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Proceedings of Immunodiagnosis of Tuberculosis: New Questions, New Tools

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INTRODUCTION

I1

Immunodiagnosis of tuberculosis: new questions, new tools conference 2008

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Introduction: The first international meeting on new biomarkers and tools for the immunodiagnosis of *M. tuberculosis* infection and disease, entitled *Immunodiagnosis of Tuberculosis: New Questions, New Tools*, was held September 21 to 23, 2008 in Virginia Beach, Virginia, United States. The objective of the conference was to integrate the most recent knowledge generated through basic, translational, and clinical research that could lead to novel, improved immunodiagnostic tools for tuberculosis (TB). About 150 researchers from academia and the private sector attended the seminar from over 26 countries, including many developing nations. The agenda included 20 scientific presentations and a keynote address by the director of the Stop TB Department at the World Health Organization (WHO).

TB is still a major killer: one person dies from this infectious disease every 15 seconds, somewhere in the world. The interaction between the human host and the causative agent of TB, the intracellular pathogen *Mycobacterium tuberculosis*, is characterized by the ability of the host immune response to control infection without causing sterilization and of the pathogen to withstand expression of host immunity by changing its metabolic and growth state. The result is an asymptomatic, chronic (latent) infection that may last for the lifetime of the host. In 5-10% of immunocompetent, infected individuals the host immune response loses control of infection at some time in the life of the individual: tubercle bacilli resume growth, and tuberculosis (TB) develops. Diseased individuals are usually infectious some time during disease. Their timely identification is critical to the success of chemotherapy and to curbing transmission of infection. Consequently, the development of disease in latently infected individuals is a major problem for TB control, as it means that new disease foci can arise outside of identified at-risk populations.

Until recently, century-old, often inaccurate methods have been the mainstay of diagnosis of active disease and latent infection [1]. Recent effort has resulted in new immunologic tests for latent infection, while diagnosis of active disease typically relies on detecting tubercle bacilli or their products in the patient's escreate (sputum). Neither old nor new tests, however, can predict progression from latent infection to active disease, a crucial stage for treatment and infection control. The need for better diagnostics for TB has been strongly advocated by The Global Plan to Stop TB 2006-2015 (http://www.stoptb.org/global/plan), which calls for universal access to high-quality diagnosis and research towards new diagnostics. Moreover, academia, industry, governmental agencies, and private agencies have expressed a renewed interest in TB diagnosis. However, achieving new, accurate TB diagnostics faces formidable challenges that are both scientific and operational. Overcoming them requires effective multi-disciplinary communication and collaboration within the scientific community.

The international conference "*Immunodiagnosis of Tuberculosis: New Questions, New Tools*" was planned as an authoritative venue for establishing partnerships and consortia leading to the next generation of TB immunodiagnostics. The speaker roster was of world-class quality, and the unique combination of themes concerning basic and translational research, field studies, and novel technologies was designed to achieve the conference goals. With its structure of plenary sessions and poster sessions, the meeting encouraged discussion and fostered collaboration among scientists in basic research, translational research, field studies, and industry to link research on biomarker discovery to a deeper understanding of the biology of the pathogen, the immune response of the host, and the host-pathogen interactions leading to the expression of immunological markers specific for various stages of infection (latent infection, progression, and disease). Moreover, the conference aimed at establishing links between basic and translational research (i) by discussing how new markers of infection and disease identified by basic research could be evaluated in field studies and (ii) by reviewing cutting-edge technologies that could lead to a new generation of immunodiagnostic tests. The goals of the conference were accomplished by integrating (i) discussion of the biological events associated with expression of immune markers of recent infection, latent chronic infection, progression, and active disease, (ii) the identification of translational research opportunities, (iii) the presentation of potential innovative new assay formats, and (iv) the definition of challenges at the preclinical and clinical stages of test development. Particular attention was given to pediatric TB, which poses unique diagnostic challenges, since children typically present a different disease spectrum than adults and are likely express immune markers differently from adults due to the immaturity of the child's immune response.

Summary of oral presentations during the plenary sessions: Keynote address: Tuberculosis is a global health issue: Mario Raviglione, director of the World Health Organization's (WHO) Stop TB program, opened the conference with WHO's perspective on the global TB situation, discussing the status of the epidemic, progress that has been made toward eliminating this disease as a major public health problem worldwide by 2050, and the critical and urgent need to increase funding for research to achieve this goal [2]. Raviglione emphasized that a ten-year funding gap for global TB research and development has been a major impediment to scientific progress. A "TB Research Movement" has been conceived to accelerate progress, the goal of which is to stimulate

investments, support efforts, accelerate implementation, and expand the existing portfolio across the research continuum to ensure development of tools that will be conducive to global elimination of TB by 2050. [2] Its objectives are 1) to provide leadership and advocacy to mobilize increased resources in support of a coherent and comprehensive global TB research agenda, and 2) to provide a forum for funders and implementers of TB research to coordinate plans and actions, with the result of ensuring that research needs are addressed, opportunities prioritized, and gaps filled [2].

Basic TB biology: current knowledge, bottlenecks and challenges: The meeting began with a review of basic TB biology that focused on current bottlenecks and challenges in basic research. Douglas Young opened the session with a discussion of the biology of latent TB, which he argued is essential to understanding the fundamental biology of TB. He specifically addressed current knowledge about why *M. tuberculosis* is able to resist the host's ability to eradicate it over a long period of time [3]. The commonly-used definition of latent TB—antigen-specific T-cell response without clinical symptoms—is very broad and, Young argued, may in fact lead us away from the consideration that latent TB may not act the same way in every patient. [3] Young also discussed key scientific questions including whether everyone infected with latent TB harbors viable bacteria, and if so, where these bacteria are located in the body and what they are doing [3].

Markus Wenk then discussed the cell biology of intracellular pathogens that has characterized the importance of lipids at various stages in host-pathogen interactions [4]. Lipids appear to be "gatekeepers" in important chemical reactions involving cell signaling during pathogen docking, invasion, and movement in and out of cells. Lipids may be viable targets for regulation of the host-pathogen interaction [4].

Sebastien Gagnuex discussed evolutionary forces that have shaped the genetic diversity in *M. tuberculosis*, in particular natural selection, host migration, and changes in host demography [5]. JohnJoe McFadden then discussed the value of system approaches to uncover characteristics of the TB bacillus grown *in vivo* that could ultimately be useful as targets for new immunodiagnostic approaches. This approach, he argued, requires an *in silico* model of linked metabolic pathways to identify genes and phenotypes and predict their interactions [6].

Lastly, Steven Elledge presented a functional genomics approach to viral-host interactions with HIV. New screening methodologies developed over the past five years, based on the novel understanding of the biology of double-stranded DNA and RNA interference, he argued, now provide scientists with the tools to carry out genetic screens of mammalian cells to identify host functions involved in the interaction with a particular pathogen [7].

Basic TB immunology: current knowledge, bottlenecks and challenges: The second portion of the conference focused on the host side of TB, covering challenges in TB immunology. Stefan Kauffman presented research related to using the metabolic profile of the host response to TB as a basis for the rational design of vaccines and biomarkers [8]. Mark Doherty spoke about the potential for identifying biomarkers of infection, citing examples that indicated how they could be useful in separating latent and acute TB [9]. Padmini Salgame posed the question of how helminth infections, which are endemic in areas most affected by TB, affect progression to active TB or re-activation of latent TB. She discussed her work with the Th1/Th2 paradigm to further examine this relationship. Maria Gennaro spoke about how high-throughput methods have facilitated the study of antibody responses on the genome-scale, and she discussed implications of this technology for monitoring disease progression using serology and profiling the biology of the tubercle bacilli in the human host [10]. Muireann Coen discussed the potential of translating metabolomics discoveries into tools for diagnosis and systems monitoring, and new technologies in biomarker discovery [11].

Pediatric tuberculosis: Joseph Bellanti introduced the differences in the pediatric immune system that make children more likely to develop severe TB, and he cited the need for obtaining new knowledge about the relationship between immune maturation and developmental immune deficiencies associated with infection [12]. Anneke Hesseling then spoke about pediatric TB from a clinical epidemiological perspective, based on her studies in a highly endemic setting in South Africa. She emphasized the need for improved diagnostics, improved programs to prevent TB and TB/HIV co-infection, and shorter treatment regimens that target children [13].

Field studies/clinical trials: present and future: Richard Menzies critically analyzed the methods used to evaluate the effectiveness of new diagnostic tests in the field, and he outlined a standardized approach that should be used to provide the necessary evidence of safety and efficacy [14] .Sandra Arend discussed the properties of a tuberculosis-specific skin test that showed promise in preliminary studies as a safe diagnostic that produced a readable skin test response and did not easily sensitize [15]. Peter Andersen discussed the value of IGRA-based diagnosis of infection and prediction of active disease, particularly in asymptomatically infected people [16].

New technologies: diagnostic assays for resource-rich and resource-limited settings: Mark Perkins discussed the potential and limitations of seroimmunodiagnosis for case detection in the context of global disease care and control, including the need for either sensitivity or specificity in order for seroimmunodiagnostics to be useful globally. Philip Felgner discussed the use of microarray chip analysis to profile immunity to infectious diseases. His research demonstrates the usefulness of the technology to differentiate immune states during infection. His goal is to develop multi-variant microarray chips that contain several antigens. Mario Roederer discussed the application of many of his team's techniques for HIV/SIV vaccine development to TB, particularly with flow cytometry's ability to measure immune responses at the T cell subset level [17]. Abraham Lee presented novel microfluidic technologies that could be of great value for use in TB serodiagnosis or as platforms for cell and biomolecular assays [18]. These technologies have properties that may make them globally useful. They include low costs, precision, speed, and integration capabilities to assess multiple parameters per sample [18]. Lastly, Adrian Ozinsky discussed the strengths and weaknesses of an application of microfluidic tools utilizing parallel single cell assays for TB diagnostics. While the application shows potential with its sensitivity, more research is needed to further define correlates of immunity and identify relevant cells for measurement.

Research recommendations that emerged from the conference:

Research priorities:

- *Diagnostic tools that differentiate latent M. tuberculosis infection from acute infection*
- *Diagnostic tools that predict durable cure during therapy and risk of relapse after therapy*
- *Rapid, point of care identification of drug-resistant TB*
- *Prognostic tools to predict risk of reactivation or progress to disease*
- *Biomarkers predicting vaccine efficacy/ correlates of protection*

Areas for innovation and partnerships and cooperation:

- *Leverage existing and planned clinical trials to test potential biomarkers of disease and protection*
- *Leverage new screening methodologies developed over the past five years for genetic screens of mammalian cells*
- *Learn from HIV vaccine development*
- *Leverage technology such as microfluidics to improve field diagnostic capabilities*
- *Establish systems biology partnerships with an emphasis on metabolomics to identify novel biomarkers*

Conclusions: The recommendations above highlight issues that are critical for research required to provide new tools for TB diagnosis. The conference also acknowledged that "turning research into tools" requires acknowledging and correcting the limited success (or even outright failure) experienced with the deployment of technologies to the field. Recent advances in TB diagnosis have produced tools that were not designed with pricing or performance characteristics suitable for developing countries. In addition, we need to recognize that market forces have typically failed to deliver products that are primarily needed for poor patients in resource-limited countries; we need to develop pricing mechanisms or supply strategies to address this. Moreover, the experience of HIV and malaria, two global diseases for which cheap, simple, point-of-care tests are available, has revealed the many weaknesses of the developing countries' healthcare systems in management, financial and human resources, laboratory capacity, and quality assurance. Thus, public-private partnerships need to be supported to move forward technology in areas where private initiatives have not emerged (for example, simple point-of-care diagnostics) and existing diagnostic capacity needs to be identified and strengthened.

To carry out research, development, and deployment of new diagnostics, additional financial resources need to be identified. The Global Plan to Stop TB 2006–2015 estimated that at least 516 million USD are needed to strengthen TB diagnostics, yet TB diagnostics development received only a tenth of that, according to the 2009 Treatment Action Group and Stop TB Partnership reports [19]. While expanded funding might seem unrealistic at a time of financial stress, it should be considered that TB prevention would be many times cheaper than the current – unsuccessful – strategy of treating the disease as it is found. Consequently, failure to invest in TB control now will cost us dearly in the future. Many public-private initiatives are currently working in the right direction. The Global Laboratory Initiative is leading plans for a large expansion of laboratory services for TB, with partners such as UNITAID and PEPFAR providing tens of millions of dollars in funding for a program called EXPAND-TB that will supply rapid MDR-TB diagnostics to 27 high-burden countries [20]. A "TB Research Movement" has been conceived to accelerate progress, the goal of which is to stimulate investments, support efforts, accelerate implementation, and expand the existing portfolio across the research continuum to ensure development of those tools that will be conducive to global elimination of TB by 2050 [2]. Its objectives are to provide leadership and advocacy that will mobilize increased resources in support of a coherent and comprehensive global TB research agenda and to provide a forum for funders and implementers of TB research to coordinate plans and actions, with the result of ensuring that research needs are addressed, opportunities prioritized, and gaps filled [2]. Finally, emerging economies such as Brazil, Russia, India, and China are taking action to increase their investments in tuberculosis, realizing that (i) they account for a large proportion of the global tuberculosis burden, and (ii) locally-manufactured, low-cost tuberculosis diagnostics are viable products for their emerging pharmaceutical industries.

The "*Immunodiagnosis of Tuberculosis: New Questions, New Tools*" conference successfully fostered interactions between basic scientists and clinical investigators, research and implementation communities, and academia and industry. It also included participation of government agencies, TB research and development partnerships, intergovernmental organizations, and funding agencies promoting research and policy making in global health. Furthermore, the participation of a diverse audience from a global arena made it possible to convey within the conference viewpoints and needs expressed from worldwide communities. Similar future initiatives are expected to greatly contribute to attracting researchers in academia and industry to TB research, to fostering communication between research and implementation communities, and to promoting formation of intergovernmental working groups to tackle neglected areas that are critical for TB control.

Competing interests: The authors declare that they have no competing interests.

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We thank the Meeting Scientific Committee (MSC) for the meeting for helping create a novel, interdisciplinary program and contribute to the success of the conference. The MSC members were: Philip L. Felgner, University of California, Irvine, USA; Anneke Hesseling of Stellenbosch University, Stellenbosch, South Africa; Stefan H.E. Kaufmann, Max Planck Institute for Infection Biology, Berlin, Germany; Richard Menzies, McGill University, Montreal, Quebec, Canada; Mario Raviglione, Stop TB World Health Organization, Geneva, Switzerland; Mario Roederer, National Institutes of Health, Bethesda, Maryland, USA; and Douglas Young, Imperial College, London, United Kingdom.

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ORAL PRESENTATIONS

O1

Tuberculosis is a global health issue: challenges and need for new tools

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Every year, more than 9 million people are affected by tuberculosis (TB) and 1.8 million die from it. Developing countries are heavily affected. The highest rates per capita are in Africa, but the largest numbers are in Asia, with nearly 60% of the global burden. TB associated with HIV/AIDS is a huge challenge in Africa and multidrug-resistant TB (MDR-TB) is frequent in the former USSR and parts of Asia [1].

The United Nations Millennium Development Goal (MDG) related to TB control aims to halt and begin to reverse the incidence of TB by 2015, and the STOP TB Partnership targets elimination of TB as a major public health problem worldwide by 2050 [2].

The World Health Organization (WHO) has developed a six-point Stop TB Strategy to reach the global targets: (i) pursue high-quality DOTS expansion and enhancement; (ii) address TB/HIV, MDR-TB and other challenges; (iii) contribute to strengthening health systems; (iv) engage all care providers; (v) empower patients with TB; and (vi) enable and promote research [3].

Progress has been made to date toward achieving the global targets. Compared to performance of control programmes in the mid-1990s, up to 6 million human lives have been saved through the implementation of DOTS, later enhanced to the Stop TB Strategy, since 1995. The global incidence of TB has peaked in 2004, resulting in an earlier achievement of the MDG related to TB control. However, the decline since then has been less than 1% per year, which is insufficient to seriously target elimination by 2050 [1].

Projections show that, even with the optimization of the current control efforts promoted by the Stop TB Strategy using the tools available today, efforts will result in an incidence of TB by 2050 that is much reduced compared to today, but about 100 times higher than the elimination target. Thus, this target cannot be achieved without addressing critical and urgent needs, such as that interrupting transmission focusing on early case detection among vulnerable groups and accelerating

innovation for increased access to the best tools available. Furthermore, new tools are needed if elimination is to be targeted assertively. Today in much of the world, the tool most commonly used to diagnose TB, sputum smear microscopy, is more than 100 years old, detects only half of the TB cases and is often ineffective in diagnosing TB in people with HIV. Rapid molecular tests for drug resistance, although already available, are not widely available in the field. Drugs for treatment, which are decades old, are not compatible with some anti-retrovirals and the treatments for MDR-TB are lengthy, expensive, and toxic. The only vaccine, Bacillus Calmette-Guérin (BCG), that is more than 85 years old, protects infants from the disseminated forms of TB, but provides unreliable protection against adult pulmonary TB – the main source of transmission – and, as a result, has had little impact on the global epidemic.

Therefore, diagnostics must be developed to provide faster detection of all forms of TB (smear positive, smear negative, and MDR-TB) and be useable at point of care. They must provide faster results, and be more sensitive and simpler than microscopy to make them useable. Shorter treatment regimens and more effective MDR-TB care regimens are priorities for drug development. An ideal candidate would shorten the treatment from the current six months to less than two months; have a novel mechanism of action; not interact with anti-retrovirals; be taken orally once daily or intermittently; and be low in cost. The vaccine development pipeline, which is focused on both pre and/or post-infection vaccines, is still weak in research needed for clinical trial activation.

The priorities to achieve success must include expanding financing for TB research and development partnerships; attracting more researchers in academia and industry into the TB research field; fostering more communication between research and implementation communities; and promoting formation of intergovernmental working groups to expand links across neglected critical areas for health research. The constraints include a recent no-growth in financing from Europe and North America for critical research and development [4]; limited incentives for the private sector to engage more broadly; a lack of clinical trial and manufacturing capacity; and limited operational research capacity and financing.

A "TB Research Movement" has been conceived, the goal of which is to stimulate investments, support efforts, accelerate implementation, and expand the existing portfolio across the research continuum to ensure development of those tools that will be conducive to global elimination of TB by 2050. Its objectives are to provide leadership and advocacy to mobilize increased resources in support of a coherent and comprehensive global TB research agenda; and to provide a forum for funders and implementers of TB research to coordinate plans and actions, with the result of ensuring that research needs are addressed, opportunities prioritized, and gaps filled.

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O2

The diversity of latent TB

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During the second half of the twentieth century, researchers have been focused on breaking down science into small pieces, and in doing so have learned that understanding the small pieces does not necessarily translate into a greater understanding of the whole. Systems biology has emerged as a way of understanding these systems in total, but requires scientists to work through complexity to arrive at a greater understanding. To understand the fundamental biology of TB, scientists must first understand the biology of latent TB: why the bacteria are able to resist the host's ability to eradicate them over a long period of time. The commonly-used definition of latent TB—antigen-specific T-cell response without clinical symptoms—is very broad and may in fact lead us away from the consideration that latent TB may not be the same in every patient. Does everyone infected with latent TB harbor viable bacteria? Where are these bacteria in the body and what are they doing?

As part of the Grand Challenges in Global Health Program, a Gates Foundation-funded initiative to develop drugs for latent TB, our team is working with a hypoxia model, based on the idea that the bacteria encounters an environment (hypoxia) in the host which is hostile. In response, the bacteria may switch to a slowly-replicating persistent phenotype that makes them more drug-tolerant. We are also using live-imaging to study lesions in active latent TB in both humans and primates to identify what the bacteria are doing in different types of lesions, why some bacteria are immunologically active and others dormant.

Based on our findings to date, biomarkers in addition to T-cell response must be identified in order to develop a better understanding of what is happening in patients with latent TB. We hypothesize that latent TB encompasses a broad spectrum of responses in patients, from the infection being eliminated without priming antigen-specific T cells, through the infection being controlled with some bacteria persisting but reduced replication, to some bacterial replication being maintained at a subclinical immune response. Our theory is that the infection in patients with latent TB can move along this spectrum over the course of time. With additional biomarkers—such as ones measuring bacterial load or cell surface markers—subsets of patients can be identified who would benefit from preventive therapy, making this a more realistic and effective approach in the goal of eliminating TB by 2050.

O3

Lipidomics in biomarker development

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Research into the cell biology of intracellular pathogens has provided information on host-pathogen interactions and on the importance of lipids at various stages in the interactions. Lipids are "gatekeepers" in important chemical reactions involving cell signaling during pathogen docking, invasion and traffic in and out of cells. Working with the Novartis Institute for Tropical Diseases, our team is exploring how TB takes advantage of normal cellular processes by using its own lipids to subvert the host's lipid signaling - allowing it to enter the host cell and survive there. Our focus is on developing host and pathogen biomarkers that correlate with TB infection in laboratory animals and in humans. We are performing mass spectrometry-based multi-marker quantification of lipids in tissue and body fluids (blood, urine, sputum), specifically host lipids (e.g. phospholipids and ceramides) and pathogen-derived lipids (e.g. mycolates, LAM, etc.). Our research is focused on laboratory animals and TB patients in clinical trials, to be used both for drug efficacy studies as well as case detection and monitoring of treatment response. For analysis of the host response, lipids are extracted from infected tissues and cells, isolated and analyzed by mass spectrometry to develop a lipid profile, which is used to identify the metabolic pathways involved in the infection and the enzyme/protein that facilitates the process. This technique is very sensitive - a drop of blood is potentially enough to perform the analysis. For pathogen biomarker discovery, mycobacterial lipids are isolated directly from small volumes of infected body fluids such as sputum and subsequently quantified via mass spectrometry. We expect this research will be (i) useful in providing a better description of mycobacterial biosynthetic activity, which will be helpful in developing new diagnostics, drugs and vaccines, and (ii) for the development of lipid biomarker for TB case detection.

O4

Evolutionary forces in *Mycobacterium tuberculosis*: implications for product development

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Research on the evolutionary forces that have shaped the genetic diversity in *Mycobacterium tuberculosis* (M.tb), in particular natural selection, random genetic drift, migration, and changes in demography have important implications for product development. Until now, scientists have not been able to quantify the genetic differences among M.tb groups. By tracking genomic deletions, six major lineages of M.tb have been identified in humans, with distinct geographical distributions. The Asian M.tb lineages cause the highest global TB burden, but most research has been done on Euro-American strains. Our team has performed a multi-locus sequencing analysis of 89 genes in 99 strains of human M.tb, eight strains of animal-adapted strains and the distantly-related *M. canettii*. We have worked to identify the sources of diversity in human-adapted M.tb and the evolutionary forces that shape this diversity. Using the DNA sequences of the 89 selected genes as a basis for a comparison, then we constructed a phylogentic tree which supported the M.tb groupings defined earlier by genomic deletion analyses. However, using DNA sequence data, actual genetic distances could be computed that suggested there was as much difference between some human strains as between M. bovis and some human M.tb strains. Our research has also determined that the six human-adapted M.tb lineages are part of two main clades, an evolutionary "ancient" clade found in the Philippines, around the Indian Ocean, and in West Africa, and an evolutionary "modern" clade with a much wider geographic distribution.

Additionally, the role of selection in M.tb evolution was analyzed through the ratio per site of non-synonymous nucleotide substitutions (dN), which change the amino acid sequence, to synonymous nucleotide substitutions (dS) that do not result in a change in the amino acid sequence (dN/dS). The dN/dS in M.tb was 0.57, much higher than most other bacteria. We explored three possible reasons for this high dN/dS: 1) M.tb is a recently-evolved pathogen and insufficient time has occurred for natural selection to remove non-synonymous single nucleotide polymorphism (nSNP) mutations which are likely to be deleterious, 2) the host immune system is selecting for variability in M.tb to evade the host immune responses, or 3) natural selection against nSNPs is reduced. Our team concluded that the latter explanation was the most likely as we found that 58% of non-synonymous changes in M.tb occurred in regions of proteins that were highly conserved in other mycobacterial species. This suggests that purifying selection against nSNPs is reduced in M.tb compared to other bacteria, and as a consequence many of these nSNPs are likely to impact protein function.

O5

Systems approaches to uncovering *in vivo* state of the TB bacillus

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Research related to system approaches to uncovering the characteristics of the TB bacillus grown *in vivo* could ultimately be useful in discovering new immunodiagnostic approaches. There are important differences between a classic molecular biology view of genes and phenotypes (one gene/one enzyme) and the systems-level vision which focuses on identifying all the interactions between genes and phenotypes. The systems approach requires an *in silico* model to identify all the possible genes and phenotypes. We have constructed such a model and are using it to investigate differences between *in vivo Mycobacterium tuberculosis* (M.tb) and *in vitro* M.tb. It is known that *in vitro* M.tb behaves differently from *in vivo* M.tb. For instance, M.tb *in vitro* is relatively easy to rapidly kill with a single drug. Not *in vivo*, however: the latter gives a two-hit pattern, suggesting that a subpopulation of the bacteria is harder to kill, even though they are still genetically susceptible. Is this due to different gene expression patterns? If so, how do we identify these? The genes that are specific to *in vivo* TB may be useful in distinguishing between infection and exposure and may be promising targets for new drugs or imunodiagnostics. Using a systems biology approach, our team has developed an approach that could be used to identify these antigens.

We found some aspects of the *in vivo* state could be simulated *in vitro*, using a culture system called a chemostat with a glycerol substrate to control the growth rate of the bacillus. At a slow rate, the bacillus developed phenotypic drug tolerance. We then developed an *in silico* genome-scale model, basically a mathematical model of linked metabolic pathways to predict how genes interact to generate the metabolism of the cell. The model was tested by comparing gene essentiality predictions with experimental data where it was shown that the *in silico* model generated 78% correct predictions. It was also able to predict the existence of a novel metabolic pathway operating in slow-growing M.tb. The M.tb model can also be used to interrogate transcriptome date to identify signal metabolites. Our research indicates that a systems approach may prove useful in identifying novel immunodiagnostics for determining whether a patient is infected with active or latent TB. However, development of such immunodiagnostics will first require identification of differences between M.tb growing in acute and chronic lesions. Systems-based approaches, such as we have described here, could be used to identify such differences.

O6

A functional genomics approach to viral-host interactions for HIV

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New screening methodologies developed over the past five years, based on a revolution in understanding the biology of double-stranded DNA and RNA interference, provide the tools for genetic screening of mammalian cells. HIV is a straightforward RNA virus, and the challenge our team has tackled is to identify the host proteins the virus needs to replicate using short strands of small interfering RNA (siRNA) to block their function. The siRNA screen is an arrayed format—siRNAs to one gene per well—using liquid-handling robots. Cells are transiently transfected with siRNAs, producing protein knock-down in a gene-specific manner that is effective for about a week. Using a SMARTpool library produced by Dharmacon with four siRNAs per pool and 21,121 genes, we completed a validation round with the four individual siRNAs.

Seventy-two hours after transecting the cells with siRNA, infected cells and supernatant were harvested, and the viral load measured. The cellular analysis indicated how well the virus had infected the cell and expressed a key capsid protein p24 protein; the supernatant indicated how efficiently the virus budded out of the cell in a functional form. We discovered several proteins that appear to be genetically important to the HIV lifecycle. Using bioinformatics, we were able to map out the role the host factors played in the viral life cycle, from the virus getting into the cell, to its integration into the DNA of the cell's nucleus, to the messenger RNA (mRNA) processing of the virus; and to its export into the cytoplasm and out of the cell. Genetic screening results provided a rediscovery of all known host dependency factors in the HIV lifecycle states and confirmation of Host Dependency Factors (Rab6, Vps53, TNPO3, Med28), suggesting the model is effective.

One of the proteins identified in the screen (TPNO3), a karyopherin, is likely to be the host factor that permits HIV access to our nucleus for integration into our genome. Our research demonstrates that when TPNO3 was knocked out by any one of eight siRNAs, the virus was prevented from infecting the cell and integrating into the genome. Utilizing unbiased whole genome screens - which can be used for any pathogen, to identify host proteins that actively support replication - could provide new drug targets. The way the screen is designed can profoundly influence which genes are uncovered and different screening platforms will yield different results, making the technique potentially very flexible.

O7

Host response to tuberculosis as basis for rational design of vaccines and biomarkers

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The current diagnostic techniques are not effective in key areas of tuberculosis (TB) research, such as predicting susceptibility, treatment outcome, and monitoring the effectiveness of drug or vaccine trials. Hence, more emphasis is to be placed on biomarker research. A biomarker is a characteristic feature that is objectively measured and evaluated as an indicator of a biological or pathological process or a response to therapy or prevention. Peripheral blood cells may not provide an accurate picture of what is happening in a TB patient at the site of disease manifestation, i.e., the granuloma. The fact that granulomas reach a point where they become largely autonomous and the immune response controls itself in the granuloma, has presented challenges. To address this point, our team infected splenectomized lymphotoxin β-receptor-deficient mice, which have no secondary lymphoid organs, with a small dose of *Mycobacterium tuberculosis (M.tb)*. Without lymph nodes and spleen, the mice mounted an immune response and survived. Upon further analysis, CD8 and CD4 T-cells were generated in the granulomas as well as central memory and multifunctional T cells. Mice were then infected with *M.tb*, cured with antibiotics and then reinfected. When reinfected mice were compared to mice with a primary infection, we found that protective memory had developed. We conclude that granulomas can develop an immune response in the absence of lymph nodes and spleen. Moreover, in a study of TB lesions in humans, we found that the same appears to occur.
Using metabolomics, and working with the company Metabalon, we examined a combination of metabolites in latently infected individuals and patients with active TB disease to define a profile to differentiate healthy infected from diseased individuals. Diagnoses made by metabolomics profiles were equally accurate as clinical diagnoses. Metabolomics was also used to identify markers for cell exhaustion, which can lead to reinfection. In particular, we studied the metabolite tryptophan, the degradation of which is involved in suppressive mechanisms. These studies were complemented using transcriptomics. Dendritic cells were stimulated with ManLam, a bacterial cell wall lipid component that binds to DC-SIGN, a host cell receptor known to be involved in immunosupression. This binding also caused the cells to over-express the enzymes involved in the degradation of tryptophan. We consider this pathway to be involved in reactivation of TB and sustenance of active disease.

O8

Separating latent and acute TB

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In theory, immunodiagnosis should be able to discriminate between latent and acute TB, because the immunopathoogic nature of the disease means there must be significant differences in either the type and/or magnitude of immune response that develop. But so far we have not been able to pinpoint them. Our investigations of immune responses after infection suggest that in a substantial number of former TB patients, strong antigen recognition persists at least 20 years post-treatment without signs of recurrent illness. In addition, antigen-specific CD4 and CD8 memory T-cells could be detected for at least two years post-infection. In a study published in 2001, we found that healthy household contacts who responded most strongly to ESAT-6 were more likely to progress to disease in a short time, but others who showed changes in their X-rays (meaning that they had been infected and even developed some pathology) remained otherwise healthy. An important question is whether this group is representative of natural latent infection. If that is so, similar people should be detectable in the general population and so our team looked at ESAT-6 responses in community controls. We found that although the responses to ESAT-6 were lower overall in the general population, a substantial subgroup were strongly positive. Our team's assumption is that these individuals have latent disease.
Given the results of our research, we have developed a model for analyzing latent TB infection. After initial exposure, two-thirds of the individuals exposed to TB do not apparently skin-test convert nor become ESAT-6 positive: if they have had an infection, early bacterial growth was presumably arrested fairly quickly. It is not possible to tell if they have latent TB because immunologically, they cannot be distinguished from uninfected individuals. Of the remaining individuals exposed, roughly five percent experience early bacterial growth that is not contained and, if not treated, progresses to clinical illness. In the remainder, subsequent bacterial growth is contained, symptoms abate, but latent infection is established and approximately two percent experience re-activation of the disease at a later time. The rest remain healthy, but have latent infection, which is discernible because immunologically these individuals tend to express elevated levels of interferon-gamma (IFN-γ), interleukin-12 (IL-12), IL-4 and IL-482 and strongly recognize latency antigens. These individuals are the focus of intense study because they represent natural immunity in action.

O9

Genome-scale antibody responses in TB

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High-throughput methods have made it possible to generate microarrays carrying all ~4,000 proteins of *MMycobacterium tuberculosis (M.tb)*. With this platform, large numbers of individual sera from infected humans and animals can be tested for antibody detection. Preliminary results of probing *M.tb* proteome microarrays with sera collected under a uniform study protocol at various sites worldwide from > 500 pulmonary TB suspects who received a final diagnosis of active TB or non-TB-disease (NTBD) showed that approximately 500 *M. tb* proteins reacted with at least one serum, while the rest of the proteome reacted to none. Thus the immunoproteome of *M.tb* is approximately one-tenth the size of the total proteome. It was also found that, among the ~500 reactive proteins, only a fraction reacted differentially to active TB sera versus NTBD sera. It is intuitive that serodiagnostic antigens belong to this antigen subset. Additionally, no obvious pattern of reactivity was observed among active TB sera. This result indicates that, within the pool of seroreactive antigens, target recognition greatly varies from individual to individual. We also explored the association between proteome-scale antibody responses and clinical and demographic covariates, such as patients' age and gender, country of origin, bacillary burden (expressed as sputum smear grade), HIV status, etc. In particular, we found that the antibody response to some antigens positively correlated with sputum smear grade, while the response to others was independent of smear grade. The previously reported positive correlation between reactivity to the 38 kDa antigen and bacillary burden supports the proteome-wide results. We also observed that, while antibody profiles varied among sera, antigens exhibiting the highest differential ability were recognized across geographical sites. Thus, it should be possible to identify serodiagnostic antigens that can be utilized worldwide. As previously reported, HIV co-infection dampened the antibody response. Moreover, results of protein class analysis showed that reactivity to the glyoxylate bypass proteins was associated with active TB. This metabolic pathway is essential for *M.tb* growth and survival in mice, and is up-regulated in tubercle bacilli found in human sputa. Thus, the analysis of antibody responses at the proteome scale can be informative of the biology of tubercle bacilli in the human host. In addition, proteome microarray probing with sera from experimentally infected macaques showed that antibody responses vary with progression to disease, supporting the possibility that disease progression can be monitored by serology.

O10

Translation of metabolite profiling to infectious diseases

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The field of metabonomics involves generation of complex metabolic profiles of tissues and biofluids using analytical platforms such as nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). Metabolic profiles can reflect changes following an intervention, such as administration of a drug or onset of a disease. Multivariate modeling tools are applied to discriminate profiles and identify biomarkers that differentiate between phenotypes of interest such as control and diseased states. One advantage of metabonomics is that it is a 'top-down' approach that reflects an integrated systems response as it takes into account genetic and environmental status. A variety of analytical technologies can be utilized; however NMR spectroscopy and MS represent the most powerful and widely used tools as they can capture information on thousands of metabolites in a biofluid in a single analytical run. The challenge of extracting relevant metabolic information from the complex spectral data sets is tackled using pattern recognition tools that reduce the dimensionality of the data, such as principal components analysis (PCA) and partial least squares regression (PLS) and many novel statistically-driven approaches have been developed in recent years.

An example of the application of metabonomics to differentiate cerebrospinal fluid (CSF) samples from clinical bacterial and viral meningitis cases was presented. The application of NMR-based metabolic profiling greatly improved the speed and specificity of sample classification. In addition, patterns of CSF metabolites that altered following bacterial and viral meningitis infection were identifed. Furthermore, gram-stain negative samples of meningococcal meningitis were clearly differentiated from all other cases of bacterial meningitis. A larger study is intended to validate these findings and to also incorporate metabolic profiling of non-invasively collected urine rather than CSF.

In terms of diagnostics, metabonomics has many diverse application potentials and could be useful in studying the metabolic signature reflecting host response, in identifying pathogen-derived metabolites, and in differentiating single infection versus co-infection. It may prove a powerful tool for providing systems level insight into mechanisms of pathology associated with infectious diseases, such as Mycobacterium tuberculosis. With respect to therapeutic monitoring, both in vitro and in vivo metabonomic-led approaches could offer insight into the response of both the pathogen and host to intervention, drug resistance, therapeutic efficacy and toxicology.

O11

The child's immune system and pediatric tuberculosis

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That children are more likely develop a severe form of TB is reflective of the differences in the maturational stages of their immune systems, but a paucity of data is available about how this system matures and what the relationship of these developmental immune deficiencies are with infection. Maturational deficiencies in the adaptive and innate immune systems in infants and young children may result in immature macrophage and DC function; Th1-type responses to pathogens; and a propensity to develop Th2-type CD4 T-cells in response to immunogens.

In vitro responses of two groups of TST positive children at risk for TB were examined by comparing Enzyme-Linked ImmunoSorbent Assay (ELISA)-based IGRAs with clinical and TST findings. Age-related changes in the immune capacity for specific and mitogen-induced IFN-γ production was also examined in these two groups. In the original guidelines for the use of QFT-G, the US Centers for Disease Control and Prevention recommended that additional studies were needed, especially in children under five years of age, both to establish the validity of the assay as a diagnostic tool in the younger age group and to compare the accuracy of the test with the TST for diagnosing active and latent TB. Of TST positive US children, 10 of the 196 (5%) were found to be QFT-G positive; nine had an indeterminate response; of the 130 children from the Philippines who had been immunized with BCG, 115 were TST positive, and seven were also QFT-G positive. In BCG immunized children, all were TST positive and BCG-immunized and because of a presumptive diagnosis of TB, all had received anti-TB therapy; of the 30 children, 14 (47%) were found to be QFT-G-IT positive and one had an indeterminate response. For the study of variations in age-related immune capacity, the capacity of IFN-γ production was measured in various age groups of children in response to specific TB peptides (ESAT-6, CFP-10 and TB7.7) as well as to mitogen in amounts used in the QFT-G and the QFT-G-IT assay kits that had been provided. Both studies suggest that ELISA QFT-G and QFT-G-IT assays are useful for diagnosing TB in children and that adequate IFN-γ production was observed in all children in both groups following lymphocyte stimulation by either purified TB peptides or mitogen including those less than 5 years of age.

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O12

Pediatric tuberculosis: clinical and epidemiological reflections from a highly endemic setting

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Pediatric TB provides unique opportunities to study TB disease epidemiology. Improved diagnostics are of key importance in order to address many of the challenges posed by TB in children. In the Western Cape Province in South Africa, the TB notification rate was more than 600 out of 100,000 for children aged 0-14 years in 2007; the maternal HIV prevalence was 15%, with a good vertical preventative program. The HIV transmission rate was 4-5%; 99% of neonates routinely receive BCG vaccination at birth. Isoniazid prophylaxis, shown to be effective in the prevention of TB in young children, is seldom initiated and many missed opportunities for preventive therapy exist. In children admitted to hospital, mycobacterial culture is routinely done for TB in children less than five years of age through gastric aspiration of stomach contents in this setting; the culture yield is 30-40% in children with clinically suspected TB.

In a recent study of infants admitted to the three Cape Town hospitals over a three-year period, 55% of children born to HIV positive mothers had a household contact with TB, indicating a high level of TB exposure. A high risk of serious BCG vaccine complications exists in HIV-positive infants in the form of disseminated BCG disease, with an incidence of 992 per 100 000 (95% CI: 567-1495). More research is needed to determine if BCG vaccination protects against TB in infants born to HIV-infected women based on the high risk in the excessively high TB incidence (more than 20-fold higher) in HIV-infected infants.

Because scientists have improved techniques for isolating *Mycobacterium tuberculosis* in children and increasing the yield, it is possible to identify the strains from infected children, which can impact diagnosis and treatment. Our team has developed a protein-enriched broth that increases yield and decreases the time to detection. Pediatric TB also offers an opportunity to look at the emergence of drug resistant TB strains. In three DST surveys conducted in Cape Town (1997 through 2007) results indicated a significant increase in both drug and multi-drug

resistant cases of TB in children. Examination of genotypes in 400 culture positive children showed the most prevalent form to be the *Beijing* strain, thought to have the greatest probability of progressing from TB infection to disease and with distinct disease forms. Programs to prevent TB and TB/HIV co-infection in children and adults must be improved; and safer vaccines, better diagnostics and shorter treatment regimens developed targeting children.

O13

Evaluating new diagnostic tests in the field – are we doing it right?

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Recent systematic reviews of diagnostic studies have reported frequent methodological shortcomings. These include failure to report participants' age and gender, state of disease, and study eligibility criteria, as well as use of inappropriate study design, and lack of appropriate blinding. Studies with adequate design should clearly define the reference standard for the study including definitions of disease and non-disease states. The reference standard should be well-accepted and reproducible, have cut-points that are defined before-hand, and should not incorporate the results of the test being evaluated. The study should assess the tests in patients who have not yet received treatment unless it is certain that treatment does not affect test results. Those performoing the test should be blinded to the clinical diagnoses and while those making the clinical diagnoses should be blinded to test results. The analysis should use a 2x2 table that can evaluate sensitivity/specificity, predictive value and likelihood ratio.

Evaluation of a new diagnostic tool should follow a standardized approach with four phases, similar to the evaluation for testing new medications to provide the evidence needed for FDA licensing. Phase one studies should provide information on technical aspects of the test, including its reproducibility, as well as the expected range of values when the test is performed in normal healthy subjects. Participants should be healthy volunteers although gender, age and ethnic origin should be considered when selecting these volunteers. Phase two studies are of two types: Phase 2a is a case control study in which the test is performed in patients with disease and healthy controls. Phase 2B is a cohort study; consecutive patients who are being investigated for the disease or condition which the new test is intended to diagnose are enrolled. Case control studies are essentially proof of concept studies, as they will over-estimate test accuracy - results in practice are usually worse than in case-control studies due to spectrum bias. The cohort design should be prospective and the population enrolled should be suspected of having the disease and should be a consecutive series, or randomly selected patients. No exclusions, due to a failed test or unclear diagnosis, should be permitted. Phase three studies are randomized trials. Patients suspected of having the disease, are randomized to receive the test, or not. Unfortunately these studies pose several ethical issues, and are not well accepted by provider and patients - often resulting in a highly selected study sample. Phase four studies should provide information from a societal perspective—cost effectiveness, disease modeling, and data base studies.

O14

A specific skin test: the best for both worlds?

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For a new tuberculin skin test (TST) to be considered useful, it must meet several requirements including safety, a readable skin response, affordability, a high predictive value, and it cannot easily sensitize, depend on patient characteristics, nor depend on the *Mycobacterium tuberculosis* isolate. After review of previously conducted animal model studies, we designed a double-blind randomized Phase 1 study comparing recombinant dimer ESAT-6 (rdESAT-6, Statens Serum Institut, Copenhagen, Denmark) to tuberculin as a skin test reagent in the diagnosis of TB. The goals of the study were to assess the safety of an intradermal method of administering the test and to determine the appropriate human dose. The study compared the administration of 2.0 TU PPD (standard dose of RT 23) to four doses of rdESAT-6 (0.01, 0.1, 1, and 10μg). The protocol also called for intra-subject randomization of the left and right forearm. Participants were enrolled in eight different groups. Four groups comprised five healthy controls each for the purpose of testing safety and sensitization issues. The other four groups comprised patients who had been treated for TB in order to determine safety and optimal dose. Several exclusion criteria were applied, including individuals who had received a TST test within the past year or had a known immune deficiency. During the 28-day trial, the guidelines called for 2 hours of close observation after administering the test on Day 0; clinical parameters, photography on Days 1 to 4 with quantiFERON testing of control subjects on Day 2 and lab and urine tests on Day 4; diary keeping on Days 5 to 28 with physical exam, lab and urine tests on Day 28 and quantiFERON testing of control groups.

In control groups, all four dosages of rdESAT-6 were administered with no serious side effects; transient redness occurred at 24 hours at the 10 μg dose; and no indication of sensitization was measured *in vitro*. In the treated TB group, only doses of 0.01 and 0.1 of rdESAT-6 were well-tolerated; equivalent responses to PPD and 0.1 μg rdESAT-6 were observed. Participants reported significant local side effects at 1 μg of rdESAT-6. Results indicate that the new skin test is safe, produced a readable skin test response, did not easily sensitize, and is robust in all practical aspects. Further studies must be conducted on subjects ranging from individuals with active and latent TB, children, pregnant women, and patients who are immunocompromised.

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O15

IGRA based diagnosis of infection and prediction of disease

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Can IGRA assays, used in the highly specific and sensitive quantiFERON and T-spot tests, predict the development of disease in individuals who are infected but currently display no symptoms? High ESAT-6 reactivity may predict disease because ESAT-6 is a marker for bacterial burden. We found that vaccinated cattle for which the vaccine did not offer protection displayed high reactivity to ESAT-6 early in infection; cattle that controlled the infection displayed low ESAT-6 reactivity. By evaluating the response in guinea pigs both vaccinated and not vaccinated with BCG, we found that the animals with a large skin test result (high reactivity) after infection with *Mycobacterium tuberculosis* did not have a long survival time. In mouse vaccination studies ESAT-6 reactivity dropped as the vaccine controlled bacterial activation, which indicates that ESAT-6 reactivity correlates with the dynamics of infection.

For humans, we developed a template to use as a cut-off or conversion model for predicting three possible scenarios for individuals post-exposure. The model, based on IFN-γ levels in response to ESAT-6, delineates three possible reactions: people who control initial bacterial replication and remain ESAT-6 negative; people who fail to control initial replication, but eventually control the infection, becoming ESAT-6 positive and latently infected; people who fail to control replication, become ESAT-6 positive and later develop clinical TB. A large study with serial quantitative IGRA testing is necessary to be able to make a statistically robust ROC curve.

ESAT-6/CFP10 has great value as a predictor of TB disease. In low/meso-endemic regions, ESAT-6/CFP10 predicts progression to disease with higher accuracy than PPD, resulting in more precise targeting, preventive

therapy and less treatment. In high endemic regions, the potential for TB prediction may depend on the establishment of a cut-off or QFT conversion that would allow the identification of QFT positive individuals at the highest risk of progression. If longitudinal monitoring of ESAT-6 reactivity levels is used as a biomarker of bacterial replication, it can also be useful as a clinical endpoint, allowing for much shorter clinical trials of both vaccines and novel drugs.

O16

TB immunodiagnosis in context of global disease care and control

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The persistence of the tuberculosis epidemic depends upon ongoing transmission from undetected and untreated cases. Unfortunately, though high cure rates have now been achieved in many countries, globally case detection is delayed and incomplete, both because diagnostic testing is relatively hard to access, and because it relies on inadequate technologies. Fewer than half of all TB cases are thought to be detectable by microscopy even if all suspects were appropriately examined. Delays in diagnosis are common, and patients are often ill for 4 to 8 months before they are detected and treated. During this delay, morbidity accrues and transmission to household and community contacts continues.
To improve TB case detection, new technologies are needed which are more rapid than culture and that are either highly sensitive, such as new-generation molecular tests, or simple enough to use that they can be widely available, even if imperfectly sensitive. Lateral flow immunoassays are among the simplest clinical diagnostic tests to perform, and are routinely used outside laboratory settings to detect conditions such as pregnancy and malaria. The availability of a well-performing seroimmunodiagnostic test for tuberculosis on a lateral flow platform could vastly simplify case detection and improve TB care and control if testing. Such assays are relatively simple to develop, and in fact more than two dozen such tests are marketed in the private sector, primarily in Asia. Unfortunately, of the many commercially-manufactured tests currently available to detect anti-TB antibody responses, none are accurate enough to be recommended for use in disease control programs. A limited number of TB antigens, however, have been explored for their diagnostic potential, and the on-going discovery of new antigens may allow development of improved serodiagnostic tests. To this end, FIND is collaborating with a team of scientists to interrogate the entire TB proteome to identify antigen candidates for new tests.
In collaboration with Felgner and colleagues (Antigen Discovery, Inc.), microarrays containing the entire TB proteome have been created using a high-throughput cloning and expression system. At PHRI, Gennaro and colleagues have used these arrays to screen serum samples from TB patients with and without concomitant HIV infection, and appropriate controls. A subset of the most promising antigens will be taken forward for development of an optimized seroimmunodiagnostic. The goal is a test that is either sensitive enough to exclude TB in test-negative symptomatic individuals, or specific enough to direct therapy in those who are positive.

O17

Profiling the immune response to TB infection on a genome-wide scale with protein microarrays

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The process for fabrication of a microarray from genomic DNA for whole proteome microarrays for *B. pseudomallei* may have application for *Mycobacterium tuberculosis* (M.tb). In the process, PCR is done on the DNA to amplify the open reading frames, genes are cloned by in vivo recombination, and mini-preps are made, isolating the plasmids produced by the recombination process. Then, *in vitro* transcription and translation reactions occur. Lastly, the reactions are brought to a microarray printer and the microarray chips are printed, using nitrocellulose-coated slides and without doing protein purification. The heart of the process is the high throughput cloning approach our team developed. Twenty nucleotide extensions are added to the PCR products so that the genes, although all different, have the same N-terminal and C-terminal sequences. The gene-specific primers are homologous with 33 nucleotide vector-specific 'adapter' sequences. The plasmids are transformed into chemically competent *E. coli* and grown overnight. The protein produced in the in vitro transcription and translation reactions, driven by a T-7 promoter, has both a HIS tag and a HA tag. The efficiency rate for producing clones by this method is greater than 95 percent, based on an analysis of 4,109 gene targets that produced 3,998 cloned genes. To test protein expression efficiency, we probed two arrays with monoclonal antibodies and then a secondary antibody. Out of 11 print runs, 2,265 chips produced 4,530 arrays, or a greater than 90 percent protein expression efficiency rate. Finally we tested batch-to-batch reproducibility by taking two batches of chips and track the signal of the HIS tag.
In a case-control M.tb study, our lab examined smear positive culture test results in individuals from endemic countries and from healthy individuals in non-endemic countries. In a series of projects involving 25 infectious disease agents, our lab has produced 18,000 proteins, printed 17,000 arrays, and probed 8,000 sera. The goal of this research, is to develop multi-variant microarray chips that contain several antigens, so that only one blood sample is required from an individual to determine a diagnosis.

O18

Multifunctional analysis of antigen-specific T cells: correlates of vaccine efficiency

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A new era for vaccine development and evaluation has begun with vaccines that generate cellular immune responses. If vaccine development is going to successfully engender T-cell responses for vaccine protection, researchers must consider that T-cells are capable of a large repertoire of cellular functions and then decide which functions they want to engage. The functions include killing by CD8 and CD4 T-cells, proliferation, and secretion of effector molecules (cytokines) that orchestrate immune responses, induction of inflammation and killing of target cells.
Using flow cytometry, functions can be measured on a cell-by-cell basis to quantify the different types of effector T-cells present. Findings in HIV and Hepatitis B vaccine research may inform vaccine work for TB. For example, an analysis of the Hepatitis B booster vaccine showed the current practice of measuring a single parameter to test vaccine efficiency is not sufficient. Measurement of IFN-γ and IL-2 produced by CD4 T-cells showed that many antigen-specific T-cells made IL-2 but not IFN-γ. In fact, only about half of the T-cells responding to the vaccine produced IFN-γ and so if only this cytokine was measured, the magnitude of the vaccine response would be undercounted by a factor of two. Further, when five cell functions were measured in HIV-infected progressors and non-progressors no single measurement identified the majority of HIV-specific CD4 T-cells. MIP-1Beta dominated CD8 T-cell response and long-term non-progressors seemed to have slightly greater cytokine responses overall. Analyzing cytokines by each T-cell subset showed that HIV-specific CD8+ T-cells appear to be multifunctional; the magnitude of the overall CD8+ T-cell response to HIV does not correlate with protection from disease progression, but long-term non-progressors maintain higher levels of polyfunctional (IL-2+, TNF-a + and IFN-g+) HIV-specific CD8+ T-cells than progressors; and polyfunctional T-cells correlate inversely with viral load. These findings define a correlate of protection against which vaccines may be evaluated.
Flow cytometry is the only platform that can provide multi-parametric analysis of the immune responses necessary for the most sensitive quantification of immunogenicity, identification of functional correlates, and adequate comparison of different regimens. In fact, flow cytometry is fundamentally changing vaccine development in terms of measuring cell response.

O19

Novel microfluidic technologies for portable diagnostics systems

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Novel microfluidic technologies being developed for portable diagnostic systems have potential for use in TB serodiagnosis or as platforms for cell and biomolecular assays. Using microfluidics, with its miniaturized channels and reservoirs for portable devices, offers many advantages, including: high surface-to-volume ratio; a low Reynolds number; increased speed of reaction; reduced cost of reagents; decreased cost of power consumption; precise mixing/dosage and heating. Its integration capability offers advantages as well, such as low manufacturing cost and multiplex capability, i.e. an increased number of parameters can be monitored per sample.

Two microfluidic platforms may be helpful in protein microarray analysis. Microfluidic electrical sensing platforms are capable of detecting antigen/antibody binding in real time. The development of this platform will allow for faster detection of certain diseases with a patient's sample compared to standard techniques such as ELISA. This platform requires microchannel-sealed microarrays with electrical detection capability. By measuring electrical charges, the scientists can determine protein-binding activity. Additionally, microfluidic colorimetric platforms are being developed using acoustic micromixing instead of fluorescence scanning. The process uses a scanner that detects an enzyme–based change of color that allows measurement of the protein-binding event. Our team is developing these platforms using components that have very small energy requirements, which means the device may not require batteries to operate.

A 'lab-in-a-droplet' technique using digital microfluidics rather than using microarrays, which produce a static substrate, diffuses samples into as many as millions of droplets, presenting a three-dimensional picoliter reactor array. This process has several advantages including: rapid mixing, homogeneous reactions, precise control of volumes and concentrations, and an ability to mimic cellular reactions. Our team is using this technique in the development of lipoplexes and to encapsulate a cell in a single droplet. Lastly, a platform is under development for cell sorting using electrodes to track cells in order to determine what function the cell will take on.

In addition to its value in the diagnosis of TB, microfluidics technology has several other applications, including: broader distribution of new assays to biologic labs, rapid analyses in the field for epidemiologic studies, and the ability to study hybridization of molecules on microarrays. For example, Lab-on-a-chip technologies are an excellent vehicle for integrating diagnostic and therapeutic functions. Digital (droplet) microfluidics enables high throughput cellular and molecular assays and cell sorting technologies enable more practical cellular analyses and therapies.

O20

Can parallel single cell assays support diagnostics in tuberculosis?

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Current biological tools to measure protein and mRNA abundance average the responses of the large number of cells present in a sample, thereby concealing cell-cell heterogeneity and limiting the analysis of rare cell types. While single cell analysis may be more desirable, the tools and methods to do these measurements are not widely available. We have developed a qRT-PCR approach to measure mRNA expression from single cell samples, and are developing a microfluidic ELISA tool to measure multiple proteins simultaneously from single cell samples. These techniques have been used to probe the spectrum and influence of cell-cell heterogeneity on immune responses by defining coordinate response patterns from individual cells and from multiple cells in parallel.

Requirement for success in achieving single cell measurements include: 1) precise nanoliter-volume fluid handling; 2) sufficient sensitivity to detect single cell responses; 3) multiplexing to measure more than one analyte from each cell sample; 4) a means to biochemically process single cell samples; and 5) methods to be able to process multiple samples in parallel. Remarkably, there are technical solutions to these challenges. However, a major limitation is the current lack of understanding of the biological significance of the very heterogeneous properties of individual cells in a population. Biological understanding of cell behavior will require reference datasets to be established so that normal/diseased responses can be classified, and the significance of the absolute abundance of a given protein within a given cell can be understood. Also, current methods focus on candidate genes and proteins, and an additional challenge is identifying which analytes are most appropriately measured to be useful for TB diagnostics and monitoring responses to therapy and vaccination.