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Selection and identification of specific glycoproteins and glycan biomarkers of macrophages involved in *Mycobacterium tuberculosis* infection

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ABSTRACT

Macrophages are the primary host target cells of *Mycobacterium tuberculosis* (*M.tb*). However, little is known about the changes of membrane glycopatterns of macrophages in response to *M. tb* infection. Using lectin microarrays we compared the differential expression of glycopatterns of macrophages upon stimulation with the heat-inactivated virulent *M.tb* H37Rv or attenuate *M.tb H37Ra*. We found that widespread alteration of macrophage membrane glycopatterns were induced by the heat-inactivated virulent *M. tb* H37Rv, as shown by the significantly changed binding abilities of 11 lectins (sugar binding proteins) among 40 lectins tested. The binding ability of the lectin ABA to macrophages showed the greatest increase after virulent *M. tb* H37Rv treatment, which suggests that the expression of N-acetyl-D-lactosamine (ABA binding ligand Galβ1-3GalNAc, *O*-link glycan) is mainly increased on macrophages during virulent *M.tb* infection. Addition of ABA blocked the attachment/engulfment of *M. tb* H37Rv, but not H37Ra, to macrophages. Further, increased glycosylated CD44, one of ABA-binding glycoproteins on macrophages, was identified by pull-down assays with ABA-agarose, followed by mass spectrometry and western blotting. ABA directly binds with Galβ1-3GalNAc-glycosylated CD44 on macrophage, and inhibits *M. tb* mannose-capped lipoarabinomannan (ManLAM) binding to glycosylated CD44. Moreover, ABA increases IL-6, but reduces IL-10 production of ManLAM-treated macrophages and inhibits *M. tb* H37Rv-induced necrosis in macrophages. Our study will help to reveal the mechanism of pathogenicity and virulence of *M. tb* from a new perspective and provide a potential new diagnostic and therapeutic strategy for tuberculosis based on glycopatterns, ABA and its ligand Galβ1-3GalNAc-glycosylated CD44 target molecule on macrophage.

1. Introduction

Tuberculosis (TB) remains to be a leading cause of death from infectious diseases worldwide, particularly in developing countries [1,2]. The World Health Organization (WHO) estimated that approximately 9 million new cases and nearly 1.5 million deaths of TB occurred in 2014 [2]. The emergence of multidrug-resistant TB (MDR-TB) and co-infection with human immunodeficiency virus (HIV) makes TB control even more urgent [3,4]. An attenuated strain vaccine of *Mycobacterium bovis* (termed Bacillus Calmette–Guérin, BCG), the only available TB vaccine, decreases the risk of TB in children but gives little protection against TB in adults [5].

Macrophages are primary host cells during *Mycobacterium tuberculosis* (*M. tb*) infection. *M. tb* binds and enters into macrophages possibly through several receptors, including mannose receptor (MR), toll-like receptors 2 (TLR2) and CD44 [3,6]. For

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example, the mannose-capped lipoarabinomannan (ManLAM), the major surface lipoglycan of virulent *M. tb*, directly binds to MR and thus promotes macrophages toward a M2 or anti-inflammatory phenotype, which is characterized by expression of anti-inflammatory cytokines (e.g., IL-10) [7]. In our previous report, ManLAM also binds to CD44 on macrophages and thus promotes the cells toward M1 polarization, which is characterized by expression of inducible nitric oxide synthase (iNOS) expression [3].

Two strains of *M. tb* H37Rv and H37Ra are all derived from their parent virulent strain H37 [8]. H37Rv is a standard laboratory virulent strain, while attenuated strain H37Ra loses its certain virulence after several passages [8]. Virulent strain of *M. tb* H37Rv can block macrophage apoptosis, decrease inflammatory responses and facilitate bacterial growth in the cells at early stage of infection, while certain attenuated *M.tb* H37Ra promotes macrophages apoptosis and inhibits bacterial survival in the cells [9–11]. At late stage of infection, virulent strains of *M. tb* H37Rv induces an atypical cell death to escape from infected macrophages and thus can infect surrounding cells [12]. This atypical death mode provides a mechanism for viable bacilli to exit host macrophages for spreading infection resulting in advanced pulmonary tuberculosis, however, attenuated strain *M. tb* H37Ra cause small part of macrophage death [12,13].

Nearly all membrane proteins are glycosylated and cell surface glycans constitute the major portion of the cell membrane (termed glycocalyx) [14]. Recently, changes of glycopatterns of host cells in response to several important bacterial pathogens have been widely investigated [15–18]. However, little is known about the changes of membrane glycopatterns of macrophages in response to virulent and attenuated *M. tb*. The exploration of altered glycan profiles of macrophages stimulated with *M. tb* strains (H37Rv and H37Ra) will help to reveal the mechanism of pathogenicity and virulence of *M.tb* from a new perspective.

In this work, we identified the altered glycan profiles of membrane proteins and target molecules on murine macrophages stimulated by virulent *M. tb* strain H37Rv and its non-virulent counterpart H37Ra using lectin microarray and mass spectrometry techniques. Our results reveal the virulence-associated differences in the glycopattern of membrane glycoproteins in *M. tb*-stimulated macrophages. These data will not only provide useful information to explore the function of host cell glycoproteins, but also could provide new diagnostic and therapeutic target molecules and strategy for the treatment of tuberculosis.

2. Materials and methods

2.1. Bacteria, cells and animals

M. tb H37Rv (strain American Type Culture Collection (ATCC) 93009) were purchased from the Beijing Biological Product Institute (Beijing, China) [3,19]. *M. tb* H37Ra strain was kindly provided by Prof. Xiong-Lin Fan (Huazhong University of Science and Technology, Wuhan, China). *M. tb* H37Rv and H37Ra were maintained in Lowenstein-Jensen medium and harvested while in the log phase of growth. Bacilli were inactivated at 65 °C for 2 h (namely iH37Rv/iH37Ra) and washed in PBS containing 0.05% Tween-80 and triturated uniformly before use, as we previously described [19].

Human monocytic cell line THP-1 (GDC100) were obtained from China Center for Type Culture Collection (CCTCC, Wuhan, China). THP-1 cells were cultured in RPMI (Life Technologies) with 10% FBS and differentiated by the addition of 100 nM phorbol 12- myristate 13-acetate (PMA) (Sigma-Aldrich) to the culture mediumfor 48 h at 37 °C. Bone marrow cells were collected from the femurs and tibias of BALB/c mice. The bone marrow cells were cultured for 7 days in DMEM supplemented with 10% FBS, 50 ng/ml M-CSF, 100 U/ml penicillin and 100 μg/ml streptomycin. The adherent cells were used in the experiments.

Female BALB/c mice (~8 weeks old) were provided by the Animal Laboratory Center of Wuhan University. The animal protocols were performed in compliance with all guidelines and approved by the Institutional Animal Care and Use Committee of Wuhan University.

2.2. Extraction of membrane proteins of macrophages

Murine resident peritoneal macrophages were harvested and cultured as described previously [20]. Thioglycolate-elicited macrophages were prepared by injecting BALB/c mice with 3.5 ml 3% sterile thioglycolate media (Difco, BD Biosciences). On day 4, cells were harvested by lavage, and macrophages were isolated after adhesion and stained with anti-F4/80 antibody for purity analysis. Isolated murine peritoneal macrophages were stimulated with heat-inactivated *M.tb* H37Rv (iH37Rv) or heat-inactivated *M.tb* H37Ra (iH37Ra, 5×10^6 CFUs per 5×10^5 cells) for 24 h. Then, membrane proteins of macrophages were extracted using membrane and cytosol protein extraction kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. Briefly, $2–5 \times 10^7$ macrophages were collected and washed with ice bath phosphate-buffered saline (PBS). 1 ml of membrane protein extraction reagent A were added to the cell pellets, and the cells were gently resuspended. After incubation at 4 °C for 10–15 min, the cells were homogenized. After centrifugation (700 g, 4 °C for 10min), the supernatants were transferred to a new tube and nucleus and cell debris were discarded. Then the supernatants were centrifuged at 14000 g (4 °C for 30min) to precipitate the cell membrane fragments. After the centrifugation, the supernatants were discard, and 200s μl of protein extraction reagent B was added onto the pellets of membrane fragments on the bottom of the tube. After resuspending the pellets, the mixtures were centrifuged at 14000 g (4 °C for 30min), and the supernatant (containing membrane protein) was collected. The concentration of the prepared membrane proteins was measured by BCA kit (Beyotime, Shanghai, China).

2.3. Lectin microarray

Lectin microarray analysis was performed as previously described [21,22]. 40 commercial lectins were immobilized onto a black opaque 384-wells microplate. Membrane proteins (2 mg) were then labelled with NHS-Cy3 (lumiprobe, Florida, USA) and purified with Sephadex G-25 columns according to the manufacturer's instructions [22]. Next, purified Cy3-labelled membrane proteins from macrophages stimulated with iH37Rv/iH37Ra were applied in triplicate to the lectin microplate. The Mean Fluorescence Intensity (MFI) at 570 nm were determined on a SpectraMax® i3x microplate reader (Molecular Devices, Sunnyvale, CA). The background was subtracted from the data.

2.4. Pull-down assay

ABA pull-down assay was performed according to previous publications [23,24]. Membrane proteins (2 mg) from iH37Rv-stimulated macrophages were incubated with ABA-agarose (Vector Laboratories, Burlingame, CA) (each 40 μl of 50%) at 4 °C for 16 h. The precipitated samples were washed with glycoprotein eluting solution (Vector Laboratories, Burlingame, CA) and then analyzed with SDS-PAGE. The bands of proteins were extracted from SDS gel and prepared for proteins identification with LC-mass spectrometry (MS)/MS analysis (Easy nLC1000, Q-Exactive, Thermo, USA).

In ManLAM-beads pull-down assay, epoxy magnetic beads