SUPPLEMENTARY FIGURES

Photocrosslinkable pMHC monomers stain T cells specifically and cause ligand-bound TCRs to be 'preferentially' transported to the cSMAC

Jianming Xie, Johannes B. Huppa, Evan W. Newell, Jun Huang,

Peter J. R. Ebert, Qi-Jing Li & Mark M. Davis

Correspondence should be addressed to M.M.D. (mmdavis@stanford.edu).

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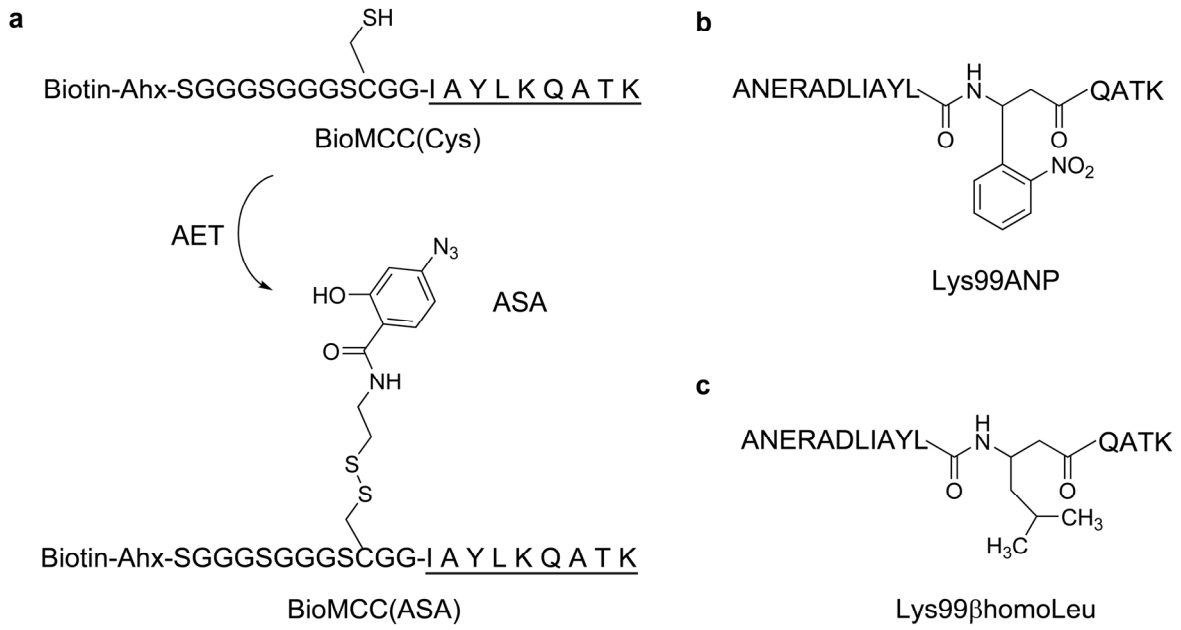

Supplementary Figure 1. Structure and synthesis of representative peptides used in this study. (**a**) BioMCC(ASA) is a photocrosslinkable derivative of MCC peptide, synthesized by solid-phase peptide synthesis and cysteine modification. Underlined is the MHC-binding and TCR-recognition region. AET = S-[2-(4-Azidosalicylamido)ethylthio]-2-thiopyridine. Ahx = 6-aminohexanoic acid, which is a flexible linker. (**b**) The exchangeable peptide Lys99ANP is a Lys99→ANP mutant of MCC peptide. (**c**) Another exchangeable derivative of MCC peptide in which Lys99 is replaced with β-homoleucine.

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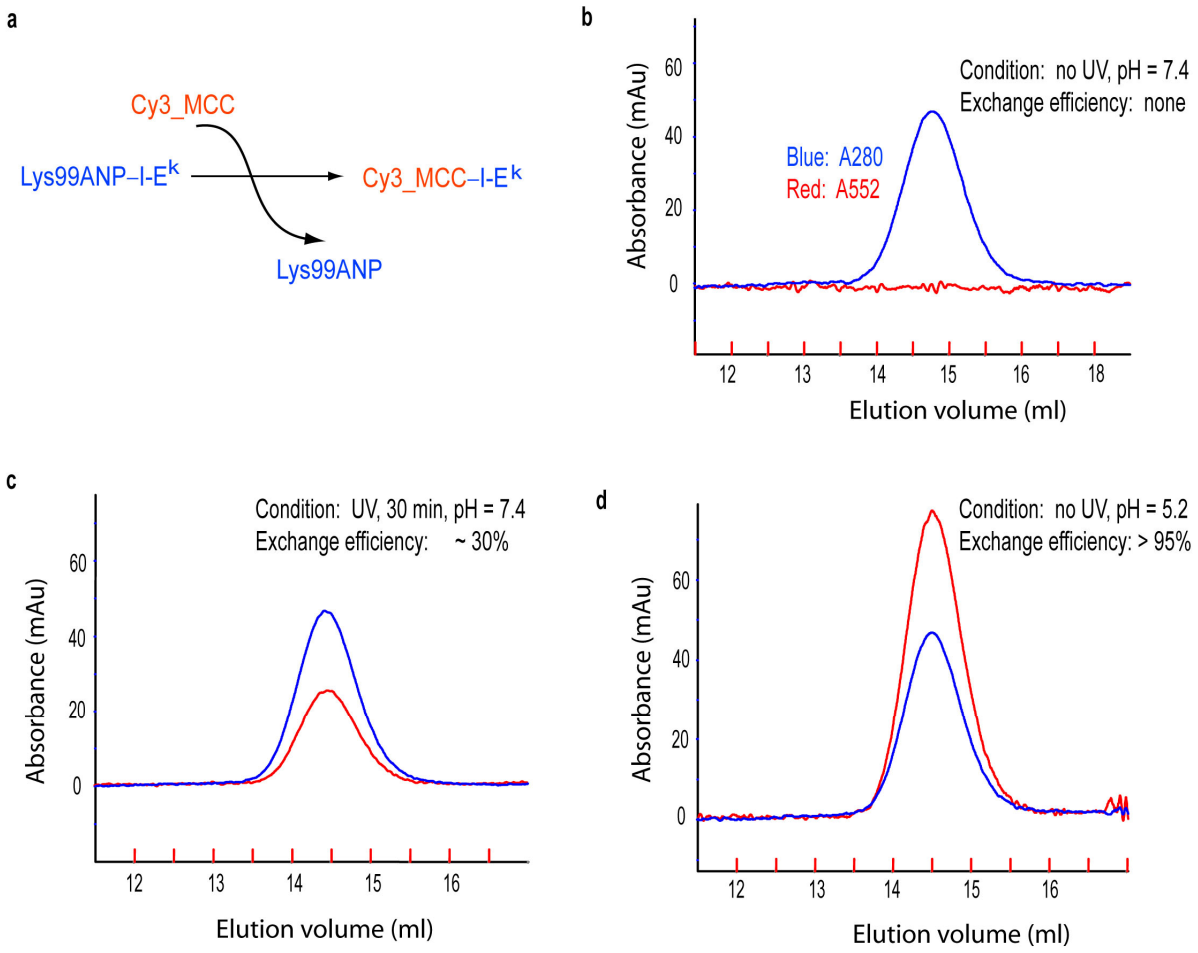

Supplementary Figure 2. A highly efficient, acid-induced peptide exchange method for I-E^k. (**a**) A schematic illustration of the peptide-exchange reaction between Lys99ANP–I-E^k and Cy3_MCC. After the exchange, peptide–I-E^k complexes and peptides were separated by Superdex 200 size-exclusion chromatography. The percentage of peptide exchange was determined by the absorption of purified proteins at 552nm (for Cy3_MCC–I-E^k) and at 280 nm (for all peptide–I-E^k). (**b**) Lys99ANP–I-E^k and Cy3_MCC were mixed in PBS buffer and incubated in the dark at 25 ^{o}C for 24 hours. The FPLC chromatography showed that the peak for peptide–I-E^k had no absorption at 552 nm, indicating that no peptide exchange occurred. (**c**) The same mixture was exposed to UV light (5 x 15 watt bulbs, 365 nm) for 30 min, and then incubated at 25 ^{o}C for 12 hours. Subsequent FPLC chromatography showed that the peak for peptide–I-E^k had some absorption at 552 nm. The calculated exchange yield was about 30%. (**d**) The same mixture was incubated at pH 5.2 for 24 hours at 25 ^{o}C without any UV irradiation. The peptide exchange was almost quantitative.

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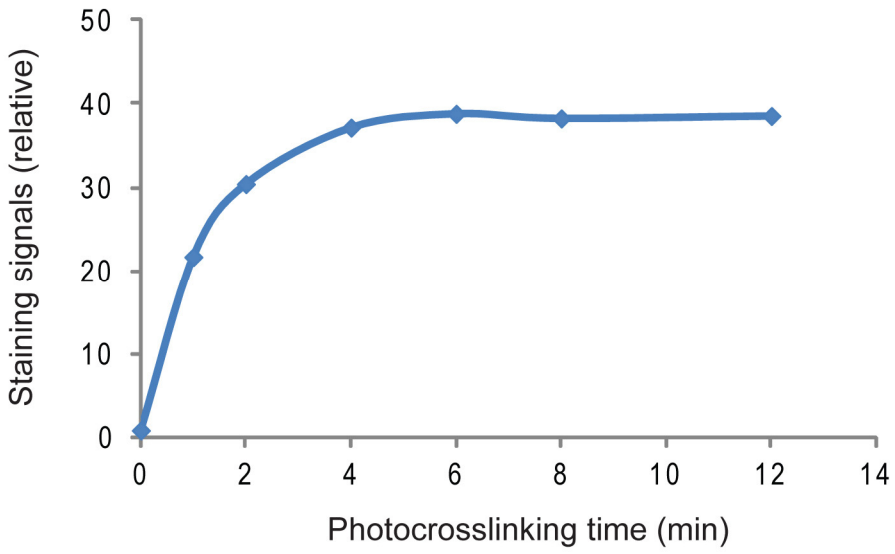

Supplementary Figure 3. Optimization of the photocrosslinking time for T cell staining using photocrosslinkable pMHC monomers. 5C.C7 T cell blasts were mixed with BioMCC(ASA)–I-E^k (0.5 μM). Aliquots of cells were exposed to UV lights at a 20 cm distance for varied periods of time (from 0-12 minutes) in a Stratalinker 2400 UV crosslinker equipped with 5 x 15 watt 365-nm UV lamps. Cells were then washed, stained with PE-SAv, and analyzed by flow cytometry. The average PE fluorescence intensity of T cells was plotted as a function of UV irradiation time. Results showed that the photocrosslinking efficiency increased significantly in the first 4 minutes, but essentially not with additional UV irradiation. This result suggests that the photoactivation is complete after 4 minutes. Accordingly, we chose to photoactivate for 5 minutes for all other experiments reported here. Data are representative of two independent experiments with similar results.

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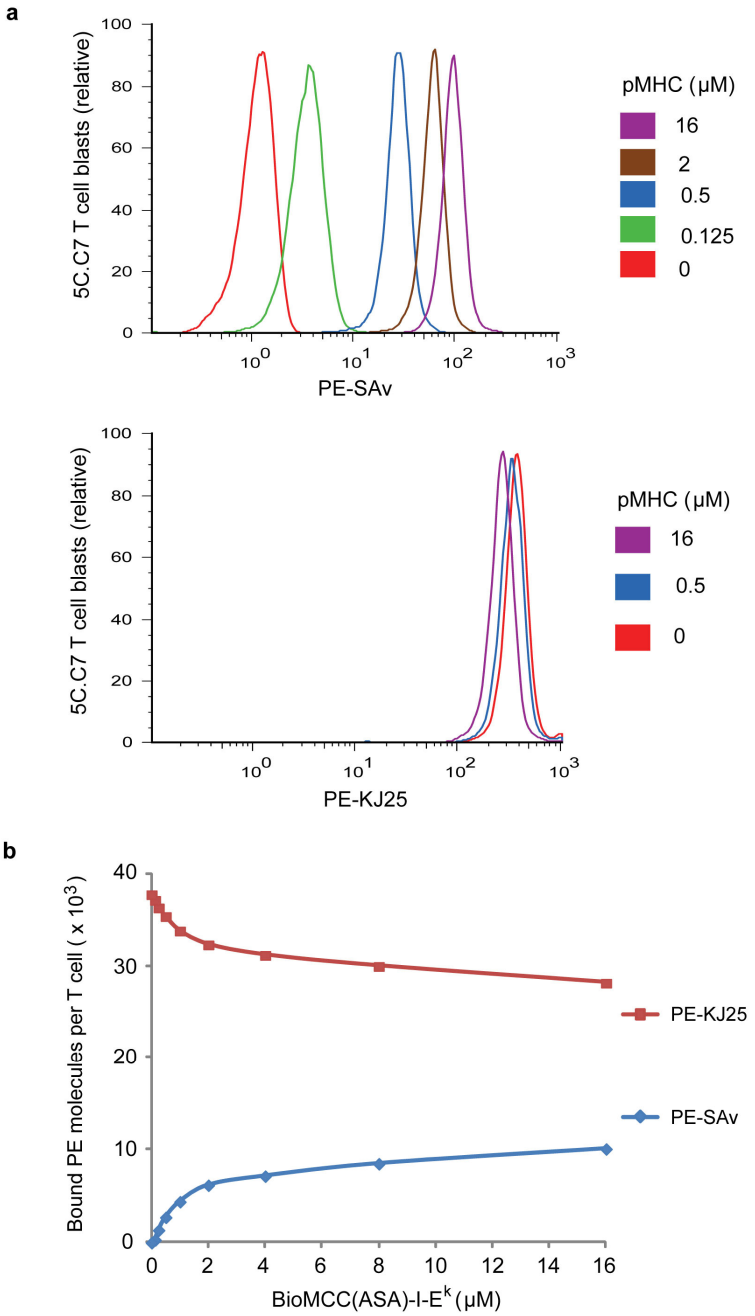

Supplementary Figure 4. Flow cytometric analysis shows that pMHC-TCR crosslinking and KJ25-TCR recognition are mutually exclusive. (**a**) 5C.C7 T cell blasts were photocrosslinked with BioMCC(ASA)–I-E^k at a series of concentrations (from 0 to 16 μM), and stained by PE-SAv and PE-KJ25, respectively, followed by flow cytometric analysis. (**b**) The numbers of bound PE-SAv and PE-KJ25 per cell were determined based on the fluorescence intensity of T cells and that of beads coated with known numbers of PE molecules, and then plotted against the concentration of BioMCC(ASA)–I-E^k used in the photocrosslinking. Data are representative of three independent experiments.

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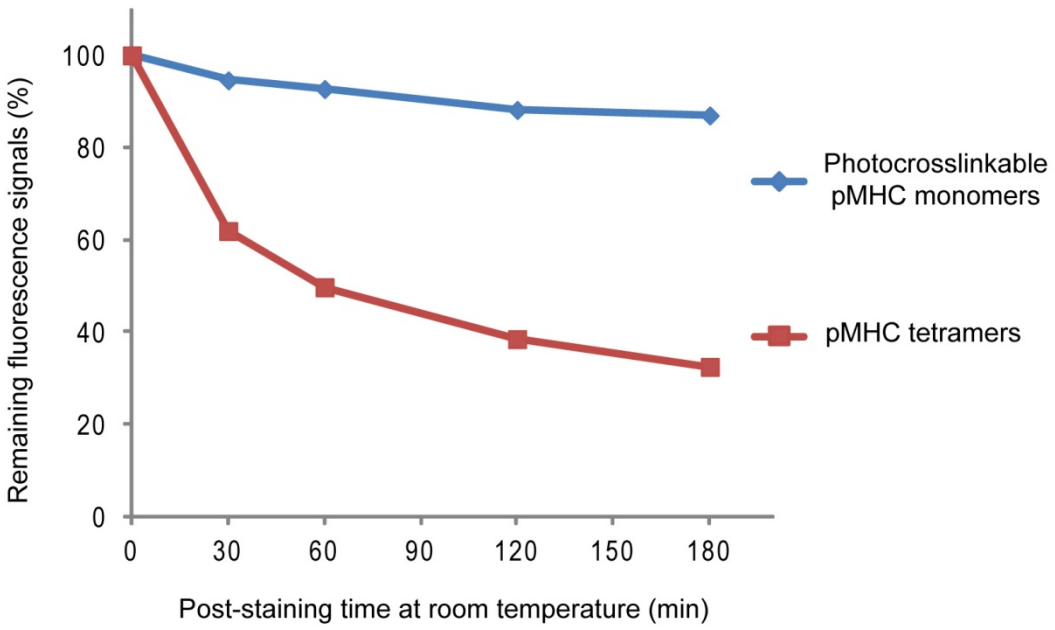

Supplementary Figure 5. The signal from photocrosslinkable pMHC monomers is more stable than pMHC tetramers. 5C.C7 T cell blasts were split into two aliquots. One aliquot was stained with PE-conjugated MCC–I-E^k tetramers, the other aliquot was photocrosslinked to BioMCC(ASA)–I-E^k and labeled with PE-SAv. T cells were washed to remove unbound ligand, and then incubated at room temperature. Both T cell samples were subjected to flow cytometric analysis at the indicated time points, and the relative fluorescence intensity was plotted against post-staining time.

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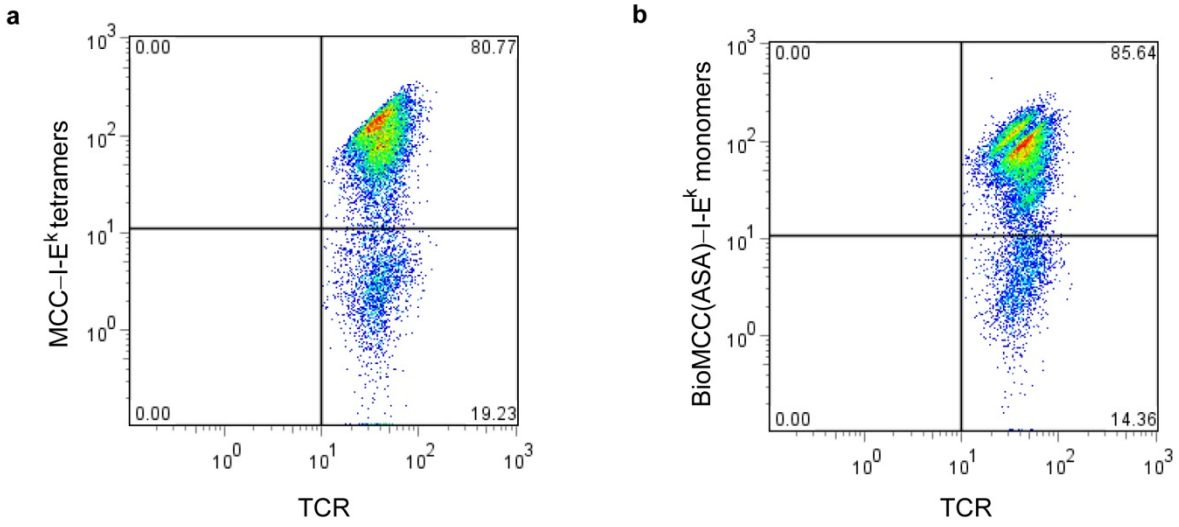

Supplementary Figure 6. Both a photocrosslinkable pMHC monomer and a standard pMHC tetramer can be used to detect T cell populations with differing affinities in a mixture. T cells extracted from 5C.C7 TCR β chain transgenic mice were primed with the MCC peptide, and cultured for 8 days. T cell blasts were then stained by H57-FITC and either a PE-conjugated MCC–I-E^k tetramer (**a**) or a photocrosslinkable BioMCC(ASA)–I-E^k monomer (**b**), and subjected to flow cytometric analysis. Data are representative of three independent experiments.

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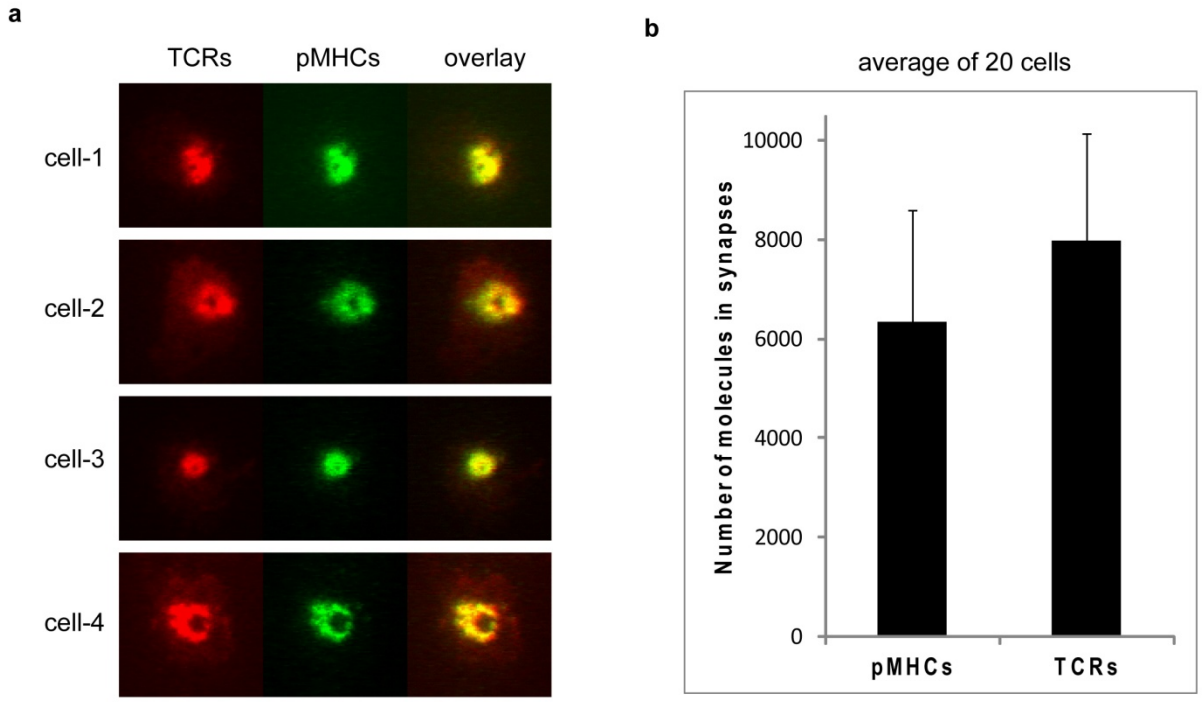

Supplementary Figure 7. Quantification of synaptic pMHC and TCR molecules in T cells photocrosslinked with pMHC ligands. (**a**) 5C.C7 T cells photocrosslinked with BioMCC(ASA)–I-E^k formed immunological synapses after contacting planar lipid bilayers presenting ICAM-1 and B7-1. Shown here are four representative T cells forming immunological synapses. TCRs were labeled with H57-scFv-A647, and pMHCs were labeled with monoSAv-A555. (**b**) Average numbers of synaptic pMHCs and TCRs (from 20 representative T cells) were determined by fluorescence single-molecule counting assays. Data are representative of two independent experiments (**a, b**; mean ± s.d. in **b**)

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