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IMMUNOLOGICAL ASPECTS

Identification of novel *Mycobacterium tuberculosis* CD4 T-cell antigens via high throughput proteome screening

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SUMMARY

Elicitation of CD4 IFN-gamma T cell responses to *Mycobacterium tuberculosis* (MTB) is a rational vaccine strategy to prevent clinical tuberculosis. Diagnosis of MTB infection is based on T-cell immune memory to MTB antigens. The MTB proteome contains over four thousand open reading frames (ORFs). We conducted a pilot antigen identification study using 164 MTB proteins and MTB-specific T-cells expanded *in vitro* from 12 persons with latent MTB infection. Enrichment of MTB-reactive T-cells from PBMC used cell sorting or an alternate system compatible with limited resources. MTB proteins were used as single antigens or combinatorial matrices in proliferation and cytokine secretion readouts. Overall, our study found that 44 MTB proteins were antigenic, including 27 not previously characterized as CD4 T-cell antigens. Antigen truncation, peptide, NTM homology, and HLA class II tetramer studies confirmed malate synthase G (encoded by gene Rv1837) as a CD4 T-cell antigen. This simple, scalable system has potential utility for the identification of candidate MTB vaccine and biomarker antigens.

Abbreviations: MTB, *Mycobacterium tuberculosis*; ORF, open reading frame; IVTT, *in vitro* transcription translation; ATBI, active *Mycobacterium tuberculosis* infection; LTBI, latent *Mycobacterium tuberculosis* infection; TST, tuberculin skin test; PPD, purified protein derivative; IGRA, interferon gamma release assay; ICC, intracellular cytokine cytometry; IFN-gamma, interferon gamma; IL-2, interleukin 2; PBMC, peripheral blood mononuclear cell; CFSE, carboxyfluorescein succinimidyl ester; TCR, T cell receptor; PCR, polymerase chain reaction; RTS, rapid translation system; DMSO, dimethylsulfoxide; CFP, cultured filtrate protein; ESAT, early secretory antigen target; PHA, phyto-hemagglutinin; PBMC, peripheral blood mononuclear cells; SEB, Staphylococcal enterotoxin B; IB, inclusion body; mAb, monoclonal antibody; TCM, T-cell medium; MCB, master cell bank; SDS, sodium dodecyl sulfate; PE, phycoerythrin; EBV, Epstein–Barr virus; LCL, lymphocyte continuous line.

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1. Introduction

MTB causes 10^7 active tuberculosis infections (ATBI) and kills about 1.3×10^6 persons annually [27]. Globally, 1 in 3 people have latent tuberculosis infection (LTBI) [48]. The licensed BCG vaccine is poorly active against adult disease and there is a global effort to improve vaccines [37]. The immune response to MTB infection includes CD4 T-cells with rearranged T-cell receptor (TCR) alpha beta receptors that recognize peptides derived from MTB-encoded proteins bound to human leukocyte antigen (HLA) class II. Notwithstanding disappointing results from a recent vaccine clinical trial of one MTB antigen [74] the elicitation or boosting of CD4 T-cells remains a valid proposed mechanism of action for candidate vaccines [77]. It has been proposed that antigen and epitope choice may be important in the context of bacterial gene expression during different phases of MTB pathogenesis. The IFN-gamma axis is vital for host defense against MTB and CD4 T-cell decline in HIV infection is associated with severe MTB outcomes [18]. Memory T-cells also form the basis for tests for MTB infection. The tuberculosis skin test (TST) measures *in vivo* leukocyte infiltration in response to a filtrate of MTB cultures, while licensed interferon-gamma release assays (IGRA) measure IFN-gamma production in response to MTB peptides from three or fewer MTB open reading frames (ORFs) [7,50]. Neither test discriminates between latent and active TB infection or predicts risk of progression from a latent to active state. There is an unmet need for biomarkers in this area.

MTB T-cell antigen discovery is thus relevant to vaccines and diagnostics. The complexity of the MTB proteome, encoded by 4000 annotated genes, has hindered systematic screening of potential antigens in MTB. Approaches, as recently reviewed [25] have included expression libraries of MTB DNA fragments [52], prediction of HLA-binding peptides MTB ORFs [81], and expression of targeted subsets of MTB ORFs based on criteria such as phase- or nutrient-dependent gene expression [10,26] or sequence motifs associated with protein secretion [9].

Advances in high throughput *in vivo* recombination and *E. coli* lysate-based *in vitro* transcription/translation (IVTT) allow expression of essentially the entire translated proteome of large-genome pathogens [6,17,21,34]. The proteins are useful for probing humoral responses [53]. Our lab adapted these protein collections for CD4 T-cell research for viruses encoding up to 240 proteins [32,34]. IVTT proteins are suitable for CD4 T-cell studies because these immune cells typically detect microbial proteins after they are digested to linear peptides of 8 to about 20 amino acids. The peptides are not post-translationally modified, with recognized exceptions [55]. Here, we report a novel approach to MTB CD4 T-cell antigen discovery that uses a proteome set [45,46] originally created for antibody studies to probe the reactivity of polyclonal MTB-specific CD4 T-cell lines. We further developed modifications of the workflow to adapt to a resource-constrained, MTB-endemic region, obtaining adequate assay performance to confirm and extend MTB antigen discovery.

2. Materials and methods

2.1. Subjects and specimens

Persons requiring LTBI evaluation for employee health in the US were screened with Quantiferon™ Gold In-Tube (QFT) (Qiagen, Germantown, MD) and participated in an institutional ethics committee-approved protocol and gave informed consent. Heparinized peripheral blood was obtained. For studies in India, institutional ethics committee approval was obtained to recover leukocyte buffy coats from blood donated by anonymous healthy donors at a blood bank, with no medical, demographic, or personal identifying information available. PBMC isolated by Ficoll-Hypaque density gradient centrifugation from blood or buffy coat were cryopreserved at $1–3 \times 10^7$ cells/vial. When QFT could not be done, thawed PBMC, with living cells re-isolated in some instances by Ficoll-Hypaque centrifugation, were assayed for LTBI using enzyme-linked spot assay (ELISPOT) [31]. Plates were coated with monoclonal antibody (mAb) 1D1K (mAbTech, Mariemont, OH) specific for IFN-gamma. After washing, 3×10^5 cells/well were added in a final volume of 100 μL T-cell medium (TCM). Stimuli included media negative and 1.6 μg/ml phytohemagglutinin (PHA) positive control. Peptides covering the sequence of MTB proteins early secretory antigen target (ESAT)-6 and cultured filtrate protein (CFP)-10 from BEI Resources (Manassas, VA) were added to 1 μg/ml final concentration each in 0.2% DMSO final as pools of 20 or 25 peptides. After 24 h, cells were removed and IFN-gamma detected with biotinylated mAb 7-B6-1 (mAbTech), avidin-peroxidase, and AEC substrate with intermediate washes. Samples with >10 spots/well for ESAT-6 and/or CFP-10 minus DMSO control were considered positive for LTBI [56]. HLA typing was performed at the Puget Sound Blood Center, Seattle, Washington, USA.

2.2. Expansion of MT-reactive cell populations from PBMC

Initial experiments adopted a strategy of sorting MTB-reactive cells using a surface activation marker, followed by non-specific polyclonal expansion. PBMC were plated at 4×10^6/well in 24-well plates in 2 ml T-cell medium TCM [41] with 1:400 native whole MTB antigen. After 18 h, cells were stained with anti-CD3-phycoerythrin (PE), anti-CD4-fluorescien isothiocyanate, anti-CD137-allophycocyanin (Becton Dickinson, San Jose, CA), and 1 μg/ml 7-aminoactinomycin D (Life Technologies, Grand Island, NY). Viable CD3+/CD4+ lymphocytes, either $CD137^{high}$ or $CD137^{negative}$, were sorted (FACSAria III, Becton Dickinson) [35]. At least 1000 sorted cells were expanded with PHA, feeder cells and 32 units/ml natural human (hn) IL-2 [41]. A portion of the resultant bulk 1st generation (termed B1) cell lines were tested for whole MTB reactivity after 14 days. Remaining cells were expanded further using anti-CD3 mAb as mitogen, feeder cells and human recombinant IL-2 [42] for bulk 2nd generation (termed B2) cell lines that were cryopreserved in aliquots for testing. To make CD4 T-cell clones, 1.5×10^6 B2 cells from subject US3 were stimulated with an equal number of autologous PBMC as APC and 1 μg/ml peptide **Rv1837** 278–292 (AVDAADKVLGYRNWL) in 2 ml TCM in a 24-well plate. At 18 h, live CD3+ CD4+ CD137 + cells were sorted and cloned at 1 cell/well [39]. Candidates were screened for ^{3}H thymidine incorporation responses to 1 μg/ml **Rv1837** 278–292 or whole 1:200 MTB antigen in separate wells using autologous gamma-irradiated PBMC as APC. Clones reactive to both peptide and MTB were further expanded [39,47]. To adapt these assays without a requirement for cell sorting or radioisotope use in a resource-limited setting in India, we modified the protocol in such a way that 5 days after initial plating of PBMC and MTB antigen, hnIL-2 was added at 32 units/ml. Cells were fed at least every other day with half-volume TCM/IL-2 and expanded as necessary. After 14–16 days, cells were expanded for a second 14–16 day cycle with PHA, IL-2, and irradiated random allogeneic PBMC [41]. Epstein–Barr virus (EBV)-transformed lymphocyte continuous lines (LCL) were cultured as described [39].

2.3. T-cell assays

For direct *ex vivo* PBMC cytokine responses, thawed cells were incubated with MTB antigens or staphylococcal enterotoxin B (SEB)