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Versatility of using major histocompatibility complex class II dextramers for derivation and characterization of antigen-specific, autoreactive T cell hybridomas

Bharathi Krishnan [a], Chandirasegaran Massilamany [a], Rakesh H. Basavalingappa [a], Rajkumar A. Rajasekaran [a], Charles Kuszynski [b], Barbara Switzer [c], Daniel A. Peterson [d], Jay Reddy [a,*]

[a] School of Veterinary Medicine and Biomedical Sciences, University of Nebraska-Lincoln, Lincoln, NE 68583, United States
[b] Nebraska Center for Virology, University of Nebraska-Lincoln, Lincoln, NE 68583, United States
[c] College of Medicine, Dean's Research Laboratory, University of Nebraska Medical Center, Omaha, NE 68918, United States
[d] Department of Pathology, Johns Hopkins School of Medicine, Baltimore, MD 21205, United States

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ABSTRACT

Antigen-specific, T cell hybridomas are useful to study the cellular, molecular and functional events, but their generation is a lengthy process. Thus, there is a need to develop robust methods to generate the hybridoma clones rapidly in a short period of time. To this end, we have demonstrated a novel approach using major histocompatibility complex (MHC) class II dextramers to generate T cell hybridomas for an autoantigen, proteolipid protein (PLP) 139–151. Using MHC class II dextramers assembled with PLP 139–151 as screening and sorting tools, we successfully obtained mono antigen-specific clones within seven to eight weeks. In conjunction with other T cell markers, dextramers permitted phenotypic characterization of hybridoma clones for their antigen specificity in a single step by flow cytometry. Importantly, we achieved successful fusions using dextramer^{+} cells sorted by flow cytometry as a starting population, resulting in direct identification of multiple antigen-specific clones. Characterization of selected clones led us to identify chemokine receptor, CCR4^{+} to be expressed consistently, but their cytokine-producing ability was variable. Our work provides a proof-of principle that the antigen-specific, CD4 T cell hybridoma clones can be generated directly using MHC class II dextramers. The availability of hybridoma clones that bind dextramers may serve as useful tools for various *in vitro* and *in vivo* applications.

1. Introduction

T cell hybridomas are useful tools for studying antigen-specific cellular, molecular, and functional events at a monoclonal level (Kubota and Iwabuchi, 2014; Rock, 1985; Rock and Benacerraf, 1983; Schrader and Clark-Lewis, 1982; White et al., 2000; Kruisbeek, 2001). Unlike primary T cell clones, which may eventually lose their antigen specificity over a period of time if left unstimulated, T cell hybridomas can maintain antigen specificity for extended periods of time because of their inherent ability to grow continuously in cultures (Rock, 1985; White et al., 2000; Kruisbeek, 2001).

T cell hybridomas are generated through fusion of antigen-sensitized effector T cells with mouse thymoma-derived BW5147 αβ T cell receptor (TCR)−/− cells, leading to the expression of only the TCRs specific to the fused T cells (Rock, 1985; White et al., 2000; Kruisbeek, 2001; Kubota, 2006). However, the screening procedure for antigen specificity is a laborious process (Rock, 1985). Generally, CD4 T cell hybridomas are screened based on cytokine secretion in response to antigen stimulations, and individual clones are then obtained mainly by limiting dilution cloning (LDC) (Rock, 1985; Kruisbeek, 2001; Peterson et al., 1999). Other methods include dispersion in soft agar, micromanipulation and flow cytometry (Rock, 1985). All these methods require expertise with the individual screening systems, making them both less practical and less desirable. For example, LDC is a labor-intensive, time-consuming procedure, because the cells need to be diluted to a low density (2 to 3 cells/ml), followed by screening of individual clones for their antigen specificity (Rock, 1985; Lefkovits and Waldmann, 1984). At least 25 such clones must be verified repeatedly to obtain a single antigen-specific clone, and the entire process to derive antigen-specific hybridoma clones can take up to three months or longer, or, the fusions may need to be repeated (Rock, 1985; Kruisbeek, 2001). Thus, there is a critical need to develop robust methods to derive T cell hybridoma clones quickly and efficiently in a shorter period of time.

Abbreviations: MHC, major histocompatibility complex; PLP, proteolipid protein; TCR, T cell receptor; LDC, limiting dilution cloning; TMEV, Theiler's murine encephalomyelitis virus; CFA, complete Freund's adjuvant; M.tb, *Mycobacterium tuberculosis*; RT, room temperature; LNCs, lymph node cells; IL, interleukin; FBS, fetal bovine serum; Con-A, concanavalin-A; PEG, polyethylene glycol; HAT, hypoxanthine/aminopterin/thymidine; HT, hypoxanthine/thymidine; 7-AAD, 7-aminoactinomycin-D; ^{3}[H], tritiated; CCRs, chemokine receptors; PMA, phorbol 12-myristate-13 acetate; IFN, interferon; TNF, tumor necrosis factor.

* Corresponding author at: Room 202, Bldg VBS, School of Veterinary Medicine and Biomedical Sciences, University of Nebraska-Lincoln, Lincoln, NE 68583, United States.
E-mail address: nreddy2@unl.edu (J. Reddy).

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We previously reported creation of the next generation of major histocompatibility complex (MHC) class II tetramers, designated "dextramers," which enabled us to detect and enumerate the precursor frequencies of autoreactive, antigen-specific CD4 T cells in a variety of systems (Massilamany et al., 2011a, 2014a, 2014b). Structurally, dextramers contain dextran molecules (polymers of glucose), each carrying up to seven streptavidin moieties to which multiple biotinylated MHC/peptide monomers can be assembled. Thus, dextramers can engage more TCRs than that could be achieved with tetramers (Massilamany et al., 2011a). We demonstrated that MHC class II dextramers exhibited greater sensitivity and specificity than could be achieved with MHC class II tetramers in several autoantigens, such as proteolipid protein (PLP) 139–151, myelin oligodendrocyte glycoprotein 35–55, and cardiac myosin heavy chain-α 334–352 (Massilamany et al., 2011a). Using PLP 139–151 dextramers as screening tools, we have devised a novel approach for rapidly deriving antigen-specific T cell hybridoma clones within 7 to 8 weeks. Importantly, dextramer-assisted sorting of antigen-specific clones allowed us to evaluate the expression of T cell markers, TCRs, and their vβ-usage in a single step by flow cytometry, including cytokine secretion and chemokine receptor expression.

2. Materials and methods

2.1. Ethics statement

Five-to-six-week-old female SJL/J (H-2^s) mice were procured from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained in accordance with the animal protocol guidelines of the University of Nebraska-Lincoln, Lincoln, NE, USA. The study was conducted in accordance with the National Institutes of Health guidelines for the use of experimental animals, and the protocols were specifically approved by the University of Nebraska-Lincoln Institutional Animal Care and Use Committee (permit number: A3459-01; protocol # 999).

2.2. Peptide synthesis and immunization procedures

PLP 139–151 (HSLGKWLGHPDKF) and Theiler's murine encephalomyelitis virus (TMEV) 70–86 (WTTSQEAFSHIRIPLP) were synthesized on 9-fluorenylmethyloxycarbonyl chemistry (Neopeptide, Cambridge, MA, USA). TMEV 70–86 peptide previously has been shown to bind MHC class II allele, IA^s molecule in SJL mice (Massilamany et al., 2010, 2011a, 2011b; Lorenz et al., 1988; Reddy et al., 2003). Thus, we used TMEV 70–86 as a control peptide for PLP 139–151 in all assays. The peptides were HPLC-purified (>90%), identity-confirmed by mass spectroscopy, and dissolved in sterile 1× PBS prior to use. Peptide emulsions involving PLP 139–151 were prepared in complete Freund's adjuvant (CFA) containing *Mycobacterium tuberculosis* (M.tb, 1 mg/ml) H37RA extract (Difco Laboratories, Detroit, MI, USA), and administered subcutaneously into SJL mice (100 μg/mouse; n = 3) (Massilamany et al., 2010). At termination, animals were euthanized using a CO_2 chamber prefilled with 2% CO_2.

2.3. Generation of MHC class II dextramers

Dextramer reagents comprised of IA^s/PLP 139–151 and IA^s/TMEV 70–86 (control) were generated as described previously (Massilamany et al., 2011a). We have used IA^s/TMEV 70–86 dextramers as controls to ascertain TCR-binding specificity of IA^s/PLP 139–151 dextramers, in all dextramer staining reactions (Massilamany et al., 2011a). Briefly, the α and β constructs of IA^s allele along with the peptide of interest were expressed together using baculovirus expression systems in SF9 insect cells (Invitrogen, Carlsbad, CA). Soluble MHC class II monomers of IA^s were then purified, concentrated, and biotinylated using biotin ligase (25 μg/10 nmol of substrate; Avidity, Denver, CO) (Massilamany et al., 2010, 2011a, 2011b). The biotinylated monomers were assembled to fluorophore conjugated dextran molecules (kindly provided by Immudex, Copenhagen, Denmark) at a molar ratio of 20:1 in 1× Tris HCl 0.05 M, pH 7.2, by incubating in the dark for 30 min at room temperature (RT) (Massilamany et al., 2011a). The dextramer reagents were aliquoted and stored at 4 °C until use.

2.4. Generation of antigen-sensitized primary T cells

Ten days post-immunization with PLP 139–151, the draining lymph nodes (mandibular, axillary, inguinal, and popliteal) were collected and single cell suspensions were prepared. Lymph node cells (LNCs) were stimulated with PLP 139–151 (20 μg/ml) at a density of 5×10^6 cells/ml for two days in clone medium (RPMI medium supplemented with 10% fetal bovine serum [FBS], 1 mM sodium pyruvate, 4 mM L-glutamine, 1× each of non-essential amino acids and vitamin mixture, and 100 U/ml penicillin–streptomycin [Lonza, Walkersville, MD]) (Massilamany et al., 2010, 2011b, 2011c). After two days, the cultures were supplemented with clone medium containing interleukin (IL)-2 (hereafter called IL-2 medium) and maintained for an additional two days. Viable lymphoblasts were harvested on day 4 and maintained in IL-2 medium until fusion. In some experiments, LNCs obtained from immunized mice were expanded with concanavalin-A (Con-A; 1 μg/ml) at a density of 2×10^6 cells/ml for two days before fusion (Massilamany et al., 2014c).

2.5. Fusion with BW5147 αβ−/− cells

Three approaches were adopted for the generation of antigen-specific T cell hybridoma clones (Fig. 1).

2.5.1. Approach 1: derivation of T cell hybridomas using Con-A-stimulated T cells generated in immunized mice

LNCs stimulated with Con-A were harvested after 48 h, and cells were washed twice with DMEM (1× DMEM [HyClone laboratories, South Logan, UT] containing 10% FBS, 1 mM sodium pyruvate, 7.5 mM L-glutamine, 0.66 M L-arginine [Fisher BioReagents, Fair Lawn, NJ], 0.27 M L-asparagine [MP Biomedicals, LLC Solon, OH], 24 mM sodium bicarbonate [Sigma-Aldrich, St. Louis, MO], 10 mM HEPES [Roche Life Sciences, Indianapolis, IN], and 100 U/ml penicillin–streptomycin, 0.05 mM β-mercaptoethanol [PMD Biosciences, La Jolla, CA]). Cells were then mixed with BW5147 αβ−/− cells at a ratio of 1:4, washed once, and fused as described earlier (White et al., 2000; Kruisbeek, 2001; Canaday, 2013; Kohler et al., 1977). The tube containing the cell pellet was placed in a 37 °C water bath, and 0.4 ml of 50% polyethylene glycol (PEG) in 75 mM HEPES (Roche Life Sciences) was gently added in a circular motion over a 1-minute period. After stirring the pellet for an additional minute, a total of 10 ml of pre-warmed DMEM with 10% FBS (hereafter called hybridoma medium) was delivered, 1 ml during the first minute, followed by another ml during the second minute, and the rest (8 ml) during the next 2 min (1 ml/15 s) as the mixture was gently stirred continuously (White et al., 2000; Kruisbeek, 2001; Canaday, 2013). After washing with hybridoma medium, cells were plated in 96-well plates at a density of 3.6×10^5 cells/ml (5×10^4 cells/140 μl/well; Fig. 1).

2.5.2. Approach 2: generation of T cell hybridomas using antigen-stimulated T cells generated in vitro

Viable lymphoblasts were harvested on day 4 poststimulation with PLP 139–151 and fused with BW5147 αβ−/− cells at a ratio of 1:3; the cells were plated as above in 96-well plates (Fig. 1).

2.5.3. Approach 3: generation of T cell hybridomas using the MHC class II dextramer$^+$ cells sorted by flow cytometry

Viable lymphoblasts were harvested on day 4 poststimulation with PLP 139–151 and rested in IL-2 medium for two days. Cells were then stained with the dextramers (IA^s/PLP 139–151 and control dextramers)