Induction and analysis of antigen-specific T cell responses in melanoma patients and animal model

Bins, A.D.

CHAPTER 7

Design and use of conditional MHC class I ligands

Mireille Toebes[1], Adriaan Bins[1,4], Miriam Coccoris[1,4], Boris Rodenko[2], Raquel Gomez[1], Nella J. Nieuwkoop[3], Willeke van de Kasteele[1], Guus F. Rimmelzwaan[3], John B.A.G. Haanen[1], Huib Ovaa[2] and Ton N.M. Schumacher[1]

[1] Division of Immunology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX, Amsterdam, The Netherlands.

[2] Division of Cellular Biochemistry, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX, Amsterdam, The Netherlands.

[3] Department of Virology and WHO National Influenza Center, Erasmus Medical Center, PO Box 1738, 3000 DR Rotterdam, The Netherlands.

[4] These authors contributed equally

Correspondence should be addressed to T.N.M.S. (t.schumacher@nki.nl) or H.O

(h.ovaa@nki.nl)

Abstract

Major histocompatibility complex (MHC) class I molecules associate with a large variety of peptide ligands during biosynthesis and present these ligands on the cell surface for recognition by cytotoxic T cells. We have designed conditional MHC ligands that form stable complexes with MHC molecules, but that degrade on command, by exposure to a defined photostimulus. "Empty MHC molecules" generated in this manner can be loaded with arrays of peptide ligands to determine MHC binding properties and to monitor antigen-specific T cell responses in a high-throughput manner. The value of this approach is documented by the identification of cytotoxic T cell epitopes within the H5N1 influenza A/Vietnam/1194/04 genome.

Introduction

MHC class I molecules are heterotrimers that consist of an invariant light chain, a polymorphic heavy chain and an eight to ten amino acid peptide ligand. The peptide forms an essential subunit of the MHC class I complex, as MHC class I molecules that fail to associate with peptide ligand are unstable[1,2]. Association of peptides with MHC class I molecules is in large part based on shape and electrostatic complementarity between two amino acid side chains at the anchor positions of the peptide and MHC allele-specific pockets[3,4]. In addition, binding of peptide ligands depends on the interaction of the MHC molecule with the terminal α-NH_2 and carboxyl groups of the peptide[5,6]. Because the sequence requirements for binding to MHC are largely restricted to two dominant anchor residues[7], the average protein contains several dozen potential T cell epitopes and, as an example, the approximately 100 open reading frames of a member of the herpesviridae family contain an estimated 4,000 potential T cell epitopes. Definition of tumor and pathogen-encoded MHC ligands and detection of T cell responses specific for such ligands remains a major challenge. The visualization of antigen-specific T cell responses was first made possible with the development of tetrameric MHC reagents by Altman et al.[8]. In this strategy, soluble MHC monomers complexed with a peptide of interest are biotinylated and converted to tetravalent structures by binding to fluorochrome-conjugated streptavidin or avidin. The resulting MHC tetramers have become essential reagents for the detection of antigen-specific $CD4^+$ and $CD8^+$ T cells by flow cytometry. More recent work indicates that definition of antigen-specific T cell responses by MHC microarray-based strategies is also feasible[9,10]. Furthermore, MHC-based selection of antigen-specific T cells has been proposed as a strategy to boost melanoma-specific T cell responses in melanoma patients[11] and to provide defined minor histocompatibility antigen-specific and virus-specific T cells in recipients of allogeneic stem cell transplants and other immunocompromised individuals[12-17]. At present, the major limitation of MHC tetramer-based technologies is formed by the involved nature of the refolding and purification steps that are required for every individual peptide/MHC complex. Consequently, it has not been feasible to apply MHC tetramer technology for high-throughput applications. To address this issue, we have explored the possibility of identifying conditional MHC ligands that can be used to generate peptide-receptive MHC class I molecules at will.

Results

Design of conditional MHC class I ligands

We set out to create MHC class I ligands that would disintegrate on command, while bound to the MHC complex. As a building block for such conditional ligands, we synthesized a 9-fluorenylmethyloxycarbonyl (Fmoc)-derivative of 3-amino-3-(2-nitro)phenyl-propionic acid (J). Subsequently, we used this building block to generate variants of the human leukocyte antigen (HLA)-A2.1-restricted influenza A matrix $(M1)_{58-66}$ epitope (sequence GILGFVFTL). An example of such

a variant, in which the Thr8 is replaced by 3-amino-3-(2-nitro)phenyl-propionic acid (resulting in compound I; GILGFVFJL), is given in Fig. 1a. Consistent with prior literature[18,19], this compound disintegrates upon exposure to UV light (Fig. 1a), and following UV exposure, a molecule with a mass of 751.4 becomes apparent, corresponding to the mass of the heptameric peptide acetamide fragment (Fig. 1a, compound II, Fig. 1b). The small, carboxy-terminal peptide fragment that is also generated during this cleavage reaction was not further studied.

To establish whether UV-sensitive ligands can be used to generate peptide/MHC complexes, we used two $M1_{58-66}$ variants with 3-amino-3-(2-nitro)phenyl-propionic acid incorporated at position four (compound III; GILJFVFTL, Suppl. Fig. 1) or position eight (compound I, Fig. 1a) in MHC class I refolding reactions[20]. MHC refolding reactions with either GILJFVFTL or GILGFVFJL produced high yields of HLA-A2.1 complexes. As a first crude test of the sensitivity of the non-natural MHC-bound ligands towards UV-mediated cleavage, we exposed HLA-A2.1 complexes containing either the unmodified influenza A $M1_{58-66}$ epitope, or the GILJFVFTL or GILGFVFJL variant to UV and analyzed these reactions by gel-filtration chromatography. Whereas the conventional HLA-A2.1/peptide complex is not affected by exposure to 366 nm light, exposure of either HLA-A2.1-GILJFVFTL (not shown) or HLA-A2.1-GILGFVFJL (Suppl. Fig. 2a) leads to a substantial reduction in MHC recovery. Furthermore, when HLA-A2.1 complexes are prepared with an influenza A $M1_{58-66}$ derivative in which the UV-resistant building block 3-amino-3-phenyl-propionic acid[21] is incorporated, the resulting MHC complexes are insensitive to UV exposure, demonstrating that the UV-induced decay of HLA-A2.1-GILJFVFTL and HLA-A2.1-GILGFVFJL requires the presence of the nitrophenyl moiety. Notably, the observed loss of folded MHC upon UV exposure of the HLA-A2.1-(GILGFVFJL) complex can be prevented by inclusion of HLA-A2.1 binding peptides during the cleavage reaction, but not by inclusion of a control HLA-A3 binding peptide (Suppl. Fig. 2b), suggesting efficient peptide exchange.

While these data demonstrate the UV-sensitivity of HLA complexes containing 3-amino-3-(2-nitro)phenyl-propionic acid-based peptide ligands, it is difficult to establish the efficiency of this cleavage reaction by gel-filtration chromatography (see Legend to Suppl. Fig. 2a). As a more stringent test for replacement of the conditional ligand by the newly added peptide, we exposed HLA-A2.1-GILGFVFJL complexes to UV light in the presence of the cytomegalovirus (CMV) $pp65_{495-503}$ epitope, and following this reaction, purified the peptide/MHC complexes and analyzed these complexes by mass spectrometry. The major peptide mass that is visible prior to UV exposure corresponds to the Na^+ ion of GILGFVFJL, providing formal proof that this ligand forms a stable complex with HLA-A2.1 (Fig. 2). Following UV-mediated cleavage, no detectable amount of GILGFVFJL remains associated with HLA-A2.1. Furthermore, also the levels of cleavage product II complexed with HLA-A2.1 are below background, suggesting that dissociation of this heptameric peptide fragment is essentially complete. Instead, upon UV-

mediated cleavage the sole peptide mass visibly associated with HLA-A2.1 corresponds to the mass of the CMV pp65$_{495-503}$ epitope (Fig. 2). Collectively, these experiments indicate that conditional MHC ligands can be defined that are released from MHC molecules at will and that MHC molecules generated in this process can be loaded with epitopes of choice. To extend these data to other conditional HLA A2.1 ligands, we synthesized a peptide that is predicted to bind avidly to HLA-A2.1[22], with the UV-sensitive building block J incorporated at position eight (ILAETVAJV). Refolding reactions with this conditional ligand gave high yields of folded HLA-A2.1 complexes and, analogous to the data obtained with HLA-A2.1-GILGFVFJL complexes, these complexes disintegrate following UV exposure (data not shown).

MHC exchange tetramers

To test the potential value of this MHC exchange technique for the visualization of antigen-specific T cells we performed MHC exchange reactions with biotinylated HLA-A.2.1- GILGFVFJL or HLA-A.2.1-ILAETVAJV complexes. Subsequently, phycoerythrin (PE)-streptavidin was added, and the resulting MHC tetramers (hereafter referred to as MHC exchange tetramers) were used to detect antigen-specific T cells by flow cytometry. MHC exchange tetramers containing the high affinity (A2L) variant of the Melan-A/MART-1$_{26-35}$ epitope[23] stain a MART-1-specific CTL clone as efficiently as conventional MHC class I tetramers (Fig. 3a). Likewise, MHC exchange tetramers can be used to detect low magnitude T cell responses in peripheral blood samples (Fig. 3b, Suppl. Fig. 3a). UV-induced cleavage of MHC-bound ligands also allows the synthesis and use of MHC tetramers that contain the naturally occurring MART-1$_{26-35}$ peptide that binds to MHC class I with low affinity, due to the absence of a leucine/ methionine residue at the P2 anchor position (Fig. 3a). This indicates that UV-induced peptide exchange is sufficiently robust to screen collections of putative T cell epitopes without a priori knowledge of MHC binding affinities. Importantly, MHC tetramers prepared from HLA-A2.1-GILGFVFJL complexes or A2K^b-GILGFVFJL complexes (see further) that have not been exposed to UV light, and that are therefore uniformly occupied by the UV-sensitive M1$_{58-66}$ variant, do not stain polyclonal influenza A M1$_{58-66}$-specific T cells (Suppl. Fig. 3b and 3c). This indicates that either the alteration in the peptide backbone due to the introduction of an unnatural β-amino acid, or the replacement of the threonine side chain is incompatible with T cell recognition by M1$_{58-66}$–specific T cells. Consequently, even in settings where release of conditional ligand would not be optimal, MHC exchange reagents are not expected to display background reactivity due to the presence of residual conditional ligand. It is noted that if conditional ligand-MHC complexes for other alleles would display background reactivity, it should be straightforward to prevent such reactivity by modification of T cell receptor-exposed sidechains. Notably, MHC tetramers generated by UV exchange compare favorably to MHC-Ig dimers generated by passive peptide exchange[24,25], both with respect to signal intensity and signal-to-noise ratios (Suppl. Fig. 4a, 4b).

To test whether conditional ligands may readily be identified for other MHC class I alleles we synthesized four variants of the H2-D^b-restricted ASNENMETM influenza A $NP_{366-374}$ epitope. Two of those variants, IV and V (Suppl. Fig. 1), fulfilled both criteria in that H2-D^b complexes could be generated with these conditional ligands and that these ligands could be cleaved in the MHC-bound state. Consistent with the data obtained for HLA-A2.1, H-2D^b exchange tetramers prepared from UV-sensitive H-2D^b-ASNENJETM complexes stain antigen-specific T cells with high specificity (Fig. 3c). Furthermore, as is the case for HLA-A2.1-GILGFVFJL tetramers, H-2D^b-ASNENJETM tetramers that are uniformly occupied by the UV-sensitive variant of $NP_{366-374}$, do not stain influenza A $NP_{366-374}$-specific T cells (Fig. 3c).

High-throughput screening with MHC exchange reagents

As a first test of the potential of MHC exchange for high-throughput epitope mapping, we cloned and sequenced the four genes encoding the immunodominant proteins of A/Vietnam/1194/04, an influenza A H5N1 strain isolated from a fatal human case in Vietnam, and scanned the encoded proteins for potential HLA-A2.1 binding peptides. Within the hemagglutinin (HA), neuraminidase (NA), matrix-1 (M1) and nucleoprotein (NP) gene products, we identified 132 potential epitopes with a score for predicted MHC binding of ≥ 20[22], and these peptides were produced by micro-scale synthesis. In parallel, we used the same set of genes to prepare vectors for DNA vaccination and groups of HLA-A2.1 transgenic mice[26] were vaccinated by DNA tattoo[27]. At the peak of the vaccination-induced T cell response, we generated a collection of MHC exchange tetramers by performing 132 parallel UV-mediated exchange reactions on A2K^b-GILGFVFJL complexes[28] in microtiter format, and the resulting MHC tetramer collection was used to screen peripheral blood samples of vaccinated mice. This analysis demonstrated the presence of two T cell epitopes within these four gene products of A/Vietnam/1194/04. Specifically, this screen confirmed the immunogenicity of the known influenza A $M1_{58-66}$ epitope that is conserved between the majority of influenza A strains. In addition, this scan revealed the presence of a previously unknown HLA-A2.1-restricted T cell epitope located in the A/Vietnam/1194/04 nucleoprotein (Fig. 4). Interestingly, in this HLA-A2.1 transgenic mouse model, the immunogenicity of this epitope ($NP_{373-381}$) is substantially higher than that of the classical $M1_{58-66}$ epitope ($M1_{58-66}$: one out of five responding mice after primary vaccination, three out of five responding mice after secondary vaccination; $NP_{373-381}$: five out of five responding mice after primary vaccination). This novel T cell epitope is shared between H5N1 strains of the past years but is distinct in older influenza A strains.

Discussion

Here we have described conditional MHC ligands that can disintegrate in the MHC bound state under conditions that do not affect the integrity of the MHC molecule, thereby permitting the reloading of assembled MHC molecules with epitopes of choice. Importantly, the peptide ligands that are bound to the MHC

in these reactions are unmodified, nor is the MHC backbone altered. In line with this, MHC complexes generated by this exchange technology display the predicted binding specificity in all cases tested. This strategy and related chemical cleavage strategies should be of substantial use in the high-throughput identification of both MHC ligands and cytotoxic T cell responses. In this strategy, a single batch of UV-sensitive MHC complex is prepared by the classical *in vitro* MHC class I refolding and purification protocols, and this UV-sensitive MHC complex is subsequently used to generate large arrays of desired pMHC complexes in 1 h exchange reactions.

MHC-Ig dimers purified from eukaryotic cells have previously been used to generate peptide/MHC reagents, by performing exchange reactions with exogenously added peptide[24,25]. While the overall goal of this technology is similar to that of UV-induced peptide exchange, the technologies differ at essential points. Specifically, while the MHC-Ig dimer technology depends on the slow release of a pool of unknown endogenous peptides, and is facilitated by conditions (e.g. low or high pH) that also destabilize the MHC molecule, UV-induced peptide exchange is based on the release of a single ligand, by exposure to a defined trigger that does not affect the integrity of the MHC molecule. Furthermore, the capacity of MHC-Ig based reagents to reveal antigen-specific T cell responses is limited as compared to MHC exchange tetramers (Suppl. Fig. 4).

The observation that ligands that disintegrate on command could readily be identified for both tested MHC alleles suggests that it will be straightforward to identify 2-nitrophenyl-based conditional ligands for other MHC class I alleles. Conditional ligands can be designed by replacement of amino acids in either a known peptide ligand, or in a predicted high affinity ligand based on the peptide binding motif for this allele, and for HLA A2.1 both approaches proved successful. For MHC alleles for which structural information is available, the water-accessibility of sidechains may also be used as a criterion to select positions at which the UV-sensitive building block can be incorporated.

Various types of functional assays for antigen-specific T cell detection such as intracellular cytokine staining and cytokine capture have been developed in the past few years and these assays may be utilized for high-throughput analysis of T cells that display a given effector function. MHC exchange multimer technology should complement these technologies by allowing high-throughput analysis of T cell responses, irrespective of the capacity of T cells to produce a given cytokine. Also the combination of the two technologies, in which high complexity MHC multimer arrays are used to probe T cell reactivity by monitoring cytokine production may be particularly useful. On-command cleavage of MHC ligands also seems attractive for the production of sets of clinical grade MHC reagents for adoptive T cell therapy[13]. Specifically, the generation of a single large batch of clinical grade MHC molecules complexed with conditional ligand should allow the straightforward assembly of an MHC reagent desired for clinical use, by performing MHC exchange reactions with the relevant peptide ligands. Such high-grade MHC

reagents should be particularly attractive for the isolation of melanoma-specific T cells[29], and for the isolation of defined minor histocompatibility antigen-specific T cells[12]. Finally, in addition to the use of conditional MHC class I ligands for high-throughput diagnostic screening and for adoptive T cell therapy, we speculate that cleavage of ligands bound to MHC molecules in the crystalline state[30] might be used to obtain the elusive structure of empty MHC class I.

Methods

Peptide synthesis and preparation of recombinant MHC. We obtained the UV-sensitive building block for peptide synthesis (N-fluorenylmethyloxycar bonyl 3-amino-3-(2-nitro)phenyl-propionic acid) by protection of 3-amino-3-(2-nitro)phenyl-propionic acid (Lancaster) with fluorenylmethyl chloroformate (Sigma-Aldrich) in dioxane/ 10% aqueous Na_2CO_3 3/2 v/v according to a published procedure[21]. We synthesized naturally occurring peptides and UV-sensitive peptide variants by standard Fmoc synthesis. We performed MHC class I refolding reactions as described[20] and we purified refolded MHC class I molecules by gel-filtration chromatography on a Phenomenex Biosep SEC S3000 column (Phenomenex) in 20 mM Tris-HCl pH 7.0/ 150 mM NaCl. Purified MHC class I complexes are stored at -20 °C in 20 mM Tris-HCl pH 7.0/ 150 mM NaCl and 16% glycerol.

MHC exchange reactions. To produce single or small sets of MHC reagents by MHC exchange, we exposed biotinylated HLA-A2.1- GILGFVFJL, HLA-A2.1-ILAETVAJV or H-2D^b-ASNENJETM complexes (0.5 μM in 20 mM Tris-HCl pH 7.0/ 150 mM NaCl/ 0.5 mM dithiothreitol (DTT)) to UV (366 nm UV lamp, CAMAG) in the presence of 50 μM of the indicated peptides for 1-2 h on ice. Following exchange, samples were spun at 16,000g for 5 min, (PE)-streptavidin was added and we used the resulting MHC exchange tetramers for T cell staining without further purification.

To generate the collection of H5N1 A2K^b tetramers, we predicted potential peptide epitopes within four gene segments of influenza A/Vietnam/1194/04 using the SYFPEITHI prediction program[22] and all peptides with a SYFPEITHI score ≥20 were produced by micro-scale (60 nmole) synthesis (JPT Peptide Technologies GmbH). Of the 132 potential epitopes, 116 terminated in either a valine, leucine or isoleucine residue and these were synthesized with the naturally occurring carboxy-terminal amino acid. The remaining 16 peptides, terminating in various non-aliphatic amino acids, were all synthesized with a carboxy-terminal isoleucine to facilitate peptide production. We prepared MHC exchange tetramers by performing parallel small scale exchange reactions on biotinylated A2K^b-GILGFVFJL class I complexes (0.5 μM)[28] with the 132 candidate influenza A/Vietnam/1194/04 epitopes and a set of control peptides (all peptides at 50 μM), by exposure to UV (366 nm UV lamp, CAMAG) for 1 h on ice in 20 mM Tris-HCl pH 7.0/ 150 mM NaCl/ 0.5 mM DTT in 96 well tissue culture V-bottom polystyrene plates (NUNC). Subsequently, samples were spun at 3,300g for 5 min, (PE)-streptavidin (10 μg/ml

final concentration) was added, and we used the resulting MHC exchange tetramers for T cell staining without further purification.

Mice and vaccinations.

We obtained C57BL/6 mice and mice transgenic for the HLA-A2K^b fusion gene[26] from the animal department of the Netherlands Cancer Institute. All animal experiments were carried out in accordance with institutional and national guidelines and were approved by the Experimental Animal Committee of the Netherlands Cancer Institute (DEC).

For live influenza A infections, we intranasally administered 50 µl of HEPES-buffered saline solution (Life Technologies) containing 200 hemagglutinating units influenza A/HKx31 virus to anesthetized mice. For vaccination of HLA-A2 transgenic mice with H5N1 gene segments, we obtained the indicated gene segments from influenza A/Vietnam/1194/04, isolated from a human case in Vietnam and cloned these into pVAX. Groups of four to six mice were vaccinated by DNA tattoo[27] on day 0, 3 and 6 with 20 µg of pVAX expressing either the influenza A/Vietnam/1194/04 M1, HA, NA or NP gene under control of the CMV promoter.

Cells and Flow cytometry. For analysis of MHC multimer binding and T cell responses in mouse samples, we obtained peripheral blood and erythrocytes were removed by incubation in erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4) on ice. Cells were stained with antibody to CD8α (BD Biosciences) and the indicated MHC tetramers for 10–15 min at room temperature.

For analysis of MHC multimer binding and T cell responses in human samples, we obtained peripheral blood mononuclear cells of healthy volunteers by Ficoll gradient separation. The MART-1_{26-35} specific CTL clone was obtained by repetitive stimulation and cloning of tumor-infiltrating lymphocytes of a melanoma patient. Cells were stained with the indicated MHC tetramers for 5 min at 37 °C. Subsequently, CD8-specific antibody (BD Biosciences) was added and cells were incubated for 10–15 min at room temperature. Data acquisition and analysis was carried out on a FacsCalibur (Becton Dickinson) using CellQuest software.

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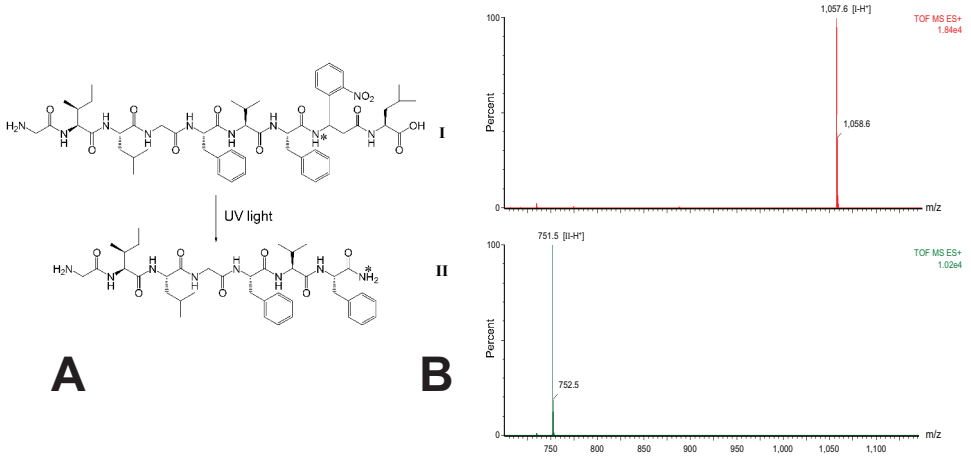


Figure 1: Photocleavage strategy. (a). Top: Structure of I, the photocleavable analog of the influenza A $M1_{58-66}$ epitope obtained as an approximate one to one mixture of diastereoisomers. Single amino acid sequence: GILGFVFJL. Bottom: Structure of II, the heptameric peptide fragment generated upon UV-induced degradation of I. Asterisks in I and II indicate the preferred cleavage site. (b). Liquid chromatography-mass spectrometry (LC-MS) of I prior to and following exposure to 366 nm light (CAMAG) for 60 min, in the presence of 0.5 mM DTT. Expected masses: single-protonated I: 1,057.6; single-protonated II: 751.4.

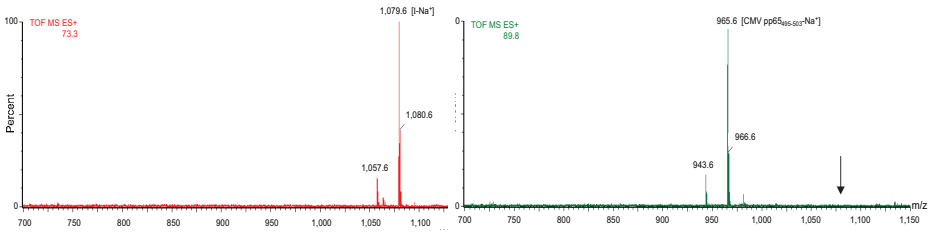


Figure 2: UV-mediated peptide exchange. Mass spectrometry of HLA-A2.1-associated peptide of HLA-A2.1-GILGFVFJL complexes prior to (left panel) and following (right panel) UV exposure in the presence of CMV $pp65_{495-503}$ peptide. Observed masses and assignments are indicated. Expected masses: Na^+ ion of I: 1,079.6; Na^+ ion of CMV $pp65_{495-503}$: 965.5. Note the absence of detectable I-Na^+ following UV-induce peptide exchange (arrow). 25 µM HLA-A2.1-GILGFVFJL in 20 mM Tris-HCl pH 7.0/ 150 mM NaCl and 0.5 mM DTT was exposed to 366 nm light for 60 min on ice in the presence of 500 µM CMV $pp65_{495-503}$ peptide. Subsequently, HLA-A2.1-peptide complexes were purified by gel-filtration chromatography on a Biosep SEC S-3000 column in 25 mM NH_4OAc pH 7.0 and directly used for analysis on a Waters LCT ESI mass spectrometer by direct infusion under optimized conditions (160 V cone voltage, 160 °C desolvation temperature). Note that the use of high cone voltage/ high temperature results in the detection of predominantly sodiated species.

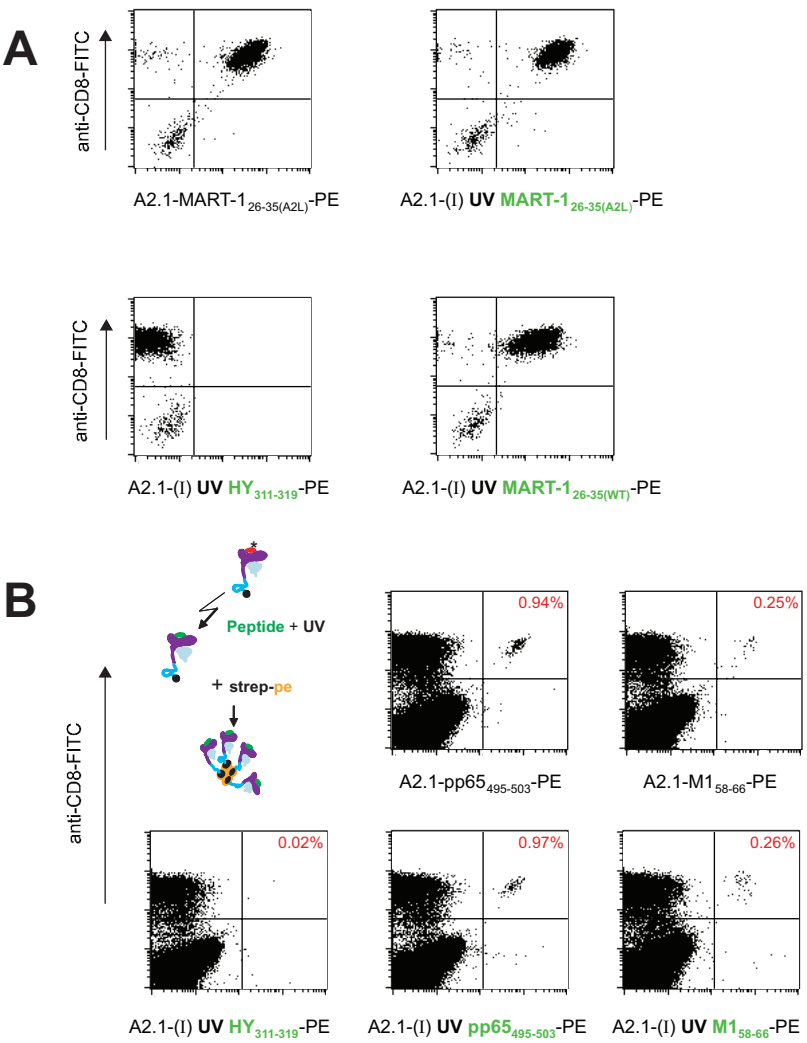
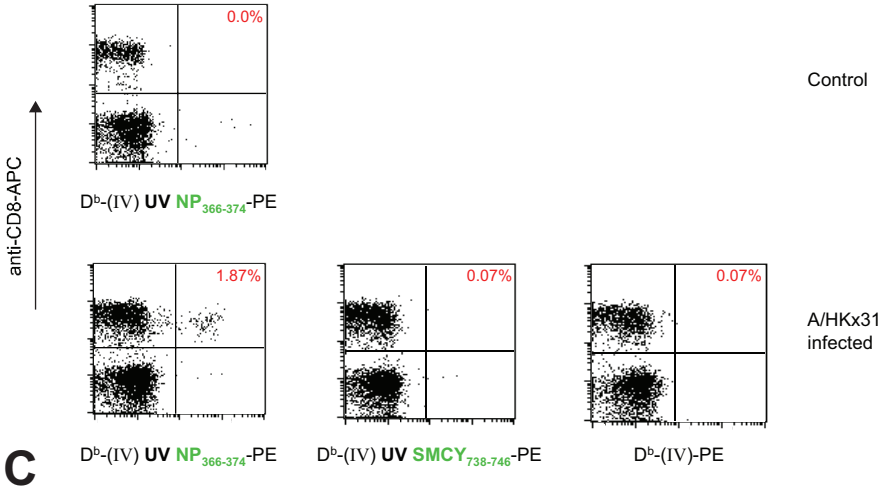


Figure 3: T cell staining with MHC exchange tetramers. (a). Flow cytometric analysis of MHC tetramer staining of a MART-1_{26-35}-specific CTL clone with classical MHC tetramers containing the MART-$1_{26-35(A2L)}$ epitope (top left panel), or MHC exchange tetramers containing the MART-$1_{26-35(A2L)}$ epitope (top right panel), a control peptide ($HY_{311-319}$, bottom left panel), or the naturally occurring low affinity MART-1_{26-35} epitope (bottom right panel). (b). Flow cytometric analysis of peripheral blood mononuclear cells from an HLA-A2.1 positive individual stained with classical MHC tetramers containing the CMV $pp65_{495-503}$ epitope (top left panel) or influenza A $M1_{58-66}$ epitope (top right panel), or with MHC exchange tetramers containing a control peptide ($HY_{311-319}$, bottom left panel), the CMV $pp65_{495-503}$ epitope (bottom middle panel) or the influenza A $M1_{58-66}$ epitope (bottom right panel).



(c). Flow cytometric analysis of MHC tetramer staining of peripheral blood cells of a C57BL/6 mouse (top panel) or a C57BL/6 mouse eight days post intranasal infection with influenza A/HKx31, encoding the A/PR8/34 $NP_{366-374}$ASNENMETM epitope (bottom panels). Analysis was performed with H-2D^b exchange tetramers containing the A/PR8/34 $NP_{366-374}$ epitope (left panels), a control peptide ($SMCY_{738-746}$ bottom middle panel), or H-2D^b tetramers prepared from biotinylated H-2D^b-ASNENJETM monomers that had not undergone exchange reactions (bottom right panel).

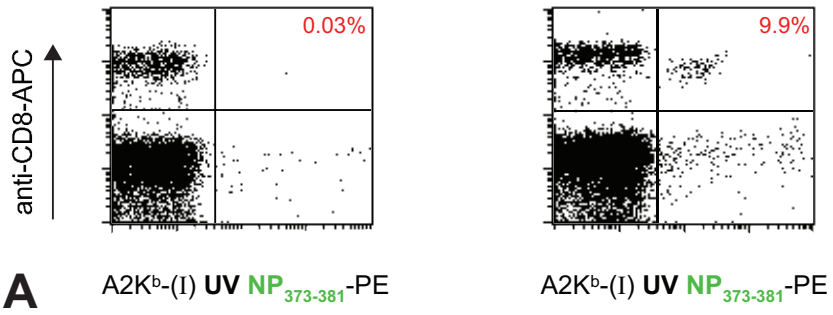
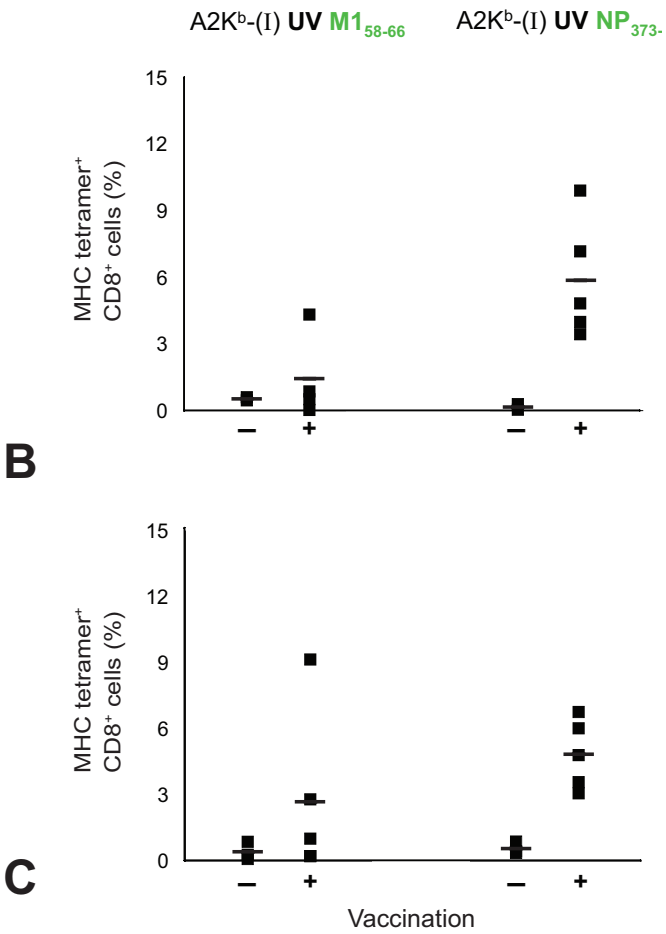
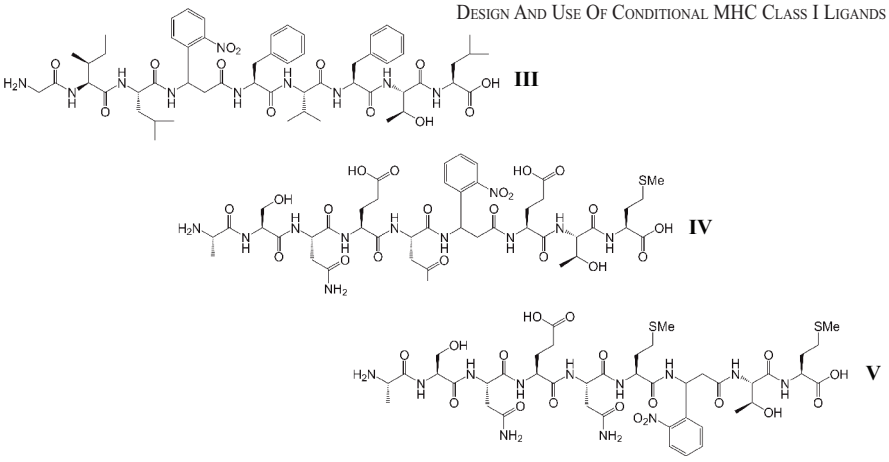


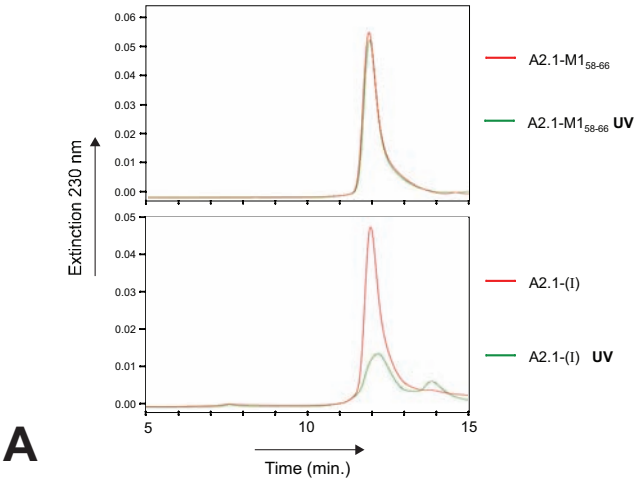
Figure 4: High-throughput screen of H5N1 T cell epitopes. (a). Flow cytometric analysis of mouse peripheral blood mononuclear cells stained with A2K^b-$NP_{373-381}$ exchange tetramers of either a non-vaccinated HLA-A2.1 transgenic mouse (left) or of an HLA-A2.1 transgenic mouse vaccinated at days 0, 3, 6 with 20 µg of A/Vietnam/1194/04 NP-encoding DNA.



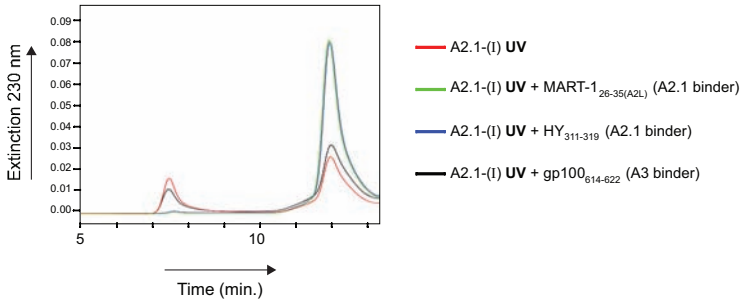
(b). Top: T cell responses of individual mice (closed squares) and average T cell responses (stripes) of non-vaccinated mice (–) and mice vaccinated with the influenza A/Vietnam/1194/04 M1 (left, +) and NP (right, +) genes, analyzed with the indicated A2K^b exchange tetramers at day 13 post primary vaccination. At the peak of the vaccination-induced T cell response, peripheral blood was drawn. Blood of mice in each of the four groups was pooled and analyzed by MHC tetramer staining. MHC tetramers that scored positive were used for re-analysis of individual mice. Bottom: T cell responses of individual mice (closed squares) and average T cell responses (stripes) of non-vaccinated mice (–) and mice vaccinated with the influenza A/Vietnam/1194/04 M1 (left, +) and NP (right, +) genes, analyzed with the indicated A2K^b exchange tetramers at day 11 post secondary vaccination. The identity of the NP$_{373-381}$ epitope was confirmed by synthesis of this sequence on a preparative scale followed by HPLC purification and screening of vaccinated mice by MHC exchange tetramer staining and intracellular interferon-γ staining (data not shown).



Supplementary Figure 1: Conditional ligands for HLA-A2.1 and H-2D^{b}. Top: Structure of III, the photocleavable analog of the influenza A $M1_{58-66}$ epitope in which Gly4 has been replaced by photolabile 3-amino-3-(2-nitro)phenyl-propionic acid (single letter sequence: GILJFVFTL). Middle: Structure of IV, the photocleavable analog of the influenza A/PR8/34 $NP_{366-374}$ epitope in which Met6 has been replaced by 3-amino-3-(2-nitro)phenyl-propionic acid (single letter sequence: ASNENJETM). Bottom: Structure of V, the photocleavable analog of the influenza A/PR8/34 $NP_{366-374}$ in which Glu7 has been replaced by 3-amino-3-(2-nitro)phenyl-propionic acid (single letter sequence: ASNENMJTM).

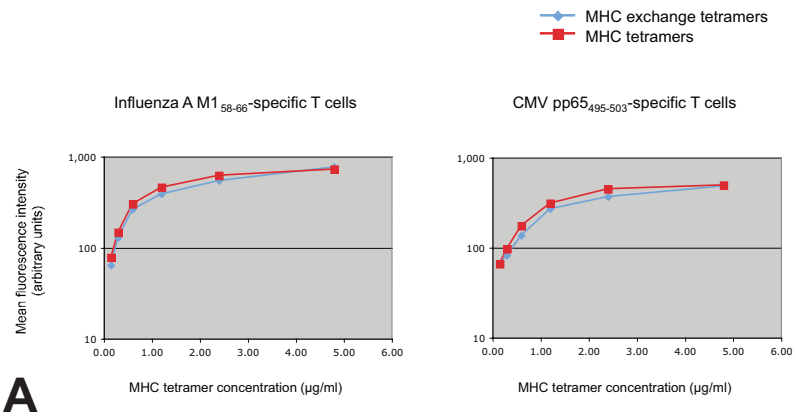


Supplementary Figure 2: Conditional peptide/MHC complexes are sensitive to UV. (a). Gel-filtration chromatography of HLA-A2.1 complexes occupied with the parental $M1_{58-66}$ epitope (top) or HLA-A2.1 complexes occupied with the UV-sensitive compound GILGFVFJL (bottom), with (green) or without (red) prior exposure to UV for 1 h on ice in 20 mM Tris-HCl pH 7.0/ 150 mM NaCl and 0.5 mM DTT.



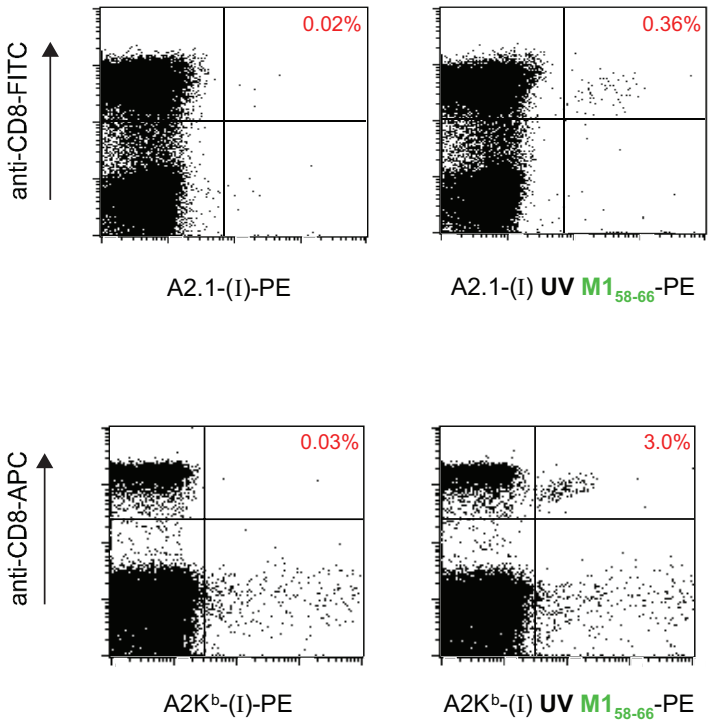
B

(b). Gel-filtration chromatography of HLA-A2.1 complexes occupied with the UV-sensitive peptide GILGFVFJL exposed to UV for 1 h on ice in 20 mM Tris-HCl pH 7.0/ 150 mM NaCl and 0.5 mM DTT without peptide (red), or in the presence of 100 µM of the HLA-A2.1-binding peptides MART-$1_{26-35(A2L)}$ (green) or $HY_{311-319}$ (blue), or the HLA-A3-binding peptide $gp100_{614-62}$ (black). Note: Free MHC class I heavy chains have a propensity to aggregate and this aggregation of free MHC class I heavy chains results in a loss of material eluting with a retention time of 11.8-12 min. This analysis underestimates the extent of UV-induced MHC destruction, as aggregation of MHC class I heavy chains is incomplete and separation of MHC class I complexes and free MHC class I heavy chains by gel-filtration chromatography is inefficient.



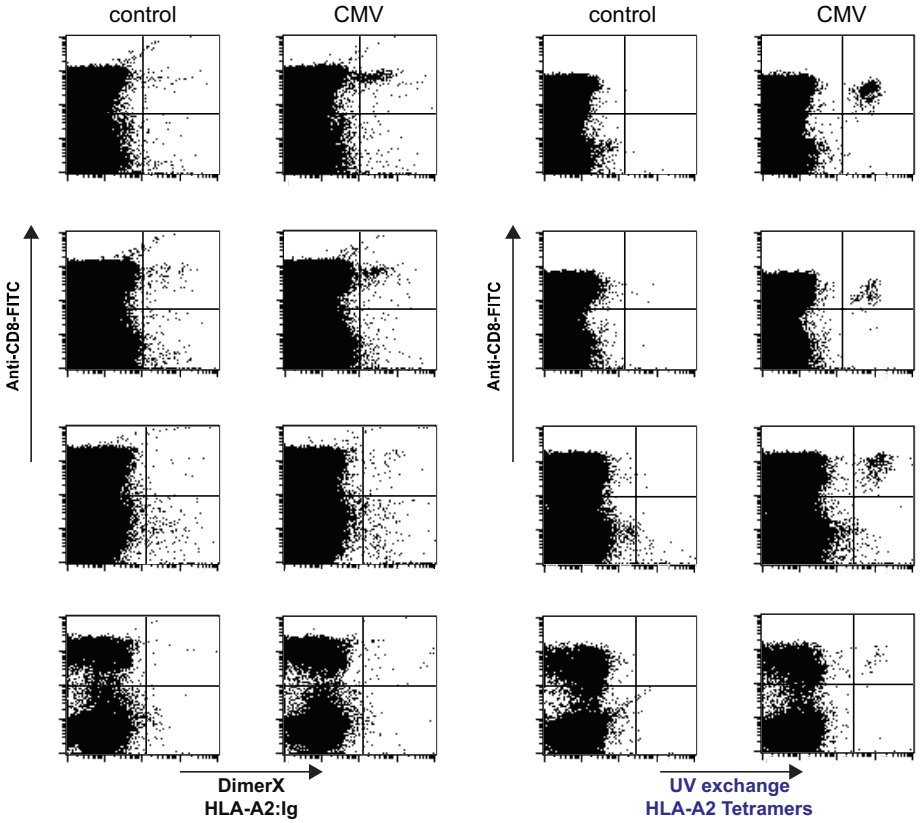
A

Supplementary Figure 3: Sensitivity and specificity of MHC exchange tetramers. (a). Peripheral blood mononuclear cells of an HLA-A2.1 positive individual were stained with the indicated concentrations of conventional HLA-A2.1 tetramers or with HLA-A2.1 exchange tetramers containing either the influenza A $M1_{58-66}$ epitope or the CMV $pp65_{495-503}$ epitope. Mean fluorescence intensity of the MHC tetramer$^+$ cells was determined and is plotted as a function of MHC tetramer concentration.



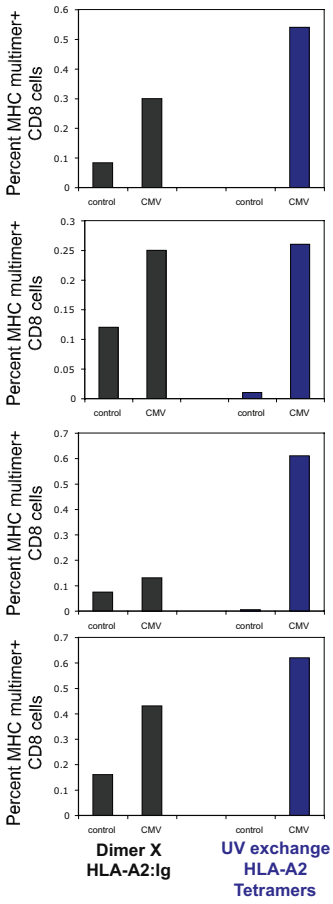
B

(b). Flow cytometric analysis of peripheral blood mononuclear cells of an HLA-A2.1 positive individual with HLA-A2.1 tetramers containing the UV-sensitive peptide GILGFVFJL (left panel), or the parental $M1_{58-66}$ epitope (right panel). (c). Flow cytometric analysis of peripheral blood mononuclear cells of an HLA-A2.1 transgenic mouse vaccinated with the influenza A/Vietnam/1194/04 M1-encoding expression plasmid with HLA-$A2K^b$ tetramers containing the UV-sensitive peptide GILGFVFJL (left panel), or the parental $M1_{58-66}$ epitope (right panel).



A

Supplementary Figure 4: Detection of antigen specific T cells with MHC-Ig dimers and MHC exchange tetramers. (a). Flow cytometric analysis of peripheral blood mononuclear cells of four CMV-positive HLA-A2.1 positive individuals with MHC-Ig (DimerX) reagents (left) prepared with a control peptide ($HY_{311-319}$) or the CMV $pp65_{495-503}$ peptide, or with HLA-A2.1 exchange tetramers (right) prepared with a control peptide ($HY_{311-319}$) or the CMV $pp65_{495-503}$ peptide.



(b). Plots of the background and specific staining obtained with MHC-Ig reagents and MHC exchange tetramers in four donors. Note that the intensity of staining of CMV $pp65_{495\text{-}503}$ specific T cells is higher with HLA-A2.1 exchange tetramers than with MHC-Ig dimers and that the background observed with control MHC multimers is substantially lower than the background observed with MHC-Ig dimers. MHC-Ig dimers for HLA-A2.1 (BD Biosciences) were loaded with the $HY_{311\text{-}319}$ or CMV $pp65_{495\text{-}503}$ peptide per manufacturer's protocol. In brief, DimerX reagents were prepared overnight at 37 °C, using a 640-fold molar excess of peptide over MHC-Ig (protocol 1, peptide concentration of 750 µM). Cells were stained with 1.5 µg of DimerX reagent for 1 h at 4 °C, followed by staining with PE-labeled anti-mouse IgG_1 (clone A85-1, BD Biosciences) plus FITC-labeled CD8-specific antibody (clone G42-8 BD Biosciences) for 30 min at room temperature (protocol 2).

MHC exchange tetramers with the $HY_{311\text{-}319}$ or CMV $pp65_{495\text{-}503}$ peptide were prepared by performing 1 h UV-exchange reactions on HLA-A2.1-GILGFVFJL monomers in the presence of 50 µM of the indicated peptide on ice. Subsequently, samples were spun at 3,300g for 5 min, (PE)-streptavidin (10 µg/ml final concentration) was added, and the resulting MHC exchange tetramers were used for T cell staining without further purification.

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