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IMMUNOLOGICAL ASPECTS

Immunogenicity of mycobacterial vesicles in humans: Identification of a new tuberculosis antibody biomarker

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SUMMARY

Biomarkers for active tuberculosis (TB) are urgently needed. Mycobacteria produce membrane vesicles (MVs) that contain concentrated immune-modulatory factors that are released into the host. We evaluated the human immune responses to BCG and *Mycobacterium tuberculosis* MVs to characterize the antibody responses and identify potentially novel TB biomarkers. Serological responses to MVs were evaluated by ELISAs and immunoblots with sera from 16 sputum smear-positive, 12 smear-negative HIV uninfected pulmonary TB patients and 16 BCG vaccinated Tuberculin skin-test positive controls with and without latent tuberculosis infection. MVs from both BCG and *M. tuberculosis* induced similar responses and were strongly immunogenic in TB patients but not in controls. Several MV-associated antigens appear to induce robust antibody responses, in particular the arabinomanan portion of the cell wall glycolipid lipoarabinomannan. Three proteins at ~36, 25, and 23 kDa were simultaneously recognized by sera from 16/16 smear-positive, 9/12 smear-negative TB patients and 0/16 controls. These results provide promise and encouragement that antibody responses to proteins enriched in MVs of pathogenic mycobacteria may constitute a novel TB biomarker signature that could have diagnostic information.

1. Introduction

The identification of easily detectable biomarkers for active tuberculosis (TB) is a global health priority.[1,2] TB remains a worldwide public health problem underscored by an estimated 8.7 million new cases in 2011 with almost one million TB-associated deaths among HIV- and ~0.43 million among HIV+ people.[3] Rapid TB diagnosis and treatment leads to reduced transmission, morbidity and mortality but is often delayed, especially in resource-limited settings where the vast majority of people with TB reside. Thus, TB biomarkers that can lead to simple rapid point-of-care (POC) tests are urgently needed.

The gold standard test for TB diagnosis remains the detection of *Mycobacterium tuberculosis* in culture.[4] However, culture methods necessitate a laboratory infrastructure and entail incubation times of weeks to months. Molecular methods for detecting *M. tuberculosis*-specific nucleic acids, especially the recently WHO endorsed GeneXpert M.TB/RIF, have revolutionized the rapid diagnosis of drug-sensitive and resistant TB.[5–8] However, they are costly and require technological investment. Therefore, although limited by a sensitivity of around 50%,[9–11] microscopy remains the most widely used method for rapid TB diagnosis, and often is the only test available in resource-limited settings. Despite ongoing research efforts a simple inexpensive POC test, applicable in all settings, is still not available.[8,12]

Serum antibodies (Abs) can be detected by rapid "dip-stick" formats suitable for POC testing,[13–15] but no accurate serodiagnostic tests for TB have been developed to date.[16–18] We have recently reported that pathogenic mycobacteria produce membrane vesicles (MVs) that are released into the extracellular space and contribute to mycobacterial virulence in mice.[19] These MVs vary in diameter between 60 and 300 nm and their composition includes glycolipids and a large number of lipoproteins. MVs provide an effective way for intra-cellular bacteria to release concentrated immune-modulatory factors into the host. Hence, the assessment of the host immune response to MVs provides a unique

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opportunity for identification of novel biomarkers. The objective of this study was to evaluate the serological responses to mycobacterial MVs in human TB cases and controls. We demonstrate that MVs from *M. tuberculosis* and *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) elicit strong Ab responses in humans that include reactivity with a set of MV proteins to produce a serological profile that is highly sensitive and specific for TB and thus potentially constitutes a new TB biomarker.

2. Subjects, materials and methods

2.1. Subjects and study design

Subjects were 21–80 years old and enrolled at 4 public hospitals in New York City from 2007 to 2010. All subjects were HIV uninfected and either had pulmonary TB ($n = 28$) or were healthy asymptomatic controls with a positive tuberculin skin-test (TST+; $n = 16$). TB cases were confirmed by a positive respiratory culture for *M. tuberculosis* (gold standard) and enrolled prior to, or within the first 7 days, of antituberculous treatment. They were further categorized by sputum smear microscopy results and considered smear-positive if one of the initial three sputum smears were positive regardless of number of acid-fast bacilli (AFB) detected. Controls were asymptomatic TST+ health care providers who were all BCG vaccinated and reported a positive exposure history to patients with TB. TST+ controls had no abnormalities on chest X-ray and were further categorized based on results for an interferon-gamma release assay (IGRA; QuantiFERON®-TB Gold, Celestis, Australia). Nine/16 controls had a negative IGRA result and were considered TST+ due to a history of BCG vaccination. Seven/16 had a positive IGRA result and were considered to have latent tuberculosis infection (LTBI). All subjects provided written informed consent prior to enrollment. Approval for human subjects' research was obtained from the Internal Review Boards at the New York University School of Medicine, NY, NY, and the Albert Einstein College of Medicine, Bronx, NY.

2.2. Mycobacterial MV preparation

Vesicles were isolated through a series of gradient filtration and centrifugation steps as previously described.[19] Essentially, *M. tuberculosis* (strain H37Rv), obtained from the Trudeau Institute (Saranac Lake, NY), or *M. bovis* BCG (Pasteur strain), obtained from the Statens Serum Institute (Copenhagen, Denmark), were grown in mid-logarithmic phase at 37 °C in roller bottles containing minimal media. Mycobacteria were harvested after 10 days of growth and pelleted to remove cell fractions. The supernatant was then filtered through a 0.45 μm polyvinylidene difluoride membrane filter (Millipore, MA) and concentrated using a 100-kDa exclusion filter with an Amicon Ultrafiltration System (Millipore, MA). The concentrate was ultracentrifuged at 60,000 rpm for 1 h at 4 °C to sediment the vesicular fraction into a pellet which was resuspended in PBS. The protein concentration of the MV preparation was determined using a BCA Protein Assay Kit (Thermo Scientific, IL).

2.3. Antibody detection assays

Antibody reactivity to MVs was determined via enzyme-linked immunosorbent assay (ELISA) as described.[20,21] Briefly, 96-well microtiter plates (Immulon 2HB, Fisher Scientific, NY) were coated with either 4 μg/ml protein concentration of MVs, 10 μg/ml of lipoarabinomannan (LAM) or arabinomannan (AM), or 4 μg/ml of antigen 85B (Ag 85B) for 1 h and then blocked with 3% BSA/0.1% PBST over night. LAM prepared from the Mtb strain H37Rv and Ag

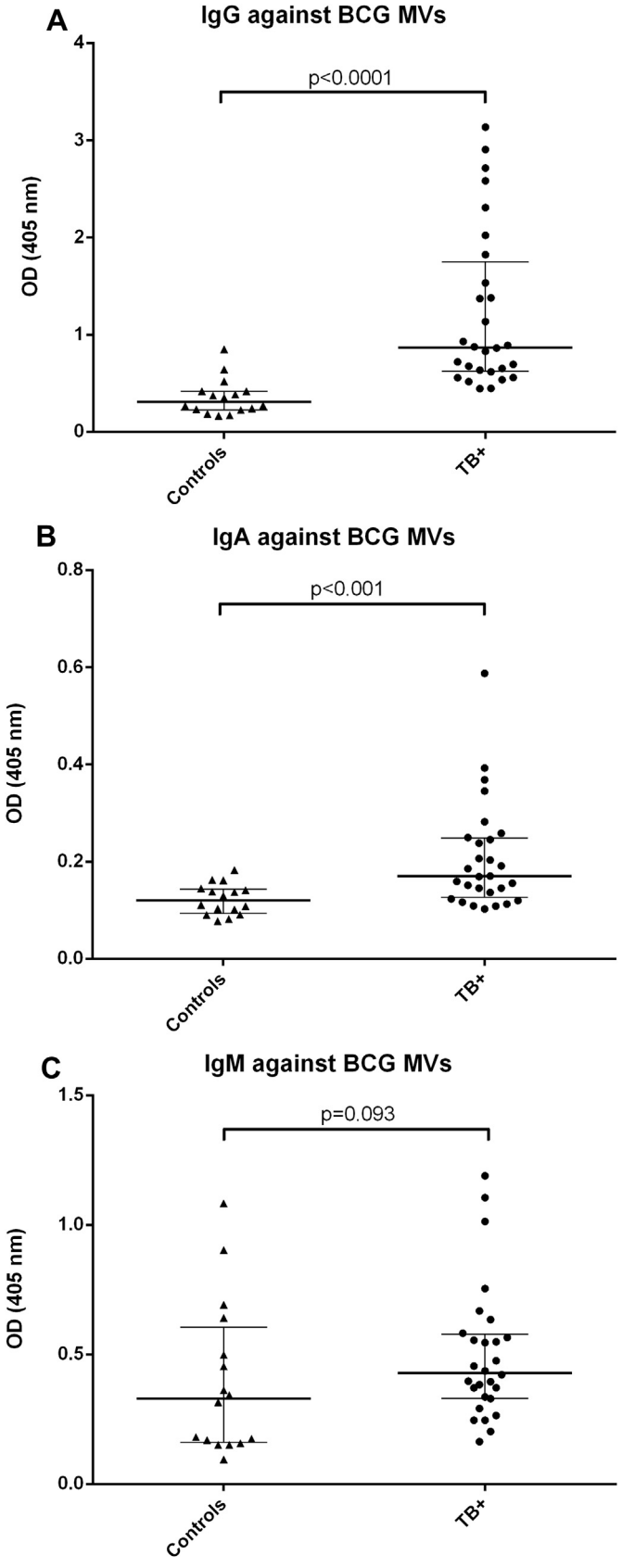

Figure 1. Antibody isotype responses against BCG MVs in TB patients and TST+ controls. A. IgG responses against BCG MVs; B. IgA responses against BCG MVs; C. IgM responses against BCG MVs. Statistical analysis with Mann–Whitney *U* test. Bars show median values with interquartile range.