CD4-Directed Peptide Vaccination Augments an Antitumor Response, but Efficacy Is Limited by the Number of CD8+ T Cell Precursors

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CD4-Directed Peptide Vaccination Augments an Antitumor Response, but Efficacy Is Limited by the Number of CD8\(^{+}\) T Cell Precursors\(^1\)

Holly L. Hanson,\(^2,3\) Silvia S. Kang,\(^2\) Lyse A. Norian, Ken Matsui, Leigh A. O’Mara, and Paul M. Allen\(^4\)

Peptide vaccination is an immunotherapeutic strategy being pursued as a method of enhancing Ag-specific antitumor responses. To date, most studies have focused on the use of MHC class I-restricted peptides, and have not shown a correlation between Ag-specific CD8\(^{+}\) T cell expansion and the generation of protective immune responses. We investigated the effects of CD4-directed peptide vaccination on the ability of CD8\(^{+}\) T cells to mount protective antitumor responses in the DUC18/CMS5 tumor model system. To accomplish this, we extended the amino acid sequence of the known MHC class I-restricted DUC18 rejection epitope from CMS5 to allow binding to MHC class II molecules. Immunization with this peptide (tumor-derived extracellular signal-regulated kinase-II (tERK-II)) induced Ag-specific CD4\(^{+}\) T cell effector function, but did not directly prime CD8\(^{+}\) T cells. Approximately 31\% of BALB/c mice immunized with tERK-II were protected from subsequent tumor challenge in a CD40-dependent manner. Priming of endogenous CD8\(^{+}\) T cells in immunized mice was detected only after CMS5 challenge. Heightened CD4\(^{+}\) Th cell function in response to tERK II vaccination allowed a 12-fold reduction in the number of adoptively transferred CD8\(^{+}\) DUC18 T cells needed to protect recipients against tumor challenge as compared with previous studies using unimmunized mice. Furthermore, tERK-II immunization led to a more rapid and transient expansion of transferred DUC18 T cells than was seen in unimmunized mice. These findings illustrate that CD4-directed peptide vaccination augments antitumor immunity, but that the number of tumor-specific precursor CD8\(^{+}\) T cells will ultimately dictate the success of immunotherapy. The Journal of Immunology, 2004, 172: 4215–4224.
illustrated that both Ag-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells were induced in response to vaccination, and that protective antitumor immunity could be generated as a result (38). Two studies that identified unique MHC class II-presented murine TAAs provided evidence that the CD4<sup>+</sup> tumor-specific T cell response stimulated by these Ags can be tumor protective (29, 30). At this time, however, far fewer MHC class II-restricted TAAs have been described as compared with their MHC class I-restricted counterparts, and consequently, development of CD4-specific vaccination strategies remains difficult.

We sought to examine the effects of a CD4-directed vaccination strategy on CD8-mediated tumor rejection, by evaluating effector function of both endogenous CD8<sup>+</sup> T cells and limited numbers of transferred TCR transgenic tumor-specific precursor CTLs. To accomplish this, we generated a synthetic 17-aa peptide by extending the sequence of a defined class I-restricted epitope to allow for binding to the MHC class II molecules present in our previously described DUC18/CM5 tumor model system (39). CM5 is a MHC class I<sup>+</sup>/class II<sup>+</sup> fibrosarcoma line derived from a BALB/c tumor. CD8<sup>+</sup> T cells from DUC18 TCR transgenic mice express an αβ TCR specific for a CM5 tumor rejection Ag that arises from a point mutation in extracellular signal-regulated kinase 2 (ERK2). The specific CTL epitope comprises aa 136–144 of the mutated tumor ERK2 (tERK) bound to H-2K<sup>d</sup> and is designated tERK-I. Transfer of as few as 12,000 naive DUC18 T cells is sufficient to protect BALB/c mice from subsequent challenge with CM5 (39).

In this study, we find that prophylactic vaccination of BALB/c mice with a CD4-directed peptide, designated tERK-II, induces an Ag-specific CD4<sup>+</sup> T cell response as evidenced by proliferation and IFN-γ production upon peptide-specific restimulation. Vaccination with tERK-II is sufficient to protect 31% of BALB/c mice from subsequent challenge with CM5. In contrast, vaccination with the 9-aa class I-restricted tERK-I peptide has no protective effects in vivo. Protection mediated by tERK-II and the resultant Ag-specific CD4<sup>+</sup> T cell response is due to enhanced priming of endogenous CD8<sup>+</sup> T cells, and relies upon CD40/CD40L interactions in the host. Increased CD4<sup>+</sup> Th cell function following tERK-II vaccination allowed transfer of fewer tumor-specific precursor CD8<sup>+</sup> T cells to prevent tumor growth in vaccinated recipient mice. Our results suggest that this simple strategy of extending known MHC class I-restricted tumor Ag epitopes to allow presentation by MHC class II molecules is an effective method for generating CD4-directed peptide vaccines that are able to boost tumor-specific CTL function in vivo.

Materials and Methods

Tumor cells and in vivo tumor challenge

CM5 fibrosarcoma (40) cells were propagated in vitro in RPMI 1640 supplemented with 10% FCS (HyClone Laboratories, Logan, UT), 1 mM Glutamax (Life Technologies, Gaithersburg, MD), and 50 μg/ml gentamicin. For in vivo tumor challenges, tumor cells were removed from culture flasks by trypsinization, washed twice in PBS, and injected s.c. Tumor growth was measured every 48 h using calipers and was recorded as the product of two orthogonal diameters (a × b). The first diameter was identified as the longest surface length (a), with the second diameter being the subsequent orthogonal width (b).

Mice

DUC18 TCR transgenic mice on the BALB/c background have been described previously (39). BALB/c mice were obtained from National Cancer Institute, and CD40 knockout (KO) mice on the BALB/c background were obtained from The Jackson Laboratory (Bar Harbor, ME). All experimental mice were age-matched females (8–12 wks). All mice were housed in a specific pathogen-free barrier facility at Washington University.

Peptides

The peptides used in this study were synthesized by standard F-moc chemistry using either a Synergy peptide synthesizer (model 432A; Applied Biosystems, Foster City, CA) or a multiple peptide synthesizer (Symphony/Multiplex; Protein Technologies, Tucson, AZ). All peptides were HPLC purified, and analyzed by mass spectrometry (Washington University Mass Spectrometry Resource) for purity. Peptide concentration was determined by amino acid analysis (model 6500; Beckman, Fullerton, CA). The amino acid sequences for the peptides used in this study are: tERK-I (136–142), QYIHSANVL; tERK-II (133–147), RQLQYIHSANVLHRDLK; native ERK-I (nERK-I) (136–142), KYIHSANVL; nERK-II (133–147), RQLKYIHSANVLHRDLK; OVA (323–339), SQYAVHAAHAEINAGR (41). tERK-I and -II denote peptides derived from the mutated tumor form of ERK, while nERK-I and -II denote the corresponding peptides containing native ERK sequences present in normal tissues. The tERK-I peptide is aimed at eliciting a CD8<sup>+</sup> CM5-specific response, whereas tERK-II is aimed at generating a CD4<sup>+</sup> T cell response. The nERK-I and nERK-II peptides are used as controls for these tumor-specific peptides. The tumor-derived peptides (tERK-I and -II) differ from their respective native controls (nERK-I and -II) only at position 136, in which there is an amino acid substitution of a Q for a K.

Intracellular cytokine staining

Single cell suspensions were made from popliteal and superficial inguinal lymph nodes from immunized mice, and 3 × 10<sup>6</sup> lymph node cells were stimulated for 18 h in the presence of 3 × 10<sup>6</sup> APCs lacking T and B cells from CB17 SCID mice (kind gift of E. Unanue (Washington University, St. Louis, MO)) with no Ag, 10 μM tERK-II, 10 μM OVA, or 10 μM tERK-I. During the last 4 h of stimulation, 10 μg of brefeldin A was added to the cultures. Cells were stained with CyChrome-conjugated anti-CD8<sup>+</sup> (BD Pharmingen, San Diego, CA) and PE-conjugated anti-CD4 (BD Pharmingen). Stained cells were then stained with FITC-conjugated anti-IFN-γ and immediately analyzed by flow cytometry on a BD Biosciences FACSCalibur (San Diego, CA) using CellQuest analysis software. For each sample, 50,000 events falling in the live lymphocyte gate were collected for analysis.

Immunization and lymph node proliferation assay

BALB/c and CD40 KO mice were immunized in the hind footpad with 20 nmol of peptide emulsified in CFA (Fisher Scientific, St. Louis, MO). After 7 days, popliteal and superficial inguinal lymph nodes were removed from immunized mice. A total of 5 × 10<sup>5</sup> cells/well was cultured in a total volume of 200 μl of RPMI 1640 supplemented with 1% normal mouse serum, 5 × 10<sup>–5</sup> M 2-ME, 1 mM Glutamax, 1% nonessential amino acids, 1% sodium pyruvate, 1% HEPES, and 50 μg/ml gentamicin. Assays were performed in a 96-well flat-bottom tissue culture plate with the indicated concentrations of each peptide. The cultures were incubated at 37°C for 72 h and then pulsed with 0.4 μCi of [3H]thymidine and harvested 18–24 h later.

Adaptive transfer of DUC18 T cells

Single cell suspensions of DUC18 splenocytes from homoyzogous transgenic mice were prepared in HBSS, washed twice, and counted. Transferred populations were normalized for Vβ8.3 and CD8<sup>+</sup> dual positivity, as determined by flow cytometry. Transferred cells were injected i.v. via tail vein injection.

Enumeration of expanded Thy-1.1 DUC18 T cells

Thy-1.2 BALB/c mice were immunized with 20 nmol of tERK-II peptide emulsified in CFA or were left unimmunized on day −7. Single cell suspension of Thy-1.1 DUC18 splenocytes were prepared in HBSS, RBC lysed, washed, and counted. Transferred populations were normalized for the number of Thy-1.1 DUC18 T cells based on Vβ8.3 and CD8<sup>+</sup> staining determined by flow cytometry. Cells were resuspended to contain 1000 naive Thy-1.1 DUC18 T cells in a 200 μl vol of HBSS and injected i.v. via the tail vein into primed or unprimed Thy-1.2 BALB/c recipients on day −1. On day 0, all mice were challenged s.c. with 3 × 10<sup>6</sup> CM55 cells. Tumor-draining lymph node spleens from individual mice were harvested on days 4, 6, and 8 posttumor challenge. Cells were counted; stained with Vβ8.3 FITC, Thy-1.1 PE, and CD8<sup>+</sup> CyChrome (BD Pharmingen); and read on the FACSCalibur. The number of transferred Thy-1.1<sup>+</sup> DUC18 T cells present in each organ was enumerated by multiplying the percentage of cells that were Vβ8.3<sup>+</sup>, Thy-1.1<sup>+</sup>, and CD8<sup>+</sup> by the total number of cells.
Results

Peptide vaccination with tERK-II primes Ag-specific proliferation

We sought to develop a technique to protect mice from tumor challenge through peptide vaccination. Initial attempts to vaccinate using the tERK-I epitope showed no therapeutic benefit, and this approach was abandoned. The failure of MHC class I-restricted vaccination in other systems has at times been attributed to the lack of CD4\(^+\) T cell help (2, 3). To develop a technique to stimulate tumor-specific CD4\(^+\) T cell help and examine its effects on the endogenous tumor-specific CD8\(^+\) T cell response, we exploited the identified K136Q mutation in ERK-2, which generates the CMS5-specific epitope recognized by DUC18 CD8\(^+\) T cells. The tERK-I peptide (aa 136–142) was extended to a total of 17 aa (aa 133–147) to make it an appropriate length to bind class II MHC. Immunization with this length of peptide in CFA is a well-established technique of generating class II-restricted, CD4\(^+\) T cell responses (42–44), and not class I-restricted responses (45). This extended peptide is referred to as tERK-II, the original K\(^2\)-restricted epitope as tERK-I, and their normal tissue counterparts as nERK-II and nERK-I, respectively (Fig. 1A). We then wanted to ascertain whether tERK-II could induce an immune response.

BALB/c mice were primed s.c. with tERK-II, nERK-II, or a control peptide (OVA), and the lymph node proliferative responses were assayed in vitro 7 days after immunization. A strong response specific for tERK-II was observed, consistent with this being presented by MHC class II. This response did not cross-react with OVA, and was only slightly cross-reactive with the nERK-II peptide at very high concentrations of peptide stimulation (Fig. 1B). Mice immunized with tERK-II did not mount a lymph node proliferative response to tERK-I or nERK-I, demonstrating that immunization with the extended peptide did not directly prime a class I-restricted CD8\(^+\) T cell response (Fig. 1B). Immunization with OVA resulted in a strong OVA-specific recall response without cross-reactivity with nERK-II or tERK-II (Fig. 1C). Immunization with nERK-II did not break in vivo self-tolerance mechanisms, as demonstrated by the inability to recall a nERK-II proliferative response in vitro (Fig. 1D). Thus, immunization using this CD4-directed peptide tERK-II is able to elicit a robust, specific, endogenous proliferative response.

In vivo priming with tERK-II/adjuvant protects BALB/c mice from subsequent tumor challenge

To determine the protective effects of vaccination using the tERK-II peptide, mice were immunized with peptide and then challenged s.c. with CMS5 7 days later. A significant percentage of mice immunized with tERK-II peptide was protected from subsequent tumor challenge. Vaccination with tERK-I, nERK-II, or OVA offered no protection (Fig. 2). In five independent experiments, we consistently observed some degree of protection in tERK-II-vaccinated mice. Collectively, 31% of the mice immunized with tERK-II were fully protected from CMS5 challenge, whereas none of the mice immunized with OVA, tERK-I, or nERK-II showed any protection (Fig. 2B). Additionally, tumors that did grow out in tERK-II-immunized mice were substantially smaller than tumors growing in untreated mice (Fig. 2A). Interestingly, although immunization with the control peptide OVA (323–339) elicited a strong CD4\(^+\) T cell proliferative response (Fig. 1C), this nonspecific T cell help was not sufficient to inhibit tumor growth. This observation illustrates that, in agreement with previous reports (46), tumor-specific CD4\(^+\) T cell proliferation and activation are required for antitumor effects of the immunization, and that nonspecific local production of cytokines or stimulation of nonspecific CD4\(^+\) T cells is not sufficient to help the antitumor effector response. Therefore, these results demonstrate that CD4-directed vaccination of mice with tERK-II was partially efficacious in protecting mice from CMS5 tumor challenge.

Vaccination with tERK-II/adjuvant does not directly prime a tERK-I-specific response

Because the tERK-II epitope contains the entire tERK-I epitope, it was necessary to determine whether the protective effect of the tERK-II immunization was due to the direct priming of CD8\(^+\) tERK-I-specific T cells. Mice immunized with the tERK-II peptide did not have an in vitro recall response to the tERK-I peptide before tumor challenge, indicating that the tERK-II immunization did not directly prime a tERK-I-reactive T cell population (Fig. 1B). Additionally, priming BALB/c mice directly with the tERK-I peptide in adjuvant was not protective against subsequent CMS5 challenge (Fig. 2A), confirming that the efficacy of the tERK-II peptide immunization was not due to inadvertent priming of a CD8\(^+\) tERK-I-specific effector cell population. Therefore, neither CD4-directed tERK-II immunization nor CD8-directed tERK-I immunization appears to directly prime endogenous tumor-specific CD8\(^+\) T cells.

The protective effect of tERK-II immunization is CD40 dependent

CD40/CD40L interactions are known to be critical for the initiation of effective tumor-specific immune responses through activation of CD4\(^+\) Th cells (47) and through activation of APCs (48). We therefore investigated the CD40 dependence of tERK-II-elicted protection in our system. Both CD40\(^{-/-}\) and BALB/c mice succumb to rapid tumor growth following CMS5 challenge (Fig. 3A).

The CD4\(^+\) T cells stimulated by tERK-II immunization could help the final CD8\(^+\) T cell effectors in a manner that is either CD40 independent (49) or depends on the interaction between CD40L on the activated CD4\(^+\) T cell and CD40 on APCs. In marked contrast to the BALB/c mice, in which tERK-II vaccination reproducibly induced partial protection from subsequent CMS5 tumor growth, none of the CD40 KO mice were protected from subsequent CMS5 challenge (Fig. 3B). This indicates that the mechanism of protection mediated by tERK-II immunization depends upon the presence of CD40.

CD40 is necessary for tERK-II immunization to elicit tERK-I-specific CD8\(^+\) T cell effectors

Because tERK-II immunization did not protect the CD40 KO mice from CMS5 tumor growth, we investigated whether tERK-II-immunized CD40\(^{-/-}\) mice were capable of priming any tERK-I-specific CD8\(^+\) T cells. We primed BALB/c and CD40-deficient mice with either tERK-II or OVA control peptide. Seven days after immunization, mice were either challenged with CMS5 or sacrificed so that the specificity of the immunization could be assayed by measuring intracellular IFN-\(\gamma\)-producing ex vivo. Popteliteal and superficial inguinal lymph node cells were cultured overnight in the presence or absence of Ag before being stained for CD4, CD8, and intracellular IFN-\(\gamma\) (Fig. 4A). CD4\(^+\) T cells from wild-type BALB/c mice immunized with tERK-II responded minimally to tERK-II stimulation, but not at all to the control peptide OVA (Fig. 4B). Importantly, there was no significant population of CD8\(^+\) IFN-\(\gamma\)-producing cells following tERK-II immunization, even...
upon restimulation with tERK-I, again indicating that the tERK-II immunization did not directly prime an effector CD8+ T cell population (Fig. 4D). The remaining mice were sacrificed 2 wk following CMS5 tumor challenge, and their lymph node cells were analyzed for cytokine production, as described above. The specificity of the CD4+ T cell response remained intact following tumor challenge. CD4+ T cells from both the BALB/c and CD40 KO animals immunized with tERK-II and challenged with CMS5 made IFN-γ only in response to tERK-II (Fig. 4C). Although no CD8+ T cells from any of the immunization groups had appreciably produced IFN-γ in response to immunization alone, tERK-II-immunized mice demonstrated a detectable population of tERK-I-specific, IFN-γ-producing, CD8+ T cells following tumor challenge. This CD8+ T cell response was absent in CD40−/− mice (Fig. 4E). The CD4+ tERK-II-specific response was diminished in the CD40−/− mice; thus, the lack of subsequent CD8+ tERK-I-specific responses could either be due to inadequate generation of CD4+ T cell help, or inadequate APC maturation. In either case, the data imply that the protective effect of tERK-II immunization is associated with the ability to

FIGURE 1. The tERK-II peptide overlaps the tERK-I epitope and stimulates a lymphoproliferative response. The tERK-II peptide derived from mutated ERK2 (133–149) contains the tumor-specific alteration from a lysine to a glutamine at position 136 and overlaps the class I-binding CMS5 tumor rejection Ag (A). Lymph node proliferation assays using 5 × 10⁵ cells/well were performed 7 days after immunization with 20 nmol of peptide emulsified in adjuvant. Cultures were incubated for 72 h before addition of [³H]thymidine for the last 18–24 h. Specific proliferative recall responses from primed mice immunized with tERK-II (B), OVA (323–339) (C), and nERK-II (D) are shown in response to peptide stimulations with tERK-II (■), nERK-II (○), OVA (▲), tERK-I (○), and nERK-I (▲). The data are representative of more than three similar experiments.
generate a tERK-I-responsive CD8\(^+\) T cell population in a CD40-dependent manner.

**Transfer of 1000 naive DUC18 T cells ablates CMS5 tumor growth in tERK-II-vaccinated mice**

Immunization with tERK-II protects \(\sim 31\%\) of BALB/c mice from subsequent CMS5 challenge. The basis for the lack of protection in the remaining mice could be due to insufficient generation of tumor-specific CD4\(^+\) or CD8\(^+\) T cells. We reasoned that a lack of endogenous tumor-specific CD8\(^+\) precursors could be corrected by the transfer of a limited number of transgenic tumor-specific T cells into immunized mice. Previously, it was determined that at least 12,000 DUC18 T cells were required to protect BALB/c mice from a subsequent challenge of \(3 \times 10^6\) CMS5 tumor cells (39).

To determine whether priming with tERK-II would result in protective immunity with a lower number of DUC18 T cells, we immunized mice with tERK-II and transferred 10, 100, or 1000 naive DUC18 T cells 6 days later. These mice and appropriate controls were challenged with CMS5 tumors 24 h following T cell transfer, and tumor growth was monitored. Sixty-four percent of mice that were immunized with tERK-II peptide alone developed tumors (Fig. 5 and Table I). Transfer of 1000 naive DUC18 T cells alone resulted in tumor outgrowth in 81% of the challenged mice, suggesting only partial protection was achieved (Fig. 5 and Table I). Impressively, tERK-II-primed mice that also received 1000 DUC18 T cells were now completely protected from CMS5 challenge (Fig. 5 and Table I). Titration of the number of transferred...
DUC18 T cells demonstrated that a low level of enhanced protection could be obtained by transferring as few as 100 DUC18 T cells in conjunction with tERK-II immunization (Table I). Interestingly, immunization with OVA peptide before transfer of 1000 naive DUC18 T cells resulted in tumor outgrowth in 100% of the challenged mice, compared with 81% of mice treated with 1000 DUC18 T cells alone (Table I). Additionally, tumor size was increased in the mice that were treated with OVA and 1000 DUC18 T cells relative to mice receiving 1000 DUC18 T cells alone (data not shown). Although the underlying mechanism for this effect has not been elucidated, it could result from the generation of a non-tumor-specific response that interferes with DUC18 T cell function.

**FIGURE 4.** Peptide immunization primes peptide-specific CD4+ T cells, but tERK-I-specific CD8+ T cells are not present until after tumor challenge. Groups of BALB/c or CD40 KO mice were examined for intracellular IFN-γ production according to the above experimental outline (A). Mice were immunized s.c. with 20 nmol of OVA (323–339) or tERK-II peptide in adjuvant. Seven days after immunization, mice were either harvested for their lymph nodes (prechallenge condition) or challenged with 1.5 × 10^6 CMS5 tumor cells and harvested for their lymph nodes 2 wk posttumor challenge (postchallenge). A total of 3 × 10^6 lymph node cells collected before CMS5 tumor challenge (prechallenge; B and D) was stimulated ex vivo for 18 h with 10 μM OVA (323–339), tERK-II, tERK-I, or no Ag. Cells were stained with IFN-γ FITC, CD4 PE, and CD8 CyChrome to assess the percentages of intracellular IFN-γ-positive cells in the CD4+ T cell compartment (B) or the CD8+ T cell compartment (D). Intracellular IFN-γ staining in lymph node CD4+ and CD8+ T cells from immunized mice was also assessed 2 wk after s.c. challenge with CMS5 tumor cells (postchallenge; C and E).
vested, counted, and stained for \( V \) lymph nodes and spleens from the mice were individually harvested on day 0. On days 4, 6, and 8 posttumor transfer, draining cells were subjected to flow cytometry to determine the percentage was then used to determine the absolute number of transferred naive DUC18 T cells in the spleen was seen by days 6 and 8 posttumor challenge. This timing coincided with the tumor rejection by days 6 and 8. This timing coincided with the tumor rejection kinetics in which rejection begins between days 6 and 8, suggesting that these Thy-1.1\(^+\) DUC18 T cells have homed into the tumor site at these time points. In contrast, minimal numbers of Thy-1.1\(^+\) DUC18 T cells were detected in the spleen of immunized mice on day 4, and a delayed, but stable increase in Thy-1.1\(^+\) DUC18 T cell numbers in the spleen was seen by days 6 and 8 posttumor challenge. Taken together, these results suggest that tERK-II priming resulted in more rapid expansion kinetics and increased trafficking efficiency to the tumor site by the Thy-1.1\(^+\) DUC18 T cells compared with unimmunized mice.

Use of vaccination plus adoptive transfer of tumor-specific CD8\(^+\) T cells resulted in a level of efficacy far above what either treatment showed alone. Thus, as has been observed in other systems, we have demonstrated that CD4\(^+\) antitumor T cells have a large capacity to augment CD8\(^+\) T cell responses. Ultimately, the number of antitumor CD8\(^+\) T cells is a determining factor in the generation of an effective antitumor response, and this threshold can be lowered by providing adequate CD4\(^+\) T cell help.

Discussion

In this study, we investigated the ability of a CD4-directed peptide vaccine to elicit protective T helper and CTL responses against a defined TAA in the murine CMS5 tumor model system. The tERK-II peptide was generated by extending the amino acid sequence of a known 9-aa CTL epitope, T\(\text{ERK}-\text{I} \)). Although this 17-aa tERK-II peptide encompassed the tERK-I sequence, no direct CD8\(^+\) T cell priming was observed as a result of vaccination, and CD8\(^+\) T cell effector function was only detectable following tumor challenge. Vaccination with tERK-I elicited no protective effects in vivo. In contrast, a low level of protection from subsequent tumor challenge was observed following tERK-II vaccination alone. Transfer of minimal numbers of tumor-specific CD8\(^+\) DUC18 T cells in conjunction with tERK-II vaccination resulted in protection from tumor challenge in 95% of treated mice. The underlying mechanisms for this combined effect may be due to increased expansion kinetics of transferred DUC18 T cells, and enhanced trafficking to the tumor site. Ag-specific priming of CD4\(^+\) T cells was required for protection, as the use of an irrelevant OVA peptide failed to stimulate any protective immune response. Therefore, the tERK-II peptide effectively generated CD8\(^+\) T cell-mediated antitumor immunity indirectly, via its effects on CD4\(^+\) Th cells.

Our current findings highlight the importance of CD4\(^+\) T cells in the establishment of antitumor immunity in the CMS5 model. However, in our initial description of the DUC18/CMS5 tumor model system, we observed that CD4\(^+\) T cells were unnecessary for rejection of CMS5 tumor challenge in DUC18 TCR transgenic mice (39). In these mice, ~35–50% of CD8\(^+\) T cells are tumor specific, and therefore the precursor frequency of antitumor CTLs is high, with millions present per mouse. A titration of naive DUC18 T cells demonstrated that adoptive transfer of 12,000–30,000 tumor-specific cells was needed to protect 90% of recipient BALB/c mice from subsequent tumor challenge (39). We now find that in the presence of enhanced CD4\(^+\) Th cell function post-tERK-II vaccination, transfer of only 1000 naive DUC18 T cells is sufficient to protect 95% of BALB/c mice against CMS5 challenge.

Table I. Effects of titrated numbers of transferred tumor-specific DUC18 T cells on subsequent tumor growth

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Tumor-Positive Mice</th>
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<tbody>
<tr>
<td>PBS</td>
<td>10/10</td>
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<tr>
<td>1000 DUC18 cells alone</td>
<td>17/21</td>
</tr>
<tr>
<td>1000 DUC18 cells plus tERK-II peptide</td>
<td>1/20</td>
</tr>
<tr>
<td>100 DUC18 cells plus tERK-II peptide</td>
<td>4/10</td>
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<tr>
<td>10 DUC 18 cells plus tERK-II peptide</td>
<td>6/10</td>
</tr>
<tr>
<td>1000 DUC18 cells plus OVA (323–339) peptide</td>
<td>19/19</td>
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*Mice were untreated or immunized with peptide plus adjuvant, and 6 days later received DUC18 T cells or PBS, as shown. Twenty-four hours after T cell transfer, mice were challenged with CMS5. The numbers of mice bearing measurable tumors by 3 wk postchallenge are shown. Data represent cumulative totals from at least two independent experiments.*
Detailed examination of peptide vaccine-induced CD8+ T cell responses in clinical trials, without detecting a corresponding objective regression to vaccination-induced expansion of Ag-specific CD8+ T cell-mediated protective immunity only indirectly (Figs. 1B and 4A). CD4/CD40L interactions were required for the generation of CTL effector function (Figs. 3A and 4B), this mechanism is likely to be operative in our CD4-directed peptide vaccination study. CD4+ T cells may also regulate CTL development independently of CD40/CD40L interactions. Although less well defined, in vitro and in vivo studies have demonstrated that CD4 cells can trigger DC maturation through a CD40-independent process, and can stimulate CD8+ T cell effector function directly through cytokine production (49). Because the protective effects of tERK-II vaccination were completely ablated in the absence of CD40 (Fig. 3B), CD40-independent routes of CD4+ T cell help for CTL generation do not appear to have a significant role in our model system. We also observed that CD40/CD40L interactions impact the generation of CD4+ Th cell function, as evidenced by the decreased levels of IFN-γ-producing CD4+ T cells in CD40-/- mice (Fig. 4A and B). This finding is in agreement with previous data from the Flavell laboratory (47), which indicated that CD40/CD40L interactions were required for optimal priming of CD4+ T cells (47).

The current study focused on the ability of CD4-directed tERK-II vaccination to augment antitumor immunity through indirect effects on tumor-specific CD8+ T cells. However, tERK-II-primed CD4+ Th cells may also function as effector cells to eliminate tumors in a more direct manner. CD4+ T cells have been shown to reject MHC class II-deficient tumor challenges through IFN-γ production, which appeared to act on host cells rather than on the tumor cells themselves (28). Additionally, the existence of a rare population of MHC class I-restricted CD4+ T cells (55) has been previously reported. These CD4+ cytotoxic cells have been shown to display tumoricidal activity in an MHC class I-restricted manner (56, 57). Thus, although the CMS55 tumor lacks MHC class II expression, IFN-γ produced by CD4+ T cells activated in response to tERK-II immunization could have some tumoricidal activity. We did not test these possibilities experimentally, because we saw optimal tumor rejection only upon transfer of CD8+ DUC18 T cells into tERK-II-vaccinated mice (Fig. 5 and Table I). Therefore, our data support a model in which the primary function of CD4+ T cells is to provide help for tumor-specific CD8+ CTLs.

Several recent reports have illustrated the benefits of eliciting a CD4+ T helper function in the context of antitumor immunity. An experimentally isolated class II-restricted TAA was shown in a similar murine fibrosarcoma model to have tumor-protective activity when provided in adjuvant (30). Additionally, adoptive transfer of class II-restricted T cell clones was also recently shown to have antitumor efficacy in two murine models (29, 30), although the exact mechanism of such antitumor activity was not clear. Work done by Melief and colleagues (38) using linked CD4 and CD8 epitopes in a single long peptide illustrated that vaccination with this peptide could induce effector function in both cell populations, and could lead to the regression of large, established tumors. A series of studies conducted in patients with Her-2/neu-overexpressing tumors demonstrated that CD8-directed peptide vaccination resulted in CTL expansion that was short-lived, but not protective; however, vaccination with 15-aa peptides containing

FIGURE 6. tERK-II priming results in a more rapid and transient expansion of transferred naive Thy-1.1 DUC18 T cells in the spleen. BALB/c Thy-1.2 mice were immunized with tERK-II (circles, △) or left unimmunized (squares, □) on day −7. On day −1, 1000 naive Thy-1.1 DUC18 cells were transferred i.v. to Thy-1.2 BALB/c recipients. The following day (day 0), both groups were challenged s.c. with 3 × 10^6 CMS55 cells. On days 4, 6, and 8 postchallenge, the tumor-draining lymph nodes and spleens were individually harvested and analyzed from each animal. Cells were counted and then stained with V98.3 FITC/Thy-1.1 PE/CD8 Cy-Chrome to determine the number of transferred Thy-1.1 DUC18 T cells that had expanded in vivo at each time point. On day 4, there are four mice per group from two separate experiments; on day 6, there are eight mice per group from three separate experiments; and on day 8, there are 15 mice in the unimmunized group and 14 mice in the tERK-II-immunized group from three separate experiments.

(With respect to the reader's understanding of the content, the figures and tables are not included in the text. However, the figures and tables are integral to the comprehension of the content and should be reviewed accordingly.)
known MHC class II-specific sequences that encompassed putative MHC class I-binding motifs induced lytic effector cells that persisted in vivo for more than 1 year (6, 35). Interestingly, in cancer patients, vaccination with MHC class II-specific peptides led to priming of MHC class I-restricted CD8+ T cells (35). In our current study, we have also observed indirect priming of CD8+ effector cells by tERK-II vaccination, which was evident only following tumor challenge (Fig. 4, D and E).

Although tERK-II vaccination successfully boosted CD4+ T helper function, this was insufficient to completely protect mice from tumor challenge unless a threshold of pCTL had been reached. Endogenous CD8+ T cell priming was helped by Ag-specific CD4+ T cells in response to vaccination, and the resultant effectors were able to eliminate tumors in ~35% of BALB/c mice challenged with CMS5 postvaccination (Fig. 2). Our titration study indicated that transfer of tumor-specific DUC18 T cells protected 95% of mice from CMS5 challenge in our model (Fig. 5 and Table I). Transfer of this number of DUC18 T cells without vaccination conferred protection on only 19% of recipient mice (Table I). When we examined possible mechanistic explanations for this discrepancy, we found that tERK-II vaccination led to an augmented expansion of DUC18 T cells in the spleen by day 4 posttumor challenge with a rapid decline in T cell numbers by days 6 and 8. In comparison, unimmunized mice demonstrated a delayed and slightly lower level of expansion that remained stable on days 6 through 8 posttumor challenge (Fig. 6). Additionally, the decline in splenic DUC18 T cell numbers in tERK-II-immunized mice coincides with the times at which CMS5 tumor size begins to decrease (Fig. 5), suggesting that the T cells have homed more efficiently to the tumor site. Thus, CD4-directed tERK-II vaccination appears to boost antitumor immunity by both enhancing tumor-specific CD8+ T cell clonal expansion, and potentially augmenting T cell trafficking to the tumor site.

Peptide vaccination protocols designed to boost both CD4+ and CD8+ immune responses simultaneously should therefore provide the increased pCTL frequency necessary for therapeutic efficacy in the presence of established tumor loads. Our findings suggest that for TAA, in which CD4+ T cell epitopes are currently unknown, the extension of CD8+ T cell epitopes to allow for MHC class II binding could lead to enhanced success rates in clinical trials in which MHC class I-presented peptides have produced promising immune responses, but minimal objective tumor regression.

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References


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