Targeting MHC-I related proteins for cancer diagnosis and therapy

Verhaar, E.R.

Chapter 7:

A monoclonal antibody that recognizes a unique 13-residue epitope in the cytoplasmic tail of HLA-E

Elisha R. Verhaar[1,2], Jin Gan[1], Susan Buhl[3], Ziao Li[4], Amir Horowitz[4], and Hidde L. Ploegh[1,2*]

[1]Program in Cellular and Molecular Medicine, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115, USA
[2]Department of Cell and Chemical Biology, Leiden University Medical Centre, Leiden, The Netherlands
[4]Department of Cell Biology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, NY 10461, USA
[4]Department of Oncological Sciences, Precision Immunology Institute, Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, New York, NY, USA.

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Abstract

The Class I MHC molecule (MHC-I) HLA-E presents peptides that are derived from the signal sequences, either those of other MHC-I products, or of viral type I membrane glycoproteins. Monoclonal antibodies with proven specificity for HLA-E, and with no cross-reactions with other MHC-I products, have yet to be described. To obtain anti-HLA-E-specific antibodies suitable for a range of applications, we generated monoclonal antibodies against a unique feature of HLA-E: its cytoplasmic tail. We created an immunogen by performing an enzymatically catalyzed transpeptidation reaction to obtain a fusion of the cytoplasmic tail of HLA-E with a nanobody that recognizes murine Class II MHC (MHC-II) products. We obtained a mouse monoclonal antibody that recognizes a 13-residue stretch in the HLA-E cytoplasmic tail. We cloned the genes that encode this antibody in expression vectors to place an LPETG sortase recognition motif at the C-terminus of the heavy and light chains. This arrangement allows the site-specific installation of fluorophores or biotin at these C-termini. The resulting immunoglobulin preparations, labeled with 4 equivalents of a fluorescent or biotinylated payload of choice, can then be used for direct immunofluorescence or detection of the tag by fluorescence or by streptavidin-based methods. We also show that the 13-residue sequence can serve as an epitope tag, independent of the site of its placement within a protein's sequence. The antibody can be used diagnostically to stain HLA-E on patient tumor samples, as an antibody-epitope tag for extracellular proteins, and to research the unique role of the cytoplasmic tail of HLA-E.

Introduction

Class I MHC proteins are composed of a membrane-embedded glycoprotein heavy chain in tight, non-covalent association with the soluble light chain beta2-microglobulin. Class I MHC molecules (MHC-I) are found on the surface of all nucleated cells in vertebrates and present fragments of intracellular antigens in the form of peptides to $CD8^+$ cytotoxic T lymphocytes (CTL) to enable elimination of intracellular pathogens. Virus-infected and malignantly transformed cells can escape immune cell recognition by down-regulation of MHC-I products, which can be achieved transcriptionally and post-transcriptionally[626]. MHC-I molecule HLA-E presents a unique case, as it is specialized in the presentation of peptides that are derived from the signal sequences of other MHC-I products, or of viral type I membrane glycoproteins[252–259]. HLA-E is frequently overexpressed on tumors and on virus-infected cells, where it serves as a ligand for CD94/NKG2A, -B, and –C on NK and T cells, thereby regulating their

cytotoxic activity[258,269–277]. Thus, even if a virus were to succeed in down-regulation of the classical Class I HLA-A, -B and -C products to escape detection by CTLs, their signal peptides would continue to be produced and could serve as peptide cargo for HLA-E, rendering the infected cell more resistant to lysis by NK and T cells.

HLA-E-specific monoclonal antibodies have been used to detect expression in tumors and normal tissues, but the available reagents that are in wide use to detect HLA-E (3-D12 and MEM-E/02) have been reported to cross-react with certain allelic products of the HLA-B and HLA-C[627]. Here, we set out to generate monoclonal antibodies that are specific for HLA-E, with no anticipated cross-reactions with conventional MHC-I products. The ectodomains of the MHC-I products, including those of HLA-E, are highly homologous. There are few locus-specific features present in the ectodomains that would allow an unambiguous assignment of a sequence to the HLA-A, -B or -C locus, and locus-specific tools for use in immunochemistry are comparatively rare[628–632]. In contrast, the cytoplasmic tails of the classical MHC-I products do show locus-specific features, shared among virtually all alleles at that locus (Figure 1). The cytoplasmic tail of HLA-E is highly conserved and shows no allelic variation. The two known HLA-E alleles, HLA-E*01:01 and HLA-E*01:03, vary only by a single replacement substitution of an arginine to a glycine at position 107 in exon 3 (underscored in Figure 1)[633,634].

The cytoplasmic tail of MHC-I is involved in trafficking peptide-bound MHC-I from the endoplasmic reticulum to the cell membrane. In addition, the cytoplasmic tail is believed to play a role in the relocation of HLA-E to late and recycling endosomes [281,282]. Antibodies against the HLA-E cytoplasmic tail would thus provide a useful tool for studying the cytoplasmic tail interactions, as well as for other purposes where detection of HLA-E is called for, such as staining of tumor tissues by conventional immunohistochemistry.

We used an immunization strategy that exploits a nanobody that targets mouse Class II MHC$^+$ antigen presenting cells[420,466,558] (VHH_{MHCII}) fused to the HLA-E C-terminal sequence (GGCSKAEWSDSAQGSESHSL, hereafter referred to as "$HLA\text{-}E_{tail}$") by means of a sortase reaction[553,635]. We obtained 23 monoclonal antibodies and selected three with unique sequences for further analysis. All of them recognize a 13-residue stretch in the HLA-E cytoplasmic tail.

To enhance the applicability of the HLA-E specific monoclonal antibody, we site-specifically modified the IgG molecules with 4 moles of fluorophore or biotin by installing sortase recognition motifs (LPETG) at the C-termini of its

IgG heavy and light chains, thus avoiding the need for secondary antibodies as staining agents. The monoclonal antibody detects HLA-E in immunoblots and immunoprecipitation on HLA-E positive cell lysates, formalin-fixed, paraffin-embedded tissue sections, and can be used for immunofluorescence and flow cytometry of permeabilized cells. The 13-residue sequence and the monoclonal antibody that recognizes it also serves as an effective epitope tag/detection pair, regardless of its location in the protein of interest, in an otherwise HLA-E negative environment. We show that antigen-specific elution with the synthetic cytoplasmic tail peptide is an effective means of retrieval of the tagged protein.

Figure 1. Alignment of the consensus amino sequences of HLA-E, -A, -B, -C, -F, and -G. *In blue are highlighted the amino acids that differ from the consensus sequence. The ectodomains show very few locus-specific features to which antibodies could be directed. In contrast, the cytoplasmic tails of the classical Class I MHC products, highlighted in the black box, do show locus-specific features, and could therefore be used to generate HLA-E specific antibodies. The cytoplasmic tail of HLA-E is highly conserved and shows no allelic variation. The two known HLA-E alleles, HLA-E*01:01 and HLA-E*01:03, vary only by a single replacement substitution of an arginine to a glycine (underscored in figure)*

Materials and methods

Sortase reactions to create VHH_{MHCII}-HLA-E_{tail}, GFP-HLA-E_{tail}, or 10-mer HLA-E_{tail} derivatives

Recombinant VHH_{MHCII} equipped with LPETG (an amino acid sequence recognized by sortase) and a $(His)_6$-tag was expressed by periplasmic expression in *Escherichia coli* WK6 (ATCC). Recombinant GFP-LPETG was expressed by cytoplasmic expression in *E. coli* BL21 (Thermo Scientific). The C-terminal $(His)_6$-tag allows purification of the recombinant proteins using Ni-NTA agarose beads (Qiagen), followed by FPLC purification on an S75 column by FPLC (ÄKTA, Cytiva Life Sciences). HLA-E cytoplasmic tail peptide was obtained from Genscript at ~85% purity. 10-mer peptides were produced by solid phase peptide synthesis and provided by the lab of Jacques Neefjes, Leiden University Medical Center. Each peptide carries an N-terminal Gly-Gly sequence to allow fusion to the VHH or GFP by means of a sortase reaction. Sortase reactions were performed in PBS at 4°C overnight with final reagent concentrations of 1 mg/ml protein, 500 mM GG-peptide, and 25 mM 7M-Sortase A[553]. Unmodified VHH_{MHCII} or GFP that retained the sortase motif, as well as 7M-Sortase A, all containing the $(His)_6$-tag, were removed by depletion on NiNTA beads for 20-60 minutes at 12°C. Completion of the sortase reactions was confirmed by LC-MS and SDS-PAGE.

Mice

C57BL/6J mice were purchased from the Jackson Laboratory. Mice were used at 8-12 weeks of age and were housed under specific pathogen-free conditions. Experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Boston Children's Hospital, protocol number 00001880.

Mouse immunization and hybridoma production

Mice were immunized intraperitoneally at ~10-day intervals with 40-50 µg VHH_{MHCII}-HLA-E_{tail} in Freund's adjuvant. Immune responses were monitored using ELISA on GFP-HLA-E_{tail} to measure the peptide-specific response. As the donor of the spleen used for hybridoma production, we chose one mouse whose immune response as measured by ELISA was detectable at a serum dilution of >1:40,000. Mice were given an intraperitoneal injection of ~100 µg VHH_{MHCII}-HLA-E_{tail} in PBS five days and four days prior to harvesting splenocytes to absorb free circulating antibody and boost the splenocytes, respectively. Hybridomas were produced by fusing splenocytes with the Ag8.653 myeloma cell line according to previously described protocols[636]. Hybridoma were allowed to expand in hybridoma medium (DMEM with 4.5

g/L glucose (Gibco), substituted with 20% fetal bovine serum (FBS), 10% NCTC-109 (Gibco), 1% non-essential amino acids (Gibco), 100 U/mL penicillin/streptomycin, 2% hypoxanthine-aminopterin-thymidine (HAT) (Sigma-Aldrich), substituted for 2% HT (Gibco) after ~12 days in culture). Hybridoma supernatant was tested by ELISA on GFP-HLA-$E_{tail,}$ because the immunized mice have not been exposed to GFP at any point, this screening strategy ensures selection for antibodies that recognize the attached HLA-E tail and not GFP. Positive clones were expanded, and single-cell clones were obtained through semi-solid cloning in ~0.4% SeaPlaque™ Agarose (Lonza) prepared in complete hybridoma medium substituted with 5% HyMax™ (Antibody Research Corporation). Clones that showed a positive response to GFP-HLA-E_{tail} on ELISA were expanded and positive hybridoma clones were selected.

Cloning and expression of LPETG-modified monoclonal immunoglobulins

mRNA was extracted from ~1x10^7 cells of each positive hybridoma clone, following manufacturer's protocol (Qiagen). cDNA was transcribed with RT transcriptase and a random hexamer primer (5'-NNNNNN-3'), following manufacturer's protocol (Takara SMARTScribe™ Reverse Transcriptase kit). Immunoglobulin HC and LC were amplified by PCR, using a combination of low and highly degenerate primers flanking the sequence between FR1 and FR4 (supplementary table 1). HC and LC sequences, modified to contain LPETGG-$(His)_6$ on the HC and LC, were ordered as GBlock™ gene fragments and cloned into a pcDNA4 vector by InFusion cloning, following the manufacturer's protocol (Takara In-Fusion® Snap Assembly Master Mix) into a murine IgG (for the HC) or IgKappa (for the LC) backbone. Proteins were expressed in EXPI-293 cells following the manufacturer's instructions. EXPI-293 cells were diluted to 3x10^6 cells/mL in Expi293™ Expression Medium (Gibco) and transfected with ExpiFectamine™ according to the manufacturer's instructions (ExpiFectamine™ 293 transfection kit, Gibco). Briefly, HC and LC DNA were mixed at a [1:3] ratio and incubated with ExpiFectamine™ 293 reagent for 20 minutes at room temperature. The mixture was added drop-wise to the cells followed by incubation in a shaking incubator at 37°C in a humidified 5%CO_2 atmosphere. 16-24 hours after transfection, ExpiFectamine™ 293 Transfection Enhancers were added according to the manufacturer's instructions. Cells were harvested 4 days after transfection and centrifuged for 45 minutes at 2000xg.

Immunoglobulins were purified from the culture medium on a Ni-NTA agarose column (Qiagen) and further purified by size exclusion on a HiLoad Superdex 200 column (Cytiva Life Sciences) using FPLC (ÄKTA, Cytiva Life Sciences).

DNA constructs

The Halo-Tev-Flag-Ube2v2 (#110070, Addgene) construct has been described previously [637]. This plasmid was used as a substrate for the introduction of epitope tags recognized by monoclonal antibody 19-H12 (Supplementary figure 1). For site-directed mutagenesis, a PCR mixture containing GFP-OTUB2 WT template, mutation primers, Phusion High-Fidelity PCR Master Mix, and MilliQ water in a 30 µL reaction volume was subjected to PCR amplification using the following program: 98 °C for 30s (98 °C for 10 s; 55 °C for 1 min; 72 °C for 1 min/Kb)×30 cycles; 72 °C for 5 min. PCR products were digested with 1 µL DpnI (ThermoFisher Scientific) overnight at 37 °C to remove methylated DNA template, then transformed into competent DH5α (Thermo Scientific). All mutated constructs were verified by sequencing. All primers were purchased from IDT. For primer sequences, see supplementary table 2.

Cell culture and reagents

HEK293T (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and 1% penicillin/streptomycin at 37 °C and 5% CO_2. K-562 HLA-E KO and K-562 HLA-E^+ cells were a gift from Alan Korman (VIR Biotechnology). Cells were maintained in Iscove's modification of DMEM with 10% FCS and 1% penicillin/streptomycin at 37 °C and 5% CO_2. Hybridomas were cultured in DMEM with 4.5 g/L glucose (Gibco), 20% FBS, 10% NCTC-109 (Gibco), 1% non-essential amino acids (Gibco), 1% pen/strep, and 1X HAT (Sigma-Aldrich). HAT was replaced with 1x HT (Sigma-Aldrich) after 2 weeks, and cells remained in HT^+ medium. For transfection experiments, HEK293T cells were seeded to achieve 50–60% confluence the following day and transfected using polyethylenimine (PEI), Polysciences Inc., Cat# 23966) as follows: 200 µL DMEM medium without supplements was mixed with DNA and PEI (1 mg/mL) at a ratio of 1:3 (e.g.: 1µg DNA : 3µg PEI), incubated at RT for 20 min, and added drop-wise to the cells. Cells were cultured for 24 hours prior to further analysis. The reaction mixtures were scaled to maintain a fixed component ratio, as follows: 6-well plate: 3µg DNA, 6 cm dish: 8 µg DNA, 10 cm dish: 24 µg DNA.

SDS-PAGE, in-gel fluorescence scan, and immunoblotting

Samples were resolved on 12% SDS-PAGE gels. For immunoblotting, proteins were transferred to a nitrocellulose membrane (#162-0112, 0.2 µm, Biorad) at 300 mA for 3 hours at 4°C in transfer buffer (25 mM Tris, 192 mM glycine in PBS). The membranes were blocked in 5% (w/v) skim milk (non-fat dry milk powder, #M0842, Lab Scientific) in 1× PBS, incubated with a primary antibody diluted in 5% (w/v) milk in PBS + 0.1% Tween-20 (PBS-T) overnight in a cold room, washed three times for 5 min in PBS-T. with rabbit anti-Flag (20543-1-AP, Proteintech, 0.3 µg/mL) secondary antibody diluted in 5% (w/v) milk in PBS-T for 1 hour, and washed three times again in PBS-T. The signal was visualized using a BioRad ChemiDoc MP imaging system. For silver staining of SDS-PAGE gels, Pierce™ Silver Stain Kit (#24612) was used.

Immunoprecipitation

HEK293T cells were lysed for 20 min in a lysis buffer containing 50mM Tris-HCl (pH 8.0), 150mM NaCl, 4mM EDTA, 1% Triton X-100, protease inhibitor (Roche, complete EDTA-free, Cat# 05056489001). The crude lysate was centrifuged (20 min, 4 °C, ~16,000xg) and the supernatant was incubated with the respective antibodies by rotation at 4 °C for 1 hour. Pierce™ Protein G Agarose (#20398) beads were then added and incubated with agitation at 4 °C for 4 h. Beads were washed four times in lysis buffer. After removal of the washing buffer, reducing Laemmli SDS sample buffer (Alfa Aezar, #J61337-AD) was added at 1x to the beads, followed by 7 min incubation at 95 °C. Immunoprecipitated proteins were separated by SDS-PAGE for immunoblotting.

Immunoblot on K-562 cells

One million K562 HLA-E KO or K-562 HLA-E$^+$ cells per lane of an immunoblot were lysed in 1x RIPA lysis buffer with DNAse I for 30 minutes on ice. Proteins were denatured with SDS Laemmli sample buffer (Alfa Aezar, #J61337-AD) with fresh 9% (v/v) beta-mercaptoethanol at 80-90°C for 10 minutes and resolved on a 12% SDS-PAGE gel. Proteins were transferred onto a PVDF membrane with a Trans-Blot Turbo System (BioRad). Membranes were blocked for 2 hours at room temperature in blocking buffer (5% (w/v skim milk in PBS + 0.02% Tween) and incubated overnight with 1 µg/mL purified 19-H12. The next day, membranes were incubated with 0.3 ng/mL HRP-conjugated goat-anti-mouse IgG (H+L) secondary antibody (Invitrogen) for 45 minutes at room temperature and developed with Western Lighting ECL Plus (Perkin-Elmer). Images were recorded on the ChemiDoc Imaging System (BioRad).

Flow cytometry

K-562 KO or HLA-E$^+$ cells were fixed at 4x10^5 cells per 100 µl with cold 4% PFA for 15 minutes at room temperature. Cells were washed with PBS and either kept in PBS or permeabilized at 2x10^5 cells per 100 µl of 0.1% Saponin and 2% FBS in PBS for 15 minutes at room temperature. Cells were stained with 19-H12-Cy5 at 2.5 µg/mL and 3-D12-PE (BioLegend Cat# 342604, Lot #B353119) at 1.25 µg/mL in either PBS (for non-permeabilized samples), or in permeabilization buffer (permeabilized samples) for 30 minutes on ice. Cells were washed twice with FACS buffer (2mM EDTA, 2% FBS in PBS) and analyzed on an LSR Fortessa (BD Biosciences). To control for non-specific intracellular retention of antibodies, we incubated permeabilized or non-permeabilized, fixed K-562 HLA-E KO or HLA-E$^+$ cells with irrelevant antibodies (PE-conjugated murine IgG-kappa isotype control (Biolegend, 1 µg/mL) and Cy5-conjugated anti-HA.11 epitope tag (Biolegend, 1 µg/mL) using the above staining protocol. Gating strategies for flow cytometry described in supplementary figure 4.

Immunofluorescence microscopy

For immunofluorescent staining of K-562 cells, we used an adaptation of a previously described protocol [638]. Briefly, cells were pelleted for 5 minutes at 500xg and resuspended in PBS. Cells were transferred to a 12-well plastic-bottom tissue culture plate (Corning) at 1x10^6 cells per well and left to adhere for 30 minutes at room temperature. Non-adherent cells were removed by aspiration. Adherent cells were fixed with 500 µl/well of 10% (v/v) formalin for 10 minutes at room temperature. Fixed cells were washed with PBS, followed by permeabilization for 10 minutes with 0.5% (w/v) Saponin in PBS, or left in PBS for the non-permeabilized control wells. Cells were washed with PBS and blocked with 1% (w/v) BSA in PBS for 30 minutes at room temperature and stained with staining solution containing either 1.25 µg/mL 19-H12-Cy5, 2.7 µg/mL 3-D12-PE (BioLegend), or both, in 1% (w/v) BSA in PBS for 1 hour at RT, in the dark. Cells were washed three times with PBS and nuclei were stained with Hoechst 33342 at 1 µg/mL (Life Technologies) for 10 minutes at room temperature, in the dark. Cells were washed twice with PBS to remove excess dye. Cells were submerged in PBS (500 µL/well) and imaged with a Keyence IX8 fluorescent microscope.

Immunohistochemistry staining

Formalin-fixed, paraffin embedded sections (3 µM) of healthy human tonsils or NMIBC bladder tumors from patients were prepared for immunohistochemistry by deparaffinization with xylene and rehydration in a series of graded alcohols. Heat-induced antigen retrieval was done at 95°C

with Dako Target Retrieval Solution, pH 6 following manufacturer's directions (Agilent Solutions, S2369). Slides were blocked with peroxidase suppressor (Thermo Scientific, 35000) for 10 minutes, followed by incubation with Serum-free Protein Block (Dako, X090930-2) for 5 minutes. Primary antibodies MEM-E/02 (Abcam, ab2216) or 19-H12 were incubated at the indicated concentrations for 60 minutes at room temperature. EnVision+ Single Reagent, HRP mouse (Dako, K4001) was used as secondary reagent. Sections were developed with DAB+ (Dako, K3468), counterstained with Mayer's hematoxylin (Sigma-Aldrich, MHS32), dehydrated in a series of graded alcohols, and mounted with a coverslip. Whole tissue sections on the slide were converted into high-resolution digital data using a NanoZoomer S60 Digital slide scanner (Hamamatsu).

Results

Immunization and hybridoma production yields HLA-E specific monoclonal antibodies

The intact cytoplasmic tail of HLA-E, fused by means of a sortase reaction to the mouse MHC-II specific nanobody VHH_{MHCII} was used as immunogen[436,558]. The identity of the ligation products was confirmed by mass spectrometry (Figure 2A). Mice were immunized in complete Freund's adjuvant and boosted with antigen until a serum antibody titer >1:40,000, as measured by ELISA, was reached (Figure 2B). To render the ELISA specific for the HLA-E cytoplasmic tail, plates were coated with recombinant GFP, modified at its C-terminus with the intact HLA-E_{tail}, again using a sortase reaction to install the HLA-E_{tail} peptide. The spleen from an appropriately responding mouse was used for the generation of hybridomas with assistance from Dr. Matthew D. Scharff and Ms. Susan Buhl from the Hybridoma Facility at the Albert Einstein College of Medicine[636]. Positive clones were identified by ELISA, again using GFP-HLA-E_{tail} as the target antigen. Single cell clones were expanded and the supernatants from growing clones were used as the primary antibody in immunoblotting experiments, using the GFP-HLA-E_{tail} fusion as the target. All clones tested by immunoblotting recognized this fusion protein (Figure 2C), clones 19-H12, 2-D12, and 10-C1 are highlighted in Figure 2.

The DNA sequences of the clones that were positive in ELISA and immunoblotting were determined by RT-PCR. We identified a single dominant V_H sequence, derived from the germline V_H IGHV1-72*01 sequence, in combination with the J IGHJ2*01 segment. A D element could not be unambiguously identified (Figure 3A). The VDJ sequence contains 4

mutations attributable to somatic hypermutation, as determined by reference to a consensus murine germline VH sequence. All mutations were present in the framework regions and caused amino-acid substitutions at positions 16, 51, and 62. One mutation, underscored in Figure 3A, was a silent replacement.

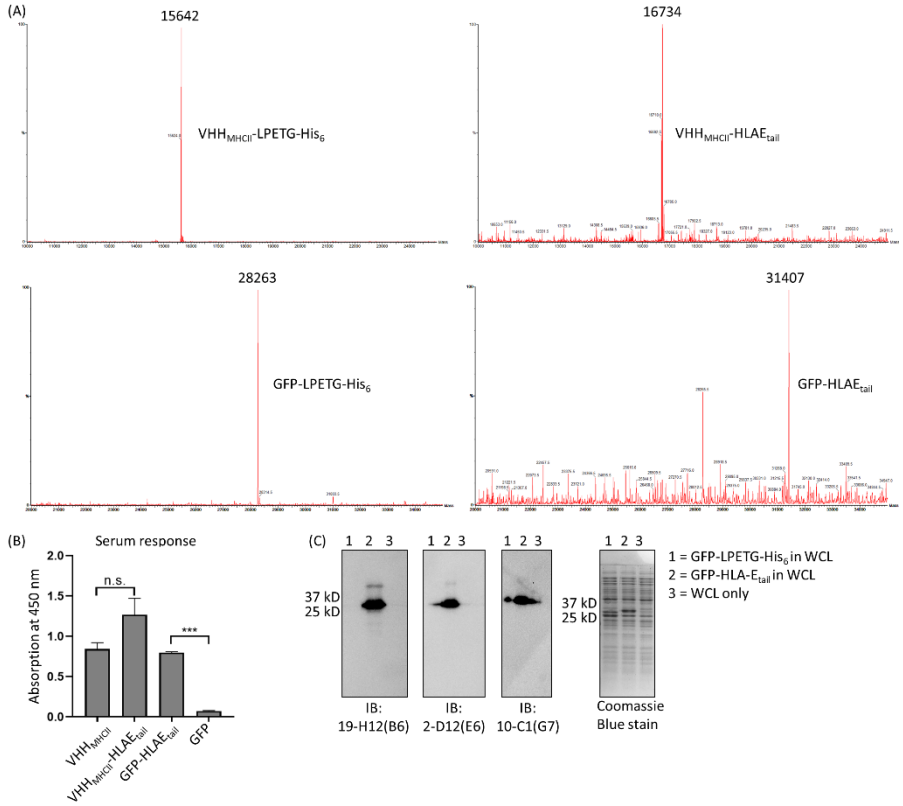

Figure 2. Immunogen production, quantification of serum titer, and immunoblot analysis of hybridoma clones. *(A) The peptide comprising the cytoplasmic tail of HLA-E was fused by means of a sortase reaction to VHH7 (anti-mouse MHC-II) or GFP. The identity of the ligation products was confirmed by mass spectrometry. Intact VHH7-LPETG-$(His)_6$ has a calculated molecular weight of 15635 g/mol, VHH7-HLA-E_{tail} has a calculated molecular weight of 16691. GFP-LPETG-$(His)_6$ has a calculated molecular weight of 28250. GFP-$HLAE_{tail}$ has a calculated molecular weight of 29306. The observed molecular weight of 31407 is attributed to the disulfide bond formed between the cysteine residues of two peptides, creating an expected molecular mass of 31329. The discrepancy between the calculated molecular weight and the observed molecular weight found with the LC-MS is within the normal range of error. LEGEND CONTINUES ON THE NEXT PAGE.*

(B) Mice were immunized in complete Freund's adjuvant and boosted with antigen until a serum antibody titer >1:40,000, as measured by ELISA, was reached. Because VHH7 is slightly immunogenic, the titer was determined on the response to GFP-HLAE$_{tail}$. A significant difference was reached in serum response to GFP-HLAEtail compared to GFP ($p = 0.00019$, calculated by multiple T-test). (C) Per lane, 500 ng of protein (lane 1: GFP-LPETG-(His)$_6$, lane 2: GFP-HLA-E$_{tail}$) was loaded. To prevent non-specific signal, proteins were mixed with unrelated E. coli whole cell lysate (WCL). Supernatant of hybridoma clones was used as primary staining agent and HRP-linked anti-mouse secondary was used as secondary agent. To verify loading, a coomassie blue gel stain was made. All the clones recognized the fusion protein. The non-specific signal in lane 1 and 3 from the 2-D12 and 10-C1 blot are spillover of the proteins into the neighboring lanes.

For the Vκ sequences we identified 3 unique occurrences, based on the usage of the germline Vκ gene IGKV1-135*01 and J-segment IGK1*01 for 19-H12, IGKV4-90*01 and IGKJ1*01 for 2-D12, and IGKV4-50*01 and IGKJ4*01 for 10-C1. 19-H12 has a V2I, N39S, and F67V mutations and 3 silent replacements. 2-D12 has mutations in the first six amino acids of the FR1, which we attribute to the primers used for amplification of the LC domain. 2-D12 has a P95Y substitution in the CDR3 region. 10-C1 has the same six amino-acid replacement in the FR1, and an S95F substitution in CDR3 (Figure 3B). We conclude that all hybridomas identified are derived from a single V_H rearrangement. Because antigen recognition is predominantly established by the identity of the V_H segment, more specifically its CDR3 region, these monoclonal antibodies are likely to all recognize the same epitope.

The DNA sequences of the clones that were positive in ELISA and immunoblotting were determined by RT-PCR. We identified a single dominant V_H sequence, derived from the germline V_H IGHV1-72*01 sequence, in combination with the J IGHJ2*01 segment. A D element could not be unambiguously identified (Figure 3A). The VDJ sequence contains 4 mutations attributable to somatic hypermutation, as determined by reference to a consensus murine germline VH sequence. All mutations were present in the framework regions and caused amino-acid substitutions at positions 16, 51, and 62. One mutation, underscored in Figure 3A, was a silent replacement.

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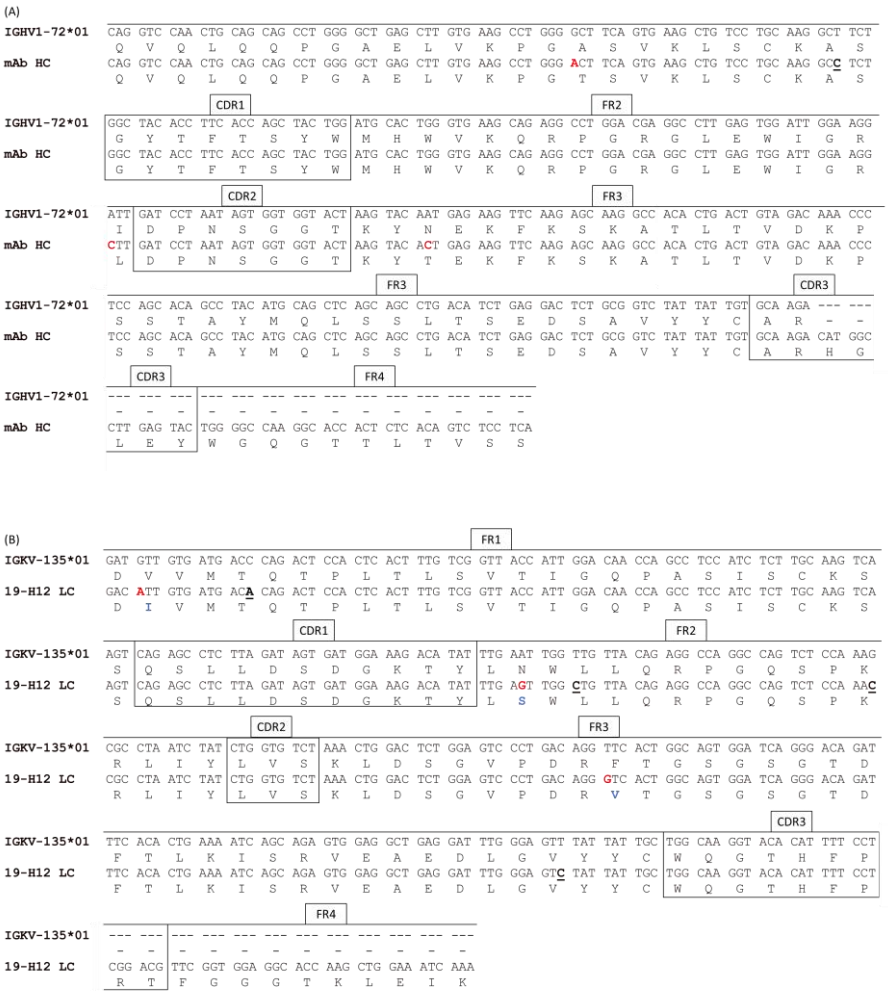

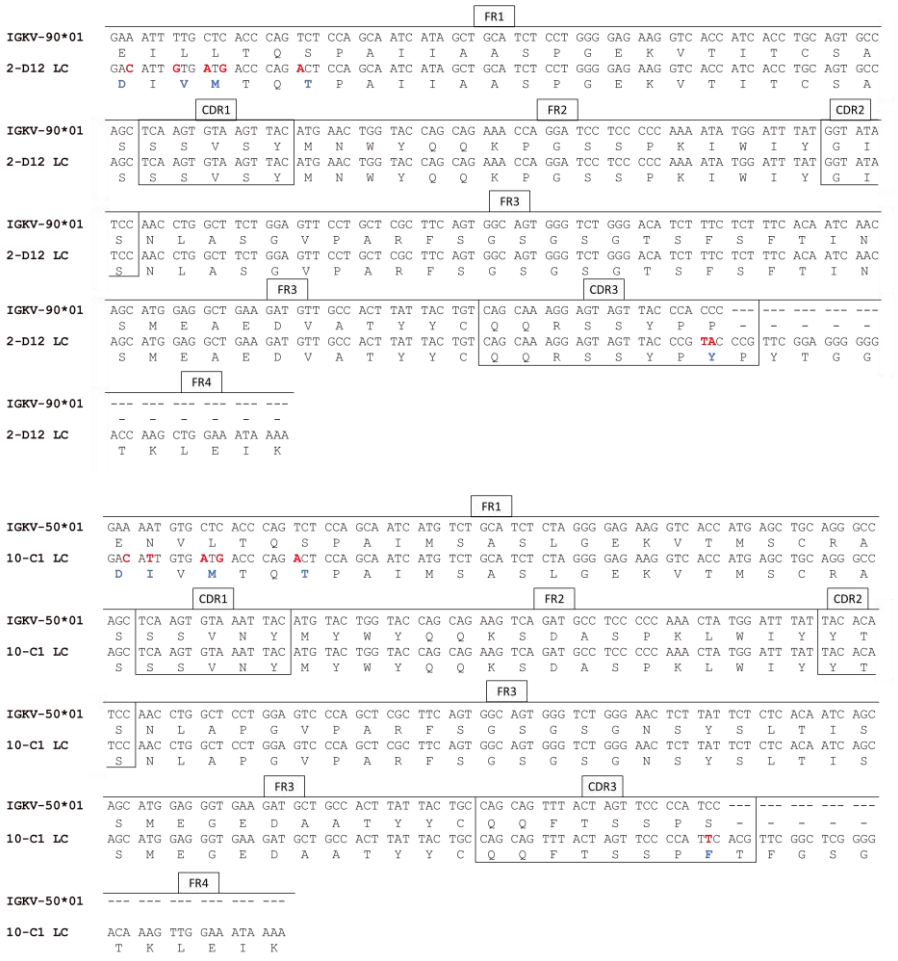

Figure 3. The DNA sequences of the clones that were positive in ELISA and immunoblotting were determined by RT-PCR. *(A) We identified a single dominant V_H sequence, derived from the germline V_H IGHV1-72*01 sequence, in combination with the J IGHJ2*01 segment. A D element could not be unambiguously identified. The VDJ sequence contains 4 mutations attributable to somatic hypermutation, as determined by reference to the germline VH sequence. All mutations, highlighted in red, were present in the framework regions and caused amino-acid substitutions (highlighted in blue). One mutation, underscored, was a silent (B) For the Vk sequences we identified 3 unique occurrences, based on the usage of the germline Vk gene IGKV1-135*01 and J-segment IGKJ1*01 for 19-H12, IGKV4-90*01 and IGKJ2*01 for 2-D12, and IGKV4-50*01 and IGKJ4*01 for 10-C1. 19-H12 has V2I, N39S, and F67V mutations and 3 silent replacements. 2-D12 has mutations in the first 6 amino acids which we attribute to the primers used for sequencing of the LC domain. The mutations P95Y in the CDR3 is attributable to somatic hypermutation. 10-C1 has this same 6 amino-acid replacement in the FR1, and an S95F mutation in the CDR3.*

We cloned the sequences spanning the FR1 to FR4 of the heavy chain and light chain backbones on a murine IgG1 (for the HC) or IgKappa (for the LC) backbone, both modified C-terminally with an LPETGG-$(His)_6$ motif. The 6x histidine tag allows easy purification of the recombinant IgG on a NiNTA matrix (Figure 4A). The LPETG motif allows sortase-mediated modification of both the heavy chain and light chain with a biotin or fluorophore. We produced the 19-H12 clone in EXPI-293T cells and modified it with biotin or Cy5 on the heavy chain and light chain for downstream applications such as cytofluorimetry or immunofluorescence (Figure 4B). Because the $(His)_6$-tag is lost upon modification of the IgG molecule with sortase, depletion of the sortase reaction mixture on a NiNTA column ensures that the final product, obtained in the NiNTA flow through, is homogeneously modified at all four C-termini. The homogeneously Cy5-modified 19-H12 was used where indicated for further experiments.

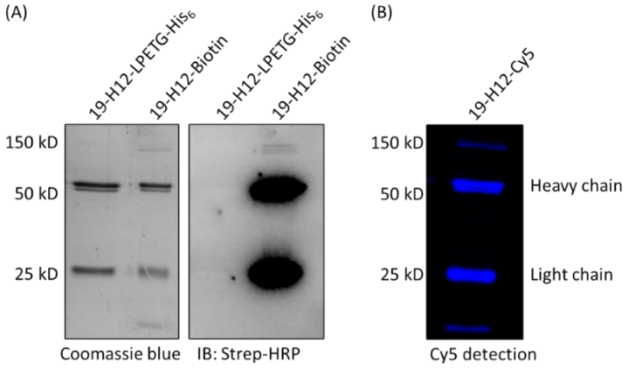

Figure 4. Production of antibody modified at the HC and LC C-terminus with an LPETG-His6 motif. *(A) We cloned the sequences spanning the FR1 to FR4 of the heavy chain and light chain in backbones on a murine IgG1 (for the HC) or IgKappa (for the LC) backbone, both modified C-terminally with LPETG-$(His)_6$ motif and transfected the plasmids into EXPI-293 cells for protein production. The $(His)_6$-tag allows for easy purification of the recombinant IgG on a NiNTA matrix. The LPETG motif allows sortase-mediated modification of both the heavy chain and light chain with a biotin or fluorophore. (B) We modified the monoclonal antibody with Cy5 on the heavy chain and light chain. The final product has an equimolar amount of Cy5 on the C-termini of both heavy chains and light chains.*

Monoclonal antibodies recognize a 13-residue HLA-E specific peptide

We mapped the epitope recognized by three monoclonal antibodies that make use of three distinct k light chains. A series of overlapping peptides with a 1-residue pitch was synthesized based on the sequence of the HLA-E_{tail}. Each peptide was extended at its N-terminus with a Gly-Gly-Ser sequence to ensure its suitability as a sortase nucleophile and to impart some degree of flexibility relative to the GFP to which the peptide is attached. Each peptide was individually ligated to a sortase-compatible, $(His)_6$-tagged GFP variant. Input $(His)_6$-tagged sortase and unreacted $(His)_6$-tagged GFP were removed from the reaction by depletion on a Ni-NTA matrix, so that the supernatant contained only the desired ligation product and free peptide, added in molar excess. Recognition of ligation products was done by immunoblot, using conditioned medium of the three monoclonal hybridomas as the primary detection agent. HRP-linked anti-mouse IgG was used as a secondary detection agent. The results unambiguously identified three overlapping peptides: (W)(S)(D)SAQGSES(H)(S)(L), thus identifying the sequence SAQGSES as the core of the epitope in the HLA-E cytoplasmic tail (Figure 5A).

To determine the smallest possible tag based on this epitope sequence, we inserted 8-mer, 10-mer, and 13-mer peptide sequences at the C-terminus of Halotag-Flag-UBE2V2 in a mammalian expression vector. The constructs were expressed in HEK-293T cells, and cell lysates were subjected to immunoblot using conditioned medium from hybridoma cultures. 19-H12 and 2-D12 clearly recognized the 13-mer at the C-terminus of the target protein. 10-C1 showed a weaker signal, which we attribute to the lower titer of the immunoglobulin in the hybridoma culture supernatant (Figure 5B). 19-H12 and 2-D12 both also recognize the 13-mer tag when positioned at the N-terminus or at the center of the protein, as validated with immunoblot on lysates of HEK-293T cells that express Halotag-Flag-UBE2V2 modified to express the 13-mer tag at the N-terminus (N13 mer) or middle (M13 mer) (Figure 5C). Immunoprecipitation further validated the interaction of purified 19-H12 mAb with the 13-mer tag (Figure 5D). Protein complexes immunoprecipitated with 19-H12 mAb can be eluted by addition of an excess of free synthetic peptide (Figure 5E, 5F).

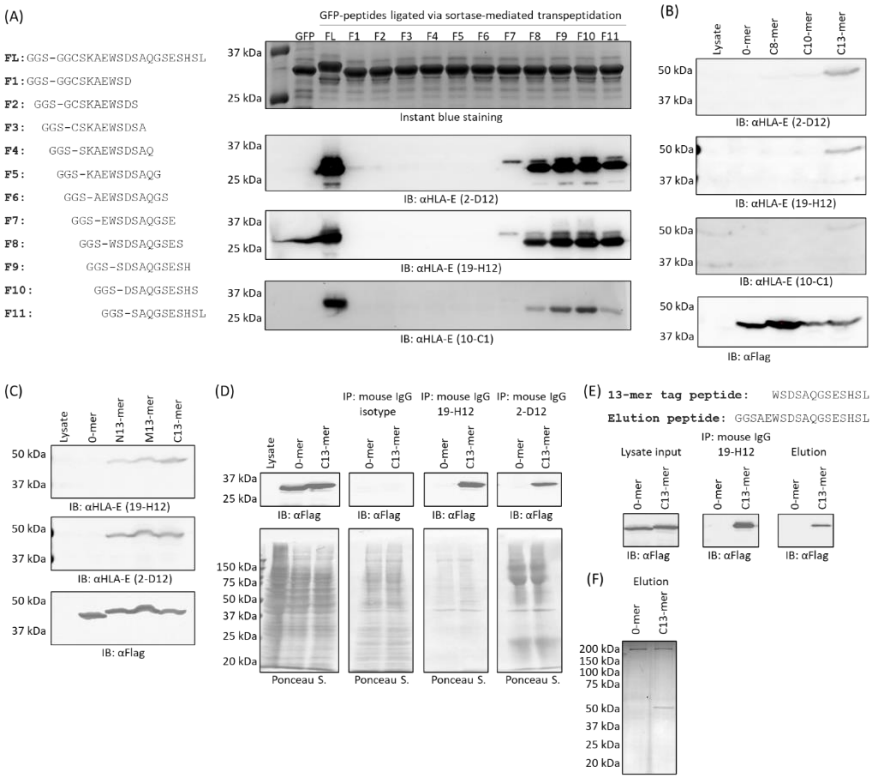

Figure 5. Monoclonal antibodies recognize a unique 13-residue HLA-E specific peptide *(A) Epitope mapping with 10mers. 10-residue HLA-E specific peptides with a pitch of one residue were chemically synthesized and ligated to GFP C-terminus via sortase-mediated transpeptidation. The ligated products were subject to SDS-PAGE and immunoblots against selected monoclonal antibodies. (B) 8-mer, 10-mer or 13-mer peptide sequences were inserted at the C-terminus of the mammalian expression vector Halotag-Flag-UBE2V2 via PCR-based site-directed mutagenesis. The constructs were exogenously expressed in HEK293T cells, the cell lysates were subject to immunoblots against indicated antibodies. (C) The 13-mer tag was inserted into the mammalian cell expression vector at the N-terminus (N13-mer), Middle (M13-mer), or C-terminus (C13-mer) of Halotag-Flag-UBE2V2 via PCR-based site-directed mutagenesis. The indicated constructs were exogenously expressed in HEK293T cells, the cell lysates were subject to immunoblots against indicated antibodies. (D) The mammalian cell expression vector Halotag-Flag-UBE2V2 (0-mer) or Halotag-Flag-UBE2V2-13mer (C13-mer) constructs were exogenously expressed in HEK293T cells, the cell lysates were subject to immunoprecipitation with indicated antibodies. The immunoprecipitated protein complex was subject to immunoblots against Rabbit anti-Flag antibody. LEGEND CONTINUES ON THE NEXT PAGE.*

(E) The mammalian cell expression vector Halotag-Flag-UBE2V2 (0-mer) or Halotag-Flag-UBE2V2-13mer (C13-mer) constructs were exogenously expressed in HEK293T cells, the cell lysates were subject to immunoprecipitation with purified 19-H12-B6 monoclonal antibody, the immunoprecipitated protein complex was competitively eluted with chemically synthesized elution peptide. (F) Silver staining of the eluted protein bands from immunoprecipitation.

Antibodies recognize HLA-E in immunoblot, flow cytometry, immunofluorescence, and immunohistochemistry

For recognition of HLA-E in cell lines, we used K-562 derivative cell lines where HLA-E was knocked out (K-562 KO), or where single chain HLA-E and beta-2-microgblobulin, complexed with the HIV Gag69 peptide, were reintroduced (K-562 HLA-E$^+$). For immunoblot, we transferred the lysate of $1x10^6$ K-562 HLA-E KO and K-562 HLA-E$^+$ cells to a PVDF membrane and blotted with purified mAb (19-H12) at 1 µg/mL using HRP-conjugated anti-mouse IgG as secondary detection agent. We noted a clear signal around 55kD, corresponding to the molecular weight of the HLA-E Gag69 trimer. The signal around 37kD corresponds to the heavy chain of HLA-E. Potentially, the β2-microglobulin got cleaved off (Figure 6A).

For flow cytometry, K-562 KO or K-562 HLA-E$^+$ cells were fixed with 4% (v/v) PFA and permeabilized with 0.1% (w/v) saponin as described in methods. Cells were stained with 19-H12-Cy5 and with the commercially available anti-HLA-E antibody 3-D12-PE as described in methods. Non-specific staining was low, as characterized by the signal from permeabilized cells stained with irrelevant antibodies (Supplementary figure 2). Fixed, permeabilized K-562 HLA-E$^+$ cells show a clear signal in the PE channel with the commercially available HLA-E antibody 3-D12, as well as in the Cy5 channel with the HLA-E specific antibody 19-H12. Permeabilized K-562 KO cells show no staining in either channel. Fixed, non-permeabilized cells were used as negative control for 19-H12, as the antibody binds an intracellular epitope. Non-permeabilized K-562 HLA-E$^+$ cells show a clear signal in the PE channel with 3-D12, but not in the Cy5 channel with 19-H12. The 19-H12 antibody thus specifically recognizes the cytoplasmic tail of HLA-E and is suitable for flow cytometry on permeabilized cells (Figure 6B). To control for non-specific intracellular retention of antibodies, we incubated permeabilized or non-permeabilized fixed K-562 HLA-E KO or HLA-E$^+$ cells with non-targeting antibodies (PE-conjugated murine IgG-kappa isotype control and Cy5-conjugated anti-HA.11 epitope tag). We see a negative signal in both the PE and Cy5 channel, confirming that any positive signal is not due to intracellular antibody retention (Supplementary figure 3)

To determine the suitability of 19-H12 in immunofluorescent imaging, we allowed K-562 KO or HLA-E$^+$ cells to adhere to the bottom of a plastic 12-well tissue culture plate. Cells were fixed with 4% PFA and either permeabilized with saponin or left intact and stained with 19-H12-Cy5 and 3-D12-PE. We observed a clear signal of the cell membrane with either antibody in the formalin-fixed, permeabilized K-562 HLA-E$^+$ cells, but not in similarly treated K-562 KO cells (Figure 6C). Cells that were fixed with formalin, but not permeabilized, showed clear staining of the cell membrane with 3-D12-PE but only little staining with 19-H12-Cy5, presumably due to some cellular damage inflicted by the small amount of methanol in the stock formaldehyde solution used for fixation. These results show that 19-H12 is suitable for immunofluorescence detection of HLA-E on the cell membrane.

For immunohistochemistry, we stained sections of healthy human tonsil and of a progressive non-muscle invasive bladder cancer (NMIBC) with 19-H12 or with the commercially available anti-HLA-E MEM-E/02. The tonsil sections show clear staining of stromal cells. We observe specific staining at 40-fold lower concentrations of 19-H12 antibody (0.25µg/mL), compared to the MEM-E/02 (10 µg/mL) (Figure 7A). Progressive or recurrent human bladder cancer is generally high in HLA-E expression and low in expression of HLA-A, -B, and -C[639]. Tissue sections from progressive NMIBC shows clear staining of cancer cells at low concentrations of 19-H12 antibody (0.25µg/mL) compared to MEM-E/02 (10 µg/mL) (Figure 7B). In both the healthy tonsil and the NMIBC sections, we observe clear staining of the cytoplasm, which we attribute to staining of HLA-E in the endoplasmic reticulum or in recycling endosomes.

Discussion

HLA-E plays a unique role in antigen presentation and target recognition by cytotoxic T cells. In humans, HLA-E is specialized in the presentation of peptides derived from the signal sequences of other Class I MHC products. The signal sequences of viral glycoproteins likewise contribute to the pool of HLA-E ligands. Antibodies against the ectodomain of HLA-E have been reported to display varying degrees of cross-reactivity with alleles of HLA-B and HLA-C[627]. An inspection of the sequences of HLA-E and its comparison with the sequences of other Class I MHC molecules shows that the amino acid sequence of the cytoplasmic tail of HLA-E is unique. In fact, assignment of a given Class I MHC sequence to a particular locus is most readily achieved by inspection of the cytoplasmic tail sequence.

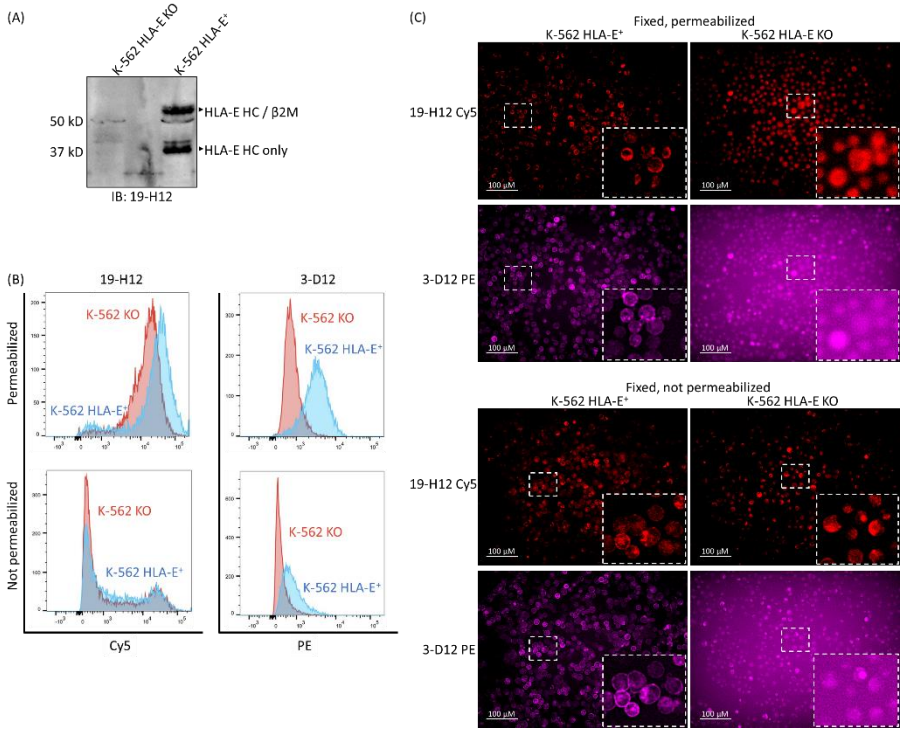

Figure 6. Cell-surface staining of HLA-E on K-562 HLA-E⁺ cells. *(A) We transferred the lysate of 1x10⁶ K-562 HLA-E KO or K-562 HLA-E⁺ cells to a PVDF membrane and blotted with purified 19-H12 mAb at 1 µg/mL. We used an HRP-conjugated anti-mouse IgG as secondary agent. We see a clear signal around 55kD, corresponding to the molecular weight of the single-chain trimer of the transfected HLA-E. The signal at ~37kD corresponds to the molecular weight of the heavy chain only. (B) K-562 KO or HLA-E⁺ cells were fixed and permeabilized and stained with PE-conjugated pan-HLA mAb 3-D12 or Cy5-conjugated HLA-E specific 19-H12. Fixed, non-permeabilized cells were used as negative control for 19-H12, as the antibody binds an intracellular epitope. Experiments were performed five times, representative data for one experiment shown here. (C) For immunofluorescence, K-562 KO or HLA-E⁺ suspension cells were adhered to a tissue culture 12-well plate by sedimentation through gravity in PBS. Cells were fixed with 4% PFA and either permeabilized with 0.1% saponin or left unpermeabilized. Cells were stained with a solution containing either 19-H12-Cy5 (1.25 µg/mL) or 3-D12-PE (2.7 µg/mL). Experiments were performed three times, representative data for one experiment shown here. Minor positive signal in the 19-H12 Cy5-stained HLA-E⁺ cells is explained by trace methanol in the formaldehyde stock solution, causing partial permeabilization of the cells.*

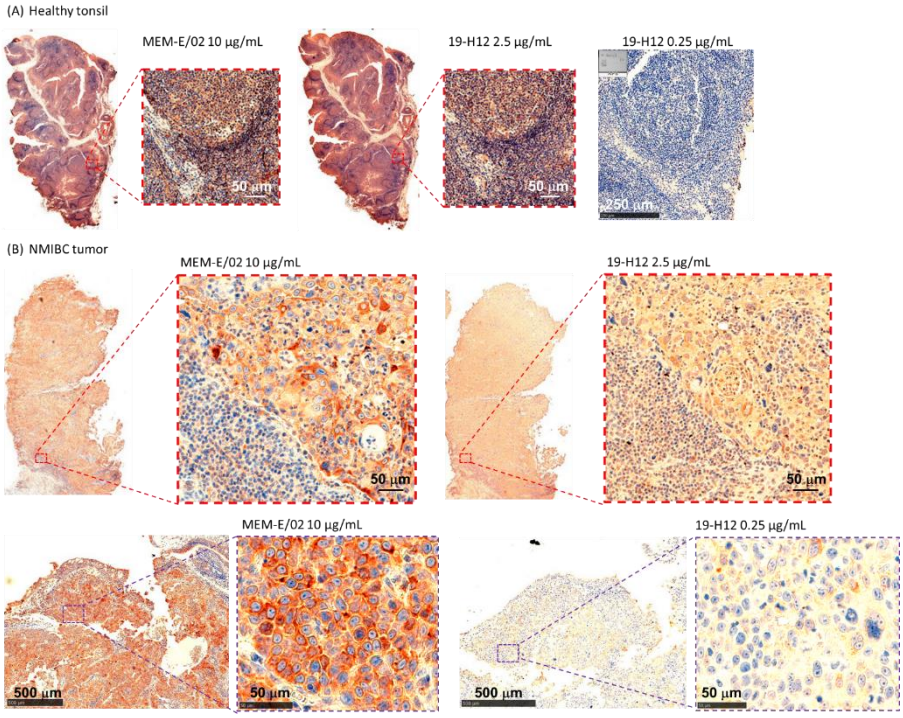

Figure 7. Immunohistochemistry on sections of human healthy tonsil and progressive non-muscle invasive bladder cancer (NMIBC). *(A) We stained sections of a healthy human tonsil with the 19-H12 antibody or the commercially available anti-HLA-E MEM-E/02. The tonsil sections show clear staining of stromal cells with 19-H12, even at lower concentration of antibody (0.25µg/mL), compared to MEM-E/02 (10 µg/mL). (B) Tissue sections from progressive NMIBC shows very clear staining of cancer cells at low concentrations of 19-H12 antibody (0.25µg/mL) compared to the standard concentration for MEM-E/02 (10 µg/mL).*

The cytoplasmic tail of class I MHC molecules is involved in trafficking peptide-bound MHC class I from the endoplasmic reticulum to the cell membrane. The cytoplasmic tail of HLA-E in particular plays a role in the internalization and the reduced stability and surface expression of peptide-bound HLA-E[281].

To target the cytoplasmic tail peptide of HLA-E (HLA-E_{tail}) for production of monoclonal antibodies, we designed an immunogen based on our prior observations that targeted delivery of antigens to antigen presenting cells elicits strong B and T cell immunity[420,466,558,640]. This is accomplished by fusing the antigen of interest to a nanobody that recognizes Class II MHC products. Fusions of this type can be obtained as genetic fusions or by a sortase-

catalyzed transpeptidation reaction, as was done here to create $VHH_{MHC\text{-}II}$-$HLAE_{tail}$. Screening of hybridomas was done by ELISA on a fusion of GFP-HLA-E_{tail}, also prepared by sortase-catalyzed transpeptidation. This ensured specificity for HLA-E_{tail} peptide in the ELISA assay, as the immunized mice were never exposed to GFP.

We obtained several hybridomas, all of which used the identical VDJ rearrangement for the Ig heavy chain locus, but with involvement of 3 distinct VJ kappa light chain rearrangements. Not surprisingly, this puts the weight of recognition of HLA-E_{tail} on the heavy chain CDRs. We mapped the epitope recognized within the HLA-E_{tail} to the core sequence SAQGSES. This core sequence was not sufficient to confer reactivity with any of the monoclonal antibodies with proteins carrying this minimal tag. Instead, we found that a 13-residue extended version of this core sequence was required for recognition in immunoblots. Given the strong reactivity of the antibody in immunoblots, this suggested its utility as an epitope tag. Indeed, by placing the WSDSAQGSESHSL sequence at the N- or C-terminus of a protein, or at an internal location, we confirmed retention of immunoreactivity with the antibody, independent of the placement of the tag. When running a search of the (W)(S)(D)SAQGSES(H)(S)(L) sequence against all available protein sequences, we found a hit only for HLA-E and its non-human primate homologs. Because in Class I MHC molecules this sequence is in the cytoplasm, use of the (W)(S)(D)SAQGSES(H)(S)(L) tag in extracellular proteins in cells of human or non-human primate origin would be possible. For all other species queried, no obvious cross-reactions of the (W)(S)(D)SAQGSES(H)(S)(L) tag with endogenous proteins is expected, thus expanding its utility.

Fluorescent labeling of antibodies is commonly done using NHS ester-fluorophores to target exposed lysine residues, or maleimide derivatives of fluorophores to target cysteine residues, either present endogenously or engineered into the antibody sequences at a particular site. These chemical modification strategies come with the attendant risk of placing fluorophores in the antibody's paratope, with possible loss of activity. Over-modification of antibodies with fluorophores can also result in an apparent loss of activity. The use of the sortase-catalyzed transpeptidation reaction ensures reproducibility, site-specificity, and produces the desired product in excellent yield, approximating >90% conversion. The sortase tags are located far away from the antigen binding site, thus minimizing the potential for loss of activity caused by the modification. While conventional methods for antibody detection, i.e., those involving the use of secondary antibodies, are

of course possible, direct modification with fluorophores or biotin eliminates the need for secondary antibodies. We modified the monoclonal antibody to carry the LPETG sortase motif on the C-termini of both heavy and light chains. The inclusion of a $(His)_6$-tag allows for easy purification after expression in EXPI-293T cells. Using 7M sortase, we successfully installed 4 moles of biotin or Cy5 on the mAb. We have shown the functionality of the modified HLA-E_{tail} specific mAb for cell staining in immunoblot, immunofluorescence, and flow cytometry.

We have also shown the use of this mAb in immunohistochemistry of paraffin embedded, formalin-fixed patient tumor samples, at much lower concentrations compared to the MEM-E/02 antibody, suggesting the use of this mAb as a possible diagnostic tool in the clinic to detect HLA-E.

In conclusion, we have developed a monoclonal antibody that targets the cytoplasmic tail of HLA-E and thus we suspect no cross-reactivity to other Class I MHC molecules. This antibody can be used diagnostically for staining HLA-E on patient (tumor) samples, as an antibody-epitope tag for extracellular proteins, and to further the research to the role of the cytoplasmic tail on HLA-E trafficking from the ER and to endosomes.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

The authors confirm their contribution to the paper as follows: E.R.V and H.L.P designed the study and supervised data collection. E.R.V performed hybridoma analysis, single-cell cloning, and downstream applications with the monoclonal antibody. J.G. collected data on epitope mapping and immunoprecipitation with the 13-mer. S.B. and M.D.S. assisted in producing the hybridomas. Z.L. and A.H. performed immunohistochemistry staining. E.R.V. and H.L.P. wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgements

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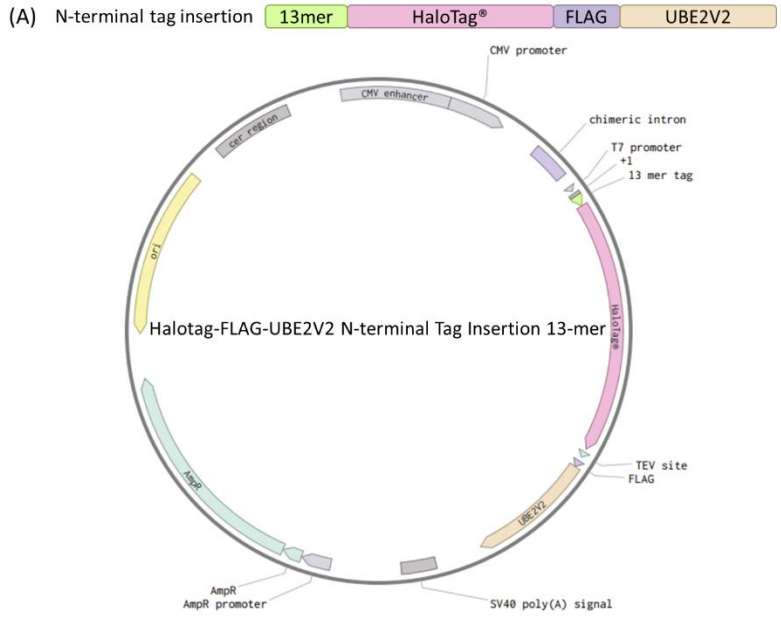
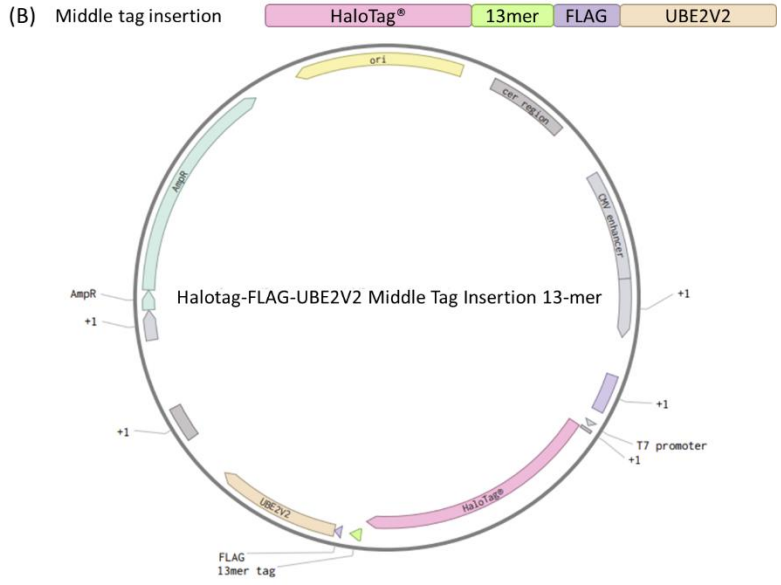
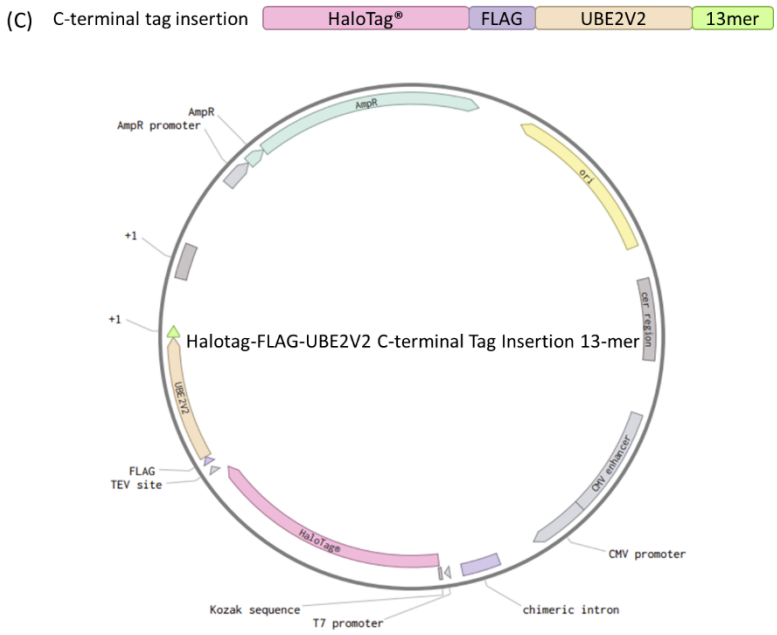
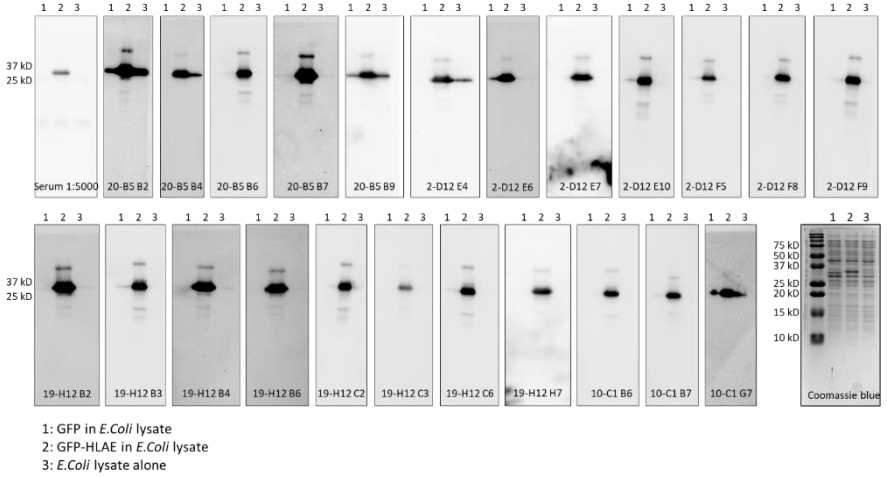
Supplementary figures





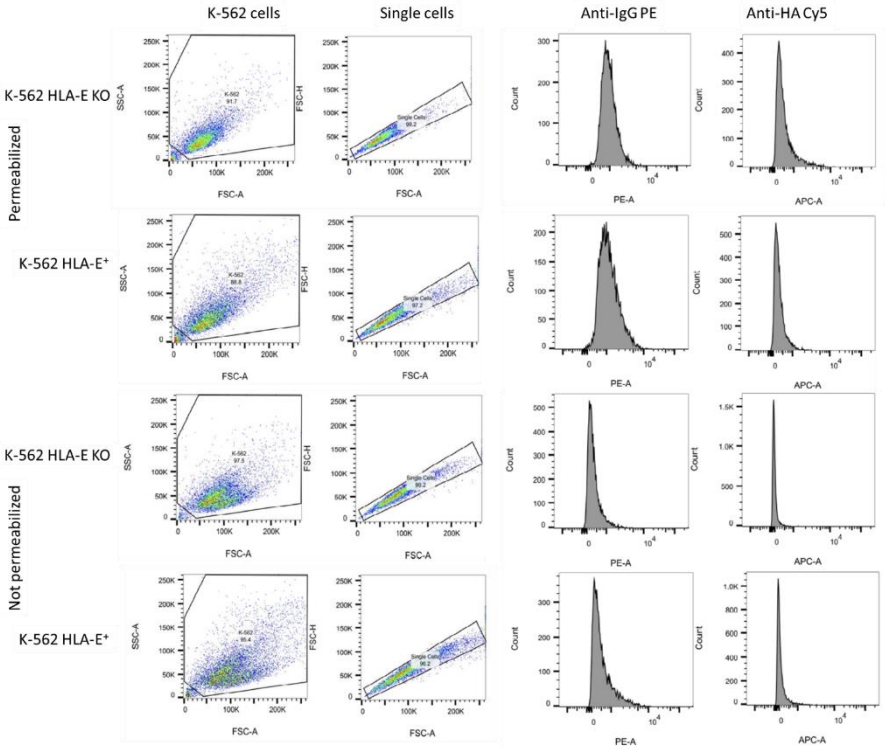


Supplementary figure 1. Schematic overview and plasmid map of the HaloTag-FLAG-UBE2V2 vector. *We inserted the 13-mer epitope sequence (WSDSAQGSESHSL) in the N-terminus (A), middle (B) or C-terminus (C) of the protein complex.*

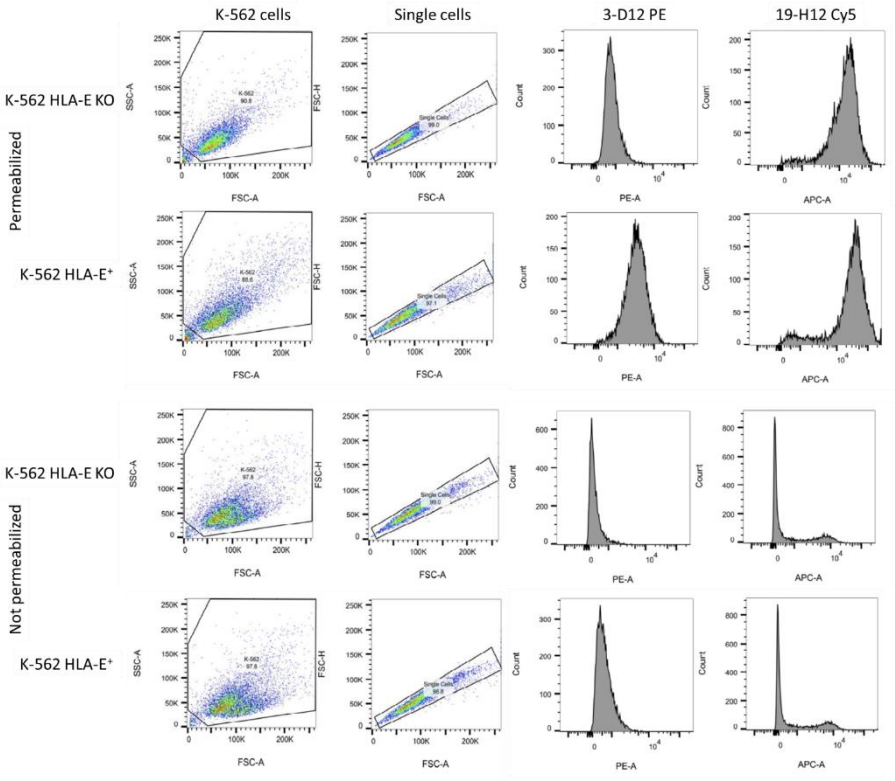


1: GFP in *E.Coli* lysate
2: GFP-HLAE in *E.Coli* lysate
3: *E.Coli* lysate alone

Supplementary figure 2. Western blot on GFP and GFP-HLAE$_{tail}$ for all hybridoma clones. *Per lane, 500 ng of protein (lane 1: GFP-LPETG-His6, lane 2: GFP-HLA-E$_{tail}$) was loaded. To prevent non-specific signal, proteins were mixed with unrelated E. Coli whole cell lysate (WCL, lane 3). Supernatant of hybridoma clones was used as primary staining agent and HRP-linked anti-mouse IgG (H+L) antibody (0.3 µg/mL, Invitrogen) was used as secondary agent. To verify loading, a coomassie blue gel stain was made. To verify a positive signal, serum from the immunized mouse was used at 1:5000 dilution. All the clones recognized the fusion protein in lane 2. The non-specific signal in lane 1 and 3 of some blots are spillover of the proteins into the neighboring lanes.*



Supplementary figure 3. K-562 cells stained with negative control antibodies. *To control for non-specific intracellular retention of antibodies, we incubated permeabilized or non-permeabilized fixed K-562 HLA-E KO or HLA-E+ cells with non-targeting antibodies (PE-conjugated murine IgG-kappa isotype control (Biolegend, 1 µg/mL) and Cy5-conjugated anti-HA.11 epitope tag (Biolegend, 1 µg/mL). We see a negative signal in both the PE and Cy5 channel, confirming that any positive signal is not due to intracellular antibody retention.*



Supplementary figure 4. Gating strategy for flow cytometry on K-562 HLA-E KO and HLA-E⁺ cells. *We fixed cells with 4% (v/v) PFA in PBS and either permeabilized cells with 0.5% (w/v) saponin in PBS or left cells intact for the non-permeabilized control. Cells were stained with 2.7 µg/mL 3-D12-PE (Biolegend) or 1.25 µg/mL 19-H12-Cy5 in 1% (w/v) BSA in PBS. For flow cytometry, we selected cells based on FSC-A and SSC-A1 and selected singled based on FSC-H and FSC-A. Positive signal coming from staining with the 3-D12-PE antibody was measured in the PE channel. Positive signal coming from staining with the 19-H12-Cy5 antibody was measured in the APC channel.*

Target	Usage	Sequence (5' -> 3')
IgG1	Reverse primer for IgG1 amplification	ATAGACAGATGGGGGTGTCGTTTTGGC
IgG2A	Reverse primer for IgG2A amplification	CTTGACCAGGCATCCTAGAGTCA
IgG2B	Reverse primer for IgG2B amplification	AGGGGCCAGTGGATAGACTGATGG
IgG3	Reverse primer for IgG3 amplification	AGGGACCAAGGGATAGACAGATGG
HC FR1 HD1	Forward primer for HC amplification (high degenerate)	SARGTNMAGCTGSAGSAGTC
HC FR1 HD2	Forward primer for HC amplification (high degenerate)	SARGTNMAGCTGSAGSAGTCWGG
HC FR1 LD1	Forward primer for HC amplification (low degenerate)	CAGGTTACTCTGAAAGWGTSTG
HC FR1 LD2	Forward primer for HC amplification (low degenerate)	GAGGTCCARCTGCAACARTC
HC FR1 LD3	Forward primer for HC amplification (low degenerate)	CAGGTCCAACTVCAGCARCC
HC FR1 LD4	Forward primer for HC amplification (low degenerate)	GAGGTGAASSTGGTGGAATC
HC FR1 LD5	Forward primer for HC amplification (low degenerate)	GATGTGAACTTGGAAGTGTC
LC Kappa	Reverse primer for Kappa LC amplification	GGATACAGTTGGTGCAGCATC
LC Kappa FR1	Forward primer for Kappa LC amplification (high degenerate)	GAYATTGTGMTSACMCARWCTMCA

Supplementary Table 1. *Primer sequences for PCR amplification of hybridoma cDNA HC (IgG1, IgG2A, IgG2B, IgG3) and LC (Kappa). A mixture of low and high degenerate primers was used.*

Target	Usage	Forward primer (5' -> 3')	Reverse (5' -> 3')
Halo-Tev-Flag-Ube2v2	C8mer	GCACAAGGATCTGAATCCCACtaaacgaattcg ggctcggtaccc	GGATTCAGATCCTTGTGCGCTattgttgtatgttt gtccttctggtggc
Halo-Tev-Flag-Ube2v2	C10mer	GCACAAGGATCTGAATCCCACAGCtaaacgaa ttcgggctcggtaccc	GGATTCAGATCCTTGTGCGCTGTCattgttgtat gtttgtccttctggtggc
Halo-Tev-Flag-Ube2v2	C13mer	GCACAAGGATCTGAATCCCACAGCCTAtaaac gaattcgggctcggtaccc	GGATTCAGATCCTTGTGCGCTGTCGCTCCAat tgttgtatgtttgtccttctggtggc
Halo-Tev-Flag-Ube2v2	N13mer	GCACAAGGATCTGAATCCCACAGCCTAgaaa tcggtactggctttccattcg	GGATTCAGATCCTTGTGCGCTGTCGCTCCAtg ccatggtggctttgctagc
Halo-Tev-Flag-Ube2v2	M13mer	GCACAAGGATCTGAATCCCACAGCCTActgta ctttcagagcgataacgcg	GGATTCAGATCCTTGTGCGCTGTCGCTCCAct cagtggttggctcgccg

Supplementary table 2. *Primer sequences for PCR amplification of Halo-Tev-Flag-Ube2v2 to insert the amino-acid sequences of the 8mer, 10mer, or 13mer (capitalized) into the C-terminus, N-terminus, or middle of the Ube2v2 protein (lowercase).*