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Cytokeratin 8-MHC class I interactions: A potential novel immune escape phenotype by a lymph node metastatic carcinoma cell line

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ABSTRACT

Defective human leukocyte antigen (HLA) class I expression in malignant cells facilitates their escape from destruction by $CD8^+$ cytotoxic T lymphocytes. In this study, a post-translational mechanism of HLA class I abnormality that does not involve defects in the HLA subunits and antigen processing machinery components was identified and characterized. The marked HLA class I downregulation phenotype of a metastatic carcinoma cell line can be readily reversed by trypsin, suggesting a masking effect by serine protease-sensitive HLA class I-interacting factors. Co-immunoprecipitation, combined with LC–tandem mass spectrometry and immunoblotting identified these factors as cytokeratin (CK) 8 and its heterodimeric partners CK18 and CK19. Ectopic CK8/18 or CK8/19 expression in HEK293 cells resulted in surface CK8 expression with an HLA class I downregulation phenotype, while redirecting CK8/18 and CK8/19 to the endoplasmic reticulum (ER) had no such effect. This observation and the failure to constrain CK8/18 and CK8/19 membrane trafficking by an ER-Golgi transport inhibitor suggested an ER-independent route for CK8 access to HLA class I molecules. Monoclonal antibody mapping revealed a potential CK8 blockade of HLA class I-CD8 and -TCR contacts. These findings, along with the emerging role of cell surface CK8 in cancer metastasis, may imply a dual strategy for tumor cell survival in the host.

1. Introduction

In animal model systems, cytotoxic T lymphocytes (CTLs) are potent immune effectors that can eliminate malignant cells through recognizing their tumor antigen-derived peptides presented by MHC class I molecules [1]. However, T cell-based immunotherapy has not been effective as anticipated in controlling cancer growth in patients with malignant disease [2]. This finding reflects, at least in part, the frequent abnormalities found in the HLA class I-peptide complex expression and/or function by malignant cells [3]. Therefore, the need to characterize the mechanisms behind these abnormal HLA class I phenotypes has been emphasized, with an expectation that this information can assist in patient stratification and optimizing the rational design of more effective immunotherapeutic strategies.

Multiple molecular mechanisms have been found to underlie HLA class I–peptide complex abnormalities in malignant cells characterized to date [3,4]. HLA class I heavy chain and β_2-microglobulin (β_2m) subunits are not expressed because of irreversible mutations, such as loss-of-heterozygosity (LOH), deletion, and missense/frame-shift mutations [3]. The assembly and transport of HLA class I–β_2m-peptide complexes are impaired because of deregulation of endoplasmic reticulum (ER)-resident antigen processing machinery (APM) components, which is often correctable by IFN-γ [5]. Furthermore, epigenetic repression of the genes encoding HLA class I allospecificities [4] and peptide transporter TAP1/2 [5] also contributes to the generation of an abnormal HLA class I phenotype. Nevertheless, little information is available concerning the post-translational mechanisms that lead to HLA class I defects in malignant cells thus far, which have yet to be explored.

In the present study, we characterized the mechanism underlying the differential HLA class I expression by the two subpopulations of a carcinoma cell line derived from a head and neck lymph node metastasis [6]. We uncovered a previously undescribed interaction of HLA class I molecules with tumor-derived cytokeratins mainly at the plasma membrane. This represents a post-translational modulation of HLA class I molecules in malignant cells, which does not directly involve HLA class I subunits and APM.

2. Materials and methods

2.1. Cell culture

UP-LN1 is a carcinoma cell line derived from a subclavicular lymph node metastasis of an unknown primary site [6]. This cell

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line contains an anchorage-dependent (AD) and an anchorage-independent (AID) population when cultured *in vitro*, and the latter population shows greater immunogenicity in SCID mice [6]. UP-LN1 AD and AID cells, human non-transformed bronchial epithelial cell line BEAS-2B (ATCC, Manassas, VA, USA), and embryonic kidney cell line HEK293 (ATCC) were cultured in RPMI-1640 (Hyclon, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL, Grand Island, NY, USA), 100 U/ml penicillin and 100 U/ml streptomycin (Gibco-BRL). Single cell suspensions were obtained by dissociation with 0.25% typsin/2 mM EDTA (Invitrogen, Carlsbad, California, USA) for 5 min at 37 °C or with EDTA (2 mM) alone for 10 min at 37 °C. Accutase was purchased from Millipore (Billerica, MA, USA). Information on other cell lines is described in Supplementary methods.

2.2. Antibodies and pharmacological inhibitors

The monoclonal antibody (mAb) W6/32, which recognizes β_2m-associated HLA-A, -B, -C, -E, and -G heavy chains [7]; HLA-A2, -A24, -A28-specific mAb CR11-351 [8]; mAb HCA-2, which recognizes β_2m-free HLA-A heavy chains [9], mAb HC-10, which recognizes β_2m-free HLA-A3, -A10, -A28, -A29, -A30, -A31, -A32, -A33, and -B (excluding -B5702, -B5804, and -B73) heavy chains [10]; TP25.99, which recognizes β_2m-free and β_2m-associated HLA class I heavy chains [11], β_2m-specific mAb L368 and NAMB-1 [12]; TAP1-specific mAb NOB-1 [13], TAP2-specfic mAb NOB-2 [14], calnexin-specific mAb TO-5 [15]; calreticulin-specific mAb TO-11 [15]; ERp57-specific mAb TO-2 [15], and tapasin-specific mAb TO-3 [15] were developed and characterized as described. Anti-Actin and anti-HA were purchased from Millipore Inc. Anti-CK8 (LP3K) and anti-CD99 were purchased from eBioscience (San Diego, CA, USA). R-phycoerythrin (R-PE)-conjugated, horseradish peroxidase (HRP)-conjugated, and DyLight™ 549-conjugated goat anti-mouse IgG Fcγ-specific F(ab')$_2$ fragments were purchased from Jackson Immunoresearch Laboratories (West Grove, PA, USA). Brefeldin A was purchased from Sigma–Aldrich (St. Louis, MO, USA). Cells were treated with brefeldin A (5, 10 μg/ml) for 4 h at 37 °C and then analyzed by cytofluorometry.

2.3. Plasmid constructs

The full-length cytokeratin (CK) 8, CK18 and CK19 coding-region cDNA was amplified from AID cells by RT-PCR with primers containing *Xba*I and *Eco*RI sites at the ends and then cloned into pcDNA3.1(−) vector (Invitrogen). Alternatively, CK cDNA was re-amplified with primers designed for including IL-2 signal sequence (IL2-ss) with an *Xba*I site, and then cloned into pcDNA3.1(−). HA-β_2m cDNA was cloned into pVAC1-mcs (Invivogen, San Diego, CA, USA) at *Bam*HI sites. HLA-A2 cDNA was subcloned into pEGFP-N1 (Clontech, Mountain View, CA, USA) at *Nhe*I and *Hin*dIII sites. All primers were synthesized by Mission Biotech, Taipei, Taiwan (Supplementary Table 1).

2.4. Flow cytometry

Cells were harvested with trypsin/EDTA or EDTA alone, washed by PBS with 1% bovine serum albumin (BSA), and analyzed as described [16]. Results are expressed as fold increase in MFI (Fold MFI).

2.5. Western blot analysis

Cell lysates were denatured with sample buffer (Bio-Rad, Hercules, CA, USA) by boiling for 10 min and proteins (50 μg) were fractionated on 10% SDS–PAGE and analyzed as described [16].

2.6. Immunoprecipitation and SDS–PAGE

Trypsinized cells or 2 mM EDTA-dissociated cells were lysed and analyzed as described [16] by 10% or 12% SDS–PAGE and visualized by silver staining.

2.7. Liquid chromatography–tandem mass spectrometry (LC–MS/MS)

Desired bands were cut for in-gel digestion with trypsin [17] and then each digested peptide mixture (5 mL) for LC/MS/MS analyses was introduced into the mass spectrometer via high-performance liquid chromatography using a 1200 series binary HPLC pump (Agilent, Palo Alto, CA, USA) and a FAMOSTM well-plate microautosampler (LC Packings). For each analysis, sample was loaded into a 2 cm–75 mm i.d. trap column packed in-house with C18 resin (Magic C18AQ, 5 mm, 200 Å; Michrom, Bioresources, CA, USA). The trap column was connected to an analytical column (11 cm–75 mm i.d.) and the columns were rigidly packed in-house with C18 resin (Magic C18AQ, 5 mm, 100 Å). Mobile phase A consisted of 0.1% formic acid and mobile phase B consisted of 0.1% formic acid in 100% ACN. The flow rate was ~250 nL/min under an in-house split flow system. Each reversed-phase step began with 5% ACN for 10 min, a gradient of 5–40% ACN for 75 min, 40–85% ACN for 5 min, 85% ACN for 10 min, and then re-equilibrated with 5% ACN for 20 min. Mass spectrometric analyses were performed on a LTQ XL linear ion trap mass spectrometer (ThermoFisher Scientific, San Jose, CA, USA). A full-mass scan was performed between m/z 350 and 2000, followed by MS/MS scans of the five highest-intensity precursor ions at 35% relative collision energy. Dynamic exclusion was enabled with a repeat count of 1, exclusion duration of 3 min, and a repeat duration of 30 s.

2.8. Protein identification

The acquired MS/MS spectra were searched against SwissProt protein database 56.8 (release of 10 February 2009) using the Mascot Daemon version 2.2.2. Peptide mass tolerance and fragment tolerance were set at 2 and 0.5 Da, respectively (Supplementary Tables 2–4). The initial search was set to allow for up to two missed tryptic cleavages. A decoy database was performed to determine false positive rates. The false positive rates were controlled below 5% by setting p Value at 0.025. All of the identified modification sites were further validated manually.

2.9. Immunofluorescence staining/confocal microscopy

Adherent cells were seeded about 90% confluency in the 24-well culture plate with glass slides. AID cells were resuspended in 1% BSA/PBS and cytospun (2×10^5 cells/ml) (Shandon) onto slides. Samples were fixed with ice-cold acetone for 5 min at RT, blocked with 1% BSA/PBST for 30 min and then incubated with primary antibodies in 1% BSA/PBST overnight at 4 °C before being incubated with secondary antibodies in 1% BSA/PBST for 1 h at RT in the dark. After being added with DAPI for 3 min, coverslips or slides were mounted, sealed and analyzed by a confocal microscope (LSM 780, Carl Zeiss, AG, Germany).

2.10. Statistical analysis

Data are expressed as mean ± S.D. Differences in means between control and experimental groups were evaluated with a Student's t test. p Value less than 0.05 ($p < 0.05$) was considered statistically significant.

3. Results

3.1. Reversal of the marked HLA class I downregulation phenotype of AID cells by the serine protease trypsin

Cytofluorometric analysis with HLA class I-specific mAb W6/32 of anchorage-independent (AID) UP-LN1 cells revealed their marked HLA class I downregulation, as compared to the autologous anchorage-dependent (AD) counterpart (Fig. 1A). AID cells were readily dissociated by EDTA (2 mM), while AD cells were detached by EDTA (2 mM) in the presence of serine protease trypsin (0.25%) (trypsin/EDTA), as this agent is known to have no enzymatic effect on HLA class I molecules [18]. Western blot analysis detected comparable levels of HLA class I heavy chain, β_2m and APM component expression in both AID and AD cell populations, implying a post-translational mechanism leading to their differential HLA class I expression (Fig. 1B). However, to our surprise, when AID cells were similarly dissociated with trypsin/EDTA, their HLA class I downregulation was markedly reversed (from Fold MFI 10.4 to 231.1, 22.2-fold) (Fig. 1C). There was also a 4.4-fold increase in the HLA class I level on AD cells dissociated with trypsin/EDTA versus EDTA alone (Fig. 1C). This trypsin-effect was not observed with BEAS-2B, a non-transformed epithelial cell line, suggesting a cell type-specific phenomenon (Fig. 1C), and appeared to be dose-dependent (Fig. 1D). Moreover, Accutase, a collagenase-rich dissociation agent, has no such effect (data not shown), suggesting some substrate specificity. These findings indicate that HLA class I molecules are equally well expressed on both AID and AD cells, but are bound by a putative trypsin-sensitive factor that interferes with the binding by mAb W6/32. To test whether the observed trypsin-effect represents a general phenomenon, we analyzed a panel of cultured tumor cell lines, including breast carcinoma MCF-7 and T47D, hepatocellular carcinoma Hep3B, HepG2, and HepG2.2.15, erythroleukemia HEL and K562, and acute monocytic leukemia THP-1, along with another non-transformed HEK293 cells. All of the tumor cell lines displayed increased mAb W6/32 reactivity following trypsin/EDTA dissociation to various extents (1.4–1.9-fold) (Fig. 1E).

3.2. Identification of cytokeratin (CK) 8, CK18, and CK19 as the factors that reduce mAb W6/32 reactivity to HLA class I molecules on AID cells

To identify this trypsin-sensitive factor that may have a negative impact on the function of HLA class I molecules, we selected AID, the most affected, and BEAS-2B, the unaffected control, as our cell models. These cells were dissociated with EDTA (2 mM) with or without trypsin (0.25%) and then their lysates were subjected to immunoprecipitation with mAb W6/32, followed by 12% SDS–PAGE and silver staining. The results revealed a band with a mass ~60 kDa only in the AID group without trypsin (Fig. 2A), suggesting the interaction of this factor with HLA class I taking place mainly at or near the cell surface. This protein, named P60, was not detected in the BEAS-2B group. Two prominent additional bands, P45 and P50, were further identified by 10% SDS–PAGE in the AID group without trypsin (Fig. 2B). P45, P50 and P60 were also present in AD cell immunoprecipitates but at lower levels (Fig. 2B), consistent with their lower degree of trypsin-mediated reversal of mAb W6/32 reactivity (Fig. 1E). There was also a

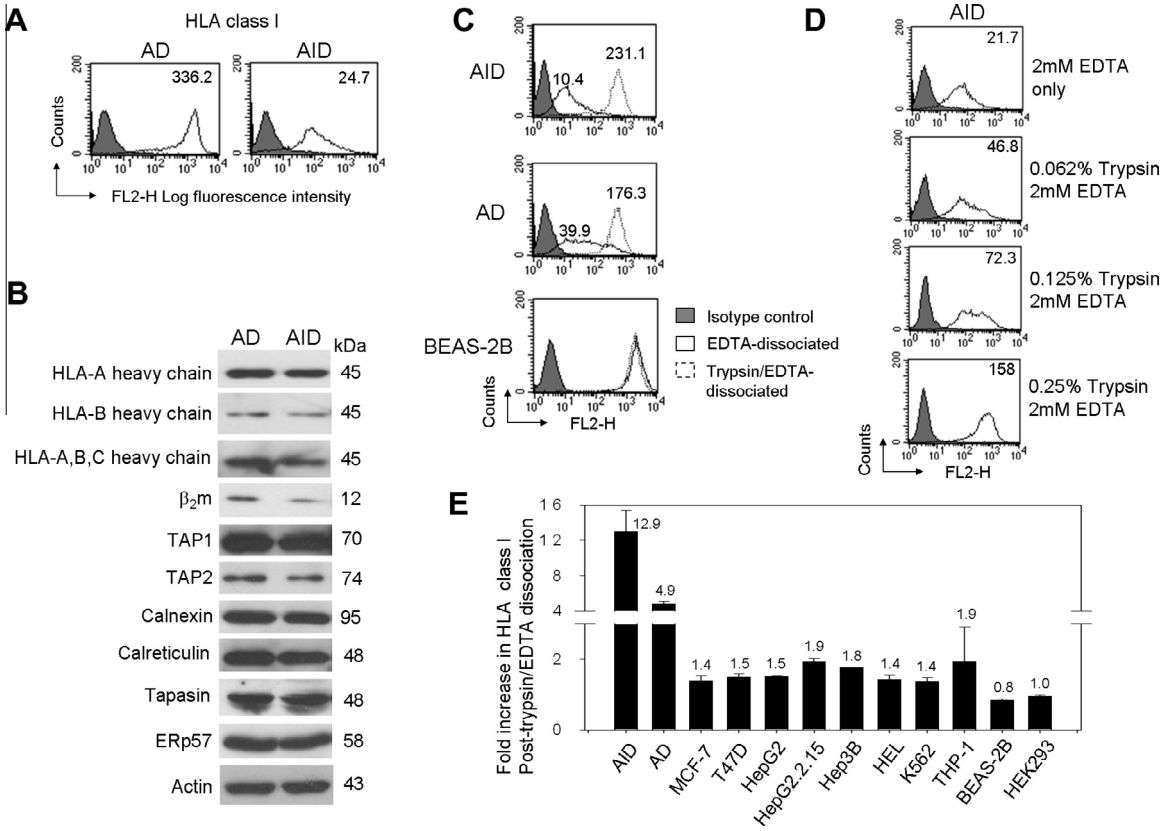

Fig. 1. Reversal of the marked HLA class I downregulation phenotype of AID cells by trypsin. AD and AID cells were analyzed by cytofluorometry with mAb W6/32 (A) and Western blotting with HLA class I subunit- and APM component-specific mAb. Cytofluorometric analysis with mAb W6/32 of (C) EDTA or trypsin/EDTA-dissociated AID, AD, and BEASE-2B cells, (D) trypsin/EDTA (at various concentrations)-dissociated AID cells or (E) EDTA or trypsin/EDTA-dissociated various indicated tumor cell lines. The figures above histograms are Fold MFI (A, C, D), representative of three experiments. EDTA (2 mM), trypsin (0.25%). Mean (Fold MFI) ± S.D. of three experiments (E).

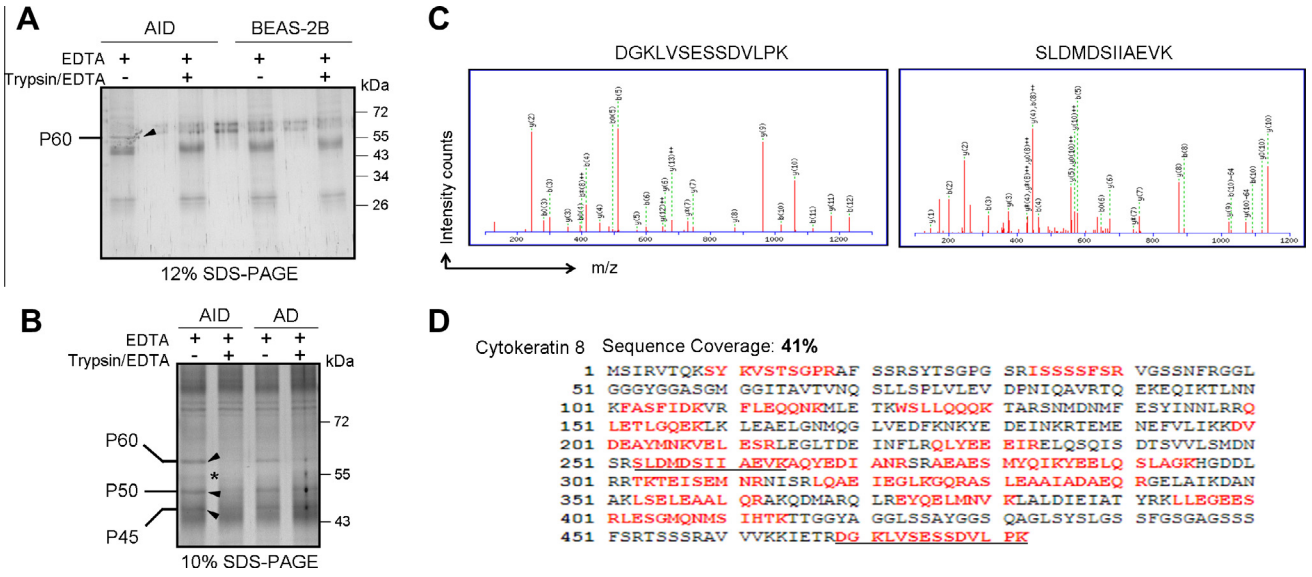

Fig. 2. Identification of CK8 by mass spectrometry. Lysates from EDTA or trypsin/EDTA-dissociated AID and BEAS-2B cells (A) or AID and AD cells (B) were subjected to immunoprecipitation with mAb W6/32 and silver staining. Arrow heads denote the bands of interest. The P60 band was cut and subjected to LC-MS/MS analysis. Star indicates a contaminant confirmed by mass spectrometry. (C) Mass spectrum of two representative CK8 peptides. (D) Matched tryptic peptides (red letters) and peptides in (C) (underlined). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

faint band revealed around 55 kDa (Fig. 2B, lane 1, star sign). LC–MS/MS analysis identified P45, P50 and P60 as cytokeratin (CK) 19 (34% coverage), CK18 (35% coverage), and CK8 (41% coverage), respectively (Fig. 2C and D, and Supplementary Tables 2–4), while indicated a contaminant from the 55 kDa signal (3% coverage) (data not shown). Cytokeratins are known to form pairs comprising a type I cytokeratin, such as CK18 and CK19, and a type II cytokeratin, such as CK8, and further form tetramers before assembling into intermediate filaments in cells [19]. Therefore, it is likely that CK8/18 and CK8/19 heterodimers form a complex with HLA class I molecules at the surface of AID cells.

The identity of CK8 was further confirmed by co-immunoprecipitation and Western blotting (Fig. 3A, left panel). Intriguingly, in the input control of the AID group with trypsin (0.25%), a ~10-kDa-truncated CK8 fragment was abundantly present (Fig. 3A, lane 2, right panel), raising the possibility that such cleavage was sufficient to impair CK8-HLA class I interactions. Whether this implies a general limited cleavage of cell surface CK8 remains to be determined. CK8-HLA class I interactions at the plasma membrane were further supported by the co-localization of transiently expressed HLA-A2-GFP with endogenous CK8 mainly at the periphery of AID cells (Fig. 3B, upper panels). Nevertheless, weak co-localization signals were also noted in BEAS-2B cells which expressed CK8 in the cytoplasm (Fig. 3B, lower panels). This result may reflect the insufficient sensitivity of co-immunoprecipitation to detect CK8-HLA class I associations in these cells (Fig. 3A, lane 3 and 4, left panel). Surface CK8 expression on AID, AD and other tumor cell lines was also demonstrated by cytofluorometry, which revealed a level positively correlated ($r = 0.6473$) with the degree of their trypsin-reversed mAb W6/32 reactivity (Fig. 3C and D).

3.3. Reduced mAb W6/32-defined HLA class I levels on HEK293 cells reconstituted with CK8/18 and CK8/19 heterodimers

To test whether CK8 surface expression was responsible for masking HLA class I molecules, HEK293 cells were transiently co-transfected with plasmids encoding CK8 and its type I partner CK18 or CK19, or mock-transfected and analyzed for surface CK8 and HLA class I expression. HKE293 cells were chosen because of their very low total level of endogenous CK8 expression (Fig. 4A, lane 2, lower panel). The results show that the transfected HEK293 cell subpopulations with low HLA class I levels expand ~3-fold (Fig. 4A and B), accompanied by a significant increase in their surface CK8 levels (Fig. 4A, left panel). In contrast, no marked expansion of the populations with low HLA class I expression was detected in HEK293 cells transfected with plasmids encoding CK8/19 or CK8/19 engineered with a potent signal peptide from interleukin 2 (IL2-ss). These findings suggest that redirecting these cytokeratins to the ER prevents their interaction with HLA class I molecules. This was contrasted by the detection on HEK293 cells of ER-redirected cytokeratins (Fig. 4C) and HA-tagged β_2m engineered with IL2-ss (Fig. 4D). A possible ER-independent CK8 trafficking pathway leading to CK8-HLA class I interactions was further supported by the failure of brefeldin A, an ER-Golgi transport inhibitor, to reduce CK8 expression on AID cells in spite of its perturbation of HLA class I transport (Fig. 4E).

To investigate the functional impact of CK8-HLA class I interactions, we mapped the HLA class I regions possibly bound by CK8, using mAb recognizing spatially distinct determinants on the HLA class I–β_2m complex. All regions were found to be potentially affected albeit to different extents (Fig. 4F). The α2 and β_2m surface junction (by mAb W6/32) was the most affected area, followed by the α2 helix (by mAb CR11-351) and the membrane proximal loop of α3 (by mAb TP25.99). The least affected region was the outer part of β_2m (by mAb NAMB-1). CD99, a membrane glycoprotein as an irrelevant control, was not affected. Given the large (>100 kDa), rod-like shape of each cytokeratin pair [19], an HLA class I–β_2m complex (~60 kDa) may be postulated to be wrapped by CK8/18 or CK8/19 heterodimers, although their stoichiometry has not been determined. These observations imply that the binding of T cell co-receptor CD8, which recognizes HLA class I α3 through α2 (PDB 1AKJ, Supplementary Fig. 1), and T cell receptor, which binds on top of α1/α2 helices (PDB 1BD2), can be affected, which may result in an impairment of CTL responses.

4. Discussion

Cytokeratins are cytoskeletal proteins that form ordered multimers to assemble into intermediate filaments in the cytosol, which

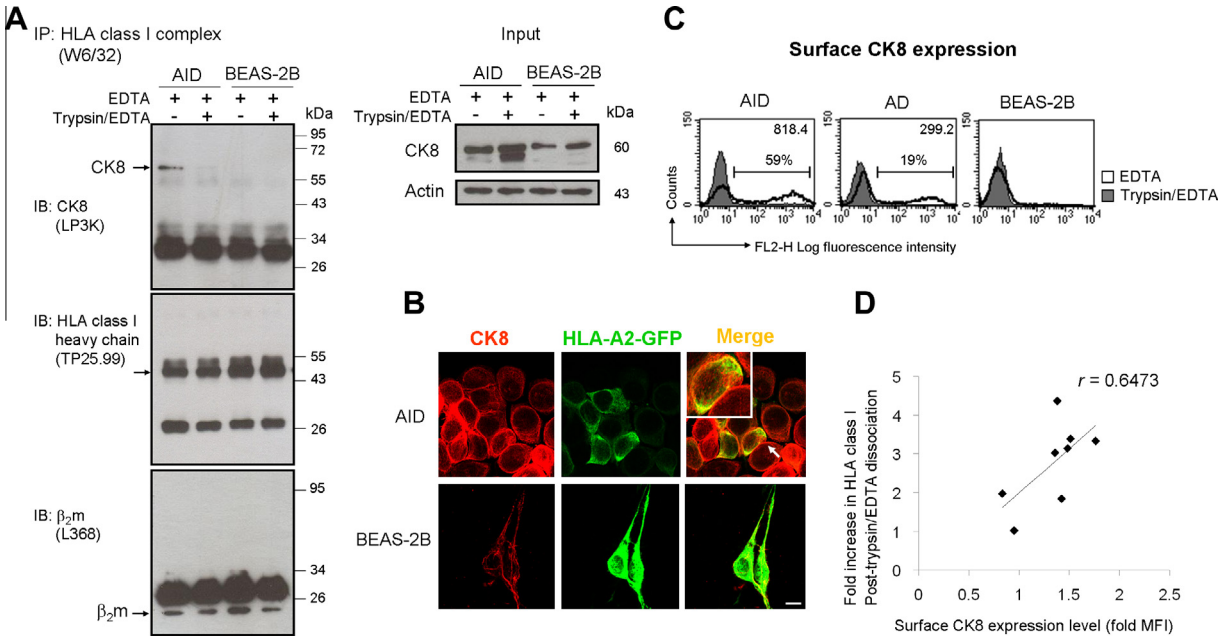

Fig. 3. Association of CK8 with HLA class I molecules at the plasma membrane. (A) Co-immunoprecipitation/immunoblot analysis of lysates from EDTA or trypsin/EDTA-dissociated AID and BEAS-2B cells. The band at ~28 kDa is the signal from anti-mouse Ig light chain antibodies. (B) Confocal microscopic analysis with CK8-specific mAb of HLA-A2-GFP- transfected AID and BEAS-2B cells. Scale bar, 20 μm. Correlation of surface CK8 expression levels and the extent of trypsin-reversed mAb W6/32 reactivity on (C) AID and AD cells and on (D) a panel of tumor cell lines.

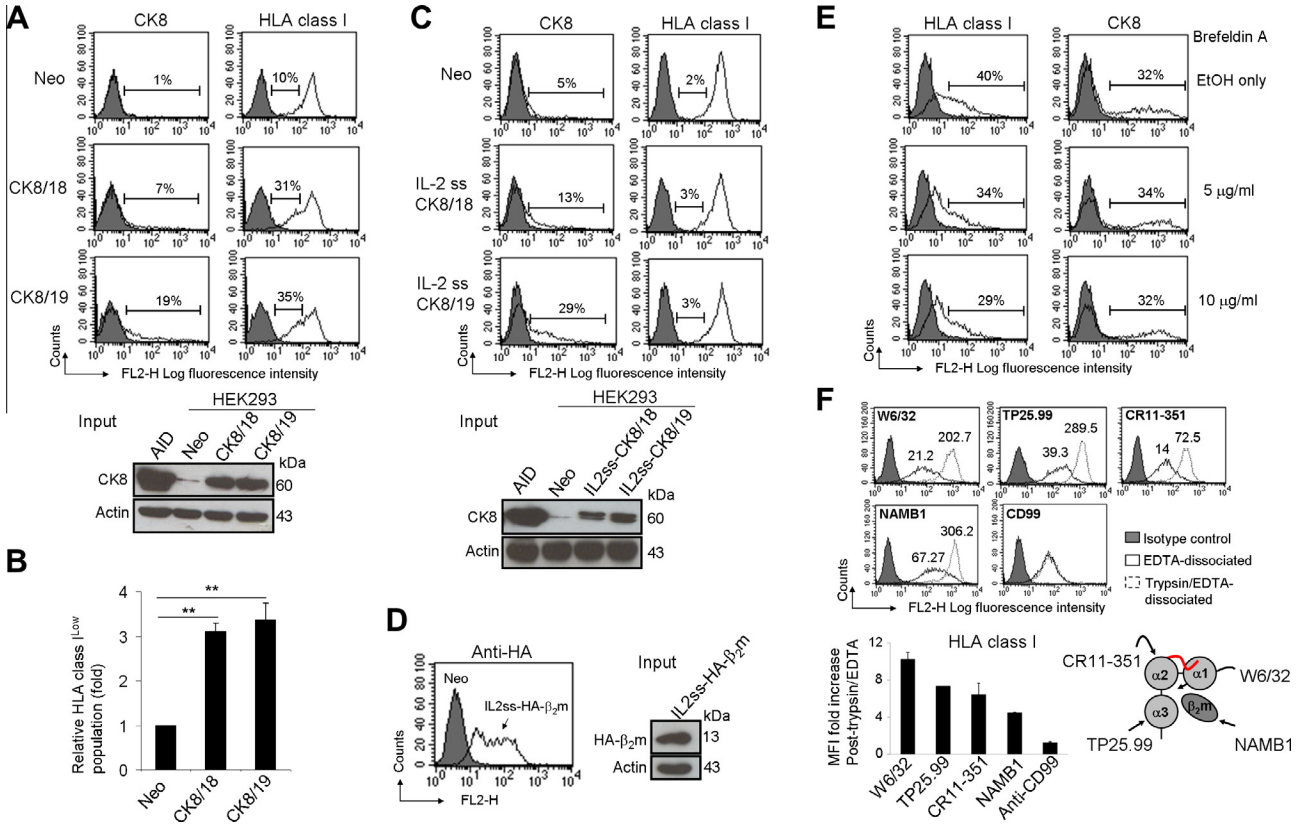

Fig. 4. Reduced mAb W6/32-defined HLA class I levels on HEK293 cells reconstituted with CK8/18 and CK8/19 heterodimers and mapping of their binding sites. Cytofluorometric analysis of HEK293 cells transiently transfected with plasmids encoding (A) CK8/18 or CK8/19, (C) IL2ss-CK8/18 or IL2ss-CK8/19 or (D) IL2ss-HA-β_2m. (B) Quantification of (A) of three experiments. Mean ± S.D. $^{**}p < 0.01$. (E) Cytofluorometric analysis of AID cells 4 h after treating with brefeldin A at 5 and 10 μg/ml. (F) Cytofluorometric analysis of EDTA or trypsin/EDTA-dissociated AID cells with a panel of mAb with defined specificity: mAb W6/32 [30], TP25.99 [11], CR11–351 [8], NAMB-1 [31]. CD99 was analyzed as an irrelevant control.

play important roles in the mechanical support, architecture and migration of cells [19]. Overexpression of cytokeratins, such as CK8, CK18, and CK19, has long been observed in malignant tissues [20], and has been used as diagnostic markers [21]. In addition, their role in cancer cell migration, invasion and metastasis has been documented [22]. Notably, cytokeratins are distributed also at the tumor cell surface [23–26] with poorly characterized functions. Hembrough et al. [27] showed that surface CK8 served as a plasminogen receptor to capture serum plasminogen, resulting in the digestion of extracellular matrix and tumor metastasis. More recently, Liu et al. [28] demonstrated that multidrug-resistant MCF-7/MX breast cancer cells overexpressed cell surface CK8 to enhance their adhesion to fibronectin and vitronectin. We show here that surface CK8, CK18 and CK19 on a head and neck lymph node metastatic carcinoma cell line interact with HLA class I molecules at domains involved in $CD8^+$ T cell activation.

Cytokeratins lack a signal peptide and a transmembrane domain. This feature suggests their non-canonical trafficking route to the plasma membrane to form a complex with HLA class I molecules. Our data exclude the ER-Golgi pathway as the route for CK8-HLA class I interactions to occur, since redirecting CK8/18 and CK8/19 to ER in HEK293 cells does not lower mAb W6/32 reactivity at their surface. Whether the endolysosomal system plays a role in CK8 trafficking remains to be determined. Nevertheless, this possibility is supported by the secretion of interleukin 1β, another leaderless protein, via a regulated exocytosis of endolysosome-related vesicles [29]. CK8 has been shown to form covalent bonds with membrane lipids and travels to the external leaflet of the plasma membrane [24]. In this regard, it remains to be tested whether CK8 interacts with other adaptors or enzymes during its trafficking to the cell surface and interaction with HLA class I molecules.

In conclusion, we have unraveled a post-translational mechanism that leads to an abnormal HLA class I phenotype in malignant cells. Through the binding by cell surface cytokeratins, the function of HLA class I molecules is potentially affected, although their real presence on the plasma membrane is not influenced. This may represent a dual strategy for cancer cells to simultaneously promote their metastasis and immune escape.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.105.

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