Supporting Information

Materials and Methods

Cell culture
HAP1 (HLA-A*02:01; HLA-B*40:01; HLA-C*03:04) and HEK293T cell lines were cultured at 37°C and 5% CO₂ in IMDM (Gibco) supplemented with 10% FCS and antibiotics (PenStrep; Invitrogen). CD8⁺ T cell clones recognizing peptides derived from the endogenously expressed proteins USP11 and SSR1[1],[2] were expanded using irradiated feeder cells (EBV-LCLs and PBMCs irradiated using 50 and 30Gy, respectively) in IMDM supplemented with antibiotics, 5% human serum (Sanquin), 5% FCS, 120U/mL IL-2 (Chiron), 0.8µg/mL PHA (HA16 Remel™, ThermoFisher) as previously described in more detail [3].

Genome editing and overexpression
The near-haploid HAP1 cell line is disomic for a portion of chromosome 15 (61.105.000-89.890.000) [4]. The genes B2M and ERp57 (PDIA3) are encoded on chromosome 15 but not in the disomic region (44.711.487 – 44.718.877 and 43.746.410 – 43.733.278, respectively). To minimize the chance of off-target effects, we selected gRNAs with the lowest off-target score and without off-target sites in coding regions using the CRISPR.mit.edu tool (now retired)[5]. gRNAs used for gene editing are listed in Supporting Information Table I. pX458 (Addgene)[6] containing a B2M targeting gRNA was kindly provided by Dr. R. Mezzadra. ERp57 and tapasin targeting gRNAs in pX330 (Addgene)[6] were generated by in frame integration of a blasticidin-resistance gene after co-transfection of pX330 with the TIA-2Ablast plasmid as previously described[7]. A gRNA targeting a conserved region in HLA-A, -B and -C (and -G) in a pX330 vector was transfected into HAP1 cells using X-tremeGENE (Sigma). To obtain knockout clones for HLA-A, -B and -C, single cells were FACS sorted based on W6/32 negativity. To improve knockout efficiency, we switched to lentiviral vectors for the remaining targets. gRNAs targeting CNX, ERAP1, CALR and GlIα in pLentiCRISPRv2 (Addgene)[8] and a gRNA targeting TAP1 in pL.CRISPR.efs.GFP (Addgene)[9] were co-transfected with packaging plasmids psPAX2 and pVSVg, and pAdVAntage (Promega) using polyethylenimine (PEI; Polyscience) into HEK293T cells for virus production. The polyclonal cell lines were independently generated using the same targeting gRNA as was used for the clonal cell lines knockout for HLA-I, CNX, GlIα, tapasin, ERp57, TAP1 or ERAP1 (Supporting Information Figure 3), but then in pLentiCRISPRv2 as described above. The gRNA GAGTAGCGCGAGCACAGCTA in pLentiCRISPRv2 was used to generate a polyclonal cell line in which B2M was targeted. Data of the TAP2- and CALR-targeted polyclonal cell lines were obtained prior to single cell cloning. All polyclonal cell lines were puromycin-selected before analysis by flow cytometry. Puc2CL6IP HLA-B*27:05/09 constructs (kind gift of Dr. S.
Springer[10]) were co-transfected using PEI with packaging plasmids pVSVg and NLBH into HEK293T cells for virus production. Plasmids encoding other HLA alleles contained a ∆NGFR marker gene (viral supernatants were a kind gift of Dr. M Griffioen) [11]. Viral supernatant was filtered and used for transduction by spinoculation in the presence of 8µg/mL protamine sulfate. Several genome-edited cells were selected using puromycin or blasticidin S, before limiting dilution cloning. TAP1 knockout cells were enriched by FACS sort on GFP positivity before limiting dilution cloning.

**Sanger sequencing**

Genomic DNA was isolated using the NucleoSpin Tissue kit (Macherey-Nagel). DNA was amplified and sequenced using BigDye v1.1 (Applied Biosystems) and specific primers listed in Supporting information Table II.

**Immunoblotting**

Proteins were separated by SDS-PAGE (Bis-Tris NuPAGE 12% gel (CALR immunoblot, 10% Bis-Tris NuPAGE gel), ThermoFisher) and transferred to a PVDF membrane (Amersham Hybond-P, GE Healthcare) (CALR, nitrocellulose, Invitrogen) at 25V for 12min by using the semidyry Trans-Blot Turbo (BioRad) system (CALR, iBlot2 semi-dry blotting system, Invitrogen). Membranes were blocked for 1h with 3% (w/v) non-fat milk powder in PBS/0.1% (v/v) Tween (PBST) (CALR, 10% Roche WBR in PBST) and incubated with primary antibody (Supporting Information Table III) overnight (CALR, 1h) at 4°C. After washing three times in PBST, the membrane was incubated with secondary antibody for 1h RT and washed again in PBST. Blots were incubated with Clarity Western ECL reagent (BioRad) (CALR, Pierce ECL plus, ThermoFisher Scientific), the signal was detected with a Lumi-Imager (Vilber, Fusion FX) (CALR, Image Lab software, Bio-Rad) and analyzed with Evolution-Capt Edge software.

**Flow cytometry**

Cells were incubated with specific antibodies diluted in PBS (Supporting Information Table III) on ice for 30min. Unconjugated antibodies were washed away in three times before secondary stain. Stained cells were sorted on a BD Aria II or fixed in PBS/1% (v/v) formaldehyde/1µM DAPI (Sigma-Aldrich) and analyzed by BD flow cytometers (Fortessa or LSR II). Data was analyzed using FlowJo (Tree Star, Inc). Events were gated on DAPI negativity, single cells, time and when applicable ∆NGFR reporter expression (Supporting Information Fig. 4).

**IFN-γ stimulation**

Cells were cultured with 0, 10, 20, 40 or 80 U/mL IFN-γ (PeproTech) for 1d or 2d before analysis by qPCR and FACS, respectively.
qPCR
RNA extraction and qPCR was performed as described previously[12]. Gene expression was determined using SYBR green and the StepOnePlus (ThermoFisher). Quantification was done using the ΔΔC_T method. 18S rRNA expression was used as an internal reference, primers are listed in Supporting Information Table II.

Pulse-chase
Cells were incubated with 100U/mL IFN-γ (BioLegend) for 24h, pulse-chase was performed as previously described[13]. Cells were metabolically labeled with 0.2mCi/mL for 30min, lysed and cleared from membrane debris at 16,200×g at 4°C for 30min. Lysates were incubated with W6/32 or MaP.ERp57 (Abcam) at 4°C for 1h in an overhead tumbler before retrieving immune complexes with protein G Sepharose (GE Healthcare). Beads were washed and complexes were treated with Endoglycosidase H (New England Biolabs) according to manufacturer’s protocol. Prior to loading on a 10-14% gradient SDS-PAGE gel, complexes were dissociated at 95°C in sample buffer (150 mM DTT).

Mass spectrometry of HLA-class I peptides.
335×10^6 cells were lysed in 3.3mL lysis buffer (50mM Tris-Cl pH 8.0, 150mM NaCl, 5mM EDTA, 0.5% Zwittergent 3-12 and protease inhibitor (Complete, Roche Applied Science) at 0°C for 2h [14]. Lysates were successively centrifuged at 2500×g for 10min and at 31,000×g for 45min to remove nuclei and other insoluble material, respectively. Lysates were passed through a 100µL CL-4B Sepharose column (in a standard yellow tip equipped with a filter) to preclear the lysate and subsequently passed through a 100µL column containing 250µg pan class I (W6/32) IgG coupled to protein A Sepharose[14]. The W6/32 column was subsequently washed with 500µL lysis buffer, 500µL of low salt buffer (20mM Tris-Cl pH 8.0, 120mM NaCl), 200µL of high salt buffer (20 mM Tris-Cl pH 8.0, 1 M NaCl), and finally with 500µL of low salt buffer. HLA α chain, B2m and peptides were eluted with 500µL of 10% acetic acid, diluted with 1µL of 0.1% TFA and purified by SPE (Oasis HLB, Waters) by sequential elution with 10%, 17.5% and 25% and 30% acetonitrile in 0.1% TFA to remove HLA protein chains.

Peptides were lyophilized, dissolved in 95/3/0.1% (v/v/v) water/acetonitrile/formic acid (WAFA) and analyzed by online C18 nanoHPLC MS/MS with a system consisting of an Ultimate3000nano gradient HPLC system (Thermo, Bremen, Germany) and an Exploris480 mass spectrometer (Thermo). Fractions were injected onto a cartridge precolumn (300µm × 5mm, C18 PepMap, 5µm, 100Å) and eluted via a homemade analytical nano-HPLC column (50cm × 75µm; Reprosil-Pur C18-AQ 1.9µm, 120Å (Dr. Maisch, Ammerbuch, Germany)). The gradient was run from 2% to 36% (v/v) solvent B (20/80/0.1 (v/v/v) WAFA) in 120min. The nano-HPLC column was drawn to a tip of ~10µm and acted as the electrospray needle of the
MS source. The MS was operated in data-dependent MS/MS mode for a cycle time of 3s, with an HCD collision energy at 30V and recording of the MS2 spectrum in the orbitrap, with a quadrupole isolation width of 1.6Da. In the master scan (MS1) the resolution was 60,000, the scan range 300-1,500, at an AGC target of 1,000,000 at maximum fill time of 120ms. A lock mass correction on the background ion m/z=445.12 was used. Precursors were dynamically excluded after n=1 with an exclusion duration of 45s, and with a precursor range of 20ppm. Charge states 1-3 were included. For MS2, the first mass was set to 110Da, and the MS2 scan resolution was 30,000 at an AGC target of 100,000 at maximum fill time of 120ms.

Raw data were first converted to peak lists using Proteome Discoverer version 2.2 (Thermo Electron), and submitted to the Uniprot Homo sapiens minimal database (20,205 entries), using Mascot v2.2.04 (www.matrixscience.com) for protein identification (10ppm precursor, 0.02Da deviation of fragment mass, no enzyme was specified). Methionine oxidation and cysteinylation on cysteine were set as a variable modification. Peptides with an FDR<1% in combination with a mascot ion score >35 were accepted. Next, peptide affinities were predicted using NetMHC4.0 for HLA-A*02:01, HLA-B*40:01 and HLA-C*03:03, which contains R91G compared to the HAP1-expressed HLA-C*03:04), and has a comparable binding motif as the expressed HLA-C*03:04[15]. Peptides were assigned to the allele for which it was predicted to have the highest affinity. All peptides with a predicted affinity above 5µM for all three alleles were discarded. Peptide sequence clustering was performed for all 9-mers using GibbsCluster-2.0 and Seq2Logo.

T cell assays

50,000 target cells were incubated with T cells in a 1:1 ratio for 18h as previously described[16]. IFN-γ or GM-CSF release in the cell culture supernatant was measured using ELISA according to the manufacturer’s protocol (Sanquin (now Diaclone) and BioLegend, respectively).

Statistical analysis

Statistical testing was done by a mixed-effects analysis followed by Dunnett’s multiple comparisons test on the normalized data (GraphPad Prism).
Supporting Information References

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Supporting Information Table I. Details of CRISPR/Cas9 mediated genome editing of PAKC clones

| Targeted gene(s) | gRNA                  | Vector used          | Resistance   | Clone name | Intact aa / (total aa) |
|------------------|-----------------------|----------------------|--------------|------------|------------------------|
| HLA-A*02:01      | CGGCTACTACACACAGACCG  | pX330                | none         | #66        | 111 (365)              |
| HLA-B*40:01      | CNX GTGGTGCTCTGTATGTAC | pLentiCRISPRv2       | puromycin    | #3F5       | 9 (592)                |
| HLA-C*03:04      | GIIα GAACAGTGGGAGTTAAACCA | pLentiCRISPRv2     | puromycin    | #A10       | 139 (966)              |
|                  | B2M CGTGAGTAAACCTGAATCTT | pX458                | none         | #14.2      | 26 (119)               |
|                  | CALR GTGTTTGGATTCGATCCAGC | pLentiCRISPRv2       | puromycin    | #1F9       | 34 (417)               |
| Tapasin          | Tapasin GTGAGGAATCGAGCGGAA | pX330                | blasticidin  | #B         | 34 (448)               |
| ERp57            | ERp57 GTGGTGAATCTAGCAGT | pX330                | blasticidin  | #B         | 26 (505)               |
| TAP1             | TAP1 ACTGCTACTTCTGGGACT | p.L.CRISPR.efs.GFP  | none         | #1         | 48/91-808 (808)        |
| TAP2             | TAP2 CTGGTGGGCTACGGGCGTCG | pLentiCRISPRv2     | puromycin    | #18        | 108 (686)              |
| ERAP1            | ERAP1 GGTGCCCATCATACGT | pLentiCRISPRv2       | puromycin    | #2C6       | 40 (941)               |
| Targeted gene | Forward primer | Reverse primer | Sequencing primer |
|--------------|----------------|----------------|-------------------|
| HLA-A        | TTCTTCACATCCCTGGTCCCG | TTCCCTCCCTCAGGACCAG | TCCCAATTGGCTCCCCTCCT |
| HLA-B        | CCGGGAGACACAGATCTCAA | CAGCTTGCTCTCCCGGGTTCT | GAGCCACTCCAGCACTC |
| HLA-C        | ACTTCATCGCAGTGGCTAC | GGATCTCAGACCGGGAGACT | ACACAGAAGTACAAGCCCA |
| CNX          | CTCTAGGCTGCCCTTCTTTATCT | GCACC GGGTATCTCTCTATAA | AAGTCTTTTTTAGCTTCGGATT |
| GIIα         | ATGCTTGGGTCTGTTTCTGG | GCCCTCTGATGCTCAAATCT | TTCCCCGGGTCTCTAAATG |
| B2M          | GGGAGAAATCGATGACCAAA | ATGTGTTGTCGAAATGCTGC | GGGAGAAATCGATGACCAAA |
| CALR         | CCGAGGATCTCTCTGAAGCCAC | GTCCTACCGTGAAACTGACCC | GCCGACAGACATAAAGCCG |
| Tapasin      | TCGCCCAAGAAGTGGGAA | CGACATGGGTCTTGTGTCCT | TGGTGATCTTCCTAGTGGCG |
| ERp57        | ATCGGGGCAATGAGTGGAG | GAATGAGAAATGTGCCGGCC | GAATGAGAAATGTGCCGGCC |
| TAP1         | CAGCCTGTGCTCTCTGGACCTT | ACTGACAAAGAGGGCGGTAG | GCTCCCCATGAGATCAGTC |
| TAP2         | GCCTGAAGGGCGCTAGAAT | ATTTTGGGAGACTGCTG | TGGAGTTAGGAAAGTGAAGACC |
| ERAP1        | CTCTTCCACGCCCTTTCTCT | GTGTTTCTGGCCTCATAATG | TACTTTCGTGGTCCTCCAGA |
| HLA-I qPCR   | TCTCCAGAGGGCACCACCA | TCTTACCTGCAGGAGATCACA | - |
| 18S qPCR     | CGGCTACCACATCCAGGAA | GCTGGAGATTACCGGGCT | - |
Supporting Information Table III. Antibodies used for immunoblotting and flow cytometry

| Antibody          | Species   | Target       | Manufacturer                                      |
|-------------------|-----------|--------------|--------------------------------------------------|
| HC10              | Mouse     | HLA-I        | Kindly provided by Dr. J. Neefjes                |
| Calnexin polyclonal | Rabbit   | CNX          | Stressgen Biotechnologies (SPA-865)              |
| B2M polyclonal    | Rabbit    | B2M          | Sigma-Aldrich (HPA006361)                        |
| Calreticulin polyclonal | Rabbit  | CALR        | Sigma-Aldrich (C4606)                            |
| 7F6               | Rat       | tapasin      | Self[17]                                         |
| MaP.Erp57         | Mouse     | ERp57        | Abcam                                            |
| 148.3             | Mouse     | TAP1         | Self[18]                                         |
| ARTS1 polyclonal  | Rabbit    | ERAP1        | Thermofisher Scientific (PA5-103065)             |
| AC-74             | Mouse     | β-actin      | Sigma-Aldrich                                    |
| DM1A              | Mouse     | α-tubulin    | Sigma-Aldrich                                    |
| Anti-Mouse-HRP polyclonal | Goat | Mouse antibody | Sigma-Aldrich                                    |
| Anti-Rabbit-HRP polyclonal | Goat | Rabbit antibody | Sigma-Aldrich                                    |
| Anti-Rat-HRP polyclonal | Goat | Rat antibody | Sigma-Aldrich                                    |
| W6/32-PerCP-eFluor710 | Mouse, IgG | HLA-A, -B, -C | eBioscience                                      |
| VDK1D12           | Human, IgM| HLA-A*01:01  | Self[19]                                         |
| OK2F3             | Human, IgM| HLA-A*03:01  | Self[19]                                         |
| BRO11F6           | Human, IgG| HLA-A*11:01  | Self[20]                                         |
| MUS4H4            | Human, IgG| HLA-A*24:02  | Self[20]                                         |
| WAR5D5            | Human, IgG| HLA-B*07:02  | Self[20]                                         |
|                   |           | HLA-B*27:05  |                                                  |
|                   |           | HLA-B*27:09  |                                                  |
| GVK4H11           | Human, IgM| HLA-B*15:01  | Self                                              |
|                   |           | HLA-B*35:01  |                                                  |
| DK7C11            | Human, IgG| HLA-B*44:03  | Self[20]                                         |
| MH16-1-DyLight650 | Mouse, IgG| Human IgG    | Sanquin, self-conjugated (Thermo)                |
| MHM-88-APC        | Mouse, IgG| Human IgM    | BioLegend                                         |
| ME20.4-PECy7      | Mouse, IgG| Human NGFR   | BioLegend                                         |
Supporting Information Figure 1

| KO Type | Chromosome A | aa | Frameshift | Chromosome B | aa | Frameshift |
|---------|--------------|----|-----------|--------------|----|-----------|
| CNX KO #3F5 | Chromosome A | aa26 | Frameshift | Chromosome B | aa25 | -19bp |
| B2M KO #14.2 | Chromosome A | aa34 | Frameshift | Chromosome B | aa25 | -19bp |
| CALR KO #1F9 | Chromosome A | aa34 | Frameshift | Chromosome B | aa25 | -19bp |
| ERp57 KO #8 | Chromosome A | aa26 | Blasticidin resistance gene | Chromosome B | aa25 | -19bp |
| TAP1 KO #1 | Chromosome A | aa40 | Frameshift | Chromosome B | aa39 | -7bp |
| TAP2 KO #18 | Chromosome A | aa40 | Frameshift | Chromosome B | aa39 | -7bp |
| ERAP1 KO #2C6 | Chromosome A | aa40 | Frameshift | Chromosome B | aa39 | -7bp |
Supporting Information Figure 1. Sanger sequencing data of the clonally derived cell lines in PAKC reveals specific targeted gene disrupting DNA mutations.

Sanger sequences of the genomic DNA around the Cas9 targeted site for each clonal cell line in PAKC. Sequencing data of the HLA-I KO, GIα KO and tapasin KO have been published elsewhere (Jongsma et al., submitted)[21]. For each clone, the wild type and knockout sequence(s) are shown. For blasticidin-resistance gene insertions or mixed sequences due to diploidy of the cells, a graphical summary is shown above the sequences (upper sequence annotation refers to chromosome A and lower to chromosome B). The number of amino acids (aa) translated before the disrupting mutation are depicted, underlined are translation start sites, green squares highlight base pair insertions. WT, wild type; KO, knockout.
Supporting Information Figure 2. Individual PAKC clones lack protein expression by the targeted gene.

Raw immunoblot data of which sections are presented in Figure 1C. (A) Immunoblots of TAP1, tapasin, ERp57, HLA-I, B2M, ERAP1 and calnexin for their respective knockout cell lines with an β-actin loading control. n=1 (B2M and ERAP1) or representative for n=2 independent experiments. (B) Immunoblot for calreticulin in wild type and calreticulin knockout cells with an α-tubulin loading control. Blot is representative of n=2 independent experiments.
Supporting Information Figure 3

Normalized count

WT  Transduced  Unstained

HLA-I KO  CNX KO  Glia KO  B2M KO  CALR KO

ERp57 KO  Tapasin KO  TAP1 KO  TAP2 KO  ERAP1 KO

WT

Transduced

Unstained

10^1  10^2  10^3  10^4  10^5

10^1  10^2  10^3  10^4  10^5

10^1  10^2  10^3  10^4  10^5

10^1  10^2  10^3  10^4  10^5

10^1  10^2  10^3  10^4  10^5

10^1  10^2  10^3  10^4  10^5

W6/32 (HLA-I)
Supporting Information Figure 3. The effect of gRNAs targeting APM components on surface HLA-I expression on HAP1 cells.

Total HLA-I surface expression (W6/32) of each polyclonal cell line was analyzed by flow cytometry after puromycin selection of transduced cells. Wild type (WT) in blue, cells transduced with a gRNA/Cas9 vector targeting the indicated gene in orange, unstained control in gray. n=1 (TAP2 and CALR) or representative for n=2 independent experiments.
Supporting Information Figure 4

Live cells

DAPI

anti-NFGR PE-Cy7

Single Cells

Single Cells Time

When applicable, NGFR+ cells
Supporting Information Figure 4. Gating strategy.

For all flow cytometric analyses, HAP1 cells were gated for DAPI negativity, single cells were selected by SSC-H vs SSC-W followed by FSC-H vs FSC-W. A time vs PE gate was used to exclude clogging artefacts. If applicable, ΔNGFR marker gene positive cells were selected by ME20.4-PE-Cy7 staining of the ΔNGFR receptor.