12. TINKERING WITH NATURE: THE TALE OF OPTIMIZING PEPTIDE BASED CANCER VACCINES

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INTRODUCTION

Methodological progress at the end of last century in the identification of tumor associated antigens specifically recognized by cytolytic T lymphocytes (CTL) made possible the characterization of numerous peptides (p) presented by class I Major Histocompatibility Complexes (MHC). These p-MHC complexes are the ligands for clonotypically distributed T cell receptors (TCR). The latter are heterodimers of α and β chains bearing structural homology with immunoglobulins. As such, they are composed of constant and variable segments. Three hypervariable regions can be identified, of which the Complementary Determining Region 3 (CDR3) is both the most variable and involved in the interactions with the peptide amino acid residues in the p-MHC complex (1, 2).

The tumor associated antigenic peptides identified thus far are derived from a large variety of cellular polypeptides. These may include regular proteins from different cellular compartments, isoforms encoded by alternatively spliced genes, the products from alternative open reading frames, mutated genes or frameshifts and may even result from the transcription of the antisense strand of DNA (3–5). In one case, the antigenic peptide was shown to be generated by a protein splicing mechanism, thus far unknown in the eukaryotic world (6). Thus, the variety of cell biological mechanisms uncovered so far as giving rise to antigenic peptides reveal the highly opportunistic nature of tumor recognition by CD8 T lymphocytes. This is the result

P1: GHM/ P2: GHM/ QC: GHM/ T1: GHM
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of two concurrent mechanisms leading to antigen recognition by T lymphocytes. On one hand, the process of antigen recognition involves a sophisticated molecular apparatus able to discriminate as few as 1–10 p-MHC complexes on the surface of the antigen presenting cell that normally display up to 10^4–10^5 p-MHC complexes (7). Thus, the system has evolved to attain an exquisite sensitivity and is endowed with powerful discrimination and amplification properties. On the other hand, the repertoire of $\alpha\beta$ T cells is shaped by positive and negative selection processes during thymic development. As a result the majority, up to 95% of immature thymocytes that successfully rearrange TCRs, are eliminated from the mature repertoire (8, 9). The coupling of these two thymic selection forces ensures that the TCRs expressed by T cells exported for immunesurveillance in the periphery possess sufficient affinity for interaction with self MHC molecules but are depleted of potentially dangerous TCRs with high affinity for self p-MHC complexes. As a result self tolerance is firmly established. However, this does not mean absence of autoreactive T cells. In fact, these cells exist in the peripheral T cell repertoire but possess a low to intermediate avidity for a high number of self antigens (10–14). Thus, despite the considerable diversity of tumor associated peptides, many of those identified thus far are derived from conventional polypeptides that are expressed by both normal and tumor cells.

Practical issues in specific therapy of cancer favour the use of those antigens that are expressed in the maximum number of patients with a given type of tumor. In this regard, most of the antigenic peptides from mutated gene products are poor candidates for widely applicable vaccines because their expression is limited to individual tumors. Consequently, the best candidates for vaccine development in terms of cancer population coverage are those derived from self antigens. Thus, an expected limitation of these generic cancer vaccines is the existence of self tolerance. Such constraints have been clearly demonstrated in studies conducted in experimental mouse models. For instance, large differences in TCR avidity for a p53-derived class I restricted T cell epitope can be measured when comparing the TCR repertoires of wild type mice and p53-genetically deficient counterparts (15). Similar findings were reported in a transgenic mouse model system for CTL recognizing a dominant viral antigen (16). Another elegant illustration of this phenomenon comes from the comparative analysis of the HLA-A2 restricted CD8 T cell repertoire for a tyrosinase-derived melanoma associated antigen in strains of mice expressing or not the *tyrosinase* gene product (17).

Attempts to vaccinate patients with such peptides result in the selection of a specific CTL response. Moreover, appropriate immunization may elicit a tumor protective response mediated by the low avidity CD8 T cell repertoire in a mouse model (18). Interestingly, it has been shown in well defined animal models that similar or even more efficient CTL responses can be obtained by immunization with peptides modified at key residues (16, 19–23). These studies clearly demonstrate that this class of peptide analogues have the ability to mobilize the intermediate/low avidity T cell repertoire and induce protective anti-tumor CTL responses. Here we review the sequence and structural basis of p-MHC complexes formation and show how antigenic peptide modifications can improve the peptide's immunogenicity.

PEPTIDE BINDING TO MHC

Sequence Analysis

To fulfil their immunological functions, MHC Class I molecules have evolved to bind with sufficient affinity a large number of peptides with widely divergent amino-acid sequences. Sequence analysis of the peptide population obtained by elution of immunoaffinity purified MHC class I molecules (24) revealed the presence of allele-specific binding motifs. For instance, *HLA A*0201* encoded molecules selectively bind peptides with L, M or I at the second position of the peptide, or P2, and V or L at PΩ, i.e. the residue occupying the carboxyl terminal position of the peptide. In contrast, P2 needs to be a Y or F and PΩ an I or an L in peptides binding to the mouse K^d molecule as well as in those binding to the human HLA-A24 molecule (24–26). Nearly identical results were obtained by a completely functional approach using substituted peptides as competitors of antigenic peptides to inhibit lysis of chromium labelled targets by H2 K^d restricted CTL clones (27). These results suggested that there are distinct amino acid residues in the peptide that are directly involved in MHC binding, while the remaining peptide residues are relatively unconstrained at the sequence level.

Since this pioneering work, large amounts of data have been collected on the nature of peptide sequences restricted to different MHC allelic products. For instance, the MHCPEP database (28) has been regularly updated and contains information on peptide binding for several MHC together with experimentally determined affinity constants for MHC. There are currently over 2500 peptide sequences known to bind HLA A2, allowing statistics to be made, though care should be taken since the peptide population is subject to certain bias (28). Table 1 shows the occurrence of each of the twenty naturally occurring amino acids at specific peptide positions for the panel of peptides known to be naturally associated with HLA-A2 in somatic cells. The first number is computed for the entire database, and the second one for high affinity peptides only (246 sequences). The main anchor residues described above are clearly still predominant and the Table provides information on which alternate residues are allowed.

Due to the strong selectivity at positions P2 and PΩ, these two residues are referred to as *main* or *primary* anchor residues and those occupying the less selective positions P1, P3 and PΩty to as *secondary* anchor residues (29). An additional regular feature of MHC class I binding peptides is their defined length of 9–10 residues. Indeed, despite the identification of occasional T cell epitopes whose optimal length is clearly at variance with this rule (e.g. (30, 31)), the large majority of known T cell epitopes as well as sequenced MHC class I-associated peptides are nona or decapeptides. Thus, relatively simple sequence motifs can be defined for sets of peptides binding to well defined class I MHC molecules, both from murine and human origin. These motifs can be defined by three components. The first, the primary anchor residues. The second, the secondary anchor residues and the third component is a defined peptide length.

The sequence motifs described here find their explanation in the specific p-MHC architecture in three dimensions, detailed in the next chapter. An immediate

Table 1. Anchor residues specificity for the different pockets of HLA A2.

Amino acid	P1	P2	P3	P(Ω-3)	P(Ω-2)	P(Ω)
A	13/6[a]	4/3	10/5	9/3	13/10	5/4
C	0/1	0/0	1/0	2/0	1/1	0/0
D	0/0	0/0	6/2	1/1	2/0	0/0
E	2/0	0/0	2/1	1/0	2/0	0/0
F	10/13	0/0	5/10	9/13	13/25	0/0
G	11/23	1/0	7/8	5/7	4/7	1/1
H	1/0	0/0	2/1	1/0	3/2	0/0
I	6/5	13/15	4/4	7/6	5/3	10/6
K	9/8	0/1	2/3	2/1	1/2	0/0
L	7/11	60/64	17/25	9/7	11/8	28/30
M	2/1	7/6	1/2	1/1	0/0	1/1
N	1/1	0/0	5/3	1/1	2/2	0/0
P	1/0	0/0	7/9	8/7	8/14	0/0
Q	2/1	0/0	1/0	2/3	2/1	0/0
R	3/4	0/0	1/0	1/0	1/0	0/0
S	6/5	0/0	5/5	5/7	3/3	0/0
T	2/2	4/3	2/1	5/4	3/2	3/2
V	4/3	3/1	5/4	15/24	9/7	42/52
W	1/2	0/0	4/4	0/0	2/2	0/0
Y	5/6	0/0	3/4	2/4	2/1	0/0

[a] The first digit represents the percentage of the particular amino acid residue in all the peptides known to bind to HLA A2, the second one represents the same quantity for the set of high affinity binding peptides only.

application of these insights has been the design of computer algorithms to identify candidate peptides in proteins of known sequence that would bind a given MHC class I molecule. Two such computer programs have gained wide recognition: the bioinformatics and molecular analysis section (BIMAS) algorithm (http://bimas.dcrt.nih.gov/molbio/hla_bind, (32, 33)) and the one based on the SYFPEITHI database (http://syfpeithi.bmi-heidelberg.com, (34)). Another algorithm with the same purpose has been reported recently (35). These algorithms have been thoroughly reviewed recently (36). Numerous studies have been performed leading to the identification of potential T cell epitopes in proteins of interest for immunotherapy of infectious or autoimmune diseases and cancer. Such an approach has been dubbed "reverse immunology" to emphasize the fact that, in contrast to the initial methods leading to CTL-defined antigen identification, the starting "reagent" is the bioinformatic tool and the end-product the isolation of the peptide-specific T cell (37).

Structural Analysis

A major step in the understanding of peptide binding to MHC molecules was the elucidation, by X-ray crystallography, of the three dimensional structure of the p-MHC complex. It allowed to derive general rules on the strategy of peptide binding by MHC, see for example (38–40). So far, the structures of around 60 different p-MHC complexes have been solved by X-ray crystallography with

resolutions ranging from 3.5 Å for the first one in 1987 (41), down to 1.4 Å (42). As for human tumor antigens, an initial attempt to elucidate their structure provided poorly resolved images (43). The first high resolution structures were solved using peptide analogues of the Melan-A/MART-1 immunodominant peptide binding to the HLA-A2 molecule (44) and another from the MAGE-A4 polypeptide binding to the same MHC class I molecule (42). Figure 1 shows the Melan-A/MART-1 peptide lodged in the groove of HLA-A2 as obtained from X-ray crystallography.

Comparison of the peptide binding modes revealed that there are three main mechanisms contributing to the overall binding affinity. While one is dependent upon the peptide's sequence, the other two are peptide sequence independent. As depicted in Figure 2, the N- and C-termini of the peptide are stabilized by a rich hydrogen bond network. They involve highly conserved MHC residues that are found in all alleles and all species. These are Y7, Y159 and Y171 for the N-terminal part and Y84, T143 and K146 for the C-terminal part. The second peptide sequence independent component of binding to MHC consists of hydrogen bonds formed between amino acid residues in the MHC molecule and the backbone of the peptide. Again, conserved MHC residues such as E63, K66 and N70 located on the α1 helix hydrogen bond to the polar groups on the backbone of P2. On the C-terminal side, residues W147 and D77 hydrogen bond to the backbone of residues PΩ and PΩ -1, respectively. The last component of the peptide binding strategy is that mediated by peptide interactions with specific pockets situated in the floor of the MHC groove, shown schematically in Figure 3. These pockets bind precisely the specific amino-acid side chains from the primary anchor residues. In contrast to the first two peptide binding components, this contribution is therefore strongly peptide sequence dependent. It is however slightly degenerated in the sense that physico-chemically similar amino-acids can be accepted by the same MHC pocket (39) See Table 1.

A description of the MHC residues forming the B, C and F pockets of HLA-A2 together with the preferred side chains selected on the peptide is listed in Table 2. The pocket composition is allele specific, and, as a result, peptide binding is observed to be strongly allele-specific (24). Pocket A does not have a strong specificity due to the fact that the P1 side chain generally points toward the solvent and does not interact strongly with the MHC. Pockets D and E, as pocket A, are not strongly selective and are not listed in the Table. Pocket F, although interacting mainly with the peptide's backbone, see above, also accommodates the side chain of the peptide's Ω residue. There is therefore a restriction on the possible amino acids that are found at the peptide's C-terminus, see Table 1. Pocket B is probably the most selective pocket in MHC Class I molecules. There is usually a strong preference for one of the amino acids, see (24) and Table 1. A three dimensional representation of the B pocket of HLA A2 is shown in Figure 4. All residues forming the pocket are shown as bonds and the solvent accessible surface is shown in blue. The anchor residue L inserted in the pocket is shown in yellow.

By adding up these different contributions, the p-MHC molecular complex is capable of reaching a large enough binding free energy, so as to keep the peptide bound even in a nearly infinite dilution at the cell surface. The relative importance

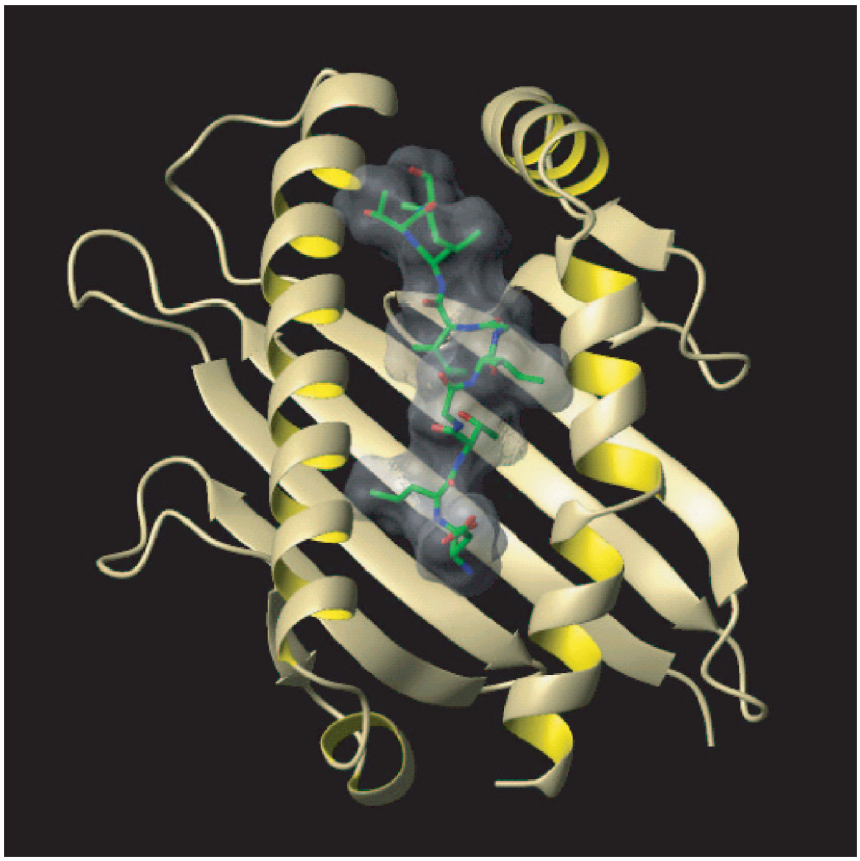

Figure 1. X-ray structure of the Melan A_{26-35} A27L peptide analogue (ELAGIGILTV) in complex with HLA-A2.

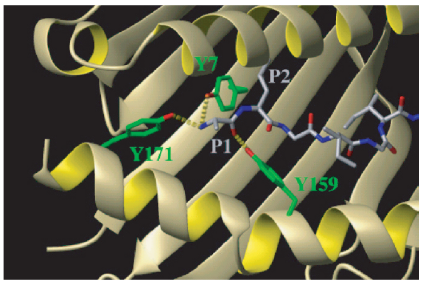

Figure 2. Canonical hydrogen bonds at the N-terminal part of the peptide: the highly conserved residues Y7, Y159 and Y171 all form strong hydrogen bonds to the amino or carbonyl group of the first amino acid residue of the peptide. A similar hydrogen bond network is found at the C-terminal part involving the conserved residues Y84, T143 and K146.

Table 2. MHC Class I pockets.

Pocket B		
MHC Class I	Involved residues[a]	Anchors (P2)[b]
H-2K^b	**Y7**, V9, E24, **V34**, Y45, **E63**, A67, (S99)[c]	—
H-2K^d	**Y7**, V9, A24, **V34**, F45, **E63**, A67, (S99)[c]	Y
HLA-A2	**Y7**, F9, A24, **V34**, M45, **E63**, V67, Y99	L
HLA-B27	**Y7**, H9, T24, **V34**, E45, **E63**, C67, Y99	R
Pocket C		
MHC Class I	Involved residues	Anchors (Ω-3)
H-2K^b	V9, V97, S99	Y,F
H-2K^d	V9, R97, F99	—
HLA-A2	F9, R97, Y99	—
HLA-B27	H9, N97, Y99	—
Pocket F		
MHC Class I	Involved residues	Anchors (PΩ)
H-2K^b	L81, I95, Y116, **Y118**, **Y123**, **I124**, **T143**, **W147**	L
H-2K^d	A81, F95, F116, **Y118**, **Y123**, **I124**, **T143**, **W147**	I,L
HLA-A2	L81, V95, Y116, **Y118**, **Y123**, **I124**, **T143**, **W147**	V
HLA-B27	L81, L95, D116, **Y118**, **Y123**, **I124**, **T143**, **W147**	K,R

[a] The amino acid residues involved in the formation of the B, C and F pockets of MHC class I are given. Residues in bold characters represent those amino acid residues conserved in all MHC listed in the table.
[b] For each MHC molecule, the preferred peptide anchor residue(s) are given.
[c] Ser 99 does not participate to pocket B formation in H-2K^b and H-2K^d and is shown in parenthesis.

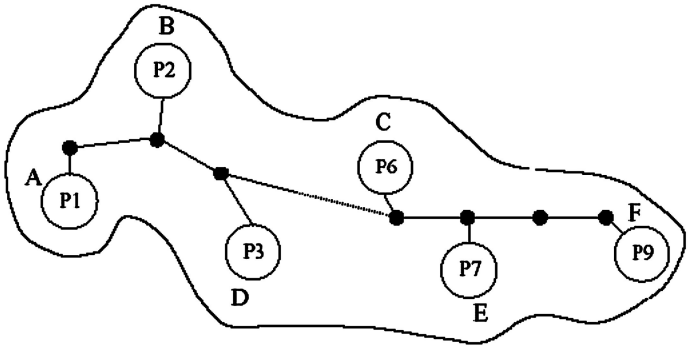

Figure 3. Schematic organization of MHC class I pockets. The side chains of the peptide anchor residues are shown as circles and the corresponding pockets shown by letters, A, B, C, D, E, and F. Schema adapted from (38).

of the different binding components has been assessed experimentally. The largest contribution to the total binding free energy comes from the N and C-terminal hydrogen bonding network which was estimated to be at around −4.6 kcal/mol for each end (45). In the second place is the contribution to binding from the primary anchor residues (P2 and PΩ) which together contribute up to −3.0 kcal/mol. These two contributions, N and C termini plus primary anchors, are necessary but not

sufficient for high affinity peptide binding (29). The rest of the binding free energy comes from non-N or C termini peptide backbone hydrogen bonding and from the secondary anchor residues. The contribution of the secondary anchor residues was estimated collectively to be on the order of 3.0 kcal/mol (29). The contribution of the backbone hydrogen bonding has not yet been estimated directly, since it would require backbone modifications of the peptide that might introduce other energetic costs which are hard to evaluate.

On the overall, the affinity of natural peptides ranges from $K_D < 50$ nM for good binders, 50 nM < K_D < 500 nM for intermediate binders and 500 nM < K_D 50 μM for weak binders (50 μM being the limit of detection for most experiments).

STRATEGIES FOR OPTIMIZATION OF CANCER VACCINES BASED ON SELF ANTIGENIC PEPTIDES

Peptide vaccines can be optimized following different avenues. As explained above, many tumor associated antigene peptides will display an intermediate to weak binding to MHC. If used as such, these peptides would have to compete against better binding peptides at the cell surface, resulting in an unfavourable chemical equilibrium. The first goal of peptide vaccine optimization is therefore to increase MHC affinity by replacing the suboptimal primary or secondary peptide anchor residues. Even optimized high affinity MHC binding peptide analogues are confronted, when delivered in vivo, with a very rapid degradation in the host due to the action of proteases. Peptide modifications aimed at reducing protease susceptibility are therefore of great interest.

Extensive probing of the specificity of antigen recognition by T cell clones using antigen peptide variants at non-anchor residues has revealed a complex picture. While most of this class of antigen peptide variants are not detectably recognized by T cells, some may trigger responses that are quantitatively stronger than the response triggered by the cognate peptide (superagonists), others trigger weaker responses and some may antagonize the response triggered by the cognate peptide. Collectively, these variant antigen peptides have been given the name of altered peptide ligands (APL, (46, 47)). The structural basis of APL effects on specific T cell activation have been studied in some well defined models. It is apparent that subtle structural changes in the non covalent complex formation between MHC/APL and TCR may account for such dramatic effects on T cell activation(48–50).

The superior therapeutic efficacy of specific T cell responses induced by immunization with a tumor APL has been illustrated in a colon tumor model in mice (51). In humans a superagonist for HLA-A2 restricted, CEA_{605}-specific CTL has been characterized (52, 53) and shown to induce tumor reactive T cells in a clinical trial with advanced cancer patients (54). Interestingly, after vaccination, two of 12 patients experienced dramatic tumor regression, one patient had a mixed response, and two had stable disease. Clinical response correlated with the expansion of CD8 HLA-A2/CEA peptide tetramer$^+$ T cells. Other human tumor antigen APLs have been characterized recently (55–57). General methods to identify superagonists or heteroclitic peptides have been proposed. One is based on conservative or

semi-conservative amino acid replacement of key "TCR" positions within antigenic peptides (58). A more comprehensive approach to APL identification involves the use of peptide libraries (59, 60). In this regard, a recent survey of APLs for human HLA-A2 restricted Melan-A specific CTL clones revealed the existence of various categories of superagonists, some which are recognized in a clone specific manner and few which are recognized by a broad array of indepenndent CTL clones of differing fine specificity (55). The latter category may be more appropriate for vaccine development based on APLs.

In the following sections we will review in detail the first two approaches for peptide vaccine optimization. The use of combinatorial peptide libraries for APL identification is beyond the scope of this chapter and it has been reviewed elsewhere (61).

Vaccine Optimization by Amino Acid Substitutions Aimed at Increasing the Peptide Affinity for MHC

Based on the detailed knowledge of the molecular interactions underlying p-MHC complex formation, it would seem rational to substitute primary and, to some extent, secondary anchor residues in tumor associated peptides in order to improve their MHC affinity. The most obvious scenario is that of a peptide lacking the primary anchor(s). For example, the Melan-A 26-35 peptide EAAGIGILTV presented by HLA-A2 has the appropriate PΩ primary anchor residue, but not the P2 the canonical residue, i.e. L. This substitution was tested experimentally and indeed resulted in a better binding to HLA A2 and a two three orders of magnitude increase in the efficiency of recognition by Melan-A-specific CTL lines and clones (62). Moreover, CTL fine specificity studies revealed that recognition by the TCR was very similar between the wild type and the mutant peptide. It is noteworthy that the major positive impact of the A27L substitution is strictly dependent on the length of the peptide. Indeed, the introduction of an A28L substitution in the context of the Melan-A 27-35 nonapeptide (AAGIGILTV) leads to a profound reduction of the peptide's antigenicity. This was despite an increase in the peptide's binding affinity for binding to HLA-A2. Interestingly, these outcomes were in keeping with the observation that the peptide species recognized by the majority of tumor reactive, Melan-A-specific CTL lines and clones, was the Melan-A 26-35 decapeptide (63, 64). The relevance of identifying the optimal peptide length for vaccine development is underlined by a recent report summarizing the results of a clinical trial in cancer patients vaccinated with two different length NY-ESO-1 peptides displaying different epitopes restricted by the HLA-A2 molecule (65). The comparison of high resolution crystal structures of HLA-A2/Melan-A 26-35 A27L and of A2/Melan-A 27-35 A28L reveals that the two complexes, though sharing common three dimensional conformations in some regions, differ in the orientation of the side chain of the I residue located in the central portion of the peptide (44). Apparently, most, if not all, of the CD8 T cell repertoire specific for the Melan-A antigen is skewed towards recognition of the epitope(s) presented by the A2/Melan-A 26-35 decapeptide complex.

Could we conclude from the previous example that substitution of main anchors, in the appropriate length peptide, will always result in an improved peptide vaccine? Probably not: it should be stressed that increasing the affinity for MHC is a necessary condition to improve peptide antigenicity, but it is not sufficient; substitution of primary or secondary anchors also need to leave the modified peptide conformation unchanged. Any conformational change might result in a change of the epitope presented to the TCR and a bias in repertoire selection. This situation has been recently observed and analyzed in detail (66). In this study, an L to V replacement in this residue PΩ of the GP2 peptide (IISAVVGIL) derived from HER-2 resulted in a very large conformational change in residues P5 and P6, as shown by X-ray crystallography data, see Figure 5. This example shows that any peptide modification, tough on a main anchor residue deeply engaged in the F pocket, can have important structural implications in distant parts of the peptide. Similar conclusions were reached in another study examining the effect of substitutions of amino acid residues in a viral peptide antigen interacting with the MHC class I C and D pockets (67).

Besides modifications in the main anchor residues it is conceivable that useful peptide analogues can be obtained through substitutions of secondary anchor peptide residues. In this regard, it has been observed for HLA-A2 binding peptides that replacement of the P1 residue by residues with an aromatic side chain generally increase the apparent affinity of the peptide (29, 63, 68), albeit with variable outcome on T cell recognition. More recently, a systematic survey of this effect on a relatively high number of weak HLA-A2 peptide binders confirmed the positive effect of replacing the P1 residue with Y and this approach was proposed as a general strategy to identify heteroclitic peptides of cryptic HLA-A2 restricted epitopes (69). Such strategy was applied to the identification of several HER-2/neu and hTERT peptide analogues that displayed enhanced immunogenicity in HLA-A2 transgenic mouse studies (70).

In any event, it is essential to insure that the proposed peptide modification does not alter the epitope presented to the TCR by Structural studies. Since it is not possible to crystallize all the modified peptides that would be of interest, there is a need for theoretical structural predictions. Such techniques make use of homology modeling and/or *ab initio* predictions to forecast the peptide's conformation in the MHC groove and assist in guiding peptide modification in a rational way (71).

Finally, preservation of the epitope presented to the TCR is a necessary condition but is, again, not sufficient. Even, keeping the exact same average conformation, the modified peptide can have properties that change the TCR affinity, and therefore bias the selected repertoire. These different properties include direct electrostatic or van der Waals interactions of the modified side chain with the TCR's CDRs, entropic terms, or modifications in peptide's induced conformational change upon TCR engagement. One possible way to assess these parameters is by use of free energy simulations that allow to compute the TCR binding free energy difference between the wild type peptide and the modified one, ΔG. These calculations did allow to accurately reproduce experimentally determined free energy

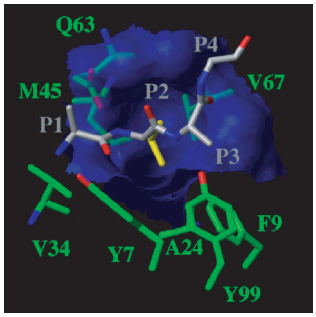

Figure 4. B pocket for HLA-A2 with a Leucine inserted, shown in yellow. The HLA-A2 amino acid residues forming the pocket are shown in green a the alpha carbon trace of the peptide in gray.

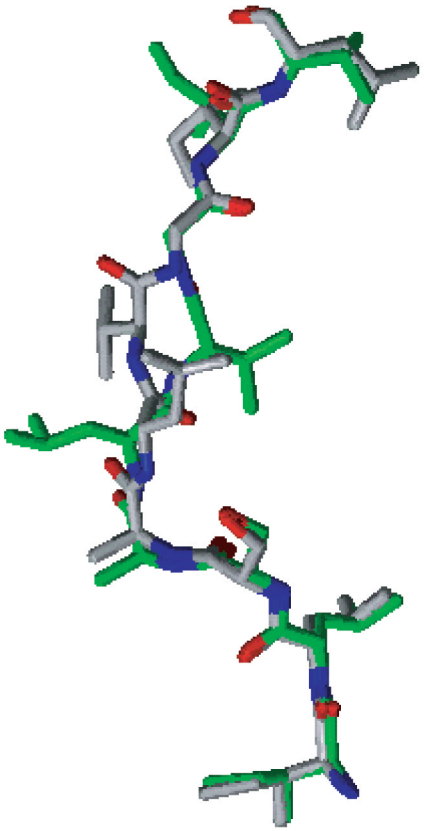

Figure 5. Structural comparison of the crystal structure of modified GP2 peptides, ILSALVGIL (grey) and ILSALVGIV (green) after optimal superimposition of the α1 domain of HLA-A2 (not represented). The C-terminal part is on the right side of the picture where the mutation occurs. Important structural deviations are found around residues L5 and V6 in the center of the peptide.

Table 3. Peptide analogues of human tumor associated antigenic peptides validated as candidates for cancer vaccines.

Tumor antigen[a]	Antigen peptide sequence[b]	Substitution(s)[c]	Reference
$gp100_{154}$	K T W G Q Y W Q V	L, V, I	(106)
$gp100_{154}$	K T W G Q Y W Q V	A	(106)
$gp100_{209}$	I T D Q V P F S V	M	(72)
$gp100_{280}$	Y L E P G P V T A	V	(72)
Melan-A_{26}	E A A G I G I L T V	L	(20, 62)
Melan-A_{27}	A A G I G I L T V	L	(62, 107)
NY-ESO-1_{157}	S S L M W I T Q C	A, S, V	(108, 109)
CEA_{605}	Y L S G A N L N L	D	(52–54)
$p53_{149}$	S T P P P G T R V	L	(110)
$p53_{139}$	K T C P V Q L W V	L	(110)
$p53_{103}$	Y Q G S Y G F R L	L	(110)
MAGE- A1	Y L E Y R Q V P D	V	(111)
HER-2/neu_{650}	P L T S I I S A V	Y	(70)
$hTERT_{988}$	D L Q V N S L Q T V	Y	(70)

[a] The name of the gene product from which the antigenic peptide is derived and the position of the first amino acid residue in the primary polypeptide sequence are given in this column.
[b] The amino acid sequence of the concerned antigenic peptide is given in the single letter code. The residue underlined corresponds to the one substituted by another natural amino acid.
[c] Amino acid residue used to substitute the naturally occurring amino acid to generate the peptide analogue.

changes for the A6 TCR binding to the Tax peptide or its P6A mutant, bound to HLA-A2 (71).

Several tumor antigenic peptide analogues have already been successfully tested in in vitro and/or in vivo experiments (see Table 3). Moreover, a handful of them were already tested in clinic trials of vaccination of cancer patients. The best studied case is that of the $gp100_{209-217}$, a peptide that mimics a dominant gp100 epitope recognized by CTL from metastatic melanoma patients. This peptide possesses a T residue at position P2 and the canonical primary anchor residue at the PΩ position. Substitution of the P2 for M leads to increased affinity ob binding to the HLA-A2 molecule and vastly improved antigenicity (72) and in vitro immunogenicity (72, 73). Vaccination of metastatic melanoma patients with this peptide analogue in conjunction with Montanide, an equivalent of the incomplete Freund's adjuvant, lead to induction of specific CD8 T cells in practically 100% of immunized individuals (74, 75). Interestingly, a second gp100 derived peptide, $gp100_{280-288}$, that is also recognized by HLA-A2 restricted CD8 T cells from melanoma patients lacks the primary anchor at its PΩ position (76). Although a peptide analogue carrying a replacement of the PΩ residue by a primary anchor for HLA-A2 gave conflicting results in the in vitro assays (72, 73, 77), recent results in the clinics clearly demonstrate a superior immunogenicity of the substituted peptide analogue (78). In this case, interestingly, the immunogen was a recombinant fowlpox virus carrying a *gp100* gene mutated to express the two HLA-A2 anchor residues mentioned above, that is M at codon 210 and V at codon 288. The Melan-A 26–35 A27L peptide analogue has also been tested in recently reported clinical trials (79–81).

Non-Natural Peptide Antigen Analogues Resistant to Biodegradation

The efficient use of antigenic peptides as therapeutic agents may be limited by the high sensitivity of peptides to degradation by peptidases present in biological fluids (82, 83). Initial *in vitro* studies showed that peptide degradation by proteases in serum could decrease the presentation of exogenous antigenic peptide by MHC on the surface of presenting cells (84, 85). In addition, the degradation of the antigenic peptide *in vitro* was correlated with a diminution of their persistence *in vivo* (86). Further studies indicated that local persistence of the antigenic peptide could be associated with the induction of an optimal immune response (87). Together, these factors could limit the immunogenicity of the antigenic peptide as tumor vaccine. Thus, rendering antigenic peptides resistant to degradation by peptidases could have important implications in the design of efficient peptide based vaccines for immunotherapeutic treatment of cancer.

To this end, an effective approach consists in introducing structural modifications to the antigenic peptide. This has been showed for both MHC class-I (88–90) or class-II (91) restricted antigenic peptides. The structural changes in the antigenic peptide involve either a variety of chemical modifications of the peptide bond (92–95) or substitutions with non-natural amino acids (a.a.) (88, 89, 96). A drawback, however, was the simultaneous appearance of dramatic negative effects on the MHC binding properties of the antigen and/or on the recognition by antigen specific T lymphocytes (88, 89). Such detrimental effects must be minimised in order to use such peptidase resistant pseudopeptides as efficient therapeutic compounds. To preserve the antigenicity and immunogenicity of the antigenic peptides, a more rational approach was taken to introduce minimal modifications of the peptide structure. In this approach, the knowledge of the degradation mechanism of the antigenic peptide guides the choice of structural modifications targeted to the appropriate position in the peptide's structure, the one(s) susceptible to proteolytic attack (88).

The detailed mechanism of tumor antigenic peptide degradation was initially determined by the analysis using an on-line HPLC mass spectrometry (HPLC/ESI-MS) method of the degradation fragments generated after incubation of the antigenic peptide in human serum for various periods of time (97). In line with the findings of this study, the analysis of the degradation of the MelanA/MART-1 related peptide $MelanA_{26–35}$ A27L indicates the involvement of aminopeptidases and di-peptidyl-carboxypeptidases (Figure 6) (94). Interestingly, the degradation of the antigenic peptides from their amino- and the carboxy-terminal ends was found to be sequential and no endopeptidase activity was involved. The kinetics of amino- and carboxy-terminal degradation can be different from one peptide to another leading to a slightly different degradation profiles. The analysis of the degradation of Melan-A/MART-1 nona- and decapeptide related peptides suggest that the nature of the amino-terminal residues has a direct or indirect effect on both amino- and carboxy-peptidase enzymatic activities (94).

The degradation model of the peptide $MelanA_{26–35}$ A27L predicts that the peptidase sensitive bonds are the first (Glu^1-Leu^2) and the eighth (Leu^8-Thr^9). Within

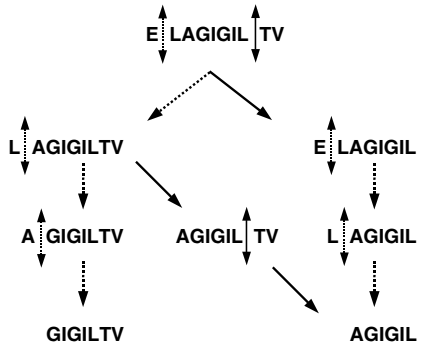

Figure 6. Degradation model of Melan-A_{26-35} A27L antigenic peptide.
The degradation model of the antigen Melan-A_{26-35} A27L (ELAGIGILTV) has been established from qualitative and quantitative data obtained from HPLC-mass spectrometry analysis. The peptide is cleaved by aminopeptidases (dotted arrows) and dipeptidyl-carboxypeptidases (full arrows). Reprinted with permission from (94).

the MelanA_{26-35} A27L sequence, the peptidase sensitive peptide bonds were targeted for the introduction of a variety of structural modifications. Protection of the N-terminal and C-terminal ends of the peptide was explored by acetylation and amidation, respectively. Peptides with backbone modifications such as reduced or retro-inverso peptide bonds were also synthesised. Finally, substitutions of peptide residues by non-natural amino acids such as the D series amino acids, β-amino acids, cyclic amino acids, N-hdroxylated amino acids or methylated amino acids (NMe-amino acids or αMe amino acids) were also investigated (94).

Although the introduction of one structural modification in only one of the sensitive peptide bonds does not significantly improve the half-life of the peptide in human serum (94), most of the structural modifications were efficient to locally protect the peptide against peptidase. The short half-life of the mono-protected analogues was related to the degradation of the non-protected end of the peptide. We have shown that only the analogues carrying both amino- and carboxy-terminal modifications of the peptide were fully protected against degradation and display a remarkable stability in the serum with a half-life superior to 24 hours, compared to 2 minutes for the natural peptide (94).

Not all the structural modifications were efficient to locally protect the peptide against peptidase activities. We have found that the amidation of the carboxy terminal end of the peptide were not fully protective against the degradation. The doubly protected analogues with amidated C-terminal ends display a half-life between 11 to 20 hours. This result confirms previous observations showing that the Angiotensin Converting Enzyme (ACE), the most abundant dipeptidylcarboxy peptidase in the serum, was able to cleave peptide with amidated carboxy-terminal ends (98, 99).

Structural modifications of antigenic peptide could have a negative impact on its immunological properties (88, 89, 96). The first important biological property

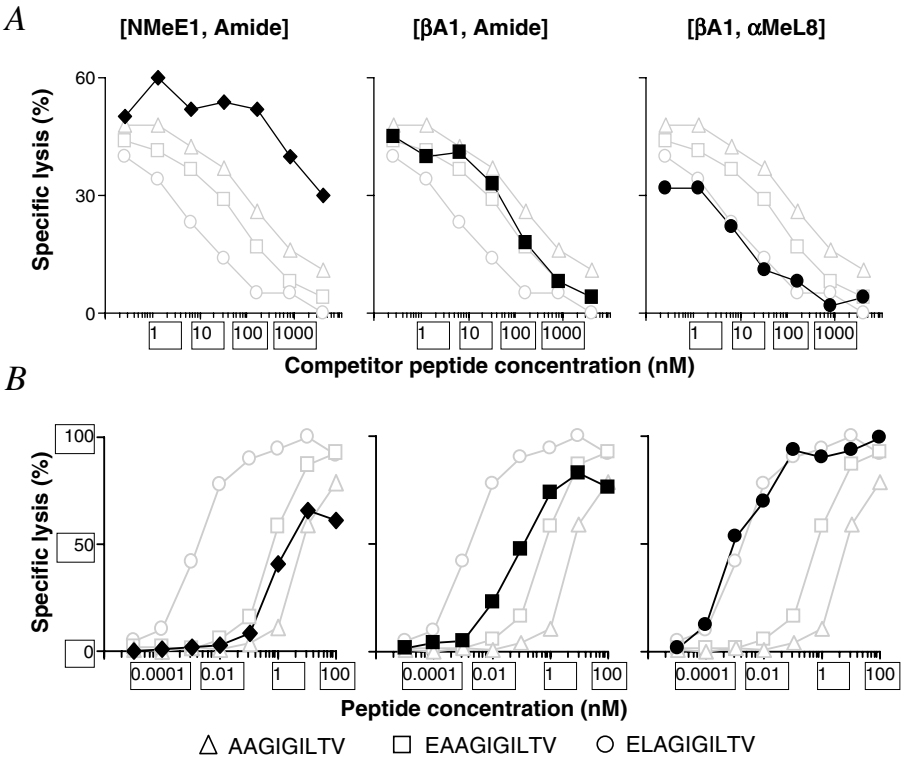

Figure 7. HLA-A*0201 binding affinity and Melan-A antigen specific CTL recognition of the Melan-A_{26-35} A27L non-natural analogues. **A**, HLA-A*0201 binding affinity of the Melan-A_{26-35} A27L non-natural analogues were determined using a competition assay based on the inhibition of the recognition of the $tyrosinase_{368-376}$ peptide by a specific T cell clone 156/34. **B**, Melan-A antigen specific CTL recognition of the Melan-A_{26-35} A27L non-natural analogues was determined using a chromium release assay. In each graph, the data corresponding to the Melan-A_{26-35} A27L protease resistant analogues, indicated in the upper part of each panel, are in black (filled symbols). The HLA-A*0201 binding (A) and antigenic (B) properties of the Melan-A related peptides are in grey (open symbols).

affected by structural modifications of the antigenic peptide is its binding to MHC molecules. We have shown that most of the structural modifications of $MelanA_{26-35}$ A27L have a negative effect on the MHC binding properties of the antigen (94) (Figure 7A). Not surprisingly, this could occur even when the MHC non-anchor residues are modified (88, 94). In case of the amino- and carboxy-modified analogues, the negative effects of the modifications are always additive. Structural modifications could modify the peptide ability to fit properly the MHC peptide-binding groove. For example, methylated or cyclic residues could increase the steric hindrance. Other studies indicate that the peptide backbone flexibility could be reduced by the introduction of retro-inverso or reduced peptide bonds (100, 101). Molecular modeling of the non-natural analogues in the MHC binding groove indicates that

the hydrogen bond network between MHC residues and the peptide backbone or the peptide ends could be disturbed (88, 100). In rare cases, structural modifications of the peptide could have a positive impact on MHC binding. Indeed, non-natural antigenic peptides including structural modifications such as β-amino acid (96) or N-hydroxylation (102) have been shown to display a better affinity for the MHC compared to the unmodified peptide. In our study, the introduction of β-amino acid or N-hydroxylation within the first peptide bond of the MelanA$_{26-35}$ A27L peptide did not significantly change binding to HLA-A*0201 (94). We have also shown that other structural modifications such as α-, or N-methylation of appropriate residues in the peptide sequence have minimal effects on the MHC binding properties of the non-natural analogues. We have identified MelanA$_{26-35}$ A27L non-natural analogues bearing both amino- and carboxy-terminal structural modifications with MHC binding properties very similar to the unmodified peptide. In addition to the MHC binding affinity, the peptide-MHC complex stability could also be considered to determine if the non-natural analogues could be efficiently presented by the MHC (96).

The second aspect of the antigenicity that could be impaired by structural modification of antigenic peptides is the efficiency of recognition by specific T cells. After normalizing the efficiency of T cell recognition to the change in binding to MHC ((94), Figure 7B), non-natural analogues could be recognised in very different ways by the T cell receptor. Previous studies, including ours, reported that non-natural antigens with reduced peptide bond, although showing similar or improved binding to MHC, were poorly recognised by antigen specific T cell clones (94, 95, 101). Other studies have shown that recognition of the non-natural analogues could be clone specific depending on the region of the peptide recognised by each clone (88, 95), illustrating how the peptide backbone and structural modifications of non-anchor residues could modify the shape adopted by the peptide in the MHC binding groove and ultimately affect the recognition by the specific T cell receptor.

Fortunately, in some cases, structurally modified antigenic peptides can be recognised efficiently by antigen specific T cells. In our studies, we have identified amino- and carboxy-modified analogues of MelanA$_{26-35}$ A27L that were efficiently recognised by a Melan-A specific T cell line ((94), Figure 7B). We have shown that the non-natural peptides bearing α-methylation, N-hydroxylation or β-amino acid were recognised by the Melan-A specific T cells within a concentration range similar to that of the non-modified peptide in cytolytic assay (94). Thus a stepwise approach to design the non-natural analogues of MelanA$_{26-35}$ A27L allows the identification of fully protected peptides against peptidases with a binding to the MHC and a recognition by the Melan-A specific T cells similar to the non-modified peptide (Figure 7).

However, even when they are efficiently recognised by antigen specific T cells, the non-natural analogues can yet trigger T cell effector functions different from those triggered by the native antigenic peptide (100, 103). Thus, structural modification of the antigenic peptide could have profound effects on the functional properties of

the antigen, and the effector functions of the antigen specific T cells recognising the non-natural analogue must be carefully checked.

An essential requirement for their use as therapeutic agents is that the non-natural peptide analogues must be able to induce an efficient antigen specific immunity after vaccination mediating recognition and elimination of tumor cells expressing the antigen. The immunogenicity of the non-natural analogues resistant to peptidase degradation was initially tested *in vitro*. Using the ELISpot method, a previous study described the cross-reactivity of T cells induced by Melan-$A_{27–35}$ non-natural analogues with the non-modified peptide (89). In our study, we used HLA-A*0201/Melan$A_{26–35}$ A27L tetramers to quantify the number of Melan-A specific cells induced after *in vitro* stimulation of PBMC from healthy donors with the structurally modified analogues. We showed that amino- and carboxy-modified analogues, efficiently presented by the MHC and recognised by the antigen specific T cells, were able to induce the expansion of Melan-A specific cells from PBMC of healthy individuals (Figure 8). Three Melan$A_{26–35}$ A27L protease resistant analogues induced a higher number of Melan-A specific cells than the non-modified peptide, indicating that protection against proteolysis could significantly enhance the *in vitro* immunogenicity of the antigenic peptide.

More importantly, the Melan-A specific $CD8^+$ T cells induced by stimulation with some of the protease resistant Melan-A peptide analogues were also able to recognise not only the unmodified Melan$A_{26–35}$ A27L peptide, but also Melan-$A_{26–35}$ or Melan$A_{27–35}$ native peptides (Figure 8). Finally, the Melan-A specific T cells induced by the non-natural Melan-A analogues showed robust cytolytic activity against Melan-A expressing melanoma tumor cell lines, indicating their ability to recognise the Melan-A antigen naturally processed and expressed by tumor cells (94). The immunogenicity of structurally modified antigenic peptides has been also shown *in vivo.* Indeed, immunization of mice with antigenic pseudopeptides with reduced bonds induced a native antigen-specific immune response and conferred a better resistance against lethal challenge with tumor cells expressing the target antigen (104).

CONCLUSION

From the above considerations, one can draw the following strategy for rational peptide modifications; the two main anchor positions (P2 and PΩ) should be considered first as they might be less prone to modify the overall peptide conformation. These positions should be replaced by the well known main anchors for the MHC molecule of interest. Alternatively, non-natural aminoacid modifications can also be used. Theoretical prediction techniques can be used to select modifications that do not alter the conformation and that do not affect TCR affinity using free energy calculations. The secondary anchor positions are much more delicate to use as they are less deeply buried than the main anchors and can interact with TCR directly or through a conformational change of the peptide. Here theoretical structure based approaches are expected to play an important role. Again natural or non-natural

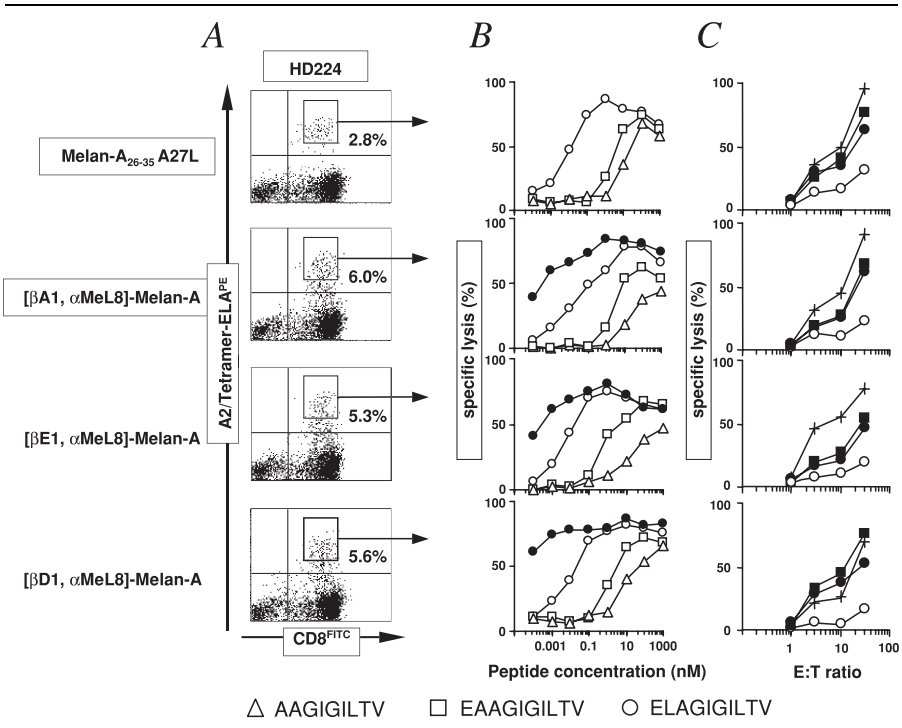

Figure 8. Antigenic specificity of and tumor recognition by CTL induced with the non-natural Melan-A analogues. After *in vitro* stimulation of PBMC from a healthy donor (HD224) with the Melan-A peptides, the tetramer$^+$ CD8$^+$ lymphocyte populations were sorted on a FACSvantage cell sorter. These populations were then expanded to sufficient cell numbers and their cytolytic activity was measured. **A**) Dot plot representation of the flow cytometry analysis of PBMC cultures after *in vitro* stimulation with Melan-A$_{26-35}$A27L or with the doubly modified analogues. The gate defined for cell sorting of the tetramer$^+$ CD8$^+$ populations is represented. **B**) Melan-A peptide recognition by the sorted tetramer$^+$ CD8$^+$ lympphocytes from the corresponding gate shown in panels A (Melan-A$_{27-35}$, open triangles; Melan-A$_{26-35}$, open squares; Melan-A$_{26-35}$A27L, open circles; Melan-A doubly modified analogue, full circles). **C**) Melanoma tumor cell recognition by the sorted tetramer$^+$CD8$^+$ lymphocytes from the corresponding gate shown in panels A. Cytolytic assay was performed with ^{51}Cr-labeled tumor cells (Me 275 and Me 290 : HLA-A*0201$^+$, Melan-A$^+$; NA8-MEL : HLA-A*0201$^+$, Melan-A$^-$) with increasing effector to target cell (E:T) ratios. (Me 275, full squares; Me 290, full circles; NA8-MEL, open circles, NA8-MEL sensitized with the Melan-A peptide, cross symbols). Reprinted with permission from (94).

substitutions can be used. Finally, backbone or side chain modifications can be used to improve peptide's protease resistance.

In the past 7 years, intensive efforts were deployed to overcome the limitation of antigenic peptide degradation in biological fluids in order to design efficient therapeutic tumor vaccines. Structurally modified antigenic peptide analogues fully resistant to exopeptidase degradation with an improved *in vitro* immunogenicity were successfully designed. Such compounds could be very attractive candidates to improve the efficacy of peptide based tumor vaccines. Nevertheless, further studies

are required to extend and validate this approach to an increased number of tumor derived antigenic peptides. Finally, to consider the use of non-natural antigenic peptides as therapeutic agents, the safety and the *in vivo* immunogenicity of these compounds should be determined more precisely in clinical studies. It can be anticipated that such stable peptides should be carefully confined in such way that they selectively reach mature professional antigen presenting cells. The risks of inducing tolerance when delivery of immunogenic peptides allows prolonged systemic persistence has been demonstrated in an animal model (105).

In summary, any peptide modification should be followed by both MHC binding assays and analysis of the CTL fine specificity of recognition for a large panel of clones. Some modifications might abrogate recognition by some of the TCR but not by others as the affected part of the epitope might be more or less important for each individual TCR. Tinkering with nature offers many opportunities but it should be carefully monitored and the many variants of natural peptides should undergo a rigorous, experimentally driven selection process.

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