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Peptide epitope identification for tumor-reactive CD4 T cells

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Abstract

Because T lymphocytes have the capacity to recognize tumor cells, significant efforts are being devoted towards the development of T cell-based immunotherapy for cancer. Most of this work has centered in the induction of anti-tumor CD8 T cells, which exhibit cytolytic activity towards tumor cells expressing tumor-specific or tumor associated antigens. Unfortunately to this day, T cell-based immunotherapy for cancer remains suboptimal. One of the possible explanations is that these immunotherapies have ignored the role that CD4 T helper lymphocytes play in the generation and persistence of CD8 T cell responses. Thus, we believe that in order to obtain clinical benefits T cell based immunotherapy must stimulate both CD8 and CD4 tumor-reactive T cell responses. During the past seven years our group has focused on the identification of CD4 T cell epitopes from tumor-associated and tumor-specific antigens that could be used to complement the already identified CD8 T cell epitopes to produce effective vaccination strategies against numerous tumor types. We will describe here the strategy we used that resulted in the identification and characterization of numerous CD4 T cell epitopes that are applicable to developing therapies against hematological malignancies and solid tumors.

Introduction

Role of CD4 T lymphocytes in anti-tumor immune responses

Most immune therapy approaches to combat malignant diseases have centered in the induction of CD8 T cell responses against tumor-associated antigens mainly because these cells can be very effective in killing tumor cells. The 2 most commonly thought therapeutic approaches based on the use of anti-tumor CD8 cytotoxic T lymphocytes (CTLs) are the use of various types of vaccines that generate *in vivo* immune responses (active immunotherapy) or the infusion of *in vitro* expanded CTLs (adoptive T cell therapy) [1]. Although CTLs have been considered to be the main protagonists in the production of anti-tumor therapeutic effects resulting from vaccines or adoptive T cell therapy, it is clear that CD4 helper T lymphocytes (HTLs) can also participate in the generation of anti-tumor responses through various mechanisms [2,3]. On one hand, HTLs are critical for the generation and persistence of CTL responses by providing help through multiple interactions with antigen-presenting cells and CD8 T cells during the induction of the immune responses in secondary lymphoid organs [4–6]. Later on, HTLs continue to sustain CTL responses through the maintenance of memory cells in the periphery or by directly providing costimulatory signals to the CTL, which enhance their function and survival at the tumor site [7–9]. In addition, tumor-infiltrating HTLs may

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function as effector cells either by the local production of cytokines that curtail tumor growth or in some instances by exhibiting anti-tumor cytotoxic activity [10,11]. Because CD4 HTLs recognize antigen as peptide epitopes presented in the context of MHC class II molecules (MHC-II), the effector activity of these cells will be circumscribed to those tumor cells that express MHC class II molecules or in those instances where conventional antigen-presenting cells (e.g., macrophages, dendritic cells) are able to capture, process and present antigens derived from tumor cells to the HTLs. In view of the above, there has been a considerable interest in identifying MHC-II epitopes for tumor-reactive HTL with the purpose of using this information to develop more effective forms of immunotherapy against malignant diseases.

The predictive approach for defining CD4 T cell epitopes from tumor-associated antigens

The identification of sequence motifs in peptides capable of binding to MHC-II molecules of various alleles has led to the development of computer-based algorithms to predict the existence of CD4 T cell epitopes within the amino acid sequence of a putative tumor-associated antigen (TAA) [12–14]. In many instances these algorithms have been validated and further refined using quantitative peptide/MHC-II binding assays. We have extensively used one of such algorithms that was designed to identify peptide binders to the products of 3 commonly found human MHC-II alleles: HLA-DR1, DR4 and DR7 [13]. In our studies we tried to select peptide sequences that scored high in the probability scale for their binding capacity for all 3 MHC-II alleles with the goal of identifying promiscuous CD4 T cell epitopes that would result a broad population coverage for a specific clinical indication. The strategy to identify promiscuous CD4 T cell epitopes using the predictive approach would follow these steps:

a) Selection of the protein antigen—There are several characteristics that would make a particular protein interesting and suitable for the analysis. The most obvious one is that the complete amino acid sequence of the target antigen must be known. Second, it is advisable to select large proteins instead of smaller ones since the probability of finding one or more potential MHC class II binding epitopes will increase. Another desirable characteristic of the protein antigen would be to select antigens that are only expressed by tumor cells and not normal cells. The reasons are that immune tolerance mechanisms operating against self-proteins will reduce the effectiveness of immune responses against tumors. In addition, in the event that strong immune responses were to be induced against a self-antigen, there would be a significant risk of autoimmune pathology towards normal tissues expressing that antigen. Unfortunately, these “tumor-specific antigens” (TSA) are not frequently encountered, so in many cases one has to rely in the use of “tumor-associated antigens (TAA) [15]. However, examples of TSA are proteins derived from oncogenic viruses that continue to be expressed by the malignant cells or products of mutated/rearranged genes that are specifically expressed by the tumor cells [16,17]. Some TAA that are desirable candidates for immunotherapy are those that belong to the “Cancer Testes” (CT) family [18]. These products are expressed in tumor cells and in germinal cells, but not in most normal tissues, limiting the deleterious effects of tolerance and autoimmunity. Other TAA candidates have limited expression in some tissues that are considered not to be vital (prostate, breast, melanocytes). Thus, depending on the tumor type, one may have a single or multiple protein candidates to choose from to submit for prediction analysis for the presence of MHC-II binding peptides.

b) Prediction algorithms for CD4 T cell epitopes—Originally these algorithms were based on the presence of specific residues (or type of residues: hydrophobic, positive/negative charged) present at particular positions within a short peptide sequence (9 to 15 residues long) that were found on T cell epitopes that had been mapped using antigen-specific T cell lines/clones. These motifs were found to be somewhat specific for individual MHC-II alleles but could not be clearly defined. In the case of MHC class I (MHC-I) binding peptides, it became feasible to identify more exact motifs by sequencing peptides eluted from purified MHC

molecules because of their restricted size (8–10 residues). On the other hand peptides that bind to MHC-II do not have a constrained size (9–20 residues), making sequencing analysis interpretation much more difficult. A major breakthrough in the design of MHC-II binding algorithms was the development of quantitative MHC-II peptide binding assays. Using this approach, Southwood and collaborators constructed a computer-based algorithm based on a large database of MHC-II binding peptides [13]. This database keeps being refined [19] and the respective algorithms to predict probability of peptides to bind to diverse human (and mouse) MHC-II alleles have been made available to researchers through the Internet (<http://www.immuneepitope.org/>). We have utilized this algorithm in numerous occasions to predict the existence of peptide sequences from TSA and TAA capable of binding to 3 frequently found MHC-II alleles (HLA-DR1, DR4 and DR7). Results obtained from these algorithms are utilized to limit the number of synthetic peptides that will be tested for their ability to induce T cell responses in labor-intensive cell cultures.

c) *In vitro* immunization of human lymphocytes using candidate CD4 T cell epitopes—Once a manageable number of peptide sequences have been selected from the algorithm analysis, synthetic peptides are made and tested in tissue culture for their ability to stimulate CD4 T cell responses. The technique to generate antigen-specific CD4 T cell lines and clones used by our group has been described in detail [20]. Briefly, the *in vitro* immunization method utilizes purified CD4 T cells isolated from peripheral blood of normal volunteers that are stimulated using peptide-pulsed autologous dendritic cells (DCs). The cultures are restimulated periodically with autologous irradiated peripheral blood mononuclear cells in the presence of IL-2. T cell lines and clones (isolated by limiting dilution) are then submitted to MHC restriction analysis using HLA-typed antigen-presenting cells (APCs) and mouse fibroblast cells (L-cells) transfected with individual human MHC-II alleles. Peptide-titration curves can be done to estimate the antigen avidity of the T cells. The most important step in this process is to assess whether the peptide-reactive CD4 T cell lines (or clones) have the capacity to recognize the TSA or TAA derived from tumor cells. Only with this validation one can be assured that the peptide in question represents a true tumor-specific CD4 T cell epitope. We believe that this validation is necessary before even considering using the peptide as a vaccine or peptide-reactive T cells for adoptive immunotherapy.

d) Assessing tumor-reactivity of peptide-reactive CD4 T cells—Two types of assays can be done to demonstrate that synthetic peptides capable of eliciting CD4 T cell responses represent real tumor-specific (or tumor-associated) epitopes. The first one is to determine the ability of a CD4 T cell line to recognize antigen presented directly by the tumor cells. For this to take place, one must utilize tumor cell lines that express MHC-II surface molecules, as it occurs with many hematological malignancies (e.g., B and T cell lymphomas). In the case of cell lines derived from solid tumors, expression of MHC-II molecules may be induced by treating the cells for 24–74 h with interferon-gamma (IFN γ), prior to performing the immunological assays. One limiting factor with this type of assay is that the tumor cells must express the same MHC-II allele that restricts the response of the CD4 T cells to peptide. In the second type of assay one estimates the capacity of autologous DCs to capture, process and present tumor antigen proteins. In some instances one may use purified recombinant DNA-produced tumor antigen if it is available. In most cases it is possible to feed the DCs with tumor cell lysates (freeze-thaw) or apoptotic (UV light-induced) tumor cells. Under these circumstances, the tumor cell lines do not require to express MHC-II molecules because the CD4 T cells will recognize the processed epitope in the context of the DC's MHC-II molecules. Antigen specificity is validated by using control tumor cell lines that do not express the tumor antigen in question and by the demonstration that antibodies to MHC-II molecules can block the reactivity of the CD4 T cells recognizing the antigen presented by the DCs. We strongly argue that only by fulfilling the above-mentioned criteria one can be certain that the peptide

epitope originally identified as an MHC-II-binding candidate truly represents a tumor-specific epitope for CD4 T cells. Without this demonstration it would be unwise and perhaps unethical to proceed with clinical development to use a CD4 T cell epitope in cancer patients.

CD4 T cell epitopes for viral-encoded TSA

Our studies have focused on 2 viruses responsible for hematological malignancies in humans, Epstein-Barr virus (EBV) and human T-cell leukemia virus-1 (HTLV-1). For these studies, we selected viral protein antigens that continue to be expressed in the malignant cells and that in most instances are involved in the establishment of the malignant phenotype. EBV is responsible for post-transplant lymphoproliferative disease in immunosuppressed individuals that can progress to B-cell lymphomas, and for other malignancies such as NK/T cell lymphoma and several lymphoepithelioma-like carcinomas including nasopharyngeal carcinoma (NPC) and gastric carcinoma. Our studies have identified CD4 T cell epitopes from 2 viral latent cycle antigens, EBNA2 and LMP1 that could be presented in the context of several MHC-II alleles (Table 1) [21,22]. The reactivity of the peptide induced CD4 T cell lines and clones was significant against various EBV-transformed cells (examples presented in Figure 1). Other studies from our group helped identify CD4 T cell epitopes from 2 antigens expressed by HTLV-1 (the causative agent of adult T cell leukemia/lymphoma), the Tax and the envelope antigens (Table I) [23,24]. Again, an important feature of the peptide-induced CD4 T cells was their ability to react with HTLV-1 expressing tumor cells (Figure 1).

CD4 T cell epitopes for TAA expressed by solid tumors

Our studies have focused in the identification of CD4 T cell epitopes for tumor types that affect large number of individuals throughout the world, such as breast, prostate, gastrointestinal cancers and malignant melanoma. Proteins that function as TAA because they are either differentiation (tissue-specific) antigens (gp100, PSMA, TARP, STEAP), tumor markers (CEA, HER2/*neu*, WT1) or belong to the family of cancer-testes antigens (MAGE-A3) were studied as potential sources for CD4 T cell epitopes. Our studies following the strategy described above resulted in the identification of several novel CD4 T cell epitopes for these antigens (Table 1) [20,25–32]. As mentioned previously, the most critical issue is to demonstrate the reactivity of peptide-induced CD4 T cells against tumor cells that express MHC-II and the TAA or alternatively, towards autologous DCs that are fed with antigens derived from tumor cells (e.g., freeze-thaw lysates). The examples presented in Figure 2 illustrate how effective was the ability of various CD4 T cell lines induced with peptides selected from MHC-II binding predictive algorithms, to recognize antigens derived from tumor cells.

CD4 T cell epitopes congregate near CD8 T cell epitopes

Throughout our studies we observed that many of the CD4 T cell epitopes that we identified contain within their sequence, or lie proximal to previously described CD8 T cell epitopes (Table I) [33–47]. Although we do not know whether the close proximity between CD4 and CD8 T cell epitopes is simply a coincidence or holds a biological significance, this observation opens the possibility of using synthetic peptides of relatively small size (15–20 residues) to stimulate CD4 and CD8 T cell responses simultaneously [47–50]. In addition, it has been proposed that large peptides containing MHC-II binding domains may be an effective way to deliver antigens to professional APCs in order to generate more effective immune responses [51].

Closing remarks

The identification of CD4 T cell epitopes for tumor antigens has attracted a high degree of interest because of the belief that immune based therapies against cancer will be more effective

if both CD8 and CD4 T lymphocytes participate in curtailing tumor cell growth. CD4 T cells not only are capable of enhancing CD8 T cell responses but may also exhibit anti-tumor effector function. Although many studies in animal models have clearly demonstrated better results when both CD4 and CD8 T cells participate in anti-tumor responses (as compared to results observed with CD8 T cells alone) [52,53], it remains to be determined whether this strategy will improve the effectiveness of immune therapy in human patients. Nevertheless, there are reports in the area of T cell adoptive therapy that the presence of CD4 T cells in cell products infused into patients, improves the persistence of CD8 T cells and the effectiveness of the therapy. Based on this knowledge we have initiated clinical studies in melanoma patients [54] using peptide gp100_{175–189} (Table I), which contains an HLA-A2-restricted CD8 T cell epitope imbedded within a promiscuous CD4 T cell epitope [25]. Similarly, our group will soon assess the immunogenicity and safety of peptide vaccines for prostate cancer patients using peptides TARP_{14–27}, which harbors adjoining CD8 and CD4 T cell epitopes and a peptide from PSMA, containing a CD8 T cell epitope, PSMA_{27–35} [55] fused to a CD4 T cell epitope from the same TAA, PSMA_{687–701} (Table I). We hope that these studies will provide some evidence of whether simultaneous CD8 and CD4 T cell responses can generate long-lasting immune responses that translate into clinical benefit.

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Figure 1.

Examples of CD4 T-cell responses to viral-encoded tumor antigens.

A, CD4 HTLs derived from 5 healthy donors (D1-D5) specific for peptide EBNA2₂₈₀₋₂₉₀ were able to recognize autologous EBV-transformed B lymphoblastoid cell lines (EBV-LCL) but not the MHC-II-negative, EBV-negative Jurkat T cell lymphoma. **B**, An HLA-DR9-restricted, EBV LMP1₁₅₉₋₁₇₅-reactive CD4⁺ HTL clone showing the capacity to directly recognize autologous EBV-LCL and an LMP1⁺ NK cell lymphoma but not MHC-II allogeneic and unrelated Burkitt's lymphoma Raji. The reactivity was inhibited by the addition of anti-HLA-DR monoclonal antibodies (α DR) indicating that the peptide-epitope is recognized through MHC-II molecules.

C, An HLA-DR9-restricted, HTLV-1Env₁₉₆₋₂₁₀-specific CD4 HTL clone recognizes peptide-pulsed autologous peripheral blood mononuclear cells (PBMC) and reacts with a DR9-expressing HTLV-1⁺ T-cell lymphoma. This reactivity was also blocked with anti-HLA-DR antibodies (α DR). **D**, An HLA-DQ9-restricted, HTLV-1Tax₃₀₅₋₃₁₉-specific CD4 HTL clone recognizes peptide-pulsed autologous PBMC and a DQ9-expressing HTLV-1⁺ T cell lymphoma. Recognition of the HTLV-1 T cell lymphoma by the HTL was blocked by anti-HLA-DQ antibodies (α DQ).

Figure 2.

Examples of CD4 T-cell responses to TAA expressed by solid tumors. HTL clones specific for HER2/*neu*₈₈₃₋₈₉₉ (A), CEA₆₅₃₋₆₆₇ (B), PSMA₃₃₄₋₃₄₈ (C), TARP₁₄₋₂₇ (D), and WT1₁₂₄₋₁₃₈-reactive (E) were shown recognize naturally processed antigens derived from recombinant protein or a cell lysate from the prostate tumor LNCaP presented by DCs in the context of MHC-II molecules. A gp100₁₇₅₋₁₈₉-reactive CD4 HTL clone (F) directly recognizes peptide presented by autologous EBV-LCL and an MHC-II+, gp100+ melanoma tumor mel697 but not the gp100 negative EBV-LCL. This recognition is inhibited by anti-HLA-DR antibodies (α DR). MAGE-A3₁₄₆₋₁₆₀-reactive (G) CD4 HTL clone recognizes directly MHC-II expressing, MAGE-A3+ (HT144 and 624mel) melanomas and react with recombinant MAGE-A3 protein presented by autologous PBMC. STEAP₁₀₂₋₁₁₆-reactive HTL (H) recognizes peptide or cell lysate from STEAP+ (LNCaP) tumor cells. In addition, this HTL can recognize directly a STEAP+, DR+ prostate tumor cell line (PC3).

Table I

Antigen ^a	Peptide Position ^b	Peptide Sequence ^c	MHC Class II Restriction ^d	Effector Function ^e	Proximal CTL Epitope (MHC-I Restriction) ^f	References ^g
EBNA2	280 – 290	TVFYNIIPML	DR1, DR7, DR16, DR52, DQ2	1, 2		[21]
EBV LMP1	159 – 175	YLQNNWWTLLVDLLWLL	DR9, DR15, DR53	1, 2	EBV LMP1 _{159–167} (A2)	[22,35]
HTLV-1 Env	196 – 210	LDHILEPSIPWKSKL	DR9	1, 2		[23]
	317 – 331	AVWLVSALAMGAGVA	DQ6	1		[23]
	384 – 398	LLFWEQGLCKALQE	DR15	1		[23]
HTLV-1 Tax	191 – 205	IEELLYKISLTGTAL	DR1, DR9	1	HTLV-1 Tax _{181–195} (B14)	[24,33]
	305 – 319	LHLLFEEYTNIPISL	DR15, DQ9	1	HTLV-1 Tax _{301–309} (A24)	[24,43]
HER2/neu	883 – 899	KVPIKWMMALESILRRRF	DR1, DR4, DR52, DR53	1		[20]
gp100	74 – 89	GPTLIGANASFIALN	DR7	1	gp100 _{70–78} (C8), gp100 _{87–95} (A3)	[25,36,37]
	175 – 189	GRAMLGTHTMEVTVY	DR53, DQ6	1, 2	gp100 _{177–186} (A2)	[25,34]
	576 – 590	SLAVVSTQLMPGQE	DR7	1	gp100 _{570–579} (A2)	[25,34]
MAGE-A3	146 – 160	FFPVIFSKASSLQL	DR4, DR7	1	MAGE-A3 _{143–151} (B52), MAGE-A3 _{159–160} (A2)	[26,39,41]
WT1	124 – 138	QARMFPNAPYLPSC	DR15, DR53	1	WT1 _{126–134} (A2)	[31,40,47]
STEAP	102 – 116	HQQYFYKIPILVINK	DR1, DR9, DR53	1	STEAP _{86–94} (A2)	[32,46]
	192 – 206	LLNWAYQQVQKNKED	DR9, DR53	1		[32]
TARP	1 – 14	MQMFPSPPLFFFLQ	DR53	1	TARP _{4–13} (A2)	[30,44]
	14 – 27	QLLKQSSRRLEHTF	DR1, DR9, DR13, DR15	1	TARP _{27–35} (A2)	[30,45]
PSMA	334 – 348	TGNFSTQKVKMHHS	DR4	1		[28]
	687 – 701	YRHVIYAPSSHNKYA	DR9, DR53	1		[28]
	730 – 744	ROIYVAAFTVQAAAE	DR53	1		[28]
CEA	625 – 639	YSWRINGIPQHTQV	DR4, DR53	1	CEA _{632–640} (B7)	[29,42]
	653 – 667	YACFVSNLATGRNNS	DR4, DR7	1	CEA _{652–660} (A2)	[27,38]

^aProtein, used in prediction algorithms to identify potential MHC-II binding peptides.

^bResidue positions of peptides used to stimulate T cell responses.

^cAmino acid sequence of T cell epitopes.

^dMHC-II restrictions established with either MHC typed human APCs or mouse L cells transfected with individual human MHC-II molecules.

^e1 = antigen induced cytokine release; 2 = cell-mediated cytotoxicity.

^fPeptide described to function as a CD8 T cell epitope and the restriction HLA class I allele, in parentheses.

^gReferences describing the CD4 and CD8 T cell epitopes.