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Journal Article

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Publication date:

2016-11

Permanent link:

<https://doi.org/10.3929/ethz-b-000120385>

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Originally published in:

Cancer Immunology, Immunotherapy 65(11), <https://doi.org/10.1007/s00262-016-1897-3>

Funding acknowledgement:

305309 - Profiling Responders In Antibody Therapies (EC)

Mass spectrometric analysis of the HLA class I peptidome of melanoma cell lines as a promising tool for the identification of putative tumor-associated HLA epitopes

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Received: 12 January 2016 / Accepted: 28 August 2016 / Published online: 6 September 2016
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Abstract Melanoma is one of the most immunogenic tumors, and extensive lists of potential tumor rejection antigens have been collected during the last decades. By isolating human leukocyte antigen (HLA) class I complexes from five melanoma cell lines (FM-82, FM-93/2, Mel-624, MeWo and SK-Mel-5) and sequencing HLA-eluted peptides by mass spectrometry, we identified over 10,000 unique peptides with high confidence. The majority of the peptides were 8–11 amino acids in length and were predicted to bind to the respective HLA alleles. Over 250 epitopes, corresponding to previously described tumor-associated antigens, were identified, suggesting that HLA peptidome analysis may facilitate the characterization of putative tumor rejection antigens. MeWo and SK-Mel-5 cell lines were further interrogated for neo-epitopes, revealing one peptide from MeWo cells carrying an amino acid mutation. We also observed a remarkable overlap between A*03:01 peptides eluted from Mel-624 cells and A*03:01 peptides recovered from soluble HLA complexes purified from two melanoma patients, shedding light on the similarity of the HLA peptidome in cell lines and in patient-derived material. The reliable characterization of the HLA class I peptidome in melanoma promises to facilitate the

identification of tumor rejection antigens and the development of immunotherapeutic strategies.

Keywords HLA · Melanoma · Immunopeptidome · Tumor-associated antigen · Mass spectrometry · Immunocapture

Abbreviations

ACN	Acetonitrile
ATCC	American Type Culture Collection
COSMIC	Catalogue of Somatic Mutations in Cancer
ESTDAB	European Searchable Tumour Line Database
ETH Zurich	Eidgenössische Technische Hochschule Zürich
FDR	False discovery rate
FPLC	Fast protein liquid chromatography
HCD	Higher-energy collisional dissociation
HLA	Human leukocyte antigen
IC ₅₀	Half maximal inhibitory concentration
LC-MS	Liquid chromatography–mass spectrometry
MS	Mass spectrometry
MS/MS	Fragment mass spectra
PMSF	Phenylmethylsulfonyl fluoride
sHLA	Soluble human leukocyte antigen
UHPLC	Ultra high performance liquid chromatography
WT	Wild type

Electronic supplementary material The online version of this article (doi:10.1007/s00262-016-1897-3) contains supplementary material, which is available to authorized users.

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Introduction

Immunotherapeutic strategies are gaining considerable importance for the treatment of various types of malignancies. Therapeutic approaches include the development of antibodies against immunological checkpoints [1, 2],

antibody–cytokine fusion proteins [3], bispecific antibodies [4], lymphokine-activated adoptive cell therapies [5, 6], as well as cancer vaccines [7].

Over the last decades, substantial evidence was collected, indicating that melanoma cells can be recognized by cytotoxic T cells through the presentation of tumor rejection antigens and neo-epitopes onto HLA class I molecules [8–10]. Thus, boosting a cancer patient's immune system represents a promising strategy for the treatment of melanoma, especially considering the high mutation rate of this cancer type [11, 12]. To understand the molecular basis for the tumor rejection process, a detailed knowledge of HLA class I peptides presented by malignant cells is essential. A detailed knowledge of the HLA peptidome facilitates the study of tumor-reactive T cell specificities, either by multiplex tetramer analysis [13, 14] or by peptide stimulation assays [15]. In principle, these investigations could be applied to cancer patients, helping profile their response to therapy. Indeed, recent reports have shown that the breadth and frequency of anti-melanoma T cell specificities increase, after therapeutic intervention with the anti-CTLA-4 antibody ipilimumab [16].

Since the first direct identification of a peptide, recognized by melanoma-specific T cells, by mass spectrometry in 1994 [17], several studies investigated the HLA peptidome of melanoma cells [18, 19]. Both studies, however, purified HLA complexes from $>10^9$ cells per analysis, which was necessary due to substantial sample loss during the purification process, estimated to be 95 % [20]. Recent advances in mass spectrometry and sample preparation protocols have made it possible to identify thousands of HLA class I-bound peptides from 10^8 cells [21, 22]. These technological improvements facilitate the investigation of tumor-associated antigens by mass spectrometry, allowing a comparison with other epitopes (e.g., peptides derived from housekeeping proteins) presented on HLA class I molecules.

In this work, we report on the confident identification of over 10,000 HLA class I-bound peptides from five human melanoma cell lines (FM-82, FM-93/2, Mel-624, MeWo and SK-Mel-5). Analysis of the identified sequences revealed the presence of more than 250 peptides from previously described tumor-associated antigens. Furthermore, we present for the first time the direct mass spectrometry-based identification of a neo-epitope purified from a human melanoma cell line. The amino acid substitution in this peptide led to an increased binding affinity to the cognate HLA allele. Finally, a comparison between peptides isolated from melanoma cell lines and from the serum of two HLA-matched melanoma patients and two healthy donors revealed similarities on the level of presented peptides.

Materials and methods

Cell lines and antibodies

Cell lines FM-82, FM-93/2, Mel-624 and MeWo were obtained from European Searchable Tumour Line Database (ESTDAB) [23], SK-Mel-5 was obtained from CLS Cell Lines Services, and A375 was obtained from American Type Culture Collection (ATCC). Cells were grown in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10 % FBS at 37 °C and 5 % CO₂. HB-95 hybridoma cells were cultivated in CD Hybridoma medium supplemented with 2 mM glutamine in a shaking incubator at 37 °C and 5 % CO₂. The W6/32 antibody was purified from HB-95 supernatant using protein A Sepharose and subsequently coupled to AminoLink Plus Coupling Resin (Thermo Fisher Scientific) following the manufacturer's instructions.

Cell lysis and affinity purification of HLA class I molecules and enrichment of HLA-bound peptides

Cells were washed with PBS, harvested using a cell scraper, washed twice again with PBS and lysed on ice at a density of 2×10^7 – 5×10^7 cells/ml lysis buffer (0.5 % IGEPAL CA-630, 0.25 % sodium deoxycholate, 1 mM EDTA, 0.2 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride (PMSF), Roche Complete Protease Inhibitor Cocktail in PBS) for 1 h. Lysates were cleared by centrifugation at 21,000g for 30 min at 4 °C and snap frozen for storage at –80 °C. HLA class I complexes were purified from cleared lysate using W6/32 antibody-coupled resin by incubation for 2 h at 4 °C. The resin was washed once with lysis buffer, then buffer A (150 mM NaCl, 20 mM Tris, pH 7.4), buffer B (400 mM NaCl, 20 mM Tris, pH 7.4), buffer A again and buffer C (20 mM Tris, pH 8.0) at 4 °C. Peptide–HLA complexes were eluted using 0.1 M acetic acid. Peptides were separated from protein by elution from C18 Macro SpinColumns (Harvard Apparatus) with 30 % acetonitrile (ACN), and subsequently dried and stored at –20 °C.

cDNA analysis of NY-ESO-1 and GAPDH

cDNA of the cell lines A375, FM-82, FM-93/2, Mel-624, MeWo and SK-Mel-5 was generated using the “SuperScript[®] III CellsDirect[™] cDNA Synthesis System” (Thermo Scientific) following the manufacturer's instructions. mRNA expression of NY-ESO-1 and GAPDH was analyzed via PCR using primers 5'-TGCTTGAGTTCTA CCTCGCCA-3' (forward) and 5'-TATGTTGCCGACA CAGTGAA-3' (reverse) for NY-ESO-1 and 5'-GA

AGGTGAAGGTCGGAGTC-3' (forward) and 5'-GAAG ATGGTATGGGATTTC-3' (reverse) for GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) and Phire polymerase (Thermo Scientific). NY-ESO-1⁺ A375 cells were used as positive control [24].

Refolding of MHC class I monomers

Extracellular domains of HLA-A*02:01 and HLA-A*26:01 were refolded in the presence of β_2 -microglobulin and peptides (Biomatik) as previously described [25]. The experimental setup was scaled down to 20 ml and performed without the addition of PMSF. In brief, 6 μ M denatured β_2 -microglobulin and 3 μ M denatured HLA alpha chain (both from bacterial inclusion body production) were refolded with 60 μ M of peptide for four days at 4 °C in a buffer containing 100 mM Tris HCl pH 8, 400 mM L-arginine hydrochloride, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, 2 mM EDTA and protease inhibitor cocktail (Roche). Refolded monomers were concentrated to 1 ml using “Vivaspin 20 (molecular weight cutoff 30,000)” (Sartorius) and subsequently 100 μ l were analyzed on a “ÄKTA Purifier” fast protein liquid chromatography (FPLC) with a “Superdex™ 200 10/300 GL” column (both GE Healthcare). Fractions (0.25 ml) of the HLA monomer peaks (peak at 15 ml) were collected and analyzed via SDS-PAGE.

Analysis of HLA class I peptides by ultra high performance liquid chromatography (UHPLC) and mass spectrometry

Liquid chromatography–mass spectrometry (LC-MS) analysis of HLA class I peptides was essentially carried out as described in Ritz et al. [22]. Peptides were analyzed by LC-MS using a Q Exactive Mass Spectrometer connected to an EASY-nLC 1000 (both Thermo Scientific). Peptides were separated by reversed-phase chromatography using an Acclaim PepMap RSLC C18, 50 μ m \times 150 mm, 2 μ m analytical column (Thermo Fisher) at a flow rate of 0.3 μ l/min, with linear gradients from 0 to 24 % ACN in 90 min. All buffers contained 0.1 % formic acid. MS spectra were recorded in full ion scan mode from 250 to 2000 *m/z*, with a resolution of 70,000 and a maximum injection time of 80 ms. Fragment mass spectra (MS/MS) were recorded at a resolution of 17,500 and an injection time of 240 ms. The most intense ten doubly or triply charged ions were selected for higher-energy collisional dissociation (HCD) fragmentation. Dynamic exclusion was set to 15 s.

The datasets were searched using the MaxQuant software (version 1.4.1.2 [26]) against the human reference proteome databases containing 89,649 human proteins downloaded from the UniProt homepage on January

20, 2015. MaxQuant parameters were essentially set as described [21]: (1) digestion mode: unspecific, (2) first search ppm 20; main search ppm 4.5, (3) fragment mass tolerance ppm 20, (4) one variable modification (oxidation of methionine), (5) no specific amino acids for the generation of the decoy databases, (6) peptide false discovery rate (FDR) 1 %, protein FDR 100 %, (7) peptide length allowed: 7–20 amino acids. Reverse hits and contaminants were removed from the “peptide.txt” output file.

To identify melanoma-associated antigens in the HLA analysis, a list of melanoma-associated antigens [10] was retrieved and compared to the identified peptides on the gene level.

HLA binding prediction of identified peptides

Peptide identifications based on the MS/MS data were combined into a final report for each cell line. Identical sequences identified multiple times due to modifications were collapsed into one identification. Peptides with a length between 8 and 11 amino acids were subjected to HLA class I binding prediction analysis using NetMHCpan 2.8 [27], taking into account the respective HLA alleles of the cell lines reported in the literature [23, 28]. The analysis was performed on a cell line by cell line basis. Each peptide was assigned the minimal predicted half maximal inhibitory concentration (IC₅₀).

Graphical representation of HLA-specific motifs

HLA-specific motifs were generated using the Seq2Logo 2.0 server using default settings [29]. Based on the binding prediction analysis, all nine mers were assigned to the allele with the lowest predicted IC₅₀-value and subsequently used for the visualization of respective HLA-specific motifs.

Identification of a neo-epitope and validation of the fragment mass spectrum

The mutational information for MeWo and SK-Mel-5 cell lines, respectively, were downloaded from the “Catalogue of Somatic Mutations in Cancer” (COSMIC) database [30] on September 3, 2015. The positional information for all protein code-changing single nucleotide polymorphisms was used to create 24 mers with the mutated amino acid at position 12. Mass spectrometric data from MeWo or SK-Mel-5 was interrogated with MaxQuant for the presence of mutated sequences using the same search parameters as for other analyses, but taking into account all protein sequences of the human reference proteome and the sequences carrying mutations. The synthetic peptide corresponding to IFRD2_{437–446}H446Y (Biomatik) was analyzed by LC-MS using the same HPLC and MS conditions as for the HLA

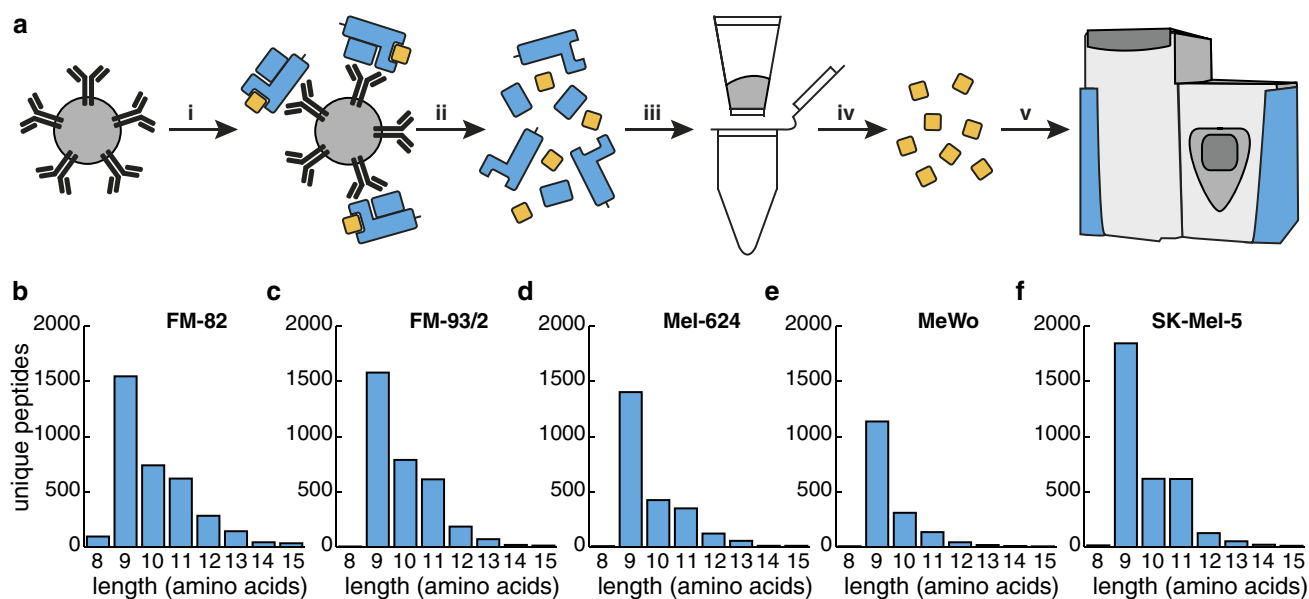


Fig. 1 Purification of HLA class I complexes and length distribution of peptides identified from melanoma cell lines. **a** Schematic representation of HLA class I peptide purification. *i* HLA complexes are captured using the pan-HLA antibody W6/32 coupled to resin. *ii* HLA complexes are eluted and denatured under acidic conditions. *iii*, *iv* HLA peptides are purified by stepwise elution from C18 resin.

v Analysis of purified peptides by LC-MS. **b–f** Length distribution of peptides identified from five melanoma cell lines. The number of identified peptides between 8 and 15 amino acids is plotted in relation to their length for **b** FM-82, **c** FM-93/2, **d** Mel-624, **e** MeWo, **f** SK-Mel-5

peptidomes. This allowed for comparison of the retention time and to identify a representative fragment mass spectrum. The fragment mass spectra of the synthetic and the endogenous peptides were extracted with Thermo Xcalibur Qual Browser 2.2 SP1 (Thermo Scientific) and plotted with Excel (Microsoft). Peptide fragments (a, b and y ions) were annotated with the MaxQuant Viewer 1.4.1.2 [26].

Results

Identification of HLA class I-bound peptides from melanoma cell lines

HLA class I peptides were purified from melanoma cell lysates, using an immunocapture procedure depicted in Fig. 1a, based on the pan-HLA class I monoclonal antibody W6/32 [31]. Peptides were subsequently eluted with acid, purified and desalted using C18 spin columns and analyzed by LC-MS. The lysates of five melanoma cell lines (FM-82, FM-93/2, Mel-624, MeWo and SK-Mel-5) were analyzed in independent quadruplicate experiments (quintuplicates for MeWo), leading to the identification of 3566, 3290, 2403, 1679 and 3321 unique peptides with a false discovery rate of 1 %, respectively (Supplementary Tables 1–5 list all identifications). Between 84 and 95 % of the identified peptides ranged between 8 and 11 amino

acids in length (Table 1; Fig. 1b–f) demonstrating compatibility with the typical length of HLA-bound peptides. HLA class I binding prediction analysis using NetMHCpan 2.8 revealed that between 79 and 91 % of these 8–11 mers were predicted to bind ($IC_{50} < 500$ nM) to the cognate HLA class I molecule, with 45–66 % predicted to be strong binders ($IC_{50} < 50$ nM) (Table 1).

Based on the binding prediction analysis of identified nine mers, individual HLA-specific motifs for the alleles of each cell line were plotted using the Seq2Logo 2.0 server [29] (Fig. 2). All cell lines shared the frequent A*02:01 allele and corresponding motifs were in excellent agreement with each other and with motifs presented in the literature [21, 32]. Distinct motifs could also be generated for all other alleles expressed by the five cell lines in agreement with the literature motifs [32, 33]. The majority of the peptides were recovered from HLA-A alleles; however, 842 and 874 nine mers were predicted to bind to HLA-B and HLA-C alleles, respectively, allowing the identification of more elusive HLA-C-specific motifs [21, 22, 33].

Expansion of the catalogue of putative tumor rejection epitopes

The list of HLA-eluted peptides was interrogated for the presence of tumor- and melanoma-associated antigens, using the detailed list described by Andersen et al. [10].

Table 1 HLA-bound peptides identified from melanoma cell lines, their length and binding prediction to respective HLA alleles

	Peptides identified	8–11 mers	% 8–11 mers	Strong binders ^a	% Strong binders of 8–11 mers	Binders ^b	% Binders of 8–11 mers	Tumor-associated antigens
FM-82	3566	3001	84.2	1545	51.5	2525	84.1	106
FM-93/2	3290	2987	90.8	1680	56.2	2610	87.4	110
Mel-624	2403	2186	91.0	980	44.8	1848	84.5	78
MeWo	1679	1589	94.6	741	46.6	1258	79.2	50
SK-Mel-5	3321	3094	93.2	2041	66.0	2824	91.3	80

^a NetMHCpan IC₅₀ < 50 nM

^b NetMHCpan IC₅₀ < 500 nM

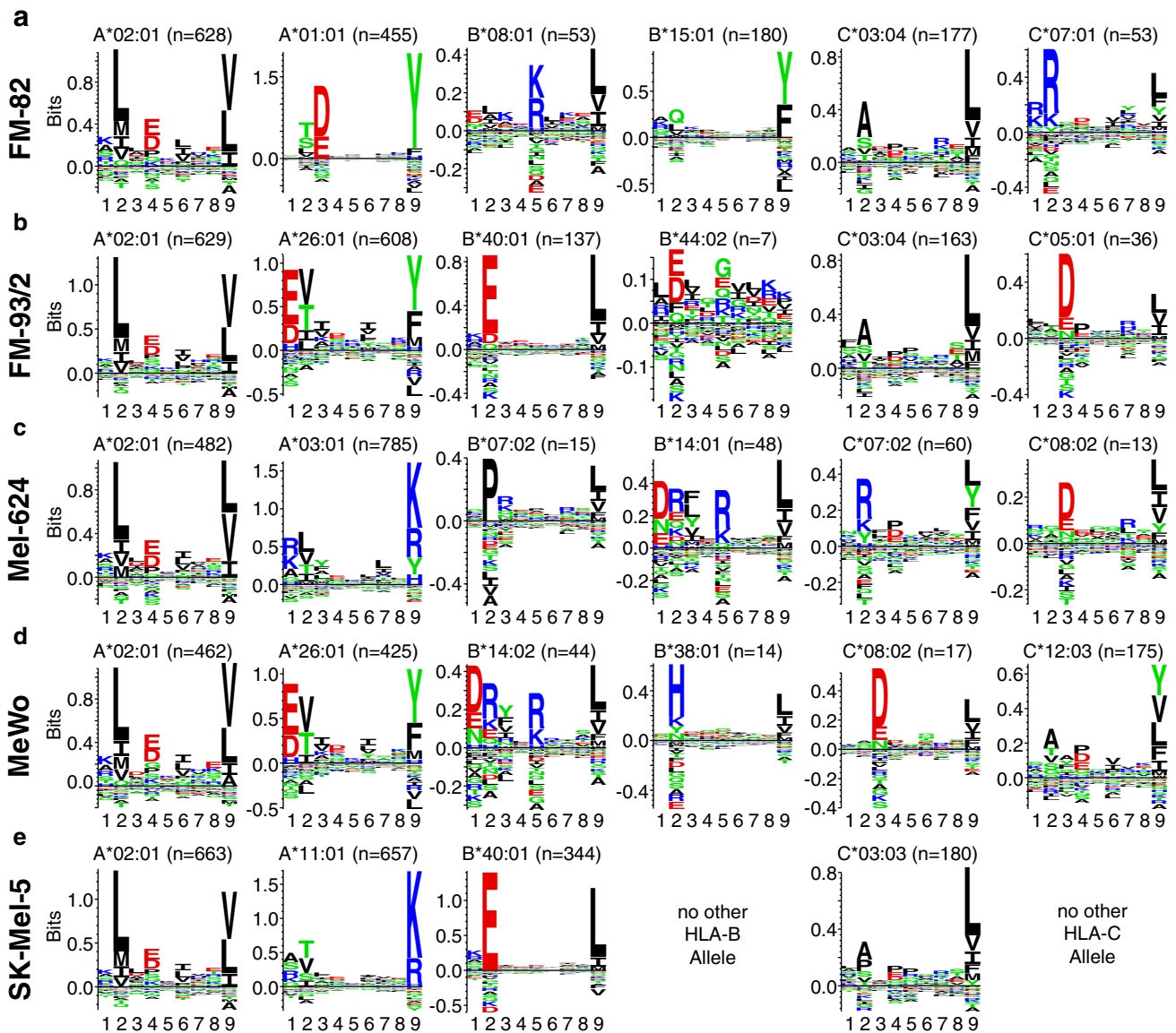


Fig. 2 Definition of HLA-specific motifs from the HLA peptidome of melanoma cell lines. IC₅₀ values of all nine mers were predicted for HLA alleles of each cell line [23, 28] using the NetMHCpan 2.8 software [27]. Each peptide was assigned to the HLA allele with the

lowest IC₅₀ value for the generation of HLA-specific motifs using Seq2Logo 2.0 [29]. HLA-specific motifs are presented for **a** FM-82, **b** FM-93/2, **c** Mel-624, **d** MeWo and **e** SK-Mel-5

Epitopes were denoted putative tumor-associated antigens if their sequence was identical to one of the genes presented in the above mentioned publication, adding up to a total of 262 epitopes from 72 antigens (Table 2). Of these, 31 demonstrated the exact same sequence as the tumor rejection antigens compiled by Andersen. Interestingly, for gp100/PMEL a total of 32 unique epitopes were identified. While many of the epitopes have been patented and/or characterized in great detail, we also identified previously undescribed epitopes, including predicted strong binders to HLA-A*02:01 originating from prominent melanoma-associated antigens [e.g., KTWDQVPFSV (gp100/PMEL) and KIWEELSVLEV (MAGE-A3/-A6)]. We also detected two length variants of gp100/PMEL epitopes (i.e., SLADTNSLAVV and AMLGTHTMEVTV; the underlined amino acids were either present or absent in the variants). A total of 85 epitopes were predicted to bind to A*02:01, which is probably a result of the high number of A*02:01-eluted peptides identified. The list further contained 22 epitopes originating from B*40:01 and 16 epitopes from C*03:04 or C*03:03, for which only one and two epitopes were presented by Andersen et al. [10], respectively. In contrast to the collection of tumor rejection antigens presented previously, our list of putative tumor rejection antigens is not biased by the frequency of a specific allele in the western population, but shows a strong linear correlation with the total number of peptides identified for a given allele ($R^2 = 0.78$, Supplementary Fig. 1).

Since some of the identified peptides were not predicted to bind to any of the cognate HLA alleles, the possibility of such peptides to form a stable complex with the HLA class I alpha chain and β_2 -microglobulin was investigated. Therefore, the putative tumor-associated antigens simultaneously identified from MeWo and FM-93/2 cells (AAAGGGGGGGRY, AMTKDNNLL, GTAAIQAHY and DSFPMEIRQY) were obtained as synthetic peptides (see Supplementary Figs. 2–5 for a comparison of synthetic and observed spectra) and added to HLA class I alpha chain and β_2 -microglobulin during refolding. FPLC profiles of the HLA complexes after refolding (Supplementary Fig. 6) and SDS-PAGE analysis (Supplementary Fig. 7) demonstrated that peptide AMTKDNNLL was able to form a stable complex with HLA-A*02:01, while peptides AAAGGGGGGGRY, GTAAIQAHY and DSFPMEIRQY facilitated the formation of a stable complex with HLA-A*26:01.

Although Table 2 contains peptides from over 70 known tumor-associated antigens, no peptide from the well-described antigen NY-ESO-1 was identified, even though NY-ESO-1 mRNA could be detected in three of the investigated cell lines (Supplementary Fig. 8).

Identification of a neo-epitope presented on the surface of MeWo cells

In order to examine whether neo-epitopes could be observed among the purified peptides, we searched the mass spectrometric raw data of the SK-Mel-5 and MeWo cell lines against publicly available catalogs of somatic mutations for these two cell lines downloaded from the COSMIC database [30] concatenated with the human reference proteome database. Out of 3911 mutations for MeWo and 128 mutations for SK-Mel-5, one mutant peptide was identified with high confidence (<1 % false discovery rate) from MeWo cells, corresponding to IFRD2_{437–446}^{H446Y} (interferon-related developmental regulator 2). The mutant peptide sequence could be confirmed by spectral comparison with a synthetic peptide (Fig. 3a). It is interesting to note that the mutated peptide was predicted to be a strong binder of HLA-A*26:01 ($IC_{50} = 34$ nM), while the wild type sequence was not predicted to bind to any of the MeWo alleles ($IC_{50} > 3000$ nM for all other alleles) (Fig. 3b).

Comparison of HLA-A*03:01-eluted peptides from melanoma cell lines and patients' sera

In a previous study [22], we had purified soluble HLA class I complexes (sHLA) from the blood of melanoma patients and healthy donors. Three of the melanoma patients possessed the HLA-A*03:01 haplotype and a significant number of peptides from two of these patients were predicted to bind to this allele. To identify whether the HLA peptidome of melanoma cell lines is comparable to the one of the melanoma patients, we investigated whether the same peptide sequences could be found both in the sHLA peptidome of patients and in the HLA peptidome of the HLA-A*03:01 positive Mel-624 cell line (Fig. 4). We observed a substantial overlap of A*03:01 peptides between the melanoma patients and the cell line (Fig. 4a), between patient and healthy donor (Fig. 4b), and between the healthy donors and the cell line (Fig. 4c). The 80 peptides shared between Mel-624 cells and one or both of the melanoma patients are reported in Supplementary Table 6. One epitope of a tumor-associated antigen was identified in the cell line Mel-624 as well as in the melanoma patient P5, but was not identified in the healthy donors (SLLTSSKGQLQK of perlipin-2 (PLIN2), also reported in Table 2).

Discussion

In this study, we present an extensive mass spectrometric characterization of the HLA peptidome of five melanoma

Table 2 List of peptides identified from tumor-associated antigens

Gene name (protein name)	Peptide sequence	FM-82	FM-93/2	Mel-624	MeWo	SK-Mel-5	HLA Alleles (IC ₅₀ in nM by NetMHCpan 2.8)
SEPT2 (septin-2)	KVNIVPVIK			X			A*03:01 (46 nM)
	RLYPWGVVEV	X	X			X	A*02:01 (5 nM)
	VEIEERG VKL		X			X	B*40:01 (47 nM)
	YIDEQFERY	X					A*01:01 (12 nM)
ALDH1A1 (retinal dehydrogenase 1)	ASERGRLLY	X					A*01:01 (35 nM)
	TMESMNGGKLY	X					No binding predicted
ARL4D (ADP-ribosylation factor-like protein 4D)	RLYEMILKR			X			A*03:01 (51 nM)
ATIC (IMP cyclohydrolase)	NLYPFVKTV	X	X				A*02:01 (41 nM)
BCAP31 (B-cell receptor-associated protein 31)	KLDVGNAEV	X	X	X	X	X	A*02:01 (27 nM)
CCNB1 (G2/mitotic-specific cyclin-B1)	VMVNQGLTKHMTV		X				A*02:01 (93 nM)
	VVMVNQGLTK			X		X	A*11:01 (27 nM), A*03:01 (95 nM)
	GEVDVEQHTL		X			X	B*40:01 (10 nM)
	TLAKYLMEL	X	X	X	X		A*02:01 (7 nM)
CCND1 (G1/S-specific cyclin-D1)	ALLESSLRQA	X		X	X		A*02:01 (90 nM)
	EVFPLAMNY		X		X		A*26:01 (22 nM)
	EVFPLAMNYL		X				A*26:01 (221 nM), C*12:03 (176 nM)
CCNI (cyclin-I)	AVLTKQLLH			X			No binding predicted
	EVIQWLAKL		X				A*26:01 (11 nM)
	LLDRFLATV	X	X	X		X	A*02:01 (8 nM)
	SLLDRFLATV					X	A*02:01 (4 nM)
CDR2 (cerebellar degeneration-related protein 2)	NSELEQQLGATGAY	X					A*01:01 (67 nM)
	RELEETNQKL		X			X	B*40:01 (19 nM)
CEP55 (centrosomal protein of 55 kDa)	AELESKTNTL					X	B*40:01 (34 nM)
	EVHNLNQLLY		X		X		A*26:01 (22 nM)
CPSF1 (cleavage and polyadenylation specificity factor subunit 1)	ALDEKLLNI					X	A*02:01 (47 nM)
	RLGNSLLLK			X			A*03:01 (17 nM)
	TLITDGMRSV	X				X	A*02:01 (76 nM)
	VSDRDRNLMVY	X					A*01:01 (13 nM)
	ALTDIDLQL	X					A*02:01 (62 nM)
CSPG4 (chondroitin sulfate proteoglycan 4)	ASSSAGTDPQLLY	X					A*01:01 (39 nM)
	SSSAGTDPQLLY	X					A*01:01 (14 nM)
	STDPQHHAY	X					A*01:01 (5 nM)
	TMLARLASA	X	X	X			A*02:01 (44 nM), B*08:01 (40 nM)
	TPNPALKNGQYW	X					No binding predicted
DCT (L-dopachrome tautomerase)	ELAPIGHNRMV		X				A*26:01 (50 nM)
	ETHLSSKRY		X		X		A*26:01 (207 nM)

Table 2 continued

Gene name (protein name)	Peptide sequence	FM-82	FM-93/2	Mel-624	MeWo	SK-Mel-5	HLA Alleles (IC ₅₀ in nM by NetMHCpan 2.8)
	GTYEGLLRR			X		X	A*03:01 (421 nM), A*11:01 (56 nM)
	KELPSLHVL					X	B*40:01 (18 nM)
	LMETHLSSK			X			A*03:01 (122 nM)
	MTVDSLVNK					X	A*11:01 (9 nM)
	REQFLGALDL		X			X	B*40:01 (13 nM)
	SLDDYNHLV		X				A*02:01 (4 nM)
	SLDDYNHLVTL	X	X	X			A*02:01 (10 nM)
	TRPWSGPYIL			X			C*07:02 (182 nM)
	TSDQLGYSY	X					A*01:01 (8 nM)
	VEETPGWPPTL		X			X	B*40:01 (61 nM)
	YYSVRDTLL			X			C*07:02 (57 nM)
EHD2 (EH domain-containing protein 2)	ALASHLIEA	X		X	X		A*02:01 (6 nM)
	GQYSTGKTSFIQY	X					B*15:01 (40 nM)
	KLPNSVLGR			X			No binding predicted
	RVHAYIISY			X			A*03:01 (59 nM)
ENAH (protein enabled homolog)	ERLERQERL			X			No binding predicted
GAGE12I (G antigen 12I)	YYWPRPRRY					X	No binding predicted
GPR143 (G-protein-coupled receptor 143)	ATSPPASVR			X		X	A*11:01 (103 nM)
	AVASLLKGR			X			No binding predicted
	GLSTILLYH			X			A*03:01 (390 nM)
	HAIPHYVTM	X	X			X	C*03:03 (5 nM), C*03:04 (5 nM)
	MLYYPVSR			X			A*03:01 (29 nM)
HMMR (hyaluronan mediated motility receptor)	KLLEYIEEI		X			X	A*02:01 (2 nM)
	KLQEELNKV		X		X		A*02:01 (12 nM)
	RLNAALREK			X			A*03:01 (62 nM)
HMOX1 (heme oxygenase 1)	EVIPYTPAM		X		X		A*26:01 (5 nM)
	EVIPYTPAMQR		X				No binding predicted
	EVIPYTPAMQRY		X				A*26:01 (4 nM)
HNRNPL (heterogeneous nuclear ribonucleoprotein L)	AAAGGGGGGGRY		X		X		No binding predicted
	AAAGGGGGGGRYYY	X					B*15:01 (159 nM)
	ETLGFLNHY		X				A*26:01 (14 nM)
	KQPAIMPGQSY	X					B*15:01 (98 nM)
	KSDALETLGFLNHY	X					No binding predicted
	MAAAGGGGGGGRY	X	X		X		A*26:01 (170 nM), B*15:01 (59 nM)
	YAVDRAITH		X		X		C*12:03 (59 nM), C*03:04 (273 nM)
HSPA1L (heat-shock 70 kDa protein 1-like)	LIFDLGGGTFD	X					No binding predicted
	TVFDAKRLIGR			X		X	A*11:01 (109 nM)
	DTERLIGDAAKNQV	X					No binding predicted
	RLIGDAAKNQV	X	X	X	X	X	A*02:01 (84 nM)
	AMTKDNNLL		X		X		No binding predicted

Table 2 continued

Gene name (protein name)	Peptide sequence	FM-82	FM-93/2	Mel-624	MeWo	SK-Mel-5	HLA Alleles (IC ₅₀ in nM by NetMHCpan 2.8)
IGF2BP3 (insulin-like growth factor 2 mRNA-binding protein 3)	EVLDSSLVQY		X				A*26:01 (47 nM)
	KIQEILTQV	X		X	X	X	A*02:01 (21 nM)
	TLYNPERTITV	X	X				A*02:01 (80 nM)
IL13RA2 (interleukin-13 receptor subunit alpha-2)	LLDTNYNLF	X					No binding predicted
	LLDTNYNLFY	X					A*01:01 (10 nM)
KDM5B (lysine-specific demethylase 5B)	ALKDSVQRA		X			X	No binding predicted
	RLIDLGVGL			X			A*02:01 (20 nM)
	RQFVTQLY	X					B*15:01 (30 nM)
	SVAQQLLNGK			X			A*03:01 (20 nM)
KIF20A (kinesin-like protein KIF20A)	KEAGNINTSL					X	B*40:01 (14 nM)
	RVFQGFFTGR			X		X	A*11:01 (22 nM), A*03:01 (72 nM)
	SEHLDTQKELL		X				B*40:01 (80 nM)
LGALS3BP (galectin-3-binding protein)	ALWKEPGSNV	X					A*02:01 (42 nM)
	NLWDLTDASVV		X				A*02:01 (19 nM)
	RIDITLSSV				X		A*02:01 (179 nM)
MAGEA1 (melanoma-associated antigen 1)	ALREEEEGV	X					No binding predicted
	EADPTGHSY	X					A*01:01 (79 nM)
	EVDYDGREHSA				X		No binding predicted
	KEADPTGHSY	X					No binding predicted
	KVLEYVIKV	X					A*02:01 (5 nM)
	LTQDLVQEKYLEY	X					A*01:01 (42 nM)
MAGEA12 (melanoma-associated antigen 12)	SAYGEPKRL	X					C*03:04 (184 nM)
	EVVRIGHLY		X				A*26:01 (7 nM)
	KMAELVHFL	X		X		X	A*02:01 (2 nM)
MAGEA12; MAGEA6; MAGEA3 (melanoma-associated antigen 12; 6; 3)	KIWEELSVLE	X					No binding predicted
MAGEA2 (melanoma-associated antigen 2)	EVFEGREDSVF		X		X		A*26:01 (72 nM)
MAGEA2; MAGEA2B (melanoma-associated antigen 2; 2B)	KMVELVHFL		X				A*02:01 (2 nM)
MAGEA1; MAGEA2; MAGEA2B; MAGEA3; MAGEA6 (melanoma-associated antigen 1; 2; 2B; 3; 6)	REPVTKAEML					X	B*40:01 (255 nM)
MAGEA3 (melanoma-associated antigen 3)	EVDPIGHLIYIF		X				No binding predicted
MAGEA4 (melanoma-associated antigen 4)	GVYDGREHTV		X				A*02:01 (225 nM)
MAGEA4; MAGEA8 (melanoma-associated antigen 4; 8)	KVLEHVVRV		X				A*02:01 (9 nM)

Table 2 continued

Gene name (protein name)	Peptide sequence	FM-82	FM-93/2	Mel-624	MeWo	SK-Mel-5	HLA Alleles (IC ₅₀ in nM by NetMHCpan 2.8)
MAGEA3; MAGE6 (melanoma-associated antigen 3; 6)	KIWEELSVLEV	X	X				A*02:01 (7 nM)
MAGEC2 (melanoma-associated antigen C2)	ALIEVGPDPHFC	X					No binding predicted
MDM2 (E3 ubiquitin–protein ligase Mdm2)	RLYDEKQQHI	X					A*02:01 (72 nM)
	RLYDEKQQHIVY	X					B*15:01 (54 nM)
MET (hepatocyte growth factor receptor)	EVIGRGRHFGCVY		X				A*26:01 (6 nM)
	KSEMNVNMKY	X					A*01:01 (193 nM)
MFGE8 (lactadherin)	EVRGDFVPSY		X				A*26:01 (15 nM)
MLANA (melanoma antigen recognized by T cells 1)	AEEAAGIGIL					X	B*40:01 (9 nM)
	ALMDKSLHV					X	A*02:01 (4 nM)
MMP2 (72 kDa type IV collagenase; PEX)	AVVDLQGGGHSY		X		X		A*26:01 (232 nM)
NELFA (negative elongation factor A)	KLLDISELDMV	X	X			X	A*02:01 (4 nM)
NOB1 (RNA-binding protein NOB1)	ALQDIGKNIYTI	X					A*02:01 (59 nM)
NPM1 (nucleophosmin)	DIKAKMQAS		X				No binding predicted
	FEITPPVVL		X			X	B*40:01 (10 nM)
	KMQASIEKA	X			X		A*02:01 (472 nM)
	RMTDQEAIQ				X	X	No binding predicted
	RMTDQEAIQDL	X	X		X	X	A*02:01 (347 nM)
	YEGSPIKVTL		X			X	B*40:01 (24 nM)
NUDCD1 (NudC domain-containing protein 1)	AEEHSIATL					X	B*40:01 (13 nM)
NUF2 (kinetochore protein Nuf2)	ATAQFKINK					X	A*11:01 (11 nM)
	EVVEKYEIY		X				A*26:01 (9 nM)
OCA2 (P protein)	KIDEKTAELK			X			No binding predicted
	HVDSTLLQ					X	No binding predicted
	HVDSTLLQV		X	X	X	X	A*02:01 (170 nM)
	KEPSEIVEL					X	B*40:01 (104 nM)
PASD1 (PAS domain-containing protein 1)	QLEERTWLL			X			A*02:01 (79 nM)
PGK1 (phosphoglycerate kinase 1)	DNGAKSVVL	X					No binding predicted
	EGKVLPGVDALSNI	X					No binding predicted
PLIN2 (perilipin-2)	RAYQQALSR			X			A*03:01 (140 nM)
	SLLTSSKGQLQK			X			A*03:01 (53 nM)
	SVASTITGV				X		A*02:01 (55 nM)
	TLLSNIQGV					X	A*02:01 (9 nM)
	TSALPIIQK					X	A*11:01 (24 nM)
PMEL (melanocyte protein PMEL; M-alpha; M-beta)	ALDGGNKHFL	X	X	X	X	X	A*02:01 (168 nM)
	ALLAVGATK			X			A*03:01 (45 nM)
	ALNFPQSQK			X			A*03:01 (21 nM)
	AMLGTHTMEV	X	X	X	X	X	A*02:01 (8 nM)

Table 2 continued

Gene name (protein name)	Peptide sequence	FM-82	FM-93/2	Mel-624	MeWo	SK-Mel-5	HLA Alleles (IC ₅₀ in nM by NetMHCpan 2.8)
	AMLGHTHTMEVTV	X		X			A*02:01 (20 nM)
	ASLIYRRRLMK					X	A*11:01 (61 nM)
	FALQLHDPSGY		X				C*03:04 (250 nM)
	FTITDQVPFSVSVSCLR	X					No binding predicted
	GTATLRLVK			X		X	A*11:01 (22 nM), A*03:01 (121 nM)
	HQILKGGSGTY	X					B*15:01 (25 nM)
	HSSSHWLRLP	X	X				No binding predicted
	HTYLEPGPVTAQ				X		No binding predicted
	ITDQVPFSV		X				C*05:01 (37 nM), A*02:01 (188 nM)
	ITDQVPFSVSVSCLR	X					No binding predicted
	KQDFSVPQL			X			A*02:01 (455 nM)
	KTWDQVPFS	X					No binding predicted
	KTWDQVPFSV	X	X	X	X	X	A*02:01 (11 nM)
	KTWDQVPFSVSV	X					A*02:01 (13 nM)
	KTWGQYWQ	X					No binding predicted
	KTWGQYWQV	X	X	X	X	X	A*02:01 (10 nM)
	LSIGTGRAM		X			X	C*03:03 (39 nM), C*03:04 (39 nM)
	LVLKRCLLH			X			No binding predicted
	MLGHTHTMEV	X	X	X		X	A*02:01 (8 nM)
	RNQPLTFALQLHDPSGY	X					No binding predicted
	RYGSFSVTL	X					C*07:02 (362 nM)
	SLADTNSLAV			X			A*02:01 (13 nM)
	SLADTNSLAVV	X	X	X	X	X	A*02:01 (12 nM)
	SVPQLPHSSSHW				X		No binding predicted
	TITDQVPFSVSVSCLR	X					No binding predicted
	VLYRYGSFSV	X	X				A*02:01 (6 nM)
	VLYRYGSFSVTL	X					A*02:01 (41 nM)
	YRYGSFSVTL			X		X	C*07:01 (10 nM), C*03:03 126 (nM)
PPIB (peptidyl-prolyl <i>cis</i> - <i>trans</i> isomerase B)	LLLPGPSAA	X	X	X	X		A*02:01 (75 nM)
PRAME (MELANOMA antigen preferentially expressed in tumors)	AAFDGRHSQTL	X	X		X	X	C*03:03 (7 nM), C*03:04 (7 nM), C*12:03 (16 nM)
	ALLERASATL	X					A*02:01 (25 nM)
	ALLPSLSHC	X	X				No binding predicted
	GQHLHLETF	X					B*15:01 (96 nM)
	QLLALLPSL			X			A*02:01 (9 nM)
	RLDQLLRHV	X	X	X		X	A*02:01 (217 nM)
	RLVELAGQSLLK			X			A*03:01 (35 nM)
	SLLQHHLIGL			X		X	A*02:01 (12 nM)
	YEDIHGTLHL		X				B*40:01 (15 nM)
PRDX5 (peroxiredoxin-5, mitochondrial)	LLDDSLVSI	X					A*02:01 (8 nM)
RAN (GTP-binding nuclear protein Ran)	AQYEHDLVA	X					A*02:01 (303 nM)
	KLIGDPNLEFV	X	X				A*02:01 (5 nM)

Table 2 continued

Gene name (protein name)	Peptide sequence	FM-82	FM-93/2	Mel-624	MeWo	SK-Mel-5	HLA Alleles (IC ₅₀ in nM by NetMHCpan 2.8)
RHOC (Rho-related GTP-binding protein RhoC)	FSIDSPDSL		X				C*03:04 (6 nM)
	IEVDGKQVEL		X			X	B*40:01 (29 nM)
RPA1 (replication protein A 70 kDa DNA-binding subunit)	DTEFPNFKY	X					A*01:01 (410 nM)
	EVFQANFRSF		X				A*26:01 (43 nM)
	GAIAAIMQK					X	A*11:01 (32 nM)
	KAYGASKTFGK			X		X	A*03:01 (32 nM), A*11:01 (35 nM)
	KVIDQQNGL				X		No binding predicted
	SGMGSTVSK					X	A*11:01 (31 nM)
	VSDFGGRSL	X	X	X	X	X	C*03:03 (438 nM), C*03:04 (438 nM), C*05:01 (89 nM)
RPL10A (replication protein A 70 kDa DNA-binding subunit)	FLASESLIKQI	X	X	X		X	A*02:01 (22 nM)
	NMVAKVDEV	X	X		X	X	A*02:01 (51 nM)
	RILGPGLNK			X			A*03:01 (21 nM)
	SLIKQIPRI		X	X		X	A*02:01 (36 nM)
	STIKFQMKK					X	A*11:01 (5 nM)
	TYEAVREV	X			X		A*02:01 (9 nM)
RPL19 (60S ribosomal protein L19)	HMYHSLYLK			X			A*03:01 (7 nM)
	ILMEHIHKL	X	X	X	X	X	A*02:01 (2 nM)
	ILMEHIHKLK			X			A*03:01 37 (nM)
	ILMEHIHKLKA		X				A*02:01 (19 nM)
	KLIKDGLIIRK			X			A*03:01 (49 nM)
	RILMEHIHKLK			X			A*03:01 (90 nM)
	RLASSVLRGK			X			A*03:01 (23 nM)
	WLDPNETNEI			X			A*02:01 (79 nM)
RPS2 (40S ribosomal protein S2)	KEWMPVTKL					X	B*40:01 (28 nM)
RPSA (40S ribosomal protein SA)	NTDSPLRY	X					A*01:01 (7 nM)
SART1 (U4/U6.U5 tri-snRNP-associated protein 1)	GLLETTVQKV	X	X				A*02:01 (9 nM)
	KSMNANTITK			X		X	A*03:01 (61 nM), A*11:01 (12 nM)
	RVKAPNKSL					X	No binding predicted
SFMBT1 (Scm-like with four MBT domains protein 1)	ESVMINGKY		X				A*26:01 (41 nM)
	SSDNYEHWLY	X					A*01:01 (5 nM)
SH3GLB2 (endophilin-B2)	EVAPPASGTR		X				No binding predicted
SOX10 (transcription factor SOX-10)	ETAGPQGPPHY		X		X		A*26:01 (13 nM)
	EVMSNMETF				X		A*26:01 (14 nM)
	FSYMGPSQRPL	X	X			X	C*03:03 (3 nM), C*03:04 (3 nM)
	GTAAIQAHY		X		X		No binding predicted

Table 2 continued

Gene name (protein name)	Peptide sequence	FM-82	FM-93/2	Mel-624	MeWo	SK-Mel-5	HLA Alleles (IC ₅₀ in nM by NetMHCpan 2.8)
STAT1 (signal transducer and activator of transcription 1-alpha/beta)	ISKPPGVAL		X		X		C*03:04 (228 nM), C*12:03 (152 nM)
	YGHSQGASGLY		X				No binding predicted
	YSDHQPSGPYY	X					A*01:01 (5 nM)
	YTDQPSTSQIAY	X					A*01:01 (4 nM)
	KLADQYPHL		X			X	A*02:01 (6 nM)
	KTLGKLWRL		X			X	A*02:01 (74 nM)
	ALLKDQQPGTFLL		X				A*02:01 (22 nM)
	DSFPMEIRQY		X			X	No binding predicted
	DSFPMEIRQYL		X				No binding predicted
	EVVHKIIEEL		X				A*26:01 (211 nM)
	KLQELNYNL	X	X	X			A*02:01 (6 nM)
	QQLDSKFLEQV	X	X				A*02:01 (176 nM)
	VLWDRTFSL	X	X	X		X	A*02:01 (3 nM)
	VMLDKQKEL	X					A*02:01 (201 nM)
SUGT1 (suppressor of G2 allele of SKP1 homolog)	ETFTREGQKL				X		No binding predicted
TOP2A (DNA topoisomerase 2-alpha)	KLAEAERVGLHK			X			A*03:01 (59 nM)
TP53 (cellular tumor antigen p53)	KLDETGNL	X			X	X	A*02:01 (68 nM)
	KLDETGNL			X			A*03:01 (153 nM)
	KLDETGNLKV		X				A*02:01 (29 nM)
	SLDKDIVAL				X		A*02:01 (59 nM)
	THFPDETEI				X		B*38:01 (175 nM)
	AEINNIKI		X			X	B*40:01 (58 nM)
	EVTVPGLY		X				A*26:01 (12 nM)
	FLYDDNQRV		X	X	X	X	A*02:01 (4 nM)
	KIFDEILVNA					X	A*02:01 (26 nM)
	LYNDFINK					X	A*11:01 (8 nM)
	GLAPPQHLIRV	X					A*02:01 (18 nM)
TTK (dual specificity protein kinase TTK)	ESFSGSLGHL		X				No binding predicted
TYMS (thymidylate synthase)	NSELSCQ	X					No binding predicted
	NSELSCQLY	X					A*01:01 (36 nM)
TYR (tyrosinase)	YMIAHITGL		X	X			A*02:01 (3 nM)
	EEYNHQSL		X			X	B*40:01 (42 nM)
	EVYPEANAPIGHN		X				No binding predicted
	HTISSDYVIPIGTY		X				A*26:01 (18 nM)
	KQLPEEKQPLL	X		X			No binding predicted
	LEEYNHQSL		X			X	B*40:01 (47 nM)
	LLMEKEDYHSL	X	X	X	X	X	A*02:01 (6 nM)
	MEKEDYHSL		X				B*40:01 (69 nM)
	NIFDLSAPEKDKFFA			X			A*02:01 (71 nM)
	SSDYVIPIGTY	X					A*01:01 (23 nM)
YVIPIGTYGQM		X				A*26:01 (37 nM)	
WNK2 (serine/threonine protein kinase WNK2)	HTRTPPIHR			X			No binding predicted

Fig. 3 Characterization of a neo-epitope identified from MeWo cells. **a** Sequence confirmation of identified neo-epitope with a synthetic peptide. (**a, upper panel**) Representative spectrum of endogenous EVLGSGMHHY peptide from the HLA peptidome analysis of MeWo cells. (**a, lower panel**) Representative spectrum of the synthetic EVLGSGMHHY peptide. **b** Binding prediction (IC_{50} in nM) for the wild type (WT) peptide and the neo-epitope to the alleles of the MeWo cell line as predicted by NetMHCpan 2.8 [27]

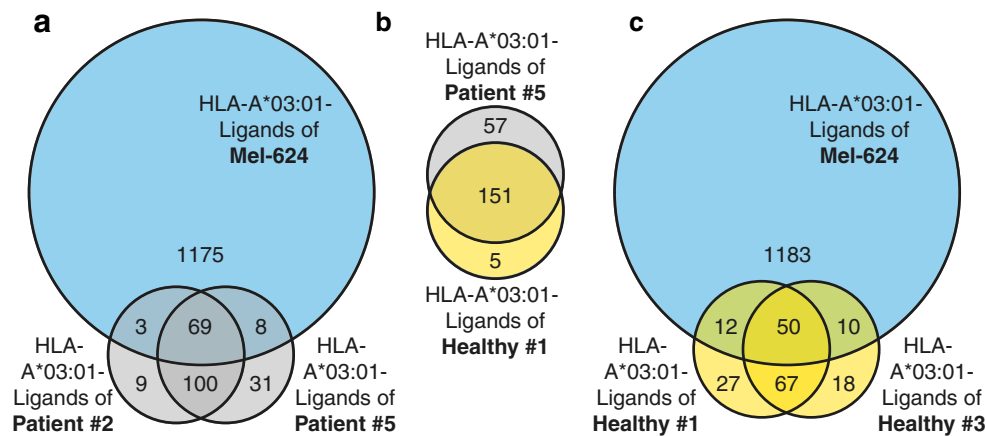
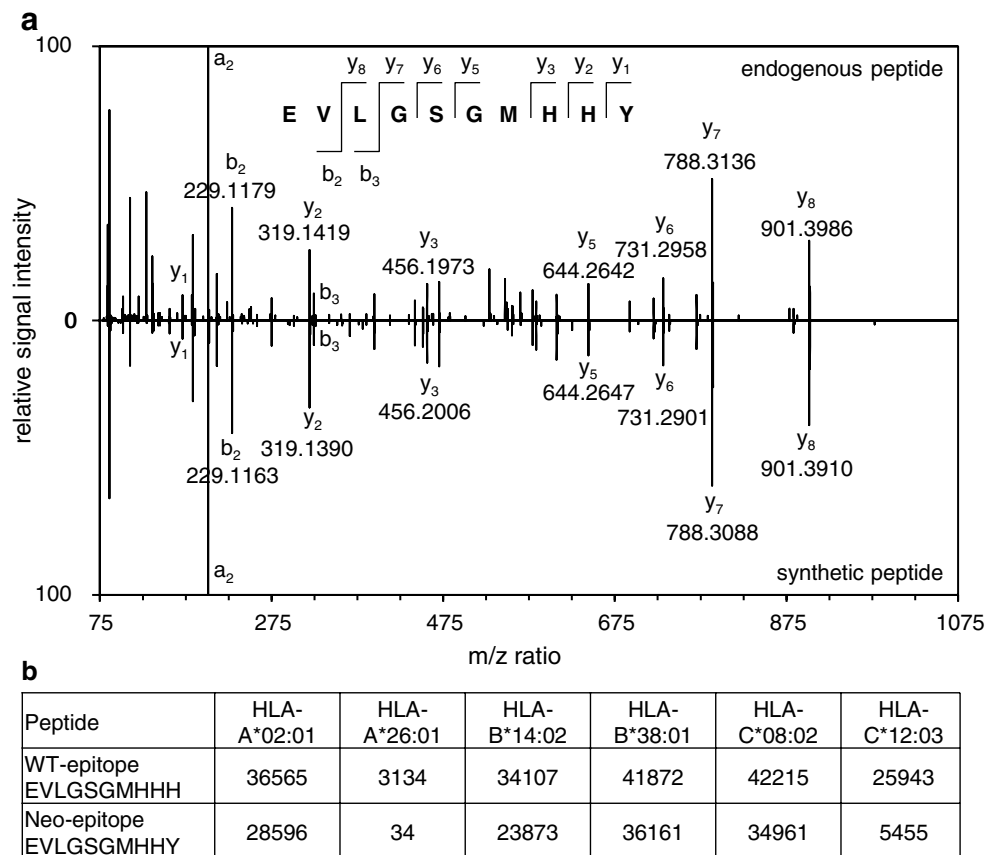


Fig. 4 Comparative analysis of HLA-A*03:01 peptides isolated from Mel-624 cells and sHLA-A*03:01 peptides isolated from the serum of melanoma patients or healthy donors. Peptides between 8 and 12 amino acids in length with a NetMHCpan 2.8 binding prediction of <math><500\text{ nM}</math> to HLA-A*03:01 isolated from Mel-624 cells were compared to peptides with the same characteristics isolated from the serum of melanoma patients or healthy donors [22]. **a** Venn diagram

depicting identifications of HLA-A*03:01 peptides isolated from Mel-624 and/or the sera of two melanoma patients. **b** Venn diagram depicting identifications of HLA-A*03:01 peptides from the serum of a healthy donor and a melanoma patient. **c** Venn diagram depicting identifications of HLA-A*03:01 peptides isolated from Mel-624 and/or the sera of two healthy donors

cell lines, which led to the identification of 10,399 unique peptides with high confidence. Comparison of these peptides with a list of tumor-associated antigens compiled by

Andersen et al. revealed that 262 peptides corresponded to antigens, which had previously been described as tumor associated [10]. A peptide containing a mutation was

identified when interrogating the MS data for a catalog of somatic mutations. Furthermore, we observed that sHLA peptides isolated from the serum of melanoma patients and from an HLA-matched melanoma cell line display a substantial overlap.

While the experimental atlas included over 10,000 HLA class I-bound peptides, identified with high confidence, it is of note that the majority of HLA ligands originated from HLA-A. A general underrepresentation of HLA-C has been described before, on the level of both surface expressed protein [34] and identified ligands in mass spectrometric experiments [21, 22]. HLA-B ligands, however, have occasionally been found at similar or even higher numbers applying the same procedure as in this paper (see MHC-specific motifs of MAVER-1 and THP-1 cells [22]), indicating that the low number of HLA-B ligands originates from a low expression of HLA-B in the investigated melanoma cell lines.

The HLA peptide atlas, further, included 262 peptides corresponding to previously described tumor-associated antigens and 31 epitopes, which had previously been reported to be potentially immunogenic (Table 2). Interestingly, some well-known tumor-associated antigens were identified with up to 19 different peptides from a single cell line (i.e., PMEL in cell line FM-82). Other antigens (i.e., NY-ESO-1, (E)AAGIGILTV of MLANA/MART-1, or YM(N/D)GTMSQV of tyrosinase) were not present in our analysis. The advent of multiplex tetramer technology, which allows the high-throughput screening of T cell specificities [13], crucially relies on the availability of the exact sequence of presented peptides. Currently, selection of epitopes is often guided by binding predictions. In this study, we demonstrate that the majority of identified peptides were predicted to bind to cognate HLA molecules. However, a substantial portion of the eluted peptides (9–21 %, depending on the cell line) were not predicted to be HLA binders, which may reflect limitations of the prediction algorithms as demonstrated by the ability of four peptides identified from both MeWo and FM-93/2 cells to form stable complexes with the HLA alpha chain and β_2 -microglobulin (Supplementary Figs. 6 & 7). We therefore believe that it is essential to complement the selection of epitopes with sequences identified by mass spectrometric characterization of HLA peptidomes. The peptidome described in this work could in principle readily be used to complement recently described studies in melanoma patients (e.g., those describing the evolution of T cells in patients, after treatment with ipilimumab, using multiplex tetramer technology [16]).

Mutational information for MeWo and SK-Mel-5 cell lines, which was available from public repositories [30], facilitated the confident identification of one neo-epitope presented by MeWo cells. The identification was confirmed

by spectral comparison with the corresponding synthetic peptide. The observation that only a small fraction of possible neo-epitopes are presented to a degree which allows identification by mass spectrometry is in line with a recent report analyzing patient-derived melanoma cell lines [19]. In that study, the authors did not observe any neo-epitope, in spite of having confidently characterized between 2701 and 6397 HLA-bound peptides from three cell lines, whose mutations had previously been identified by whole exome sequencing. This finding is intriguing, especially since melanoma represents one of the most mutated human cancers [11, 12], and since neo-epitopes have recently been proposed as a major factor in the activity of immunotherapy [35, 36]. Mass spectrometry-based methodologies may allow to experimentally measure the relative frequency of different types of peptide display (e.g., those derived from housekeeping proteins, over-expressed antigens, tumor testis antigens and mutant peptides) on HLA class I molecules. This information is likely to be crucially important for a molecular characterization of T cell-based anti-tumor immunity.

A comparison of melanoma patient-derived sHLA peptidomes and data derived from cell lines indicated the presence of shared peptides. Since tumor cell lines do not fully resemble the nature of corresponding solid tumors, as they may change their HLA peptidome under cell culture conditions, it is not surprising to observe differences between the ligandomes. Nevertheless, observation of a significant overlap between cell lines and unrelated patients sera suggests that, at least in part, the sHLA peptidome may represent peptides derived from the tumor. In the future, analysis of matched primary tumor and sHLA peptidomes will be important to understand if this methodology might indeed be useful to probe for tumor-derived antigens. If this is the case, sHLA peptidomics could represent a promising noninvasive strategy for the monitoring of patients during immunotherapeutic intervention.

Immunotherapy is gaining importance for the treatment of cancer and, in particular, for the treatment of melanoma. It is very well established that tumor-associated antigen-specific T cells are found in melanoma patients [10]. Recent advances in multiplex tetramer technology have provided unambiguous evidence that treatment with anti-CTLA-4 therapy broadens the profile and alters the frequency of the melanoma-reactive CD8+ T cell response [16]. In addition, survival of melanoma patients with tumor-associated antigen-specific T cells against NY-ESO-1 and Melan-A was found to be significantly prolonged [37]. These studies reinforce the concept that immunotherapy can be used to boost the patient's immune system against melanoma malignancies. A detailed characterization of the cancer peptidome, as presented in this article, should facilitate mechanistic studies on the quality and quantity of tumor

rejection antigens in melanoma and other cancer types. A molecular characterization of T cell specificities in patients (e.g., before and after pharmacological intervention) may shed light on clinically important issues, such as why only certain individuals respond to immunotherapy. In addition, peptides bound to HLA class I may be considered for vaccination strategies [38] and for the development of T cell receptor-engineered adoptive cell therapies [6].

Acknowledgments We thank Camilla Bacci (Philogen SpA) for providing W6/32 antibody.

Funding This work was supported financially by ETH Zürich, the Swiss National Science Foundation, the European Union's Seventh Framework Programme (FP7/2007-2013) under Grant Agreement Nos. 305309 (PRIAT) and 305608 (EUrenOmics), the Bonveda Foundation and the European Research Council (ERC advanced Grant "ZAUBERKUGEL").

Compliance with ethical standards

Conflict of interest Dario Neri is co-founder of Philogen, shareholder and member of the board. Tim Fugmann and Danilo Ritz are employees of Philochem AG. The authors declare no additional conflict of interest.

Research involving human participants and/or animals This article does not contain any studies with human participants or animals performed by any of the authors.

References

- Pardoll DM (2012) The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* 12(4):252–264. doi:10.1038/nrc3239
- Sharma P, Allison JP (2015) The future of immune checkpoint therapy. *Science* 348(6230):56–61. doi:10.1126/science.aaa8172
- Probst P, Neri D (2014) Immunocytokines for cancer therapy. *Forum Immunopathol Dis Therap* 5(1):83–99. doi:10.1615/ForumImmunDisTher.2015014013
- Kontermann RE, Brinkmann U (2015) Bispecific antibodies. *Drug Discov Today* 20(7):838–847. doi:10.1016/j.drudis.2015.02.008
- Rosenberg SA, Restifo NP (2015) Adoptive cell transfer as personalized immunotherapy for human cancer. *Science* 348(6230):62–68. doi:10.1126/science.aaa4967
- June CH, Riddell SR, Schumacher TN (2015) Adoptive cellular therapy: a race to the finish line. *Sci Transl Med* 7(280):280ps7. doi:10.1126/scitranslmed.aaa3643
- Melero I, Gaudernack G, Gerritsen W, Huber C, Parmiani G, Scholl S, Thatcher N, Wagstaff J, Zielinski C, Faulkner I, Mellstedt H (2014) Therapeutic vaccines for cancer: an overview of clinical trials. *Nat Rev Clin Oncol* 11(9):509–524. doi:10.1038/nrclinonc.2014.111
- Robbins PF, Lu YC, El-Gamil M, Li YF, Gross C, Gartner J, Lin JC, Teer JK, Cliften P, Tycksen E, Samuels Y, Rosenberg SA (2013) Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. *Nat Med* 19(6):747–752. doi:10.1038/nm.3161
- Scheibenbogen C, Sun Y, Keilholz U, Song M, Stevanovic S, Asemussen AM, Nagorsen D, Thiel E, Rammensee HG, Schendoldorf D (2002) Identification of known and novel immunogenic T-cell epitopes from tumor antigens recognized by peripheral blood T cells from patients responding to IL-2-based treatment. *Int J Cancer* 98(3):409–414
- Andersen RS, Thru CA, Junker N, Lyngaa R, Donia M, Ellebaek E, Svane IM, Schumacher TN, Thor Straten P, Hadrup SR (2012) Dissection of T-cell antigen specificity in human melanoma. *Cancer Res* 72(7):1642–1650. doi:10.1158/0008-5472.CAN-11-2614
- Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, Bignell GR, Bolli N, Borg A, Borresen-Dale AL, Boyault S, Burkhardt B, Butler AP, Caldas C, Davies HR, Desmedt C, Eils R, Eyfjord JE, Foekens JA, Greaves M, Hosoda F, Hutter B, Illic T, Imbeaud S, Imielinski M, Jager N, Jones DT, Jones D, Knappskog S, Kool M, Lakhani SR, Lopez-Otin C, Martin S, Munshi NC, Nakamura H, Northcott PA, Pajic M, Papaemmanuil E, Paradiso A, Pearson JV, Puente XS, Raine K, Ramakrishna M, Richardson AL, Richter J, Rosenstiel P, Schlessner M, Schumacher TN, Span PN, Teague JW, Totoki Y, Tutt AN, Valdes-Mas R, van Buuren MM, van't Veer L, Vincent-Salomon A, Waddell N, Yates LR, PedBrain I, Zucman-Rossi J, Futreal PA, McDermott U, Lichter P, Meyerson M, Grimmond SM, Siebert R, Campo E, Shibata T, Pfister SM, Campbell PJ, Stratton MR, Australian Pancreatic Cancer Genome I, Consortium IBC, Consortium IM-S (2013) Signatures of mutational processes in human cancer. *Nature* 500(7463):415–421. doi:10.1038/nature12477
- Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA Jr, Kinzler KW (2013) Cancer genome landscapes. *Science* 339(6127):1546–1558. doi:10.1126/science.1235122
- Hadrup SR, Schumacher TN (2010) MHC-based detection of antigen-specific CD8+ T cell responses. *Cancer Immunol Immunother* 59(9):1425–1433. doi:10.1007/s00262-010-0824-2
- Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, McMichael AJ, Davis MM (1996) Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274(5284):94–96
- Tran E, Ahmadzadeh M, Lu YC, Gros A, Turcotte S, Robbins PF, Gartner JJ, Zheng Z, Li YF, Ray S, Wunderlich JR, Somerville RP, Rosenberg SA (2015) Immunogenicity of somatic mutations in human gastrointestinal cancers. *Science* 350(6266):1387–1390. doi:10.1126/science.aad1253
- Kvistborg P, Philips D, Kelderman S, Hageman L, Ottensmeier C, Joseph-Pietras D, Welters MJ, van der Burg S, Kapiteijn E, Michielin O, Romano E, Linnemann C, Speiser D, Blank C, Haanen JB, Schumacher TN (2014) Anti-CTLA-4 therapy broadens the melanoma-reactive CD8+ T cell response. *Sci Transl Med* 6(254):254ra128. doi:10.1126/scitranslmed.3008918
- Cox AL, Skipper J, Chen Y, Henderson RA, Darrow TL, Shabanowitz J, Engelhard VH, Hunt DF, Slingluff CL Jr (1994) Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines. *Science* 264(5159):716–719
- Jarmalavicius S, Welte Y, Walden P (2012) High immunogenicity of the human leukocyte antigen peptidomes of melanoma tumor cells. *J Biol Chem* 287(40):33401–33411. doi:10.1074/jbc.M112.358903
- Pritchard AL, Hastie ML, Neller M, Gorman JJ, Schmidt CW, Hayward NK (2015) Exploration of peptides bound to MHC class I molecules in melanoma. *Pigment Cell Melanoma Res* 28(3):281–294. doi:10.1111/pcmr.12357
- Hassan C, Kester MG, de Ru AH, Hombrink P, Drijfhout JW, Nijveen H, Leunissen JA, Heemskerk MH, Falkenburg JH, van Veelen PA (2013) The human leukocyte antigen-presented

- ligandome of B lymphocytes. *Mol Cell Proteom* 12(7):1829–1843. doi:[10.1074/mcp.M112.024810](https://doi.org/10.1074/mcp.M112.024810)
21. Bassani-Sternberg M, Pletscher-Frankild S, Jensen LJ, Mann M (2015) Mass spectrometry of human leukocyte antigen class I peptidomes reveals strong effects of protein abundance and turnover on antigen presentation. *Mol Cell Proteom* 14(3):658–673. doi:[10.1074/mcp.M114.042812](https://doi.org/10.1074/mcp.M114.042812)
 22. Ritz D, Gloger A, Weide B, Garbe C, Neri D, Fugmann T (2016) High-sensitivity HLA class I peptidome analysis enables a precise definition of peptide motifs and the identification of peptides from cell lines and patients' sera. *Proteomics* 16(10):1570–1580. doi:[10.1002/pmic.201500445](https://doi.org/10.1002/pmic.201500445)
 23. Pawelec G, Marsh SG (2006) ESTDAB: a collection of immunologically characterised melanoma cell lines and searchable databank. *Cancer Immunol Immunother* 55(6):623–627. doi:[10.1007/s00262-005-0117-3](https://doi.org/10.1007/s00262-005-0117-3)
 24. Chou J, Voong LN, Mortales CL, Towler AM, Pollack SM, Chen X, Yee C, Robbins PF, Warren EH (2012) Epigenetic modulation to enable antigen-specific T-cell therapy of colorectal cancer. *J Immunother* 35(2):131–141. doi:[10.1097/CJI.0b013e31824300c7](https://doi.org/10.1097/CJI.0b013e31824300c7)
 25. Toebes M, Rodenko B, Ovaa H, Schumacher TN (2009) Generation of peptide MHC class I monomers and multimers through ligand exchange. *Curr Protoc Immunol*. doi:[10.1002/0471142735.im1816s87](https://doi.org/10.1002/0471142735.im1816s87)
 26. Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol* 26(12):1367–1372. doi:[10.1038/nbt.1511](https://doi.org/10.1038/nbt.1511)
 27. Nielsen M, Lundegaard C, Blicher T, Lamberth K, Harndahl M, Justesen S, Roder G, Peters B, Sette A, Lund O, Buus S (2007) NetMHCpan, a method for quantitative predictions of peptide binding to any HLA-A and -B locus protein of known sequence. *PLoS One* 2(8):e796. doi:[10.1371/journal.pone.0000796](https://doi.org/10.1371/journal.pone.0000796)
 28. Boegel S, Lower M, Bukur T, Sahin U, Castle JC (2014) A catalog of HLA type, HLA expression, and neo-epitope candidates in human cancer cell lines. *Oncoimmunology* 3(8):e954893. doi:[10.4161/21624011.2014.954893](https://doi.org/10.4161/21624011.2014.954893)
 29. Thomsen MC, Nielsen M (2012) Seq2Logo: a method for construction and visualization of amino acid binding motifs and sequence profiles including sequence weighting, pseudo counts and two-sided representation of amino acid enrichment and depletion. *Nucleic Acids Res* 40(Web Server issue):W281–W287. doi:[10.1093/nar/gks469](https://doi.org/10.1093/nar/gks469)
 30. Forbes SA, Bindal N, Bamford S, Cole C, Kok CY, Beare D, Jia M, Shepherd R, Leung K, Menzies A, Teague JW, Campbell PJ, Stratton MR, Futreal PA (2011) COSMIC: mining complete cancer genomes in the Catalogue of Somatic Mutations in Cancer. *Nucleic Acids Res* 39(Database issue):D945–D950. doi:[10.1093/nar/gkq929](https://doi.org/10.1093/nar/gkq929)
 31. Barnstable CJ, Bodmer WF, Brown G, Galfre G, Milstein C, Williams AF, Ziegler A (1978) Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens—new tools for genetic analysis. *Cell* 14(1):9–20
 32. Rapin N, Hoof I, Lund O, Nielsen M (2008) MHC motif viewer. *Immunogenetics* 60(12):759–765. doi:[10.1007/s00251-008-0330-2](https://doi.org/10.1007/s00251-008-0330-2)
 33. Rasmussen M, Harndahl M, Stryhn A, Boucherma R, Nielsen LL, Lemonnier FA, Nielsen M, Buus S (2014) Uncovering the peptide-binding specificities of HLA-C: a general strategy to determine the specificity of any MHC class I molecule. *J Immunol* 193(10):4790–4802. doi:[10.4049/jimmunol.1401689](https://doi.org/10.4049/jimmunol.1401689)
 34. Apps R, Meng Z, Del Prete GQ, Lifson JD, Zhou M, Carrington M (2015) Relative expression levels of the HLA class-I proteins in normal and HIV-infected cells. *J Immunol* 194(8):3594–3600. doi:[10.4049/jimmunol.1403234](https://doi.org/10.4049/jimmunol.1403234)
 35. Snyder A, Makarov V, Merghoub T, Yuan J, Zaretsky JM, Desrichard A, Walsh LA, Postow MA, Wong P, Ho TS, Hollmann TJ, Bruggeman C, Kannan K, Li Y, Elipenahli C, Liu C, Harbison CT, Wang L, Ribas A, Wolchok JD, Chan TA (2014) Genetic basis for clinical response to CTLA-4 blockade in melanoma. *N Engl J Med* 371(23):2189–2199. doi:[10.1056/NEJMoa1406498](https://doi.org/10.1056/NEJMoa1406498)
 36. Schumacher TN, Schreiber RD (2015) Neoantigens in cancer immunotherapy. *Science* 348(6230):69–74. doi:[10.1126/science.aaa4971](https://doi.org/10.1126/science.aaa4971)
 37. Weide B, Zelba H, Derhovanessian E, Pflugfelder A, Eigentler TK, Di Giacomo AM, Maio M, Aarntzen EH, de Vries IJ, Sucker A, Schadendorf D, Buttner P, Garbe C, Pawelec G (2012) Functional T cells targeting NY-ESO-1 or Melan-A are predictive for survival of patients with distant melanoma metastasis. *J Clin Oncol* 30(15):1835–1841. doi:[10.1200/JCO.2011.40.2271](https://doi.org/10.1200/JCO.2011.40.2271)
 38. Haen SP, Rammensee HG (2013) The repertoire of human tumor-associated epitopes—identification and selection of antigens and their application in clinical trials. *Curr Opin Immunol* 25(2):277–283. doi:[10.1016/j.coi.2013.03.007](https://doi.org/10.1016/j.coi.2013.03.007)