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CONDITIONAL MHC CLASS I LIGANDS AND PEPTIDE EXCHANGE TECHNOLOGY FOR THE HUMAN MHC GENE PRODUCTS HLA-A1, -A3, -A11 AND -B7

Arnold H. Bakker[1], Rieuwert Hoppes[2,5], Carsten Linnemann[1,5], Mireille Toebes[1], Boris Rodenko[2], Celia R. Berkers[2], Sine Reker Hadrup[1], Wim J.E. van Esch[3], Mirjam H.M. Heemskerk[4], Huib Ovaa[2], and Ton N.M. Schumacher[1]

[1]Division of Immunology and [2]Cellular Biochemistry, The Netherlands Cancer Institute, Amsterdam, The Netherlands.
[3]Sanquin, Amsterdam, The Netherlands.
[4]Laboratory of Experimental Hematology, Department of Hematology, Leiden University Medical Center, Leiden, The Netherlands.
[5]R.H and C.L contributed equally.

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Major histocompatibility complex (MHC) class I multimer technology has become an indispensable immunological assay system to dissect antigen-specific cytotoxic $CD8^+$ T cell responses by flow cytometry. However, the development of high-throughput assay systems, in which T cell responses against a multitude of epitopes are analyzed, has been precluded by the fact that for each T cell epitope a separate *in vitro* MHC refolding reaction is required. We have recently demonstrated that conditional ligands that disintegrate upon exposure to long-wavelength UV light can be designed for the human MHC molecule HLA-A2.

To determine whether this peptide-exchange technology can be developed into a generally applicable approach for high-throughput MHC based applications we set out to design conditional ligands for the human MHC gene products HLA-A1, -A3, -A11 and -B7. Here we describe the development and characterization of conditional ligands for this set of human MHC molecules and apply the peptide-exchange technology to identify melanoma-associated peptides that bind to HLA-A3 with high affinity. The conditional ligand technology developed here will allow high-throughput MHC-based analysis of cytotoxic T cell immunity in the vast majority of Western European individuals.

| INTRODUCTION

MHC Class I molecules are heterotrimeric complexes consisting of an invariant light chain called β2-microglobulin (β2m), a polymorphic heavy chain (HC) and an approximately 8–11 amino-acid peptide ligand. These peptide-MHC (pMHC) complexes are recognized by the T cell receptor (TCR) of $CD8^+$ T cells in a peptide-specific fashion, and this interaction forms the molecular basis of antigen recognition by $CD8^+$ T cells. In the past decade, the mapping of pathogen-specific and autoimmune- or cancer-associated T cell epitopes has been a major driving force in the development of assay systems for immunomonitoring. In addition, knowledge of such T cell epitopes forms a cornerstone in the development of vaccine-based or adoptive T cell therapies. As a first step in the mapping of disease-associated T cell epitopes, peptide fragments of disease-associated proteomes may be analyzed for binding to MHC molecules of interest, and subsequent assays can then be used to determine whether T cell reactivity against such pMHC complexes does occur. As demonstrated in a landmark study by Altman and colleagues, such antigen-specific T cell reactivity can efficiently be detected by the staining of T cell populations with recombinant fluorescent multimeric MHC molecules[1].

There is an increasing interest in the development of assay systems, such as MHC-based microarrays, that can monitor a multitude of T cell responses in parallel[2-4]. Unfortunately, current technology does not allow for the high-throughput generation of different pMHC complexes, thereby limiting the utility of these techniques. Specifically, as MHC class I complexes that are devoid of peptide are markedly unstable[5,6], current production processes for recombinant MHC complexes require inclusion of a specific T cell epitope during the initial refolding step[7], and this precludes the production of the large collections of pMHC multimers that would be needed to

analyze antigen-specific T cell responses in a comprehensive manner.

Based on these considerations it seemed valuable to devise technologies that allow the high throughput parallel generation of peptide-MHC class I complexes. As a step toward this goal, we recently designed an HLA-A2 specific peptide that contains a photo-cleavable moiety[8]. When refolding reactions of HLA-A2 heavy chain and β2m are performed with this ligand, stable HLA-A2 complexes are formed. However, upon irradiation with long-wavelength UV, the ligand is cleaved and dissociates from the HLA-A2 complex. The resulting empty HLA-A2 complexes disintegrate rapidly, unless UV exposure is performed in the presence of a "rescue peptide". In this case, the peptide-binding groove that has been vacated by UV exposure will be occupied by the rescue peptide, resulting in the formation of stable pMHC complexes with a distinct T cell specificity. The utility of this approach has been demonstrated by the identification of an HLA-A2-restricted CTL epitope from an H5N1 influenza strain isolated from a lethal case of avian influenza infection in humans[8].

To determine whether this technology can be developed into a broadly applicable high-throughput system for the dissection of human CTL responses we set out to design and test a panel of UV-sensitive ligands for the human MHC gene products HLA-A1, -A3, -A11 and -B7.

| RESULTS

Design of the conditional ligands

In an effort to test the feasibility of developing a broadly applicable high-throughput platform for MHC based detection we focused on a set of 4 gene products (HLA-A1, -A3, -A11, and -B7) with a high prevalence in the Western-European population. Using the SYFPEITHI database[9], a set of 3–6 high affinity 9-mer peptides was designed for each molecule, in which the UV-sensitive β-amino acid (+/-)-3-amino-3-(2-nitro) phenyl-propionic acid was incorporated at different positions. Amino acid sequences were based on known peptide motifs for each gene product and had a predicted SYFPEITHI peptide binding score of >25 (excluding a potential detrimental effect of the β-amino acid). In all peptides, the photo-labile building block was incorporated at positions predicted to result in solvent exposure of the 2-nitrophenyl side chain, as based on structural data[10,11], or the lack of amino acid selectivity at this position[9]. While solvent exposure of the 2-nitrophenyl side-chain is not required for the UV-mediated cleavage step, this strategy was used to reduce the likelihood that incorporation of the photo-labile amino acid would affect MHC binding capacity.

First, a series of small-scale refolding reactions was performed with each of the 19 starting ligands (**Supplementary Table 1**). The resulting pMHC complexes were subsequently analyzed by gel-filtration HPLC and MHC ELISA to determine three parameters: the efficiency of MHC refolding, the stability of the pMHC complex in the absence of UV exposure, and the UV-sensitivity of this complex. A description of the starting set of conditional ligands and a summary of the outcome of these assays

in terms of stability and UV-sensitivity of the different pMHC complexes is given in **Supplementary Table 1**.

Of the 19 ligands tested, 3 either showed no or very poor refolding with the corresponding MHC molecule, or the pMHC complex displayed a substantial instability upon storage or brief 37 °C exposure. The remaining 16 pMHC complexes were exposed to UV-light and analyzed for unfolding of the pMHC complex: 9 of these pMHC complexes displayed no or low UV-induced unfolding, while the remaining 7 displayed efficient UV-induced degradation. From this set, the ligands that yielded the highest efficiency of refolding were selected for further optimization. Specifically, to facilitate rapid release of the resulting peptide fragments upon UV exposure while maximizing complex stability under normal conditions, the anchor residues of the selected ligands were altered while keeping the UV-sensitive amino acid at the same position. After analyzing this pool of second candidate ligands for the same three parameters, an optimal conditional ligand was selected for each HLA gene product, termed p*[allele]: p*A1 = STAPGJLEY, p*A3 = RIYRJGATR, p*A11 = RVFAJSFIK, p*B7 = AARGJTLAM, where J is 3-amino-3-(2-nitro)phenyl-propionic acid.

Analysis of the conditional pMHC complexes

Large-scale refolding reactions were performed to enable more detailed analysis of the selected p* ligands for the 4 different molecules. In parallel, refolding reactions were performed with a set of known epitopes (termed pA1, pA3, pA11, pB7) lacking a photo-labile residue, for use as controls in these experiments. For all 4 HLA molecules the efficiency of refolding of the different p*MHC complexes was comparable to that of the corresponding control pMHC (HLA-A1 p*A1: 21%, HLA-A3 p*A3: 20%, HLA-A11 p*A11: 23%, HLA-B7 p*B7: 12%).

To set up a more rapid assay system for the measurement of UV-induced MHC unfolding for the different complexes, pMHC and p*MHC preparations were either exposed to UV or left untreated, and the amount of remaining folded MHC was then analyzed by ELISA[12,13] of serial dilutions (shown for HLA-A1 in **Supplementary Fig. 1**). Having established a suitable pMHC concentration to visualize the effect of UV exposure on p*MHC stability via ELISA (between 10–20 nM, **Supplementary Fig. 1**), a kinetic analysis of UV-mediated degradation was performed for each MHC product. UV-exposure of control MHC complexes refolded with UV-insensitive peptides had no effect on their stability, independent of the time of exposure. In contrast, each of the p*MHC complexes showed substantial degradation after a 1 minute UV-treatment, and the effect of UV exposure was close to complete after a 5 minute exposure (**Fig. 1a**).

To allow the use of conditional MHC complexes for various high-throughput applications, it is essential that the instable MHC molecules that are formed upon UV exposure can bind a newly added ligand and thereby be stabilized. To test whether this is the case for the 4 HLA gene products under study, exchange reactions were performed either in the absence of peptide, in the presence of an HLA-A2-restricted control peptide, or in the presence of a known peptide ligand for each complex. For all 4 HLA gene products, addition of the relevant ligand resulted in a substantial

rescue (71% to 108% of starting material, **Fig. 1b**), whereas addition of the HLA-A2-restricted control peptide was without effect. Combined, these experiments show that 1) all 4 conditional ligands refold efficiently into stable MHC complexes, 2) the resulting complexes display a similar UV-sensitivity and 3) the peptide-free MHC molecules generated upon triggering can be charged with newly added allele-specific ligands.

Peptide cleavage and exchange kinetics

A caveat of the MHC ELISA data (**Fig. 1**) is that the observed unfolding of MHC upon UV exposure is an indirect measure of peptide dissociation and

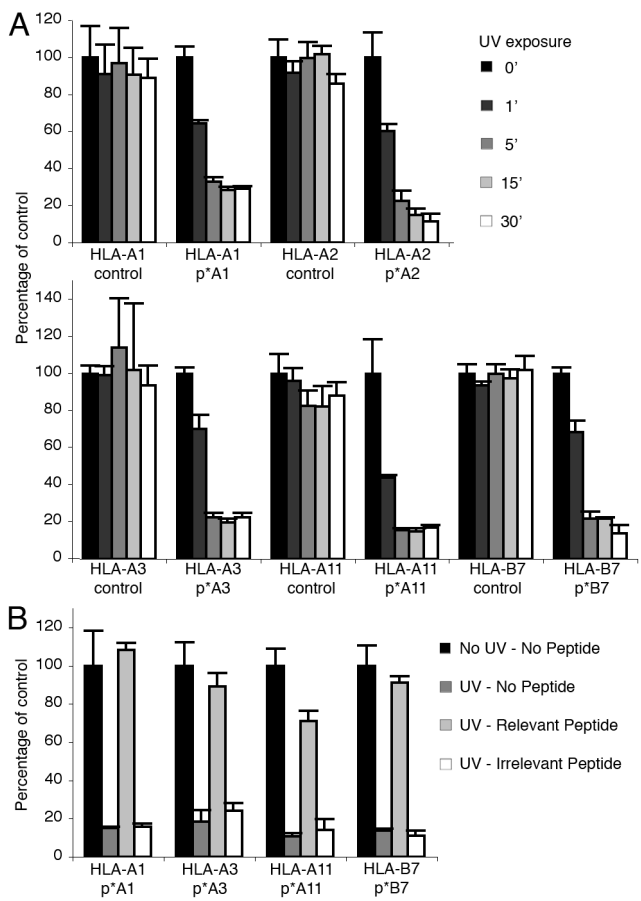

Figure 1. Characterization of conditional ligands for HLA-A1, A3, -A11 and -B7. (**a**) Control and p*-HLA complexes for each complex were exposed to UV for the indicated times and UV-induced MHC unfolding was measured by ELISA. Control peptides: pA1 VTEHDTLLY, pA2 FLWGPRALV, pA3 RLRAEAQVK, pA11 IVTDFSVIK, pB7 RPHERNGFTVL. (**b**) The indicated p*-HLA complexes were exposed to UV-light for 0 or 60 minutes in the presence of either no peptide, the HLA-A2 restricted CMV-pp_{65} epitope NLVPMVATV (irrelevant peptide), or their respective specific ligands, pA1, pA3, pA11, pB7 (sequences under (a)) and analyzed by MHC-ELISA. Values indicate mean +/- S.D. of triplicates.

may be influenced by the fact that the stability of the peptide-free state can vary between different MHC molecules[14]. To allow a more direct measurement of the dissociation of peptide fragments upon UV exposure, a fluorescently labeled variant of p*A2 was produced: KILGC(Fl)VFJV (^{Fl}p*A2). HLA-A2 complexes occupied with this fluorescent ligand were then used in exchange reactions in the presence of an unlabeled CMV pp_{65}-derived HLA-A2 ligand. When aliquots of HLA-A2 ^{Fl}p*A2 complexes are either left untreated or exposed to UV for different time periods and analyzed by gel-filtration HPLC, recovery of total MHC as reflected by absorbance at 230 nm is identical for all samples. In contrast, when the amount of remaining MHC-associated ^{Fl}p* ligand is monitored by fluorescence analysis, a clear reduction is observed (**Fig. 2a**, 88% reduction after 60 minutes UV exposure). Thus, the instability of MHC upon UV exposure of p*MHC complexes is accompanied by a parallel release of the fluorescent peptide fragment.

In the p*A2 ligand, the J residue is incorporated at p8 of the nonameric peptide. Because the side chain of p9 is buried in the F pocket of the peptide-binding groove, fluorescent labeling at a COOH-terminal position relative to the cleavage site was precluded for this peptide. To assess whether both peptide fragments that are formed upon cleavage of a UV-sensitive peptide dissociate with similar kinetics from the peptide binding groove, subsequent analyses were performed with variants of the p*B7 ligand, in which the photo-labile residue is incorporated at a more central position (p5). Two variants of the p*B7 ligand were produced with a fluorescently labeled cysteine residue at either an NH_2- or COOH-terminal position relative to J (AARC(Fl)JTLAM and AARGJTLC(Fl)M). HLA-B7 complexes refolded with these peptides were then exposed to UV in the presence of an unlabeled HLA-B7 ligand and analyzed by gel filtration HPLC. No substantial difference was observed between the rate of dissociation of fluorescently NH_2- and COOH-terminal cleavage products (**Fig. 2b,c**) and a 60 minute UV-exposure led to an 84% and 75% reduction of the fluorescent signal, respectively.

For both the fluorescently labeled NH_2-terminal fragment of p*A2 and the fluorescently labeled NH_2- and COOH-terminal fragments of p*B7 a small amount of fluorescent signal that co-migrated with the MHC complex was consistently observed after UV-exposure (**Fig. 2a–c**). Because prolonged UV exposure does not result in a substantial further decrease in this signal (data not shown), it seemed unlikely that this signal reflected the presence of uncleaved MHC-associated ligand. To test this directly, peptides were extracted from UV-exposed or untreated HLA-A2 ^{Fl}p*A2 complexes via acid elution and analyzed by reverse-phase HPLC. This analysis demonstrated that UV exposure leads to a near-complete cleavage of the starting MHC-associated material (**Fig. 2d**). Next, the fluorescent material that remained MHC-associated upon UV exposure was isolated by gel filtration HPLC, followed by peptide elution. Subsequent analysis of this material by reverse-phase HPLC demonstrates that also the fluorescent material that remains MHC-associated upon UV exposure does not contain substantial amounts of the starting ^{Fl}p *A2 ligand. The identity of the formed cleavage products was not investigated (**Fig. 2e**).

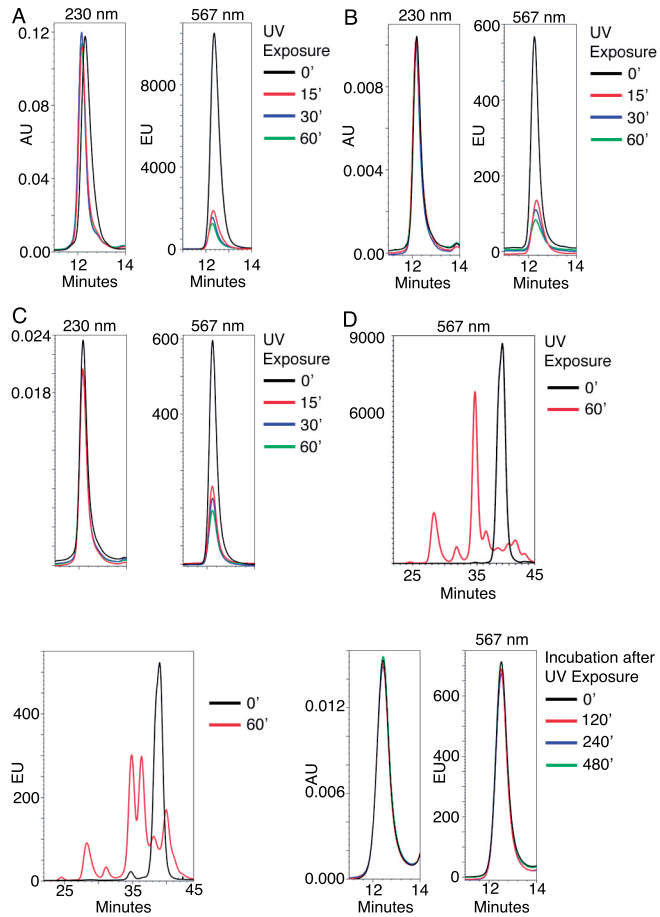

Figure 2. UV-induced peptide cleavage and exchange kinetics. (**a**) HLA-A2-monomers refolded with the fluorescent UV sensitive peptide ^{Fl}p*A2 were treated with UV in the presence of the HLA-A2 ligand NLVPMVATV for different time periods and analyzed by gel-filtration HPLC. Absorption at 230 nm (left) and fluorescence at 567 nm (right) was measured. (**b–c**) HLA B7-monomers refolded with either the fluorescent UV sensitive peptides AARC(Fl)JTLAM (**b**) and AARGJTLC(Fl)M (**c**) were treated with UV in the presence of the HLA-B7 ligand TPRVTGGGAM for different time periods and analyzed as in (a). (**d**) HLA-A2-monomers refolded with the fluorescent UV sensitive peptide ^{Fl}p*A2 were either left untreated or exposed to UV for 60 minutes. Extracted peptides were analyzed by reverse phase HPLC. Black line: untreated, red line: UV-treated. (**e**) As in (d) except that prior to peptide extraction, elution material with the retention time of pMHC molecules was isolated by gel-filtration HPLC. Black line: untreated, red line: UV-treated. (**f**) p*A2-monomers were treated with UV for 30 minutes in the presence of 0.5 μM fluorescent FLPSDC(FL)FPSV and 49.5 μM FLPSDCFPSV peptide and kept at RT for the indicated periods prior to analysis as in (a).

To directly visualize the kinetics of binding of newly added ligands to UV-exposed p*MHC complexes, HLA-A2 p*A2 complexes were exposed to UV light for 30 minutes in the presence of the fluorescent A2 ligand FLPSDC(Fl)FPSV[15] and then incubated at room temperature for different periods. Subsequently, the

amount of newly bound MHC ligand was determined by gel-filtration HPLC. A strong fluorescent signal was seen when MHC complexes were analyzed directly after UV exposure and this signal did not increase measurably upon further incubation. This indicates that binding of new ligands to the MHC complex is essentially complete during the time of UV exposure (**Fig. 2f**).

Detection of antigen-specific T cell responses with HLA-A1, -A3, -A11 and -B7 exchange tetramers

As a stringent test of the value of peptide-exchanged MHC complexes of the different gene products for the detection of antigen-specific T cell responses, exchange reactions were performed with a series of pathogen-derived epitopes and used for multimerization without further purification. Subsequently, T cell staining of these MHC exchange tetramers was compared to that of MHC tetramers generated in classical individual refolding reactions[1]. In a first set of experiments, the intensity of staining of epitope specific T cell clones restricted by HLA-A1 and -A3 was analyzed, upon incubation with different concentrations of classical MHC tetramers or MHC exchange tetramers (**Supplementary Fig. 2**). For both HLA-A1 and -A3 complexes, MHC exchange tetramers and classical MHC tetramers are indistinguishable in their capacity to stain a relevant T cell clone (**Fig. 3**). Furthermore, in both cases background staining as revealed by incubation of an irrelevant CTL clone with a high concentration of MHC tetramer is negligible (less than 0.05%, **Fig. 3**, **Supplementary Fig. 2**). To extend this analysis to clinically more relevant samples MHC exchange tetramers and classical MHC tetramers were compared with respect to the ability to detect low frequency T cell responses in peripheral blood samples. Tetrameric forms of p*MHC complexes that had not been exposed to UV and that are therefore uniformly occupied with the conditional ligand were included as controls. In all cases tested, MHC exchange tetramers stained the relevant T cell populations, and both the percentage and fluorescence intensity were directly comparable to that observed upon staining with MHC tetramers produced by individual refolding reactions (**Fig. 4**).

Identification of melanoma-associated HLA-A3 ligands

To test the feasibility of the peptide-exchange technology to rapidly screen large panels of peptides for MHC binding, we set out to identify novel HLA-A3 ligands in melanoma-associated proteins. To date, only 4 HLA-A3 restricted T cell epitopes have been identified in melanoma-associated proteins, all of them derived from the gp100 antigen[16-18]. We designed a library of peptides derived from the melanoma differentiation antigens (gp100, mart-1, tyrosinase and tyrosine-related-protein-1 and -2), plus the melanoma-associated protein Nodal[19] using 3 binding prediction algorithms for HLA-A3[9,20]. The resulting 203 peptides (**Supplementary Table 2**) were then analyzed for HLA-A3 binding in a fluorescence polarization assay. 22 peptides that showed a high inhibition of binding of the tracer peptide were selected for determination of IC_{50} values (**Supplementary Table 2**). Importantly, this set of 22 identified HLA-A3 ligands included the 4 known gp100 epitopes. **Table 1** and **Supplementary Fig. 3** show that all 22 peptides form high affinity ligands of HLA-A3, with IC_{50} values ranging between 73 and 857 nM.

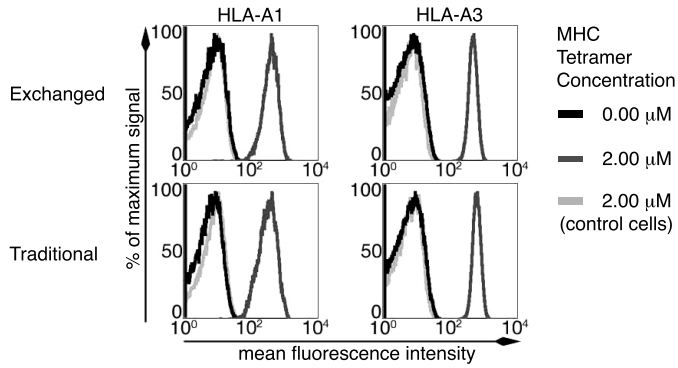

Figure 3. Staining of epitope-specific T cell clones by HLA-A1 and -A3 exchange tetramers. $CD8^+$ T cell clones specific for HLA-A1 CMV-pp_{65} (left panels) and HLA-A3 EBV-EBNA-3a (right panels) were stained with equal amounts of HLA-A1 CMV-pp_{65} or HLA-A3 EBV-EBNA-3a tetramers, generated via peptide-exchange (Exchanged, top panels) or classical refolding (Traditional, bottom panels). Control cells represent staining with the matched MHC tetramer and cross-sample controls.

Table 1. IC_{50} values of selected peptides

Protein	Peptide	Position	IC_{50} (nM)	SEM
Gp100	**IALNFPGSQK**	86-95	127	7
	LIYRRRLMK	614-622	159	18
	GTATLRLVK	460-468	204	7
	ALLAVGATK	17-25	212	5
	ALNFPGSQK	87-95	247	12
	GVSRQLRTK	34-42	343	23
	QLVLHQILK	551-559	363	22
	QLRALDGGNK	221-230	415	17
Nodal	SLYRDPLPR	46-54	73	2
	HAYIQSLLK	293-301	97	4
	KTKPLSMLY	317-325	277	6
	RVAGECWPR	175-183	735	5
Tyr	YMVPFIPLYR	425-434	384	26
	SLLCRHKRK	497-505	498	52
	VSSKNLMEK	25-33	552	14
	GLVSLLCRHK	494-503	857	42
Tyrp1	SLPYWNFATR	245-254	149	5
	ASYLIRARR	497-505	169	18
Tyrp2	TLLGPGRPYR	196-205	120	1
	GTYEGLLRR	301-309	194	14
	RMYNMVPFF	461-469	310	19
	VLLAFLQYR	521-529	578	50
Influenza NP	**ILRGSVAHK**	265-273	181	7
CMVpp65 (HLA-A2)	**NLVPMVATV**	495-503	>50.000	ND

IC_{50} values of selected peptides determined in a competitive binding FP assay after 45 hours of incubation. Bold peptides indicate previously described HLA-A3 epitopes. Values were determined in three independent experiments. ND, not determined

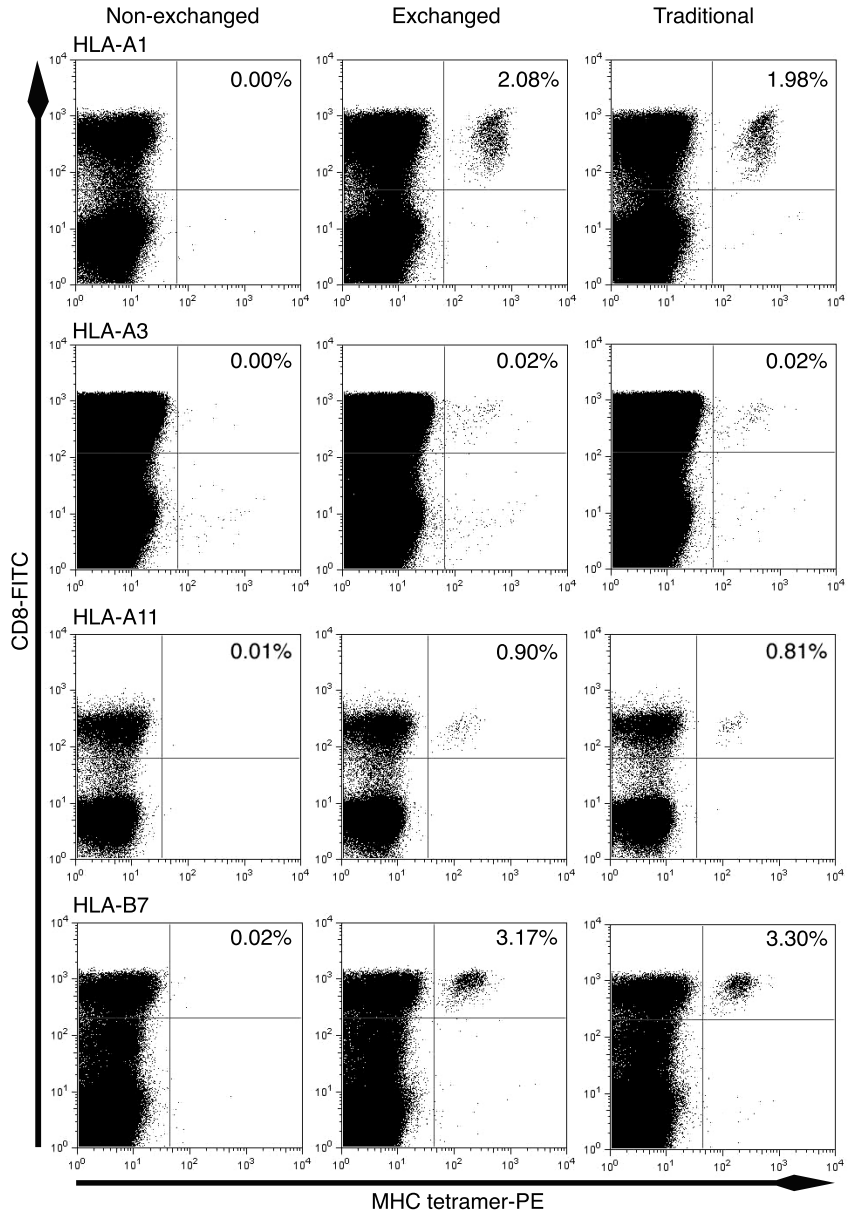

Figure 4. Detection of low-frequency T cell responses in peripheral blood by HLA-A1, -A3, A11 and -B7 exchange tetramers. Indicated pMHC complexes were prepared by classical refolding reactions or by 1h exchange reactions and converted to tetramers. As a negative control, streptavidin-conjugated non-exchanged p*-MHC complexes were used. HLA-typed PBMC were stained with the indicated pMHC tetramers and analyzed by flow cytometry: Top to bottom panels: HLA-A1 CMV-pp_{50} (VTEHDTLLY); HLA-A3 EBV-EBNA-3a (RLRAEAQVK); HLA-A11 EBV-EBNA-3b (IVTDFSVIK); HLA-B7 CMV-pp_{65} (TPRVTGGGAM) tetramers, respectively. For all complexes, stainings were performed at equal concentrations for all three panels. Numbers indicate the percentage of MHC $tetramer^+$ cells of $CD8^+$ cells.

| DISCUSSION

The use of multimeric forms of pMHC complexes has become a core immunological technique to visualize antigen-specific CD8+ T cell populations[7]. Fluorescently labeled MHC multimers are commonly used for the detection of antigen-specific T cells by flow cytometry. In addition, there is an increasing interest in the development of high-throughput assay systems, such as MHC microarrays or combinatorial coding schemes, to visualize pathogen-specific or other disease-associated immune responses in a more comprehensive manner[2,3,21]. A major obstacle in the development of these high-throughput approaches for the dissection of antigen-specific CD8+ T cell immunity has been the fact that for each specific peptide-MHC class I complex a separate production run is required[1,22], limiting the practical use of MHC multimer-based T cell detection to a few T cell specificities.

We have set out to develop technologies that enable the rapid generation of large collections of defined pMHC complexes in parallel reactions. Here, we aimed to determine whether MHC-based peptide-exchange technology can be developed into a broadly applicable platform for the screening of human cytotoxic T cell responses. To this purpose we defined conditional ligands for 4 different human MHC products: HLA-A1, -A3, -A11, and -B7 (with the conditional ligands STAPGJLEY, RIYRJGATR, RVFAJSFIK and AARGJTLAM, respectively).

Refolding reactions with these conditional ligands are efficient and result in thermo-stable p*HLA complexes that rapidly degrade upon UV-exposure. The presence of a cognate peptide ligand during UV-treatment leads to the replacement of the cleaved conditional ligand in the peptide-binding groove of the MHC molecule and thereby results in the generation of pMHC complexes of a desired specificity. In line with this, fluorescently labeled MHC tetramers generated in such exchange reactions stain antigen-specific CD8+ T cell populations with equal specificity and sensitivity as MHC tetramers prepared via individual refolding reactions. Dissociation of cleaved peptide fragments displays non-linear kinetics, with release of 75% to 95% within 15 minutes, but only limited further release upon prolonged incubation. Based on reverse phase analysis of fluorescent peptide that remains MHC-associated upon UV-exposure it is apparent that the residual MHC-bound product primarily consists of reaction products, indicating that lack of dissociation is not due to incomplete cleavage. Prior data have shown that the dissociation of full-length peptides from MHC class I molecules also occurs with biphasic kinetics[23] (although, as expected with half lives that are orders of magnitude greater). This has been interpreted as evidence for the existence of a 'closed' and 'open' conformational state of peptide-charged MHC molecules, and it is possible that the same applies to MHC complexes analyzed here.

Of more importance for the practical use of MHC reagents, the presence of a fragment of the conditional ligand in a fraction of UV-exposed MHC molecules has no measurable effect on the ability to detect antigen-specific CD8+ T cell responses. Specifically, the amount of MHC exchange multimer required to stain HLA-A1 and -A3 restricted T cells does

not substantially deviate from that required when using conventional MHC tetramer reagents (a factor of 0.8 and 1.5 for HLA-A1-CMV-pp_{65} and HLA-A3-EBV-EBNA-3a). This may be explained by the fact that of the 4 MHC complexes in a tetrameric MHC molecule a maximum of 3 is likely to be simultaneously available for binding and the identity of the 4th pMHC complex will in this case be irrelevant[24]. In addition, it is noted that also in classical MHC refolding reactions it is rather unclear to what extent MHC occupancy is homogeneous.

To demonstrate the application of peptide exchange for high throughput screening of potential T cell epitopes, a screen of melanoma-associated peptides was performed in the context of HLA-A3. The time between obtaining the peptide library and completing the final binding assay was a mere 3 weeks, including quality controls and data analysis. 22 peptides were identified with high affinity for HLA-A3, and this included the 4 previously described gp100-derived epitopes. These epitopes could be used to monitor naturally occurring T cell responses in melanoma patients. More intriguingly, this collection of epitopes may be utilized to isolate TCRs from vaccinated HLA-transgenic mice[25,26]. For those epitopes that are presented at the cell surface of melanoma cells, this would provide a strategy for the targeting of melanomas by TCR gene therapy with an expanding collection of TCRs[27].

With the collection of conditional ligands for 5 HLA gene products that is now available, coverage of the Western-European population has become substantial. Specifically, with this set of molecules, high-throughput analysis is feasible for at least one HLA-A or -B complex for greater than 90% of individuals. However, coverage of human populations in other areas is lower, with 58%, 65% and 75% for populations in Sub-Saharan Africa, North America and South-East Asia, respectively. Definition of conditional ligands for the HLA-A24, -B15 and -B58 gene products that are prevalent in these areas would be useful to increase coverage in these areas to the same level.

MHC peptide-exchange based strategies may be used for both large scale T cell epitope discovery, as demonstrated here for HLA-A3, and for T cell screening. The latter large-scale MHC-based analyses of T cell responses will generally not be feasible by standard MHC tetramer flow cytometry approaches, as the amount of patient material that is required would be prohibitive. Rather, it seems essential to develop robust platforms that can be used to analyze large series of antigen-specific cytotoxic T cell responses in parallel. Two conceptually different approaches may possibly be used for this purpose. In a first approach, parallel analysis of a large number of T cell specificities is achieved by spatial encoding schemes, in which T cells with a given specificity are selectively retained or active at defined sites. The MHC microarray platforms as developed by the Davis and Stern groups have provided evidence for the feasibility of this approach[2-4]. As an alternative approach, parallel analysis of antigen-specific T cell responses may potentially be achieved by combinatorial coding schemes, in which T cells specific for a given pMHC complex are defined by the binding of combinations of differentially labeled MHC tetramers. Dual T cell staining using MHC tetramers conjugated to PE and APC has previously proven feasible[28] and efforts to test the feasibility of large scale

combinatorial coding are in progress (A.H. Bakker, unpublished observations).

In addition to the use of peptide exchange strategies for large-scale T cell monitoring, it seems likely that this technology will also be valuable for the development of protocols for antigen-specific adoptive T cell therapy. The infusion of antigen-specific T cell populations is considered valuable to restore antiviral immunity in transplant recipients and other immunocompromised patients[29], and a first study in which the feasibility of infusion of CMV-specific T cells obtained by MHC tetramer-assisted enrichment has been reported[30]. In addition, selection of defined T cells may be used to enhance the anti-tumor effect of allogeneic hematopoietic stem cell transplantation[31], and TCR gene therapy protocols[27,32,33].

There is no conceptual difficulty in the production of MHC tetramers or reversible MHC tetramers[34] under GMP conditions. However, the production of the collection of GMP-grade pMHC tetramers required for these various applications may be cost-prohibitive, as for most of these applications, small series of pMHC reagents rather than single pMHC products would be preferred. For example, CMV-specific $CD8^+$ T cell responses in healthy individuals are directed towards on average 8 different open reading frames[35]. Extrapolating this to the other human Herpesviridae that are a cause of morbidity and mortality in transplant recipients, a cell product intended to prevent activation of HSV, VZV, CMV and EBV would perhaps ideally include reactivity against one or two dozen distinct $CD8^+$ T cell epitopes. Based on these considerations it seems attractive to develop GMP production processes for p*HLA complexes, and the use of such GMP-grade p*MHC complexes in simple exchange reactions may facilitate the clinical development of oligoclonal adoptive T cell therapy.

| MATERIALS AND METHODS

Generation of peptide-MHC complexes

All peptides were synthesized by standard Fmoc synthesis. (+/-)-3-amino-3-(2-nitro) phenyl-propionic acid was generated as previously described[12]. Fluorescent labeling of peptides was performed as described in the **Supplementary Methods** and confirmed by LC-MS. Labeled peptides were purified by reverse-phase HPLC.

Recombinant HLA-A1, -A2, -A3, A11 and -B7 heavy chains were produced in *E. coli*. MHC Class I monomer refolding reactions with *E. coli*-derived β2M were performed as described[22] and purified by gel-filtration HPLC in PBS (pH 7.4). Biotinylation and MHC tetramer formation were performed as described[12]. pMHC complexes were stored at -20 °C in PBS/ 16% glycerol, MHC tetramers were stored at -20 °C in PBS/ 16% glycerol/ 0.5% BSA.

Analysis of peptide exchange

Exchange reactions were performed by exposure of pMHC complexes (25 μg/ml in PBS) to long-wavelength UV, using a 366 nm UV lamp (Camag, Muttenz, Switzerland) in the presence or absence of 50 μM exchange peptide. After UV-exposure, pMHC complexes intended for subsequent analysis by ELISA were incubated at 37 °C for 60 minutes to promote unfolding of peptide-free

MHC molecules[6]. For gel-filtration HPLC, incubations after UV exposure were performed at room temperature. pMHC complexes intended for use in flow cytometry were multimerized by the stepwise addition of streptavidin-PE (Invitrogen Eugene, OR, USA). For gel-filtration HPLC, 300x21 and 300x7 mm Biosep SEC S3000 columns (Phenomenex, Torrance, CA, USA) were used for protein isolation and analysis, respectively. Absorbance was monitored at 230 nm and fluorescence was monitored with excitation at 550 nm and emission at 567 nm. Peptide elution and subsequent reverse phase chromatography was performed as described in the **Supplementary Methods**. Sandwich ELISAs were performed as described[12].

Cells and flow cytometry

Frozen peripheral blood mononuclear cells from individuals undergoing an HLA-matched allogeneic bone marrow transplantation were obtained after informed consent and with approval from the LUMC IRB. For flow cytometric analysis, cells were stained with PE-labeled MHC tetramers for 5 minutes followed by FITC-labeled anti-CD8 (BD Biosciences, San Jose, CA, USA) staining for 15 minutes at room temperature. Data acquisition was carried out on a FACSCalibur (Becton Dickinson, San Jose, CA, USA), Analysis was performed using FlowJo (Tree Star, Inc., San Carlos, CA, USA).

Peptide library and binding studies

Protein sequences for Nodal (NP_060525), Mart-1/Melan-a (NP_005502) Tyrosinase (AAB60319), Tyrosinase-related protein 1 (CAG28611), Tyrosinase-related protein 2 (ABI73976), and GP100/PMEL17 (NP_008859) were analyzed for potential HLA-A3 ligands using SYFPEITHI[9], and the artificial neural network (ANN) and stabilized matrix method (SMM) algorithms from IEDB (version prior to December 2007)[20]. Peptides were selected with a predicted binding value of either over 21 for SYFPEITHY (nona- and decamers), below 6000 for ANN (nonamers only) or below 600 for SMM (decamers only), resulting in 203 peptides. Synthesized peptides (Pepscan Lelystad, The Netherlands), were checked by LC-MS. HLA-A3 binding assays were performed using a fluorescence polarization (FP) assay. For this purpose, a FP assay reported for HLA A2.1[36], was modified for application with UV mediated peptide-exchange, using fluorescently labeled A3-specific KVPCALINK[37] as tracer peptide (see **Supplementary Methods**). To determine the binding capacity of peptides for HLA-A3, percentage inhibition relative to controls was determined at 5 μM in an FP competition assay with conditional p*A3. For peptides displaying >63 % inhibition at 5 μM, IC_{50} values were determined by generating dose response curves of serial peptide dilutions from 50 μM to 50 nM.

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| AUTHOR CONTRIBUTIONS

A.H.B., R.H., C.L., M.T., B.R., C.R.B., H.O., and T.N.M.S. designed research; A.H.B., R.H., C.L., M.T., and C.R.B. performed research; B.R., S.R.H., W.J.E.v.E., M.H.M.H., and H.O. contributed new reagents/ analytic tools; A.H.B., R.H., B.R., C.R.B., and T.N.M.S. analyzed data; and A.H.B. and T.N.M.S. wrote the paper.

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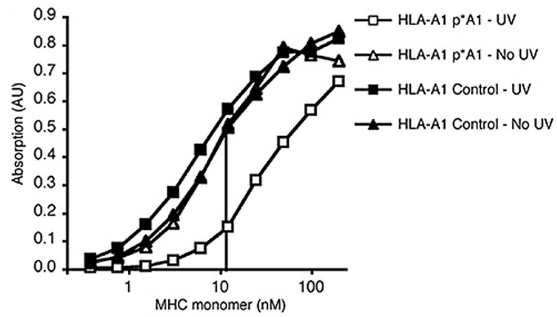
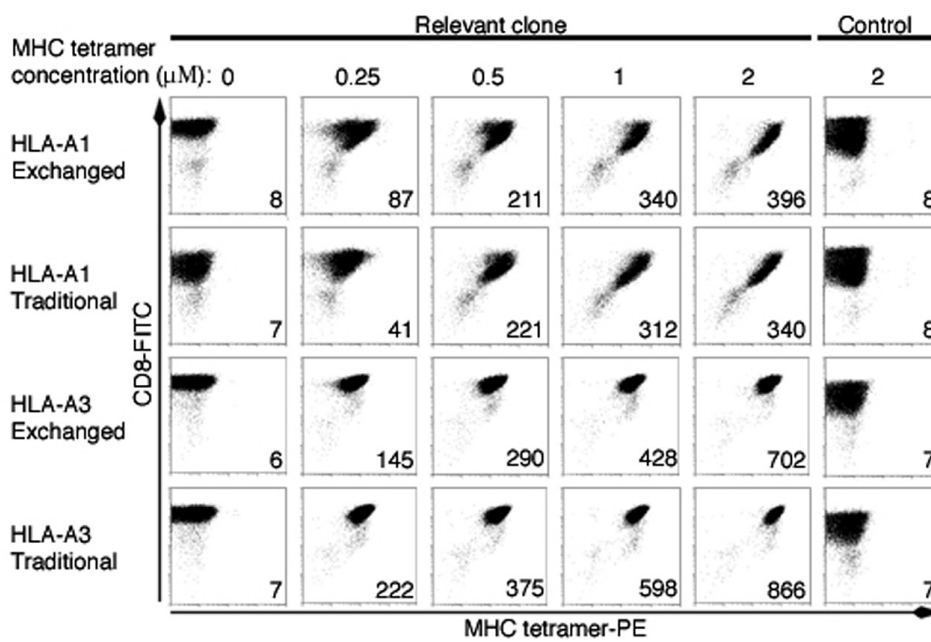
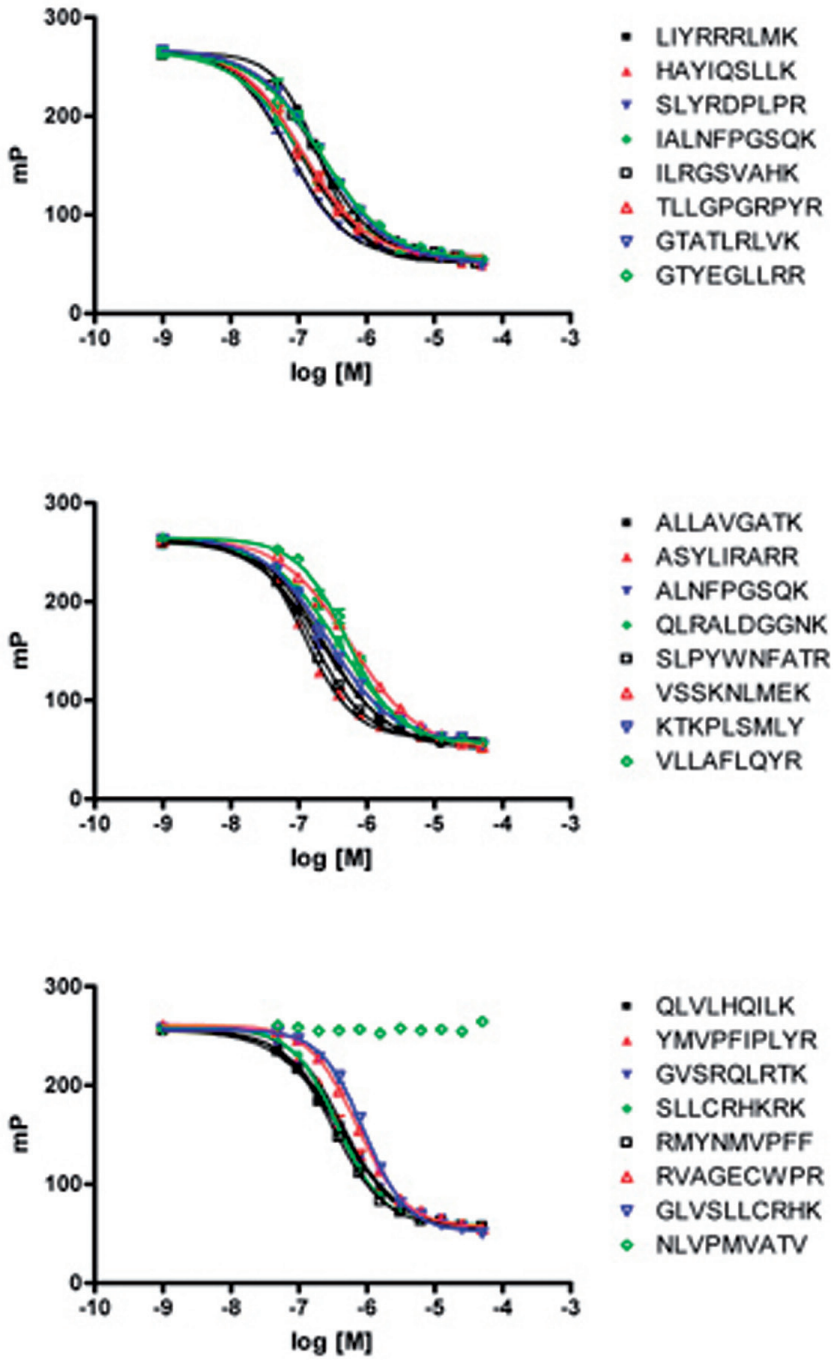
| SUPPLEMENTARY MATERIALS



Supplementary Figure 1. Establishment of an optimal MHC concentration for ELISA analysis. HLA-A1 complexes containing a control HLA-A1 ligand pA1 or the UV-sensitive ligand p*A1 were exposed to 366 nm UV for 0 or 60 minutes, and serial dilutions were analyzed by ELISA. Vertical line indicates the MHC concentration used for subsequent analyses. MHC concentrations as established for the other MHC gene products were also in the 10–20 nM range.



Supplementary Figure 2. Tetramer titration on T cell clones. $CD8^+$ T cell clones specific for HLA-A1 CMV-pp_{65} and HLA-A3 EBV-EBNA-3a were stained with the indicated amounts of HLA-A1 CMV-pp_{65} (Top) and HLA-A3 EBV-EBNA-3a (Bottom) tetramers. "Exchanged" indicates MHC tetramers generated via peptide-exchange, while "Traditional" indicates MHC tetramers generated via classical MHC refolding with the specific peptide. Panels designated "Relevant Clone" and "Control" represent staining with the matched MHC tetramer and cross-sample controls, respectively. Numbers indicate MFI of $CD8^+$ cells.



Supplementary Figure 3. Dose response curves of a competitive binding FP assay using HLA-A3 p*A3 and the indicated peptides. The FP assay was performed after 30 min UV irradiation and 45 h incubation. IC_{50} values were determined from three independent experiments. A representative experiment is shown. mP indicates millipolarization.

Supplementary Table 1.

Allele	Sequence	Refolding efficiency Thermostability UV-sensitivity
HLA-A1	STDJGVLEY	+
	STDPJVLEY	- (LD)
	STDPGJLEY	++
	STDPGVJEY	+
	STDPGVLJY	- (LD)
	STAPGJLEY	**Candidate**
	STEPGJLEK	--- (LR)
	STDPGJLEF	--- (TI)
HLA-A3	RLYJGGATK	- (LD)
	RLYRJGATK	++
	RLYRGGAJK	- (LD)
	RIYRJGATR	**Candidate**
	RGYRJGATY	--- (TI)
	RLYRJGATL	--- (LR)
HLA-A11	AIFQJSMTK	+
	AIFQSSMJK	--- (TI)
	ATFQJSMTK	--- (LR)
	ATFQSSMJK	-- (ND)
	RVFAFSFJK	-- (ND)
	RVFAJSFIK	**Candidate**
HLA-B7	APRJPTLAL	- (LD)
	APRGJTLAL	++
	APRGPJLAL	- (LD)
	APRGPTJAL	-- (ND)
	APRGPTLJL	--- (LR)
	AARGJTLAM	**Candidate**
	AARGJTLAL	++
	APRGJTLAM	++

Overview of the peptides tested for each of the four HLA molecules. **J** indicates 3-amino-3-(2-nitro) phenyl-propionic acid. Sequences in the top part of each panel reflect the peptides used in a first screen. Arrows indicate which peptides were chosen as leads for a second screen (bottom parts). For HLA-A11, a panel of high and low affinity binders was tested in a single screen with **J** at two different positions. The four conditional ligands selected for further analysis are depicted in bold. LR: low refolding efficiency, TI: thermo-instable, ND: no UV-mediated degradation, LD: low UV-mediated degradation.

Supplementary Table 2.

Protein	Sequence	Inhibition	SD	Protein	Sequence	Inhibition	SD	Protein	Sequence	Inhibition	SD
Tyrosinase related protein 2	TLLGPGRPYR	84	1	Tyrosinase	VSSKNLMEK	74	3	GP100	LIYRRRLMK	90	2
	GTYEGLLRR	83	2		YMVPFIPLYR	72	2		IALNFPGSQK	86	1
	VLLAFLQYR	74	11		SLLCRHKRK	70	2		GTATLRLVK	84	2
	RMYNMVPFF	69	6		GLVSLLCRHK	64	5		ALLAVGATK	83	1
	LMETHLSSK	62	2		SIFEQWLQR	63	4		ALNFPGSQK	80	2
	VMSLHNLVH	59	8		KSYLEQASR	60	0		QLRALDGGNK	79	0
	VLLAFLQYRR	55	4		KFFAYLTLAK	51	4		QLVLHQILK	72	3
	LLRRNQMGR	48	1		MVPFIPLYR	43	7		GVSRQLRTK	72	2
	FLLSCLGCK	47	5		TQYESGSMDK	36	3		QLRTKAWNR	62	1
	GLLRRNQMGR	46	2		FMGFNCGNCK	36	3		QVWGGQPVY	59	4
	MTVDSLVNK	42	1		CVSSKNLMEK	27	4		VSCQGGLPK	58	10
	FQNSTFSFR	41	8		LVSLLCRHK	26	7	►	VLASLIYRR	54	2
	AIFDEWMKR	39	1		FFAYLTLAK	22	1		TVSCQGGLPK	49	1
	VVISNRLLY	36	11		VSLLCRHKRK	19	1		VVLASLIYR	45	3
	ILEHVRKEK	30	1		LLRWEQEIQK	14	2		GALLAVGATK	38	1
	FLGALDLAK	27	0		ALLGGSEIWR	2	0		HTMEVTVYH	35	4
	HVRKEKATK	27	3		SMHNALHIYM	0	2		ILKGGSGTY	30	2
	PLMETHLSSK	27	3		YMVPFIPLY	0	2		SLIYRRRLMK	30	7
	QVMSLHNLVH	21	6		NIFDLSAPEK	-1	1		LVLKRCLLH	29	7
	FLGALDLAKK	19	1		IPIGTYGQMK	-3	1		SSHWLRLPR	20	1
	NSMKLPTLK	14	1		QLSGRGSCQ	-4	2		TVYHRRGSR	18	6
	SLVNKECCPR	7	1		DLSAPEKDK	-4	2		RQLRTKAWNR	15	3
	TLLGPGRPY	3	1		HIYMNGTMSQ	-4	3		QKRSFVYVWK	14	4
	LLAFLQYRR	3	4		HFPRACVSSK	-4	1		LGVSRQLRTK	11	1
	CMTVDSLVNK	2	1		PLRRNPGNH	-4	1		LIYRRRLMKQ	9	3
	GFLLSCLGCK	1	1		ALLAGLVSL	-5	2		QILKGGSGTY	7	1
	SLSPQEREQF	0	3		RLPSSADVEF	-5	2		CWRGGQVSLK	6	4
	DIRDCLSLQK	0	1		PIGTYGQMK	-5	0		AESTGMTPEK	5	2

Supplementary Table 2. *Continued*

Protein	Sequence	Inhibition	SD	Protein	Sequence	Inhibition	SD	Protein	Sequence	Inhibition	SD
	FLNGTNALPH	0	1		WLLGAAMVG	-5	2		CQLVLHQILK	3	3
	TTNILEHVRK	-1	1		VLYCLLWSFQ	-5	1		YLAEADLSY	2	1
	AFLQYRRLRK	-1	1		LLAGLVSLLC	-6	1		AVVLASLIYR	1	1
	RLRKGYTPLM	-1	3		SYMVPFIPLY	-8	1		GLSIGTGRAM	0	3
	RKFFHRTCK	-1	0		RLLVRRNIFD	-8	1		ILLVLMAVVL	-1	2
	QFLGALDLAK	-1	2		YDLFVWMHYY	-8	0		ALQLHDPSGY	-1	1
	RLGAESANVC	-2	1		PLYRNGDFF	-9	1		AVGATKVPR	-2	1
	LVTLCNGTY	-3	1		NLLSPASFF	-9	2		AVVLASLIY	-2	3
	ILPGAQGQF	-4	1		RLLVRRNIF	-9	2		TLIGANASF	-4	1
	FLQYRRLRK	-4	1	**Nodal**	**HAYIQSLLK**	**90**	**1**		AVIGALLAVG	-4	1
	FVLLAFLQY	-4	1		**SLYRDPLPR**	**88**	**1**		AVIGALLAV	-4	1
	RLIGNESFA	-4	1		**KTKPLSMLY**	**74**	**2**		VLYRYGSFS	-4	1
	ALVGLFVLLA	-5	3		**RVAGECWPR**	**67**	**3**		YLEPGPVTA	-5	1
	KILPGAQGQF	-5	1		VTRPLSKWLK	57	3		LISRALVVTH	-5	1
	ALDLAKKRVH	-6	1		ATVATALLR	56	1		LLVLMAVVL	-5	1
	TLISRNSRF	-7	1		STCCAPVKTK	54	3		TLISRALVV	-6	0
	SVYDFFVWLH	-7	0		ATNVLLMLY	44	9		QVLGGPVSG	-6	1
	RMYNMVPFFP	-7	1		LAYMLSLYR	38	8		QVPLDCVLY	-6	2
	NILEHVRKEK	-7	2		WLKRPGALEK	36	8		PLLDGTATL	-7	1
	RLLYNATTN	-8	1		HAYIQSLLKR	24	5		FLRNQPLTF	-7	2
	RLLYNATTNI	-8	1		IIYPKQYNAY	14	2		KVLPDGQVIW	-7	2
	FVWLHYYSVR	-8	2		AIEIFHQPK	12	10		KVLPDGQVI	-8	2
Tyrosinase related protein 1	**ASYLIRARR**	**83**	**2**		VLEVTRPLSK	8	1	**Mart1**	HVGTQCALTR	0	1
	SLPYWNFATR	77	**4**		LKRPGALEK	-1	1		AHFIYGYPKK	-3	2
	GLFDTPPFY	60	3		ALLQAGAAT	-3	2		VLLLIGCWY	-4	2
	LTWHRYHLLR	55	10		LLQAGAATV	-4	1		GVLLLIGCWY	-4	1
	AVFDEWLRR	53	4		GWGSWIIYPK	-4	2		RDSKVSLQEK	-4	0
	WTHYYSVKK	47	4		MLYSNLSQE	-5	1		DAHFIYGYPK	-5	1

Supplementary Table 2. *Continued*

Protein	Sequence	Inhibition	SD	Protein	Sequence	Inhibition	SD	Protein	Sequence	Inhibition	SD
	FVWTHYYSVK	42	6		ALLQAGAATV	-6	2		LLLIGCWYCR	-8	3
	LPYWNFATRK	18	7		SPLAYMLSLY	-6	3		LLIGCWYCRR	-8	2
	NVARPMVQR	17	2		AVDGQNWTF	-6	0				
	YSNSTNSFR	9	4		ALLRTRGQP	-6	2				
	ALIFGTASY	8	2		TVTLSQVTF	-7	1				
	KLLSLGCIF	2	1		LLMLYSNLSQ	-7	1				
	EIIAIAVVG	-1	4		NHAYIQSLLK	-8	2				
	FVRALDMAK	-3	1		NLIGWGSWI	-8	1				
	ALLLVALIFG	-3	0		RQLGGSTLLW	-8	2				
	TLISPNSVF	-3	2		PLAYMLSLY	-8	1				
	FLNGTGGQTH	-3	2		PLAYMLSLYR	-10	1				
	ALLLVALIF	-3	1								
	ILGPDGNTP	-4	1								
	AVRSLHNLAH	-4	3								
	NLLDLSKEEK	-4	1								
	AVVGALLLVA	-5	2								
	ALDMAKRTTH	-5	1								
	FVRALDMAKR	-5	1								
	HRYHLLRLEK	-6	1								
	CIFFPLLLFQ	-7	0								
	ALIFGTASYL	-7	2								
	SIYNYFVWTH	-8	1								

203 predicted HLA-A3 ligands from 6 melanoma-associated proteins were selected based on 3 binding prediction algorithms. In an initial screen, peptides were screened at 5 μM in a 45 hour FP competition assay format. Peptides in bold were chosen for further analysis to determine IC_{50} values. Underlined peptides represent previously described HLA-A3 ligands. Inhibition reflects the percentage reduction in polarization of the tracer peptide. Values represent mean +/- SD of triplicate samples.

| SUPPLEMENTARY METHODS

Labeling of peptides. Fluorescent labeling of peptides was performed as follows: 6 mg of peptide (50 mg/ml in DMSO) was diluted in 1.5 ml 50 mM HEPES buffer and low amounts of N,N-Dimethylformamide (DMF, Biosolve, Valkenswaard, The Netherlands) were added whenever precipitates formed. 2.5 mg TMR-5-maleimide (Tebu-Bio, Le Perray en Yvelines, France) from a 50 mg/ml DMF stock solution was added and the reaction mixture was incubated at room temperature for 36 hours on an orbital shaker. 5 µl of the reaction was analyzed by LC-MS and the reaction was terminated by the addition of DTT to a final concentration of 5 mM. Prior to purification, 1.5 ml of 10% trifluoroacetic acid (TFA, Biosolve)/ 5 mM DTT/ 50 mM HEPES was added. Labeled peptides were purified by reverse-phase HPLC (1525EF, Waters, Milford, MA, USA) with H_2O/0.05% TFA as solvent A and acetonitrile/0.05% TFA as solvent B at a flow rate of 18 ml/min on a 10 µm 30x250 mm dc18 Atlantis column (Waters). A linear gradient of 10% to 95% solvent B over 14 minutes was used for a first elution. The fractions were then analyzed by LC-MS and fractions containing fluorescently labeled peptide were further purified using a similar protocol with a linear gradient of 25% to 80% solvent B over 19 minutes for elution. Fractions were then lyophilized, dissolved in DMSO, and stored at -20°C.

Peptide Elution. HLA-A2 ^{Fl}p*A2 complexes were either left untreated or exposed to UV-light for 60 minutes. For examination of the effect of UV-treatment on the total pool of MHC-associated plus released fluorescent ligand, peptides were extracted with 10µl 10% TFA (Biosolve) for 60 minutes at room temperature. The material was then analyzed by reverse-phase chromatography with H_2O/0.05%TFA as solvent A and acetonitrile/0.05% TFA as solvent B at a flow rate of 1 ml/min on a 300x3.9 Delta Pak 15µ C18 column (Waters). A linear gradient of 10% to 60% solvent B over 60 minutes was used for elution. To analyze the composition of material remaining MHC-associated after UV-treatment, reactions were separated by gel-filtration chromatography after UV exposure and material eluting with the retention time of pMHC complexes was collected. Subsequently, samples were adjusted to contain an equal amount of fluorescent signal, and concentrated to 100 µl. 10 µl 10% TFA was added and samples were incubated for 60 minutes at room temperature. 900 µl H_2O was added and samples were analyzed by reverse phase chromatography as described above.

Fluorescence polarization assay. For all fluorescence polarization binding assays peptides were diluted in bovine gamma globulin in PBS (0.5 mg/mL, Sigma). The HLA-A3 binding peptide KVPCALINK was labeled with TMR-5-maleimide (Anaspec, San Jose, CA, USA) and purified by reverse phase HPLC as described above. This peptide (pTAMRA) was used as a tracer for fluorescence polarization (FP) experiments. Labeled peptide was standardized against a fluorescence intensity curve using free TMR-5-maleimide as a standard.

FP measurements were performed on a Perkin Elmer Wallac EnVision 2101 Multilabel Reader. Samples were measured using a 531 nm excitation filter,

579 nm S-channel emission filter, a 579 nm P-channel emission filter and a Bodipy-TMR FP Dual mirror (all filters and mirrors obtained from Perkin Elmer). FP values are given as mP (millipolarization) and calculated using the following formula: polarization (mP) = 1000*(S-*G*P)/(S+*G*P), where S and P are the fluorescence intensities measured in the S (parallel to polarization plane) and P (perpendicular to polarization plane) directions and *G* is the grating factor. The *G* factor is a correction factor for instrument (filters, mirror) dependent variations in sensitivity for measurements in the S and P directions and is determined according to the instrument manufacturer's instructions by measuring a 1 nM sample of free TAMRA. The *G* factor is then calculated using the following formula: *G* = (S/P)*(1-L/1000)/(1+L/1000), where L is the theoretical or literature polarization value for the fluorophore, here TAMRA (L = 50 mP). Typically, *G* factors of 1 to 1.1 were obtained.

Epitope screening. To determine the binding capacity of the predicted ligands for HLA-A3, percentage inhibition of binding of tracer peptide was determined at 5 μM in an FP competition assay with conditional p*A3. For this purpose, an FP assay was modified for application with UV mediated peptide exchange for HLA-A3. In a 384 well black non-binding surface assay plate (Corning, UK) each well was loaded with 10 μL of a 2.5× peptide solution (12.5 μM), 5 μL of a pTAMRA solution (5 nM) and 10 μL of a 2.5× HLA-A3 p*A3 solution (1.25 μM). For all preparations 0.25 mg/mL bovine gamma globulin in PBS was used as a buffer. The plate was spun for 1 min at 1000 g at room temperature. To start UV mediated peptide exchange the plate was placed 10 cm under a 365 nm UV lamp (2×15W blacklight blue tubes, L×W×H 505×140×117 mm, Uvitec, UK) located in a cold room (4 °C). After 30 min irradiation the plate was sealed with thermowell sealing tape (Corning, UK) and incubated at room temperature for 45 hours, when periodic readings showed no further increase in polarization, indicating the establishment of equilibrium. Controls included peptide free samples (1 % dmso) for 0 % inhibition of tracer binding and 200 μM ILRGSVAHK (A3-Flu epitope) samples for 100 % inhibition of tracer binding. All data points were determined in triplicate.

Competition assay. For peptides displaying >63 % inhibition at 5 μM IC_{50} values were determined by generating dose response curves of serial peptide dilutions covering a range of 50 μM to 50 nM using the UV mediated FP competition assay described above. The binding affinity (IC_{50} value) of each competitor peptide was defined as the concentration that inhibits 50% binding of pTAMRA tracer peptide. Data were analyzed using GraphPad Prism software (GraphPad, San Diego, CA, USA). 50% inhibitory concentrations were determined in at least three independent experiments for each peptide. Peptide concentrations used for determination of IC_{50} values were calculated assuming maximum synthetic yield (2 μmol). For 13 peptides containing tyrosine or tryptophan residues, concentrations were verified by measuring absorption at 280 nm (for calculations of extinction coefficients see: http://www.expasy.org/tools/protparam.html). Peptide concentrations as determined by absorption at 280 nm were in between 0.47 and 1.05 of the predicted values.