2 The Rational Design of T-Cell Epitopes With Enhanced Immunogenicity

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1. THE CHALLENGE OF OVERCOMING IMMUNOLOGICAL TOLERANCE

For all cancer vaccine strategies, a major challenge facing efforts to induce a clinically effective T-cell response is the necessity to break tolerance to normal, "self" antigens. To control auto-reactivity, some T cells with high avidity for tumor-associated antigen (TAA) epitope–major histocompatibility (MHCs) complexes are deleted in the thymus and the remaining T cells are controlled by peripheral tolerance *(1)*. However, several groups have demonstrated using in vitro systems that thymic-deletion of TAA-specific cytolytic T cell (CTL) is not complete *(2–4)*. More importantly, it is clear that in some patients, natural exposure to tumor or immunization with wild-type antigens or epitopes can induce CTL of sufficient avidity and functionality to infiltrate tumors in vivo and/or recognize tumor cells in vitro. Therefore, although the fundamental vaccine strategy of targeting TAA to mount tumor-specific immune responses is supported, it remains a significant challenge to design cancer vaccine strategies that consistently overcome immunological tolerance in order to effectively activate and maintain therapeutic T-cell responses. Experimentation in the late 1980s and 1990s has resulted in a detailed understanding of the molecular mechanisms controlling T-cell activation and effector function. It is now appreciated that the interaction of a T-cell receptor with a peptide epitope presented by an antigen-presenting cell (APC) in the context of an MHC molecule generates the central event (referred to as "signal 1") in the activation of naïve or memory

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T cells. Studies have also demonstrated that the specificity and affinity of peptide binding by human lymphocyte antigens (HLAs) is determined by the interactions between the side chains of the linear peptide epitope and the residues present in each HLA molecule. Insights from these studies have given rise to two strategies for the rational modification of T-cell epitopes to enhance immunogenicity and "breaking" of immunologic tolerance. The molecular mechanisms, immunological consequences, and the cancer vaccine applications of each approach will be discussed.

2. FIXED-ANCHOR EPITOPE ANALOGS

One type of modification that can be utilized to enhance the immunogenicity of wild-type TAA-derived peptides involves the substitution of a single amino acid residue to facilitate an increase in the binding affinity of the analog peptide for an HLA molecule. Although the affinity of peptide binding by an HLA molecule is essentially the product of the interactions between residues of the peptide and the residues in the peptide-binding cleft of the HLA molecule, two positions have a dominant influence on binding, and are referred to as "primary anchor positions." For most HLA-binding peptides, these primary anchor residues are position 2 and the carboxyl-terminus. The specific amino acids that most effectively function as primary anchor residues in a peptide are dependent on the composition of the binding pocket of the given HLA molecule, and the amino acids that are "preferred" and "tolerated" at each peptide position have been defined for numerous HLA molecules *(5)*. For example, for binding to HLA-A2.1, L,M and V,I,L are preferred anchors at position 2 and the C-terminus, respectively. Knowledge of these HLA-specific patterns, referred as motifs, is the foundation for the rational design of fixed-anchor analog epitopes. The primary amino acid sequence of a wild-type TAA can be analyzed using motif-based algorithms to identify peptides that harbor a preferred residue at one anchor position and a tolerated residue at the other. The HLA-binding affinity can then be enhanced by a single amino acid substitution to replace the tolerated residue, thereby "fixing" the anchor. Residues at other positions of the peptide can contribute to HLA binding, albeit more weakly, and are referred to as secondary anchor positions *(5)*. These positions can also be substituted to enhance HLA binding of a peptide.

Unlike receptor:ligand interactions that directly result in biological functions such as signal transduction, binding of a vaccine-delivered peptide to an HLA molecule has no direct biological effect. The immunological and pharmacological importance of enhancing this interaction for vaccine design lies in the observation that the affinity of an HLA–peptide interaction directly correlates with immunogenicity *(6)*. By designing epitopes that increase the stability of HLA–peptide complexes on the surface of APCs, the opportunity to effectively engage the cognate T-cell receptor (TCR) and induce a therapeutic T-cell response is enhanced. In addition, it has been hypothesized that CTL specific for a low-affinity, wild-type epitope may be less tolerized than those specific for high-affinity peptides, due to the lower amounts of cell-surface epitope–HLA complexes generated by normal processing and presentation *(7)*. Therefore, by utilizing fixed-anchor analog epitopes as high-affinity immunogens, a vaccine can be used to stimulate these potentially more reactive T-cell populations.

The validity of this approach for generating immunogens with enhanced potency was initially demonstrated in in vitro studies using fixed-anchor analogs derived from HER-2/neu and gp100, where analogs were demonstrated to be more effective for CTL induc-

tion than the wild-type peptides from which they were derived. Single amino acid substitutions to introduce optimal residues at the anchor positions of three peptides derived from the melanoma-associated antigen gp100 were demonstrated to yield analog peptides with enhanced HLA binding and immunogenicity relative to the native sequence *(8)*. In another early study, single amino acid substitutions were made at secondary anchor positions of HER-2/neu-derived peptides resulting in analog epitopes exhibiting enhanced HLA binding and immunogenicity *(9)*.

Our group has utilized HLA motifs and epitope prediction algorithms in combination with high-throughput HLA binding assays to identify novel fixed-anchor analog epitopes from CEA, p53, HER2/neu, and MAGE2/3, five TAAs that are frequently expressed in epithelial-derived tumors *(2,10)*. Initial studies focused on epitopes restricted by the HLA-A2 supertype, and included the identification of wild-type epitopes in addition to fixed-anchor epitopes. From the primary sequences of the four TAAs, approx 1650 motif-positive sequences ranging in size between 8 and 11 residues and carrying the general extended motif that has been associated with the capacity of peptides to bind HLA2.1 were identified. From these motif-positive peptides, a more refined algorithm that takes into account the influence of secondary anchors was used to select 223 peptides for further analysis. Utilizing the HLA-binding assays and this set of peptides, 82 different wild-type epitopes were demonstrated to bind HLA-A2.1 with an IC_{50} of ≤500 n*M*, an affinity previously shown to correlate with peptide epitope immunogenicity *(6)*. Importantly, 123 fixed-anchor analogs with optimized HLA-binding capacity were also generated. From these A2.1 binding peptides, 115 were also demonstrated to bind at least one, and most often two or three additional HLA molecules from the HLA-A2 supertype. Of these HLA-A2 supertype epitopes, 22 wild-type epitopes and 21 analogs were tested for immunogenicity in CTL induction assays using normal donor peripheral blood mononuclear cells (PBMCs). As a read-out, immunoassays measuring cytolysis and/or interferon-γ production were utilized. The CTL cultures demonstrated to be positive for recognition of the immunizing peptide were then further expanded and tested for recognition of naturally processed epitope as presented by HLA- and TAA-matched tumor cell lines. The specificity of the CTL induced by in vitro immunization with peptides was also demonstrated by cold target inhibition experiments for selected epitopes *(2,10)*. For the wild-type peptides, 20 out of 22 (91%) were immunogenic for PBMC from at least one donor, and recognition of tumor cell lines expressing naturally processed antigens was noted for 16 out of 20 (65%) of these epitopes. Interestingly, no significant difference in "hit rate" was observed between MAGE, p53, CEA, and HER2/neu antigens, suggesting that for these TAAs the degree of peripheral tolerance in the CTL compartment is indistinguishable, despite the significantly different protein expression patterns reported for these TAAs.

Fixed-anchor analog epitopes substituted at one or both anchor positions were also evaluated for immunogenicity. Of the analog epitopes harboring a single primary anchor substitution, 100% of the analogs induced CTL when measured against target cells pulsed with the analog peptide. However, to be useful components of a cancer vaccine, epitope analogs must induce CTLs that recognize the wild-type peptide presented on the surface of tumor cells. Recognition of naturally processed antigen on tumor cell lines was demonstrated for 46% (6/13) of the fixed-anchor analogs, thereby validating their use as vaccine immunogens. Analog peptides substituted at both primary anchor positions were also found to be immunogenic, but the CTLs induced were less frequently associated with

recognition of endogenous antigens. These data underscore the importance of using rational design strategies that introduce minimal changes to the epitope, and serve as a cautionary note regarding the use of analog design approaches that yield multiply-substituted analogs.

It is interesting to consider that in general, for both wild-type and analog peptides, the induction of CTLs that are capable of recognizing tumor cell lines as targets is associated with high HLA-binding affinity *(2)*. This conclusion is consistent with previous work by our group and others *(4,8,10)*, although contrary to the postulate that CTL recognizing high-affinity binding epitopes are preferentially deleted from the repertoire, and that epitopes that can induce CTLs recognizing naturally processed epitopes are mostly directed against low-binding-affinity peptides *(11)*.

Several other groups have recently identified HLA-A2.1-restricted fixed-anchor analog epitopes derived from other important TAA. Two analog epitopes derived from an overlapping region of the melanoma differentiation antigen MART-1 have been reported *(12)*. In addition, fixed-anchor analogs derived from NY-ESO-1 *(13)* and Ep-CAM *(14)* have been identified. For each of these analog epitopes, superior immunogenicity relative to the cognate wild-type sequence was demonstrated using human PBMC in an in vitro CTL induction system.

One fixed-anchor analog tested in clinical studies is the gp100.209 (210 M) epitope, which has been utilized as a synthetic peptide delivered to melanoma patients. This analog epitope was reported to be markedly more effective at in vivo CTL induction than was the gp100.209 wild-type peptide when each was administered separately to melanoma patients as a synthetic peptide in Montanide® ISA 51 adjuvant in conjunction with IL-2 *(3)*. In this study, the analog and wild-type peptides induced wild-type peptide-specific CTL responses in 10/11 and 2/8 patients, respectively. Although not a direct comparison of wild-type and analog peptides, two other clinical studies by Weber and colleagues *(15)* and Banchereau and colleagues *(16)* have also demonstrated the immunogenicity of the gp100.209 (210 M) analog, with the latter reporting vaccine-induced CTL recall responses in 12/16 patients. Detailed analyses of the CTL responses induced by this fixed-anchor analog epitope have been conducted *(17,18)*.

All of the fixed-anchor epitope analogs reported to date are restricted by the HLA-A2 supertype. Although this HLA supertype is relatively frequently expressed in patients, 45% on average, vaccines produced using HLA-A2 epitopes are inherently limited in applicability. To expand the application of this rational vaccine design approach, our group is using the same strategy described above to identify CEA-, p53-, HER-2/neu-, and MAGE 2/3-derived fixed-anchor analog epitopes that are restricted by three other common HLA supertypes, -A1, -A3, and -A24 *(19)*. The design of vaccines that combine epitopes restricted by these four HLA supertypes will provide essentially complete population coverage for all patients, regardless of ethnic background.

3. HETEROCLITIC ANALOGS

A second epitope modification strategy involves the introduction of selected single amino acid substitutions at selected positions other than the main HLA anchors of the peptide. The resulting peptides, referred to as heteroclitic analog epitopes, are capable of stimulating unexpectedly potent T-cell responses. A number of different reports have illustrated that heteroclitic analogs are associated with T-cell hyperstimulation, and that

this more potent response is, in fact, mediated by increased binding of the peptide-HLA complex to the TCR *(20,21)*. Importantly for cancer vaccine development, heteroclitic analogs have also been associated with a striking capacity to break tolerance, as shown in a variety of different studies *(22,23)*. Schlom and colleagues identified the first HLA-restricted heteroclitic analog epitope derived from a clinically important human TAA. This modified CEA peptide, designated CAP1-6D, harbors a single substitution of aspartic acid for asparagine at position 6 of the nine amino acid sequence *(24)*. This substitution did not increase the HLA-A2 binding affinity of the peptide, but did result in the hyperstimulation of wild-type specific CTL when the analog peptide was presented on target cells. Importantly, under in vitro conditions where the wild-type peptide was not immunogenic, the CAP1-6D was demonstrated to induce human CTLs capable of recognizing HLA-$A2^+$, CEA^+ tumor cell lines. These data provided support for the use of CAP1-6D in human vaccine design (*see* later discussion), and more generally supported the concept of identification and use of heteroclitic analogs derived from human TAA.

For our initial studies to develop and characterize heteroclitic analogs *(23)*, three wild-type, TAA-derived, HLA-A2 epitopes identified in the screening studies described earlier were selected as targets: MAGE-3.112, CEA.691, HER2/neu.157. For each epitope, conserved, semiconserved, and nonconserved substitutions were introduced at all positions in the peptide excluding the MHC anchor positions. These analogs, a total of approximately 350, were screened for heteroclicity by performing antigenicity and dose titration analyses with a CTL line specific for the cognate wild-type peptide. The magnitude of response and shifts in dose responses induced by each analog relative to the wild-type peptide were evaluated, and striking increases of the order of 6–7 logs were detected for some substituted peptides. Importantly for use of these epitopes in vaccine development, the human CTLs generated by in vitro immunization with heteroclitic analogs were able to recognize naturally processed wild-type epitope expressed on tumor cell lines, and were of higher avidity than CTLs induced with the parent peptide. Also, as compared to the wild-type parent peptide, heteroclitic analogs were found to more consistently induce CTLs in vitro. Further, a model heteroclitic analog epitope derived from the murine p53 epitope using this same analog identification strategy was demonstrated to break tolerance to "self" and activate CTLs that recognize tumor cell lines when utilized for in vivo immunization of HLA-$A2/K^b$ transgenic mice.

From these studies, novel heteroclitic analog epitopes were identified, and clinical studies utilizing a subset of these heteroclitic epitopes are currently planned. In addition, several observations relating to the nature and function of epitopes displaying heteroclicity were made. First, it was noted that the amino acid substitutions generally associated with heteroclicity were conservative or semiconservative and occurred in the middle of the peptide at position 3, 5, or 7. Since odd-numbered positions in the middle of an HLA-bound peptide may be pointing up toward the T-cell receptor, this observation is consistent with the effect being mediated by an increased interaction with TCRs. Some previous studies implied that modulation of T-cell responses by heteroclitic analogs involves modification of peptide residues that directly contact the TCRs *(20,24)*, but this finding was not corroborated by our study *(23)*, which indicated that heteroclicity is likely to be generated by subtle alterations in epitope conformation rather than by gross alterations of TCR contacts or MHC binding capacity. Salazar et al. have reported increased phosphorylation of Zap-70 in heteroclitic analog-stimulated T cells *(22)*, but the structural and signaling mechanisms involved in heteroclicity require further investigation.

From a practical standpoint, our studies have important implications for vaccine development. By identifying the substitution patterns most frequently associated with heteroclicity, "rules" were developed that can streamline the analog screening process. We have found that we can successfully identify heteroclitic analogs from all epitopes, and our success rate of analog prediction is in the 20–40% range, which represents about a 20-fold increase over random prediction. By combining this targeted substitution strategy with high-throughput screening, heteroclitic analog epitopes derived from any TAA and restricted by commonly expressed HLA supertypes can be identified and utilized for cancer vaccine design.

The HLA-A2 restricted, CEA-derived CAP1-6D heteroclitic analog identified by Schlom and colleagues *(24)* has been utilized in human clinical trials. In a study by Fong and colleagues, the CAP1-6D peptide was demonstrated to effectively expand epitope-specific human CTL when delivered using dendritic cells and measured using tetrameric HLA/peptide complexes (tetramers) *(25)*. These CTL expansion data, and to a lesser extent the ELISPOT data reported, correlated with the observed clinical responses. Previous clinical studies using the wild-type CAP-1 peptide had demonstrated relatively weak immunogenicity *(26)*, underscoring the potential significance of these early results obtained using the CAP1-6D heteroclitic analog. Additional clinical studies testing the CAP1-6D epitope and the novel heteroclitic epitopes described above should provide the human immunological data needed to address the general applicability of this approach for cancer vaccine design.

4. SUMMARY

The rational design of analog epitopes with enhanced immunogenicity is a promising strategy for improving on the cancer vaccine clinical outcomes to date. The use of fixed-anchor and heteroclitic analog epitopes is compatible with most all delivery systems, and these modified antigens should expand the quality and breadth of antitumor T-cell responses achievable in humans by facilitating the stimulation of CTLs specific for epitopes that in their natural form are too weakly immunogenic to be effective vaccine components. In addition, these optimized immunogens should complement other approaches directed at enhancing T-cell induction, maintenance, and effector function *(1)*.

REFERENCES

1. Yu Z, Restifo NP. Cancer vaccines: progress reveals new complexities. J Clin Invest 2002; 110:289–294.
2. Keogh E, Fikes J, Southwood S, Celis E, Chesnut R, Sette A. Identification of new epitopes from four different tumor-associated antigens: recognition of naturally processed epitopes correlates with HLA-A*0201-binding affinity. J Immunol 2001; 167:787–796.
3. Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL, et al. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat Med 1998; 4:321–327.
4. Reynolds SR, Celis E, Sette A, Oratz R, Shapiro RL, Johnston D, et al. Identification of HLA-A*03, A*11 and B*07-restricted melanoma-associated peptides that are immunogenic in vivo by vaccine-induced immune response (VIIR) analysis. J Immunol Methods 2000; 244:59–67.
5. Ruppert J, Sidney J, Celis E, Kubo RT, Grey HM, Sette A. Prominent role of secondary anchor residues in peptide binding to HLA-A2.1 molecules. Cell 1993; 74:929–937.
6. Sette A, Vitiello A, Reherman B, Fowler P, Nayersina R, Kast WM, et al. The relationship between class I binding affinity and immunogenicity of potential cytotoxic T cell epitopes. J Immunol 1994; 153:5586–5592.

7. Cibotti R, Kanellopoulos JM, Cabaniols JP, Halle-Panenko O, Kosmatopoulos K, Sercarz E, et al. Tolerance to a self-protein involves its immunodominant but does not involve its subdominant determinants. Proc Natl Acad Sci USA 1992; 89:416–420.
8. Parkhurst MR, Salgaller ML, Southwood S, Robbins PF, Sette A, Rosenberg SA, et al. Improved induction of melanoma-reactive CTL with peptides from the melanoma antigen gp100 modified at HLA-A*0201-binding residues. J Immunol 1996; 157:2539–2548.
9. Fisk B, Savary C, Hudson JM, O'Brian CA , Murray JL, Wharton JT, et al. Changes in an HER-2 peptide upregulating HLA-A2 expression affect both conformational epitopes and CTL recognition: implications for optimization of antigen presentation and tumor-specific CTL induction. J Immunother 1996; 18:197–209.
10. Kawashima I, Hudson SJ, Tsai V, Southwood S, Takesako K, Appella E, et al. The multi-epitope approach for immunotherapy for cancer: identification of several CTL epitopes from various tumor-associated antigens expressed on solid epithelial tumors. Hum Immunol 1998; 59:1–14.
11. Kappler JW, Roehm N, Marrack P. T cell tolerance by clonal elimination in the thymus. Cell 1987; 49:273–280.
12. Rivoltini L, Squarcina P, Loftus DJ, Castelli C, Tarsini P, Mazzocchi A, et al. A superagonist variant of peptide MART1/Melan A27-35 elicits anti-melanoma CD8+ T cells with enhanced functional characteristics: implication for more effective immunotherapy. Cancer Res 1999; 59:301–306.
13. Chen JL, Dunbar PR, Gileadi U, Jager E, Gnjatic S, Nagata Y, et al. Identification of NY-ESO-1 peptide analogues capable of improved stimulation of tumor-reactive CTL. J Immunol 2000; 165:948–955.
14. Trojan A, Witzens M, Schultze JL, Vonderheide RH, Harig S, Krackhardt AM, et al. Generation of cytotoxic T lymphocytes against native and altered peptides of human leukocyte antigen-A*0201 restricted epitopes from the human epithelial cell adhesion molecule. Cancer Res 2001; 61:4761–4765.
15. Lee P, Wang F, Kuniyoshi J, Rubio V, Stuges T, Groshen S, et al. Effects of interleukin-12 on the immune response to a multipeptide vaccine for resected metastatic melanoma. J Clin Oncol 2001; 19:3836–3847.
16. Banchereau J, Palucka AK, Dhodapkar M, Burkeholder S, Taquet N, Rolland A, et al. Immune and clinical responses in patients with metastatic melanoma to CD34(+) progenitor-derived dendritic cell vaccine. Cancer Res 2001; 61:6451–6458.
17. Yang S, Linette GP, Longerich S, Haluska FG. Antimelanoma activity of CTL generated from peripheral blood mononuclear cells after stimulation with autologous dendritic cells pulsed with melanoma gp100 peptide G209-2M is correlated to TCR avidity. J Immunol 2002; 169:531–539.
18. Lee KH, Wang E, Nielsen MB, Wunderlich J, Migueles S, Connors M, et al. Increased vaccine-specific T cell frequency after peptide-based vaccination correlates with increased susceptibility to in vitro stimulation but does not lead to tumor regression. J Immunol 1999; 163:6292–6300.
19. Sette A, Sidney J. Nine major HLA class I supertypes account for the vast preponderance of HLA-A and -B polymorphism. Immunogenetics 1999; 50:201–212.
20. Slansky JE, Rattis FM, Boyd LF, Fahmy T , Jaffee EM, Schneck JP, et al. Enhanced antigen-specific antitumor immunity with altered peptide ligands that stabilize the MHC-peptide-TCR complex. Immunity 2000; 13:529–538.
21. England RD, Kullberg MC, Cornette JL, Berzofsky JA. Molecular analysis of a heteroclitic T cell response to the immunodominant epitope of sperm whale myoglobin. Implications for peptide partial agonists. J Immunol 1995; 155:4295–4306.
22. Salazar E, Zaremba S, Arlen PM, Tsang KY, Schlom J. Agonist peptide from a cytotoxic T-lymphocyte epitope of human carcinoembryonic antigen stimulates production of TC1-type cytokines and increases tyrosine phosphorylation more efficiently than cognate peptide. Int J Cancer 2000; 85:829–838.
23. Tangri S, Ishioka GY, Huang X, Sidney J , Southwood S, Fikes J, et al. Structural features of peptide analogs of human histocompatibility leukocyte antigen class I epitopes that are more potent and immunogenic than wild-type peptide. J Exp Med 2001; 194:833–846.
24. Zaremba S, Barzaga E, Zhu MZ, Soares N, Tsang KY, Schlom J. Identification of an enhancer agonist cytotoxic T lymphocyte peptide from human carcinoembryonic antigen. Cancer Res 1997; 57:4570–4577.
25. Fong L, Hou Y, Rivas A, Benike C, Yuen A, Fisher GA, et al. Altered peptide ligand vaccination with Flt3 ligand expanded dendritic cells for tumor immunotherapy. Proc Natl Acad Sci USA 2001; 98:8809–8814.
26. Morse MA, Deng Y, Coleman D, Hull S, Kitrell-Fisher E, Nair S, Schlon J, Ryback ME, Lyerly HK. A Phase I study of active immunotherapy with carcinoembryonic antigen peptide (CAP-1)-pulsed, autologous human cultured dendritic cells in patients with metastatic malignancies expressing carcinoembryonic antigen. Clin Cancer Res. 1999;5:1331–1338.