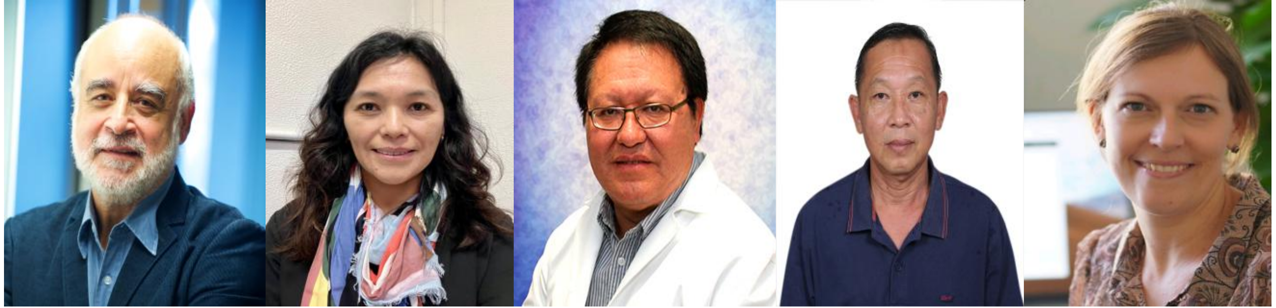
ImmunoTools *FlowISiAM* Award 2025



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Fluorescent staining of *Mycobacterium tuberculosis* antigens in Circulating Monocytes as a Point-of-Care Assay for the Diagnosis of Tuberculosis.

Background

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (Mtb). The World Health Organization (WHO) estimates that there will be 10.8 million new cases of TB worldwide in 2023, including 400,000 caused by drug-resistant strains that are resistant to rifampicin or rifampicin in combination with isoniazid (1). Developing tools for early diagnosis is critical to improving TB control worldwide.

Mtb has a complex, antigen-rich cell wall. Lipoarabinomannan (LAM), a glycolipid, makes up about 15% of the total bacterial mass. Virulent strains produce LAM with additional mannose residues (manLAM) (2,3). *In vitro* studies show that LAM integrates into T cell lipid rafts and impairs the maturation of monocytes into macrophages, reducing their ability to control Mtb growth (4-6).

LAM's ability to modulate the immune response is well documented, but LAM is not restricted to the site of infection; it is detectable in circulating blood and urine, making it a promising target for novel point-of-care diagnostic assays to improve TB detection (7).

Current TB diagnostic methods, including GeneXpert, acid-fast bacilli (AFB) stain and culture, are considered gold standards. However, these methods have significant limitations: difficulty in obtaining sputum samples, reliance on specialised equipment and long turnaround times. Emerging diagnostic platforms such as photonic biosensors, nanoparticle conjugate immunoassays (8), immune polymerase chain reaction (I-PCR) (9) and gas chromatography/mass spectrometry (GC/MS) (10) promise improved sensitivity (50-1,000-fold; 11). However, high costs limit their feasibility as point-of-care tools.

AlereLAM and Fujifilm SILVAMP TB LAM (FujiLAM) are commercially available tests for the detection of LAM by ELISA. However, these tests have suboptimal sensitivity and are dependent on $CD4^+$ T-cell counts, particularly in immunosuppressed individuals (above 200 cells/mm^3). In addition, clinical misinterpretation has been reported (12,13).

Using confocal microscopy, our group has shown that peripheral blood mononuclear cells (PBMCs) from TB patients and household contacts with latent TB are positive for LAM (manuscript in preparation). Although this technique eliminates the need for sputum samples, it remains time consuming. Preliminary data from our group indicate that PBMC LAM^+ can also be detected by flow cytometry. As monocytes are phagocytic cells, flow cytometry has the advantage of distinguishing between cell surface and intracellular LAM. This method is highly sensitive, provides rapid results and has significant potential to facilitate early diagnosis of TB. As a proof of concept, blood samples will be obtained from Balb/c mice infected by the intratracheal route with a high dose of reference strain H37Rv, a well-characterized model of pulmonary. This model has been extensively characterized since the immunopathological response and can contribute to detecting LAM during the early and late phases of active pulmonary disease (14).

Aim

This study aims to develop a point-of-care assay for diagnosing TB by identifying the fluorescent staining of mycobacterial antigens in circulating monocytes using flow cytometry in infected individuals with different genetic background.

Experimental Design and Methods

This project will be conducted in two cohorts of TB-infected individuals recruited in Cambodia and Mexico.

Patients :

Cambodia site :

One hundred thirty adults with tuberculosis (TB) infection will be enrolled from the Tuberculosis Clinical Center of the National Center for Tuberculosis and Leprosy Control (CENAT, http://www.cenat.gov.kh/cenat/km), located in Phnom Penh capital of Cambodia. We will recruit adult patients with bacteriologically confirmed positive acid-fast bacilli (AFB)

smear and/or GeneXpert MTB/RIF (n = 50), along with their household contacts (n = 80), comprising both individuals with positive (n = 50) and negative (n = 30) QuantiFERON-TB (QTF) test results. Individuals with negative QTF results will serve as controls.
The inclusion criteria are as follows:

1. **For patients:** Age ≥ 18 years; evidence of positive AFB smear and/or positive GeneXpert MTB/RIF result; HIV seronegative status.
2. **For TB household contacts:** Age ≥ 18 years; residence in the same household and/or direct contact with a documented active pulmonary TB patient; agreement to undergo QTF screening for TB infection.

The exclusion criteria are pregnant or breastfeeding women and individuals under the age of 18 years.
For the experiment, we will collect 5 mL of venous blood in a heparinized tube once, at the time of TB diagnosis and before the initiation of anti-tuberculosis treatment.

Mexico city site :
This part of the project will be conducted in a cohort of TB-infected individuals recruited in Mexico City. Individuals must be over 18 years of age and enrolment will be at the time of diagnosis (prior to any anti-TB therapy). Protocol approval numbers (INER): B20-22 and B37-24.
1.- Collect peripheral blood samples from three groups of patients (n=40 per group):
- Household contacts negative for Quantiferon (uninfected).
- Quantiferon-positive household contacts (latent Mtb infection).
- Individuals with active pulmonary tuberculosis.
- The exclusion criteria are pregnant or breastfeeding women and individuals under the age of 18 years

2.- The test will be performed in parallel, either in whole blood or in isolated mononuclear cells (PBMC), and cytometric phenotyping (see below) will be performed. Correlation between the two tests is performed to validate the results. A faster diagnostic method may be chosen.
3. As a proof of concept, peripheral blood cells will be obtained from the tail by vein puncture of Balb/c mice after 1, 21, 60, and 120 days of intratracheal infection with a high dose of Mtb strain reference H37Rv (2.5x10-5), which induces progressive pulmonary TB.

Methods :
Phenotyping to human cells: The identify monocytes positive for TB antigens using the following gating strategy (whole blood or PBMC), haematopoietic cells will be identified by CD45 marker. After gating in viable cells and single events, the cells will be incubated with a cocktail of antibodies (Lin negative), including CD2, CD3, CD4, CD8, CD19, CD56. To characterize monocytes, the cells will be stained with CD14, CD16, HLA-DR, and LAM (for extracellular detection). For the intracellular staining, we will use the FlowISiAM technique and platform to analyze the results, and staining will be done with monoclonal antibodies against Mtb proteins (from Lionex, diagnostics, and therapeutics) and anti-Lam antibodies.

Phenotyping to mouse cells: The identify monocytes positive for TB antigens using the following gating strategy (whole blood). After gating in viable cells and single events, the cells will be incubated with a cocktail of antibodies (Lin negative), including CD2, CD3, CD4, CD8. To characterize monocytes, the cells will be stained with F4/80 and LAM (for extracellular detection). For the intracellular staining, we will use FlowISiAM technique and platform to analyze the results, and staining will be done with monoclonal antibodies against Mtb proteins (from Lionex, diagnostics, and therapeutics) and anti-Lam antibodies.

NOTE: The LAM staining will be performed using two schedules: a) primary antibody Mouse Anti-LAM Recombinant Antibody (clone CS35) + secondary antibody Alexa Fluor™ 546 Goat anti-Mouse IgG, and b) anti-LAM conjugated with APC. The best option to use for flow cytometry is schedule "b"; however, due to the expense of the conjugated antibody, option "a" will be considered.

Data Analysis objectives :

Compare the diagnostic efficiency of "Fluorescent Staining of Mtb antigens in Circulating Monocytes" with current gold-standard methods.

Evaluate the feasibility and reliability of using flow cytometry for rapid TB diagnosis.

References

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ImmunoTools *FlowISiAM* AWARD for **Daniel Scott-Algara, Leslie Chávez-Galán, Rogelio Hernández-Pando, Polidy Pean, and Tineke Cantaert** includes some antibodies for *FlowISiAM*, know how transfer and protocol. Together with INVIGATE, we try to support regarding selection of specific antibodies against specific biomarkers, antibody conjugation, expert assistance in evaluating the results obtained, and integration into the ImmunoTools *FlowISiAM* network.

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