Heteroclitic Immunization Induces Tumor Immunity

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Summary

In tumor transplantation models in mice, cytotoxic T lymphocytes (CTLs) are typically the primary effector cells. CTLs recognize major histocompatibility complex (MHC) class I-associated peptides expressed by tumors, leading to tumor rejection. Peptides presented by cancer cells can originate from viral proteins, normal self-proteins regulated during differentiation, or altered proteins derived from genetic alterations. However, many tumor peptides recognized by CTLs are poor immunogens, unable to induce activation and differentiation of effector CTLs. We used MHC binding motifs and the knowledge of class I:peptide:TCR structure to design heteroclitic CTL vaccines that exploit the expression of poorly immunogenic tumor peptides. The in vivo potency of this approach was demonstrated using viral and self-(differentiation) antigens as models. First, a synthetic variant of a viral antigen was expressed as a tumor antigen, and heteroclitic immunization with peptides and DNA was used to protect against tumor challenge and elicit regression of 3-d tumors. Second, a peptide from a relevant self-antigen of the tyrosinase family expressed by melanoma cells was used to design a heteroclitic peptide vaccine that successfully induced tumor protection. These results establish the in vivo applicability of heteroclitic immunization against tumors, including immunity to poorly immunogenic self-proteins.

Key words: cytotoxic T lymphocyte • tumors • peptide vaccines • heteroclitic peptides • major histocompatibility complex class I

Cytotoxic T lymphocytes (CTLs) can play a central role in rejecting tumors (1). Tumor antigens recognized by CTL generally originate from three sources: (a) viruses; (b) self-proteins expressed during development or differentiation; and (c) mutant or aberrantly expressed proteins (1). Because many, if not most, tumor antigens are products of normal or altered cellular genes, they are typically not efficient at initiating immune responses. Thus, a central problem in cancer immunotherapy is how to efficiently prime CTLs against poorly immunogenic tumor antigens.

CTLs recognize target antigens in the form of short intracellularly processed peptides, presented by self-MHC-encoded class I molecules (pep: class I). Upon binding of the antigen-specific TCRs on a CTL to its cognate peptide-MHC complex on the tumor cell, the target cell is lysed and the tumor eliminated. To develop into effector CTLs capable of tumor lysis, naive precursor CTLs (pCTLs)1 have to be activated. This pCTL activation requires two signals: the first, or stimulatory signal (signal 1), transmitted via the TCR–CD3 complex, and the second, or costimulatory signal (signal 2), delivered by professional APCs (2–4). In the thymus, a strong signal 1 will induce negative selection of immature thymocytes, regardless of signal 2 (5, 6). In the periphery, the same strong signal 1 will induce immunity (including pCTL → CTL differentiation) or anergy, depending on the presence or absence of signal 2 (2–4). By contrast, even a weak signal 1 without signal 2 can be sufficient for tumor cell lysis by differentiated CTLs (7). This means that a whole class of antigenic peptides exists that, although poorly immunogenic (i.e., unable to induce CTL immunity), can readily serve as molecular targets for lysis by differentiated effector CTLs. Such antigenic, but poorly immunogenic, peptides remain invisible to the naive pCTL.

1Abbreviations used in this paper: ER, endoplasmic reticulum; ERIS, ER insertion sequence; pCTL, precursor CTL; SEI, peptide SEIEFARL; a variant of SSI; SSI, Herpes simplex virus glycoprotein B496-505 peptide SSIEFARL; TAY, peptide TAYRYHLL, an engineered variant of TWH; TM, synthetic adjuvant TiterMax; TWH, melanoma gp75222-229 peptide, TWHRYHLL.
Heteroclitic CTL Vaccines for Tumor Immunity

One strategy to exploit the presence of such poorly immunogenic or nonimmunogenic peptides at the surface of tumor cells is to design immunogenic variants of these peptides that would prime CTL response that cross-reacts to the original targeting peptide. If successful, this strategy could be attractive, since the relative invisibility of poorly immunogenic self-peptides to the immune system could be advantageous. Namely, unlike self-peptides that provide strong signal 1 (8), poorly immunogenic peptides would most likely fail to induce tolerance, and the T cell repertoire reactive against them should be intact and available for activation with immunogenic peptide variants. According to the nomenclature used for variants of a pigeon cytochrome C peptide (9), peptides of higher biological potency than the original peptide were called heteroclitic. Crystal structure analysis revealed that, of the 8-10 amino acid residues of a class I-bound peptide, roughly half point into the solvent and can interact directly with the TCR via their side chains (10-12). The other half are buried by class I and are not directly accessible to the TCR (10-12). Heterocliticity has been achieved by substituting amino acids that contact class I, the TCR, or both (13, 14).

The aim of this study was to produce heteroclitic immunogens that elicit antitumor CTL responses that cross-react to the original poorly immunogenic antigens. Therefore, to design peptides that are heteroclitic for polyclonal CTL responses, one would like to optimize pep:class I binding, since this property correlates with immunogenicity (7, 15). At the same time, the peptide:TCR contact should not be disturbed, to maximize the potential crossreactivity between the heteroclitic and the original, nonimmunogenic targeting peptide. We sought to test whether this strategy could induce antitumor CTLs reactive to nonimmunogenic targeting peptides. We demonstrate successful in vivo induction of cross-reactive CTLs in two tumor models, using an engineered viral peptide variant expressed as a tumor antigen (a model of a tumor antigen of viral origin) and a self-antigen expressed in melanomas (a model of nonmutated, differentiation antigen). Induced CTLs were biologically active in vivo, and were able to effect rejection of both newly implanted and established day 3 tumors.

Materials and Methods

Mice. Female C57BL/6 (B6) mice were purchased from the National Cancer Institute breeding program (Frederick, MD). B6.C-H-2<sup>bm8</sup> (bm8) mice were bred in the MSKCC vivarium from a breeding stock obtained from The Jackson Laboratory (Bar Harbor, ME) via Dr. J. Sprent (The Scripps Research Institute, La Jolla, CA). All mice entered the study between 7 and 10 wk of age.

Antibodies. In vivo CD8 depletion, Flow cytometry and Class I Stabilization Assays. The anti-CD8 mAb, 53.6.7 (rat IgG) and the anti-K<sup>b</sup> mAb, Y3 (mouse IgG<sub>2b</sub>), both obtained from the American Type Culture Collection (Rockville, MD) were produced as acetic fluid in our lab. For in vivo CD8 depletion, 100 µl of acetic fluid was injected intraperitoneally on days -7 and -3 relative to tumor challenge, which was denoted as day 0. PE-conjugated anti-mouse IgG<sub>2b</sub> was purchased from Fisher Biotech (Malvern, PA). Flow cytometry and the class I stabilization assays were performed exactly as previously described (16) using a FACScan<sup>®</sup> instrument equipped with Lysys II software (Becton Dickinson, Mountain View, CA).

Construction of Minigenes. Inserts coding for the endoplasmic reticulum (ER) insertion sequence (17, 18) (amino acid sequence: MRYMLGLLALAAVCSA) followed by the peptides SEI (SEIEFARL) and SSI (SSEIFARL), based on the immunodominant sequence 498-505 of the Herpes simplex virus glycoprotein B, or peptides TWH (TWHRYHLL) or TAY (TAYRYHLL), based upon the sequence 222-229 of the melanoma gp70 protein, were produced by multistep PCR. All oligonucleotides were purchased from Retrogen (San Diego, CA). For the construction of pERIS-SSI and pERIS-SEI minigenes, the PCR reactions were done with two common oligonomers C1 (GGG AGG CTG ACC ATG AGA TAC ATG ATC CTG GGC CTG CTG, C2 (GGG CTG CTG GCC CTG GCC GCC GTG TGC AGC GCT GCC AGC), and the specific oligomers SSI (TTT CTC GAG TCA CCT GCT GGC GAA CTA CTA GCT GCT GCC AGC AGC) or SEI (TTT CTC GAG TCA CCT GCT GCC GAA CTA CTA GCT GCT GCC AGC AGC). C2 and SSI or SEI were first joined in 50-µl reactions consisting of 300 µM dNTPs and 20 µg/ml of primers for 30 cycles at 95°C for 30 s, 60°C for 60 s and 72°C for 60 s, µl of the product was added to 45 µl of C1 and either SSI or SEI at the same primer and dNTP concentrations, and another PCR was performed for 10 cycles as above, followed by 30 cycles at 95°C for 1 min and 72°C for 2 min. For the construction of pERIS-TAY and pERIS-TWH, the common oligomers C1 (GGG AGG CTG ACC ATG AGA TAC ATG ATC CTG GGC CTG CTG GCC GCC GC) and C2 (GGG CTG CTG GCC CTG GCC GCC GTG TGC AGC GCT GCC) were used with the specific oligomers TAY (TTT CTC GAG TCA CCT GCT GCT GTG TGC AGC GCT GCC AGC GCC AGC) or TWH (TTT CTC GAG TCA CCT GCT GCT GTG TGC AGC GCT GCC AGC GCC AGC) or TWH (TTT CTC GAG TCA CCT GCT GCT GTG TGC AGC GCT GCC AGC GCC AGC). The first step was exactly as described above. However, the second step was done for 40 cycles at 94°C for 20 s followed by 60°C for 20 s and 72°C for 45 s. Products thus engineered contain the HindIII and Xhol sites, which were used for cloning into a LacZ-containing pCR2 cloning vector (Invitrogen, San Diego, CA). Clones that scored positive by blue/white screening were digested by HindIII and Xhol, and the inserts were recloned into pCDNA3 to obtain the appropriate expression constructs pERIS-SSI, pERIS-SEI, pERIS-TAY, and pERIS-TWH. The transfer was confirmed by sequencing.

Cell Transfection. 10 µg of linearized plasmid DNA was electroporated into 10<sup>6</sup> R MA-S cells in 500 µl of OptiMEM (GIBCO BRL, Gaithersburg, MD) containing 5% FCS, using a Gene Pulser (Bio-Rad, Hercules, CA) set at 220 V and 960 µF. 48 h later, the cells were incubated in the presence of 600 µg/ml of G418 (GIBCO BRL). G418-resistant clones were selected from the 96-well plates with ≤30% positive wells.

Peptide and Gene Gun Immunization, In Vivo CTL Restimulation, and CTL Assays. The five peptides used in this study, HIV-10 (RGPGRAFVTI), SSI (SSIEFARL), SEI (SEIEFARL), TWH (TWHRYHLL), and TAY (TAYRYHLL), were synthesized by the Memorial Sloan Kettering Cancer Center Microchemistry Core Facility, and were HPLC purified to >98% purity. Peptide immunization using the synthetic immune adjuvant TiterMax (CytRx Inc., Norcross, GA), referred to as pep/TM in the text, CTL restimulation, and 3Cr-release assays were performed as previously described (19). In brief, mice were immu-
nized in the footpad with 10 μl of the pep/TM emulsion (mixed according to the manufacturer’s instructions) containing 5 μg of the indicated peptide. 7 d later, splenic cells from the immunized mice were restimulated in vitro with syngeneic, irradiated (30 Gy), peptide-coated (1 μg/ml, 2 ml/spleen, 1 h at 37°C followed by three washes) cells. 5 d later, cytolytic activity was assessed in a standard 51Cr-release assay. Genetic immunization using DNA-coated gold particles was performed exactly as previously described using a gene gun provided by Powderject, Inc. (Middleton, WI) (20). 100 μg of DNA from the plasmids described in the previous section was mixed with 0.95–2.6-μm diameter gold particles, in the presence of 0.05–0.1 μM spermidine. CaCl2 (1.5 mM) was added in a dropwise fashion to this mixture during vortexing. After precipitation, the gold plasmid DNA complex was washed three times in 100% ethanol and 7 ml of ethanol was added to achieve a bead-loading rate of 0.5 mg of gold to 1.0 μg plasmid DNA per injection. This solution was instilled into plastic Tefzel tubing, the ethanol was gently drawn off, and the tube was purged under nitrogen gas at 400 ml/min for drying. The tube was then cut into 0.5-inch bullets. The gold particles in the “bullets” were injected into the skin of anesthetized mice using a helium-driven gene gun (Powderject, Inc.). The skin was shaved and depilated before injection (20). Four injections at 400 pounds/square inch were delivered to each mouse, one to each of the abdominal quadrants, for a total of 4 μg of plasmid DNA per mouse. 7 d later, spleen cells were restimulated and CTL activity was determined as described for pep/TM.

To establish the principle of heteroclitic immunization against tumors, we used an engineered peptide based on the viral origin. This peptide, called SEI (sequence SEIEFARL), binds poorly to the murine MHC I molecule H-2Kb due to the electrostatic repulsion between the negatively charged glutamic acid residues at the buried position 2 of Kβ (P2E) in position 2. SSI would be predicted to remove the S mutation that enables strong SEI binding in vivo (16). Due to poor binding, this peptide cannot elicit a CTL response in Kβ-bearing C57BL/6 (B6) mice after immunization with peptide/adjuvant (Fig. 1 A, circles, and Table 1) or with genetic immunization using DNA delivered by particle bombardment (Table 1). However, SEI was a good immunogen in B6.C-H-2.Kbm8 (bm8) mice (Fig. 1 B, circles), which express a natural Kβ variant, Kβ bm8. This class I molecule has an E24→S mutation that enables strong SEI binding (16). These results demonstrated that the immunogenicity of the above peptide correlated directly to peptide binding, and that the absence of a response in B6 was not a result of deficient vaccine formulation.

The natural viral peptide from which H2E was derived, SSI (SEIEFARL, also referred to as HSV-8), differs from SEI by having a serine (P2S) instead of the glutamic acid (P2E) in position 2. SSI would be predicted to remove the electrostatic repulsion between the peptide and Kβ. Indeed, SSI bound 100-fold better than SEI to Kβ (16) and was strongly immunogenic for B6 CTLs (Fig. 1 A, squares, and Table 1). We next asked whether SSI-induced CTLs could lyse cells bearing the SEI peptide in a cross-reactive fashion, and found that this was the case when target cells were coated with high concentrations of SEI (>10 μM) in vitro (Dyall, R., unpublished data).

Although these in vitro results were encouraging, their in vivo relevance for tumor immunity was obscure. In particular, it was unclear whether intracellulary expressed weak MHC binders, such as SEI, would be processed and presented efficiently enough for target cell lysis. To address this issue, we expressed SEI in a B cell lymphoma, RMA-S (21). RMA-S has a chemically induced deletion of one of its transporter associated with peptide processing (TAP) genes, Tap-2. This deletion prevents the vast majority of cytosolically processed peptides from entering the ER.
binding to empty class I molecules, which leads to decreased expression of stable class I molecules at the surface of RMA-S cells. The TAP defect was circumvented using a minigene encoding an ER insertion sequence (ERIS) (17) followed by the SEI peptide. Fusion proteins encoded by such ERIS-containing minigenes have been shown previously to insert the attached class I binding peptides into the ER, thereby bypassing the TAP defect and partially restoring the surface expression of pep:class I (17, 18). Indeed, the experimental tumor line, R S-SEI (RMA-S cells transfected with the pERIS-SEI plasmid), had a higher surface level of K^b than R S-Null cells (transfected with the "empty" control plasmid, pcDNA3), as measured by flow cytometry. The mean relative K^b fluorescence intensity for R S-SEI was 123 as compared to only 79 for R S-null (Dyall, R., unpublished data). These observations were consistent with the efficient ERIS-mediated import of SEI into the ER. The fact that R S-SEI was lysed by anti-SSI CTL line, whereas R S-Null was not (Fig. 2), not only confirmed this conclusion, but also demonstrated that CTLs induced by the heteroclitic vaccine cross-reacted on the SEI:K^b expressed by the minigene-transfected cells.

To test the potency of SSI as a heteroclitic vaccine in vivo, mice were immunized with either the heteroclitic or the parental peptide as previously described (19) and challenged with R S-SEI or R S-Null tumor lines. Tumor growth was then assessed for 90 d. Tumor growth curves among the different groups of challenged mice for a typical experiment are shown in Fig. 2 A and results from all experiments are shown in Table 2. The only group protected was the one vaccinated with the heteroclitic vaccine (SSI) and challenged with the tumor line expressing SEI. Numbers shown above each figure show cumulative tumor survival for all mice within the indicated experiment. These results clearly demonstrate the antigenic specificity of the response, and confirm the in vitro findings that SEI cannot induce a protective immune response in B6 mice (Fig. 3). Heteroclitic protection was dependent on CD8^+ cells, because mice depleted of CD8^+ cells by antibody treatment were not protected (Fig. 3, Table 2). Identical results were obtained with genetic immunization (Table 2). The ability of the heteroclitic immunogen to induce rejection of 3-d tumors was next investigated, using both peptide (19) and genetic (20) vaccination. Successful rejection was achieved by genetic immunization only (Fig. 4 and Table 2), consistent with our previous findings that genetic vaccination...
may be more potent than peptide vaccination (20). These results establish the in vivo relevance of heteroclitic immunization.

Heteroclitic Vaccination against a Tyrosinase Family Epitope of a Melanoma. We next sought to test the applicability of this approach using naturally occurring tumor antigens. As a model, we selected the brown locus product, also known as the tyrosinase-related protein 1, or gp75. This glycoprotein is a lineage-specific self-antigen, present in melanocytes and expressed in melanomas (22, 23). The product of the brown locus is a relevant cancer antigen, recognized by both antibodies and T cells in patients with melanoma (22–24). In mice, passive and active immunization against gp75 results in both melanoma rejection and manifestations of autoimmunity (25, 26). However, in the mouse model, CTL immunity against gp75-expressing melanoma cells was not induced by immunization with tumor cells plus adjuvant, nor tumor cells engineered to express cytokines, or with purified gp75 protein (26).

Using the canonical K b-binding motif (27) to scan the amino acid sequence of gp 75 (28), five potential epitopes were identified. Synthetic peptides corresponding to these epitopes were used in both CTL inhibition and class I stabilization assays to determine their ability to bind to Kb (Moroi, Y., and R. Dyall, unpublished data). Peptide TWH, corresponding to gp75 residues 222–229 (TWHRYHLL), exhibited similar binding characteristics to SEI and was selected for further experiments. Since this peptide has two bulky side chains not commonly found at the buried, MHC-contacting positions 2 and 3 (P2W and P3H), we suspected that they might sterically impair pep–K b binding. Based on the available sequence/motif information for K b-binding peptides, and on the crystallographic data on the pep:K b structures (10–12, 27), we changed these two residues to A and Y, respectively. The variant designed in this manner, named TAY (TAYRYHLL), turned out to be an excellent Kb binder, comparable to SSI (Fig. 5).

We next immunized B6 mice with the TAY and TWH peptides using both pep/TM and genetic methods. For both immunization protocols, successful CTL priming was obtained only with the engineered, and not the native, peptide (Table 1). Importantly, the anti-TAY CTLs lysed target cells pulsed with TWH in vitro, revealing that TAY exhibits the heteroclitic properties for TWH (Fig. 6 A). The well-characterized melanoma, B16 (29), and its radiation-induced gp75 loss mutant, B78H.1 (29), were next used as in vitro targets for the anti-TAY CTL lines (Fig. 6 B). Despite comparable surface expression of K b after induction with IFN-g (data not shown), B16 (Fig. 6 B, closed...
CTLs against Kb-expressing target cells pulsed with TWH. CTL activity of anti-TAY melanoma cell surface to serve as targets for CTL attack. Immunogenicity of the tumors, and other factors. Together with previous studies in vitro (30–32), the above results confirm the principles and establish the applicability of rationally designed heteroclitic vaccination to tumor immunity in vivo.

**Discussion**

These results demonstrate the applicability and the in vivo efficacy of heteroclitic CTL vaccines. The approach to substitute the buried, MHC-contacting residues of a poorly binding peptide, but leave the solvent-exposed TCR-contacting residues intact, was previously used to generate CTLs that killed virally infected (30) or tumor (31, 32) targets expressing poorly immunogenic peptides in vitro. Furthermore, heteroclitic peptides of the human tyrosinase were shown to be more effective in stimulating CD4+ cells in vitro (31). Most recently, in a heterologous vaccination model, a fortuitous presence of a peptide with heteroclitic properties primed T cells that exhibited antitumor activity upon in vivo adoptive transfer (33). Using rational epitope identification and engineering and in vivo tumor challenge, we now demonstrate the in vivo potency of this strategy. The amino acid sequences of tumor antigens can be easily examined to select candidate MHC-binding peptides that can target the tumor for CTL attack. Once the best candidates are identified, they can be used individually or in a cocktail vaccine (reference 19 and Dyall, R., and L. Weber, unpublished data). The latter would maximize the odds of success and minimize the risk.

### Table 1. Summary of In Vitro CTL Responses in C57BL/6

| Immunogen | Exp. No. | DNA | Peptide* |
|-----------|----------|-----|---------|
| SSI       | 1        | 5/5 | 5/5     |
|           | 2        | 5/5 | 5/5     |
|           | 3        | 10/10 | 5/5 |
| SEI       | 1        | 0/5 | 0/3     |
|           | 2        | 0/5 | 0/5     |
|           | 3        | N D | 0/5     |
| TAY       | 1        | 5/5 | 3/5     |
|           | 2        | 3/3 | 3/3     |
|           | 3        | N D | 5/5     |
| TWH       | 1        | 0/5 | 0/5     |
|           | 2        | 0/3 | 0/3     |
|           | 3        | N D | 0/5     |

The responses were assayed 5 d after in vitro stimulation, in a Cr-release assay as previously described (19). Only mice with specific lysis of >35% were scored as responders. Groups responding to the immunogen are shown in bold. N D, Not done.

* Refers to pep/ TM immunization.
of tumor escape by epitope mutation. Of course, similar principles can be applied equally well against intracellular pathogens.

Our data also touch upon the issue of self-tolerance and tumor immunotherapy. As recently discussed (8), it is now clear that many self-differentiation antigens do not induce complete tolerance through deletion of self-reactive lymphocytes from the immune repertoire. In that regard, an important advantage of poor MHC binders derived from self-proteins may be that they are unlikely to tolerize T cells (as the signal 1 they provide is not strong enough), sparing a precursor CTL population that can be activated by an appropriate heteroclitic vaccine. Our results with the gp75 peptide TWH lend experimental support to this view, suggesting that this class of weak differentiation antigens could become a potential target for tumor therapy. Of interest, in the experiments described here, we did not notice any overt signs of autoimmunity (including depigmentation) that one might expect if a melanocyte antigen, such as gp75, is used to target the tumor for lysis by heteroclitic CTLs. Indeed, such autoimmune manifestations frequently accompany productive antimelanoma immunity in mice and have been suggested in humans (26 and the references contained therein). At present, it is unclear whether CTLs induced by heteroclitic vaccination are less prone to induce autoimmunity or whether the results observed were due to the particular peptide chosen. This issue is currently being addressed experimentally.

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