



Tuberculosis Research Units (TBRU)

A critical component of NIAID's tuberculosis program has been the Tuberculosis Research Unit (TBRU) which was first established in 1994. The purpose of the TBRU has been to integrate scientific and clinical research disciplines to study human TB in endemic countries. The program was expanded in 2014/2015 to four multi-project awards (U19) to study TB latency and persistence and their relation to active TB disease in humans and supported by animal models.

Transformative advances for how this complex disease can be optimally managed continue to be hampered by a limited understanding of the stages of TB that precede active pulmonary disease (latency) or are implicated as the reason for prolonged antibiotic treatment (persistence). Latency and persistence are characterized by low levels of bacteria in secretions and likely other sites of infection (paucibacillary stages) are inherently difficult to study and have limited our understanding of the dynamic nature of TB and the design of innovative preventive, diagnostic and therapeutic interventions.

The awarded multi-disciplinary, multinational TBRU awards will work as a collaborative network to address these complex questions and are expected to drive innovation in TB research.

Awards

Emory University (TBRU-ASTRa)

<http://tbru.emory.edu/>

PI: Henry Blumberg (Emory University)

PI: Joel Ernst (New York University)

Boston Medical Center (TBRU-BURU)

<http://www.bumc.bu.edu/tbru/>

PI: Jerrold Ellner

PI: David Alland (Rutgers University)

PI: Padmini Salgame (Rutgers University)

Brigham and Women's Hospital (TBRU-LIMAA)

PI: D. Branch Moody

PI: Megan Murray (Harvard School of Public Health)

Weill Cornell Medical College (Tri-I TBRU)

PI: Carl Nathan

Co-PI: Michael Glickman (Sloan Kettering Institute for Cancer Research)

TBRU ASTRa

Dual PIs: Henry Blumberg, MD (Emory University) (henry.m.blumberg@emory.edu)

Joel Ernst, MD (New York University [NYU]) (joel.ernst@med.nyu.edu)

Overview

Our TBRU focuses on antigen-specific T cell immunity to *M. tuberculosis* (*Mtb*), to test the overall hypothesis that latent TB infection (LTBI) exists as a spectrum of bacterial and immunologic states, that at least three immunological states can be distinguished and correspond to: 1) past, but cleared *Mtb* infection; 2) stable LTBI with a low risk of progression to active disease; or 3) LTBI with a high risk of progression to active TB disease. To test this hypothesis, we will identify T cell signatures, consisting of the breadth of antigens recognized, the phenotypes, and the functions of *Mtb* antigen-specific CD4 and CD8 T cells, with correlations to outcomes determined in unique prospective cohort studies in humans and in experimentally-infected nonhuman primates. In addition to unique prospective studies, we will use a novel “Response Spectrum Assay” (RSA) that includes 60 strategically-selected *Mtb* antigens and epitope peptides, to assay the quantitative breadth and specific pattern of antigen recognition in LTBI subjects with distinct risks and rates of progression to active TB. In addition, antigens and epitopes identified in the RSA will be used to design HLA-peptide tetramers for high-resolution studies of the phenotypes and functional capabilities of *Mtb* epitope-specific T cells. The results of these studies, which employ advanced technologies including mass cytometry (CyTOF), together with the results of the RSA, will be used to identify T cell signatures characteristic of distinct risk categories of LTBI. Certain validated T cell signatures will correspond to efficacious immune responses that are desirable targets of TB vaccine development, while others will be useful for identifying individuals with LTBI that are at highest risk of progression to active TB, so these individuals can be prioritized for preventive interventions. Our human studies will be conducted in Atlanta, GA, USA, and in collaboration with investigators at the Kenya Medical Research Institute/U.S. Centers for Disease Control (KEMRI/CDC); studies of TB in nonhuman primates will be done by experts at two national primate research centers (Yerkes and Tulane). The team we have assembled possesses a wide range of knowledge and expertise, and is poised to generate improved understanding of TB immunity to contribute to the elimination of TB. Collaborating institutions include Emory University, NYU, Tulane, the La Jolla Institute of Allergy and Immunology (LIAI), Aeras, the DeKalb County (Georgia) Board of Health, KEMRI/CDC, and CDC.

Project #1: Identification of human *Mtb*-specific T cell signatures that are associated with resolved and persistent *Mtb* infection

Co-Program Directors: Jyothi Rengarajan, PhD and Cheryl L. Day, PhD (Emory University)

Project 1 will test the hypothesis that distinct *Mtb*-specific memory T cell profiles are associated with bacterial clearance or persistence. This is supported by data showing that distinct antigen-specific memory T cell phenotypes and functions are associated with LTBI, active TB disease, and clinically resolved TB. We propose to use chemotherapy-mediated clearance to model immune-mediated clearance of *Mtb*, as the treatment regimen for LTBI should result in significant reduction or elimination of bacteria. By combining studies from low-exposure and high-exposure settings, it is anticipated that these studies will provide insight into protective immunity to TB and new tools to evaluate *Mtb* persistence or clearance in LTBI. Research proposed in Project 1 also synergistically supports work proposed in Project 2 and Project 3.

Project #2: Human T cell responses permissive for progression to active TB disease

Program Director: Joel Ernst, MD (NYU)

The goal of Project 2 is to understand why some individuals with LTBI progress to active TB disease while others do not. Although individuals with LTBI possess *M. tuberculosis* (Mtb) antigen-specific T cell responses, in some, these are qualitatively or quantitatively insufficient, and LTBI progresses to active, transmissible, TB disease. Numerous studies provide evidence for a host-protective role of T cells in TB, but differences in the T cell responses that mediate stable LTBI and T cell responses that allow progression to active TB have not yet been identified. Similarly, the role of T cell responses in preventing future episodes of TB in the setting of re-exposure has not been prospectively studied nor quantified. Aims 1 and 2 of Project 2 will seek to identify T cell responses that distinguish stable control of LTBI and those associated with progression to active TB. Another factor confounding our understanding of the progression from LTBI to active TB disease is the impact of repeated exposures to active TB cases, which is increasingly relevant in high TB incidence settings. Aim 3 will seek to better understand the potential protective effects of prior LTBI with repeated exposure. Data from Project 2 will provide important insights for vaccine development, while enhancing public health strategies for addressing the global TB epidemic. The results will also link closely to those generated in Projects 1 and 3.

Project #3: Macaque T cell signatures of Mtb control

Co-Program Directors: Deepak Kaushal, PhD (Tulane National Primate Research Center) and Jyothi Rengarajan, PhD (Emory Vaccine Center and Yerkes National Primate Center)

This project will generate detailed data to demonstrate that *Mtb*-specific T cell responses associated with chemotherapy-mediated bacterial clearance are distinct from persistent *Mtb* infection in terms of antigen recognition, phenotype, and function, both ex vivo and in vivo. In addition, clearance of *Mtb* or its failure will be tested via reactivation with co-infection with simian immunodeficiency virus (SIV), as a model of HIV/*Mtb*, reflecting a common clinical occurrence in high TB incidence areas around the world. T cell responses noted in the same monkey before and after chemotherapy for the treatment of LTBI will be compared and these profiles will then be compared with profiles from untreated and chemotherapy treated humans with LTBI derived from Projects 1 and 2. The impact of exposure of monkeys with LTBI to a heterologous *Mtb* challenge will be modeled to monitor the evolution of specific T cell signatures in monkeys maintaining LTBI status vs those who develop active TB disease, in efforts to validate the T cell signatures associated with LTBI status. The objectives of Project 3 (carried out in a non-human primate [NHP] TB model) are highly complementary to those of the other two human subjects related projects (Projects 1 and 2) within our TBRU. These studies will establish tools for studying antigen-specific T cell responses in NHP, which will make pre-clinical studies of TB vaccines in NHP more relevant and provide important insights into immune control of TB.

In addition to three projects, our TBRU has three cores outlined below which support translational research projects (Projects 1 and 2) that involve human subjects and Project 3 which involves the NHP TB model.

Core A: Administrative Core

Co-Program Directors: Henry Blumberg, MD (Emory University) and Joel Ernst, MD (NYU); Scientific Advisor: Rafi Ahmed, PhD (Director, Emory Vaccine Center)

The Administrative Core (AC) is responsible for managing, coordinating, and supervising all TBRU activities. The AC provides leadership to support scientific investigation into the immunology of LTBI and facilitate communication among investigators, research projects and cores to ensure there is translation and back translation between human subjects studies and animal studies that utilize a non-human primate TB model. The AC includes a Data Management Center (DMC) under the direction of Dr. Lance

Waller (Chair, Emory Rollins School of Public Health, Department of Biostatistics and Bioinformatics) that is responsible for collection, storage, quality control, and evaluation of all study data; the DMC also provides biostatistical and bioinformatics support to TBRU investigators. The DMC is collaborating with Aeras on the implementation of an integrated data management system for tracking of TBRU specimens from both human subjects and non-human primates (NHP). In addition to facilitating coordination and communication across projects and cores at collaborating institutions, the AC provides fiscal management and regulatory requirement oversight and evaluation activities that includes monitoring progress and assessing the degree to which TBRU goals are being met. The AC will also facilitate involvement in collaborative research opportunities across the NIH TBRU network, leveraging our unique resources including clinical sites, a NHP model for TB, and investigators with expertise in TB immunology.

Core B: Immunology Core

Program Director: John Altman, PhD (Emory University)

Our TBRU Immunology Core is based at the Emory Vaccine Center and has expertise in MHC tetramer technology, development of advanced flow cytometry methods, and development of novel viral vector antigen delivery systems for in vitro T cell assays. Through collaborations with the La Jolla Institute of Allergy and Immunology it also has expertise in epitope mapping, application of MHC peptide binding algorithms, and HLA typing. The Immunology Core of the TBRU will perform the most sophisticated-to-date analyses of the phenotypes of T cell responses to *Mycobacterium tuberculosis* (*Mtb*) in humans (Projects 1 and 2) and rhesus macaques (Project 3). The activities of the Immunology Core include both provision of centralized standard support services and reagents, as well as development of important novel reagents and research activities. Standard support services include provision of MHC typing in humans and rhesus macaques; maintenance and distribution of peptide libraries; and construction, validation, and distribution of MHC tetramers, all to be used by every TBRU project. Innovative research activities include application of a viral antigen delivery system that will enable screening of more than 60 *Mtb* antigens for T cell responses in rhesus macaques, development of novel cell lines and MHC tetramer reagents for mapping epitopes and their MHC restriction elements, and application of CyTOF mass cytometry technology—including the use of mass-tagged MHC tetramers—to enable multiparametric descriptions of *Mtb*-specific T cells that will be tested for their use as biomarkers indicative of defined disease and infection states.

Core C: Clinical Core

Program Director: Neel Gandhi, MD (Emory University)

The goal of the Clinical Core (Core C) is to provide centralized expertise and capacity to enroll well-characterized cohorts of participants infected with *M. tuberculosis* to meet the aims of the TBRU. Coordination by the Clinical Core will create internal consistency across cohorts and improve the efficiency of enrollment for the TBRU projects involving human subjects, as well as for TBRU “Collaborative Projects.” The Clinical Core is a major resource for the TBRU by providing: 1) expertise in clinical and translational research; 2) coordination of participant enrollment and follow-up to achieve maximal efficiency and synergy between projects; 3) centralized specimen collection, processing and shipping through the TBRU Data Management Center; and 4) assurance of high-quality, ethical research conducted in accordance with U.S. and international regulatory requirements.

International Sites

The Kenya Medical Research Institute (KEMRI)/U.S. Centers for Disease Control and Prevention (CDC) [KEMRI/CDC] in Kisumu, Kenya is the primary international site. KEMRI/CDC was established in 1979 and maintains a vibrant collaboration for more than three decades, operating a Field Research Station in western Kenya, near the city of Kisumu In Nyanza Province, and a research site In Nairobi's largest urban settlement, Kibera. The KEMRI/CDC Field Station in Kisumu is located on 43 acres and has over 1600 employees, contracted through a cooperative agreement from CDC to KEMRI and through other funders including NIH NIAID. The site also has close partnerships with the Kenya Ministry of Health, nongovernmental organizations, foundations (including the Bill and Melinda Gates Foundation and Aeras), universities in the U.S., Europe and Kenya, bilateral and multilateral donors, and UN agencies.

Clinical Cohorts

Kenya

- Cohort
 - Active TB Cohort (n=75)
 - LTBI Cohort (n=2000)
 - Total Study population (n=6990)
- Sample Collection
 - 50 cc blood for Response Spectrum Assay (RSA), PBMCs, plasma; Sputum collection, QFT, TST (for LTBI cohort)
- Study type
 - Observational

Atlanta, GA (DeKalb County Board of Health)

- Cohort
 - LTBI Cohort (n=150)
- Sample Collection
 - 50cc blood for RSA, PBMCs, plasma; TST, QFT, TSPOT.TB, ²H-labeled DNA
- Study type
 - Observational

Animal Model(s)

Non-human primates (NHPs): Experimentally infected macaques exhibit human-like complex pulmonary pathology, unlike mice. There is experimental evidence of asymptomatic infection similar to the latent (or non-replicating persistent) infection observed in humans. The taxonomic proximity between human and NHPs makes it feasible to extrapolate the conclusions reached from studies in macaques to the human situation. World class BSLIII and ABSLIII facilities are available to perform this work at the Tulane and Emory/Yerkes National Primate Centers.

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Boston University-Rutgers University Tuberculosis Research Unit

Principal Investigators:

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David Alland, MD (New Jersey Medical School, Rutgers University) Padmini Salgame, PhD (New Jersey Medical School, Rutgers University)

Administrative core and data management: David

Hom, MSc Clinical core: Robert Horsburgh, MD, MUS

About Us

Latent tuberculosis infection (LTBI) and persistence of *Mycobacterium tuberculosis* (Mtb) after appropriate treatment are two forms of paucibacillary tuberculosis (TB), in which organisms are not readily culturable. The first represents host control of primary infection with Mtb, whereas the latter represents persistence of viable Mtb organisms despite treatment. They both constitute hidden reservoirs for future transmission of TB and pose an obstacle to global elimination. The mechanisms that determine latency and persistence of Mtb are not completely understood. Critical gaps include biomarkers for latency and persistence of Mtb in humans and animal models of paucibacillary TB that resemble human disease – both would facilitate development of new drugs, allow tailored treatment regimens, promote cure of patients and accelerate TB control and elimination.

The Boston University (BU) – Rutgers University (RU) Tuberculosis Research Unit (TBRU) is a collaboration of scientists and clinicians from the US and TB endemic countries dedicated to the elucidation of biomarkers and mechanisms that pose a risk of progression from LTBI to active disease and of treated TB to relapse. The multidisciplinary approach includes principally microbiology, immunology, molecular genetics, and epidemiology. The collaborative network includes faculty based at Boston University, Rutgers University, Albert Einstein College of Medicine, Massachusetts Institute of Technology, and Center for Infectious Diseases Research. The principal international sites are Universidade Federal do Espírito Santo in Vitória, Brazil and the University of Cape Town in Cape Town, South Africa.

Research Strategy

The BU-RU TBRU program is composed of four research projects that are described in full below. Their goals are to:

- Discover biomarkers to stratify risk of individuals with latent and persistent TB infection and promote their use in targeting preventive therapy.
- Use the rabbit model to establish models of latent and persistent TB that are comparable to humans in rates of Mtb replication and mutation, image findings, and sites of reactivation; and validate the use of the model to study new drugs, regimens and immunotherapies, that can be translated into the clinic.
- Discover biomarkers of Mtb persistence and treatment relapse to allow individualized short course treatment regimens and as surrogate endpoints in clinical trials.
- Unravel the role of bacterial factors such as high minimum inhibitory concentrations (MIC) (within the drug susceptible range) in persistence and investigate the genetic mechanisms for loss of persistence phenotypes that can provide a new focus for development of drugs, regimens and schedules uniquely active against persisting organisms.

Biomarker Discovery | We will use cutting-edge technology that includes Illumina's Next-Generation whole genome sequencing platform and SOMAScan Technology for transcriptome-

and proteomic-based biomarker discovery through the analysis of splice variants, novel transcripts, gene fusions, and multiple human proteins. We propose a novel hypothesis that the phenotype of memory T cells can predict whether viable Mtb are still surviving in the host. We will apply multiparametric flow cytometry coupled with the computational method of Cytokine Fingerprinting to identify patterns in the effector and memory T cell subsets that will address this hypothesis. Further, we will use tetramers to isolate antigen-specific T cells for cell surface phenotyping and RNA-Seq for gene expression. This will for the first time combine the power of cytomics with genomics in host biomarker discovery in TB.

In our search for biomarkers, we will quantify and compare the levels of interferon (IFN)-gamma (γ), interleukin (IL)-17, IL-4, and IL-10 in samples from patients with different statuses of TB infection. We will also use FDG PET/CT scan (positron emission tomography-computed tomography using 18F-fluoro-2-deoxy-glucose) as a surrogate of inflammatory activity to identify patterns of imaging findings that correlate with specific outcomes (particularly relapse *versus* non-relapse and progression to infection *versus* control of the latent focus).

Animal Model | There is a critical need to develop an animal model that reflects the human spectrum of latency. Mice and macaques have both shown limitations in filling this role. We use the New Zealand White Rabbit and an infectious Mtb strain CDC1551 as the model for LTBI in humans. Rabbits infected with this strain develop a primary infection that is rapidly contained, with Mtb soon becoming undetectable, as in human latency. To this animal model we will apply innovative approaches and techniques that include primary infection with complex bar-coded Mtb pool that will allow the study of replication dynamics and anatomical spread of latent Mtb; novel loss of persistence (LOP) mutants such as our $\Delta whiB6$ mutant, which will enable us to dissect the bacterial factors required for survival during LTBI; and innovative PET-CT imaging techniques that will allow us to study progression from infection to latency to disease. We will also use this model to test vaccine candidates and new treatment interventions. Ultimately, we hope to uncover the key to eradicating the paucibacillary state in treated TB patients and shortening TB therapy, identify the bacterial genes required for persistence, develop a dynamic model of drug-induced paucibacillary disease, and identify bacterial predictors of clinical relapse.

Project 1 – Biomarkers to stratify risk of progression from latent TB infection to disease

Project Leaders: Dr. Padmini Salgame (NJMS), Dr. Charles Horsburgh (BU), Dr. Reynaldo Dietze (HUCAM/NDI), Dr. Rodrigo Rodrigues (HUCAM/NDI); Dr. Edward Jones-López (BMC), Dr. Karen Jacobson (BMC), Dr. Robert Wilkinson (UCT), Dr. Friedrich Thienemann (UCT), Dr. Anna Coussens (UCT)

Questions addressed:

- What are the risk factors for progression to TB disease after infection with Mtb?
- Are there early markers of bacterial clearance in response to chemotherapy?
- Which immunological markers can indicate the presence (or absence) of live Mtb?
- What are the correlates of protection from active TB disease?

Specific aims:

1. To characterize and validate biomarkers of high risk of progression.
2. To characterize and validate biomarkers of low risk of progression and of cure.
3. To identify correlates of protection in tetramer-isolated antigen-specific T cells from household (HHC) contacts who self-cure.

Significance: Biomarkers of latency progression would transform TB control by allowing the targeting of preventive therapy to those who would benefit most. Biomarkers of cure can be used as surrogate endpoints in future trials of therapeutic drugs and vaccines and for treatment shortening.

Project 2 – Validation and application of a model of human TB-like latency in rabbits

Project Leaders: Dr. Veronique Dartois (NJMS), Dr. David Alland (NJMS)

Questions addressed:

- How does Mtb replicate and mutate during human and rabbit latency?
- Are there early markers of bacterial clearance in response to chemotherapy and/or a therapeutic vaccine or immune-stimulatory agent?
- What are the correlates of protection from active TB disease?
- Which bacterial factors are essential for latency? Which represent good drug targets?

Specific aims:

1. To define growth and mutation rates in Mtb during human latency.
2. To develop and characterize a rabbit model of latency.
3. To use the rabbit latency model to test immunological and drug interventions to eradicate the latent state.

Significance: A validated rabbit model of latency, along with new tools that allow for latent Mtb to be monitored for growth, persistence and decline at the clonal level will allow pre-clinical testing of new drugs and regimens and immunotherapeutics.

Project 3 – Biomarkers of persistent TB infection and TB treatment relapse

Project Leaders: Dr. Charles Horsburgh (BU), Dr. Karen Jacobson (BMC), Dr. Reynaldo Dietze (HUCAM/NDI), Dr. Rodrigo Rodrigues (HUCAM/NDI); Dr. Robert Wilkinson (UCT), Dr. Friedrich Thienemann (UCT), Dr. Anna Coussens (UCT).

Questions addressed:

- Does bacterial burden correlate with likelihood of relapse post treatment?
- Which immunological markers indicate the presence or absence of live Mtb?

Specific aims:

1. To determine the association between abnormalities on FDG-PET/CT scan and relapse of TB patients after completion of treatment.
2. To develop and validate a model to predict Mtb persistence and treatment relapse of drug-sensitive-TB patients based on changes in biomarkers.
3. To prospectively validate the most promising biomarkers as predictors of relapse in another drug-susceptible TB cohort.

Significance: Identification of anatomic locations of persisting organisms would allow potential targeting of interventions. Biomarkers predictive of residual paucibacillary disease would facilitate development of new drugs, tailoring of treatment regimens, and promote more durable cure of patients.

Project 4 – Bacterial mechanisms and host pharmacokinetic factors that determine persistence in paucibacillary TB

Project Leaders: Dr. David Alland (NJMS), Dr. Veronique Dartois (NJMS).

Questions addressed:

- What is the role of persistent, metabolically diverse Mtb populations in treatment duration and relapse?
- Which bacterial factors promote persistence, and which represent novel drug targets?
- Which metabolic products antagonize persistence and which metabolic adjuvants can enhance it?
- Does bacterial burden correlate with likelihood of developing active TB disease?

Specific aims:

1. To identify persistence mechanisms relevant to human TB treatment.
2. To determine whether Mtb strains with moderately increased (but drug-susceptible) minimum inhibitory concentrations (MIC) are more prone to relapse after treatment, and identify the underlying mechanism of these MIC differences.
3. To examine the *in vivo* relevance of persistence mutants in the rabbit disease model and the effect of different anti-TB drug concentrations (kill rates).

Significance: Identifying the factors that promote and antagonize Mtb persistence has the potential to aid in the search for optimized treatment strategies that can provide the best kill kinetics during the paucibacillary TB stage, ultimately leading to treatment shortening.

International Sites

The Núcleo de Doenças Infecciosas (NDI) (Vitória, Brazil) was created in 1990 as a branch of the Social Medicine and Epidemiology Department at the Universidade Federal do Espírito Santo (UFES). It is located within UFES University's Health Sciences campus, near the 300-bed affiliated teaching Hospital (Hospital Universitário Cassiano Antonio de Moraes - HUCAM). The NDI has a strong clinical research infrastructure that includes state-of-the-art mycobacteriology and immunology laboratories adhering to international standards of good laboratory practice.

The Clinical Infectious Diseases Research Initiative (CIDRI) at the University of Cape Town (UCT) (Cape Town, South Africa) was established by a Wellcome Trust strategic award and works in partnership with UCT's Centre for Infectious Disease Epidemiology and Research. The clinical research facilities are in Khayelitsha township at two sites that are five minutes' drive apart: 1. Khayelitsha Site B Ubuntu HIV-TB clinic, and 2. Khayelitsha Hospital (KH). These are augmented by clinical facilities at Tygerberg hospital, approximately 25 minutes' drive from Khayelitsha. Site staff are experienced in participant recruitment and equipped to conduct the necessary clinical tests and sample collection for studies. The clinical facilities are complemented by established laboratory and administrative infrastructure at the UCT Faculty of Health Sciences campus.

Clinical Cohorts

We will perform prospective observational studies of cohorts of TB patients and household contacts. A nested case-control study of biomarkers will be performed comparing those that reach the endpoint (relapse or progression to TB) and matched controls that do not.

- Cohort 1A: 250 HHC of MDR-pulmonary TB cases will be enrolled over a period of 3-4 years.

They will be followed for the development of incident TB for 18 months. All will undergo FDG-PET-CT scans at baseline, and a subset will have repeat FDG-PET-CT scans at 6 months. All will have blood draws.

- Cohort 1B: 400 HHC of DS pulmonary TB cases with positive tuberculin skin test TST will be enrolled over a period of 3-4 years. They will be followed for the development of incident TB for up to 4 years. All will have blood draws at baseline.

- Cohort 3A: 150 DS pulmonary TB will have a FDG-PET-CT scan at the end of treatment and blood drawn for biomarker studies. Follow-up for relapse will be done for 12 months after the completion of treatment.

- Cohort 3B: 300 patients with DS-TB who are at the end of treatment will undergo a blood draw. Follow-up for relapse will be done upon completion of treatment for 12 months after the completion of treatment.

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Brigham and Women's Hospital TBRU

PI: D. Branch Moody (Brigham and Women's Hospital)

Co-PI: Megan Murray (Harvard Medical School)

Overview

The absence of an effective vaccine for tuberculosis means that TB control relies on the early diagnosis and effective treatment of infectious cases, which is compromised by the relatively low sensitivity and specificity of standard diagnostic tools. Because TB infection most often results in a chronic asymptomatic state, prevention of disease by targeting those who are infected, but not yet ill, has been difficult to implement in high burden settings where more than half of the population is TB infected. The long duration of treatment necessary to achieve high cure rates and the emergence and spread of drug resistant organisms have further undermined the potential impact of national TB control programs. Our proposed plan responds to these research priorities and grows out of a series of recent research findings from our own groups and others that suggest an innovative interdisciplinary approach to the discovery of basic mechanisms. Our proposed project begins with the identification and longitudinal follow-up of patients diagnosed with active TB and their household contacts. Patients that progress to active TB disease (progressors) are followed for disease outcomes, including relapse, and household contacts are followed for evidence of TB infection and disease. This design and our extensive longitudinal follow up capabilities will allow us to identify and characterize TB index cases and their exposed household contacts through careful clinical and epidemiologic studies, human genomics (by exome sequencing) human genetics (by exome chip), transcriptomics, and metabolomics. We have established Cores in Human subjects, Bio-informatics, and Metabolomics that will work in parallel to identify targets including pathways linking human metabolism and immune response, T cells involved in Mtb response, pathogen determinants of drug resistance and pathogen-shed markers of clinical TB phenotypes. Each project includes validation of these targets in the guinea pig model. Based on our results, we will then go on to test specific interventions in the animal model, focusing in particular on pharmacologic agents that alter human metabolic and immune responses.

Project #1: A multi-disciplinary approach to the identification of host metabolic determinants of TB clinical phenotypes

Project Leader: Megan Murray (Harvard Medical School)

Project scientists: Brendan Podell, Randall Basaraba, Branch Moody, Yang Luo

The mechanisms through which metabolic factors affect clinical TB phenotypes are not known. We will carry out genome-wide studies to determine host genetic associations with a key outcome of human infection: the progression or non-progression to TB disease after an exposure. In addition, we will focus on metabolic and immune-related genes expressed in human macrophages and dendritic cells, where specific pathways involving leptin, adiponectin and the peroxisome-proliferator activating receptor- γ (PPAR- γ) have been implicated in regulating host response. Therefore, we emphasize human genetic associations known to affect these metabolic pathways, which we hypothesize may alter the natural course of TB progression. We also take advantage of newly-developed guinea pig models that allow induction of a hyperglycemic state to measure TB outcomes in intervention studies using available drugs. The goals of this work are to identify host metabolic parameters that predict progression/non-

progression to TB disease among recently exposed individuals and to evaluate the impact of putative metabolic and macrophage factors associated with TB susceptibility through pharmacological modulation of their function in guinea pigs with pre-existing hyperglycemia and Mtb-induced metabolic changes.

In year 1 we have examined the effects of hyperglycemia in mycobacterial control and are evaluating a euglycemic clamp. Although human subjects approvals are now being finalized for new recruitment, analysis of existing cohorts has focused on the role of dietary influences on outcomes including unexpected roles of fat soluble vitamins. The exome studies have commenced based on published data from the thousand genomes project from Peruvian subjects as well as new data collected through this mechanism. Analysis demonstrates that Peruvian populations have highly distinct genotypes that show native signatures that are quite distinct from other European populations. Human macrophage protocols have been updated to emphasize the use of GM-CSF and we are carrying out a pilot study to look at whole cell transcriptomics on human macrophages.

Project #2: Identify the host genetic determinants of immune response and TB control

Project Leader: Soumya Raychaudhuri (Brigham and Women's Hospital)

Project Co-leader: Ildiko Van Rhijn (Brigham and Women's Hospital)

Project scientists: Yang Luo, Branch Moody, Sarah Iwany, Roger Calderon

This project seeks to treat human T cells as spanning a complex functional spectrum that is not fully captured by the Th1, Th2, Th17, Treg or other established paradigms. Instead, we posit that progression to active TB disease is determined by complex interactions among co-receptors, homing receptors, costimulatory molecules, cytokines and non-cytokine effector molecules. Using an unbiased approach to measuring T cell function, we seek to determine the specific genes in CD4⁺ effector memory T cells that are associated with TB progression. The primary goals of this work are to determine which human effector memory T cell functions associate with tuberculosis progression by performing transcriptional profiling as well as determine the functions of invariant T cells in TB disease. This project supports Project #1 through the study of TST positive HHC and Active TB patients from different households to serve as a nested subpopulation. This project will also feed data into Project #4 for the evaluation of proposed diagnostics.

In year one we have implemented a new biosafety protocol to allow for sorting of unfixed cells after culture in Peru to exclude patients with bacteremia. In year 1 we have validated multi-color flow cytometry protocols, including those needed for analysis of MAIT cells (MR1 tetramers), GEM T cells (CD1b tetramers), gamma-delta T cells and effector memory cells. A full time flow cytometrist, Sarah Iwany, has been hired to the project and the laboratory in Peru has been outfitted with centrifuges, freezers and laboratory equipment. Roger Calderon and Megan Murry are overseeing implementation of PBMC isolation protocols with an on-site visit scheduled for April 2016.

Project #3: Diagnostics for acute and recurrent M. tuberculosis disease in humans

Project Leader: D. Branch Moody (Brigham and Women's Hospital)

We propose detection of the specific *M. tuberculosis* lipids and small molecules that can be considered biosignatures of disease in two stages involving discovery and validation. We take advantage of recently generated catalogs of 142 subclasses of mycobacteria-specific molecules and nearly universal mass detectors to achieve the key strategic goal of identifying targets with diagnostic specificity. Our proposed plan starts with bench studies to survey all detectable molecules in *Mtb* and determine those which are specifically expressed by clinically important and common strains of *Mtb*, but not non-tuberculous mycobacteria or other pathogens. Second, using recently validated systems that allow detection of up to 7,000 molecules in serum or urine, we will carry out clinical translational studies to detect mycobacterial molecules in assayable fluids *ex vivo* in TB patients using a study design that tracks patients with paucibacillary disease and captures patients with TB relapse. Also, we will detect pathogen-shed molecules and in guinea pigs during controlled infection and relapse after induction of hyperglycemic states. Both human and guinea pig studies emphasize longitudinal monitoring and *ex vivo* detection during early, resolving and relapsing TB disease states to detect those molecules specifically produced by *Mtb*. These studies leverage basic and translational discoveries highlighted in Projects 1 and 2 toward the practical goal of diagnostics development for the clinic.

In year 1 we completed biosafety protocols for MS analysis of human samples that have been inactivated, which required revalidation of MS protocols for chloroform and methanol treatment as well as filtration. Reference lipidomic datasets have been generated for human urine and serum. As part of an interTBRU program, we have implemented a solid phase mycobacterial culture method and are combining lipidomic (BWH) and metabolomic (Cornell platforms). We have completed pilot studies to optimize and define MS profiles of human urine. We have detected CD1b reactive T cells in human TB patients.

Core A: Administrative

Core Leader: D. Branch Moody and Megan Murray

The Administrative Core will house the data management center that integrates and archives the data generated in each of the projects; will ensure smooth communication between the projects and between the TBRU and other stakeholder by scheduling and managing regular communications; will monitor milestones and timelines; will develop a collaborative projects program; will ensure fiscal accountability; and will identify and help to resolve any problems that arise during the grant period. In addition, the core will manage data and other resource sharing.

Core B: Human Subjects

Core Leader: Megan Murray

The aim of the Core is to support the TBRU's scientific project by recruiting and following TB patient and household contact cohorts in Lima, Peru, obtaining relevant data and samples and ensuring the efficient transfer of data and samples to the Data Management Center in Boston. To that end, we will identify and use existing blood samples from 3000 of our initial cohort members with well-defined TB outcomes for human genotyping and will enroll 300 new TB cases and 900 household contacts who will be followed for relevant outcomes and who will provide blood and urine samples for biomarker assessment.

Core C: Bioinformatics

Core Leader: Soumya Raychaudhuri

Through the Bioinformatics Core C, this TBRU will have not only the capacity to generate high throughput data, but in addition, will be able to employ state of the art analytical methods to interpret the data. In particular, Bioinformatics Core C will support customized genotyping and sequencing strategies in Peruvian populations (Project 1), will use high-throughput transcriptional assays to query and define genetic networks for tuberculosis susceptibility (Project 2), and will expand automated cytometric data analysis tools for immune-systems biology (Project 2). Each of these goals will be complemented by analytical expertise from Dr. Soumya Raychaudhuri and that of the members of the Core analytical team. To enable these goals the Core will support (1) Human genomic assays, including next-generation sequencing and exome-chip genotyping, (2) Transcriptional profiling with the Nanostring nCounter™ assay, and (3) High-throughput automated flow-cytometric data acquisition utilizing cutting edge analysis software. The Core will be flexible in its approach to accommodate evolving technologies and computational approaches as they come online.

Core D: Animal Models

Core Leader: Randall Basaraba

The Animal Models Core will parallel human cohort studies to identify host metabolic determinants of tuberculosis disease control and progression and use metabolomics to discover host- and pathogen-derived biomarkers for tuberculosis diagnosis. The Core has developed the first ever model of Mtb

infection in guinea pigs with diet-induced insulin resistance and type 2 diabetes that accurately mimics the comorbidity of tuberculosis and emerging noncommunicable diseases. It has characterized the profound alterations in systemic and cellular metabolism in response to *Mycobacterium tuberculosis* infection alone and demonstrated how preexisting alterations in host metabolism influences host susceptibility and in vivo disease progression. Using the guinea pig model to study altered host metabolism associated with *Mycobacterium tuberculosis* infection has not only improved our understanding of the host-pathogen interaction but has identified potentially important therapeutic strategies that can be used as adjunct therapy in combination with conventional antimicrobial drug therapy.

Core E: Metabolomics Core

Core Leader: D. Branch Moody

The Metabolomics Core (B) is comprised of a Biosafety Level 3 suite for handling infectious samples, massively parallel detection of mycobacterial metabolites using Time of Flight mass spectrometry (Agilent Accurate Mass ToF 6230), specialized resources for identifying known mycobacterial compounds, and analytical capabilities to discover previously unknown compounds (Agilent Accurate Mass 6520, QToF, Thermo LXQ Advantage 2 Dimensional Ion Trap with MSn).

The Metabolomics Core will align large datasets derived from different patients, clinical isolates or genetically engineered bacteria, inhouse-designed software pipeline identifies all compounds that are changed at statistically significant levels. In a second, targeted phase, all changed compounds are ranked by biological or quantitative criteria to define compounds of interest, whose structures are solved by comparing their masses to the literature (MycoMass) and in-house (MycoMap) databases or are solved through collisional mass spectrometry.

This system has discovered previously unknown compounds, identified strain-specific mycobacterial biomarkers in vitro and from tissues and identified lipids changed after gene deletion. This overview describes expansion of the substantial existing core facilities, including a new generation of high accuracy mass spectrometry and expansion of mycobacterial databases, as well as use of the Core to discover biomarkers in drug-resistant or latent mycobacteria or biomarkers of infection.

International Sites

Socios en Salud (SES) (Lima, Peru) has run a treatment program for patients with MDR TB since 1995 through a formal agreement with the Peruvian Ministry of Health and the Office of Foreign Affairs. To date, the physicians, nurses and home health workers at SES have delivered care to thousands of patients with drug resistant TB in Lima. SES has built a highly efficient and well-organized research infrastructure that has consistently achieved high marks on required NIH monitoring visits. SES has also developed close collaborative relationships with over 100 Ministry of Health clinics where patients are first screened and diagnosed with TB. The site has an experienced, high-functioning management team, a network of over 100 TB clinics from which we recruit TB patients and identify households, a data flow system that includes electronic scheduling of visits, data entry through hand-held devices on which case report forms are programmed, and a data management system that enables auditing of data changes,

and a flexible data dictionary which allows new data items to be added without changing the underlying database structure. SES constructed and staffed a BL3 container lab.

Clinical Cohorts

Lima, Peru

- Cohort
 - Active TB & Household contacts (HHC) (n=1500 ea; n=3000 total)
 - Newly diagnosed index TB patients (n=300) and HHC (n=900)
- Sample Collection
 - blood; Sputum collection for smear, culture, DST; urine; TST/IGRA
- Study type
 - Observational and interventional

Animal Model(s) -

Guinea pig: Outbred guinea pig strains are highly susceptible to Mtb and develop a spectrum of disease that closely mimics aspects of natural occurring infection in humans, including well-organized granulomata with central caseous necrosis and fibrous encapsulation. This effort is directed by Dr. Randy Basaraba at Colorado State University and includes diet induced models of type II diabetes.

Data Manager: Zibiao Zhang

Lipidomics/ Bioinformatics: Jacob Mayfield

Weill Cornell Medical College TBRU

Tri-I TBRU: Persistence and Latency

PI: Carl Nathan

Co-PI: Michael Glickman (Sloan Kettering Institute for Cancer Research)

Overview

Mycobacterium tuberculosis (Mtb) is one of the world's most successful pathogens. WHO estimates that about one third of the world's population has a positive skin test that reflects a long-term adaptive immune response to Mtb antigens. These individuals are considered to have actual or potential latent Mtb infection (LTBI). Among them, a minority that cannot be identified prospectively will develop reactivation tuberculosis (TB) despite having apparently normal immunity. Active TB can be contagious both to those who were previously unexposed and those with LTBI and is usually lethal if untreated. Adequate numbers of CD4 T cells, tumor necrosis factor alpha (TNF α), and interferon-gamma (IFN γ) are validated determinants of control of primary TB, but the vast majority of HIV negative patients with reactivation TB do not have defined defects in these pathways. The ability of within the human host, and the related failure of the human immune system to sterilize Mtb in latently infected individuals, are poorly understood. Antimicrobial therapy for active infection by drug-sensitive Mtb is effective, but current drugs must be given for 6 months to achieve relapse-95%. The necessity for this prolonged duration of therapy is attributable to the ability of genetically drug-sensitive Mtb to adopt a phenotypically drug-tolerant, persistent state in which it is not readily sterilized by current drugs. Despite substantial efforts to understand these two critical features of Mtb infection--latency and persistence--fundamental questions remain about the genetic, immunologic, and microbiologic contributors to both. We seek to close this knowledge gap through a Tuberculosis Research Unit (TBRU) that unites investigators at Weill Cornell Medical College (WCMC), Rockefeller University (RU), and Memorial Sloan Kettering Cancer Center (MSKCC), with selected external collaborators, and draws on patients at the WMC-affiliated GHESKIO Centers in Haiti to provide insight into latency and persistence of Mtb during human infection. A CETR application involving the WCMC and MSKCC investigators plus cores at University of Medicine and Dentistry of New Jersey and University of Kansas led to a supplement to the TBRU. The supplement supports the discovery of tool compounds to test and overcome mechanisms of phenotypic tolerance.

Project #1: Human Genetics of Reactivation Pulmonary TB

Project Lead: Dr. Jean Laurent Casanova (Rockefeller University), Dr. Laurent Abel (Rockefeller University), Dr. Michael Glickman (MSKCC), Dr. Ming Li (MSKCC)

There is growing genetic epidemiological evidence that PTB has a strong genetic component in humans, but the molecular basis of susceptibility to Mtb reactivation remains largely unknown. Candidate gene and genome-wide (GW) association studies have suggested that common variants play only a modest role in the genetics of PTB, or that their role is restricted to specific subgroups. We recently showed, by an unbiased GW linkage approach, that common variants of TOX, a gene involved in T-cell and NK-cell development, are associated with PTB in young patients with a short latency duration, in two ethnically different populations. Genetic factors other than TOX may also contribute to PTB, including rare variants

of unknown genes. Our project will combine a focused in-depth dissection of the role of TOX in PTB, based on both human and mouse studies, with a GW approach investigating the role of rare variants in PTB. Study subjects will be recruited in Haiti, through the GHESKIO Center, to establish a large case/control sample (HIV-negative and HIV-positive), and a family based sample with at least two PTB-affected siblings. Mouse models will be used to investigate the role of TOX in T-cell responses to Mtb infection, together with analyses of endogenous T-cell responses in mice with T cell-specific TOX gene deletion.

Project #2: Innate, transcriptomic, and microbiomic correlates of TB infection

Project Lead: Dr Michael Glickman (MSKCC)

The life cycle of Mtb within the human host is marked by critical transition points between the major phases of infection: initial infection, latent disease, active tuberculosis, and resolution with antibiotic therapy. The vast majority of HIV negative TB patients do not have defined deficiencies in the immune factors known to be associated with each phase and therefore the host determinants of the heterogeneous ability of TB patients to control Mtb infection, either in the context of initial infection after exposure, reactivation of latent infection, or relapse-free elimination of Mtb after antimicrobial therapy, are unknown. In addition, there is a paucity of longitudinal studies in which the transition from uninfected > infected, latently infected > active infection, and active infection > cured are studied in the same patient, thereby limiting our knowledge of the microbiologic and immunologic events that accompany these transition points. This gap in our knowledge of predictive biomarkers severely limits our ability to optimally manage this infection. To address these knowledge gaps, we have assembled a set of patient cohorts to examine three interrelated host factors and biomarkers that we hypothesize may contribute to the heterogeneity in host control of Mtb during the transition from uninfected > infected, LTBI > active disease, and infected > cured. We will examine the role of Mucosal Associated Invariant T (MAIT) cells, innate lymphocytes that respond to TB at mucosal surfaces, in TB defense. We will expand our use of whole blood transcriptomics to derive transcriptional patterns of innate resistance and successful TB therapy. Finally, we will examine the role of stool microbiomic composition, and its perturbation by antibiotics, in Mtb infection.

Project #3: Viable but Nonculturable Mtb

Project Leader: Dr. Carl Nathan (Weill Medical College of Cornell University)

We hypothesize that non-replicating (NR) Mtb represent a major population of the "persisters" that survive the first months of chemotherapy. One subset of NR Mtb is termed "viable but nonculturable" (VBNC) because they elude detection as colony forming units (CFU) on agar. Because these cells can sometimes be cultured by other means, we now describe them as "differentially detectable" (DD) Mtb. The nonculturability of DD Mtb has left us with almost no knowledge of their essential genes, metabolic pathways or susceptibilities to specific drugs and drug candidates. We have now improved a limiting dilution assay for DD Mtb and used it to confirm that an average of 87% of the viable Mtb detected in sputum from 33% of the untreated TB patients studied were DD forms missed both by CFU assays and by liquid culture in the BACTEC MGIT assay. In close collaboration with Project 5, Project 2, and the Clinical Core, we will further improve and apply the resuscitation assay to characterize DD Mtb by chemical genomics in vitro, by genetics in the mouse, and by their presence in sputum from patients before and after standard therapy.

Project #4: Biomarkers of PZA activity and its target populations

Project Leader: Dr. Kyu Rhee (Weill Medical College of Cornell University)

Unlike other TB drugs, PZA lacks activity against Mtb under conditions of standard in vitro growth, and, instead, requires the use of mildly acidic pH conditions that render Mtb phenotypically resistant or tolerant to most other drugs. This in vitro characteristic has led to the hypothesis that PZA targets non- or slowly replicating (NR) drug tolerant Mtb populations in acidic in vivo environments such as intra- and extracellular inflammatory lesions. However, mounting evidence suggests that the chemical microenvironments and accompanying drug tolerant NR states occupied by Mtb within the host may encompass a broader range of conditions and states associated with PZA activity than those modeled by acid alone. We propose to identify physiologic biomarkers of PZA activity and susceptibility through a combined metabolomic and transcriptomic approach. These studies will integrate experimental studies of PZA in vitro activity with in vivo studies of PZA activity as reported by the transcriptional profiles of Mtb recovered from the lungs of infected mice or sputa of culture confirmed patients.

Project #5: Mouse models for paucibacillary persistence

Project Leaders: Dr. Sabine Ehrt (Weill Medical College of Cornell University) and Dr. Dirk Schnappinger (Weill Medical College of Cornell University)

Drug treatment and silencing of *in vivo* essential genes can cure acute and chronic Mtb infections in mice to the extent that CFU can no longer be detected on agar plates. However, as in humans, Mtb in these mice is often not sterilized, and paucibacillary persistence eventually may result in relapse of TB. We propose to identify the molecular processes that Mtb requires to persist following apparent eradication and to develop a new mouse model to study paucibacillary persistence. In collaboration with Project 3 we will test the hypothesis that "viable but nonculturable" (VBNC) Mtb are present during acute, chronic and paucibacillary infections and that VBNC populations contribute to the duration of chemotherapy, paucibacillary persistence and relapse TB. To determine correlates of transition from paucibacillary to active disease and to integrate the animal studies with the clinical activities of the TBRU, we will identify host blood mRNA signatures associated with paucibacillary TB in mice and compare them to the RNA profiles from people with latent TB obtained in Project 2 and in the Clinical Cohorts Core. We will further attempt to identify early biomarkers predictive of relapse TB in mice.

Core A: Administrative. Core leaders, Dr. Carl Nathan (WCMC) and Dr. Michael Glickman (MSKCC)

Core B: Clinical . Core leader: Dr. Daniel Fitzgerald (WCMC)

Core C: Chemistry (CETR). Core leader: Dr. Jeffrey Aubé (Univ. of North Carolina)

Core D: Pharmacology (CETR). Core leader: Dr. Veronique Dartois (Univ. of Medicine and Dentistry of New Jersey)

Centers of Excellence in Translational Research (CETR)

The TBRU award was administratively combined with the relevant scientific projects that were selected for funding under RFA-AI-12-044 - Centers of Excellence in Translational Research (CETR) (U19) and that were complementary to this TBRU award. The goals of the Supplemental projects are to bring tool compounds and chemical probes to bear on the problem of persistence by *Mycobacterium tuberculosis*

(Mtb). We will identify candidate drugs and chemical probes that inhibit Mtb's ability to persist *in vitro*, *in vivo* in mouse models of paucibacillary disease and *ex vivo* in human sputum and then use these compounds to identify the targets whose inhibition or activation thwarts Mtb's ability to persist. Almost all existing anti-mycobacterial agents were screened for activity against replicating populations of Mtb and, in fact, only kill Mtb when it is replicating. Thus existing anti-mycobacterial agents do not constitute a suitable panel of tool compounds with which to explore persistence, which is manifest in non-replicating Mtb. These Supplemental activities integrate with and augment the work in Projects #2, #3, #4, and #5, by using candidate drugs and chemical probes to identify pathways on which Mtb depends for survival in the host to sustain persistence and latency. The susceptibility of these mechanisms to chemical inhibition will ultimately allow development of medical interventions and definitive tests of their clinical relevance. Candidate drugs and chemical probes developed and tested through this supplement will be made available to the other TBRU projects, where appropriate.

International Sites

Groupe Haitien d'Etude du Sarcome de Kaposi et des Infections Opportunistes (GHESKIO) (Port-au-Prince, Haiti) serves 200,000 patients and provides large populations for recruitment, including adults and adolescents with active TB, patients with MDR TB, healthy controls, and families with multiple members with TB. All GHESKIO clinical research complies with US, Haitian Government, and NIH regulations. The GHESKIO institutional review board (IRB) was established in 1984 and has a US Federal Wide Assurance. Cornell-GHESKIO has published seminal papers on the ethical conduct of research in resource poor settings. The GHESKIO Data Management Center has established strategies and software programs for the screening, recruitment, and retention of volunteers. Once subjects are enrolled, an electronic calendar of patient visits, case report forms, and sample collection is generated based upon the protocol schedule.

Clinical Cohorts

Port-au-Prince, Haiti

- Cohort
 - HIV-negative, Active TB (n=600; 400 patients with PTB before 25 years of age, and 200 who had PTB later in life)
 - HIV-negative, Healthy Controls (n=600)
 - Families with at least two PTB-affected siblings (n=90)
 - HIV-positive, Active TB (n=300)
 - Active TB Patients for EBA study (n=90)
 - MDR-TB Patients (n=60)
- Sample Collection
 - DNA, blood draw, sputum, PPD, IGRA, Stool,
- Study type
 - Observational
 - EBA

Animal Model(s)

Mouse: Mice are the smallest, least expensive experimental animals in which the biology of the Mtb has been characterized. We have developed a TCR transgenic mouse that expresses a T-cell receptor specific for the Mtb immunodominant antigen ESAT-6 (ESX-1) [58, 59], which we have used to investigate the kinetics and molecular determinants of CD4⁺ T cell-mediated Mtb control.

Administrative Program Manager: Dr. Jamie Bean (MSKCC) serves as data coordinator for the Infection Biology components of the TBRU. Email: beanj@mskcc.org

Data Manager: Dr David Zhang (WCMC) serves as data coordinator of the Drug Discovery components of the TBRU supplement, formerly the CETR. Email: juz2005@med.cornell.edu