Preparation of peptide–MHC and T-cell receptor dextramers by biotinylated dextran doping

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Peptide–major histocompatibility complex (pMHC) multimers enable the detection, characterization, and isolation of antigen-specific T-cell subsets at the single-cell level via flow cytometry and fluorescence microscopy. These labeling reagents exploit a multivalent scaffold to increase the avidity of individually weak T-cell receptor (TCR)-pMHC interactions. Dextramers are an improvement over the original streptavidin-based tetramer technology because they are more multivalent, improving sensitivity for rare, low-avidity T cells, including self/tumor-reactive clones. However, commercial pMHC dextramers are expensive, and in-house production is very involved for a typical biology research laboratory. Here, we present a simple, inexpensive protocol for preparing pMHC dextramers by doping in biotinylated dextran during conventional tetramer preparation. We use these pMHC dextramers to identify patient-derived, tumor-reactive T cells. We apply the same dextran doping technique to prepare TCR dextramers and use these novel reagents to yield new insight into MHC I–mediated antigen presentation.

T cells are the central mediators of adaptive immunity. The unique specificity of each naïve T cell is determined by the T-cell receptor (TCR), which binds antigenic peptides presented on the surface of a target cell by major histocompatibility complex (MHC) molecules. The affinity of the TCR for a monomeric peptide–MHC ($K_d \approx$ 0.1–400 μM) is much weaker than the nanomolar affinity of a typical antibody–antigen interaction (1). To enable direct and stable labeling of antigen-specific T cells, Davis and colleagues assembled four biotinylated peptide–MHC (pMHC) monomers non-covalently around a fluorescently labeled, tetrameric streptavidin core (2) (Figure 1A). This pMHC tetramer exploits avidity to reduce the off-rate of TCR–pMHC interactions, enabling detection and monitoring of antigen-specific T-cell subsets via multicolor flow cytometry. However, T cells bearing low-affinity TCRs (>80 μM) are poorly detected using pMHC tetramers (3). This is particularly problematic for detection of T cells mediating autoimmunity and anti-tumor immunity, as thymic selection eliminates T cells with high affinity for non-mutated self-antigens (1,4).

Increasing the avidity of pMHC multimers beyond that of tetramers improves detection of low-affinity T cells. For instance, high-valency pMHC multimers such as dextramers and the recently described dodecamers enable detection of 2- to 5-fold more specific $CD8^+$ T cells than do corresponding tetramers (5,6). Currently, commercial pMHC dextramers (Immudex) are constructed by assembling biotinylated pMHC monomers around a dextran scaffold functionalized with both streptavidin and fluorochrome (Figure 1A). This dual-functionalized dextran is not available as a commercial product, and ready-to-use dextramers are expensive reagents ($1250/50 tests; Immudex). Additionally, the covalent conjugation of streptavidin and fluorochrome to the dextran scaffold precludes the flexibility afforded by commercial streptavidin reagents (i.e., the large variety of fluorochrome-streptavidin conjugates available).

Here, we describe a simple and inexpensive protocol for producing dextramers. Streptavidin-fluorochrome is bound to a sub-stoichiometric number of biotinylated pMHCs or biotinylated TCRs, leaving some streptavidin sites available for binding additional biotin groups. Biotinylated high-molecular-weight dextran is then doped into the binding reaction, resulting in the assembly of high-valency dextramers. We demonstrate that the resulting pMHC dextramers or

METHOD SUMMARY

Here, we present a simple, inexpensive protocol for preparing peptide–major histocompatibility complex (pMHC) dextramers. Streptavidin is assembled with a sub-stoichiometric number of biotinylated pMHC molecules, and the remaining available biotin-binding sites enable subsequent high-valency assembly on a biotinylated dextran scaffold. This flexible protocol can be used with various fluorochrome-conjugated streptavidins as well as with other biotinylated molecules, including T-cell receptors (TCRs).

TCR dextramers can be used to detect and monitor low-affinity (tumor) antigen-reactive T-cell subsets or to study antigen presentation.

Materials and methods

Primers were purchased from Integrated DNA Technologies (Coralville, IA). KOD Hot Start Master Mix (containing KOD Hot Start Polymerase) and R-1003-G Polybrene Infection/Transfection Reagent were purchased from EMD Millipore (Darmstadt, Germany). Sequencing was performed by Retrogen Inc. (San Diego, CA). Fluorescently labeled antibodies used for flow cytometry were purchased from Biolegend (San Diego, CA). Anti-CD3 (OKT3) and anti-CD28 (CD28.2) activating antibodies were purchased from eBioscience (San Diego, CA). Cytokines were purchased from Peprotech, Inc. (Rocky Hill, NJ). Concanavalin A was purchased from Sigma-Aldrich (St. Louis, MO). RetroNectin was purchased from Clontech Laboratories (Mountain View, CA). BioT transfection reagent was purchased from Bioland Scientific (Paramount, CA). Cell culture media, antibiotics, and fetal bovine serum (FBS) were purchased from Corning (Corning, NY). Human AB serum was purchased from Omega Scientific (Tarzana, CA). Peptides were purchased from Anaspec (San Jose, CA). Biotin ligase (BirA) was prepared and used to biotinylate pMHC and TCR molecules as described previously (7).

Cell lines and primary cells

HEK 293T/17, Jurkat E6–1, and K562 cells were obtained from the American Type Culture Collection (Manassas, VA). Their identities were authenticated by STR analysis, and they were confirmed to be mycoplasma-free (DDC Medical, Fairfield, OH). HEK 293T/17 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) FBS and the antibiotics penicillin/streptomycin (pen/strep; 100 IU/mL pen, 100 μg/mL strep). Jurkat and K562 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) FBS, 50 μM β-mercaptoethanol, 1× MEM non-essential amino acids (NEAA), 10 mM HEPES, 1 mM sodium pyruvate, and antibiotics (pen/strep). The HLA-A2$^+$ K562 line (Kv701) was derived from the K562 line by transducing the cells with a lentiviral vector encoding HLA-A2 and ZsGreen, and then sorting for these markers. All cell lines were grown at 37°C with 5% atmospheric CO_2, and were split every 2–3 days. Primary human peripheral blood mononuclear cells (PBMCs) were purchased from the CFAR Virology Core Lab at the UCLA AIDS Institute. PBMCs from a patient with advanced melanoma were obtained through leukapheresis under UCLA IRB approval 10–001598 and viably cryopreserved in aliquots until use as previously described (8).

Peptide–MHC multimers

Biotinylated H-2K^b/ovalbumin$_{257–264}$ monomers were provided by the NIH Tetramer Core Facility (Atlanta, GA). HLA-A2 and β2-microglobulin were expressed in *E. coli*, refolded in the presence of peptide [MART1$_{26–35(A27L)}$ (ELAGIGILTV); NYESO1$_{157–165(C165V)}$ (SLLMWITQV); or UV-cleavable J-ligand for HLA-A2 (GILGFVF**J**L; **J** = 3-amino-3-(2-nitro)phenyl-propionic acid)], purified, and biotinylated as previously described (7). Peptide–MHC monomers were stored at -20°C or -80°C until use.

Peptide–MHC tetramers were prepared from monomers using fluorescently labeled streptavidin from Molecular Probes (Eugene, OR) according to the NIH Tetramer Core Facility protocol. One equivalent (eq) (2.5 μM final) of streptavidin-fluorochrome [either phycoerythrin (PE) or allophycocyanin (APC)] was added over 10 × 10 min time intervals to 4 eq biotin-pMHC (10 μM final). Peptide–MHC dextramers were prepared by doping the tetramer assembly reaction with bioti-

nylated dextran. Briefly, peptide–MHC and fluorescently labeled streptavidin were added at a molar ratio of 3:1 (7.5 μM : 2.5 μM final) and incubated 10 min. Biotinylated dextran, molecular weight (MW) 500 kDa (Molecular Probes) was then added to the incubation at 1:20 with respect to streptavidin (125 nM final) and incubated an additional 10 min. Peptide–MHC multimers were prepared fresh or stored briefly (<1 week) at 4°C. We attempted to use high-performance size-exclusion chromatography/multiangle light-scattering (HPSEC-MALS) to measure the average number of streptavidin molecules bound per dextran scaffold, but due to the large size, shape, and heterogeneity of the commercial dextran molecules, we were unable to determine this parameter.

Commercial dextramers for H-2K^b/ovalbumin$_{257–264}$ (SIINFEKL), HLA-A2/MART1$_{26–35(A27L)}$ (ELAGIGILTV), and HLA-A2/MART1$_{26–35(A27L)}$ (ELAGIGILTV) were purchased from Immudex (Copenhagen, Denmark) and used within 1 month of receipt.

TCR multimers

Soluble TCR monomers were prepared as described (9), with several modifications. A His$_6$ tag was fused to the C terminus of the TCRα ectodomain to enable purification by Ni-NTA (QIAGEN, Hilden, Germany), and the 15-amino-acid AviTag BirA substrate sequence (10) was fused to the C terminus of the TCRβ ectodomain to enable biotinylation. TCRα and TCRβ inclusion body pellets from *E. coli* were dissolved in inclusion body solubilization buffer (8 M urea, 25 mM MES pH 6.0, 10 mM EDTA, 0.1 mM DTT) and stored at -80°C until refolding. M1 TCRα dissolved poorly in 8 M urea and was instead dissolved in solubilization buffer containing 6 M guanidine hydrochloride (GnHCl). Thirty milligrams each of TCRα and TCRβ were diluted into 500 mL refolding buffer [3 M urea, 0.2 M Arg-HCl, 150 mM Tris-HCl pH 8.0, 1.5 mM reduced glutathione, 0.15 mM oxidized glutathione, protease inhibitor cocktail (Roche, Basel, Switzerland), and 1 mM PMSF (added daily)] and stirred at 4°C for 72 h. Refolded TCR was dialyzed in SnakeSkin dialysis tubing (10,000 Da MWCO) (cat#68100; ThermoFisher, Waltham, MA) at 4°C for 24 h in 4 L dialysis buffer (10 mM Tris pH 8.5, 50 mM NaCl) and then for an additional 24 h in fresh 4 L dialysis buffer. Dialyzed TCR was both concentrated and initially purified using Ni-NTA, further concentrated with centrifugal filters (30,000 MWCO, EMD Millipore), and then purified by size-exclusion chromatography. Purified TCR monomers were enzymatically biotinylated with BirA as described for pMHC (7), purified by size-exclusion chromatography, concentrated, supplemented with glycerol to 18%, and stored at -20°C or -80°C until use. TCR tetramers and dextramers were assembled analogously to pMHC tetramers and dextramers.

Determination of TCR–pMHC binding affinity

The affinities of the M1, 1G4, and F5 TCRs for their respective pMHC ligands were determined by surface plasmon resonance using a Biacore T200 biosensor instrument (Uppsala, Sweden). Streptavidin was immobilized via amine coupling on a Series S Sensor chip CM5 General Electric (Fairfield, CT) and then biotinylated peptide–MHC complex was bound to the chip at 100 nM [Cell 1: no pMHC; Cell 2: HLA-A2/InfluenzaM1$_{58–66}$ (GILGFVFTL); Cell 3: HLA-A2/MART1$_{26–35(A27L)}$ (ELAGIGILTV); and Cell 4: HLA-A2/NYESO1$_{157–165(C165V)}$ (SLLMWITQV)]. The chip was flooded with 1 mM d-biotin to block the remaining streptavidin, and then varied concentrations (15 μM–0.47 μM) of biotinylated TCR were flowed over all cells of the chip to measure TCR–pMHC binding. Cell 1 (blocked streptavidin only; no pMHC) was used for background subtraction, and kinetic constants were determined by fitting the data to a 1:1 Langmuir curve. The K_d values determined for

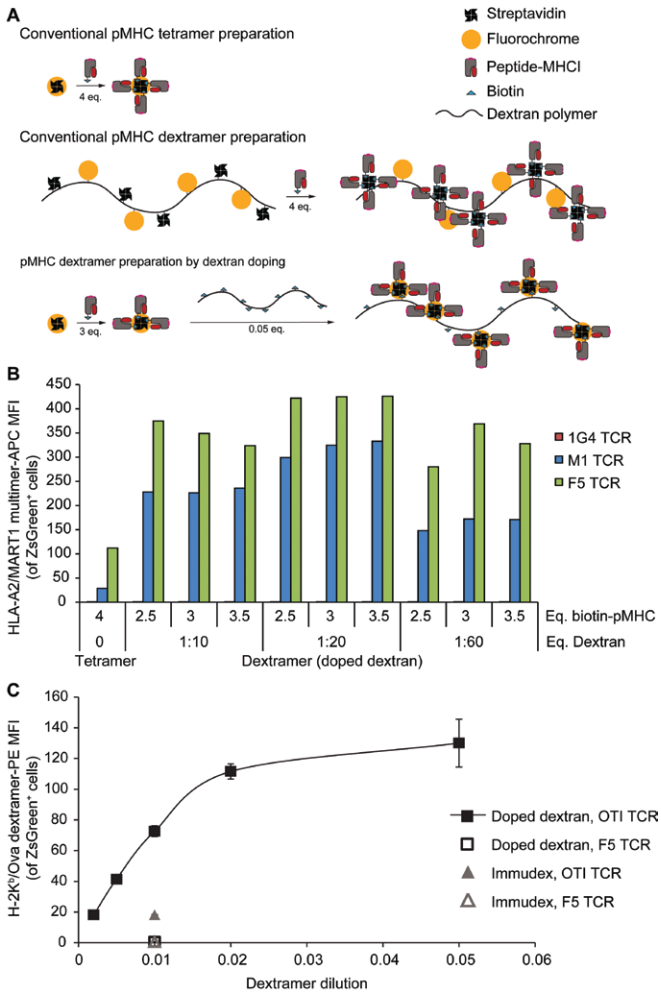

Figure 1. Design and optimization of dextran doping strategy for dextramer assembly. (A) Schematic comparing dextran doping to existing approaches for preparing peptide–major histocompatibility complex (pMHC) multimers. Equivalents (eq) are with respect to streptavidin. (B) Flow cytometry analysis of pMHC (HLA-A2/MART1) multimer binding by CD3$^+$ HEK 293T/17 cells transfected with a cognate (high-affinity F5 or low-affinity M1) or control (1G4) TCR. Multimers were prepared with the indicated molar equivalents of biotin-pMHC and dextran relative to streptavidin-fluorochrome conjugate [allophycocyanin (APC)]. (C) Flow cytometry analysis of pMHC dextramer H-2K^b/Ova binding by CD3$^+$ HEK 293T/17 cells transfected with the cognate (OTI) or control (F5) TCR. Data are triplicate measurements from a single experiment.

F5 TCR/HLA-A2/MART1$_{26–35(A27L)}$ (ELAGIGILTV) (5.4 μM) and 1G4 TCR/HLA-A2/NYESO1$_{157–165(C165V)}$ (SLLMWITQV) (4.3 μM) were similar to those previously reported (11,12). Antigen-specific binding was detectable for M1 TCR/HLA-A2/MART1$_{26–35(A27L)}$ (ELAGIGILTV), but an exact K_d was not determined (reported as >15 μM). Cross-reactivity of M1 or F5 for A2/NYESO1 or of 1G4 for A2/MART1 was not detectable.

TCR and pMHC single-chain trimer (SCT) viral vector constructs

OTI TCR, M1 TCR, 1G4 TCR, and F5 TCR retroviral constructs were generated as described (13). Peptide–MHC single-chain trimer (SCT) lentiviral constructs of NYESO1(SLLMWITQV)-β2m-HLA-A2 and MART1(ELAGIGILTV)-β2m-HLA-A2 were prepared with a disulfide trap modification as described(14).

Optimization of pMHC dextramer assembly and staining protocol

Tetramer preparation was performed per the standard NIH protocol, as detailed above. Dextramer preparation was performed as above except the ratios of biotinylated pMHC and biotinylated dextran to

streptavidin-allophycocyanin (APC) were varied as shown. HEK 293T/17 cells (50,000 cells/96-well plate well) were transfected with 0.1 μg DNA (0.02 μg each of plasmids encoding TCRαβ-IRES-ZsGreen, CD3δ, CD3ε, CD3γ, and CD3ζ) using BioT transfection reagent according to manufacturer's instructions. Medium was refreshed after 24 h. Cells were de-adhered from the plate 48 h after transfection using 2 mM EDTA in PBS and then stained for flow cytometry analysis with tetramer or dextramer preparations diluted 1:50 in FACS buffer (2% FBS in PBS).

To optimize the staining protocol, HEK 293T/17 cells were transfected as described above with 0.1 μg DNA [0.05 μg each of plasmids encoding TCRαβ-IRES-ZsGreen (murine OTI or human F5) and murine CD3(δεγζ)]. Transfected cells were stained for flow cytometry analysis with varying dilutions of H-2K^{b}/Ova$_{257–264}$ (SIINFEKL) dextramer purchased from Immudex or prepared in-house by biotinylated dextran doping.

Multimer staining of TCR-transduced primary human T cells

Murine stem cell virus (MSCV)-based retroviral vectors encoding LNGFRΔ-P2A-TCRα-F2A-TCRβ (LNGFRΔ is a cell-surface transduction marker) were produced in HEK 293T/17 cells as described previously (13). Primary T cells from human PBMCs were transduced in the presence of 10 μg/mL polybrene following activation with 1 μg/mL plated anti-CD3 (OKT3), 1 μg/mL anti-CD28, and 300 U/mL IL-2 in T-cell media [AIM-V media supplemented with 5% (v/v) human AB serum and antibiotics (pen/strep)]. Transduced primary T cells were stained with antibodies and either pMHC tetramers or pMHC dextramers. Stained CD8^{+} and CD8^{-} T cells were analyzed for multimer binding by flow cytometry.

UV-exchange dextramer preparation and testing

Biotinylated HLA-A2/J-ligand (GILGFVFJL) was diluted to 2 μM in PBS and exposed to UV light (365 nm) for 1 h in the presence of 200 μM exchange peptide as described previously (7). Following UV exchange, pMHCs were assembled into tetramers through 4 additions (at 10 min intervals) of streptavidin-APC to a final ratio of 4:1::pMHC:streptavidin. Alternately, streptavidin-APC was added in a single step to a final ratio of 3:1::pMHC:streptavidin and incubated 10 min; biotinylated dextran was then added to a final ratio of 0.05:1::dextran:streptavidin. Jurkat T cells transduced with retrovirus encoding LNGFRΔ-P2A-TCRα-F2A-TCRβ (either 1G4 TCR or F5 TCR) were stained for flow cytometry with UV-exchanged tetramers or dextramers.

Detection of low-frequency tumor-specific T cells from PBMCs

PBMCs were obtained through leukapheresis from a patient with metastatic melanoma, which had been previously described to contain up to 5% MART-1-specific CD8^{+} T cells (8). Viably frozen cells were thawed and resuspended in FBS containing APC-labeled commercial dextramer (1:20 dilution), tetramer prepared in-house (1:200 dilution), or dextramer prepared in-house by dextran doping (1:200 dilution). After 15 min at room temperature, an antibody mix was added containing anti-CD3ε(clone OKT3)-Brilliant Violet 421, anti-CD8α(clone SK1)-Brilliant Violet 510, anti-CD16(clone 3G8)-FITC, anti-CD19(clone HIB19)-FITC, and anti-CD4(clone RPA-T4)-APC-Cy7, all diluted to 1:100. Cells were incubated with antibody for 15 min on ice, then washed, centrifuged, and resuspended in FACS buffer containing 7-aminoactinomycin D (7-AAD) (00-6993-50; eBioscience, Inc., San Diego, CA). Cells were analyzed by flow cytometry to measure the percentage of live CD3^{+}CD8^{+} T cells specific for A2/MART1.

Detection of surface pMHC on peptide-loaded K562 cells using TCR dextramers

HLA-A2+ K562 cells were incubated with varying concentrations (16 nM–50 μM) of peptide for 2 h at 37°C. The peptide-loaded HLA-A2+ cells were then washed, stained with APC-labeled TCR tetramer or TCR dextramer (1:50 dilution), and then analyzed by flow cytometry.

Detection and resolution of single-chain trimer pMHC clusters using TCR dextramers

K562 cells were transduced with lentiviral vectors encoding peptide–MHC single-chain trimers (NYESO1(SLLMWITQV)-β2m-HLA-A2, MART1(ELAGIGILTV)-β2m-HLA-A2, or both vectors). Transduced cells were stained with 1G4 TCR dextramer-PE and F5 TCR dextramer-APC, and then analyzed by flow cytometry. For microscopy experiments, SCT-transduced K562 cells were stained with 1G4 TCR dextramer-AlexaFluor555 and F5 TCR dextramer-AlexaFluor647. Cell images were taken with an objective-type total internal reflection fluorescence microscope (Olympus X81; Olympus Life Science Solutions, Center Valley, PA) and analyzed with ImageJ.

Statistics

Significance analysis of tumor-specific T-cell multimers staining was conducted with one-way analysis of variance and Tukey's post-test for multiple comparisons. A statistical probability of $P < 0.05$ was considered significant. Descriptive statistics are presented throughout the figures as mean ± SD of replicate assays.

Results and discussion

We devised a simple strategy to produce pMHC dextramers: Streptavidin-fluorochromes assembled with sub-stoichiometric amounts of biotinylated pMHC molecules are subsequently assembled via their remaining open binding sites on a biotinylated dextran scaffold (Figure 1A). This dextran doping strategy has several advantages over the current protocol for pMHC-dextramer assembly. First, it is inexpensive and uses only commercially available reagents and biotinylated monomers that can either be made in-house or obtained without charge from the NIH Tetramer Core Facility. Second, the streptavidin and fluorochrome are not conjugated to the dextran core. Thus, dextran doping can be used with a wide variety of commercially available streptavidin-fluorochrome conjugates. Used in concert with UV-mediated peptide exchange (7) and multicolor combinatorial encoding (15), dextran doping enables construction of panels of pMHC dextramers for parallel detection of multiple low-affinity T-cell specificities. Third, this general protocol can be used to multimerize other interesting molecules (e.g., the TCR) to construct reagents that are not available commercially.

To optimize the dextramer assembly protocol, we varied the ratio of biotinylated pMHC (HLA-A2/MART1$_{ELAGIGILTV}$) molecules per fluorescent streptavidin molecule, then doped in various equivalents of biotinylated dextran. The resulting reagents were tested for their capacity to label HEK 293T/17 cells displaying either cognate TCR (A2/MART1-specific M1 and F5) or non-cognate TCR (A2/NYESO1-specific 1G4) (Figure 1B). Four conclusions can be drawn from this experiment. First, dextran doping increased the intensity of labeling compared with conventional tetramer staining for cognate TCR–expressing HEK 293T/17 cells. Second, the difference in staining intensity between tetramer and various dextramer preparations was larger for the low-affinity M1 TCR (5.5–11.7 fold) than that for the high-affinity F5 TCR (2.5–3.8 fold). Third, neither the A2/MART1 tetramer nor any of the preparations of dextramer bound to non-cognate 1G4 TCR–transfected HEK 293T/17 cells, indicating that dextran doping does not impair the specificity of binding. Finally, the staining intensity did not vary greatly across dextramer preparations (<2 fold), indicating that the high avidity resulting from dextran doping renders staining intensity robust toward the number of biotinylated-pMHC bound to each individual streptavidin. Notwithstanding this, we identified ratios of 3:1::pMHC:streptavidin and 0.05:1::dextran:streptavidin as optimal for dextramer assembly.

We next sought to evaluate the efficiency and specificity of cell staining with dextramers prepared by dextran doping. We prepared murine H-2K^b/Ova dextramers according to the optimized protocol described above and varied the dextramer dilution used to stain HEK 293T/17 cells displaying either cognate TCR (H-2K^b/Ova-specific OTI) or non-cognate TCR (F5) (Figure 1C). H-2K^b/Ova dextramer staining was specific and titratable, demonstrating that dextran doping can be

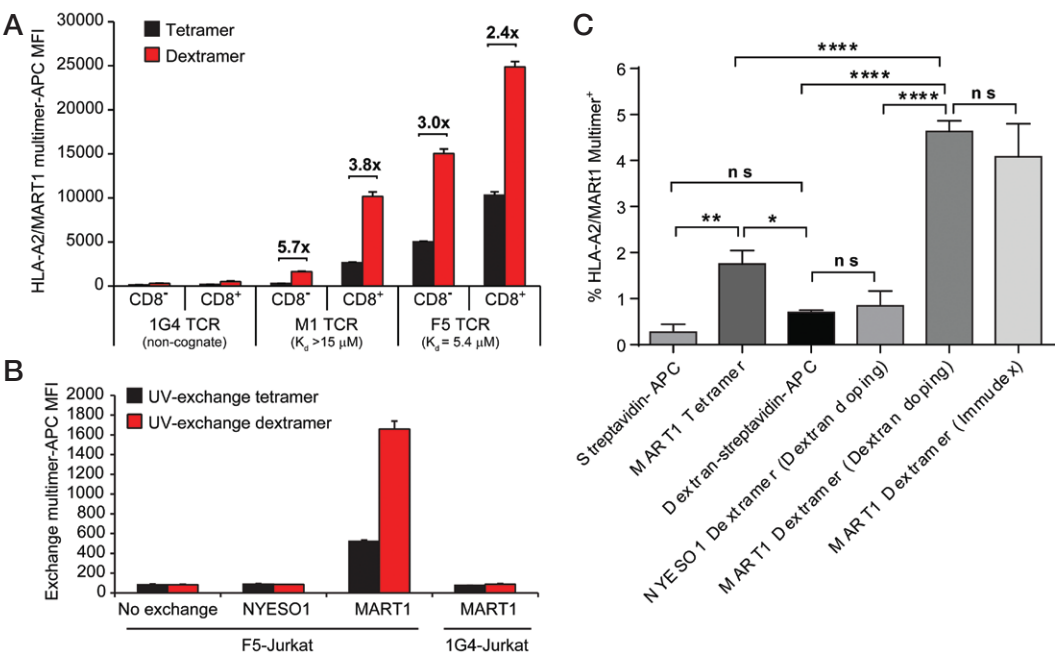

Figure 2. Dextran doping improves detection of low-affinity/tumor-reactive T cells. (A) Flow cytometry of peptide–major histocompatibility complex (pMHC) (HLA-A2/MART1) multimer binding by primary T cells transduced with cognate (high-affinity F5 or low-affinity M1) or control (1G4) T-cell receptor (TCR). (B) Flow cytometry comparing exchange pMHC multimer binding by Jurkat T cells transduced with cognate (F5) or control (1G4) TCR. Tetramers and dextramers were assembled following UV-mediated exchange of HLA-A2/J-ligand with 100-fold excess of the indicated peptide. Means ± SD for three replicate transductions are shown. (C) Flow cytometry of melanoma-reactive primary T cells collected by leukapheresis from a patient with melanoma. Means ± SD for three replicate experiments are shown. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$; ns, not significant.

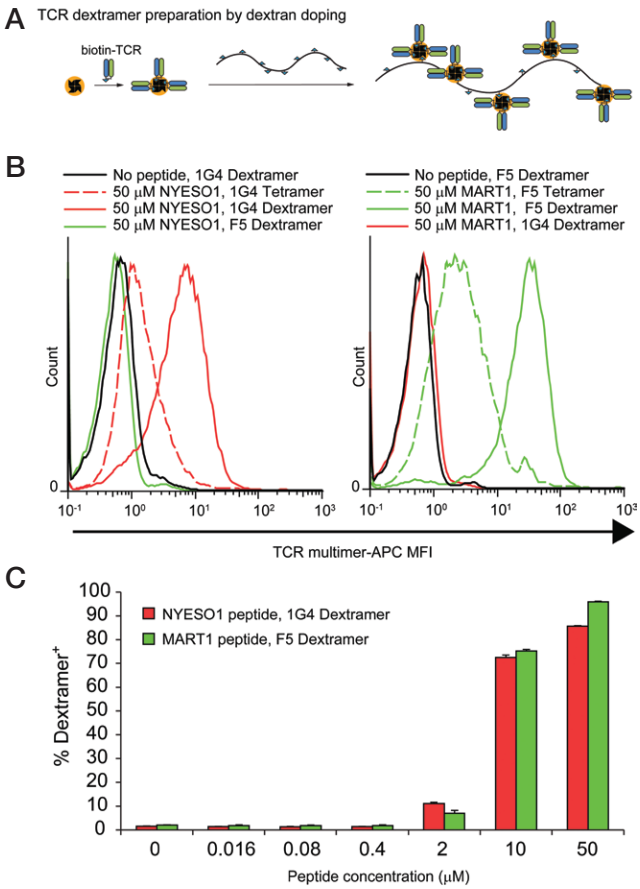

Figure 3. T-cell receptor (TCR) dextramers improve antigen-specific target-cell binding. (A) Schematic depicting TCR dextramer preparation using dextran doping. TCR tetramer preparation is performed analogously to peptide–major histocompatibility complex (pMHC) tetramer preparation as depicted in Figure 1A. (B) Overlay of flow cytometry histograms showing TCR (1G4 or F5) multimer binding by $A2^+$ K562 cells pulsed with 50 μM $NYESO1_{SLLMWITQV}$ (left) or $MART1_{ELAGIGILTV}$ (right) peptide. Data are representative of three replicate experiments. (C) Flow cytometry of TCR (1G4 or F5) dextramer binding by $A2^+$ K562 cells pulsed with varying concentrations of $NYESO1_{SLLMWITQV}$ or $MART1_{ELAGIGILTV}$ peptide. Means ± SD for three replicate experiments are shown.

applied to the assembly of murine pMHC multimers. We determined that a dilution of 1:200–1:50 is optimal for dextramers generated by dextran doping, yielding staining intensity comparable to or better than commercial H-$2K^b$/Ova dextramer. To compare non-specific background staining, primary human $CD8^+$ T cells were stained with A2/MART1 and A2/NYESO1 dextramers prepared in-house or purchased from Immudex. Background staining with in-house dextramers was not significantly higher than with commercial dextramers (0.11 ± 0.02% versus 0.06 ± 0.07%, $P = 0.31$ for A2/MART1 dextramer; 0.24 ± 0.18% versus 0.02 ± 0.04%, $P = 0.17$ for A2/NYESO1).

Straightforward assembly of dextramers will be particularly useful for the fields of cancer immunology and immunotherapy. Dextramers of class II pMHC molecules improve detection of antigen-specific $CD4^+$ T cells (16), which are emerging as important components of anti-tumor immunity (17,18) and are generally lower-affinity than $CD8^+$ T cells(19). Moreover, TCRs derived from tumor-reactive $CD8^+$ T cells are constrained by thymic selection to a low-affinity range (1,4) and are generally assisted in pMHC recognition by CD8:MHC I interaction (20). We transduced PBMCs with TCRs of varying affinity for A2/MART1 to determine the extent to which dextran doping improves pMHC multimer staining of low- and high-affinity TCRs expressed on $CD8^+$ or $CD8^-$ T cells. A2/MART1 dextramers did not bind T cells transduced with the non-cognate 1G4 TCR, confirming the specificity of these reagents in the context of primary T-cell staining (Figure 2A). By contrast, dextramers improved antigen-specific staining of cognate TCR–transduced T cells relative to tetramers. The extent of this improvement ranged from 2.4-fold for high-affinity TCRs on $CD8^+$ T cells to 5.7-fold for low-affinity TCRs on $CD8^-$ T cells, the latter of which could not be discerned from background by tetramer staining.

The ability to assemble a multiplexed panel of pMHC tetramers using UV-exchangeable peptide ligands constitutes a key advance for the characterization and isolation of tumor-specific T cells (7). To determine if dextran doping enables combination of the improved sensitivity of pMHC dextramers with pMHC UV-exchange technology, we prepared biotinylated HLA-A2 monomers bound to a UV-exchangeable peptide, irradiated the monomer in the presence of MART1 or NYESO1 peptide, prepared tetramers or dextran-doped dextramers with the exchanged monomers, and then used the resulting multimers to stain TCR-transduced Jurkat T cells. Similar to conventional multimer staining of primary $CD8^-$ T cells (Figure 2A), UV-exchanged dextramers stained F5 TCR–transduced, $CD8^-$ Jurkat T cells with a 3.2-fold higher mean fluorescence intensity (MFI) than UV-exchanged tetramers (Figure 2B). Binding was dependent on UV-exchange with MART1 ligand, and no binding was observed to cells transduced with a non-cognate TCR. Thus, dextran doping can be combined with pMHC UV-exchange technology to produce multimer panels with comparable specificity to and higher sensitivity than UV-exchanged tetramers.

To determine whether dextran-doped dextramers improve detection of T cells involved in endogenous anti-tumor immunity, we used tetramers or dextramers to stain PBMCs isolated from a melanoma patient whose peripheral repertoire was previously characterized to contain MART1-specfic T cells at a frequency of ~5% (8). While MART1-specific T cells were detectable using conventional tetramers, dextran doping improved the staining of these cells 2.6-fold (Figure 2C). Staining with dextran-doped dextramers was comparable to staining with commercial dextramers, suggesting dextran doping can be used to detect low-affinity T cells with significant cost savings compared with commercial dextramers.

Dextran doping is a general approach that can be used to increase non-covalent multimerization of any biotinylated molecule. Previous results showed that TCR tetramers enable specific and quantitative staining of target cells presenting cognate antigen, competitive blockade of T-cell-mediated immune responses, and potentially antigen-directed drug delivery (21–23). Multimerized TCRs can also be used to study TCR cross-reactivity and to discover peptide ligands for orphan TCRs (24–26). Thus, we produced TCR dextramers—reagents for which there is no commercial source—to determine if these applications may be facilitated by increasing reagent valency. TCR dextramers were produced by dextran doping, analogously to the scheme used to produce pMHC dextramers (Figure 3A).

To determine whether TCR dextramers enable specific staining of targets presenting cognate antigen, the human erythroleukemic cell line K562 was transduced with HLA-A2, the derived cell line was pulsed with either NYESO1 or MART1 peptide, and peptide-pulsed cells were stained with either NYESO1-specific 1G4 TCR or MART1-specific F5 TCR multimers. TCR multimers specifically stained those cells pulsed with their cognate peptide (Figure 3B). TCR dextramers improved staining over TCR tetramers for both specificities tested (7.9-fold for F5 TCR and 2.9-fold for 1G4 TCR). Dextramer staining

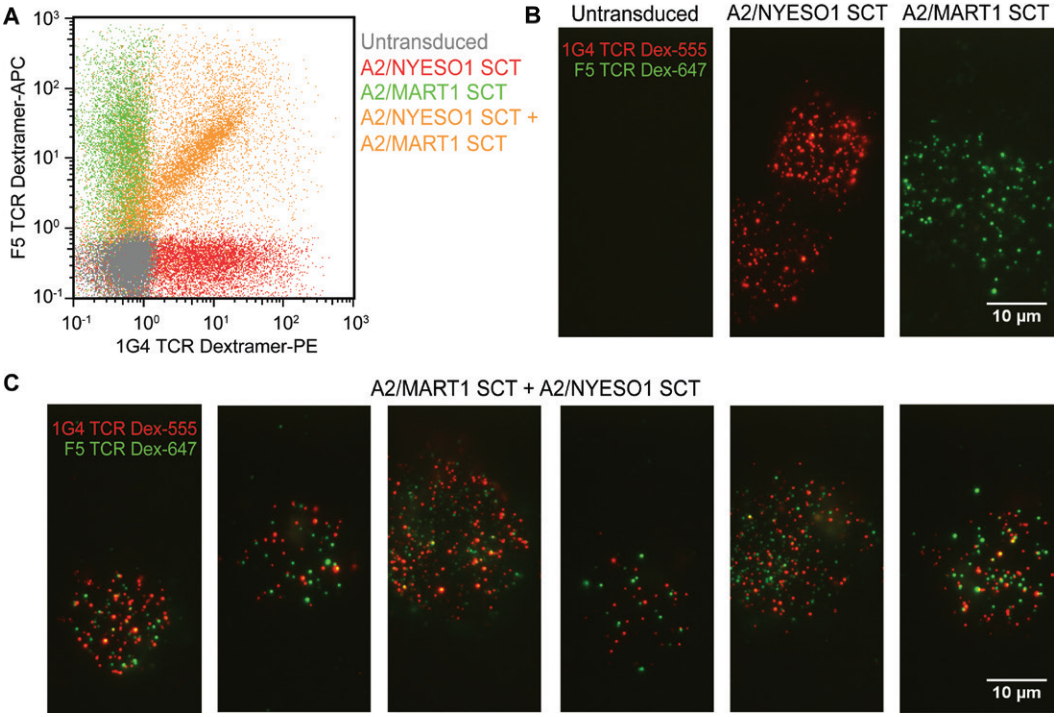

Figure 4. T-cell receptor (TCR) dextramers detect peptide-specific clustering of peptide–major histocompatibility complex (pMHC) single-chain trimers (SCTs). (A) Overlay of flow cytometry plots showing dual staining (1G4 and F5 TCR dextramers) of K562 cells transduced with mock vector, A2/NYESO1 SCT, A2/MART1 SCT, or both SCTs. Data are representative of three replicate experiments. (B) TIRF microscopy showing K562 cells mock transduced or singly transduced (A2/NYESO1 or A2/MART1 SCT) and then stained with both 1G4 and F5 TCR dextramers. Data are from a single experiment. (C) TIRF microscopy showing K562 cells transduced with both A2/NYESO1 and A2/MART1 SCTs, then stained with both 1G4 and F5 TCR dextramers. Multiple representative fields are shown from a single experiment. Images in (B) and (C) are overlays of two pseudocolored channels. Yellow indicates co-localization of TCR dextramers.

was quantitative, decreasing as the amount of pulsed peptide was titrated down (Figure 3C). Dextramer signal decreased precipitously at a peptide concentration of 2 μM and was undetectable at 0.4 μM and below. At these low levels of peptide, the density of cognate pMHC targets on the cell surface is too low for multivalent binding, so TCR dextramers are of limited utility. Importantly, these concentrations of pulsed peptide likely result in substantially more peptide presented on the cell surface than levels resulting from endogenous antigen processing. Indeed, we attempted to use F5 TCR dextramer to detect endogenously presented MART1 antigen from melanoma but were unsuccessful (data not shown). Thus, for applications requiring detection or targeting of endogenously expressed antigens, affinity-matured soluble TCR reagents are better suited than multimerized TCR reagents, regardless of valency (27,28).

Although TCR dextramers cannot detect endogenously processed antigens, they may be useful for specific studies related to antigen processing. For example, a previous study described a phenomenon of segregated clustering of class I pMHC molecules bearing different peptides on the surfaces of target cells (29). Peptide-specific pMHC clustering required endogenous expression (i.e., it was not observed following exogenous peptide pulsing), and it preceded export to the cell surface (i.e., it was detectable in the Golgi apparatus). However, it was not clear at which stage of antigen processing clustering was initiated, nor was it determined if this clustering was dependent on proteasomal processing or TAP-mediated import to the endoplasmic reticulum. Single-chain trimers (SCTs) are synthetic constructs that genetically link a particular peptide to β2-microglobulin and an MHC I heavy chain as a single polypeptide chain (14). Due to this linkage, cell-surface expression of SCTs bypasses proteasomal processing and peptide loading. Moreover, because these molecules ensure presentation of the encoded peptide on every surface-expressed SCT, we presumed they would be presented at sufficient density to engage avidly with multimerized TCR reagents. Therefore, TCR dextramers may be used to investigate whether SCTs—like pMHCs—are clustered on the target cell's surface in a peptide-specific manner.

We first determined whether TCR dextramers detect SCTs on the surfaces of target cells. We transduced K562 cells with A2/MART1 SCT, A2/NYESO1 SCT, or both and assayed these derived cell lines by flow cytometry for binding to both F5 and 1G4 TCR dextramers. As expected, untransduced cells bound neither TCR reagent, transduced cells expressing only one SCT bound specifically to the cognate TCR reagent only, and transduced cells expressing both SCTs bound both TCR reagents (Figure 4A). We then used total internal reflection fluorescence (TIRF) microscopy to investigate the clustering of these SCT molecules on the K562 cell surface. Consistent with flow cytometry results, untransduced cells did not bind either TCR reagent while singly transduced cells bound only their cognate TCR (Figure 4B). Interestingly, cells transduced with both SCTs exhibited distinct clusters of A2/MART1 and A2/NYESO1 SCT molecules, as evidenced by the lack of overlap between 1G4 and F5 TCR dextramer binding (Figure 4C). The importance of this result is two-fold. First, because SCTs bypass proteasomal processing, TAP-mediated transport to the endoplasmic reticulum, and peptide loading to the MHC, our data suggest that peptide-specific clustering of pMHC molecules occurs independently of these processes. Second, determining the rules governing pMHC clustering will require pairwise clustering analyses of many different peptides, a challenging prospect if cognate soluble TCR reagents are required for each peptide tested. SCTs may facilitate this investigation because the genetic linkage between a specific peptide and MHC molecule bypasses the need for soluble TCRs (e.g., epitope-indexed SCTs bearing different peptides can be resolved using epitope-specific antibodies).

Here, we present a simple technique for producing pMHC and TCR dextramers by assembling streptavidin-fluorochrome conjugates with sub-stoichiometric amounts of biotinylated monomers, followed by doping the assembly reaction with a highly biotinylated dextran scaffold. Like commercial dextramers, these dextran-doped dextramers improve the sensitivity of antigen-specific T-cell detection relative to pMHC tetramers. The advantages of this approach over commercial dextramers are that dextran doping is: (*i*) inexpensive; (*ii*) easy to adopt in a biology or immunology lab setting; (*iii*) compatible with state-of-the-art

techniques (e.g., UV-mediated peptide exchange and multicolor combinatorial encoding), enabling monitoring of multiple antigen-specific T-cell subsets; and (*iv*) applicable to other biotinylated molecules—such as TCRs—for which commercial sources of dextramers do not exist. The disadvantages of these reagents—which are shared with commercial dextramers—are two-fold. First, they rely on avidity and cannot bind stably to targets at a low density. For detection of endogenously loaded pMHCs, higher-affinity reagents such as affinity-matured TCRs or TCR-like antibodies are required. Second, the number of pMHC and fluorochrome molecules bound per dextran molecule is not strictly controlled. For highly quantitative studies, reagents that are molecularly defined may be better suited. Despite these caveats, our protocol presented should facilitate semi-quantitative studies requiring high-valency multimers of pMHCs or TCRs. As examples, we demonstrated the use of pMHC dextramers to detect tumor-reactive T cells from peripheral blood as well as the use of TCR dextramers to provide new insight into MHC I–mediated antigen presentation.

Author contributions

M.T.B. designed the study; M.T.B., B.C.-A., and Y.-H.H.F. performed the experiments; M.T.B. wrote the manuscript with input from B.C.-A., Y.-H.H.F., A.R., and D.B.; B.C.-A. and A.R. provided blood cells from a patient with metastatic melanoma; D.B. supervised the study.

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Competing interests

The authors declare no competing interests.

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