Archivum Immunologiae et Therapiae Experimentalis, 2001, **49**, 271–277
PL ISSN 0004-069X

Review

Enhancing Cytotoxic T Cell Responses with Altered-Peptide Ligands

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Abstract. Interest in class I MHC-mediated immunotherapy is growing rapidly. In order to fight a virus or cancer effectively, a successful immunotherapeutic must activate a large number of specific $CD8^+$ T cells and also generate immunological memory. Attempts to generate immune responses towards tumor- or virus-derived peptides have frequently been frustrated by the nature of the peptide antigen itself. Either the peptide does not bind well to its cognate MHC, or the T cells directed towards it have been functionally inactivated *in vivo*. Altered--peptide ligands (APL) are an effective way to circumvent these problems. However, generating enhanced binding of altered peptides to class I MHC while still maintaining recognition of the wild-type peptide is not straightforward. Many groups design enhanced binding peptides by substituting the observed anchor residues with those that are most preferred by the class I MHC molecule. For many antigenic peptides, this approach does not work. Furthermore, if a higher affinity peptide is designed, the substitutions may result in reduced recognition by $CD8^+$ T cells. Therefore, the design of APL requires careful testing of each candidate therapeutic in terms of affinity for class I MHC and immunological reactivity. Lastly, immunotherapy using class I MHC must also take into account the large genetic heterogeneity in the population. A therapeutic that is only effective for 5–10% of the population is not as attractive as one that works for over 90% of the population. The use of MHC supertypes (groups of class I MHC allotypes that share similar peptide-binding characteristics) shows great promise in overcoming this problem.

Key words: class I MHC; T cell receptor; ligand design; immunotherapy; x-ray crystallography.

Introduction

The interaction between the T cell receptor (TcR) and the peptide/class I MHC complex (pMHC) is critical to activating cellular immune responses. In an effort to understand this interaction, peptide analogs of wild--type antigenic peptides have been generated in a variety of antigen systems by introducing amino acid substitutions (for review, see[42]). The term "altered-peptide ligand" (APL) was coined to represent these peptide

Abbreviations used: MHC – major histocompatibility complex, pMHC – peptide/class I MHC complex, CTL – cytotoxic T lymphocyte, TcR – T cell receptor, APL – altered-peptide ligand, CDR – complementary determining region, PBMC – peripheral blood mononuclear cells, DC – dendritic cell, CD – circular dichroism spectropolarimetry, B7 supertype members – B*0702, B*3501, B*5101, B*5301, B*5401; B7, B35, B51, B53, B54: gene products of B*0702, B*3501, B*5101, B*5301, B*5401.

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analogs[12]. These APL demonstrate that, instead of being an "all or nothing" event, a continuum of different T cell responses can be elicited with APL bound to class I MHC. APL may be agonist or super-agonist peptides that are capable of triggering similar or even better cytotoxic T lymphocyte (CTL) responses compared with the original peptide antigen. They may be partial agonists that lead to partial activation of the cytotoxic T cells. This partial activation may be manifested by either reduced cytotoxicity or the failure to produce one or more cytokines important for a vigorous response. APL may be null peptides that completely fail to stimulate any response. Lastly, APL may be antagonists which inhibit the ability of CTL to kill cells bearing the original, unsubstituted peptide.

The complexity of the interaction between pMHC and TcR is demonstrated by the various T cell responses to peptides with subtle changes. It also raises hope that APL may be useful for immunotherapy. The goal of immunotherapy is to stimulate a specific T cell response towards a virus, parasite or tumor, or to inactivate a specific response in the case of self-reactive T cells in autoimmune disease. Peptide-based immunotherapies are often frustrated by the fact that the peptide antigens derived from virus or tumor frequently bind MHC too poorly to mount a useful immune response. In some other cases, T cells directed toward these peptide antigens have been functionally inactivated. APL constitute a promising approach to overcome these problems. This review summarizes our current understanding of peptide binding to class I MHC specifically with respect to using APL to generate enhanced CTL responses.

What Is the Best Peptide to Choose as a Target for Modification?

The goal of class I MHC-mediated immunotherapy is to generate large numbers of specific activated $CD8^+$ T cells. Clearly, the best choice of peptide antigen to modify is one with the largest population of potentially reactive T cells in the host. This population could be derived from a previously expanded population of T cells or from a large naïve precursor population. When targeting a tumor-associated antigen, the situation is complicated by the fact that the antigen is a self-peptide. In order to reduce the potential for autoimmune disease (or premature inactivation of the response by antigen-induced cell death), the chosen peptide should not be presented ubiquitously in the host, or at the very least it should be expressed at significantly higher levels in the tumor. Logically, to have a large $CD8^+$ T cell precursor population, this peptide would be one that has **not** been negatively selected against during thymic education. In this sense the best target would be a peptide with low affinity for class I MHC. If it has low affinity (or a short half-life), the peptide would not be present on the antigen-presenting cells significantly during negative selection in the thymus. It would also not be present at high levels in the periphery, so that the self-reactive T cells would be functionally naïve. Poor-affinity peptides are also the best targets for chronic viral therapies. If the high-affinity, highly presented peptides were immunogenic, they would facilitate removal of the virally infected cells. However, poor-affinity peptides generate significant challenges for immunotherapy. The poor affinity of the peptide often makes the peptide weakly immunogenic when compared with the dominant antigens in the proteins from which the peptides are derived[11, 31, 32]. One approach to increase the immunogenicity of these peptides is to design an APL with increased binding affinity to MHC. The goal is for the APL to stimulate the activation and proliferation of T cells that recognize the cells presenting low levels of the wild-type peptide. So, what do we know about peptide binding affinity and class I MHC that we can use to design an APL?

Peptide-Binding to Class I MHC

Class I MHC normally binds peptides of 8–11 amino acids in length. These peptides are derived from proteins in the cytoplasm that are degraded by the proteasome and other proteases. Specific peptide transporters (TAPs) transport the peptides into the endoplasmic reticulum, where they bind to immature class I MHC. Mature class I/peptide complexes are then exported to the plasma membrane for examination by circulating T cells[35].

The earliest crystal structures revealed how class I MHC molecules are capable of binding peptides with diverse sequences[16, 28]. The class I MHC makes conserved hydrogen bonds with the peptidic amino and carboxyl termini. The construction of the peptide-binding groove of the MHC limits the length of the peptides that may bind. The termini of the peptide bind in buried pockets in the peptide-binding cleft. Thus, longer peptides may zigzag[27] or bulge[4] in the center to accommodate the increased length. Long peptides may also extend out of the carboxyl end of the cleft, although there is an energetic penalty to pay for such binding[5, 29]. The amino terminal binding pocket is buried much more

deeply than the carboxylate-binding pocket. This deep pocket precludes the extension of the peptide out the amino terminus ([29] and COLLINS, unpublished data).

A specific class I allotype usually has a strong preference for a few amino acids at one or two positions in the peptide; the amino acids in these positions are called anchors. Crystal structures of pMHC reveal that polymorphic residues from class I MHC form specificity pockets that accommodate the chemical nature of the corresponding anchor residues of the peptide[19]. In addition to the primary anchors, there are secondary anchors that may enhance the binding of the peptide[36]. It is becoming increasingly obvious that the total binding energy is a summation of the positive and negative contributions from all residues in the peptide. There are many examples where a peptide has the required primary anchors but nevertheless binds poorly to class I MHC (for example, see[24]). There are also examples where a peptide does not have the required primary anchors yet binds with reasonable affinity. In these cases, the peptide utilizes other residues to complement the absent anchor residue (for example see[47]).

Empirically based prediction algorithms, such as described by PARKER et al.[32], have proven useful in the search for epitopes within target proteins. However, the algorithm fails to accurately predict the half-lives of many peptides. When it does fail, it is likely to do so because one of the precepts of the algorithm is that each peptide residue binds independently of the others. Substitution experiments with GP2 (ILSAVVGIL) have distinctly shown that the individual residues do not bind independently. Singly substituted APL GP2-I2L*, GP2-V5L, and GP2-L9V each have enhanced binding affinities. However, the affinities of the doubly substituted peptides GP2-I2L/V5L and GP2-V5L/L9V are less than that of the any of the singly substituted peptides (KUHNS, unpublished data). Therefore, there are interactions between residues that lead to the non-additive phenomenon of peptide-binding affinity.

Designing an APL with Increased Binding Affinity to MHC

Although there has been tremendous progress in our understanding of peptide-binding to class I MHC, a large gap still exists between this understanding and our ability to rationally design a more tightly binding peptide. There is no specific set of rules that one may use to design ligands for class I MHC at this time. The guidelines that exist now are the same as they were when peptide-binding motifs were first discovered[13]. Using the ranked preferences of amino acids for the allotype-specific anchor positions, we can make substitutions and test binding affinity. Affinity may be measured by circular dichroism (CD) with recombinant protein[2, 4], cell surface assembly assay[24], a competitor assay with radiolabeled peptide[37] or using radiolabeled β_2m[33]. These assays give comparable results most of the time. When peptide solubility is an issue, the CD assay seems to be more reliable (COLLINS, unpublished data). Since relatively conservative anchor substitutions can affect recognition by T cell clones, the immunological reactivity of each APL must also be examined.

One frequently ignored aspect of peptide binding is the rate of peptide dissociation from class I MHC. The half-life of the peptide bound to MHC may be a more critical aspect of TcR recognition than the affinity of the peptide to MHC. If the peptide/MHC has a half-life of 20 min *in vivo*, it will not be presented to T cells very well because it takes roughly the same amount of time to be transported to the plasma membrane from the endoplasmic reticulum (ER). Currently, it is not clear if some critical time-threshold is required to generate immunoreactivity. A more careful study of immunological reactivity based on the half-life of the peptide bound to MHC is underway in our laboratory. The off-rate of the peptide may be measured using radiolabeled peptide[3] or using a modification of the cell surface binding assay[34].

It should be noted that there do exist successful examples where substituting anchor residues to the most favored amino acids generated more immunogenic peptides[10, 22, 30]. However, this approach does not always work.

When Substituting the Anchor Residues Does Not Increase Affinity

The GP2 peptide (HN654-662 IISAVVGIL) is derived from HER-2/neu (C-erbB-2), a receptor tyrosine kinase that is over-expressed in roughly 30% of adenocarcinomas, including lung, breast and ovarian tumors. GP2 binds very poorly to HLA-A2.1[24], yet it is recognized by CTL derived from tumor-infiltrating lymphocytes[14, 15, 23, 45]. The low affinity of GP2 for A2 has been used as an explanation for its poor immunoge-

* Peptide substitutions are shown in terms of the wild-type amino acid, the position of the amino acid within the peptide and the substituting amino acid. Therefore, GP2-I2L is I**L**SAVVGIL.

nicity. Substituting the anchors of GP2 with those most preferred by HLA-A2.1 (L at position 2 and V at position 9) does not significantly increase the binding affinity, as shown by CD and cell surface binding experiments. The crystal structure of the A2/GP2 complex illustrates that, unlike other pMHC structures, the center of the GP2 peptide is highly disordered and does not form stabilizing interactions with the MHC molecule[24].

Two hypotheses were proposed to account for the flexibility seen in GP2. The first hypothesis was that the central residues of the peptide, positions 5–7 (VVG), are too small to fill a large cavity in the hydrophobic peptide-binding groove of class I MHC. Substitutions of these residues would result in higher affinity if the amino acids were larger and made specific interactions. Each position was substituted with D, K, F, or G independently. These substitutions were chosen, to represent a broad selection of size and charges. Within the four amino acid substitutions chosen, there is an amino acid that is small nonpolar, large nonpolar, positive or negatively charged. No improvement in affinity was seen for these peptides except for V5L, and V5L was designed from the crystal structure of A2/GP2[24]. The second hypothesis was that the secondary anchor at position 3 (serine) was too small and nonpolar. HLA-A2.1 prefers a large aliphatic chain at peptide position 3 (P3) to fill the "D" pocket[36]. However, none of the peptides substituted with D, K, F, or G at P3 bind with higher affinity than GP2. Thus, there is some combination of amino acids that are not preferred in this peptide that makes it bind poorly (KUHNS, unpublished data).

In addition to reducing the affinity to the MHC, the flexibility at the center of the peptide may also have a direct impact on its immunogenicity. On the one hand, the flexibility decreases the already small concentration of the specific molecular surface that can interact with TcR on a circulating T cell. On the other hand, multiple peptide conformations generate more molecular surfaces that can be potentially recognized by a larger number of circulating T cells. Perhaps in the context of a peptide that binds well, increased flexibility is more immunogenic. However, in the context of a poorly-binding peptide, increased flexibility does not increase immunogenicity because of the reduced concentration effect. It is likely that other tumor antigens exist which bind to MHC poorly and for which affinity cannot be improved by anchor residue substitutions. Flexibility at the center of the peptide may be a general phenomenon in these peptides. Therefore, substituting residues at the center of the peptide to reduce peptide flexibility may constitute another useful strategy for increasing peptide immunogenicity, but clearly much more work must be done to decide whether it is possible.

The Interaction Between pMHC and TcR

Technically, a more immunogenic peptide can be designed not only to increase the binding affinity to MHC, but also by optimizing the affinity between pMHC and TcR. This process would undoubtedly increase the efficiency of immunogenic APL design to that particular T cell clone. To do so, however, requires a thorough understanding of the interaction between pMHC and TcR.

Several co-crystal structures of class I pMHC/TcR complexes have recently been determined[7, 8, 17, 43]. These crystal structures reveal a similar, diagonal orientation of the TcR over the peptide-binding cleft of each pMHC complex. They also reveal that plasticity seems to be an inherent part of the interaction between these pMHC and TcR complexes. The center of the Tax peptide adjusts its conformation upon binding of A6 and B7 TcRs[8]. Conversely, the peptide retains the same conformation in the dEV8 structure, but the CDR loops of the 2C TcR show shifts relative to their positions in the structure of the uncomplexed TcR[17].

One might expect that, because of the very fine specificity of recognition between pMHC and TcR, the recognition surfaces would be very complementary. For example, the addition of a single hydroxyl group (RT--I1Y versus RT-I1F) in APL of HIV RT peptide (ILKEPVHGV) makes an agonist peptide become a null peptide when tested with a T cell line[21, 34]. Surprisingly, the complementarity between the pMHC and TcR is poor in the crystal structures of pMHC/TcR determined to date[17, 18, 46]. In other cases, the TcR appears to have the ability to recognize drastically different molecular surfaces. A xeno-reactive mouse T cell clone, AHIII 12.2, is able to recognize both the murine pMHC D^b/p1027 (FAPGVFPYM) and the human pMHC A2/p1049 (ALWGPPFVL)[25]. The crystal structures show no similarities (molecular mimicry) in the TcR footprint regions of the two pMHC surfaces[47]. Therefore, functional mimicry rather than molecular mimicry plays a role in T cell xeno-reactivity. This mechanism is likely to be general for all cross-reactive TcR.

In general, no gross conformational changes are seen in the co-crystal structures of pMHC/TcR complexes upon binding[17]. Likewise, no significant conformational changes in the TcR are triggered by engagement of an agonist versus partial agonist or antagonist

that would explain their differing biological outcomes[7]. In light of these observations, quantitative models that relate T cell responsiveness to affinity, avidity or kinetics of TcR and class I/peptide binding are becoming more attractive. Although the story is not complete, the majority of the evidence gathered using surface plasmon resonance supports a kinetic model of discrimination[1, 7, 20, 26], that is, pMHC that remain bound to TcR for long periods of time are able to generate the best T cell response. Those pMHC complexes that fall off TcR rapidly do not activate the T cell.

Although it is appealing to be able to rationally design APL that are optimized for affinity of the pMHC to TcR, the co-crystal structures determined to date suggest that this is not immediately feasible. Furthermore, even if it could be accomplished, designing APL to increase recognition by one TcR would only increase reactivity of this single clone. It is most likely that the best T cell response in a host would be polyclonal. Therefore, each candidate APL must be tested for immunogenicity directly. There is no way to predict immunogenicity.

The Use of MHC Supertypes

One major difficulty associated with class I MHC-linked therapies is the genetic diversity associated with the MHC. The MHC is the most polymorphic locus in vertebrates; more than 50 alleles have been identified to date. If a MHC-based therapy only works for one allele (for example, HLA-A*0201), then its usefulness is limited. The worldwide phenotypic frequency of HLA-A*0201 is approximately 29.4% and is by far the most prevalent. Most allotypes are present in less than 5–10% of the population. Recently, it was observed that **sets** of class I MHC allotypes (called supertypes) have the potential to bind the same antigenic peptide[6, 40, 41]. Thus, instead of one allotype, a particular antigenic peptide may bind to a MHC supertype and expand the percentage of the population treatable with a peptide vaccine. An immunogenic peptide therapeutic that would bind to the five most prevalent members of a supertype would be effective for 20–45% of people in the world. However, a cocktail of peptides that would bind to each of the four supertypes (B7, A2, A3, B44) would be of almost universal applicability (>94% of population)[39].

To date, supertype-binding peptides have been found, not designed. For example, *Plasmodium falciparum* peptides have been screened for their ability to bind members of the A2, A3 and B7 supertypes. Many peptides were found that could bind to one of the five members tested, but few could bind to all five. Most bound to only one of the five[9]. Interestingly, a peptide derived from the tumor-associated antigen tyrosinase-related protein (TRP) peptide TRP197-205 binds to both A31 and A33, and T cell clones from an $A33^+$ patient with melanoma can recognize TRP197-205 bound to A31[44]. This suggests that supertype-binding peptides will generate similar T cell repertoire responses in people expressing the different members of the supertypes. Based on the understanding that T cell cross-reactivity is as unpredictable as antibody cross-reactivity[47], it is likely that this observation will not generally hold true.

It is not clear how to go about designing peptides for supertype binding. Since the rank order of preferences for specific amino acids at the anchor positions varies among the different members, a “middle of the road”, moderate-affinity peptide may be the best candidate, as opposed to a high-affinity binder that may be designed for a single allotype.

In summary, presently there is only a truism about designing peptides for immunotherapy. Every peptide must be synthesized and tested for binding affinity and immunogenicity. Currently, there are not sufficient data describing peptide binding to class I MHC to be able to predict affinity. Additionally, because of the diversity of the T cell repertoire and the complexity of pMHC/TcR interactions, it may prove to be impossible to predict the immunological consequences of even small changes to anchor positions.

Acknowledgment. We wish to thank the members of the Frelinger and Collins laboratories for stimulating conversations and critical reading of this manuscript. This work was supported by the Department of Defense DAMD17-97-1-7052 and National Institutes of Health A120288 and A129324.

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Received in March 2000
Accepted in May 2000