Supporting Information

A multimodal targeting nanoparticle for selectively labeling T cells**

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Peptide-MHC Tetramer Preparation

The peptide-MHC tetramer was synthesized as previously described.[1] Briefly, recombinant MHC Class-I heavy chain (in this case, D^b) and b2-microblobulin were expressed in E. coli and purified from the inclusion body. The $gp100_{25\text{-}33}$ pmel-1 peptide was folded into the MHC complex by dilution of the proteins, and the peptide-MHC complex purified by gel-filtration. The product was then biotinylated using the BirA enzyme (Affinity, LLC, Denver, CO) and re-purified by gel filtration. The tetramer was formed by mixing biotinylated peptide-MHC complex with Alexa Fluor 647-conjugated Streptavidin (Invitrogen) at 4:1 ratio.

Splenocyte Isolation

Pmel-1 is a transgenic mouse strain on a C57BL/6 background obtained from Jackson Laboratories. The transgene encodes a $gp100_{25–33}$-specific, H-2Db–restricted CD8+ TCR.[2] Pmel-1 mice were bred and housed at the Fred Hutchison Cancer Research Center animal facilities in a specific pathogen-free environment. Splenocytes were obtained from 6–10 week old Pmel-1 mice and B6 wild-type mice, filtered by passage through a 25g needle and incubated in RPMI 1640 with 10% heat-inactivated fetal bovine serum, 2mM L-glutamine, 25mM Hepes, 1mM sodium pyruvate, 100 μg/ml streptomycin, and 100 μg/ml penicillin.

Nanoconjugate Preparation

All chemicals were purchased from Sigma Chemicals (St. Louis, MO) unless otherwise specified. Magnetite iron oxide nanoparticles were prepared as previously described,[3] and the surface was modified with a heterobifunctional poly(ethylene glycol) chain (MW 600) following a procedure developed previously.[4] Nanoparticles were isolated on a rare-earth magnet and washed twice in 150 mM boric acid pH 8.0. To 2g Fe nanoparticles (1 mg/mL) was added 200 μL of *N*-Succinimidyl iodoacetate (SIA; Molecular Biosciences, Boulder, CO; 1mg/mL in anhydrous DMSO), and the mixture was placed on a shaker at room temperature for 2 hrs. Unreacted SIA was removed with a Sephacryl S-200 HR column (GE Healthcare, Piscataway, NJ) against 150 mM boric acid pH 8.0.

Neutravidin (10 mg; Molecular Probes, Eugene, OR) was dissolved in 1 mL PBS and reacted with 43 μL Alexa Fluor 647 monosuccinimidyl ester (10 mg/mL in anhydrous DMSO; Molecular Probes). The mixture was placed on a shaker and reacted at room temperature for 1 hr. Unreacted dye was removed with a PD-10 desalting column equilibrated with 50 mM Na Bicarbonate pH 8.5.

Fluorophore-labeled neutravidin (1 mg in 500 μL) was mixed with 11.8 μL N-Succinimidyl-S-acetylthioacetate (SATA; Molecular Biosciences; 0.6 mg/mL in anhydrous DMSO) and allowed to react for 2 hrs at room temperature. The neutravidin-SATA solution was then mixed with a deprotection solution (55 μL of 0.5M hydroxylamine and 25 mM EDTA, pH 7.2) for 40 min at room temperature. The mixture was then passed through a Zeba spin column equilibrated with 100 mM boric acid pH 8. Isolated neutravidin was mixed with 2 mg Fe SIA-modified nanoparticles overnight.

Nanoparticles were passed through a Sephacryl S-200 HR column equilibrated against 0.1 M boric acid pH 8.0, then isolated on a rare earth magnet, and redispersed in the same buffer. Nanoparticle concentration was determined by inductively coupled plasma atomic emission spectroscopy, and 100 μg peptide-MHC was mixed with 288 μg Fe nanoparticles (700 μL total volume) for 30 min.

Cell Labeling and Analysis

Primary cell cultures were incubated in media at ~3×10^6 cells/mL. Peptide-MHC labeled nanoparticles and unlabeled nanoparticles (control) were incubated with cells at 0.1 mg/mL Fe for 1 or 3 hrs. Alternatively, cells were incubated with 35 μL Peptide-MHC tetramer (0.12 mg/mL) for 1 hr at 37°C. Cells were washed of unbound nanoparticles or tetramer 3× with 0.2% FBS by centrifugation, and incubated with anti $CD8^+$-FITC antibody for 15 min at room temperature. Cells were again washed 3× with 0.2% FBS by centrifugation. CD69 analysis was conducted 18 hr post nanoparticle/tetramer incubation. Here, cells were incubated with fluorochrome-conjugated anti-CD69 for 15 min, followed by 3× washes with 0.2% FBS. Flow cytometry analysis was performed on a BD LSR II; data analysis was performed with the FlowJo software package. A minimum of 10,000 cells were counted for each sample. Cell samples for TEM analysis were prepared by fluorescence-assisted cell sorting (FACS) in the same manner as for flow cytometry analysis. Cells labeled with anti $CD8^+$ (FITC) antibody were separated from the splenocyte population using a BD FACS Aria cell sorter.

Fluorescent Imaging

After flow cytometry, 2×10^5 cells were plated on cover slips and fixed with a 4% paraformaldehyde solution. After fixation, cells were stained with 4',6-diamidino-2-phenyindole (DAPI) per the manufacturer's instructions. Confocal images were acquired on a DeltaVision SA3.1 wide-field deconvolution microscope (Applied Percision, Inc., Issaquah, WA) with DAPI and Cy5

filters. SoftWoRx (Applied Precision, Inc.) was used for image processing, including normalization of fluorescence intensity.

MR and TEM Imaging

Isolated splenocytes were incubated with either CTL-targeting (anti-CD8 antibody coated) or non-CTL-targeting magnetic nanoparticles (specific to alternative cell markers; Miltenyi, Auburn, CA). Each population was passed through an autoMACS magnetic column to remove labeled cells, and separate untouched CTLs and non-CTLs. These cells were incubated with peptide-MHC-conjugated nanoparticles for 3 hrs, washed 3× with PBS, and equilibrated to 1.5 million cells per sample. Cells were suspended in an agarose cast, as previously reported,[5] and visualized with a 4.7-T Varian MR spectrometer (Varian, Inc., Palo Alto, CA) and a Bruker magnet (Bruker Medical Systems, Germany) equipped with a 5-cm volume coil. A spin-echo multisection pulse sequence was selected to acquire MR phantom images. Repetition time (TR) of 3000 msec and variable echo times (TE) of 15–90 msec were used. The spatial resolution parameters were as follows: an acquisition matrix of 256 × 128, field of view of 4 × 4 cm, section thickness of 1 mm, and 2 averages. Regions of interest (ROIs) of 5.0 mm in diameter were placed in the center of each sample image to obtain signal intensity measurements using NIH ImageJ. T2 values were obtained using VnmrJ "t2" fit program to generate a T2 map of the acquired images. Cells incubated with peptide-MHC labeled nanoparticles were imaged with a Philips CM100 TEM at 100kV with a Gatan 689 digital slow scan camera.

Neutravidin

While the PEG coating on nanoparticles limits unwanted interactions, a small fraction of cells eliciting nonspecific nanoparticle association is not unexpected. To maintain minimal particle-cell interaction outside of the MHC/peptide presentation, neutravidin was exploited for its low isoelectric point and the lack of an expressed RYD sequence (present in streptavidin). Targeted nanoparticle labeling was high (74%) after incubation for 3 hrs compared to alternative loading schemes that require incubation times of over 24 hr, indicating efficient cell tagging.

Fluorescence Quantitation

Fluorescence quantitation of labeled neutravidin yielded an estimated 2.59 AF647 dyes per protein. Subsequent analysis of neutravidin-AF647 modified nanoparticle fluorescence against AF647 dilutions in a nanoparticle mixture (common nanoparticle concentration of 50 μg Fe/mL) gave an estimated

fluorophore concentration of 44.9 μg/mL, corresponding to 13.0 neutravidins per nanoparticle (Figure S1). As each neutravidin protein has four biotin binding sites, conjugated nanoparticles retain an estimated 52 biotin binding sites for conjugation with biotinylated peptide-MHC targeting complexes.

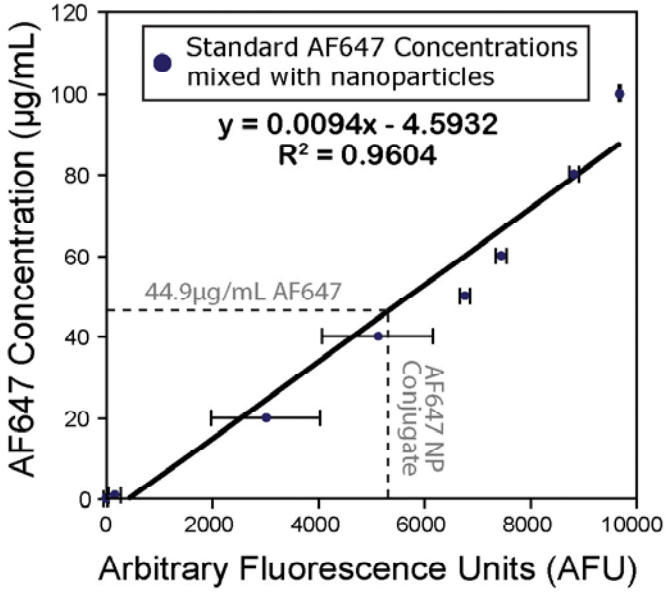

Figure S1. Fluorescence of AF647-conjugated nanoparticles mapped onto a standard curve of AF647 dilutions mixed with PEG-coated nanoparticles.

Active PEG Quantitation

Conjugation of the SPDP linker molecule to the amine-terminated nanoparticles allowed for quantitation of the activated groups after treatment with a reducing agent (dithiothreitol). Each bound SPDP group releases a cyclical 2-pyridine thione (2-PT) group with an absorption at 343 nm. UV/Vis analysis of separated 2-PT groups from a nanoparticle stock of known concentration (Figure S2) yielded an estimated 26.26 terminal-active PEG chains per nanoparticle.

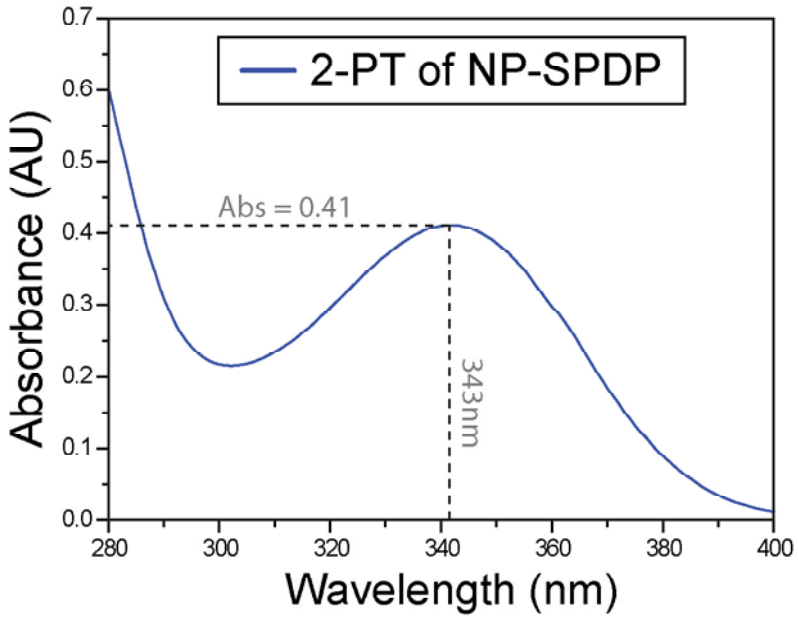

Figure S2. 2-Pyridine Thione (2-PT) absorbance of reduced nanoparticle-bound SPDP molecules indicating ~26 PEG chains per nanoparticle.

Fourier Transform Infrared (FTIR) Spectroscopy

Nanoparticle coating and surface functionalization with peptide-MHC monomers was confirmed by FTIR (Figure 1b). All analyzed nanoparticles showed a broad –OH stretch above 3000 cm^{-1} distinctive of the iron oxide surface. PEG-silane modified nanoparticles (NP-PEG-SIA) showed characteristic carbonyl (1642 and 1546 cm^{-1}) and methylene bands (2916 and 2860 cm^{-1}) of the immobilized polymer, and a Si-O peak (1105 cm^{-1}) indicating covalent binding of PEG to the nanoparticle surface. Complete nanoparticle constructs displaying the peptide-MHC monomers (NP-PEG-MHC-AF647), likewise displayed characteristic PEG peaks as well as amide I and amide II peaks (1650 and 1480 cm^{-1}, respectively) indicating the protein immobilization at the particle surface.

Control Nanoparticle Testing

Additional study of splenocyte populations incubated with non-targeting nanoparticles (NP-PEG-AF647) by fluorescence and electron microscopy was conducted to complement flow cytometry studies. Transmission electron microscopy verified little to no non-specific binding of neutravidin-only coated nanoparticles to CTLs ($CD8^+$; Figure S3b). Nanoparticle accumulation was typically found at the membrane surfaces of CTLs when cells were exposed to peptide-MHC targeting nanoparticles (Figure 3b). Similar binding was not observed with control nanoparticles that lacked the peptide-MHC complexes (Figure S3b), further verifying the flow cytometry studies. Confocal fluorescence

microscopy additionally showed little or no observed fluorescence activity of NP-PEG-AF647 (emission: 655 nm) inside, or at the surface, of CTL cells (Figure S3a). Here, cell nuclei were stained with DAPI, CTLs labeled with a FITC-conjugated anti-CD8+ antibody, and the nanoparticles were colorized in a red pseudocolor. These micrographs corroborated the results of the flow cytometry studies whereby limited nonspecific attachment of non-targeting neutravidin-coated nanoparticles was observed.

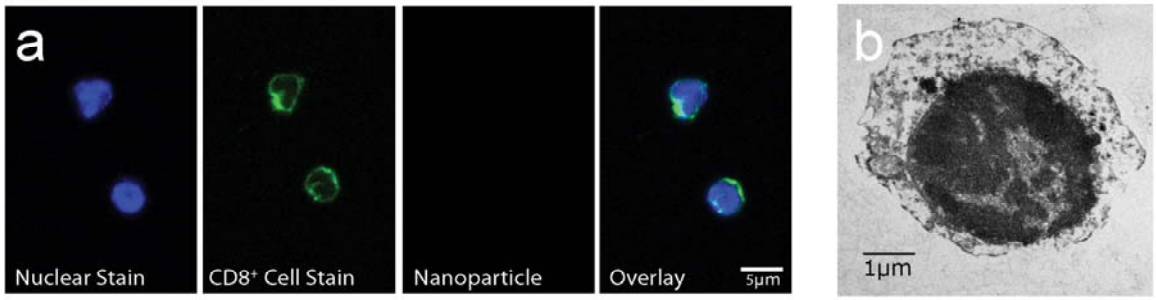

Figure S3. (a) Fluorescence and (b) electron microscopy analysis of CTLs incubated with neutravidin-conjugated control nanoparticles (NP-PEG-AF647). CTLs showed little or no nanoparticle binding.

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