HLA-A24 ligandome analysis of colon and lung cancer cells identifies a novel cancer-testis antigen and a neoantigen that elicits specific and strong CTL responses

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ABSTRACT

This study focused on HLA-A24 and comprehensively analyzed the ligandome of colon and lung cancer cells without the use of MHC-binding in silico prediction algorithms. Affinity purification using the antibody specific to HLA-A24 followed by LC-MS/MS sequencing was used to detect peptides, which harbored the known characteristics of HLA-A24 peptides in terms of length and anchor motifs. Ligandome analysis demonstrated the natural presentation of two different types of novel tumor-associated antigens. The ligandome contained a peptide derived from SUV39H2, a gene found to be expressed in a variety of cancers but not in normal tissues (except for the testis). The SUV39H2 peptide is immunogenic and elicits cytotoxic CD8+ T-cell (CTL) responses against cancer cells and is thus a novel cancer-testis antigen. Moreover, we found that microsatellite instability (MSI)-colon cancer cells displayed a neoepitope with an amino-acid substitution, while microsatellite stable (MSS)-colon and lung cancer cells displayed its counterpart peptide without the substitution. Structure modeling of peptide-HLA-A24 complexes predicted that the mutated residue at P8 was accessible to T-cell receptors. The neoepitope readily elicited CTL responses, which discriminated it from its wild-type counterpart, and the CTLs exhibited considerably high cytotoxicity against MSS-colon cancer cells carrying the responsible gene mutation. The specific and strong CTL lysis observed in this study fosters our understanding of immune surveillance against neoantigens.

Introduction

The remarkable clinical effects of immune checkpoint blockades observed in a wide variety of cancer patients have promised to prolong survival and demonstrated that T cells of cancer patients can potentially respond to and eliminate cancer cells. These promising results fueled strong interest in CD8+ T-cell-based immunotherapy and called for identification of the antigens that are presented by the HLA class I of cancer cells, which are targeted by cytotoxic CD8+ T cells (CTLs), and are responsible for the eradication of cancer cells in clinical settings. Although an increasing number of tumor associated antigens (TAAs) continue to be identified using reverse immunology techniques, recent proteomics approaches provide a comprehensive way to analyze whole HLA ligandomes containing various types of TAAs. It is now widely accepted that direct peptide isolation from live cells using antibodies directed against HLA molecules followed by LC–MS/MS sequencing is an ideal strategy to map and screen natural T-cell epitopes presented by cancer cells. Many HLA ligandome studies have isolated mixtures of peptides using an anti-pan-HLA class I W6/32 antibody and subsequently assigned the peptides to their corresponding HLA types using MHC-binding algorithms. The use of the W6/32 antibody allows the isolation of peptides presented by multiple classes of HLA class I and often increases the total number of detected peptides per a sample, which are highly advantageous for maximizing the amount of information that can be derived from limited clinical samples. One drawback of this strategy is however that allocation prediction is neither completely accurate nor consistently able to assign every ligand to its corresponding HLA type because the eluted peptides are comprised of a complex mixture of HLA-A, -B, and -C ligands. As a result, peptides are left unassigned, and additional experiments are required for verification. In contrast, the use of a single HLA type-specific antibody for peptide isolation no longer relies on prediction algorithms and does not require allocation; thus, the outcome would directly represent the ligandome presented by a
particular HLA type. In this study, we specifically focused on HLA-A24, the most frequent MHC class I type found in East Asian populations, and established the HLA-A24-specific system of ligandome analysis, which allows selective detection of natural HLA-A24 ligands, even from cancer cells expressing HLA-class I types other than HLA-A24. We mapped the HLA-A24 ligandomes of three colon and two lung human cancer cell lines. We found that the peptide encoded in the SUV39H2 gene was displayed by HLA-A24 of colon cancer cells, and SUV39H2 serves as a cancer-testis antigen.

Meanwhile, the effects of immune checkpoint inhibitors in the clinical setting have been shown across different histological types of malignancies, such as melanoma, non-small cell lung cancer (NSCSC), bladder cancer, Hodgkin’s lymphoma, and renal cell carcinoma.9–13 In particular, melanoma, NSCSC, and bladder cancer are known to frequently harbor genetic mutations; therefore, neoepitopes that arise from mutations are considered as major targets of T-cell responses leading to antitumor effects.14,15 In addition, PD-1 blockade exclusively showed durable clinical effects on microsatellite-instability (MSI) colon cancer patients, while leaving microsatellite-stable (MSS) colon cancers refractory.16 The screening of neoepitopes in charge of CTL responses against tumors has been challenging; however, two seminal works revealed the neoepitope repertoire of mouse model tumors using an elegant method combining mass spectrometry and exome sequencing.17,18 The recent study also reported the successful detection of neoepitopes in the HLA class-I ligandome of native melanoma tissues.19 In this study, we demonstrated that the HLA-A24 ligandome of an MSI-colon cancer line, HCT15, contained a neoepitope, AKF9, which arose from an AP2SI gene mutation. The induced CTLs discriminated AKF9 from its wild-type counterpart ANF9 and exhibited considerably high cytotoxicity against HCT15 cells that carries the responsible gene mutation. Considering that identification of neoepitopes based solely on tumor exome or transcriptome analysis followed by HLA binding prediction requires a laborious effort of screening many candidates as well as an effort of demonstrating the natural presentation of predicted epitopes, our findings also indicate an advantage of HLA ligandome analysis in neoantigen discovery for therapeutic use.

Materials and methods

Cells and antibodies

K562, K562-A24 (K562 stably expressing HLA-A’24:02), and T2-A24 (T2 stably expressing HLA-A’24:02) were maintained in RPMI 1640 medium. Human colon carcinoma cell lines SW480, HCT-116, HCT-15, and Colo320, the lung adenocarcinoma line LHK2, and lung squamous-cell carcinoma Sq-1 were grown in DMEM medium. Because the original HCT-15 line lacked β 2-microglobulin (β2m) expression, we used HCT-15 cells stably expressing β2m (HCT-15/β2m) throughout the study. Both RPMI 1640 and DMEM were supplemented with 10% FBS and 1% antibiotic. Monoclonal antibodies (mAb) W6/32 (AbD Serotec) and C7709A2 (gift from Dr. P.G. Coulie) hybridomas were grown in Hybridoma-SFM (Gibco) supplemented with 1% penicillin/streptomycin. The supernatant was collected via centrifugation and further condensed by reverse osmosis in cellulose tubes (Viscase Companies, Inc.) against polyethylene glycol (PEG-20,000; Wako Chemicals). The prepared mAbs were supplemented with 0.03% sodium azide and complete protease inhibitors (Roche Diagnostics) and stored at 4°C until use.

Isolation of HLA-A24-bound peptides

We used an established procedure to isolate detergent-solubilized peptide-HLA-A24 complexes.20,21 Briefly, protein Absorbeose beads were covalently linked with the mAb using dimethyl pimelimidate (DMP) dihydrochloride (Sigma). Cancer-cell lysates (∼1 × 10⁹ cells each) were clarified through a series of centrifugations and incubated with the beads covalently linked to the C7709A2 mAb. The beads were then sequentially washed, and peptides bound to HLA-A24 were eluted with 10% acetic acid, followed by 3 kDa cut-off ultrafiltration (Amicon Ultra-15; Millipore). The resulting flow-through was condensed and submitted for the LC–MS/MS analysis.

HPLC, mass spectrometry, and MS/MS data analysis

Peptides were separated in a HiQ sil C18W-3 column using a DiNa system (KYA Technologies) coupled to a MALDI spotting device. Elution solvent A was 0.1% trifluoroacetic acid (TFA), and solvent B was 0.1% TFA in 70% acetonitrile (ACN). The gradient was 5–50% for solvent B over 80 min at a flow rate of 300 nL/min. Peptide fractions (150 nL) were collected every 30 sec and overlaid with 700 nL of 4 mg/mL α-cyano-4-hydroxycinnamic acid in 70% ACN/0.1% TFA and 80 μg/mL diammonium hydrogen citrate. Mass spectrometry analysis was performed on a 4800 Plus MALDI-TOF/TOF Analyzer (AB Sciex) with 4000 Series Explorer software (ver. 3.5.3). The human International Protein Index (IPI) ver. 3.71 (86,739 sequences), ver. 3.87 (91,444 sequences), and the human UniProt (88993 sequences as of July 2014) or XMAn databases were used.22 The MS/MS spectrum of each peptide was manually checked for correspondence between the fragments (y- and b-ions) and the amino acid sequences. False identifications were removed from the results. Peptide sequence alignments were performed using WebLogo.23

Peptides, binding assay, and flow cytometry

Synthetic peptides of following purity were purchased from PH Japan (RF8, 94.3%; ANF9, 90.7%; AKF9, 89.5%). Peptides in a range of indicated concentrations were pulsed onto T2-A24 cells, incubated for 3 h at 27°C, and incubated for 2.5 h at 37°C. Cells were incubated with C7709A2 and a secondary FITC-conjugated antibody and analyzed using flow cytometry. The peptide sequences are as follows: KDM2A, KYANFVTF; ZNF254, EQPYKWEKF; IPMK, EYNNNFHVL; EEF2, RYFDPANGKF; SSR4, LYADVGGKQF; VASP, IYHNPTANSF; BMS1, VYYDLGGSHVF; BTBD3, KYSFDGSSNFTF; GKI2, GIYSPFYINTSK. The difference in mean fluorescence intensity (∆MFI) indicates the difference in MFI values between samples with and without the primary antibody.
RT-PCR

A panel of human cDNA was extracted from Clontech. Total RNA from human cancer cell lines was extracted using the RNeasy Mini Kit (Qiagen), and cDNA was synthesized using oligo(dT) primers and SuperScript III reverse transcriptase (Invitrogen). The following primers were used to detect AKF9/ANF9

\[ \text{G3PDH} \text{ gene expression:} \ \\
\text{5'}-\text{GACGTGG-3'} \text{ and} \ 5'-\text{CAATGCTATTCGGGGAAGACG-3'} \]

\[ \text{3'}-\text{CAGGAGAATCACTTGAACC-5'} \text{ and} \ 5'-\text{TCCACCACCCCTGTTGCTGTA-3'} \]

DNA sequencing

Genomic DNA was extracted from SW480, Colo320, HCT15/b2m, LHK2, and Sq-1 cells using DNeasy kit (Qiagen) and amplified by PCR using primers flanking the AKF9/ANF9 sequence (5'-TGCATCTGAGCAGGGTAG-3' and 5'-GCTGAGGCAGGAGAATCCT-3'). Residual primers were removed from the PCR mixtures using ExoSAP-IT (Affymetrix), and the amplified DNA was sequenced using an ABI PRISM3130 instrument according to the manufacturer's instructions. The following primers were used for sequencing AKF9/ANF9:

\[ 5'-\text{GACGACATGGATGGAGTCAA-3'} \text{ and} \ 5'-\text{CAGGAGATCTTTGAACC-3'} \]

TAA-specific CTL induction and single CTL cloning

Peripheral blood mononuclear cells (PBMCs) from HLA-A24:02-positive healthy donors were cultured in AIM-V medium (Life Technologies) containing 10% human serum and 50 U/mL human IL-2 for 2 weeks and repeatedly stimulated with 20 μM of synthetic peptide (RF8, RYGNVSHF or AKF9, AYLEA1HKF). A single cell double-positive to anti-CD8+ mAb (Beckman Coulter) and HLA-A24 tetramers (bound to RF8 or AKF9 synthetic peptide, MBL, Japan) was isolated using FACS Aria II (BD) and expanded in AIM-V medium containing 100 U/mL IL-2, 1 μg/mL PHA, and X-ray-irradiated PBMCs from healthy volunteers.

ELISPOT IFNγ assay

Tetramer-positive CTLs (100 μL at 2 × 10^6 cells/mL) were added to ELISPOT plates coated with anti-Human IFNγ antibodies (BD Biosciences). T2-A24 (100 μL at 1 × 10^6 cells/mL) or the indi-
cated cancer lines were added to the corresponding wells. T2-A24 was pre-incubated at room temperature for 2 h with 50 μM RF8, AKF9, or an irrelevant HIV peptide. After incubation in a 5% CO2-incubator at 37°C for 24 h, the wells were incubated with a biotinylated anti-human IFNγ antibody for 2 h at room temperature, followed by the ELISPOT Streptavidin-HRP antibody for 1 h. Spots were visualized using an ELISPOT AEC Substrate Set according to the manufacturer’s instructions (BD Biosciences).

LDH cytotoxicity assay

The amount of lactate dehydrogenase (LDH) released from lysed target cells pre-incubated with CTL clones was measured using LDH cytotoxicity detection kit according to the manufacturer’s instructions (TaKaRa, Japan). The percentage of LDH release (cytotoxicity) was calculated as follows:

\[ \text{%release} = \frac{\text{LDH release}}{\text{maximal LDH release}} \times 100 \]

Results

Efficient peptide isolation with an HLA-A24 specific-antibody

The HLA-A24 ligandome studies that have been performed so far used a pan-HLA class I W6/32 antibody for affinity purification, followed by peptide-HLA allocation using MHC-binding algorithms.6,26,27 To establish the HLA-A24-specific pepti-
dulation system without relying on algorithmic prediction, we tested the efficacy of a monoclonal C7709A2 antibody to isolate peptide-HLA-A24 complexes (pHLA-A24) from extracts of living cells.28 First, C7709A2 and W6/32 antibodies were able to detect HLA-A24 on the surface of K562 cells stably expressing the HLA-A*24:02 gene (K562-A24) (Fig. 1A). Peptides bound to HLA class I molecules were eluted as described previously and sequenced using LC–MS/MS (Fig. 1B). Elution using C7709A2 and W6/32 did not capture peptides from K562 cells but yielded 27 and 15 peptides from K562-A24 cells, respectively, out of which approximately 35% (11/31) peptides were shared (Fig 1C). The majority of the sequenced peptides were 9-mers with conserved Tyr at P2 and Phe or Leu at P9, consistent with the known length and motifs of HLA-A24 bound peptides (Fig. 1D and E).29 We thus concluded that C7709A2 had the same or higher affinity for capturing peptide-HLA-A24 complexes compared with W6/32.

The HLA-A24 ligandome of human colon and lung cancer cells

Colon and lung cancers are the leading cause of cancer-related deaths and together account for about 31% and 26% of male and female cancer patients in Japan, respectively. For HLA-A24 ligandome analysis, we prepared four colon and three lung cancer cell lines, which include HLA-A24-positive colon (Colo 320, SW480, and HCT15/β2m), HLA-A24-negative colon (HCT116), HLA-A24-positive lung (Sq-1 and LHK2), and HLA-A24-negative lung (1—87) cancer lines, all of which...
expressed HLA class I molecules other than HLA-A24 (Fig. 2A). HLA-A24 ligands were eluted from each cell with C7709A2 and sequenced according to the workflow described in Fig. 2B and methods as described. In summary, we detected >100 peptides from each of the HLA-A24-positive cancer lines but eluted only 6 and 11 peptides from 2 HLA-A24-negative lines (Fig. 2C). Given that HCT116 and 1–87 are HLA-A24-negative but express HLA-A*02:01 and A*02:07, respectively;
the clear gap in the number of eluted peptides between HLA-A24-positive and negative lines validates the specificity of the C7709A2 antibody against the HLA-A24 molecule. Thus, the few peptides obtained from two A24-negative lines were regarded as contaminants, and identical sequences were subtracted from a series of HLA-A24-positive peptides, leaving 187 and 304 non-redundant sequences representing the peptides that were naturally processed and presented by HLA-A24 in the three colon and two lung HLA-A24-positive cancer lines, respectively (Table S1).

Most of the detected peptides were 9-mers and harbored Tyr (Y) at P2 and Phe (F), Leu (L), or Iso (I) at P9 (Fig. 3A and B), consistent with the known length and dominant anchor motifs of previously reported HLA-A24 binding peptides.29 The list of isolated peptides also included a small number of unusually short and long peptides containing HLA-A24 binding motifs at P2 and P9 (Fig. 3C). The positions of two binding anchors were fixed at P2 and P9 regardless of peptide lengths, implying that the peptide-HLA-A24 complexes exist in a stretching or bulging conformation in vivo. We randomly sampled and synthesized representative peptides with anchor motifs (three 9-mers, three 10-mers, and two 11-mers). Results of the T2-A24 binding assay demonstrated that in addition to canonical 9-mers, the 10-mer and 11-mer peptides also successfully increased the surface levels of HLA-A24 in a dose-dependent manner, thereby validating the capability of the peptides detected in our ligandome analysis to bind to HLA-A24 molecules on the surface of live cells (Fig. 3D).

**SUV39H2 is a novel HLA-A24 restricted cancer-testis antigen**

The 403 HLA-A24 ligands naturally displayed by colon and lung cancer cells could represent ideal targets for CTL-based cancer immunotherapy. We screened source-gene expression of each ligand in normal and cancer cells and ultimately found...
that the suppressor of variegation 3–9 homolog 2 (SUV39H2) gene was not expressed in any of the normal tissues (except for the testis) but was expressed in a wide variety of malignant cell lines, including colon, lung, breast, oral, pancreas, and prostate cancers, as well as melanoma and leukemia (Fig 4A). Cancer-specific protein expression of SUV39H2 is also registered in the Human Protein Atlas; this protein is not detected in a panel of normal tissues (except for in the testis) but is displayed in breast, stomach, liver, kidney, prostate, and pancreatic cancer tissues when subjected to weak to moderate staining (Fig. 4B and C).30

Fig. 5A shows the MS/MS spectrum of an 8-mer peptide (RYGNVSHF, termed RF8) detected in the SW480 lysate in this study. The peptide is encoded in the three known SUV39H2 isoforms (Uniprot #Q9H5I1). The ΔMFI values of T2-A24 cells pulsed with a synthetic RF8 peptide and stained with C7709A2 mAb increased in a peptide dose dependent manner (Fig. 5B). In addition, a tetramer composed of the RF8 peptide and HLA-A24 was successfully prepared, empirically demonstrating that the RF8 is capable of binding to HLA-A24. After stimulating HLA-A*24:02-positive healthy donor PBMCs with the synthetic RF8 peptide, we obtained an RF8/HLA-A24 tetramer-positive T-cell population and ultimately cloned a specific-CD8+ T cell via single-cell sorting (Fig. 5C). This clone, termed clone 11, specifically responded to T2-A24 cells pulsed with RF8 and produced IFNγ in the ELISPOT assay (Fig. 5D). Clone 11 also responded to SW480 from which the RF8 was originally eluted, demonstrating its nature as a naturally presented peptide. Finally, results from an LDH release assay demonstrated that clone 11 was cytotoxic to T2-A24 cells pulsed with RF8, as well as SW480, Sq-1, and LHK2 cells, all of which are HLA-A24-positive and express the SUV39H2 gene (Fig. 5E). Thus, SUV39H2 is a novel cancer-testis antigen and serves as an attractive candidate for CTL-based vaccination therapy.

**HLA-A24 ligandome analysis identifies a neoepitope presented by MSI-colon cancer cells**

In this study, we analyzed the HLA-A24 ligandomes of MSI-colon cancer (HCT15/β2m) and microsatellite stable (MSS)-colon cancer (SW480 and Colo320) cell lines in addition to lung cancer lines.31 To further search our MS/MS spectra obtained from the cancer cell lines for neoepitopes, we used a reference database called XMAn, which includes mutated-peptide sequences translated from gene-level mutations registered in public resources (COSMIC, IARC P53, OMIM, and UniprotKB).22 Spectral matching with XMAn allowed us to identify a 9-mer peptide (AYLEAIHKF, AKF9) with a single amino acid substitution from Asn (N) to Lys (K) at P8. The MS/MS spectrum was shifted from wild type as a result of the substitution (Fig. 6A). The wild-type peptide (AYLEAIHNF, ANF9) is encoded in the AP2S1 gene, a component of the adaptor protein complex 2 (AP-2) that is widely and abundantly expressed in normal organs (http://www.gtexportal.org/home/gene/AP2S1).32 The mutated AKF9 was detected in MSI-colon cancer HCT15/β2m cells, whereas ANF9 was detected from MSS-colon cancer SW480 cells, as well as LHK2 and Sq-1 cells. The c.258C>G mutation in the AP2S1 gene is responsible for the missense amino-acid substitution (N86K) and is registered in
COSMIC as a somatic mutation found in HCT15 (http://cancer.sanger.ac.uk/cosmic/mutation/overview?id=1681155). Our sequence data also demonstrated that HCT15/b2m cells carried a c.258C>G mutation in one of the AP2S1 alleles but the other cell lines did not (Fig. 6B). Thus, the somatic mutation in the AP2S1 gene in HCT15/b2m yielded a mutated HLA-A24 ligand, and our system identified the neoepitope and its wild-type counterpart peptide from MSI- and MSS-colon cancer lines, respectively. AKF9 was the only neoepitope detected based on the analysis of three colon and two lung cancer cell lines using the XMAn reference database (Table 1). A CTL clone recognizing the neoepitope AKF9 is highly cytotoxic against HCT15 cells

T-cell receptors (TCRs) recognize peptide–MHC complexes, and conformational changes in the complexes yield immunogenicity. In particular, TCRs mainly recognize the middle of peptides (P3–P7) because both N- and C-terminal ends of peptides are often buried inside MHC pockets. To predict the conformational change caused by the single amino-acid substitution (N86K) found in this study, we used the reported crystal structure of a 9-mer peptide-HLA-A24 complex and modeled ANF9 and AKF9-HLA-A24 complexes using a Rosetta-based algorithm. Structure modeling predicted that the mutated lysine residue at P8 was oriented toward the solvent interface and was therefore likely accessible to TCRs (Fig. 7A and B). The result suggests the potential immunogenicity of the neoepitope, AKF9.

We next asked if the amino-acid substitution influenced the ability of AKF9 and ANF9 to bind to HLA-A24. HLA-A24 levels were equally observed to increase with the amount of AKF9 and ANF9 peptides pulsed into T2-A24 cells in a dose-dependent manner. This suggests that N86K at P8 did not influence binding affinity and that both peptides could fit into the HLA-A24 binding groove to form stable peptide-HLA-A24 complexes on the cell surface (Fig. 8A). We further stimulated PBMCs from a healthy donor with either the synthetic AKF9 or ANF9 peptides and measured the strength of T-cell induction. In contrast to ANF9, stimulation with AKF9 yielded a CD8+ cell fraction producing IFNγ, from which AKF9-specific T-cell frequency accounted approximately 0.21% of stimulated CD8+ T cells (Fig. 8B). Single-cell sorting of the specific T cells using an AKF9/HLA-A24 tetramer resulted in successful expansion of 16 T-cell clones, all of which presented AKF9/A24 tetramer- and CD8-double positivity at higher than 99% of the cell population (Fig. 8C). Three selected clones (clones 2, 7, and 11) responded equally to T2-A24 cells pulsed with AKF9 and produced IFNγ, whereas no responses were observed in T2-A24 alone or T2-A24 pulsed with an HIV control peptide (Fig. 8D). Signals from clones 2 and 11, if any, suggested very weak cross-reaction to T2-A24 pulsed with
wild-type ANF9. However, the numbers of ANF9 spots were significantly less than AKF9 in all three clones. Consistent with ELISPOT data, clone 2 recognized and specifically lysed T2-A24 cells pulsed with AKF9 in a dose-dependent manner (Fig. 8E). Moreover, clone 2 also lysed HCT15/β2m, an MSI-colon cancer, without lysing other cancer cell lines, including two MSS-colon cancer lines. Thus, AKF9-specific CTLs are not only inducible but also highly specific and cytotoxic against cancer cells carrying the responsible gene mutation.

Discussion

To our knowledge, the present study is the first to perform a large-scale and comprehensive study of the HLA-A24 ligandome presented by human cancer cells without relying on in silico prediction or allocation algorithms. So far, more than 80 HLA-A24 ligands that have been reported were detected mainly using conventional reverse immunology techniques.35,36 Our ligandome data contained only one of the reported HLA-A24 ligands and is encoded in the TP53 tumor suppressor gene.37 This inconsistency between our results and previously reported HLA-A24 ligands can be explained by differences in gene

Table 1. Summary of mutated and non-mutated HLA-A24 peptides

|       | Colo320 | SW480 | HCT15 | Sq-1 | LHK2 |
|-------|---------|-------|-------|------|------|
| Status| MSS     | MSS   | MSI   |      |      |
| Total peptides | 104   | 104   | 122   | 259  | 127  |
| AKF9 (mutated)   | 0     | 0     | 1     | 0    | 0    |
| ANF9 (wild type) | 1     | 0     | 0     | 1    | 1    |

Abbreviations: AKF9, AYLEAIHKF; ANF9, AYLEAIHNF; MSI, microsatellite instability; MSS, microsatellite stable; total peptide, total numbers of peptides isolated from that cell line.

Figure 6. Identification of a neoepitope and its wild-type counterpart naturally presented by HLA-A24 of cancer cells. (A) The MS/MS spectra of AYLEAIHKF (AKF9) at 1091.61 m/z and AYLEAIHNF (ANF9) at 1077.50 m/z. Corresponding b- and y-ions are connected between two spectra with dashed lines. (B) DNA sequence Trace of the AP2S1 gene in SW480, HCT15/β2m, Colo320, LHK2, and Sq-1 cells. Underlined nucleotides code amino acids noted below.
expression patterns across various source organs because analysis performed in this study was limited to colon and lung cancers. Also, ligandome analysis uses affinity purification with mAb followed by MS/MS sequencing. Thus, the methodology used in this study may have excluded ligands that were processed and presented in colon and lung cancer lines but were below detectable levels. Notably, we found that 47% of the ligands detected in this study were unlikely to be predicted as HLA-A24 ligands from whole source-protein sequences using NetMHC4.0 because they were not included in the top-10 ligand candidates (Fig. S1). These results demonstrate the difficulty of predicting HLA-A24 ligands at the gene or protein level and highlight the advantage of HLA ligandome analysis in comprehensive screening of T-cell epitopes for use in cancer.

Figure 7. Modeling of ANF9- and AKF9-HLA-A24 complexes. The complexes were modeled using the existing crystal structure from the Protein Data Bank as starting models, and the conformation of bound peptides was optimized using the FlexPepDock web server (http://flexpepdock.furmanlab.cs.huji.ac.il). ANF9- and AKF9-HLA-A24 models are shown in (A) and (B), respectively. HLA-A24 represented as a ribbon diagram is shown in gray. The main and side chains of modeled peptides represented as stick models are shown in green, with the mutated (Lys) or wild type (Asn) residue highlighted in yellow. Surface models were generated using PyMOL (Schrödinger, LLC) and shown in light gray.

Figure 8. An AKF9-specific CTL clone lyses the HCT15/b2m MSS-colon cancer cell line. (A) Peptide binding assay with T2-A24 cells pulsed with indicated synthetic peptides. (B) Flow cytometry of healthy donor PBMCs stimulated with AKF9 peptides. Cells were stained with AKF9- and HIV-HLA-A24 tetramers along with CD8 mAb. Right panel shows CD8 population. The number indicates percentage of AKF9 tetramer single-positive cells in total CD8 cells. (C) Flow cytometry of a representative of AKF9-specific CTL clones (clone 2) stained with AKF9-HLA-A24 tetramer along with CD8 mAb. Panels are shown in the same manner as above. (D) IFN- ELISPOT assay of three AKF9-specific CTL clones. Target cells are T2-A24 cells pulsed with the indicated synthetic peptides. A bar chart below indicates the summarized number of IFN- positive spots per a well. (E) LDH-release cytotoxicity assay of CTL clone 2. T2-A24 pulsed with indicated synthetic peptides (left panel) or indicated cancer cell lines (right panel) were used as targets releasing LDH. The x-axis represents effector/target ratio (E/T); the y-axis represents cytotoxicity (%) and indicates the ratio of LDH released by clone 2 to LDH released by NP40-induced cell death.
immunotherapy. Of note, we found that LC–MS/MS successfully detected and sequenced 1 fmol of synthetic peptides as a lower limit included in samples, which suggests the sensitivity of our system.

Here, we identified two different types of novel HLA-A24 TAAs. The first one is a cancer-testis antigen, SUV39H2. We demonstrated the natural presentation of the RF8 epitope of SUV39H2 by HLA-A24 of the colon cancer SW480 cell line, as well as SUV39H2 expression across a wide variety of cancers. Moreover, the epitope elicited CTL responses specific to cancer cells expressing SUV39H2 along with HLA-A24. SUV39H2 was originally reported as a histone H3 methyltransferase exhibiting testis-specific expression.38 SUV39H2 is required for controlling telomere length through the trimethylation of histone 3 at lysine 9 (H3-K9), and its overexpression in human lung cancer tissues regulating radio/chemosensitivity by histone H2AX methylation has been reported.39–41 Additionally, we recently isolated a cell population from SW480 using Hoechst dye staining.42 The cell population showed significant sphere formation in vitro and vigorous tumor growth in vivo in mouse xenograft models, demonstrating a major characteristic of cancer stem cells. The SUV39H2 gene was strongly expressed in the cancer-stem cell-like cell population, as well as differentiated cells (Fig. S2). Taken together, the results highlight the potential of SUV39H2 as a novel target for CTL-based immunotherapy.

Moreover, we demonstrated that the HLA-A24 ligandome of a human MSI-colon cancer line, HCT15, contained a neoepitope, AKF9, which was caused by an AP2S1 gene mutation. In contrast to HCT15, an MSS-colon cancer (SW480), as well as lung cancer lines (LHK2 and Sq-1), presented its wild-type counterpart ANF9. The responsible c.258 C>G substitution in the AP2S1 gene was detected in a single allele of HCT15/β2m but not in the other cancer lines, validating that the substitution is a somatic mutation specific to HCT15. This unique pair of non-mutated/mutated epitopes did not demonstrate a difference in terms of HLA-A24-binding capacity; however, AKF9-stimulation successfully induced CTL responses from healthy-donor PBMCs. Surprisingly, the cytotoxicity of AKF-9-specific CTLs was notably high. Cytotoxicity (%) against HCT15/β2m was between 30% and 87% at E/T ratios ranging 1–9, corresponding to 62% and 87% cytotoxicity against T2-A24 cells pulsed with 20 μM AKF9 synthetic peptide (Fig. 8E). The cytotoxicity (%) of an RF8-specific CTL clone against SW480 ranged from 8% to 18%, corresponding to 16% and 27% cytotoxicity against 20 μM RF8 synthetic peptide, even though the HLA-A24 surface level of SW480 was higher than that of HCT15/β2m (Figs. 5E and 2A). Why did the neoepitope elicit a strong CTL response? First, the AKF9-specific CTL clones were able to distinguish between AKF9 and ANF9, suggesting that the single amino-acid substitution at P8 interacted and modified TCR recognition without reducing the binding affinity to HLA-A24. Although TCR interaction with P8 residue of peptides is not common, the Rosetta-based structure modeling predicted the mutated residue at P8 was oriented toward solvent interface of the HLA-A24 binding groove (Fig. 7).25,43 Thus, the substitution generated a foreign HLA-A24 ligand that is not found in normal cells and subsequently activated CTLs that escaped from negative selection or tolerance. Second, we emphasize that the natural presentation of the wild-type epitope was observed in multiple cancer lines harboring no mutation. Considering the nature of mass spectrometry detection, which is biased toward most abundant peptides, ANF9 was an abundantly presented self-peptide. The positive correlation between the stability of peptide–MHC complexes and CTL immunogenicity has been known in non-mutated antigens, and the principle can be applied to neoantigens.44,45 Of note, the IC50 values were calculated by NetMHC4.0 and both AKF9 and ANF9 exhibited high-binding affinity prediction (IC50 = 23.82 and 22.00, respectively).

In summary, we identified two different types of novel TAAs through HLA-A24 ligandome analysis of three colon and two lung cancer cell lines. SUV39H2 is a cancer-testis antigen whose source gene and protein are expressed across a variety of cancer tissues, thereby serving as an attractive target for future CTL-based immunotherapy. Meanwhile, the AKF9 neoepitope is cell-line specific and is not appropriate for universal therapeutic use. Nevertheless, the considerably high cytotoxicity of AKF9-specific CTLs against the AKF9-presenting cancer cells caused by a single amino-acid substitution illustrates the potential of CTL-based immunotherapies that target neoantigens, and fosters our understanding of clinical effects of immune-checkpoint blockade observed in MSI-colon cancer patients.16 One remaining questions is a relation between clinical effects and HLA haplotypes. Heterogeneity of neoantigen expression in a tumor mass is an issue of importance, too.46 These matters should be further investigated using primary tissues from colon-cancer patients.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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