Therapeutic targeting of immune escaped cancers

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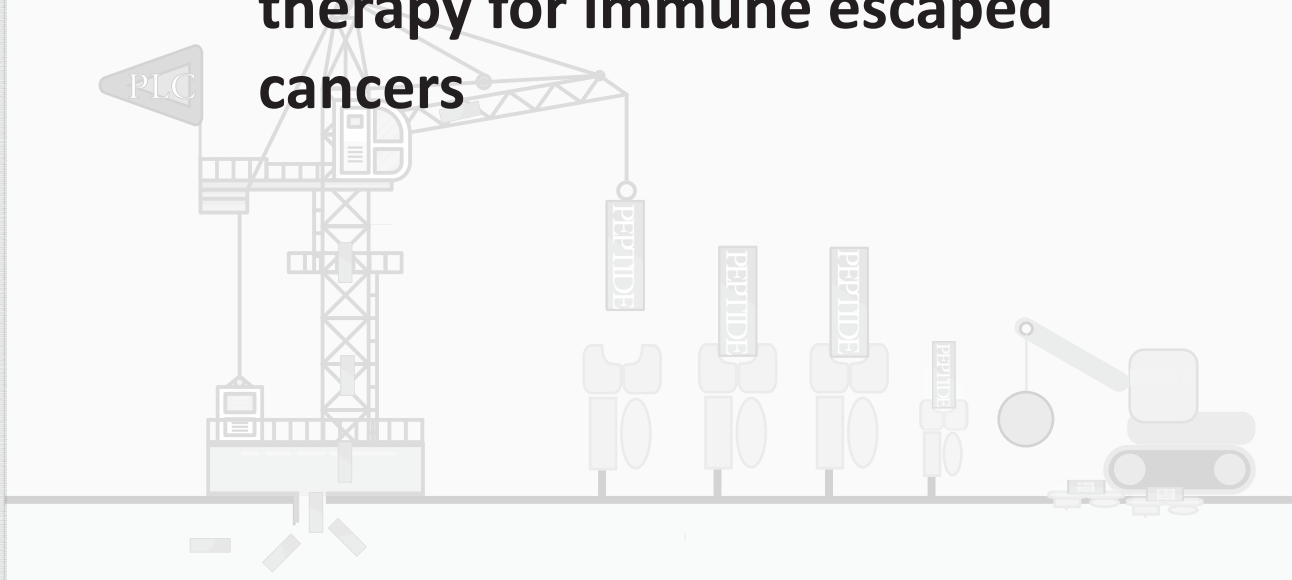
Chapter 6

Dendritic cell vaccination therapy for immune escaped cancers



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Manuscript in preparation (2020)

Abstract

TEIPP antigens are presented on cancer cells with defects in the antigen-processing pathway, in particular the peptide pump TAP. Therefore, CD8 T cell immunity to TEIPP peptides might constitute a salvage therapy for immune-escaped cancers. However, clinical application for TEIPPs have yet to be developed. Here, we describe the pre-clinical development of synthetic long peptide (SLP) vaccine for the most immunogenic TEIPP antigen identified thus far, derived from the LRPAP1 signal peptide. We show that dendritic cells were unable to cross-present a long version of the HLA-A*0201 presented $LRPAP_{21-30}$ epitope when elongated with its natural flanking sequence. Remarkably, amino acid exchange of the C-terminal anchor residue from a serine (S) to a valine (V) resulted in an enhanced T cell stimulation. $LRPAP_{21-30}$-specific CD8+ T cells isolated with multimers presenting either the S- or V-variant could be co-stained with the other multimer to the same extent, recognized the other peptide as well as recognized TAP-deficient tumor cells. Similar findings were obtained when CD8+ T cells were transfected with an isolated $LRPAP_{21-30}$-specific T cell receptor. Importantly, *in vitro* vaccination with the V-variant SLP resulted in cross-presentation of the peptide vaccine and in polyclonal LRPAP1-specific CD8 T cell cultures isolated from the normal T cell repertoire. Expanded CD8 T cell clones from these cultures not only recognized the natural S-containing peptide but also displayed a highly selective capacity to recognize TAP-deficient melanoma cells. We are currently developing TEIPP epitope vaccines for clinical testing in patients with immune escaped, TAP-impaired cancers.

Introduction

The success of T cell targeted immunotherapy relies on the presentation of tumor antigens on the cell surface of cancer cells. However, cancer cells often downregulate components of the antigen processing machinery to prevent the presentation of tumor-associated and tumor-specific antigens by HLA class I molecules[1-3]. One critical step in this intracellular process is the transport of liberated peptides over the ER membrane by the dedicated pump TAP, which functions as a bottleneck and delivers peptides for all HLA class I molecules[2-4]. Such processing defects allow the tumor to escape from CD8 T cell immunity and are frequently observed in human cancers. We have previously described a novel subset of tumor antigens that are selectively presented by cancers with down modulated TAP expression and called these TEIPP (Tumor Epitopes associated with Impaired Peptide Processing)[5-7]. In a recent study, we reported on the molecular identification of 16 HLA-A*0201 presented human TEIPP antigens by means of a novel hybrid forward-reverse approach[7]. The identified antigen from the signal sequence of the LRPAP1 protein was presented on multiple HLA-A*0201 positive TAP-deficient cancers, including renal cell carcinoma, lymphoma, melanoma, and colon carcinoma. Additionally, LRPAP1-specific T cells were detectable in all tested healthy donors and this repertoire could be activated *in vitro* to target these immune escaped cancers.

We now aim to exploit these findings for the benefit of cancer patients and develop therapeutic formats for immunotherapy. Although interest in cancer vaccines waned long ago due to a sheer lack of objective clinical responses in hundreds of trials, they recently regained attention since novel platforms demonstrated efficacy to induce broad CD4 and CD8 anti-tumor T cell immunity, increase immune infiltration of human cancers and eradicate pre-malignant lesions[8-10]. Moreover, vaccination therapy seems to combine very well with immune checkpoint blockade in that relapsed vaccinated patients responded extremely well to PD-1 therapy and, importantly, the addition of a long peptide vaccine to a standardized PD-1 treatment schedule improved the overall response rate and median overall survival [11,12].

All T cell-geared vaccination platforms depend on delivery of tumor antigens to the host and on the exceptional capability of dendritic cells to cross-present these tumor antigens in HLA class I and II molecules for subsequent T cell activation. Many parameters are important for successful development a therapeutic cancer vaccine, including delivery systems, route of administration and adjuvants, which are supposed to activate the innate immune system and induce T cell co-stimulatory molecules. Our group developed the synthetic long peptide (SLP) vaccination platform and showed that long peptides (20-35 amino acids) possess the capacity to trigger CD4 and CD8 T cell responses, and results in eradication

of premalignant lesions[9,13,14] as well as improves overall survival of patients with cancer when vaccinated during chemotherapy. Cross-presentation of long peptides by host dendritic cells depends on uptake and processing the proteasome, the dominant proteolytic enzyme, and TAP-mediated transport resulting in HLA class I loading[14-17]. However, most identified TEIPP tumor antigens derive from signal peptides at the N-terminus of nascent proteins and are thus processed in a proteasome- and TAP-independent way[7,18,19]. Thus, TEIPP antigens might need modifications in order to be exploited as SLP.

Here, we describe the pre-clinical development of the most prominent human TEIPP antigen derived from the LRPAP1 protein[7]. In short, we demonstrate that a single amino acid exchange in the sequence of this signal peptide allows application in the SLP platform. The alteration at position 9 resulted in a higher binding affinity to HLA-A*0201 and more efficient cross-presentation by monocyte-derived dendritic cells. *In vitro* vaccination with the altered peptide, led to the generation of CD8 T cells that exhibit an indistinguishable reactivity to the altered peptide sequence as well as the natural peptide, and were highly selective in recogning TAP-negative tumor cells. These data indicate that small alterations to signal peptide-epitopes retains immunogenicity of TEIPP antigens and render them suitable candidates for the SLP format.

Results

The signal peptide of LRPAP1 is not cross-presented by dendritic cells when provided as long peptide

We previously showed that the TEIPP antigen derived from the signal peptide of the ubiquitously expressed LRPAP1 protein is presented in HLA-A*0201 on a wide variety of TAP-deficient cancer types[7]. Here, we set out to exploit the TEIPP concept for vaccination strategies, in particular the synthetic long peptide (SLP) platform that we developed for viral-induced cancers. We assessed the efficiency of cross-presentation of a long version of this signal peptide $LRPAP_{21-30}$ in dendritic cells. Three different SLP variants were synthesized with natural flanking amino acids extending the amino-terminus, the carboxy-terminus or at both ends (figure 1a). We incubated these SLPs together with monocyte-derived dendritic cells (moDC), and used a LRPAP1-specific CD8 T cell clone to assess correct processing and presentation of the minimal TEIPP epitope. Cytokine release was measured and showed that none of the three SLPs were cross-presented to T cells, whereas exogenous pulsing of the short $LRPAP_{21-30}$ peptide did stimulate the T cells (figure 1a). These results suggested that cross-presentation of the $LRPAP_{21-30}$ epitope from its longer peptide stretch is not efficient and had to be optimized for vaccine applications.

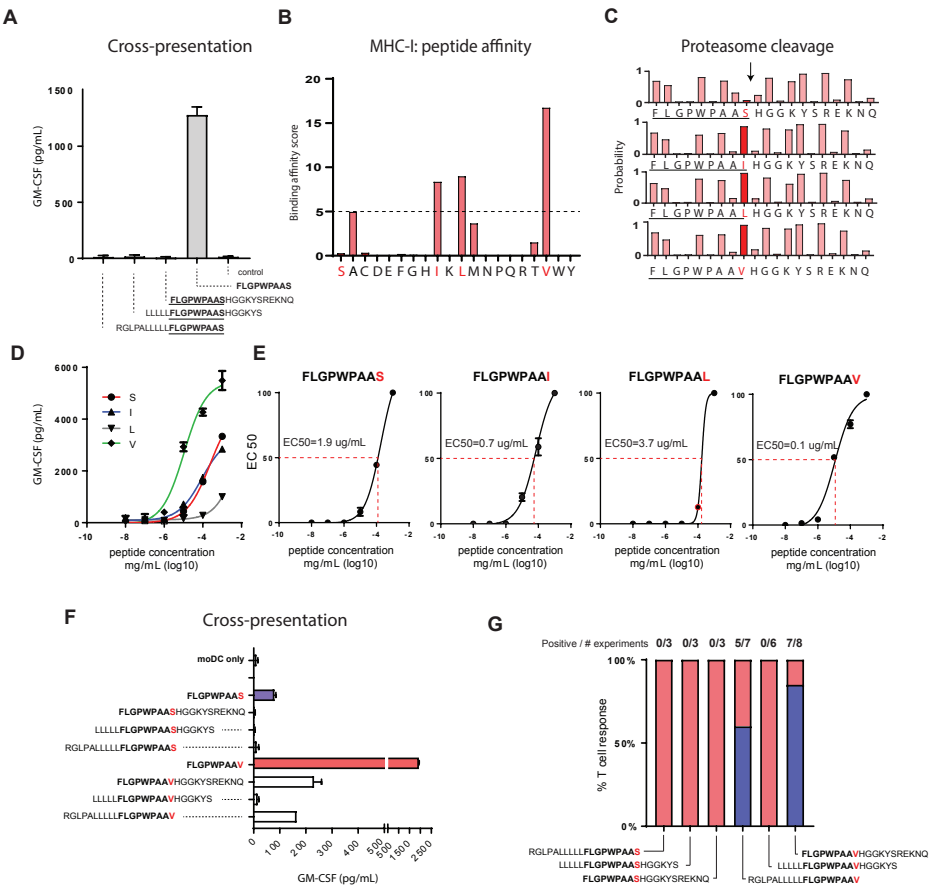


Figure 1: Substitution of the C-terminal amino acid of the signal peptide of LRPAP1 allows for cross-presentation by dendritic cells.

(A) Monocyte-derived dendritic cells were incubated with long peptides of the LRPAP1 epitope and co-cultured with a TEIPP-specific CD8 T cell clone. Natural flanking amino acids were used to elongate the minimal epitope. GM-CSF secretion by the TEIPP-specific T cell clone was used to determine cross-presentation efficiency and pulsing with short peptide was used as positive control. (B) Predicted HLA-A2*01 binding affinity scores of LRPAP1 peptides with substituted amino acids at the C-terminal p9 (MHCnet 4.1 algorithm). Binding affinity score was calculated on basis of the IC50 values and ranking percentage (see Table 1 for exact values). (C) Predicted proteasome cleavage activity for four different LRPAP1 peptides using NetCHop 3.1 algorithm. A score of 1 is maximal and predicts cleavage by the proteasome after the indicated amino acid. Arrow indicates the C-terminus of the LRPAP1 epitope. (D) Functional T cell avidity was measured as GM-CSF secretion by TEIPP-specific T cell clone stimulated with short peptide–pulsed EBV-JY cells in serial dilutions of the peptides. Mean and SD are plotted from one out of three experiments with similar outcome. (E) EC50 values were calculated from values obtained in D. (F) Monocyte-derived dendritic cells were cultured with different long S- and V-peptides of the LRPAP1 sequence. Cross-presentation of the long peptides was determined by T cell clone. Pulsed short peptide served as positive controls. (G) Overview of cross-presentation experiments using dendritic cells from eight different donors.

Serine to Valine substitution of the C-terminal anchor allows efficient binding and cross-presentation

Cross-presentation of long peptides by dendritic cells involves multiple sequential steps, including uptake via endocytosis, cytosolic cleavage of the SLP into short peptides by the proteasome, transport over the ER membrane by TAP and loading onto MHC-I molecules[15]. Previous studies have shown that the $LRPAP_{21-30}$ epitope has a moderate binding affinity onto HLA-A*0201[7], since the C-terminal serine is not a preferred anchor residue for binding into this allele[7]. This anchor residue is important for binding to MHC-I molecules and can be replaced because T cell receptors (TCR) do not interact with these residues. We investigated if serine exchange into another amino acid would result in a more efficiently processed epitope. First, the binding affinity to HLA-A*0201 of all possible peptide sequences with varying amino acids at position 9 was estimated using an *in silico* algorithm (table 1, figure 1b). The C-terminal serine had indeed a low predicted binding score and ranking (affinity=364nM, %rank=2.50, respectively). However, substitution of serine (S) into, isoleucine (I), leucine (L), or valine (V) resulted in strongly enhanced predicted binding affinities. Replacement with a valine resulted in an affinity of 6nM and rank percentage of 0.05%. Additionally, proteasome cleavage probability analysis using netCHOP, revealed a probability score close to the maximum of 1, for isoleucine (I), leucine (L), and valine (V), whereas the natural serine (S) at the C-terminus had a cleavage probability score of almost 0 (figure 1c). These *in silico* analyses indicated that these two important parameters might be strongly improved by substitution of the serine (S) by an isoleucine (I), leucine (L), or valine (V) at the C-terminus.

To examine whether these substitutions would interfere with LRPAP1-specific T cell recognition, we exogenously pulsed short peptide variants of the exact epitope in titrated concentrations on HLA-A*0201 positive T2 cells and measured T cell activation (figure 1d). Unexpectedly, the I- and L-variant peptides induced similar or worse cytokine responses when compared to the S-peptide, while the V-peptide induced a more potent IFNy response (figure 1d). Calculation of the EC50 values confirmed that the V-peptide variant elicited the strongest T cells response at limiting peptide concentrations (EC50 in ug/mL= V: 0.1, S: 1.9, I: 0.7, L: 3.7) (figure 1e). We concluded that substitution of serine (S) to valine (V) at the C-terminus of the $LRPAP_{21-30}$ peptide resulted in better MHC-I binding affinity and a 19-fold better T cell activation. We then evaluated cross-presentation of the V-peptide variant as SLP. moDCs were incubated with SLPs containing the S (S-SLP) or the V (V-SLP) variant of the TEIPP epitope. After uptake and processing of the SLPs, the moDCs were co-cultured with a LRPAP1-specific T cell clone and cytokine production was measured (figure 1f). While the three S-SLP variants again failed to activate T cells, the C- and the N-terminal extended peptide of V-SLP, but not the variant with elongations at both ends, were efficiently processed

Peptide sequence	NetMHC		
	Affinity (nM)	%Rank	[1]
FLGPWPAAS	364.80	2.50	WB
FLGPWPAAA	20.23	0.30	SB
FLGPWPAAC	303.14	2.50	WB
FLGPWPAAD	6719.97	12.00	NB
FLGPWPAAE	4516.70	9.00	NB
FLGPWPAAF	521.16	3.00	NB
FLGPWPAAG	984.43	4.00	NB
FLGPWPAAH	7052.81	12.00	NB
FLGPWPAAI	11.98	0.15	SB
FLGPWPAAK	4183.99	8.50	NB
FLGPWPAAL	11.14	0.15	SB
FLGPWPAAM	27.46	0.40	WB
FLGPWPAAN	4995.00	9.50	NB
FLGPWPAAP	1762.34	5.50	NB
FLGPWPAAQ	3029.38	7.50	NB
FLGPWPAAR	3690.59	8.00	NB
FLGPWPAAT	66.98	0.80	WB
FLGPWPAAV	5.98	0.05	SB
FLGPWPAAW	2198.56	6.00	NB
FLGPWPAAY	3105.78	7.50	NB

[1] SB, strong binder; WB, weak binder; ND, non-binder

Table 1: HLA-A*02:01 peptide binding scores of the LRPAP1 epitope

Overview of predicted binding affinity of p14 peptide variants where the anchor residue at position 9 is substituted with all other known amino acids using NetMHC 4.1. Peptide variants highlighted in green are predicted as strong binders in HLA-A*02:01.

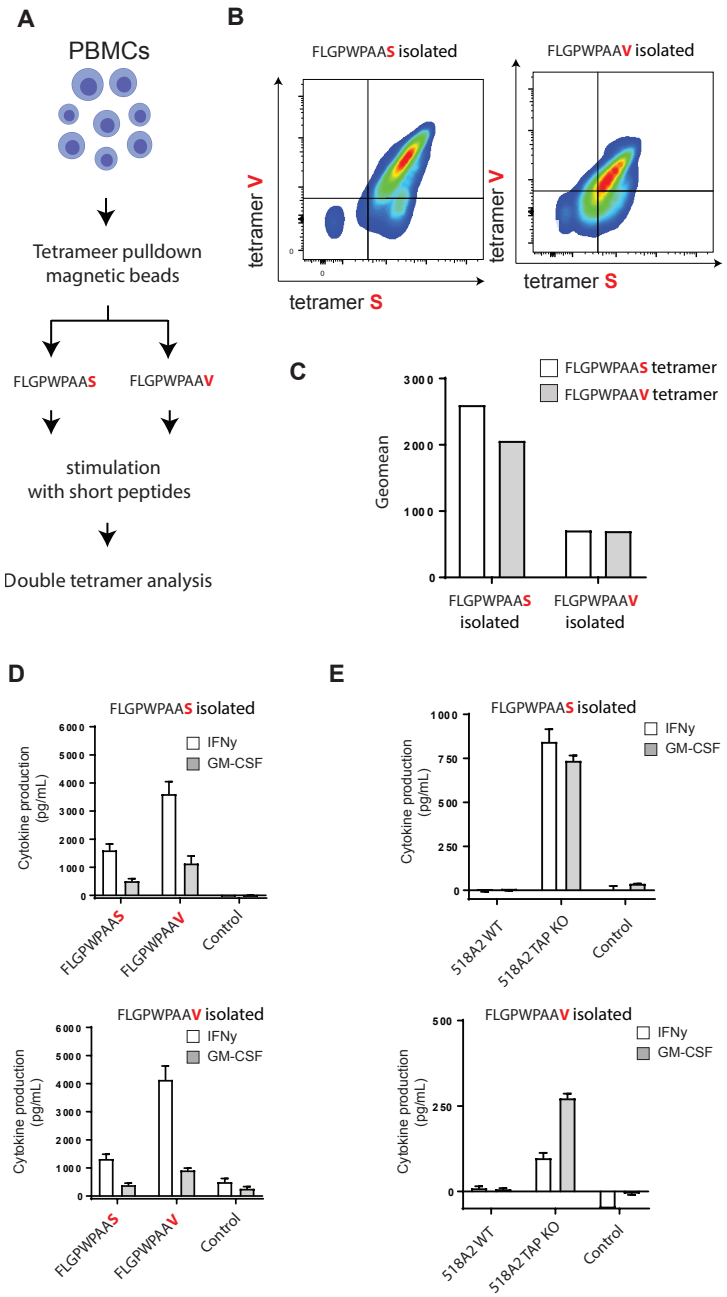
and presented by the moDCs (figure 1f). These results were reproducible in nearly all of the independent experiments with different moDC donors and revealed that the C-terminal extension was most efficiently processed (7/8 donors) (figure 1g). To summarize, these data showed that the substitution of serine (S) to valine (V) at the C-terminus of the LRPAP1-derived TEIPP antigens allows for application in the SLP vaccination platform.

Characterization of CD8 T cell repertoire isolated with the optimized TEIPP peptide

Next, we evaluated the cross-reactivity of the CD8 T cell repertoire, isolated and expanded with the V-peptide, to the wild type $LRPAP_{21-30}$ peptide, as an SLP vaccine should ultimately generate T cell reactivity towards the natural peptide sequence as presented by TAP-deficient tumors. Therefore, we generated CD8 T cell cultures using our previously described approach with HLA-A*0201 tetramer pull-down and subsequent expansion by peptide stimulations[7]. This protocol resulted in the generation of polyclonal, LRPAP1-specific CD8 T cell cultures induced by the V-variant or the natural S-variant (figure 2a). Combined tetramer staining revealed that both T cell cultures bound tetramers with the S-variant as well as the V-variant, indicating that these T cell repertoires were indistinguishable in specificity (figure 2b). The CD8 T cell repertoire isolated and stimulated with V-peptide variant seemed to bind the tetramers with somewhat lower affinity, as mean fluorescence intensities were lower (figure 2b, c). This might reflect the weaker binding capacity of the S-peptide whereby only high-affinity TCRs are recruited from the total repertoire, whereas the strongly binding V-peptide was able to also recruit lower-affinity TCRs.

Figure 2: Isolation of LRPAP1-specific T cells with HLA-A2*01 tetramers containing the short V-substituted peptide.

(A) Schematic overview of the tetramer pull-down approach and subsequent stimulation with short peptides. (B) Specificity of polyclonal T cells measured by double tetramer staining of FLGPWPAAS peptide isolated T cells (upper panel) or FLGPWPAAV peptide isolated T cells (bottom panel) by flow cytometry. (C) Geomeans for double tetramer positive cells on FLGPWPAAS or FLGPWPAAV peptide isolated T cells. (D) Reactivity of polyclonal T cell bulks by measurement of IFNγ and GM-CSF production upon peptide pulsed EBV-JY cells. Means and SD are plotted of one out of three independent experiments. (E) Reactivity of these polyclonal T cell bulks to 518A2 melanoma panel.


A
PBMCs
Tetrameer pulldown
magnetic beads
FLGPWPAAS
FLGPWPAAV
stimulation
with short peptides
Double tetramer analysis
B
FLGPWPAAS isolated
FLGPWPAAV isolated
tetramer V
tetramer S
C
FLGPWPAAS tetramer
FLGPWPAAV tetramer
Geomean
FLGPWPAAS isolated
FLGPWPAAV isolated
D
FLGPWPAAS isolated
IFNy
GM-CSF
Cytokine production (pg/mL)
FLGPWPAAS
FLGPWPAAV
Control
FLGPWPAAV isolated
E
FLGPWPAAS isolated
518A2 WT
518A2 TAP KO
Control
FLGPWPAAV isolated

To test the functionality of these T cell bulks, we measured cytokine responses towards both short peptides pulsed on immortalized HLA-A*0201 positive B cells (figure 2d). Both T cell bulks responded to both peptides by secretion of IFNy and GM-CSF, indicating that the T cell repertoire selected via the high affinity binding V-peptide is cross-reactive to the natural S-peptide. Finally, we tested the recognition of naturally presented S-peptides on the surface of the TAP-negative melanoma 518A2 cell line (figure 2e). Importantly, V-peptide induced polyclonal T cell cultures exhibited preferred recognition of the TAP-negative tumor line as compared to the TAP-proficient counterpart. These data are in line with previous findings that the C-terminal amino acid is an anchor position for binding to HLA-A2*01 molecules and is not directly involved in the TCR interface. We concluded that the exchange of C-terminus into a valine of the LRPAP1-derived TEIPP antigen results in the isolation of a comparable peptide-specific CD8 T cell repertoire from the total pool of CD8 T cells.

TCR gene transfer confers LRPAP1-specificity

Vaccination with synthetic long peptides aims to elicit T cell reactivity from the natural T cell repertoire. TCR gene transfer from TEIPP-specific CD8 T cells constitutes an alternative immunotherapeutic approach to introduce T cell immunity to TAP-deficient cancers. We examined this with the rearranged TCR from the previously described CD8 T cell clone 1A8 (figure 3). DNA sequencing of both TCR-alpha and TCR-beta chains revealed the rearranged sequences and the TCR-Vβ2 usage was confirmed by flow cytometry (table 2 and figure 3a). Retroviral constructs of this TCR with mouse C-domains to improve correct pairing of the transduces genes resulted in successful generation of TCR-transduced CD8 T cells as measured by an antibody to mouse TCR-Cβ domain (figure 3b). Tetramer staining confirmed that both TCR chains were expressed and recognized both S-variant and V-variant peptides, indicating that the specificity T cell clone 1A8 was preserved (figure 3c). Moreover, T cell reactivity was conferred by TCR gene transfer in that stronger cytokine responses were observed against the V-variant peptide than to the S-variant (figure 3d). Finally, TCR transduced T cells selectively recognized TAP-deficient melanomas in a comparable way as the original T cell clone (figure 3e)[7]. Together, these proof-of-concept data demonstrated the feasibility of TCR gene transfer as a mode of immunotherapy for TEIPP antigens and suggests that vaccination with the V-SLP may provide *in vivo* help to prevent T cell contraction.

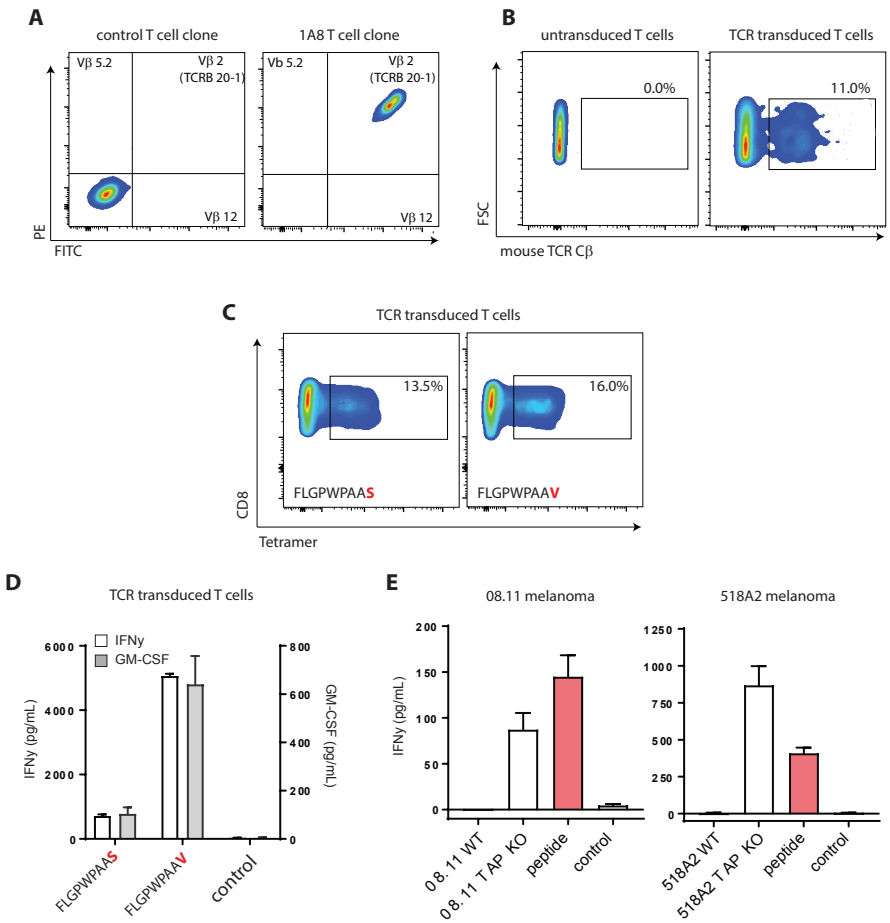


Figure 3: T cell receptor gene transfer of LRPAP1-specific CD8 T cells.

(A) Flow cytometry staining of LRPAP1-specific T cell clone 1A8 using three different Vβ antibodies revealing expression of TCR Vβ2 usage. (B) TCRαβ gene transduction into T cells from a healthy donor. The introduced genes contain the murine TCR-Cβ domain which enhances correct pairing of transgenic alpha and beta chains. (C) Tetramer staining of TCRαβ transduced CD8 T cells. (D) Functional reactivity to peptide pulsed EBV-JY cells of tetramer-enriched TCR-transduced T cells. T cell activation was measured by IFNγ or GM-CSF production. Means and SD are depicted from one out of three experiments with similar outcome. (D) TCR transduced T cells were co-cultured with two melanoma cell lines, which were rendered TAP-deficient by CRISPR/CAS9 technology.

In vitro vaccination with V-SLP promotes the expansion of LRPAP$_{21-30}$-specific TEIPP T cells.

The validate the concept of V-exchanged SLP vaccination for the induction of LRPAP1-directed TEIPP T cell responses a so-called *in vitro* vaccination protocol was used[20,21]. SLP-loaded moDC were co-cultured for two sequential stimulations with tetramer-enriched autologous T cells (figure 4a). Already after one round of stimulation a great expansion of LRPAP1-specific T cells was observed compared to control cultures, as measured by tetramer analysis (16.6% versus 1.5%, respectively) (figure 4b). This specific expansion was even more pronounced after the second stimulation (38.5% versus 0.2%, respectively) (figure 4b), indicating that professional antigen-presenting cells are capable to cross-present the V-SLP and activate TEIPP-specific T cells. These results were remarkable in the light of our previous finding that all LRPAP1-specific CD8 T cells were still in the naïve state of healthy donors[7] and suggest that we observed real *in vitro* priming in our co-cultures.

Next, we generated CD8 T cell clones in order to determine their reactivity towards TAP-deficient cancers. Tetramer-positive T cells were sorted by flow cytometry as single cells and expanded in an antigen-unrelated way using the mitogen phytohaemagglutinin (PHA). T cell clones were analyzed for their specificity by tetramer analysis (figure 4c). Five new T cell clones showed equal staining for both V-peptide and S-peptide tetramers, comparable to the previously isolated clone 1A8[7]. Importantly, two TAP-deficient melanomas were efficiently recognized by three (2H11, 2B9, and 1A10) of these five SLP-induced CD8 T cell clones, in a manner very similar to the previously established clone 1A8 (figure 4d). Collectively, these observations demonstrated that the V-SLP constitutes a functional TEIPP vaccine ready to be exploited for the induction of LRPAP1-specific T cell immunity.

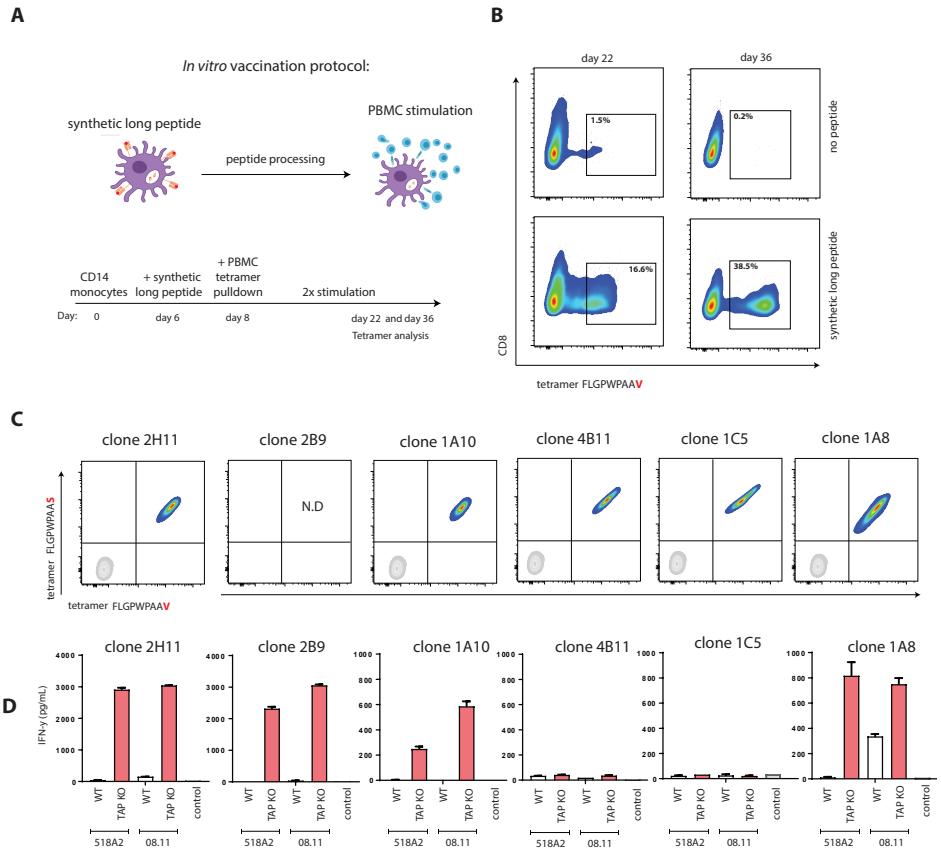


Figure 4: In vitro vaccination with V-SLP results in stimulation of LRPAP1-specific T cell repertoires.

(A) Schematic overview of the *in vitro* vaccination protocol using two V-variant peptides (C-terminal and N-terminal extended) as synthetic long peptides that require cross-presentation by monocyte-derived dendritic cells for priming of CD8 T cells. (B) *In vitro* vaccination protocol resulted in T cell bulk cultures containing LRPAP1-specific CD8 T cells as determined with tetramer staining. Control co-cultures were incubated without any long peptide. (C) LRPAP1-specific T cell clones were generated from single cell FACS sort and tested for reactivity to the natural S-variant peptide. Clone 1A8 was isolated from a previous study[7], and used as a reference clone. (D) LRPAP1-specific T cells clones were examined for recognition of TAP-deficient melanoma cell lines and their wild-type counterparts. Means and SD are shown from one out of two experiments with similar outcome.

Discussion

The aim of this study was to design a synthetic long peptide (SLP) vaccine for the TEIPP antigen derived from the LRPAP1 protein. The HLA-A*0201 presented peptide-epitope $LRPAP_{21-30}$ (FLGPWPAAS) is encoded by the signal peptide (also referred to as 'leader sequence') which functions to dock protein translational products to the sec61 translocation channel in the ER membrane[22]. These signal peptides are usually cleaved from nascent proteins by the protease Signal Peptidase (SPase), resulting in a small protein transmembrane remnant. These signal peptide stubs are liberated from the ER membrane by the protease Signal Peptide Peptidase (SPPase) and cleaves within the lipid bilayer[19]. A part of the signal peptide thus enters the ER in a TAP-independent fashion and this is the reason why signal peptides are overrepresented in the HLA class I binding repertoires of TAP-deficient cells[23-25]. Although not formally demonstrated, the liberation of the $LRPAP_{21-30}$ peptide is most likely not mediated by the proteasome, which is involved in proteolytic cleavage of the majority of HLA class I presented peptides[4]. Indeed, the *in silico* probability algorithm NetCHop predicted that cleavage after the natural serine at p9 of the $LRPAP_{21-30}$ sequence is not likely (figure 1c). We therefore conclude that this signal peptide is processed in a proteasome- and TAP-independent way. Application of synthetic long peptide vaccines, however, depends on uptake by host dendritic cells and processing via the classical route, involving proteasomes and the peptide transporter TAP[26]. We demonstrated that the long peptide of LRPAP1 encompassing the minimal peptide-epitope fails to be cross-presented in dendritic cells (figure 1). The single amino acid substitution from serine to a valine at p9 of the epitope rendered the long peptide sensitive for proteasomal cleavage and, in addition, improved binding affinity to HLA-A*0201. We hypothesize that this single amino acid exchange may alter the processing pathway of this signal sequence peptide, from SPase- and SPPase-mediated to proteasome-mediated.

Table 2: Specification of TCR the LRPAP1-specific T cell clone 1A8

	TCRa chain	TCRb chain
V-GENE and allele	TRAV12-1*01F	TRBV20-1*01F
J-GENE and allele	TRAJ26*01F	TRBJ2-3*01F
(D)-GENE and allele	-	TRBD1*01F
CDR3 region		
Nucleotide sequence	tgt gtg gtg atg ggc tat ggt cag aat ttt gtc ttt	tgc agt gct atg ggg aga cag agc aca gat acg cag tat ttt
Amino acid sequence	CVVMGYGQNFVF	CSAMGRQSTDTQYF
V(D)J region sequence	cggaaggaggtggagcaggatcctggacccttcaatgttccaga gggagccactgtcgctttcaactgtacttacagcaacagtgcttct cagtctttcttctggtacagacaggattgcaggaaagaacctaag ttgctgatgtccgtatactccagtggtaatgaagatggaaggttt acagcacagctcaatagagccagccagtatatttccctgctcatc agagactccaagctcagtgattcagccacctacctctgtgtggtg atgggctatggtcagaattttgtctttggtcccggaaccagattgt ccgtgctgccct	cggaagatgctgctgcttctgctgcttctggggccaggctccggg cttggtgctgtcgtctctcaacatccgagctgggttatctgtaaga gtggaacctctgtgaagatcgagtgccgttccctggactttcagg ccacaactatgttttggtatcgtcagttcccgaaacagagtctcat gctgatggcaacttccaatgagggctccaaggccacatacgagc aaggcgtcgagaaggacaagtttctcatcaaccatgcaagcctg accttgtccactctgacagtgaccagtgcccatcctgaagacagc agcttctacatctgcagtgctatggggagacagagcacagatac gcagtattttggcccaggcacccggctgacagtgctcgaggacct gaaaaacgtgttccca

Side-by-side comparison of S- and V-peptide induced T cell responses with the use of an *in vitro* vaccination protocol revealed that both repertoires were comparable concerning cross-reactivity and functionality (figure 4). Stimulation with the short V-peptide seemed to recruit a CD8 T cell repertoire with lower affinity as witnessed by less intense staining with tetramers (figure 2b). However, stimulation with dendritic cells loaded with long peptides, which require intracellular cross-presentation, resulted in polyclonal CD8 T cell bulks and clones with high affinity and strong capacity to recognize the natural S-variant on TAP-deficient melanomas (figure 4). These findings imply that vaccination with the optimized V-SLP would result in the generation of LRPAP1-specific T cells with high affinity TCRs. This advantage of SLP over vaccination with short minimal epitopes is in line with our previous investigations in pre-clinical mouse models and suggest that the SLP platform is well suited to recruit a high affinity TCR repertoire[14,17].

Interestingly, we previously showed that LRPAP1-specific T cells all reside in the naïve repertoire of healthy blood donors, indicating that our *in vitro* vaccination protocol actually primed CD8 T cells and not merely reactivated memory T cells[7]. The differentiation status of LRPAP-1-specific T cells in cancer patients and in particular those harboring TAP-deficient tumor cells needs further analysis, however, data from our mouse tumor model revealed that TEIPP-directed CD8 T cells are still naïve in these situations[6,27]. We found that TAP-deficient tumors failed to prime TEIPP T cells and also host dendritic cells were unable to pick up TEIPP antigens and cross-prime them. Consequently, TEIPP immunity might need to be installed by active immunizations, like we suggested here via SLP vaccines, or by TCR gene transfer into host T cells. Thus, the optimized long peptide of the signal peptide of LRPAP1 containing one amino acid exchange constitute an ideal vaccine candidate to induce TEIPP immunity in cancer patients. Although cancer vaccines lack potential to induce cancer remission as standalone therapy[8], combinations with checkpoint blockade or other immunomodulatory compounds might lead to successful immune attack to immune-escaped TAP-deficient cancers, as has been shown for the combination of cancer-virus vaccination with PD-1 blockade[11].

Material and methods

Cell culture

Tumor cells were cultured in DMEM medium (Gibco) supplemented with 100ug/mL streptomycin, 100 U/mL penicillin, 2mM L-glutamine (Invitrogen) and 10% FCS (Gibco). Genetic disruption of the TAP1 gene in human tumor cell lines was performed with CRISPR/CAS9 and described before[7]. T cells were cultured in IMDM medium (Gibco) supplemented with 2mM L-glutamine, 10% human serum (Sanquin), and 50U/mL IL-2 (proleukine, Novartis). T cells were stimulated every 10-14 days using synthetic short peptide (in house synthesis) or 800 ng/ml PHA (Phytohaemagglutinin) (Murex Biotech), supplemented with 100 U/ml IL-2 and IL-7 (5ng/mL), and a feeder mix containing irradiated PBMCs (1×10^6 cells, 80 Gy), and EBV-JY cells (1×10^5 cells, 100 Gy). All cell types were maintained in humidified air incubator at 37°C and 5% CO_2.

In vitro vaccination protocol

HLA-A*02:01 positive PBMCs were isolated from buffy-coats from consented donors (Sanquin bloodbank, Amsterdam), using a gradient ficoll layer. PBMCs were incubated with anti-CD14 magnetic beads for 20 min at 4 °C and the CD14 positive monocytes were isolated using magnetic separation columns (miltenyi). CD14+ monocyte were cultured in RPMI medium supplemented with 10% FCS, GM-CSF (800 units/ml), and IL-4 (500 units/ml) for 6 days to generate immature monocyte-derived dendritic cells. On day 6, the immature moDCs were incubated with synthetic long peptide (20 µg/ml, in house synthesized) for 24h, and matured with LPS (20 ng/ml) stimulation on day 7. Differentiation of monocytes to matured moDCs was verified by flow cytometry analysis. Matured moDCs were co-cultured with tetramer enriched T cell bulks in complete T cell medium. T cell bulks were stimulated a second time after 14 days. T cell specificity and reactivity were analyzed by flow cytometry.

T cell clone isolation from expanded T cell bulks

Expanded CD8 T cells were single cell sorted on tetramer positive cells in 96-well plates, using an Aria III machine (BD). Following FACS sorting, single T cells were non-specifically stimulated using PHA (800ng/mL), a feeder mix containing irradiated PBMCs (1×10^6 cells, 80 Gy), and EBV-JY cells (1×10^5 cells, 100 Gy), supplemented with IL-2 (100 units/mL) and IL-7 (5ng/mL) every 10-14 days. Expanded T cell clones were analyzed on tetramer specificity and further expanded in T25 culture flasks using the non-specific (PHA) T cell expansion protocol.

T cell receptor sequencing

Monoclonal T cells ($2x10^6$) were washed in cold PBS/BSA and pelleted by centrifugation. mRNA from T cell clones were isolated using the Dynabeads mRNA purification kit (Thermofisher). Full-length cDNA from the TCRα and TCRβ was generated using SMARTscribe reverse transcriptase with oligo's binding the constant domain of the TCRs[28]. Amplification of the cDNA transcript was done by standard PCR reaction using nested primers and high fidelity Taq polymerase. The PCR reaction mix was purified over a DNA purification column and nucleotide sequence analysis was done using Sanger sequencing (in house sequence facility). TCR sequencing results were analyzed using the T cell receptor sequence alignment software (V-quest) from IMGT (http://www.imgt.org/). Full-length codon optimized cDNA transcripts for murinized TCRs for both TCR-alpha and TCR-beta chains were cloned into a retroviral pMP71 flex expression vector[28].

Retrovirus production and T cell transductions

Platinum-Amphotropic retrovirus production (Plat-A) retroviral packing cells (Cell Biolabs) were used for retrovirus production. Plat-A cells were seeded in 6-well plates and incubated overnight until fully attached. Next, the cells were transfected with 2 µg pMP71_1A8 TCR vector using lipofectamine 2000. Retrovirus supernatant was collected on 24h and

48h after transfection, spun down to remove cells, and stored in -80 ^{0}C. CD8 T cells were purified from PBMC using magnetic bead isolation (Miltenyi), and a-specifically activated by aCD3/aCD28 beads (Thermofisher). After 48h, 1x10^{6} activated CD8 T cells were plated in a retronectin (Takara) coated 24-well together with 0.5mL retrovirus supernatant. Subsequently, CD8 T cells and retrovirus containing supernatant was spun down for 120min at 1300g to increase the efficiency of transduction. 48h after transduction, the T cells were placed in a cell culture incubator for another 48h.

Flow cytometry analysis

Tetramer staining on CD8 T cells was performed by 15min incubation at 4°C and washed three times with cold PBS/BSA prior to cell surface staining. T cells were stained with anti-CD3 (clone SK-7, BD), anti-CD4 (clone SK-3, BD), anti-CD8 (clone SK-1, BD) antibodies for 30min at 4°C and washed three times with cold PBS/BSA. T cell activation was measured by intracellular IFNγ staining (XMF1.2, Biolegend) using an ICS kit (BioLegend) according to manufactures protocol. moDCs were stained with anti-CD1a (clone HI149, BD), anti-CD14 (clone M5E2, BD), anti-CD80 (clone L307.4, BD), anti-CD83 (clone HB15e, BD), anti-CD86 (clone IT2.2, biolegend), and anti HLA-DR (clone G46-6, BD) antibodies for 30min at 4°C and washed three times with cold PBS/BSA. Samples were acquired using a BD LSRFortessa™ flow cytometry system and analyzed using FlowJo software (Tree Star). Single cell sorting was done using a BD Aria III™ FACS.

Statistics

Statistical analysis was calculated using the T-test (paired, two-tailed) with welch correction to determine the statistical significance of the differences. A minimum of two technical replicates was used in all experiments. All experiments were at least performed two times. Differences were considered statistically significant at $p < 0.05$. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Acknowledgements

We thank the flow cytometry facility for their support with the FACS based work. Also, we thank G. Moray for his creative insights. This work was financially supported by the Dutch Cancer Society (grant KWF 2013-6142).

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