ARTICLE IN PRESS

Journal of Immunological Methods xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Validation of a CD1b tetramer assay for studies of human mycobacterial infection or vaccination

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ARTICLE INFO

Keywords:
Human
T cells
CD1b
Tetramer
Mycobacteria
Assay validation

ABSTRACT

CD1 tetramers loaded with lipid antigens facilitate the identification of rare lipid-antigen specific T cells present in human blood and tissue. Because CD1 proteins are structurally non-polymorphic, these tetramers can be applied to genetically diverse human populations, unlike MHC-I and MHC-II tetramers. However, there are no standardized assays to quantify and characterize lipid antigen-specific T cells present within clinical samples. We incorporated CD1b tetramers loaded with the mycobacterial lipid glucose monomycolate (GMM) into a multi-parameter flow cytometry assay. Using a GMM-specific T-cell line, we demonstrate that the assay is linear, reproducible, repeatable, precise, accurate, and has a limit of detection of approximately 0.007%. Having formally validated this assay, we performed a cross-sectional study of healthy U.S. controls and South African adolescents with and without latent tuberculosis infection (LTBI). We show that GMM-specific T cells are specifically detected in South African subjects with LTBI and not in U.S. healthy controls. This assay can be expanded to include additional tetramers or phenotypic markers to characterize GMM-specific T cells in studies of mycobacterial infection, disease, or vaccination.

1. Introduction

T cells respond to mycobacterial cell wall lipids presented by functionally non-polymorphic CD1 molecules (Beckman et al., 1994; Gilleron et al., 2004; Layre et al., 2009; Moody et al., 2004, 2000a,b, 1997). The human CD1 locus contains five genes (CD1A, CD1B, CD1C, CD1D, and CD1E) encoding five proteins (CD1a, CD1b, CD1c, CD1d, and CD1e) capable of processing and presenting lipid antigens to T cells. These proteins vary in the configuration of their binding grooves, patterns of cellular expression, and subcellular trafficking (Van Rhijn et al., 2013b). Canonically, CD1a binds lipopeptides, CD1b binds glycolipids, and CD1c binds phospholipids. T-cell responses to these lipids are detectable in the blood of *Mycobacterium tuberculosis* (M.tb)-infected humans (Gilleron et al., 2004; Layre et al., 2009; Montamat-Sicotte et al., 2011; Moody et al., 2000a,b; Seshadri et al., 2015). However, it is currently unknown whether lipid-specific T-cells become activated or expand as a result of mycobacterial vaccination. A major barrier to progress has been the lack of formally validated assays to quantify and characterize T-cell responses to lipid antigens.

Tetramers take advantage of multimerization to generate high avidity reagents that can bind to and track rare antigen-specific T cells within a larger mixed population of T cells. Tetramers based on the major histocompatibility complex (MHC) Class I and Class II proteins have significantly advanced our understanding of T cell responses to peptide antigens but are limited by the highly polymorphic nature of MHC (Altman et al., 1996). On the other hand, CD1 genes are structurally non-polymorphic, so a CD1 tetramer can in principle be used on everyone, thus permitting a truly global analysis of antigen-specific T cell responses for the first time. The development of soluble lipid-loaded CD1 tetramers changed the landscape for ex-vivo investigation of T-cell phenotypes and functions (Benlagha et al., 2000; Karadimitris et al., 2001; Matsuda et al., 2000). These tetramers allowed engagement of more than one copy of the T cell receptor (TCR) on the surface of a T cell, resulting in increased avidity of the interaction and allowing identification of antigen-specific T cells by flow cytometry, even those present at low frequencies. Initially developed for CD1d, tetramers have

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https://doi.org/10.1016/j.jim.2018.04.004
Received 6 February 2018; Received in revised form 2 April 2018; Accepted 18 April 2018

now been extended to CD1a, CD1b, and CD1c, including those loaded with mycobacterial lipid antigens to facilitate studies in patients with latent and active tuberculosis (James et al., 2018; Kasmar et al., 2013, 2011; Ly et al., 2013). However, these reagents have not yet found their way into validated end point assays that could be employed in clinical settings.

Here, we present the formal validation of an assay using CD1b tetramers loaded with glucose monomycolate (GMM), a major component of the mycobacterial cell wall (Brennan et al., 1970). GMM comprises up to 2% of total extractable lipid and is produced by many mycobacterial species, including *Mycobacterium bovis BCG, M. tuberculosis, M. smegmatis, M. leprae,* and *M. phlei* (Brennan et al., 1970; Moody et al., 2000a, 1997; Silva, 1985). Because the glucose moiety is host-derived, GMM in infected tissues signals the presence of pathogenic mycobacteria and provides an antigenic target for T cells (Moody et al., 2000a). Thus, GMM has been observed to be an immunodominant antigen in experimental infection of cattle and studies of humans with latent tuberculosis (Nguyen et al., 2009; Seshadri et al., 2015).

We used GMM-specific T-cell lines to establish the operating characteristics in a flow cytometry assay and to optimize and validate the tetramer assay according to the following parameters: linearity, range, limit of detection, repeatability, reproducibility, intermediate precision, and accuracy. We used this assay to study a cohort of healthy subjects and find that GMM-CD1b tetramer positive cells are specifically detected in South African adolescents at high risk for M.tb exposure but not in U.S. subjects at low risk for exposure. We expect that this assay will find utility in natural history studies of M.tb exposure and disease as well as investigations into the immunogenicity of novel whole cell mycobacterial vaccines.

2. Methods

2.1. Culture media

Media (R10) for washing peripheral blood mononuclear cells (PBMC) consisted of RPMI 1640 (Gibco, Waltham, MA) supplemented with 10% fetal calf serum (Hyclone, Logan, UT). Our base T cell media (TCM) consisted of RPMI 1640 supplemented with 10% fetal calf serum, 100 U/mL Penicillin, 100 mg/mL Streptomycin, 55 mM 2-mercaptoethanol, 0.3× Essential Amino Acids, 60 mM Non-essential Amino Acids, 11 mM HEPES, and 800 mM L-Glutamine (Gibco, Waltham, MA) sterile-filtered. Our TCM containing human serum (TCM/HS) consisted of 10% human serum (derived from healthy donors), 100 U/mL Penicillin, 100 mg/mL Streptomycin, and 400 mM L-Glutamine (Gibco, Waltham, MA).

2.2. Preparation and storage of GMM lipids

Glucose monomycolate (C32-GMM) isolated from *Rhodococcus equi* was generously provided by the laboratory of D. Branch Moody. Stock GMM was solvated in chloroform:methanol (2:1, v:v) at a concentration of 1 mg/mL and sonicated for 3 min in a 37 °C water bath to resuspend the lipid. The resulting suspension was divided into single-use glass vial aliquots (21 μg/vial) as repeated sonication may lead to solvent evaporation and increased concentration of the reagent. The solvent from each aliquot was then evaporated under a stream of nitrogen, and the vials were stored at −20 °C.

2.3. Generation of GMM-loaded CD1b tetramers

Soluble biotinylated CD1b monomers were provided by the National Institutes of Health Tetramer Core Facility (Emory University, Atlanta, GA). The CD1b monomer loading protocol for GMM was derived from a previously published protocol (Kasmar et al., 2011). Briefly, one aliquot of 21 μg of GMM was sonicated into 41 μL of a 50 mM sodium citrate buffer at pH 4, containing 0.25% with 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) (Sigma, St. Louis, MO) for 2 min at 37 °C. In parallel, mock, unloaded tetramer was generated similarly to the loaded tetramer but in the absence of lipid. Subsequently, 9 μL of CD1b monomer (2 mg/mL) was added, and the resulting suspension contained GMM at 100-fold molar excess of CD1b monomer. The acidic buffer facilitates monomer loading by allowing the lipid to access the antigen-binding groove. This mixture of lipid and monomer was incubated in a 37 °C water bath for 2 h with vortexing every 30 min. At the end of the incubation, the solution was neutralized to pH 7.4 using 6 μL of 1 M Tris pH 9. Returning the solution to physiological pH prevents dissociation of the lipid-monomer complex. Equal amounts of a streptavidin conjugate were then added every 10 min for 100 min until a final volume of 15.43 μL streptavidin allophycocyanin (Life Technologies, Carlsbad, CA) or 28.83 μL of streptavidin phycoerythrin (Life Technologies, Carlsbad, CA) had been added. The stepwise addition of streptavidin ensured that each molecule bound to the maximum number of monomers and optimized for tetramerization. The final product was filtered through a SpinX column (Sigma, St. Louis, MO) to remove aggregates. The reagent was then stored at 4 °C until use.

Biotinylated hCD1b monomer has a molecular weight of 57,106.2 daltons (Da), and 9 μL of a 2 mg/mL stock solution is used, which is equal to $3.15 * 10^{-10}$ moles (mol). C32 GMM is used at 100-fold molar (M) excess and has a molecular weight of 659 Da (Moody et al., 1997). Thus, $3.15 * 10^{-8}$ mol of C32 GMM is needed.

$$3.15{*}10^{-8}\ \text{mol}{*}659\ \text{Da} = 20.76\ \text{ug}$$

Our GMM stock is stored at 1 mg/mL, so 20.76 μL is needed for this reaction. Streptavidin is a complex made up of four subunits, and each has one binding site for biotin. For every four molecules of hCD1b monomer, one molecule of streptavidin is needed, and streptavidin is used at a 25% excess to ensure each hCD1b monomer is bound.

$$\frac{(3.15{*}10^{-10}\ \text{mol})}{4}{*}1.25 = 9.844{*}10^{-11}\ \text{mol streptavidin}$$

Streptavidin conjugates are provided at 1 mg/mL (Life Technologies, Carlsbad, CA), and the weight of the fluorophore is included. Streptavidin-APC has a molecular weight of 156,800 Da ($6.378 * 10^{-6}$ M), and streptavidin-PE has a molecular weight of 292,800 Da ($3.415 * 10^{-6} M$).

$$\frac{9.844{*}10^{-11}\ \text{mol}}{6.378{*}10^{-6}\ \text{M}} = 15.44\ \mu\text{L streptavidin} - \text{APC}$$

$$\frac{9.844{*}10^{-11}\ \text{mol}}{3.415{*}10^{-6}\ \text{M}} = 28.83\ \mu\text{L streptavidin} - \text{PE}$$

2.4. Derivation of T cell lines

PBMC were isolated from healthy South African adults. The cells were depleted of CD14-expressing monocytes for a separate study and cryopreserved. For this study, the monocyte-depleted PBMC were thawed, washed in warm, sterile-filtered R10 supplemented with Benzonase (Millipore, Billerica, MA) at 10 μL/mL, and enumerated using Trypan blue exclusion. The cells were rested overnight in a 37 °C humidified incubator supplemented with 5% CO_2 at a density of three million cells per well in a 24-well plate in the presence of TCM. The following day, PBMC were washed and blocked with human serum (Valley Biomedical, Winchester, VA) in FACS buffer (1× phosphate-buffered saline (PBS) (Gibco, Waltham, MA) supplemented with 0.2% bovine serum albumin (BSA) (Sigma, St. Louis, MO)) mixed 1:1 for 10 min at 4 °C. The samples were washed twice with PBS and stained with Aqua Live/Dead stain (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Following two additional PBS washes, cells were resuspended in 50 mL FACS buffer and 1 μL of either unloaded CD1b tetramer or GMM-loaded CD1b tetramer and incubated