Alternative HLA class-I peptide presentation in processing deficient tumors

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CD8 T-cell responses against TAP-inhibited cells are readily detected in the human population

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Abstract

Target cell recognition by Cytotoxic T Lymphocytes (CTL) depends on the presentation of peptides by Human Leukocyte Antigens class I (HLA-I) molecules. Tumors and herpes viruses have adopted strategies to greatly hamper this peptide presentation at the important bottleneck, the peptide transporter TAP. Previously, we have described the existence of a $CD8^+$ CTL subpopulation that selectively recognizes such TAP-deficient cells in mouse models. Here, we show that the human counterpart of this CTL subset is readily detectable in healthy subjects. Autologous PBMC cultures were initiated with dendritic cells rendered TAP-impairment by gene transfer of the viral evasion molecule UL49.5. Strikingly, specific reactivity to B-LCLs expressing one of the other viral TAP-inhibitors (US6, ICP47 or BNLF2a) was already observed after three rounds of stimulation. These short term T-cell cultures and isolated $CD8^+$ CTL clones derived thereof did not recognize the normal B-LCL, indicating that the cognate peptide-epitopes emerge at the cell surface upon an inhibition in the MHC class I processing pathway. A diverse set of T-cell receptors were used by the clones and the cellular reactivity was TCR-dependent and HLA-I-restricted, implying the involvement of a broad antigenic peptide repertoire. Our data indicate that the human $CD8^+$ T-cell pool comprises a diverse reactivity to target cells with impairments in the intracellular processing pathway and that these might be exploited for cancers that are associated with such defects and for infections with immune-evading herpesviruses.

Introduction

$CD8^+$ Cytotoxic T-Lymphocytes (CTLs) have an important role in the immune response against infections and outgrowth of tumors. They recognize antigenic peptides presented at the cell surface by class I molecules of the Major Histocompatibility complex (MHC-I). Inhibition of the intracellular MHC-I antigen presentation pathway is therefore an efficient strategy to avoid CTL recognition. Indeed, both tumors and viruses are able to escape CTL-mediated immune control through MHC-I down modulation [1-6]. Several mechanisms have been described that lead to loss of peptide presentation and these involve targeting of the MHC class I heavy chain, the β2-microglobulin chain, and components of the peptide loading complex (PLC). One common target in the PLC that results in efficient impairment of MHC-I presentation is the Transporter associated with Antigen Processing (TAP), which transports peptides generated by the proteasome from the cytosol into the Endoplasmic Reticulum (ER). Deficiency in TAP expression is frequently found in human cancers and several viral proteins have been indentified that bind to TAP and block its peptide transport function [2,7]. As the heterodimeric TAP1/TAP2 proteins constitute the bottleneck of the MHC-I processing pathway, a blockade at this step results in efficient and total decrease of antigen presentation at the cell surface, and as a consequence, resistance to antigen-specific CTL.

Within the family of *Herpesviridae* four variants of viral TAP inhibitors have been characterized. Herpesviruses cause life-long latent infections in their hosts, and occasionally reactivate. Therefore, immune evasion is an important survival strategy. UL49.5 is a protein encoded by herpesviruses belonging to the genus *Varicellovirus*. In this paper the bovine herpesvirus-1 (BHV-1) variant of UL49.5 is used, which efficiently inactivates TAP by arresting it into a translocation-incompetent state, and, moreover, promotes the degradation of TAP [8-10]. The TAP inhibitor ICP47 is found in herpes simplex virus type I and II and binds in a stable fashion to the cytosolic site of the TAP complex, thereby acting as a high-affinity competitor for peptide binding [11-14]. The human cytomegalovirus protein US6 is an ER resident protein, which prevents ATP binding to TAP and thereby blocking peptide transport [15,16]. Recently a new TAP inhibitor was indentified in Epstein-Bar Virus (EBV), BNLF2a, which prevents both peptide- and ATP binding to TAP [17,18].

TAP impairment is found in a broad range of tumor types with a frequency between 10-74%, and is regularly found in metastatic leasons [1,3]. It is the most frequent cause of decreased expression of Human Leukocyte Antigens class I (HLA-I), the MHC-I in man, and is associated with a poor prognosis [1,19,20]. Loss of antigen presentation as a result of TAP down regulation leads to failure of recognition and elimination by tumor-specific CTLs [21-24]. Interestingly, the residual peptides that are still presented at the surface of TAP-impaired cells are dramatically different from the normal peptide repertoire. In 1992, two studies described that the peptide repertoire complexity of the TAP-negative T2 cell-line was much more limited compared to that of the TAP–positive counterpart cell-line [25,26]. Weinzierl *et al* characterized these peptides in more detail using a differential tandem mass-spectrometry approach and reported a list of peptides which were much more pronounced in the repertoire of TAP-negative B-LCL [27]. These studies demonstrated that blockades in the intracellular antigen processing pathway lead to alterations in the peptide repertoire presented by HLA-I at the cell surface.

In a mouse model we recently described that some of the differentially presented peptides are immunogenic and that a subpopulation of T cells is able to target these TAP-independent peptides [28-31]. This category

of T cells eliminated tumors with a defect in MHC-I antigen processing, while cells with no processing defects were not eliminated. Such peptides were found to be presented by classical MHC-I molecules, but also by the conserved non-classical MHC-I Qa-1 [28]. The peptide-epitopes, which were recognized by the cognate T cells, were 'self' peptides which only emerge at the cell surface once there is a defect in the antigen presentation pathway. We named these new peptides TEIPP, for T cell Epitopes associated with Impaired Peptide Processing [28-31]. Because TEIPP peptides are derived from housekeeping proteins, their presentation is also induced by TAP-deficiencies in normal, non-transformed cells. Indeed, introduction of the viral TAP inhibitor UL49.5 in myeloid dendritic cells (DC), which are professional antigen presenting cells capable of activating naïve T cells, sensitized these cells for recognition by TEIPP-specific CTL [30]. These TAP-inhibited DCs were equally well recognized by TEIPP T-cells, as DCs from $TAP1^{-/-}$ mouse. Importantly, vaccination with these TAP-impaired DCs elicited strong TEIPP T-cell responses *in vivo*, which were able to prevent outgrowth of TAP-loss lymphoma and fibrosarcoma tumor variants [30].

Thus far studies on TEIPP antigens have been limited to mouse models. Here, we present the first evidence for the existence of a human T-cell subset with TEIPP-specificity. In analogy with our mouse model, we rendered monocyte-derived DC TAP-deficient by gene transfer of viral evasion molecules that block the peptide transporter TAP. These TAP-deficient DC were used as stimulator cells in cultures with autologous T cells. This approach revealed an efficient outgrowth of $CD8^+$ T-cells with selective reactivity against TAP-deficient target cells. Polyclonal cultures and T-cell clones exhibited TCR-dependent and HLA-I restricted lytic activity against TAP-inhibited targets, but not against their non-inhibited counterparts. Collectively, our data indicate that the $CD8^+$ T-cell repertoire in humans contains reactivity against peptides presented by cells with a defect or block of the MHC-I antigen-processing route.

Material and methods

Cell lines and primary cell cultures

B-LCLs modo, hodo and HD10 (see Table I) were transduced with retroviruses based on the pLZRS vector, which contains an internal ribosome entry site, followed by GFP, and one of the viral TAP inhibitors. This system is recently reported [32] and the viral TAP inhibitors UL49.5 (from BHV-1), ICP47, US6 and BNLF2a were used, which were previously described [8-18]. Cells were sorted on GFP expression by FACS to ensure homogenous and high expression of the various TAP inhibitors. All B-LCL and K562 were cultured in complete IMDM medium (Invitrogen, Carlsbad, CA) containing 8% heat-inactivated FCS, 100 units/ml penicillin, 100µg/ml streptomycin (Life technologies, Rockerville, MD) and 2mM glutamine (Invitrogen).

We obtained blood from healthy donors (HDs), which were partly HLA-matched with Modo or Hodo B-LCLs (Table I). From these donors Peripheral Blood Mononuclear Cells (PBMCs) were isolated, and positive selection was performed on monocytes and B-cells, using respectively $CD14^+$ and $CD19^+$ MACS beads (Miltenyi Biotec, Gladbach, Germany). $CD14^+$ monocytes were MoDCs in a concentration of $2x10^6$ cells/well in a 6 wells plate (Costar, Cambridge, MA) in RPMI 1640 (Invitrogen) containing 8% heat-inactivated FCS, 100 units/ml penicillin, 100µg/ml, streptomycin and 2mM glutamine, 800 units/ml GM-GSF (Invitrogen), and 500 units/ml IL-4 (Invitrogen) at 37ºC, 5%CO_2. After electroporation 250 ng/ml LPS (Sigma-Aldrich, St Louis, MO, *Escherichia coli* 055:B5) was added for 24h to ensure maturation into mature dendritic cells, which were then washed and used as stimulators for T-cells. B-cells ($2x10^6$ cells/well) were cultured with irradiated CD40 ligand (CD40L) L-cells, $0.5x10^6$ cells/well in 6-wells plates with 500 units/ml IL-4 and ITES supplement (Lonza group) in complete IMDM medium, with 8% heat-inactivated FCS. B-cells were stimulated once a week and after electroporation the B-cells were cultured overnight in complete IMDM medium including 500 units/ml IL-4 before usage as stimulators.

RNA electroporation of MoDCs and B-cells

RNA was synthesized from the pGem4Z vector containing the UL49.5 gene from BHV-1. Vector DNA was linearized for 1h at 37ºC, with SpeI (Roche, Basel, Switzerland). RNA of UL49.5 was then synthesized with the High Yield Capped RNA transcription Kit (T7; Ambion, Austin, TX).

MoDCs cells were cultured for 6 days before electroporation with 20µg synthesized RNA. For each electroporation a maximum of $1x10^7$ cells in 100 µl Optimem media (Invitrogen) was used. Cells were collected, washed and taken up in Opti-Mem media and pulsed with a blockpulser (Biorad, Hercules, CA), in a 2mM cuvette (Biorad), at 300V, 150 µCF [33]. Afterward cells were taken up in X-vivo medium (Lonza) without phenol red for 15 min at 37ºC, 5%CO_2, and then incubated overnight in complete RPMI 1640 medium, with 8% heat inactivated FCS, 250 ng/ml LPS, 800 units/ml GM-GSF, and 500 units/ml IL-4. For electroporation of B-cells, $5x10^6$ cells were stimulated with CD40L expressing L-cells, in a concentration of $0.5x10^6$ cells/well, and 500 units/ml IL-4 the day before. A maximum of $5x10^6$ cells were electroporated with 20µg synthesized RNA with the Nucleofector II, program U-008

(Amaxa, Lonza, Cologne, Germany), using the human B-cell nucleofector kit (Amaxa). After electroporation cells were kept in X-vivo medium without phenol red for 15 min at 37ºC, 5%CO_2 and then cultured overnight in complete IMDM medium, with 8% heat-inactivated FCS containing 500 units/ml IL-4.

T-cell induction protocol

Polyclonal T-cell cultures with a concentration of 6x10^5 cells/ml were induced, in a U-bottom 96 wells plate (Costar), by co-cultures with electroporated MoDCs (week 0 and 4) or B-cells (week 2 and 6) in a concentration of 2x10^5 cells/ml, in complete IMDM medium containing 8% human AB serum (Greiner, Bio-One, Alphen aan den Rijn, the Netherlands), 10 ng/ml IL-7 (only week 0), and 60 units/ml IL-2. An alternating scheme is used as the amount of MoDCs, obtained from our healthy donors, is limited.

T-cell clones were generated by limiting dilution at week 4 or 6. Polyclonal cultures were first depleted for CD4$^+$ cells and stimulated with 1x10^5 cells/ml TAP inhibited B-LCLs (Modo or Hodo), 1x10^6 cells/ml pool PBMCs and 60 units/ml IL-2 in complete IMDM medium with 8% heat-inactivated FCS. After one week, T-cell cultures were restimulation with similar conditions. Clones were selected and restimulated every other week with 1x10^5 cells/ml a mixture of various TAP inhibited B-LCLs (Modo or Hodo), 1x10^6 cells/ml pool PBMCs, 60 units/ml IL-2, and 5 ng/ml IL-15 (Invitrogen Coroperation) in U-bottom wells.

Peptide transport assay

To ensure inhibition of TAP, a peptide transport assay was done as described previously [9,10]. In brief cells where permeabilized, and incubated with the fluoresceinconjugated synthetic peptide CVNKTERAY in the presence or absence of 10mM ATP. Peptide translocation was terminated by adding ice-cold lysis buffer, and after the removal of cell-debris incubation with ConA beads was done to isolated glycosylated peptides. The fluorescence intensity was measured and peptide transport is expressed a percentage of translocation, which is relative to the translocation in control cells.

Western blot

Cells were lysed in 0.5% Nonidet P-40 lysisbuffer (50 mM Tris, 150 mM NaCl (pH 8.0), 0.5% NP40, 25 mg/ml Leupeptin, 25 mg/ml 4-(2-amino-ethyl)-benzene-sulfonyl fluoride hydrochloride (Sigma-Aldrich, Zwijndrecht, the Netherlands. Depending on the protein, either 16.5% Tricine-PAGE (for UL49.5) or 10% SDS page gels (for β-actin and TAP1) were made [9]. The gels were loaded, and BenchMark prestained ladder (Invitrogen) was used as marker. For Tricine-PAGE gels, gels ran for 3-4h at 25mA/gel. The 10% SDS page gel ran for 1.5h at 15mA/gel. Afterwards the gel was blotted in a semi-dry manner, (Hep-1 OWL Panter; Thermo Scientific, Etten-Leur, the Netherlands) in semi-dry blot buffer (25mM Tris-Base, 192mM Glycine), for 45 min at 10V onto a Hybond-PVDF transfer membrane (GE healthcare, Zoetermeer, the Netherlands), which was activated for 1 min in pure methanol. After blotting the membrane was incubated overnight at 4ºC in either 5% elk (for UL49.5 and β-actin) or 0.6% BSA (for TAP-1) in TBST (10mM Tris-base, 150mM NaCl, 0.05% tween20, pH 7.4).

The next day the blots were stained with one of the following antibodies, H11Az (anti BHV-1 UL49.5) [34], 148.3 (anti TAP-1) [35,36], or anti-β-actin (Sigma) in either 0.6 % BSA in TBST (anti TAP1) or 1% elk in TBST (anti UL49.5 and anti-β-actin). After the staining, the membrane was washed 3 times for 10 min in TBST, followed by 3 times 10 min washing in TBS (10mM Tris-Base, 150mM NaCl, pH 7.4). HRP labeled secondary antibodies were used: anti-rabbit Ig (DakoCytomation, Carpinteria, CA) for UL49.5, and anti-mouse Ig for TAP1 and β-actin (Dako), followed by 3 times washing in TBST and 3 times TBS. The western blot was developed on film using ECL Plus Western Blotting Dectection Reagents (GE Healthcare).

Flow cytometry

For flow cytometry the following antibodies were used from BD bioscience: CD3 (SK7, FITC), CD137 (4B4-1, APC), CD8 (SK1, PE or APC), CD4 (SK3, PE) from Serotech: anti-HLA A/B/C (W6/32, APC), and from Beckman Coulter the Vβ Repetoire Analyses kit was used. Cells were analysed using a FACS Calibur, and Cellquest software (Becton Dickinson, USA), or Flowjo software (Tree star, Inc).

CD8+ T-cells clones were stained for the following NK and KIR receptors: CD226 (clone DX11), NKG2A (clone Z199PE), NKG2C (clone 134591), NKG2D (clone 149810), CD56 (clone MY-31), CD94 (clone DX22), 3DL1 (clone DX9), 2DL3 (clone GL183), 2DS1/2DL1 (clone EB6), 2DS4 (clone FES 172), 3DS1 (clone Z27), IgG1 (clone A687), 2DL4 (clone A687), 2DL5 (clone A687), all kindly provided by Dr J. van Bergen (Leiden).

T-cell reactivity assays

IFNγ production by T-cells was analysed after 24h or 48h incubation via ELISA (PeliKine compact human IFN ELISA kit, Sanquin Amsterdam). ELISA plates were developed with TMB (3, 3', 5, 5'-Tetramethylbenzidine Liquid Substrate Supersensitive, for ELISA, Sigma) and measured at 450nm. On each plate a standard curve was added to calculate the IFNγ production (pg/ml).

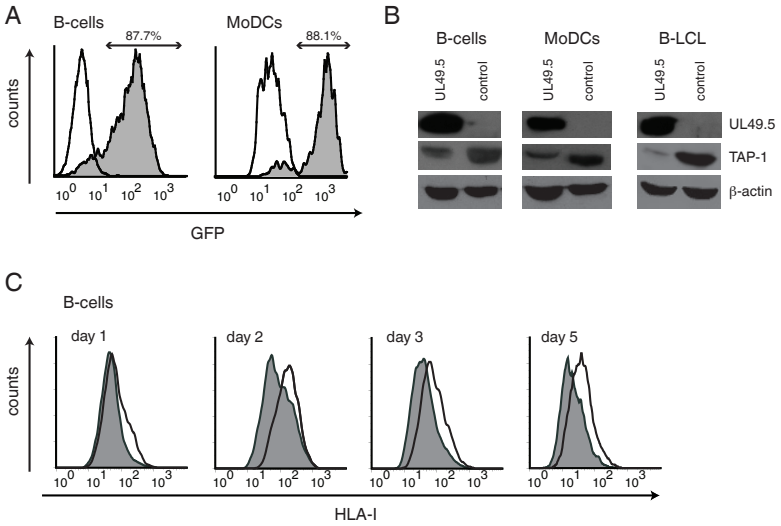

Figure 1: RNA electroporation with UL49.5 into primary B cells leads to MHC-I downregulation. (A) Primary cultures of B-cells or MoDCs were electroporated with RNA encoding GFP. Mock electroporations served as negative controls. After 24h incubation for B-cells and MoDCs ~90% of the cells where successfully transfected with GFP. (B) Primary cultures of B-cells or MoDCs were electroporated with RNA encoding the viral TAP-inhibitor UL49.5. Mock electroporations served as negative controls. After electroporation, cells were lysed and expression of UL49.5 (after 24 h) and TAP1 (48 h) was analysed by western blot. B-LCL with stable expression of the UL49.5 protein served as positive control. (C) Electroporated primary B-cells were stained with anti HLA-I antibody at different time points. HLA-I surface expression is depicted of B cells electroporated with UL49.5 RNA (filled histograms) and with control RNA (open histograms). A decrease of HLA-I at the cell-surface was seen up till day 5 after electroporation. Data are representative for three independent experiments. Monocyte-derived dendritic cells showed similar results (data not shown).

To confirm the receptor dependency of the T-cells, several antibodies were used: W6/32 (HB-95, anti HLA), BB7.2 (anti HLA-A2) and, B123.2.1 (anti HLA-B/C) (kindly provided by Dr. E. Verdegaal) and FK18 (anti CD8) (kindly provided by Dr. A. Mulder). Cells were pre-incubated at 37ºC, with 50µl of the antibody and IFNγ release was measured after overnight incubation.

Lytic activity of $CD8^+$ T-cell clones was determined in a 4h ^{51}Cr release assay as previously described [37]. $1x10^6$ target cells were labeled with 100µl ^{51}Cr for 1h at 37Cº. After labeling the cells were washed 3 times with PBS. The target cells were incubated for 4h at 37ºC, 5% CO_2, with different concentrations of $CD8^+$ T-cells, after which the chrome release was measured. The percentage of specific lysis was calculated as follows: ((specific lysis test well – spontaneous lysis)/(maximum lysis – spontaneous lysis)) x 100.

A $CD8^+$ T-cell reactive against the TAP-dependent peptide HA-1 (clone HA-1, kindly provided by Dr. E. Goulmy) was used to ensure TAP inhibition in the B-LCL modo TAP inhibited targets [38].

Results

RNA electroporation of UL49.5 leads to effective TAP inhibition

Previously, we demonstrated that inhibition of peptide transport through TAP induces the HLA-I-mediated presentation of TEIPP antigens by DCs [30]. In analogy with this approach in our mouse model, we engineered TAP-inhibition in human monocyte-derived DC (MoDC) and B cells by the introduction of the TAP inhibitor UL49.5 of the BHV-1 virus [8]. Gene transfer was accomplished through electroporation with synthetic RNA in order to prevent artificial vector sequences in these professional stimulator cells. The UL49.5 gene was chosen from the four characterized viral TAP-blocking molecules, since we previously found that this protein is the most efficient inhibitor and actively breaks down TAP proteins [32]. Introduction of synthetic RNA encoding for GFP showed that ~90% of both B-cells and MoDC were successfully transfected with GFP (fig 1a). Electroporation with synthetic RNA of UL49.5 resulted in strong expression of UL49.5 in MoDC and B-cells from healthy blood donors (fig. 1b). The high expression levels of the viral protein resulted in a specific TAP degradation, comparable to B-LCL that were stably transfected with the gene (fig 1b), and this TAP degradation was still present in B-cells 5 days after electroporation (data not shown). Importantly, the introduction of UL49.5 in the primary MoDC (12-20% reduction in intensity two days after electroporation) and B-cells (fig. 1c) led to a decrease of HLA-I molecules at the cell surface, illustrating that the inhibition of TAP function indeed limited the availability of peptides in the ER. Therefore, we concluded that

electroporation with synthetic RNA encoding the viral UL49.5 is an effective method to generate TAP-inhibited Anitgen Presenting Cells (APCs). These TAP impaired professional APCs were used for the induction of TEIPP-specific T-cells *in vitro*.

Construction of a target cell panel for the analysis of TEIPP-specific T cells

In order to determine the specificity of the T-cell cultures resulting from stimulations with TAP-inhibited APCs, we constructed target cell panels on the basis of stable B-LCL cell-lines. B-LCLs hodo and modo cell panels were used as these two cell-lines cover around 80% of the Caucasian population, including HLA-A1, -A2, -B8 and -B44 (Table I) (39). TAP was inhibited in these cell-lines via stable expression of the inhibitors UL49.5, ICP47, US6 and BNLF2a and as a control the empty pLZRS vector was used. Flow cytometry analysis was performed to determine surface HLA-I expression using an anti-HLA-A/B/C mAb. The B-LCL displayed decreased HLA-I levels after introduction of one of the TAP inhibitors, albeit to variable extent (fig 2a). Subsequently, we determined the impairment of TAP function in these B-LCLs, with a peptide transport assay. The efficiency of TAP inhibition was found to vary between 70 to 90%, with UL49.5 giving the strongest decrease in TAP transport (fig 2b). These results confirmed that all viral proteins were capable of efficiently impair TAP function, although the changes in total HLA-I surface levels were not compelling. In accordance, earlier studies indeed point at a discrepancy between TAP blockade and the effects in HLA-I expression levels at the cell surface (32), implying that alternative peptides substitute for the TAP-mediated peptide repertoire. To examine this notion, the recognition of the minor histocompatibility antigen HA-1 presented by HLA-A2 in a TAP-dependent way was determined with a previously established CD8+ CTL clone (32,38). IFNg production and cytotoxicity by this CTL clone upon incubation with the HLA-A2 positive B-LCL modo and its TAP-inhibited variants clearly indicated that this peptide-epitope is virtually absent from the TAP-inhibited B-LCLs (fig 2c and d). We

Tabel I. HLA-I serotype of cell-lines and donors

		HLA typing					
Sort	**Name**	**HLA-A**		**HLA-B**		**HLA-C**	
Cell-line	B-LCL hodo	1	11	8	60	w3	w7
	B-LCL modo	2	2	44	60	w5	w10
	B-LCL HD10	1	68	44	58	w3	w7
Donor	HD1	2	23	44	44	w5	w4
	HD2	11	31	7	44	w5	w15
	HD3	2	2	44	18	w5	w7
	HD4	1	2	8	44	w5	w7
	HD5	2	24	44	39	w5	w7
	HD6	1	11	8	40	w2	w7
	HD7	2	3	44	57	w5	w6
	HD8	1	1	8	58	w7	w7
	HD9	2	23	44	44	w5	w4

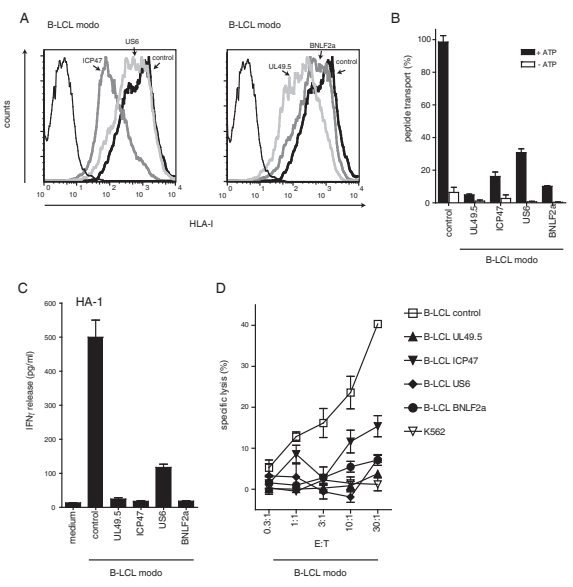

Figure 2: B-LCL panel with stable expression of each of the four known viral TAP inhibitors. (A) The genes of UL49.5, ICP47, US6 or BNLF2a were introduced into B-LCL modo using retroviral vectors. MHC-I cell-surface display was measured with a pan-HLA-I antibody. All cell lines were stained in the same experiments, but are separated in two histogram plots for clarity. Isotype control (thin lines) and modo B-LCL transfected with vector alone ('control', thick lines) are the same in both panels. Similar results were obtained in three other experiments. (B) All viral proteins efficiently inhibit TAP function, as tested in a peptide transport assay. Percentage of TAP transport was calculated against B-LCL modo control which was set at 100%. Means and standard deviations shown are representative of two assays with similar outcome. Comparable results were observed for B-LCL hodo (data not shown). (C-D) IFNγ release (C) and lytic activity (D) by an established CTL clone, specific for the HA-1 antigen, was measured to ensure that the viral TAP-inhibitors were able to block surface presentation of TAP-dependent peptide-epitopes. The HA-1 antigen is endogenously expressed by modo B-LCL and is presented by HLA-A2. Means and standard deviations are shown from one out of three experiments with similar outcome

Tabel II. IFNγ release by polyclonal T-cell cultures from different donors

	Target	Medium		Control[a]		UL49.5		ICP47		US6	
Donor	cell-line	mean[b]	SD[c]	mean	SD	mean	SD	mean	SD	mean	SD
HD1	Modo	39[d]	10	354	50	1098	176	1308	81	1407	141
HD2	Modo	44	6	68	16	145	24	198	32	164	4
	Hodo	44	6	72	3	354	76	368	78	225	16
HD3	Modo	NT[e]	NT	2	2	51	49	121	131	61	26
HD4	Modo	10	0	205	34	122	27	240	47	213	59
	Hodo	10	0	418	53	629	98	NT	NT	NT	NT
HD5	Modo	NT	NT	155	32	269	85	811	120	NT	NT
HD6	Hodo	30	4	54	1	114	24	62	1	52	19
HD7	Modo	14	0	134	28	507	49	793	215	466	187
HD8	Hodo	181	14	529	83	737	39	623	27	610	86
HD9	Modo	2	1	4	5	744	109	814	69	744	12

a B-LCLs are transduced with either an empty vector (control) or a vector containing one of the viral TAP inhibitors (UL49.5, ICP47 or US6).
b mean is calculated from triplicate samples
c SD is Standard Deviation
d IFNγ release (pg/ml)
e NT is Not Tested

therefore concluded that the control B-LCL presents a TAP-positive peptide repertoire and that the variants expressing one of the viral TAP-inhibitors predominantly present a different, TAP-independent peptide repertoire in their broad array of HLA alleles. These two cell panels constituted ideal target cells to determine TEIPP-reactivity by the T cell cultures induced with the UL49.5 RNA electroporated APCs .

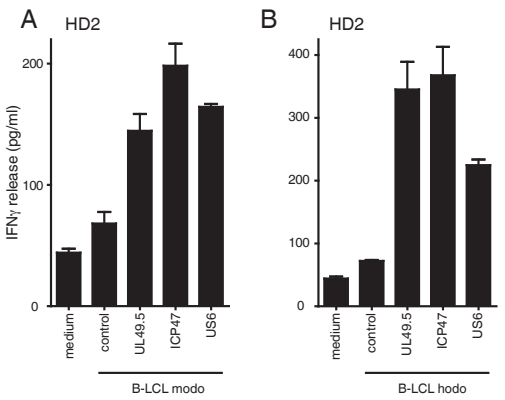

Figure 3: Polyclonal T-cell cultures exhibit TEIPP-specificity. (A-B) Short term T cell cultures from PBMC were stimulated with UL49.5 expressing autologous dendritic cells and tested four to six weeks after initial stimulation against two panels of TAP-inhibited B-LCL. Healthy subjects were selected on basis of sharing some HLA-I alleles with the modo or hodo B-LCL target cell lines (see Table I for HLA typing). IFNγ release by one T cell cultures from donor HD2 (A-B) is shown. HD2 shared HLA-I alleles with both B-LCL modo (A) and hodo (B) and was tested against both panels. Effector to target ratio was 2:1 for all experiments. (see Table II for results of all donors).

Polyclonal T-cell cultures display TEIPP-specificity

Polyclonal T-cell cultures were initiated from PBMC of healthy donors whom were selected to match HLA-I alleles with the modo or hodo B-LCL (Table I). MoDC or B-cells, with an impaired TAP function through UL49.5 RNA electroporation, were added to autologous T-cell cultures in a bi-weekly alternating scheme in order to stimulate the outgrowth of T-cells with TEIPP-specificity. Specificity of the T-cell cultures was examined after three to four *in vitro* stimulations by measuring IFNγ release upon incubation with cells from the partly HLA-matched modo and hodo B-LCL panels. Strikingly, these short term cultures already displayed selective reactivity to the TAP-inhibited variants of the B-LCL (fig 3). One donor (HD2) which shared HLA-I alleles with both B-LCL lines, also exhibited reactivity against both TAP-inhibited cell lines (fig 3), suggesting that this T-cell culture contained multiple TEIPP-specificities presented by at least two different HLA-I molecules. Complete mismatched targets showed no reactivity, and the HLA-I negative target K562 was also not recognized (data not shown). IFNγ release by the polyclonal T-cells was observed against TAP-inhibited B-LCL, irrespective of which viral TAP-targeting protein was expressed. Since the viral evasion molecules exert different mechanisms for TAP impairment [8-18], these data suggested that the T-cell recognition was induced by general TAP-impairment in the target cells rather than by a particular molecular strategy. An overview of the results from all nine polyclonal T-cell cultures is depicted in Table II. All cultures, from different blood donors, displayed selective reactivity against the TAP-inhibited B-LCL. These surprisingly broad T-cell responses from short-term cultures prompted us to

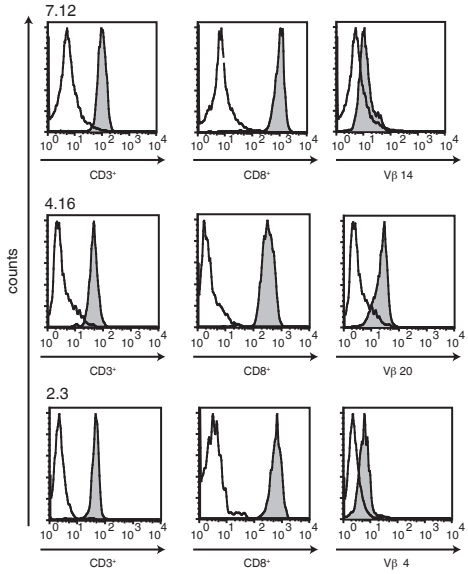

Figure 4: Clones 7.12, 4.16 and 2.3 are CD3+, CD8+ and each has their own TCR Vβ usage. All three clones were stained for CD3, CD8 and 24 different TCR Vβ. Flow cytometry analysis revealed that clone 7.12 has TCR Vβ 14, clone 4.16 Vβ 20 and clone 2.3 Vβ 4, confirming their independent origin. All clones are CD3+ and CD8+.

exclude a super-antigen like response, reflected by strong T-cell expansions of one particular TCR Vβ-positive subpopulation. Staining with a panel of Vβ-specific antibodies demonstrated a wide array of TCR Vβ usage (data not shown), indicating that the T-cell cultures were clearly polyclonal and were not the result of super-antigen mediated expansion. Together, these data suggested that TEIPP-specific T-cells can readily be detected in short-term T-cell cultures from PBMC of healthy blood donors.

Isolation of TEIPP-specific CD8+ T-cell clones

The short-term polyclonal T-cell cultures resulting from three stimulations with RNA electroporated TEIPP-presenting cells contained both CD4+ and CD8+ T cells. CD8+ T-cell clones were isolated from these cultures by limiting dilution, stimulating CD4-depleted cultures with TAP-inhibited modo or hodo B-LCL. Approximately 10-20% of the isolated clones showed higher reactivity against TAP-inhibited B-LCLs than control B-LCL. Further expansion of these T-cell clones resulted in the isolation of three stably growing T-cell clones from different donors. These three clones (clone 7.12, clone 4.16, and clone 2.3) expressed CD3, CD8 and TCR molecules from different Vβ families (fig. 4), confirming their independent origin.

Flow cytometry profiling of natural killer receptors demonstrated that the TEIPP-specific CD8+ clones did not display an aberrant expression of these receptors. Comparable to the vast majority of activated CTL, the lectin receptors CD94, NKG2A and NKG2D were expressed, but the KIR family was absent (supp. fig 1).

All three CD8+ T-cell clones recapitulated the TEIPP-specificity of the polyclonal T-cell cultures and recognized all TAP-inhibited B-LCL, but not control the B-LCL (fig. 5a-c). This indicates that all viral TAP-inhibitors present an overlapping peptide repertoire which is different than its non-inhibited counterpart, and that the viral proteins themselves were not recognized by our T-cell clones. Of note, the reactivity pattern is completely opposed to that of the HA-1-specific CTL (fig. 2c), illustrating the distinct peptide repertoires presented by TAP-proficient and TAP-impaired target cells [32]. The TAP-inhibited B-LCLs were recognized with different efficiencies by the CD8+ T-cell clones (fig 5). Interestingly, the B-LCLs inhibited with US6 had the least TAP-blocking effect (see fig 2b

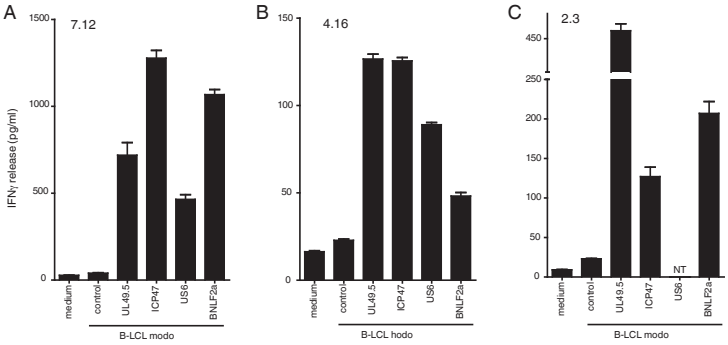

Figure 5: CD8+ T-cell clones exhibit TEIPP-specificity. (A-C) CTL clones were obtained by limiting dilution and selected on reactivity against TAP-inhibited B-LCL. Approximately 10-20% of all isolated CTL clones showed preferred reactivity against TAP-inhibited B-LCL, similar to the three CD8+ CTL clones shown here for 7.12 (A), 4.16 (B) and 2.3 (C). IFNγ release by CTL clones upon incubation with the B-LCL panels is shown. Means and standard deviations are shown from one out of four similar assays. *NT* means not tested.

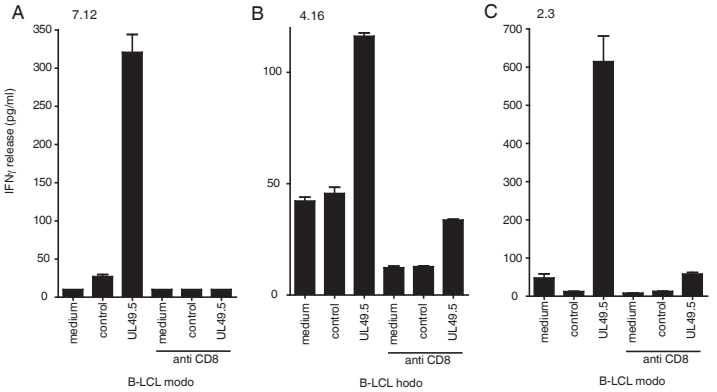

Figure 6: Recognition of TAP-inhibited target cells is TCR-mediated. (A-C) CTL clones were pre-incubated with a blocking anti-CD8 antibody before incubation with B-LCL target cells. IFNγ release for all three CTL clones is shown: 7.12 (A), 4.16 (B) and 2.3 (C). Isotype control antibodies did not alter the recognition of TAP-inhibited B-LCL (not shown).

for modo) and were also the least recognized by the T cells, suggesting that a correlation exist between the degree of TAP-inhibition and the level of HLA-I mediated TEIPP antigen presentation in HLA-I at the cell surface.

Target cell recognition is TCR-mediated and HLA-restricted

We previously reported in mouse tumor models that TEIPP-specific CTL exert their reactivity strictly via their T-cell receptors and that the TEIPP antigens can be presented on classical as well as non-classical MHC-I molecules [28,29]. Since the isolated human TEIPP-specific CD8+ T-cell clones described here displayed comparable specificity against TAP-inhibited targets, we anticipated that also the human CD8+ T-cell clones mediated target cell recognition in a TCR- and HLA-I dependent fashion. To examine this, all three CD8+ T-cell clones were incubated with a blocking antibody against CD8 during the incubation with the B-LCL panel. The anti-CD8 antibody caused a complete block of CTL-reactivity, strongly indicating that TAP-impaired targets were recognized via the TCR (fig 6). Secondly, to determine the HLA-I restriction of the human T-cell clones, we generated an additional TAP-inhibited B-LCL, which expresses a complementary set of HLA alleles (B-LCL HD10). All three CTL clones were tested for IFNγ production against B-LCL hodo, modo and HD10 (Table III). Importantly, the CTL clones did not show reactivity against completely HLA-I mismatched target cells (Table III), even when the UL49.5 TAP-inhibitor was properly expressed, clearly indicating a dependency on specific HLA class I molecules. With the use of these target cell panels we were capable to determine the shared HLA-restriction element that determined the restriction of the three CTL clones: HLA-A2, HLA-B8 and HLA-B44 (Tabel III). Some ambiguity was left for the CTL clone 7.12 concerning HLA-I restriction, since both A2 and Cw5 were still candidates (Tabel III). The HLA-A2 allele could, however, be appointed as restriction element for this CTL clone, because CTL reactivity was blocked using a HLA-A2-specific antibody, whereas an antibody against HLA-B and -C molecules did not block the recognition (supl fig 2). The fact that each T-cell clone used a different HLA-restriction element for recognition of target cells indi-

Tabel III: HLA-I restriction of CD8+ T-cell clones

		CD8+ T-cell		
B-LCL target		**clone 7.12**	**clone 4.16**	**clone 2.3**
Modo	shared HLA-I[a]	**A2**, B44, **Cw5**[b]	A2, B44, Cw5	**B44**, Cw5
	T-cell recognition[c]	654[d]	17	474
Hodo	shared HLA-I	none	A1, **B8**, Cw7	A11
	T-cell recognition	4	244	23
HD10	shared HLA-I	B44	A1, B44, Cw7	**B44**
	T-cell recognition	11	3	121

a HLA-I expressed by both the CTL of the donor and the B-LCL
b In bold are possible restriction molecules for CD8+ T-cell clones
c Specific IFNγ release (pg/ml) by the T cell clones calculated by the IFNγ release against B-LCL UL49.5 minus the IFNγ release against the B-LCL control.
d mean value of IFNγ of triplicate samples, SD < 50 pg/ml in all cases

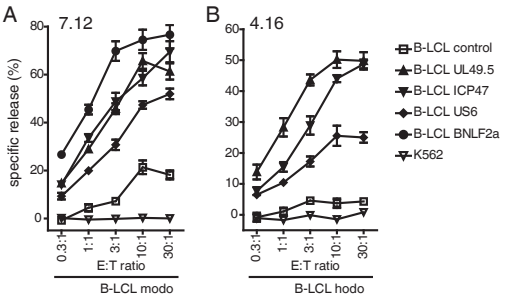

Figure 7: CD8⁺ T-cell clones lyse TAP-inhibited B-LCL. (A-B) The B-LCL cell panels were loaded with chromium and used as targets for CTL clones 7.12 (A) and 4.16 (B). K562 cells are HLA-negative target cells and were not lysed by the CTL clones, indicating the absence of natural killer-like reactivity. Different effector to target (E:T) ratios were tested and data points represent means and standard deviations from triplicate wells. Similar results were observed in two independent experiments.

cated that multiple different peptide-epitopes are involved in the TEIPP-specific response of healthy subjects. The broadness of TEIPP-specific T-cells was further supported by the finding that the polyclonal T-cell culture of donor HD2 displayed reactivity against both hodo (A11 matched) and modo (B44 matched) B-LCLs (fig. 3c), whereas the modo stimulated CTL clone derived thereof was solely restricted by the B44-allele (Tabel III). All together, these data demonstrated that the TEIPP-directed T-cell response in humans is broad and involves multiple HLA-I presented peptide-epitopes.

CD8+ T-cell clones lyse TAP-impaired B-LCL

To examine if the CD8⁺ T-cell clones were also capable to lyse TAP-deficient targets, a chromium release assays was preformed. The two tested T-cell clones 7.12 and 4.16 efficiently lysed all four TAP-inhibited target cells, whereas their TAP-proficient counterparts were not killed (fig 7a,b). The specificity pattern of the T-cell clones matched that of the IFNγ release assays (fig 5a,b). To determine the contribution of NK-like reactivity to this lysis, the K562 cell line, which is completely HLA-I negative, was included as a target. K562 cells were not killed by the T-cell clones, indicating that our cytotoxic T-cell clones did not exhibit HLA-I-independent killing activity (fig 7a,b). We speculate that our T-cell clones constitute the first examples of TEIPP-reactive CTL in humans, and that this CD8⁺ T-cell subset is well represented in PBMC of healthy subjects.

Discussion

Here we present the first evidence for the existence of TEIPP-specific CTL in humans. These CD8⁺ T cells selectively recognize TAP-inhibited target cells, which are generally resistant to detection and lysis by conventional CTL. TEIPP-specific CTL were already detectable in short term T-cell cultures that received only three rounds of stimulation, indicating that TEIPP-CTL are well represented in the PBMC of healthy subjects. All PBMC cultures exhibited reactivity against B-LCL expressing a viral TAP-inhibitor, but not against the normal B-LCL, suggesting that TEIPP-CTL are generally present in the human population and target a broad repertoire of peptides which are presented upon TAP-inhibition. Analyses of TEIPP-specific CTL clones revealed that target cell recognition depends on the T-cell receptor and that several different classical HLA-I molecules present these cognate peptides. These data are in complete analogy with our previous findings in mouse models [28-31].

TEIPP-specific T cells were isolated by stimulations of PBMC from healthy donors with autologous dendritic cells that were electroporated with RNA encoding the BHV-1 gene UL49.5. The protein from this bovine herpesvirus efficiently inhibits the peptide transporter TAP (see fig 1) through induction of a conformational arrest and proteasomal degradation [8-10]. Using this autologous culture system, we minimized the risk of inducing artificial T-cell specificities, e.g. allogenic-HLA antigens, minor histocompatibility antigens and vector-derived antigens. The generated T-cell cultures were tested against panels of B-LCL stably expressing one of four different well characterized viral TAP inhibitors (UL49.5, ICP47, US6 and BNLF2a). The polyclonal T- cell cultures, and also the isolated T-cell clones responded to all four TAP-inhibited cell lines, excluding the UL49.5 protein, which was present during the culture period, as the source of target peptides. Although RNA electroporation is only transient and not completely inhibiting TAP function (fig 1b), it is sufficient enough to alter the repertoire of peptides as such that CTLs which are specific for TAP-inhibited cells are induced. This is in line with our previous studies in which both TAP-knockout and TAP-inhibited cells where recognized [28-30].

The CTL clones isolated display different HLA-I restrictions for their TEIPP epitope. CTL 7.12 is HLA-A2 restricted, CTL 4.16 is most likely HLA-B8 restricted and CTL 2.3 is HLA-B44 restricted. HLA-I molecules associate in the peptide loading complex with TAP in order to directly load peptides which are

there pumped into the ER. The binding of HLA-I to TAP is not well understood, but it is shown that position 116 (p116) in the HLA-I molecule has a great effect on association with TAP and/or tapasin [40-42]. This is well illustrated by the two closely related alleles HLA-B4402 and B4405 [40-42]. Where B4402, with aspartic acid at p116, is highly dependent on TAP, B4405 with tyrosine at this position is completely independent of TAP [40-42]. Detailed analyses showed that peptide loading of HLA-A2, -A23, -B7, -B8, and -B4405 molecules is largely independent of TAP [25,26,43-47], while the TAP-dependent pathway is the main processing route for HLA-A1, -A3, -A11, -A24, -B15, -B27 and -B4402 [40-46]. It could be hypothesized that TEIPP peptides are predominantly presented by HLA-I molecules that are normally dependent on TAP for their peptide loading, since the transition to TAP-deficiency would then select for a completely distinct peptide repertoire. However, we isolated CTL with HLA-I restrictions from both categories (HLA-A2, HLA-B8, and HLA-B4402), suggesting that TAP associations are not predictive for presentation of TEIPP peptide-epitope, and that all alleles could display TEIPP upon inhibition of TAP.

Apart from presenting TEIPP at the cell-surface of TAP-deficient cells another explanation for reactivity against TAP-deficient targets could the involvement of the CD94/NKG2a complex which is expressed on some of the $CD8^+$ T-cells. Indeed our CTL 7.12 also expresses this complex, while it is absent on CTL 4.16 (supl fig 1). CD94/NKG2a can bind to surface HLA-E molecules loaded with peptide, which then acts as an inhibitory molecule [48]. Since TAP-deficient cells express these HLA-E molecules and TAP-deficients cells in lower amounts it could be possible that the TAP-independent peptide, which is recognized by the TCR of CTL 7.12, is already presented at TAP^+ but non-reactive due to the presence of HLA-E and NKG2a/CD94. However, in our previous studies in mice blocking the NKG2a had no effect on the reactivity of the CTLs [28], and we therefore have no reason to believe that this would be the case for our CTL 7.12 which is expressing NKG2a/CD94.

Molecular identification of human TEIPP antigens would enable immunotherapeutic approaches for the treatment of tumors harboring antigen processing defects. Since processing-deficient tumors are resistant to conventional anti-tumor CTL and represent a major percentage of all tumors, alternative T-cell based strategies, like TEIPP antigens, are more than welcome. In an attempt to indentify the nature of the involved peptide-epitopes, we tested HLA-A2 binding, TAP-independently processed peptides that were described by the group of Dr. Stevanovic (Tuebingen, Germany) [27] for recognition by our TEIPP T-cell clones. However, none of the peptides from this list stimulated our T-cells, and also the T2 cell-line from which these peptides were eluted was not recognized by our HLA-A2 specific CTL. Despite the fact that the exact identity of our human TEIPP peptides remains unknown, TAP-impaired DC could constitute an alternative in vaccination studies. RNA electroporation of MoDCs or B-cells with a viral TAP inhibitor is feasible in clinical settings. RNA electroporation has the advantage that the nucleotides are not integrated in the host genome, like viral vectors [33,49-53]. In our mouse model, TAP-deficient dendritic cells were capable to prevent the outgrow of TAP-deficient tumors [30]. Several improvements still need to be made to fully optimize dendritic cell vaccination in cancer patients, but the process will remain tailored and laborious as each patient needs their own manufactured vaccine. Therefore, we argue for the molecular identification of human TEIPP in order to enable peptide vaccination, which holds great promise for the future as a cancer treatment.

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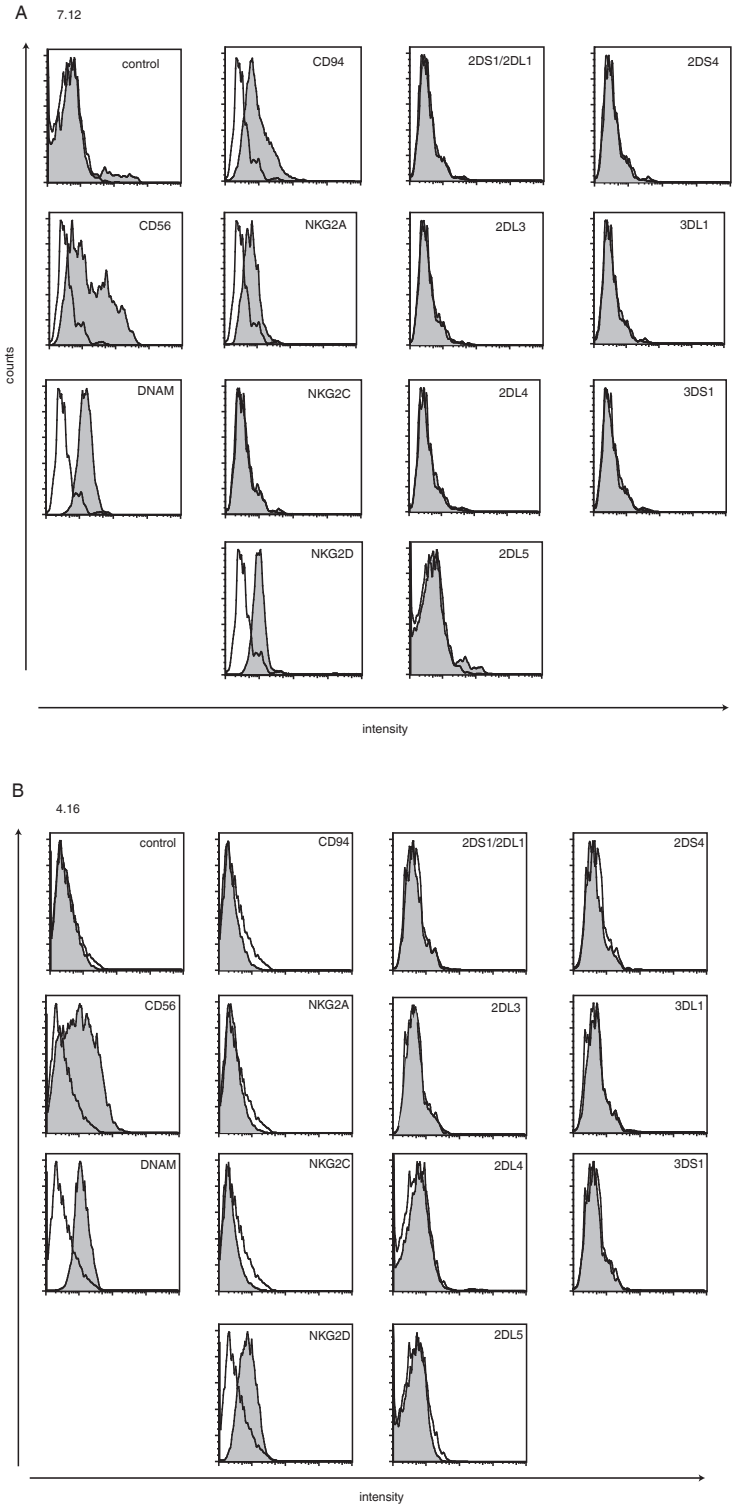
Reference List

1. Chang, C. C., M. Campoli, and S. Ferrone. 2003. HLA class I defects in malignant lesions: what have we learned? *Keio J. Med.* 52: 220-229.
2. Hansen, T. H., and M. Bouvier. 2009. MHC class I antigen presentation: learning from viral evasion strategies. *Nat. Rev. Immunol.* 503-513.
3. Hicklin, D. J., F. M. Marincola, and S. Ferrone. 1999. HLA class I antigen downregulation in human cancers: T-cell immunotherapy revives an old story. *Mol. Med. Today* 5: 178-186.
4. Garrido, F., and I. Algarra. 2001. MHC antigens and tumor escape from immune surveillance. *Adv. Cancer Res.* 83: 117-158.
5. Seliger, B., M. J. Maeurer, and S. Ferrone. 2000. Antigen-processing machinery breakdown and tumor growth. *Immunol. Today* 21: 455-464.
6. Seliger, B., D. Atkins, M. Bock, U. Ritz, S. Ferrone, C. Huber, and S. Storkel. 2003. Characterization of human lymphocyte antigen class I antigen-processing machinery defects in renal cell carcinoma lesions with special emphasis on transporter-associated with antigen-processing down-regulation. *Clin. Cancer Res.* 9: 1721-1727.
7. Seliger, B. 2008. Molecular mechanisms of MHC class I abnormalities and APM components in human tumors. *Cancer Immunol. Immunother.* 57: 1719-1726.
8. Koppers-Lalic, D., E. A. Reits, M. E. Ressing, A. D. Lipinska, R. Abele, J. Koch, R. M. Marcondes, P. Admiraal, L. D. van, K. Bienkowska-Szewczyk, T. C. Mettenleiter, F. A. Rijsewijk, R. Tampe, J. Neefjes, and E. J. Wiertz. 2005. Varicelloviruses avoid T cell recognition by UL49.5-mediated inactivation of the transporter associated with antigen processing. *Proc. Natl. Acad. Sci. U. S. A* 102: 5144-5149.
9. Verweij, M. C., D. Koppers-Lalic, S. Loch, F. Klauschies, H. de la Salle, E. Quinten, P. J. Lehner, A. Mulder, M. R. Knittler, R. Tampe, J. Koch, M. E. Ressing, and E. J. Wiertz. 2008. The varicellovirus UL49.5 protein blocks the transporter associated with antigen processing (TAP) by inhibiting essential conformational transitions in the 6+6 transmembrane TAP core complex. *J. Immunol.* 181: 4894-4907.
10. Koppers-Lalic, D., M. C. Verweij, A. D. Lipinska, Y. Wang, E. Quinten, E. A. Reits, J. Koch, S. Loch, R. M. Marcondes, F. Daus, K. Bienkowska-Szewczyk, N. Osterrieder, T. C. Mettenleiter, M. H. Heemskerk, R. Tampe, J. J. Neefjes, S. I. Chowdhury, M. E. Ressing, F. A. Rijsewijk, and E. J. Wiertz. 2008. Varicellovirus UL 49.5 proteins differentially affect the function of the transporter associated with antigen processing, TAP. *PLoS. Pathog.* 4: e1000080.
11. Ahn, K., T. H. Meyer, S. Uebel, P. Sempe, H. Djaballah, Y. Yang, P. A. Peterson, K. Fruh, and R. Tampe. 1996. Molecular mechanism and species specificity of TAP inhibition by herpes simplex virus ICP47. *EMBO J.* 15: 3247-3255.
12. Hill, A., P. Jugovic, I. York, G. Russ, J. Bennink, J. Yewdell, H. Ploegh, and D. Johnson. 1995. Herpes simplex virus turns off the TAP to evade host immunity. *Nature* 375: 411-415.
13. Tomazin, R., A. B. Hill, P. Jugovic, I. York, E. P. van, H. L. Ploegh, D. W. Andrews, and D. C. Johnson. 1996. Stable binding of the herpes simplex virus ICP47 protein to the peptide binding site of TAP. *EMBO J.* 15: 3256-3266.
14. York, I. A., C. Roop, D. W. Andrews, S. R. Riddell, F. L. Graham, and D. C. Johnson. 1994. A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes. *Cell* 77: 525-535.
15. Dugan, G. E., and E. W. Hewitt. 2008. Structural and Functional Dissection of the Human Cytomegalovirus Immune Evasion Protein US6. *J. Virol.* 82: 3271-3282.
16. Hewitt, E. W., S. S. Gupta, and P. J. Lehner. 2001. The human cytomegalovirus gene product US6 inhibits ATP binding by TAP. *EMBO J.* 20: 387-396.
17. Hislop, A. D., M. E. Ressing, L. D. van, V. A. Pudney, D. Horst, D. Koppers-Lalic, N. P. Croft, J. J. Neefjes, A. B. Rickinson, and E. J. Wiertz. 2007. A CD8+ T cell immune evasion protein specific to Epstein-Barr virus and its close relatives in Old World primates. *J. Exp. Med.* 204: 1863-1873.
18. Horst, D., L. D. van, N. P. Croft, M. A. Garstka, A. D. Hislop, E. Kremmer, A. B. Rickinson, E. J. Wiertz, and M. E. Ressing. 2009. Specific targeting of the EBV lytic phase protein BNLF2a to the transporter associated with antigen processing results in impairment of HLA class I-restricted antigen presentation. *J. Immunol.* 182: 2313-2324.
19. Jordanova, E. S., A. Gorter, O. Ayachi, F. Prins, L. G. Durrant, G. G. Kenter, S. H. Van Der Burg, and G. J. Fleuren. 2008. Human Leukocyte Antigen Class I, MHC Class I Chain-Related Molecule A, and CD8+/Regulatory T-Cell Ratio: Which Variable Determines Survival of Cervical Cancer Patients? *Clin. Cancer Res.* 14: 2028-2035.
20. Kamarashev, J., S. Ferrone, B. Seifert, R. Boni, F. O. Nestle, G. Burg, and R. Dummer. 2001. TAP1 down-regulation in primary melanoma lesions: an independent marker of poor prognosis. *Int. J. Cancer* 95: 23-28.
21. Alimonti, J., Q. J. Zhang, R. Gabathuler, G. Reid, S. S. Chen, and W. A. Jefferies. 2000. TAP expression provides a general method for improving the recognition of malignant cells in vivo. *Nat. Biotechnol.* 18: 515-520.
22. Chen, H. L., D. Gabrilovich, R. Tampe, K. R. Girgis, S. Nadaf, and D. P. Carbone. 1996. A functionally defective allele of TAP1 results in loss of MHC class I antigen presentation in a human lung cancer. *Nat. Genet.* 13: 210-213.
23. Evans, M., L. K. Borysiewicz, A. S. Evans, M. Rowe, M. Jones, U. Gileadi, V. Cerundolo, and S. Man. 2001. Antigen processing defects in cervical carcinomas limit the presentation of a CTL epitope from human papillomavirus 16 E6. *J. Immunol.* 167: 5420-5428.
24. Lou, Y., T. Z. Vitalis, G. Basha, B. Cai, S. S. Chen, K. B. Choi, A. P. Jeffries, W. M. Elliott, D. Atkins, B. Seliger, and W. A. Jefferies. 2005. Restoration of the expression of transporters associated with antigen processing in lung carcinoma increases tumor-specific immune responses and survival. *Cancer Res.* 65: 7926-7933.
25. Henderson, R. A., H. Michel, K. Sakaguchi, J. Shabanowitz, E. Appella, D. F. Hunt, and V. H. Engelhard. 1992. HLA-A2.1-associated peptides from a mutant cell line: a second pathway of

antigen presentation. *Science* 255: 1264-1266.
26. Wei, M. L., and P. Cresswell. 1992. HLA-A2 molecules in an antigen-processing mutant cell contain signal sequence-derived peptides. *Nature* 356: 443-446.
27. Weinzierl, A. O., D. Rudolf, N. Hillen, S. Tenzer, E. P. van, H. Schild, H. G. Rammensee, and S. Stevanovic. 2008. Features of TAP-independent MHC class I ligands revealed by quantitative mass spectrometry. *Eur. J. Immunol.* 38: 1503-1510.
28. Oliveira, C. C., P. A. van Veelen, B. Querido, R. A. de, M. Sluijter, S. Laban, S. H. Van Der Burg, R. Offringa, and T. van Hall. 2010. The nonpolymorphic MHC Qa-1b mediates CD8+ T cell surveillance of antigen-processing defects. *J. Exp. Med.* 207: 207-221.
29. van Hall, T., E. Z. Wolpert, V. P. van, S. Laban, d. van, V, M. Roseboom, S. Bres, P. Grufman, R. A. de, H. Meiring, J. A. de, K. Franken, A. Teixeira, R. Valentijn, J. W. Drijfhout, F. Koning, M. Camps, F. Ossendorp, K. Karre, H. G. Ljunggren, C. J. Melief, and R. Offringa. 2006. Selective cytotoxic T-lymphocyte targeting of tumor immune escape variants. *Nat. Med.* 12: 417-424.
30. Chambers, B., P. Grufman, V. Fredriksson, K. Andersson, M. Roseboom, S. Laban, M. Camps, E. Z. Wolpert, E. J. Wiertz, R. Offringa, H. G. Ljunggren, and T. van Hall. 2007. Induction of protective CTL immunity against peptide transporter TAP-deficient tumors through dendritic cell vaccination. *Cancer Res.* 67: 8450-8455.
31. van Hall, T., S. Laban, D. Koppers-Lalic, J. Koch, C. Precup, P. Asmawidjaja, R. Offringa, and E. J. Wiertz. 2007. The varicellovirus-encoded TAP inhibitor UL49.5 regulates the presentation of CTL epitopes by Qa-1b1. *J. Immunol.* 178: 657-662.
32. Oosten, L. E., D. Koppers-Lalic, E. Blokland, A. Mulder, M. E. Ressing, T. Mutis, A. G. van Halteren, E. J. Wiertz, and E. Goulmy. 2007. TAP-inhibiting proteins US6, ICP47 and UL49.5 differentially affect minor and major histocompatibility antigen-specific recognition by cytotoxic T lymphocytes. *Int. Immunol.* 19: 1115-1122.
33. Schuurhuis, D. H., P. Verdijk, G. Schreibelt, E. H. Aarntzen, N. Scharenborg, B. A. de, M. W. van de Rakt, M. Kerkhoff, M. J. Gerritsen, F. Eijckeler, J. J. Bonenkamp, W. Blokx, J. H. van Krieken, O. C. Boerman, W. J. Oyen, C. J. Punt, C. G. Figdor, G. J. Adema, and V. de, I. 2009. In situ expression of tumor antigens by messenger RNA-electroporated dendritic cells in lymph nodes of melanoma patients. *Cancer Res.* 69: 2927-2934.
34. Lipinska, A. D., D. Koppers-Lalic, M. Rychlowski, P. Admiraal, F. A. Rijsewijk, K. Bienkowska-Szewczyk, and E. J. Wiertz. 2006. Bovine herpesvirus 1 UL49.5 protein inhibits the transporter associated with antigen processing despite complex formation with glycoprotein M. *J. Virol.* 80: 5822-5832.
35. Meyer, T. H., P. M. Van Endert, S. Uebel, B. Ehring, and R. Tampe. 1994. Functional expression and purification of the ABC transporter complex associated with antigen processing (TAP) in insect cells. *FEBS Lett.* 351: 443-447.
36. Plewnia, G., K. Schulze, C. Hunte, R. Tampe, and J. Koch. 2007. Modulation of the antigenic peptide transporter TAP by recombinant antibodies binding to the last five residues of TAP1. *J. Mol. Biol.* 369: 95-107.
37. Verdegaal, E. M., C. Hoogstraten, M. H. Sandel, P. J. Kuppen, A. A. Brink, F. H. Claas, M. C. Gorsira, J. F. Graadt van Roggen, and S. Osanto. 2007. Functional CD8+ T cells infiltrate into nonsmall cell lung carcinoma. *Cancer Immunol. Immunother.* 56: 587-600.
38. den Haan, J. M., L. M. Meadows, W. Wang, J. Pool, E. Blokland, T. L. Bishop, C. Reinhardus, J. Shabanowitz, R. Offringa, D. F. Hunt, V. H. Engelhard, and E. Goulmy. 1998. The minor histocompatibility antigen HA-1: a diallelic gene with a single amino acid polymorphism. *Science* 279: 1054-1057.
39. Bui, H. H., J. Sidney, K. Dinh, S. Southwood, M. J. Newman, and A. Sette. 2006. Predicting population coverage of T-cell epitope-based diagnostics and vaccines. *BMC. Bioinformatics.* 7: 153.
40. Hillen, N., G. Mester, C. Lemmel, A. O. Weinzierl, M. Muller, D. Wernet, J. Hennenlotter, A. Stenzl, H. G. Rammensee, and S. Stevanovic. 2008. Essential differences in ligand presentation and T cell epitope recognition among HLA molecules of the HLA-B44 supertype. *Eur. J. Immunol.* 38: 2993-3003.
41. Thammavongsa, V., G. Raghuraman, T. M. Filzen, K. L. Collins, and M. Raghavan. 2006. HLA-B44 polymorphisms at position 116 of the heavy chain influence TAP complex binding via an effect on peptide occupancy. *J. Immunol.* 177: 3150-3161.
42. Zernich, D., A. W. Purcell, W. A. Macdonald, L. Kjer-Nielsen, L. K. Ely, N. Laham, T. Crockford, N. A. Mifsud, M. Bharadwaj, L. Chang, B. D. Tait, R. Holdsworth, A. G. Brooks, S. P. Bottomley, T. Beddoe, C. A. Peh, J. Rossjohn, and J. McCluskey. 2004. Natural HLA class I polymorphism controls the pathway of antigen presentation and susceptibility to viral evasion. *J. Exp. Med.* 200: 13-24.
43. de la Salle, H., E. Houssaint, M. A. Peyrat, D. Arnold, J. Salamero, D. Pinczon, S. Stevanovic, H. Bausinger, D. Fricker, E. Gomard, W. Biddison, P. Lehner, F. UytdeHaag, M. Sasportes, L. Donato, H. G. Rammensee, J. P. Cazenave, D. Hanau, M. M. Tongio, and M. Bonneville. 1997. Human peptide transporter deficiency: importance of HLA-B in the presentation of TAP-independent EBV antigens. *J. Immunol.* 158: 4555-4563.
44. Doytchinova, I., S. Hemsley, and D. R. Flower. 2004. Transporter associated with antigen processing preselection of peptides binding to the MHC: a bioinformatic evaluation. *J. Immunol.* 173: 6813-6819.
45. Grandea, A. G., III, M. J. Androlewicz, R. S. Athwal, D. E. Geraghty, and T. Spies. 1995. Dependence of peptide binding by MHC class I molecules on their interaction with TAP. *Science* 270: 105-108.
46. Luckey, C. J., J. A. Marto, M. Partridge, E. Hall, F. M. White, J. D. Lippolis, J. Shabanowitz, D. F. Hunt, and V. H. Engelhard. 2001. Differences in the expression of human class I MHC alleles and their associated peptides in the presence of proteasome inhibitors. *J. Immunol.* 167: 1212-1221.
47. Neisig, A., R. Wubbolts, X. Zang, C. Melief, and J. Neefjes. 1996. Allele-specific differences in the interaction of MHC class I molecules with transporters associated with antigen processing. *J. Immunol.* 156: 3196-3206.
48. Sarantopoulos, S., L. Lu, and H. Cantor. 2004. Qa-1 restriction of CD8+ suppressor T cells. *J. Clin. Invest* 114: 1218-1221.
49. Bontkes, H. J., D. Kramer, J. J. Ruizendaal, C. J. Meijer, and E. Hooijberg. 2008. Tumor associated antigen and interleukin-12

mRNA transfected dendritic cells enhance effector function of natural killer cells and antigen specific T-cells. *Clin. Immunol.* 375-384.

50. Kyte, J. A., and G. Gaudernack. 2006. Immuno-gene therapy of cancer with tumour-mRNA transfected dendritic cells. *Cancer Immunol. Immunother.* 55: 1432-1442.
51. Milano, F., J. W. van Baal, A. M. Rygiel, J. J. Bergman, S. J. Van Deventer, M. L. Kapsenberg, M. P. Peppelenbosch, and K. K. Krishnadath. 2007. An improved protocol for generation of immuno-potent dendritic cells through direct electroporation of CD14+ monocytes. *J. Immunol. Methods* 321: 94-106.
52. Saeboe-Larssen, S., E. Fossberg, and G. Gaudernack. 2002. mRNA-based electrotransfection of human dendritic cells and induction of cytotoxic T lymphocyte responses against the telomerase catalytic subunit (hTERT). *J. Immunol. Methods* 259: 191-203.
53. Sousa-Canavez, J. M., F. C. Canavez, K. R. Leite, and L. H. Camara-Lopes. 2008. Therapeutic Dendritic cell vaccine preparation using tumor RNA transfection: A promise approach to treat prostate cancer. *Genet. Vaccines. Ther.* 6: 2.



Supplementary figure 1: Clones 7.12 and 4.16 have a normal T-cell phenotype. CTL clone 7.12 (A) and clone 4.16 (B) were stained for well known T-cell, NK and KIR receptors. Clone 7.12 was positive for CD56, CD94, DNAM, NKG2A and NKG2D all markers which are expressed on a subet of T-cells. None of the KIR receptors was found at its cell surface. Clone 4.16 was positive for CD56, DNAM and NKG2D and again no KIR receptor expression was observed.

Supplementary figure 2: TEIPP-CTL clone 7.12 is HLA-A2 restricted. Target cells modo B-LCL and UL49.5 expressing modo B-LCL were pre-incubated with anti HLA-I, anti HLA-A2, and anti HLA-B/C antibodies, before clone 7.12 was added. IFNγ release by CTL was measured. Two panels represent independent experiments, which show that total HLA-I blocking and specific HLA-A2 blocking prevented CTL activation. These results corroborate those from figure 7 on the HLA-A2 restriction of TEIPP-CTL clone 7.12.