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Optimal organization of a polypeptide-based candidate cancer vaccine composed of cryptic tumor peptides with enhanced immunogenicity

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Abstract

Polyspecific tumor vaccination should offer broad control of tumor cells and reduce the risk of emergence of immune escape variants. Here, we evaluated the capacity of a polypeptide composed of optimized cryptic peptides derived from three different universal tumor antigens ($TERT_{988Y}$, HER-2/neu_{402Y} and MAGE-A_{248V9}) to induce a polyspecific CD8 cell response both in vivo in HHD mice and in vitro in humans. A mixture of $TERT_{988Y}$, HER-2/neu_{402Y} and MAGE-A_{248V9} peptides failed to induce a trispecific response. In contrast, a polypeptide composed of the three peptides stimulated a trispecific immune response. Interestingly, the capacity of the polypeptide to induce a trispecific response depended on its internal organization. Six different polypeptide variants corresponding to all possible combinations of the three peptides were tested. Only one variant, named Poly-6, elicited an immune response simultaneously targeting all three peptides.

Keywords: Polypeptide; Tumor vaccination; Cryptic peptides

1. Introduction

The recent identification of tumor-associated antigens targeted by antitumor cytotoxic T lymphocytes (CTL) has opened the way to cancer vaccine approaches aimed at stimulating the tumor-specific CTL repertoire.

Experimental antitumor vaccines take many forms, including free peptides, dendritic cells loaded with peptides or tumor lysates and DNA. Although peptide-based vaccines are very attractive over other forms in term of feasibility, many studies, with dominant tumor peptides were found to elicit only weak immunological and clinical responses, with strong inter-patient variability [1]. Several factors may explain these relatively disappointing results. First, most tumor antigens are non-mutated self proteins also expressed by normal tissues, including the thymus. This raises issues of tolerance of the tumor-specific CTL repertoire [2,3], which involves dominant rather than cryptic peptides [4–6]. In fact, we recently demonstrated that cryptic peptides induced antitumor immunity more efficiently than dominant peptides [7]. Second, approaches based on single epitopes induce an HLA-restricted CTL response against only one antigen, which owing to the genetic instability of tumors, may be not expressed by all tumor cells [8,9]. Approaches eliciting CTL responses to multiple antigens would have several potential advantages. In particular, expression of at least one target antigen should be sufficient to trigger cytotoxicity and tumor cells are unlikely to lose all the target antigens simultaneously, especially when the antigens in question are essential for cell survival and tumor growth. We have previously shown that this approach can elicit strong immune responses [10].

Finally, broad-spectrum cancer immunotherapy should target universal tumor antigens, such as TERT, HER-2/neu, MUC-1 and MAGE-A that are over-expressed by a wide variety of tumors [11–17]. Most of these antigens are involved in tumor cell survival and tumorigenicity and

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their down-regulation to escape the immune response may therefore, have deleterious effect on tumor growth.

In this study we combined three universal tumor-antigen-derived optimized cryptic peptides ($TERT_{988Y}$, HER-2/neu_{402Y} and MAGE-A_{248V9}) in a 28-aminoacid polypeptide and evaluated the capacity of the polypeptide to induce an immune response against all three peptides simultaneously, both in vivo in HLA-A*0201 transgenic (HHD) mice and in vitro in healthy human donor. Each of the three peptides had previously been shown to elicit an antitumor response in vivo and in vitro [7,18]. Interestingly, CTL elicited by MAGE-A_{248V9} targeted all MAGE-A antigens (-A1, -A2, -A3, -A4, -A6, -A10, -A12) [19].

Here we report that (a) a polypeptide consisting of $TERT_{988Y}$, HER-2/neu_{402Y} and MAGE-A_{248V9} elicits a polyspecific CD8 response, contrary to a simple mixture of the three peptides; (b) the capacity of the polypeptide to induce a polyspecific CD8 response depends on its internal organization: among the six variants corresponding to all possible arrangements of the three peptides, only one produced a trispecific CTL response in almost all experiments with mice and human cells.

2. Materials and methods

2.1. Animals

HLA-A*0201 transgenic HHD mice have been described elsewhere [20]. They are β_2-microglobulin and H-2D^b double knockout ($\beta_2m^{-/-}$ and $D^{b-/-}$) and express an HLA-A*0201 monochain composed of a chimeric heavy chain (HLA-A*0201 α1 and α2 domains and the mouse H-2D^b α3 and intracellular domains of D^b allele) linked by its NH_2 terminus to the COOH terminus of the human β_2m by a 15-amino acid long peptide.

2.2. Cells

Murine RMAS/HHD cells have been described elsewhere [20]. Briefly, the TAP-deficient RMA-S cell line was transfected with the HLA-A*0201 monochain composed of the chimeric heavy chain (HLA-A*0201 α1 and α2 domains and mouse H-2D^b α3, transmembrane and cytoplasmic domains) linked to the human β_2m. HLA-A*0201-expressing human tumor T2 cells are TAP1/2-deficient. All cells were grown in RPMI 1640 or DMEM medium supplemented with 10% fetal calf serum (FCS).

2.3. Selected peptides

The peptides were synthesized by Epytop (Nîmes, France). Three peptides were selected to be included in the polypeptide (Table 1). HER-2/neu_{402Y} and $TERT_{988Y}$ are the optimized variants of the low-HLA-A*0201-affinity cryptic peptides HER-2/neu_{402} and $TERT_{988}$, themselves derived from the widely expressed tumor antigens HER-2/neu and TERT [18]. They differ from the native peptides at position 1, where the native residue is replaced by a Y. This substitution enhances the affinity of HLA-A*0201-restricted cryptic peptides [21]. MAGE-A_{248V9} is the optimized variant of the low-HLA-A*0201-affinity MAGE-$A_{248D9/G9}$ that is common to all MAGE-A molecules. It differs from the native peptides at position 9, where the amino acids D/G are replaced by the primary anchor residue V. This substitution also enhances the affinity of peptide for HLA-A*0201 molecule [19].

Table 1
Affinity for HLA-A*0201 of native and optimised MAGE-A_{248}, HER-2/neu_{402} and $TERT_{988}$ peptides

Peptide	Sequence	RA[a]	DC_{50}[b]
Native			
$TERT_{988}$	DLQVNSLQTV	28.6	<2
HER-2/neu_{402}	TLEEITGYL	19.0	<2
MAGE-A_{248D9}	YLEYRQVPD	22.5	<2
MAGE-A_{248G9}	YLEYRQVPG	>27	<2
Optimized			
$TERT_{988Y}$	YLQVNSLQTV	2.1	>6
HER-2/neu_{402Y}	YLEEITGYL	3.6	4
MAGE-A_{248V9}	YLEYRQVPV	1.8	4

The substituted amino acid is underlined.
[a] RA, relative affinity; concentration of experimental peptide/concentration of reference peptide that induced 20% of HLA-A*0201 obtained by 100 μM of the reference peptide. Affinity of the reference peptide = 1.
[b] DC_{50}: dissociation complex 50; half life of the HLA/peptide complex (hours).

All three peptides exhibited high HLA-A*0201 binding affinity (RA < 5) and formed stable HLA/peptide complexes (DC_{50} > 2 h) [18,19]. As previously shown, all three peptides were immunogenic in HLA-A*0201 transgenic HHD mice [18,19]. More importantly, mouse CTL lines specific for optimized variants cross-recognized and killed RMAS/HHD targets loaded with the corresponding native peptide [18,19].

2.4. Flow cytometric immunofluorescence analysis

For tetramer labeling, cells from inguinal and para-aortic lymph nodes (LN) of immunized mice were stained with 15 μg/ml PE-coupled HLA-A2/$TERT_{988Y}$, HLA-A2/MAGE-A_{248V9} and HLA-A2/HER-2/neu_{402Y} tetramers (Proimmune, Oxford, UK) in the presence of an anti-Fc receptor antibody (clone 2.4 G2) in 20 μl of PBS, 2% FCS for 1 h at room temperature. The cells were washed once in PBS, 2% FCS and then stained with anti-CD44-FITC (clone 1M.178), anti-TCRβ-CyChrome™ (clone H57) and anti-CD8α-APC (clone 53.6.7) (BD Biosciences, Le Pont de Claix, France) in 50 μl of PBS, 2% FCS for 30 min at 4 °C. The cells were then washed once in PBS, 2% FCS and immediately analyzed in a FACSCalibur® flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.5. Generation of CD8 cells from human peripheral blood mononuclear cells (PBMC)

PBMC were collected by leukapheresis from healthy HLA-A*0201 volunteers. Monocyte-derived dendritic cells were produced from adherent cells (2×10^6 cells/ml) cultured for seven days with 500 IU/ml GM-CSF (Leucomax®, Schering-Plough, Kenilworth, NJ, USA) and 500 IU/ml IL-4 (R&D Systems, Minneapolis, MN, USA) in complete medium (RPMI 1640 supplemented with 10% heat-inactivated human AB serum, 2 μM L-glutamine and antibiotics). On Day 7, monocyte-derived dendritic cells were pulsed with 10 μM peptides or polypeptides for 2 h; the maturation agents Poly I:C (Sigma, Oakville, Canada) at 100 ng/ml, and anti-CD40 mAb (clone G28-5, ATCC, Manassas, VA, USA) at 2 μg/ml were added to the culture and monocyte-derived dendritic cells were further incubated at 37 °C overnight or for up to 48 h. Mature monocyte-derived dendritic cells were then irradiated (3500 rads). $CD8^+$ cells were purified by positive selection with CD8 MicroBeads (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions. $CD8^+$ cells (2×10^5) + $CD8^-$ cells (6×10^4) were stimulated with 2×10^4 peptide-pulsed monocyte-derived dendritic cells in complete culture medium supplemented with 1000 IU/ml IL-6 and 5 IU/ml IL-12 (R&D Systems, Minneapolis, MN, USA) in round-bottomed 96-well plates. From Day 7, cultures were restimulated weekly with peptide-loaded monocyte-derived dendritic cells in the presence of 20 IU/ml IL-2 (Proleukin, Chiron Corp., Emeryville, CA, USA) and 10 ng/ml IL-7 (R&D Systems, Minneapolis, MN, USA). After the third restimulation, CD8 cells were tested in an IFN-γ release assay.

2.6. Intracellular IFN-γ labeling

T cells (10^5) were incubated with 2×10^5 T2 cells loaded with stimulating peptide in the presence of 20 μg/ml Brefeldin-A (Sigma, Oakville, Canada). Six hours later they were washed, stained with r-phycoerythrin-conjugated anti-CD8 antibody (Caltag Laboratories, Burlingame, CA, USA) in PBS for 25 min at 4 °C, washed again, and fixed with 4% PFA. The cells were then permeabilized with PBS, 0.5% BSA, 0.2% saponin (Sigma, Oakville, Canada), and labeled with an allophycocyanin-conjugated anti-IFN-γ mAb (PharMingen, Mississauga, Canada) for 25 min at 4 °C before analysis with a FACSCalibur® flow cytometer.

3. Results

3.1. Immune responses to the peptide mixture

The simplest way to stimulate a polyspecific CD8 response in vivo would be to inject a mixture of the relevant peptides.

Table 2
CD8 T cell responses against MAGE-A_{248V9}, HER-2/neu_{402Y} and $TERT_{988Y}$ in individual mice immunized with an equimolar peptide mixture

Mouse number	Specific T CD8 response against		
	MAGE-A_{248V9}	HER-2/neu_{402Y}	$TERT_{988Y}$
1	++	–	–
2	+	+	–
3	+	–	–
4	+	–	–
5	–	++	+
6	–	++	++
7	+	–	++
8	+	++	–

"+": The percentage of tetramer-positive CD8 T cells was between one and two-fold the cutoff as defined in Section 2 (0.16% for MAGE-A_{248v9}, 0.13% for HER-2/neu_{402Y}, and 0.16% for $TERT_{988Y}$); "++": the percentage of tetramer-positive CD8 T cells was more than twice the cutoff.

We therefore, examined whether HHD mice vaccinated with an equimolar mixture of peptides HER-2/neu_{402Y}, $TERT_{988Y}$ and MAGE-A_{248V9} developed a polyspecific response in vivo. The immune response was evaluated by measuring the frequency of peptide-specific CD8 T cells in the lymph nodes draining the injection site 7 days after vaccination, using specific tetramers. Before use, each tetramer was validated with peptide-specific CTL lines as previously described [22]. A positive response was recorded when the percentage of tetramer-positive CD8 cells was higher than the mean percentage + 3 standard deviations of tetramer-positive CD8 cells in six naive mice (0.16% for MAGE-A_{248V9}, 0.13% for HER-2/neu_{402Y} and 0.16% for $TERT_{988Y}$). Eight mice were vaccinated with the peptide mixture in two independent experiments (Table 2). None of the eight mice responded simultaneously to all three peptides. Three mice responded to one peptide and five responded to two peptides. Responses to MAGE-A_{248V9} were more frequent (6/8 mice) than responses to HER-2/neu_{402Y} (4/8 mice) or $TERT_{988Y}$ (3/8 mice). The inability of the peptide mixture to stimulate a trispecific CD8 T cell response was confirmed in vitro with human cells. PBMC from three HLA-A*0201 donors were stimulated in vitro with a mixture of MAGE-A_{248V9}, HER-2/neu_{402Y} and $TERT_{988Y}$ and after four cycles of restimulation, were tested for their capacity to recognize and be activated by stimulator cells loaded with each peptide. PBMC activation was evaluated by measuring the percentage of IFN-γ-producing CD8 cells by means of intracellular labeling. A positive response was recorded when the percentage of activated PBMC was at least twice that obtained with an irrelevant peptide. None of the three donors developed a specific CD8 T cell response against all three peptides (Table 3). Donor no. D5725 responded to MAGE-A_{248V9}/HER-2/neu_{402Y}, donor no. D7225 responded to HER-2/neu_{402Y}, and donor no. D7241 responded to MAGE-A_{248V9}/$TERT_{988Y}$.

These results demonstrated that vaccination with a simple mixture of immunogenic peptides did not generate a polyspecific response.

Table 3
Peptide-specific CD8 T cells induced by stimulation of healthy donor PBMC with the peptide mixture

PBMC donor	% of IFN-γ-producing CD8 cells in response to			
	MAGE-A_{248V9}	HER-2/neu_{402Y}	$TERT_{988Y}$	Irrelevant
D5725	**0.29**	**0.33**	0.09	0.09
D7225	0.32	**0.54**	0.24	0.27
D7241	**2.84**	0.36	**1.21**	0.22

Peptide-specific CD8 T cells were generated by in vitro stimulation of PBMC from three healthy donors with an equimolar mixture of MAGE-A_{248V9}, HER-2/neu_{402Y} and $TERT_{988Y}$ peptides. The specificity of induced CD8 T cells was evaluated by measuring the % of IFN-γ-producing CD8 cells after stimulation with peptide-loaded T2 cells as described in Section 2. Values more than twice the negative control value, indicating a positive response, are shown in bold.

3.2. Polypeptide immunogenicity

We then examined whether vaccination with polypeptides composed of MAGE-A_{248V9}, HER-2/neu_{402Y} and $TERT_{988Y}$ elicited a trispecific CD8 T cell response. Six polypeptide variants, designated Poly-1 to Poly-6, encompassed all possible peptide arrangements (Table 4A). First, we investigated if the best candidate vaccine within the six polypeptide combinations could be selected in silico. For that purpose, we took into account the processing rules of each peptide at its C-terminal position and the generation of junctional peptides with high affinity for HLA-A*0201. Processing at the C-terminal position was evaluated by using two online predictive models of proteasome cleavage (Netchop: http://www.cbs.dtu.dk/services/NetChop/, PAProc: http://www.uni-tuebingen.de/uni/kxi/) [23–25]. A peptide was arbitrarily considered to be processed if its cleavage was predicted by both models. The affinity of the new junctional peptides was evaluated by using the Bimas predictive model [26]. None of the six variants was associated with cleavage of all three peptides in the predictive models (Table 4B). Moreover, Poly-1, Poly-3, Poly-4 and Poly-5 generated junctional peptides with high predictive scores for binding to HLA-A*0201 (Table 4A). The junctional epitope YLYLQVNSL predicted in Poly-1 and -3 matches a self peptide derived from the variable heavy chain region of human immunoglobulin.

As this predictive approach failed to identify the polypeptide variant with the highest theoretical efficiency, we experimentally tested the variants for their capacity to generate a trispecific CD8 T cell response in vivo in HHD mice and in vitro with PBMCs derived from healthy HLA-A*0201 donors. HHD mice were vaccinated with each polypeptide, and CD8 T cells specific for the individual peptides were identified in draining lymph nodes by using specific tetramers. All six polypeptide variants were immunogenic in HHD mice (i.e. they generated a response to at least one peptide) but the

Table 4
In silico analysis of the six possible polypeptide variants

(A) Generation of junctional peptides predicted to have high HLA-A*0201 affinity

Polypeptide	Sequence	Sequence of junctional epitope	Bimas score
Poly-1 M-N-T	YLEYRQVPV-YLEEITG*YL-YLQVNSL*QTV	YLYLQVNSL	723.245
Poly-2 M-T-N	YLEYRQVPV-YLQVNSLQTV-YLEEITGYL		
Poly-3 N-T-M	YLEEITG*YL-YLQVNSL*QTV-YLEYRQVPV	YLYLQVNSLQ	723.245
Poly-4 N-M-T	YLEEITG*YL-YLEYRQV*PV-YLQVNSLQTV	YLYLEYRQV	307.142
Poly-5 T-N-M	YLQVNSLQTV-YLEEITG*YL-YLEYRQV*PV	YLYLEYRQV	307.142
Poly-6 T-M-N	YLQVNSLQTV-YLEYRQVPV-YLEEITGYL		

(B) Prediction of proteasome cleavage positions in the six possible polypeptide configurations

	Cleavage prediction algorithm	Sequence and site of cleavage (| and (|))	Number of predicted processed peptides[c]
Poly-1	Paproc[a]	YLEYRQV|PVY|L|E|EITGY|L|Y(|)L|QV|NSLQTV	0
	Netchopp[b]	Y|L|EY|RQVPVY|L|EEITGY|L|Y|L|QVNSL|QTV	0
Poly-2	Paproc	YLEYRQV|PVY|L|QV|N(|)SLQT|VYLE|EITGYL	0
	Netchopp	Y|L|EY|RQVPVY|L|QV|NSL|QT|V|Y|LEEITGYL	0
Poly-3	Paproc	YLEEITGY|L|Y(|)L|QV|NSLQT|VYLE|Y(|)RQVPV	0
	Netchopp	YLEEITGY|L|Y|L|QVNSL|QTV|Y|L|EY|RQVPV	0
Poly-4	Paproc	YLEEITGY|L|YL|EYRQV|PVY|L|QV|N(|)SLQTV	0
	Netchopp	YLEEITGY|L|Y|L|EYRQVPVY|L|QV|NSL|QTV	0
Poly-5	Paproc	YLQVNSLQT|VYLE|EITGY|L|YL|EYRQVPV	0
	Netchopp	Y|L|QV|NSL|QTV|Y|L|EEITGY|L|Y|L|EYRQVPV	0
Poly-6	Paproc	YLQVNSLQT|VYLE|Y(|)RQV|PVY|L|E|EITGYL	0
	Netchopp	Y|L|QV|NSL|QTV|Y|L|EY|RQVPVY|L|EEITGYL	0

[a] For paproc (|) symbolizes a low probability of cleavage.
[b] For netchopp, the threshold was set at 0.5 and the network used was: "C-term 1.0. C-term 2.0 and 20 S".
[c] Cleavage prediction by both models was required to consider that a peptide would be processed.

frequency of responding mice varied from one variant to the other. The most immunogenic variants were Poly-1, Poly-3 and Poly-6, with 100, 87 and 83% of responding mice, respectively (Table 5). Poly-2, Poly-4 and Poly-5 induced a response in 57, 62 and 62% of vaccinated mice, respectively. The frequency of strong responses (% of tetramer-positive CD8 cells at least twice the cutoff value; designated ++) was highest with Poly-6 (41% of all responses), Poly-3 (30% of all

Table 5
CD8 T cell response against MAGE-A_{248V9}, HER-2/neu_{402Y} and $TERT_{988Y}$ in individual mice immunized with the different polypeptides.

	Specific T CD8 response against		
	MAGE-A_{248V9}	HER-2/neu_{402Y}	$TERT_{988Y}$
Poly-1	+	++	+
	-	+	-
	+	++	+
	-	+	-
	+	+	-
	+	-	-
	++	-	-
Poly-2	-	+	+
	-	++	+
	-	+	-
	-	-	-
	-	-	-
	-	-	-
	+	-	-
Poly-3	+	+	-
	-	+	-
	++	++	+
	++	-	-
	+	-	-
	+	-	-
	+	-	-
	-	-	-
Poly-4	-	-	+
	+	+	+
	-	-	+
	-	-	-
	+	++	+
	-	-	-
	+	+	+
	-	-	-
Poly-5	-	+	-
	-	-	-
	+	+	+
	+	+	+
	+	+	-
	-	-	-
	-	-	-
	+	-	-
Poly-6	+	++	+
	+	++	+
	+	+	+
	+	-	+
	+	++	+
	-	-	-
	-	-	-
	+	+	+
	-	+	-
	++	++	++
	++	++	+
	++	++	++

The immune response was evaluated by measuring the % of tetramer-positive CD8 T cells in the draining lymph nodes of vaccinated mice. "−": Percentage of tetramer positive CD8 T cells below the cutoff, as defined in Section 2 (0.16% for MAGE-A_{248v9}, 0.13% for HER-2/neu_{402Y}, and 0.16% for $TERT_{988Y}$); "+": percentage one- to two-fold the cutoff; "++": percentage more than twice the cutoff. The shaded lines correspond to mice responding to all three peptides.

Table 6
Peptide-specific CD8 T cells induced by polypeptide stimulation of healthy human donor PBMC

Polypeptide	Donor	Specific T CD8 response against		
		MAGE-A_{248V9}	HER-2/neu_{402Y}	$TERT_{988Y}$
Poly-1	D9442	++	++	-
	D0204	++	+	-
	D7131	+	++	+
	D1100	-	-	+
Poly-3	D9242	++	+	-
	D3031	-	+	-
Poly-4	D3031	-	-	+
	D9242	-	-	+
	D7131	+	-	+
Poly-5	D7771	-	+	+
	D0204	+	+	-
	D7017	+	-	+
Poly-6	D7744	+	+++	+
	D4212	+	+	+
	D7017	+++	+	+
	D7225	+++	+	+
	D7601	-	-	-

Peptide-specific CD8 T cells were generated by in vitro stimulation of PBMC from healthy donors with the different polypeptides. The specificity of induced CD8 T cells was evaluated by measuring the % of IFN-γ-producing CD8 cells after stimulation with peptide-loaded T2 cells as described in Section 2. "−": % of IFN-γ-positive cells less than two-fold the negative control (irrelevant peptide); "+": % of IFN-γ-positive CD8 cells two-fold higher than the negative control; "++": % of IFN-γ-positive CD8 cells 2- to 10-fold higher than the negative control; "+++": % of IFN-γ-positive CD8 cells more than 10-fold higher than the negative control. The shaded lines correspond to donor cells responding to all three peptides.

responses) and Poly-1 (25% of all responses). The responses were directed against MAGE-A_{248V9} in 74% of responding mice, HER-2/neu_{402Y} in 71% and $TERT_{988Y}$ in 55%. Analysis of the immune responses in individual mice showed that Poly-6 induced a trispecific response in 67% of vaccinated mice, followed by Poly-4 (37.5%), Poly-1 (28.5%), Poly-5 (25%) and Poly-3 (12.5%). Poly-2 did not induce a trispecific response in any mice. These results were confirmed with human cells in vitro. Each polypeptide (except for Poly-2, which was very weakly immunogenic in HHD mice) was tested with cells from 2 to 5 healthy donors. In vitro immune responses were assessed by measuring the percentage of CD8 cells producing IFN-γ after specific peptide activation. All five polypeptides stimulated T cells to respond to at least one peptide in 80–100% of donors. However, only Poly-6 and Poly-1 induced trispecific CD8 responses. Poly-6 induced responses to all three peptides in 80% of donors, compared to only 25% of donors with Poly-1 (Table 6). Poly-6 also elicited the strongest responses (against MAGE-A_{248V9} in donor no. D7017 and D7225; and against HER-2/neu_{402Y} in donor no. D7744).

Together, these results showed that Poly-6 induced frequent and strong trispecific CD8 T cell responses both in vivo (HHD mice) and in vitro (human PBMC).

4. Discussion

This study of polypeptides composed of three HLA-A*0201-restricted optimized cryptic peptides derived from the universal tumor antigens hTERT, HER-2/neu and MAGE-A identified a polypeptide, named Poly-6, that induced a CD8 response against all three component peptides both in HLA-A*0201-expressing transgenic HHD mice and in healthy human donor cells.

Several parameters may influence the capacity of polypeptide vaccines to induce polyspecific responses, such as the presence of a Th peptide, peptide affinity for MHC molecules, optimal proteasome cleavage (which depends on the peptide arrangement), the presence of inter-peptide spacers and creation of new junctional high HLA affinity peptides. However, there is no consensus on the influence of most of these parameters on polypeptide vaccine efficiency.

Results of Mateo et al. [27] suggest that there is a correlation between the affinity and the immunogenicity of peptides included in a polypeptide melanoma vaccine. In this polypeptide, composed of 10 peptides, the seven immunogenic peptides showed high HLA-A*0201 affinity, contrary to two of the three non-immunogenic peptides ($MelanA_{32}$ and $tyrosinase_1$). Moreover, Palmowski et al. [29] found that affinity diversity among peptides included within a polypeptide construct was crucial for skewing the immune response and for CTL immunodominance in HLA-A*0201 transgenic mice. In contrast, Ishioka et al. [28] tested a polypeptide HIV vaccine and found that peptide affinity did not influence its capacity to induce a CTL response. Ishioka et al. [28] found that inclusion of the Th peptide PADRE improved vaccine efficiency, while Velders et al. [30] found no difference in antitumor immunity between normal and CD4-deficient mice vaccinated with a polypeptide containing a tetanus toxin-derived Th peptide.

There is broad agreement on the influence of polypeptide organization (peptide arrangement, addition of spacers), which should ideally permit appropriate cleavage of all the component peptides and avoid the creation of new junctional peptides with high affinity for the relevant HLA molecule. Several studies have shown that the presence of spacers between peptides increases vaccine efficiency by promoting the cleavage of individual peptides [31–33]. Moreover, Ishioka et al. found that the position of a peptide within a polypeptide can affect its immunogenicity. This highlights the importance of the global configuration of the polyepitope [28]. Our results support these findings, as one of the six polypeptide arrangements we tested was highly immunogenic, while another was minimally effective. This is the first direct demonstration that polypeptide organization must be optimized in order to obtain maximal immunogenicity. Our results also show that this optimal organization cannot be foreseen by using current predictive models of proteasome cleavage. Indeed, none of the six candidate polypeptides was predicted to be more efficiently cleaved than the others. Yet, it should be noticed that we cannot exclude that the generation of $TERT_{988}$, HER-2/Neu_{402} and $Mage_{248}$ epitopes from their native sequence is independent on proteasome but rather involves alternative processing pathways. Finally, our results indicate that the generation of junctional peptides predicted to have high affinity for HLA-A*0201 in the Bimas model system cannot predict the efficiency of the polypeptide as a vaccine. Indeed, a junctional epitope with a high HLA affinity binding is predicted in Poly-6 which is the most immunogenic polypeptide whereas, no junctional peptide has been predicted in Poly-2 which failed to elicit a polyspecific response.

We also found that vaccination with a mixture of the three peptides was far less efficient than polypeptide vaccination at eliciting a polyspecific response. Interestingly, cells from human donor D7225 responded to all three peptides after stimulation with Poly-6 ex vivo, but only to HER-2/neu_{402Y} after stimulation with the peptide mixture. Similar results have been obtained with another polypeptide composed of Hsp70-derived peptides (Faure et al., submitted for publication). The use of exogenous peptides has the drawback that the number of peptide/MHC I complexes decays with the same kinetics as the exogenous peptide concentration [34]. The short half-life of these complexes would lead to a marked loss of priming efficiency [35]. Because of their length which excludes a direct binding to MHC class I molecules, the polypeptides present the advantage to be taken up by professional APC that are able to process exogenously derived Ags for presentation in MHC class I molecules. The cross presentation of these polypeptides constitute an endogenous source of peptides to be presented in much slower and sustained kinetics. This long peptide strategy has been shown to result in an enhanced immunogenicity over the short peptide form [36].

Besides the ability of Poly-6 to induce a polyspecific immune response, it might also present the advantage to be able to recruit a high avidity T cell repertoire specific for cryptic peptides. Indeed, each of the three optimized cryptic peptides used in the present study have previously been shown to be more immunogenic than their native counterpart, to induce specific CTL which cross recognize the relevant native epitope and to elicit an antitumor response in vivo and in vitro [7,18]. Preliminary studies suggest that a first immunization with an optimized cryptic peptide followed by an antigenic challenge with the native counterpart would be more efficient in generating a high functional avidity T cell repertoire specific for the native cryptic peptide. In this context, the possibility to use polypeptides composed of native cryptic peptides to challenge the immune response initiated against polypeptides composed of modified cryptic peptides is currently under investigation.

In conclusion, we show that a polypeptide (Poly-6) composed of three optimized cryptic tumor peptides derived from universal tumor antigens (HER-2/neu_{402Y}, $TERT_{988Y}$ and MAGE-$1A_{248V9}$) induces a polyspecific response in HLA-A*0201-expressing HHD mice and in human cells ex vivo. This polypeptide has the potential for broad-spectrum tumor vaccination of cancer patients.

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