Metal-Conjugated NeutrAvidin for MHC Multimer Assays Using Mass Cytometry

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Introduction

Antigen-specific T cells in blood can be detected through the use of soluble MHC-peptide ligands that engage αβTCR. T cell receptors (TCRs) expressed on T cells specifically recognize and bind to complexes of major histocompatibility complex (MHC) molecules and peptide fragments. CD8+ T cells, also called cytotoxic T lymphocytes (CTL), recognize complexes of MHC class I molecules and specific peptides. The fluorescent tetramer assay, developed in part by John Altman et al. (1) of the Vaccine Research Center, has become a standard tool for immunologists. Evan Newell et al. (2) adapted this MHC-peptide tetramer technology to mass cytometry (3) for the purpose of screening of up to 109 different peptide–MHC tetramers in a single human blood sample, as well as analyzing another 23 markers of T cell phenotype and function using a recombinant form of streptavidin conjugated to metal tags (Fluidigm Maxpar® kits). We will describe the workflow for enumeration and identification of CMV-specific CD8+ T cells with a) NeutrAvidin™-metal reagent complexed with HLA-A*0201 CMV pp65 biotinylated monomer (MBL International), b) metal-labeled surface markers, c) cisplatin dead cell identifier, and d) metal barcoding (4) of several samples. There are 198 different biotinylated monomers, commercially available from MBL International, which can be combined with up to 35 isotope-tagged NeutrAvidin™ reagents to design a highly multiparametric assay.

Methods and Materials

Source of PBMCs: CMV-positive characterized cryopreserved human PBMCs were obtained from Cellular Technology Limited (CTL). Cells were isolated from three donors by Ficoll-Paque™ gradient density centrifugation. One day prior to staining with tetramers, PBMCs were rested overnight in CTL test medium supplemented with L-glutamine.

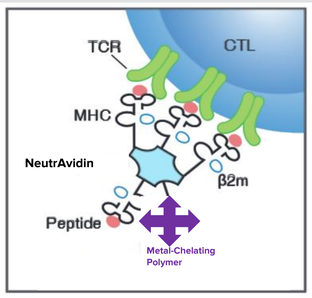
Source of monomers: HLA-A*0201 CMV pp65 (NLVPMVATV) Monomer Cat. No. MR01009; Negative Monomer Cat. No.MR01044 from MBL International.

Metal-conjugated Neutravidin: NeutrAvidin™ (Thermo Scientific™ Cat. No. PI-31000) was labeled with Fluidigm proprietary amine-reactive metal-chelating polymer according to an optimized protocol.

Cell staining: PBMCs rested overnight in CTL test medium supplemented with L-glutamine were stained with dead cell discriminator Cell-ID™ Cisplatin-198Pt (Fluidigm Cat. No. 201198) according to the technical data sheet. This was followed by the tetramer cocktail made from MBL monomers and NeutrAvidin-metal conjugates (Table 2). PBMCs were then stained with metal-labeled antibodies against surface markers (Table 1). Cells were fixed overnight in 1.6% formaldehyde and incubated with palladium barcoding reagents to combine all samples into one tube and discriminate doublets (6). Finally, the barcoded sample was stained with Intercalator-Ir (Fluidigm, Cat. No. 201192A) to identify nucleated cells.

Mass cytometry: Following DNA intercalation, samples were prepared for mass cytometry analysis by washing twice with Maxpar Cell Staining Buffer then once with Maxpar Water. Immediately prior to sample acquisition on the CyTOF® 2 mass cytometer, cells were resuspended in 4 mL of EQ Four Element Calibration Beads diluted to 0.1X in Maxpar Water. Samples were filtered through cell strainer cap tubes and injected into the mass cytometer for acquisition of approximately 250,000 events. All of the channels indicated in Table 1 were collected in addition to 198Pt for Cell-ID Cisplatin viability stain, 191Ir and 193Ir for nucleated cell identification, and 140Ce, 151Eu and 153Eu, 165Ho, and 175Lu for data normalization on EQ beads.

Data analysis: FCS files were normalized to the EQ beads using the CyTOF software. For conventional cytometric analysis of antigen-specific T cells FCS files were imported into FlowJo and Fluidigm Cytobank analysis software (5).



CTL-cytotoxic T lymphocyte; TCR -T cell receptor

MHC class I heavy chain, ß2-microglobulin (ß2m), and peptide are folded to generate a MHC class I/peptide complex (monomer), a process that is monitored daily for several days, depending on the particular peptide/MHC combination. The lysine residue in the C-terminus of MHC class I heavy chain is biotinylated using the BirA enzyme, and the monomers are purified by column purification. E. coli-expressed recombinant MHC class I heavy chain and ß2m are folded in the presence of a peptide antigen to generate a soluble monomeric MHC class I/peptide complex (monomer). The monomer is then biotinylated by the biotin ligase enzyme BirA at a lysine residue present in the biotinylation sequence that has been added to the C-terminus of MHC class I heavy chain of the complex. The biotinylated monomer is purified by column chromatography. The purified biotinylated monomers are tetramerized by mixing with a metal-labeled NeutrAvidin to make MHC metal-conjugated tetramer reagents. Schematic is modified from MBL International: www.mblintl.com/tetramer/classITetramers.aspx

Table 1. Mass Cytometry panel

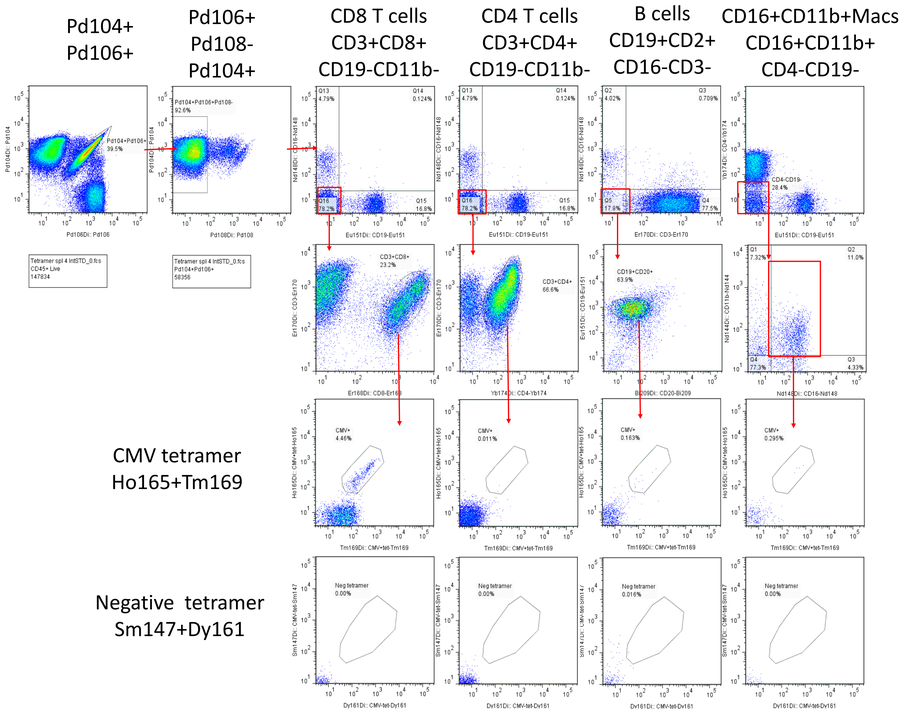
Channel	Isotope	Marker	Marker used for identification
144	Nd	CD11b	Cell population
148	Nd	CD16	Cell population
151	Eu	CD19	Cell population
168	Er	CD8	Cell population
170	Er	CD3	Cell population
174	Yb	CD4	Cell population
209	Bi	CD20	Cell population
147	Sm	CMV-neg.tetramer	Antigen-specific T cell
161	Dy	CMV-neg.tetramer	Antigen-specific T cell
165	Ho	CMV-pos.tetramer	Antigen-specific T cell
169	Tm	CMV-pos.tetramer	Antigen-specific T cell
171	Yb	CMV tetramers	Antigen-specific T cell
104	Pd	Barcode	Sample ID
106	Pd	Barcode	Sample ID
108	Pd	Barcode	Sample ID
198	Pt	Cell-ID stain	Dead cells

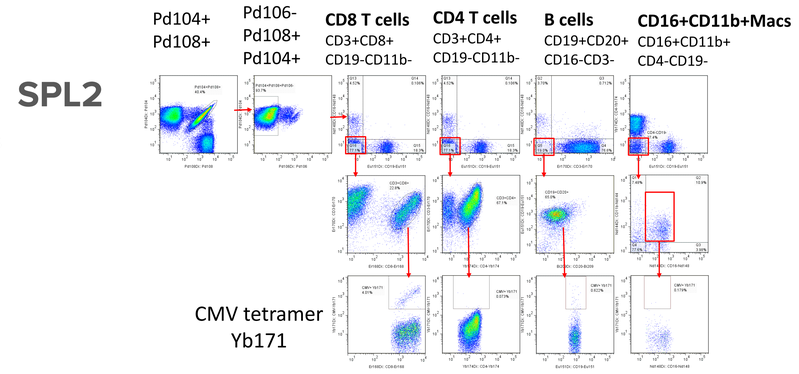
Results

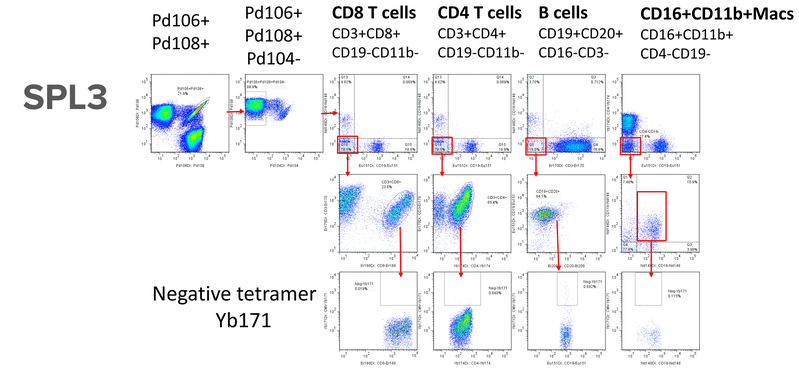
Table 2. Barcoding and tetramer staining of PBMCs samples

Sample	Barcodes	Tetramer identity	Tetramer tags
SPL1	Pd104, Pd106	CMV	Ho165 + Tm169
		Negative	Sm147 + Dy161
SPL2	Pd104, Pd108	CMV	Yb171
SPL3	Pd106, Pd108	Negative	Yb171

All samples were stained with the same surface markers. Plots are presented with data normalized to EQ beads and gated on CD45-positive, Cisplatin-negative cells







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Conclusion

- Using 10 markers for cell phenotyping and discrimination of dead cells, in combination with metal-tagged tetramer staining, enabled identification of CMV-specific cytotoxic T lymphocytes in donor PBMCs samples.
- Using the dual-barcoding system enabled a decrease in background staining of metal-tagged tetramers (e.g., B-cells)
- Protocol for metal-tagged tetramer assay was optimized, validated, and shown to be easy to use.

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