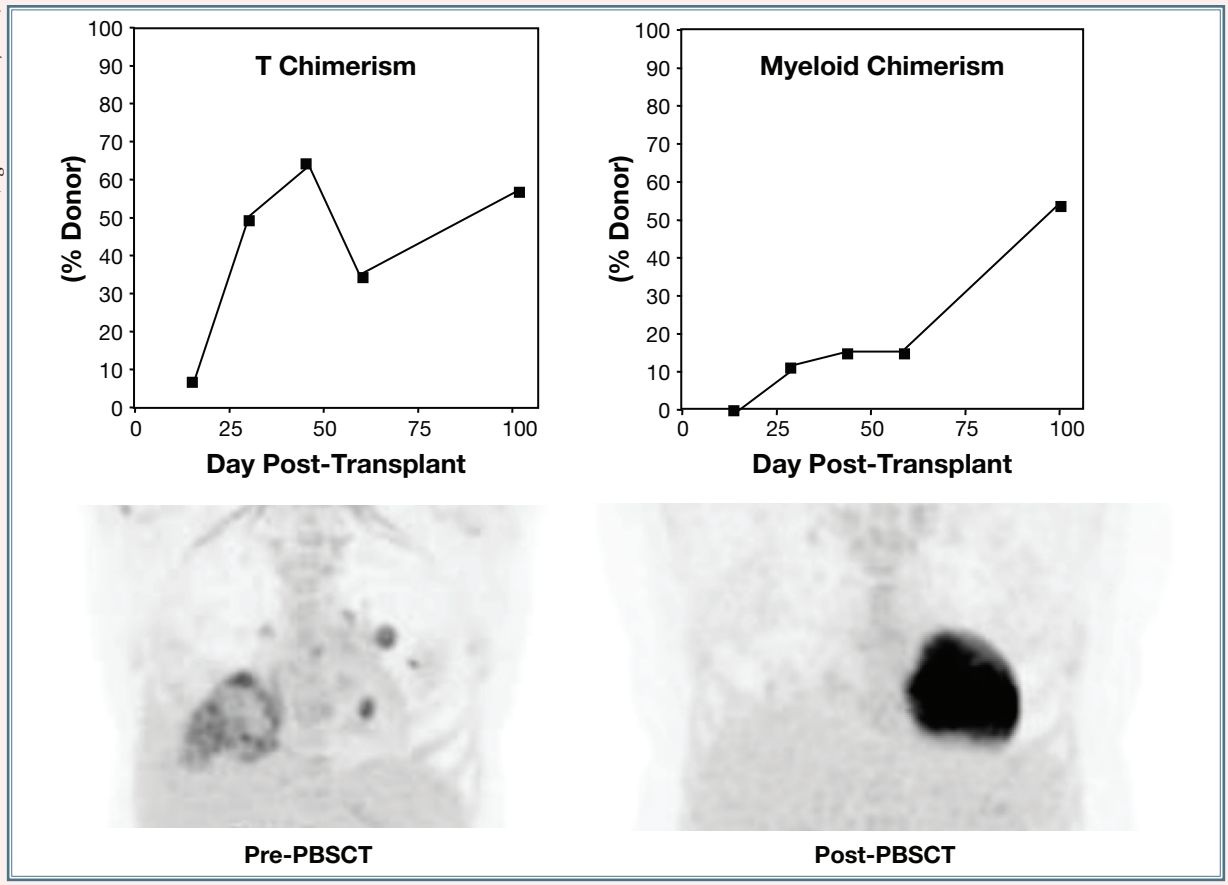


Figure 2. Zero-intensity preparation (ZIP) allogeneic peripheral blood stem cell transplant (PBSCT) yields exaggerated mixed chimerism and generates antitumor responses. Top panels show engraftment results from a representative patient transplanted without a preparative regimen. At day 14 post-transplant, both T cell and myeloid cell populations are overwhelmingly of host origin. However, donor elements increase rapidly after infusion of T-Rapa cells at day 14 post-transplant. Within the first 100 days post-transplant, during the state of mixed chimerism, the patient enters complete remission from diffuse large B-cell non-Hodgkin's lymphoma.

T-cell Immunotherapy and a Zero-Intensity Prep Future

At this point, most of our patients have advanced stages of hematologic malignancy that is refractory to conventional therapy and all have received transplants from HLA-matched siblings. Going forward, we envision that the zero-intensity prep transplant, by increasing the safety of the procedure, would allow transplantation to be used earlier in disease treatment algorithms, for patients who do not have an HLA-matched sibling, and for patients of advanced age or organ impairment.

We are also continuing our laboratory research to discover new approaches to further improve the safety and efficacy of transplantation through the modulation of T-cell function. I am very fortunate to have an outstanding laboratory team and clinical research team in ETIB. We have also collaborated with Scott Rowley, M.D., at the Hackensack University Medical Center in New Jersey to treat approximately 30 patients using our T-Rapa cells. This has served as a good proof-of-principle that we can manufacture T-Rapa cells at the NIH and then ship them out to other centers, thus expanding our work into the extramural community. As such, we are on our way to developing safer and more effective transplant approaches that can hopefully soon be implemented in standard practice.

> "we envision that the zero-intensity prep transplant, by increasing the safety of the procedure, would allow transplantation to be used earlier in disease treatment algorithms"

To learn more about Dr. Fowler's research, please visit his CCR website at https://ccr.cancer.gov/daniel-h-fowler.

The Art of Living

(Photo: R. Baer)

Annette Abrams stands by a mural she created while undergoing treatment. This art is displayed in the NIH Clinical Center.

In June 2007, Annette Abrams decided to make a change in her life. After teaching preschool for 11 years, she took a leave of absence to devote more time to the practice and teaching of her art. Two months later, she was diagnosed with a T-cell lymphoma. "I had what I thought was a swollen gland in my neck for at least six months before I got worried," said Abrams.

At Georgetown Medical Center, her tumor initially responded to treatment but then became resistant. Her biopsy was sent to the NIH where, the Head of the Lymphoma Therapeutics Section of CCR's Lymphoid Malignancies Branch, Wyndham Wilson, M.D., Ph.D., reviewed it and recommended a hematopoietic stem cell transplant.

Abrams and her husband met with Dan Fowler, M.D., who walked them through the protocol. "He was so low key, easy to talk to, easy to listen to, and full of information!" said Abrams. "He said we could do a transplant if there was a donor match. Fortunately, I have four siblings and two were matches."

Fowler and his team began treating Abrams at the NIH Clinical Center in mid-October 2007. Despite their best efforts, the first transplant, from Abrams' brother, performed at the end of November, was not successful due to rapid tumor progression. "Dr. Fowler was working on other protocols, so they kept treating me with localized radiation and chemotherapy until I could receive the next transplant from my sister, at the end of May 2008."

The second transplant (which used low-intensity preparation before the stem cell transplant and infusion of T-Rapa cells) proceeded without toxicity or GVHD, but there was also persistent lymphoma. Accordingly, the post-transplant course was altered by several infusions of additional donor cells either with or without additional chemotherapy. Then six weeks into the treatment, Abrams developed a high fever and swollen lymph nodes. She was admitted to the hospital, and after the fever broke, the tumor started to die. "My tumor was a big, ugly, bloody looking thing; I always wore a bandage over it. When my lymph nodes started popping, I think it was the last stand for the cancer. They were fighting."

Abrams has been cancer-free for six years. Stem cell transplants are not without side effects and Abrams developed a few symptoms of chronic Graft versus Host Disease (GVHD), an effect of the donor immune system attacking the patient's healthy cells. Abrams is currently enrolled in two NIH experimental protocols for treatment of these complications.

Beyond her contribution to clinical research, Abrams also contributes her talents directly to the NIH community. Her mosaics are featured in the Clinical Center and she volunteers on the art committee at the Children's Inn. Once a month she supervises the artistic endeavors of the pediatric cancer patients.

Abrams also shares her experience with cancer both on an ad hoc basis with new patients and in the form of a children's book. "It's about a little girl who has cancer—it's really me—and all the feelings she is going through. She realizes she has a whole team of helper heroes: doctors, nurses, friends, and family as guardian angels to help her get better."

"Before my husband and I first went to meet with the team at NIH, we thought we would look at our options, maybe check out MD Anderson or Johns Hopkins," said Abrams. "But after our conversation, I knew I was meant to be at the NIH."

CCR connections is available online at http://home.ccr.cancer.gov/connections.

Web Sites with More Information about CCR

Center for Cancer Research
http://ccr.cancer.gov

Office of the Director
https://ccr.cancer.gov/office-of-the-director

CCR News
https://ccr.cancer.gov/ccr-news

Office of Training and Education
https://ccr.cancer.gov/training-office-of-training-and-education

Patient Information on Cancer and Clinical Trials

Open NCI Clinical Trials
http://www.cancer.gov/clinicaltrials/search

How to Refer a Patient
https://ccr.cancer.gov/physicians

NCI Cancer Information Service
http://www.cancer.gov/aboutnci/cis
1-800-4-CANCER (1-800-422-6237)

CCR Clinical Cancer Trials in Bethesda, Md.
https://ccr.cancer.gov/clinical-trials-search-start

Additional Links

National Cancer Institute (NCI)
http://www.cancer.gov

Working at NCI
http://www.cancer.gov/aboutnci/working

National Institutes of Health (NIH)
http://www.nih.gov

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