Therapy of Established Tumors in a Novel Murine Model Transgenic for Human Carcinoembryonic Antigen and HLA-A2 with a Combination of Anti-idiotype Vaccine and CTL Peptides of Carcinoembryonic Antigen

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Abstract

Induction of potent and sustained antitumor immunity depends on the efficient activation of CD8+ and CD4+ T cells. Immunization using dendritic cells loaded with tumor antigens constitute a powerful platform for stimulating cellular immunity. Our previous studies suggested that vaccination with an anti-idiotype antibody 3H1, which mimics a specific epitope of carcinoembryonic antigen (CEA), has the potential to break immune tolerance to CEA and induce anti-CEA antibody as well as CEA-specific CD4+ T-helper responses in colon cancer patients as well as in mice transgenic for human CEA. Here, we have combined the anti-idiotype 3H1 with the CTL peptides of CEA to augment both T-helper and CTL responses in a clinically relevant mouse model, which is transgenic for both CEA and HLA-A2. We have evaluated the potential of two different HLA-A2–restricted epitopes of CEA pulsed into dendritic cells in a therapeutic setting. The overall immune responses and survival were enhanced in groups of mice immunized with agonist peptide for CEA691 (YMIGMLVGV)–pulsed dendritic cells or CAP1-6D (YLS-GADLNL, agonist peptide for CAP-1)–pulsed dendritic cells. Mice immunized with peptide-pulsed dendritic cells along with 3H1-pulsed dendritic cells resulted in significant increase in survival compared with mice immunized with peptide-pulsed dendritic cells alone (P < 0.02). IFN-γ ELISPOT and ⁵¹Cr-release assays showed that HLA-A2–restricted, CEA-specific CTL responses were augmented by combined dendritic cell vaccinations. The combined vaccination strategy resulted in increased antigen-specific proliferation of splenocytes and secretion of Th1 cytokines by CD4+ T cells that correlated with increased survival. These results suggest the potential use of this vaccination strategy for future clinical applications. [Cancer Res 2007;67(6):2881–92]

Introduction

CD8+ CTLs are a major component of antitumor immune responses. CTL responses have been shown to be effective in the elimination of tumors in a number of experimental models (1). CTLs can directly lyse tumor cells and also secrete cytokines such as interleukin 2 (IL-2), tumor necrosis factor, granulocyte macrophage colony-stimulating factor, and IFN-γ, which likely have indirect antitumor effects. CTLs constitute a major component of tumor-infiltrating lymphocytes. These cells have been associated with spontaneous tumor regression in humans (2). Adoptive transfer experiments in humans have also shown the efficacy of antitumor CTLs (3). Clinical trials of cancer patients have shown that epitope-specific CTLs can be induced in these patients, and their induction correlated, in multiple instances, with partial or complete tumor responses (4–6). Hence, vaccination with antigens recognized by tumor-specific CTLs represents an effective strategy for cancer immunotherapy.

CD4+ T cells are required for the generation and maintenance of CTLs (7) and are generally believed to be essential for the generation of both cellular and humoral antitumor immune responses (8–11). However, the contribution of CD4+ T cells to the development, expression, and maintenance of antitumor immunity is still the subject of intense investigation. Using active-specific immunotherapy, several studies have shown that CD4+ T cells play a critical role in the development of a therapeutic antitumor immune response (8, 11, 12). In addition, CD4+ T cells are essential for generating CD8+ T memory cells (13, 14). Therefore, CD4+ T cells are central to many significant aspects of antitumor immunity, and their efficient activation is likely to be a key element for cancer vaccine efficacy.

Development of effective cancer vaccines relies not only on the identification of target antigens, but approaches to deliver these antigens to generate immunity as well. Dendritic cells represent unique antigen-presenting cells that are capable of sensitizing T cells to both new and recall antigens (15, 16). In recent years, several groups have explored the use of dendritic cells loaded in vitro with tumor-associated antigens (TAA) as therapeutic vaccines (17). Dendritic cell vaccination has produced promising results primarily in patients with immunogenic tumors; however, little success has been observed in poorly immunogenic tumors such as colorectal and lung cancer.

We have chosen carcinoembryonic antigen (CEA) as an index antigen for evaluating immune responses because of its low expression on normal colon epithelium and overexpression on epithelial tumors, such as lung, colon, gastric, and breast carcinomas (18). Because of its unique expression pattern, CEA has been targeted as a TAA to induce CEA-specific antibodies, CD4+ T-helper cells, and CD8+ CTLs to treat CEA+ tumors (19). Our previous studies suggest that murine monoclonal anti-idiotype antibody 3H1 (20) can be used as a surrogate antigen for CEA and

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induced anti-CEA immunity in CEA-transgenic mice (21–23) as well as in humans (reviewed in ref. 24). 3H1 showed promise as a potential vaccine candidate in the phase I/II clinical trials of colon cancer patients. Anti-idiotypic 3H1 could easily break immune tolerance to CEA and induced high-titer, anti-CEA antibody as well as CD4+ T-helper (Th1) responses in mice and cancer patients (21, 22, 24).

The objective of the present study was to assess the ability of dendritic cell–based vaccine consisting of CTL peptide of CEA in combination with 3H1 as a potential vaccine candidate to induce antitumor immunity in a clinically relevant mouse model that expresses both CEA and HLA-A2. To this end, two different HLA-A2–restricted epitopes were selected: CEA_H1 (YMGMLGVY) or CEA_H2 (YLSGANLNL) synthesized by Synthetic Biomolecules (San Diego, CA) with a purity of 50 to 100%.

Materials and Methods

Mice and cell lines. C57BL/6-CEA and C57BL/6-CEA2Kb homozogous transgenic (Tg) breeder mice were obtained from Dr. F. James Primus (Vanderbilt University Medical Center, Nashville, TN) and were maintained as described previously (27, 28). C57BL/6-CEA-A2Kb double-transgenic mice were generated in the animal facility of the University of Cincinnati Medical Center by crossing male C57BL/6-CEA-Tg with female C57BL/6-CEA2Kb-Tg mice. The genotype of CEA-A2Kb double-transgenic mice was confirmed by PCR using mouse-tail DNA as described previously (27, 28). The HLA-A2 expression on the cell surface was also confirmed by flow cytometry after staining with FITC-conjugated anti–HLA-A2 monoclonal antibody (mAb, clone B7/2; BD Biosciences, San Diego, CA). For in vivo studies, all mice were used at 6 to 8 weeks of age. Mice were treated in accordance with Institutional Animal Care and Use Committee guidelines.

MC-38-CEA-A2Kb cells were generated by transfecting murine colon carcinoma cell line MC-38-CEA (27) with a plasmid encoding A2Kb. This double-transfected cell line was also provided by Dr. F. James Primus. These MC-38-CEA-A2Kb cells were maintained in culture with 500 μg/mL G418 and 200 μg/mL xeozin (Invitrogen, Grand Island, NY). T2, a human HLA-A2+ cell line, was kindly provided by Dr. Walter J. Storkus (University of Pittsburgh School of Medicine, Pittsburgh, PA).

Synthetic peptides. CEA_H1 (YMGMLGVY, peptide 1), agonist peptide for CEA_H1 (YMGMLGVY, peptide 2), agonist peptide for CEA_H2 (YLSGANLNL, peptide 3), CAP-1 (YLSGANLNL, peptide 4), agonist peptide for CAP-1 (YLSGANLNL, peptide 5), and HIV-1 gp160 peptide 6 (KTLPCVTL) were synthesized by Synthetic Biomolecules (San Diego, CA) with a purity of >90%. A2-restricted HIV-1 gp160 peptide 6 was used as a control.

Generation of dendritic cells. Dendritic cells were generated from bone marrow as described previously (21). On day 9 of culture, dendritic cells were incubated with 3H1 or peptide for 6 to 8 h at 37°C, in the presence of 50 to 100 μg/mL of antigen. After loading, dendritic cells were extensively washed in PBS and were injected into syngeneic C57BL/6-CEA2Kb mice for immunization.

Tumor therapy studies. C57BL/6-CEA2Kb mice were transplanted with 5 × 106 of MC-38-CEA2Kb colon carcinoma cells s.c. in the lower left flank. This dose of tumor cells is lethal in 100% of mice within 5 to 6 weeks after transplant if left untreated. Seven days after tumor transplant, when tumors were palpable (4–5 mm in diameter), mice were randomly divided into several groups (8–12 mice per group) for immunizations. Immuno-therapy was started on day 7 and repeated on days 9, 11, 14, 16, and 18 (Fig. L4) by injecting peptide-pulsed dendritic cells (2 × 106–3 × 106 cells) and/or 3H1-pulsed dendritic cells (2 × 105–3 × 105 cells) s.c. in the lower right flank. Group of mice immunized with unpulsed dendritic cells (2 × 105–3 × 106 cells) served as control. Mice were monitored twice weekly for tumor growth and survival and were sacrificed when tumors reached a diameter >20 mm; survival was recorded accordingly. In our previous studies in naïve and transgenic mice, we did close kinetic experiments using different numbers of 3H1-pulsed dendritic cells or peptide-pulsed dendritic cells (1 × 106–6 × 105 cells per injection) to induce antigen-specific immune response in vivo. Our results suggested that 2 × 105 pulsed-dendritic cells per immunization were sufficient to induce optimum antigen-specific immune response. Based on these data, in the present study, we have selected the dose of 2 × 105 to 3 × 105 antigen-pulsed dendritic cells per immunization.

In vitro cytotoxicity assay. Assays were done according to the standard protocols (29). Lymphocytes were isolated from harvested spleen of three mice per group 5 days after the final vaccination, and these cells (2 × 107/mL) were stimulated by coculture with dendritic cells (2 × 105/mL) pulsed with immunizing peptide and/or dendritic cells (2 × 105/mL) pulsed with 3H1 along with 20 IU/mL recombinant human IL-2 (Sigma, St. Louis, MO). On day 5, these in vitro stimulated cells were used as CTL effector cells, and the CTL activity was determined by a standard 6 h 31Cr-release cytotoxicity assay using a variety of target cell lines. Assays were done in triplicate wells at different effector to target (E/T) ratios as indicated in the figures. Spontaneous release was always <25% of maximum release. For inhibition experiments, CTL activity was tested in the presence of anti-mouse CD8 mAb (clone 53-6.7), anti-mouse CD4 mAb (clone H129.19), anti-mouse H-2Kb/H-2Db (clone 28-8-6), anti-mouse I-Aβ mAb (clone KH74), or anti-human HLA-A2 mAb (clone B7/2). Isotype-matched mAbs were used as control. Antibodies used in inhibition experiments were purchased from BD Biosciences and were used at a final concentration of 10 μg/mL.

In selected experiments, cells obtained from antigen-specific CTL culture were first incubated with anti-mouse CD8 mAb or anti-mouse CD4 mAb conjugated to magnetic microbeads, washed, and separated by magnetic-activated cell sorting separation columns by positive selection methods according to the manufacturer’s specifications (Miltenyi Biotec, Auburn, CA). All samples were passed through the columns twice and were >90% pure as determined by flow cytometry. The purified CD8+ or CD4+ T cells were used as effector cells in CTL assay.

IFN-γ ELISPOT assay. CEA-specific and peptide-specific immune responses were also evaluated in ELISPOT assays. Briefly, splenocytes were isolated from different groups of immunized mice 5 days after the final vaccination. After lysis of the RBC with RBC lysis buffer (Sigma), splenocytes (2 × 106) were cultured in medium with or without stimulator cells (2 × 105) at 37°C in 96-well ELISPOT plates for 18 to 20 h. The assay was done in triplicate wells and developed according to the manufacturer’s instructions (BD Biosciences). In selected experiments, immunized mice splenocytes (2 × 106) were evaluated for their cellular avidity to the respective peptides by measuring IFN-γ spot in ELISPOT assay in the presence of titrated peptide pulsed on T2 cells (2 × 105).

In vitro proliferation and cytokine ELISA. T-cell proliferation was measured by 3H-thymidine incorporation as described previously (21). Dendritic cells pulsed with immunizing peptide or dendritic cells pulsed with purified human CEA ( Fitzgerald Industries, Concord, CA) were used as stimulator cells. Assays were done in triplicate wells. Cell-free supernatants were harvested and analyzed for the production of IFN-γ, IL-2, IL-4, and IL-10 by using ELISA kits (R&D Systems, Minneapolis, MN) as described previously (21). All samples were tested in triplicates.

In vivo depletion of immune cell subsets. For antibody depletion experiments, mAb against CD4 (clone GK1.5), mAb against CD8 (clone 2.43), and mAb against NK-1.1 (clone PK136) were used. Hybridomas were grown in nude mice, and collected ascitic fluids were purified using protein G columns and were adjusted to 1 mg/mL in PBS. C57BL/6-CEA2Kb mice were transplanted with MC-38-CEA2Kb tumor cells on day 0. Therapy was started on day 7 in groups of mice with vaccine consisting of peptide-5–pulsed dendritic cells plus 3H1-pulsed dendritic cells as described above.
Mice were depleted of CD4+ and/or CD8+ T cells or natural killer (NK) cells by i.p. injections of 150 μg of antibody per mouse 1 day before each immunization and then once weekly until the end of the study. Rat IgG (Sigma) was used as control antibody. The efficiency of depletion was always >90% as determined by flow cytometry.

Rechallenge experiment and adoptive transfer experiment. C57BL/6J-CEA-A2Kb mice were challenged with MC-38-CEA-A2Kb tumor cells s.c. on day 0, and therapy was initiated on day 7 with peptide-5–pulsed dendritic cells plus 3H1-pulsed dendritic cells as described above. Tumor-free mice were used for rechallenge experiments and adoptive transfer experiments after 13 weeks of post–tumor challenge. For rechallenge experiments, mice were transplanted either with MC-38-CEA-A2Kb (5 × 10^5), MC-38 (5 × 10^5), or EL4 (1 × 10^6) tumor cells s.c. Mice bearing 7-d established tumors were divided into several treatment groups and immunized as indicated. Pooled results of four experiments. D, CEA-A2Kb-Tg mice were transplanted with 5 × 10^6 of nontransfected parental MC-38 cells s.c. Mice bearing 7-d established tumors were divided into several treatment groups and immunized as indicated. Pooled results of two experiments.

Statistical analysis. Statistical analyses were done by Student’s two-tailed *t* test or nonparametric Mann-Whitney rank-sum test using SigmaStat software (Jandel, San Rafael, CA). Survival data were plotted using the method of Kaplan-Meier and were analyzed by Fisher exact test at different time points. The data are presented as means ± SD. *P* < 0.05 was considered as significant.

Results

Generation of C57BL/6J-CEA-A2Kb double-transgenic mice. Mice expressing human CEA as a transgene have provided a potential preclinical model to assess the induction of anti-CEA immune responses (27, 30). Several human CEA-specific, HLA-A2–restricted epitopes have been identified. Therefore, our objective was to determine whether CEA-specific antigenic epitopes can be presented on human MHC class I molecules and induce CEA-specific CTL responses in a mouse model. HLA-A2-Tg mice express a1 and a2 domains of the human HLA-A2.1 molecule and therefore provide a critical preclinical screening model to develop human vaccine.

Several reports suggest that HLA-A2-Tg mice were effective for studying efficacy of human antigens and the immune response
directed against them (31–33). In this study, CEA-Tg mice were crossed with HLA-A2-Tg mice to generate CEA-A2Kb double-transgenic mice and offspring were screened for transgene DNA in the tail tissues of individual mice using PCR. PCR products indicated that F1 offspring, derived from several breeding pairs, expressed both CEA and HLA-A2 transgenes (data not shown).

Combined vaccination with known CTL epitope of CEA along with an anti-idiotype antibody mimicking CEA, resulted in increased survival of tumor-bearing CEA-A2Kb transgenic mice. Dendritic cells, the most potent antigen-presenting cells capable of priming naïve T cells to specific antigen in an HLA-restricted fashion, have been shown to induce protective T-cell-mediated immunity in tumor-bearing mice (34, 35). A study was initiated to determine the therapeutic potential of different CTL peptides pulsed into dendritic cells. Two agonist peptide for CEA_{a91} and agonist peptide for CAP-1 (CAP1-6D) were included in our studies because they were reported as more antigenic than original peptide (33, 36). In these studies, MC-38-CEA-A2Kb cells, which expressed CEA and HLA-A2, were injected s.c. into CEA-A2Kb-Tg mice on day 0. Seven days after tumor transplant, when tumors were palpable, vaccination was initiated. In our preliminary studies, mice immunized thrice (on days 7, 10, and 14) with peptide-pulsed dendritic cell–immunized group of mice was compared with other vaccinated groups.

![Figure 2](cancerres.aacrjournals.org)
tumor size was considerably reduced in experimental groups of mice compared with control group of mice (data not shown). Accumulating data from several experiments suggest that tumor regressed completely in 12.5% and 25% of the mice and those mice remained tumor-free until the end of the experiment when immunized with peptide-2– or peptide-5–pulsed dendritic cells, respectively (Fig. 1B). The difference in survival was significant compared with control group of mice \( (P < 0.02, \text{peptide } 2; P < 0.01, \text{peptide } 5) \).

We wanted to evaluate whether inclusion of CD4+ T-cell help would improve CEA-specific T-cell responses that would increase the overall survival of immunized mice. To this end, using the same vaccination schedule (Fig. 1A), mice were immunized with peptide-pulsed dendritic cells and/or 3H1-pulsed dendritic cells and the difference in survival was compared. Accumulating data from several experiments suggest that tumor regressed completely in 50% (14 of 28) and 67.5% (27 of 40) of the mice and those mice remained tumor-free for more than 90 days when immunized either with peptide-2–pulsed dendritic cells plus 3H1-pulsed dendritic cells or peptide-5–pulsed dendritic cells plus 3H1-pulsed dendritic cells, respectively, and the difference in survival was significant \( (P = 0.004 \text{ and } 0.003, \text{respectively}) \) compared with control group of mice (Fig. 1C). Whereas complete tumor regression was observed in only 10% of the mice (2 of 20) and remained tumor-free until the end of the study when immunized either with peptide-4–pulsed dendritic cells plus 3H1-pulsed dendritic cells, or 3H1-pulsed dendritic cells alone. Survival was significantly enhanced by inclusion of 3H1-pulsed dendritic cells in the vaccine for peptide-2 \( (P < 0.04) \), peptide-3 \( (P < 0.03) \), and peptide 5 \( (P < 0.003) \) on week 9 after tumor challenge. Increased survival was also present on week 13 for peptide-2 \( (P < 0.02) \) and peptide 5 \( (P < 0.003) \) when experiment was terminated (Fig. 1C). However, groups of mice transplanted with nontransfected parental MC-38 cells and immunized with peptide-pulsed dendritic cells and/or 3H1-pulsed dendritic cells were not protected from tumor growth and all mice died within 42 days (Fig. 1D).

### Induction of CD4+ and CD8+ T-cell responses in immunized mice.

To detect the induction of CTL response in vivo, immune spleen cells were stimulated in vitro with the corresponding peptide used in immunization, and CTL activity was measured against the immunizing peptide-pulsed T2 cells as targets in standard \( ^{51} \text{Cr} \)-release assays. Effector cells obtained from mice

![Figure 3](image-url)

**Figure 3.** The lysis of target cells was mediated by CD8+ T cells and was HLA-A2 restricted. Mice were immunized with peptide-5–pulsed dendritic cells along with 3H1-pulsed dendritic cells, and CTLs were generated from splenocytes as described in Materials and Methods. Lytic activity of CTLs was tested against MC-38-CEA-A2Kb cells (A–C) or peptide-5–pulsed T2 cells (D) by \( ^{51} \text{Cr} \)-release assays. The 6 h cytotoxicity assays were done in the presence of neutralizing mAb or isotype-matched control (10μg/mL) as indicated (A and B). In selected experiments, CD4+ and CD8+ T cells were purified from antigen-specific CTL culture by magnetic-activated cell sorting and were used as effector cells (C and D). Columns, mean of three individually analyzed mice per group; bars, SD. Representative of three independent experiments.
vaccinated with peptide-pulsed dendritic cell exhibited lysis of corresponding peptide-pulsed T2 cells; however, the lysis of target cells was most effective in peptide-5–pulsed dendritic cells vaccinated group at an E/T ratio of 50:1 (Fig. 2A). The differences in percentage of positive cells were not significant for surface expression of HLA-A2 when T2 cells were pulsed with different peptides at same concentration and analyzed by flow cytometry (data not shown). Thus, the differences in CTL activities are not due to the difference in binding abilities of the peptides to T2 cells. The extent of lysis of peptide-pulsed T2 cells increased significantly in the groups of mice treated with combined vaccines, and effector cells obtained from mice immunized with peptide-5–pulsed dendritic cells plus 3H1-pulsed dendritic cells proved to be the most effective (Fig. 2B). The difference in lysis was significant compared with other vaccinated groups at all E/T ratios tested (P < 0.05). Because both mouse effector cells and human T2 target cells only share HLA-A2, the observed cytotoxicity is expected to be HLA-A2 restricted. Irrespective of vaccination, the use of A2-restricted HIV-1 gp160120–128 peptide-pulsed T2 cells as target resulted in background lysis (e.g., at 50:1 ratio, lysis was <5%; data not shown).

To measure the induction of CEA-specific CTL responses, we used MC-38-CEA-A2Kb as target cells. Irrespective of vaccination, splenocytes from peptide-pulsed dendritic cell–immunized mice were able to lyse the target cells, and maximum lysis was observed in peptide-5–pulsed dendritic cell–immunized group (Fig. 2C). However, the extent of lysis increased significantly when splenocytes obtained from mice treated with combined vaccines were considered (Fig. 2D). CTLs from mice that received vaccination with peptide-5–pulsed dendritic cells plus 3H1-pulsed dendritic cells proved to be the most effective, inducing up to 50% to 60% lysis of the target cells expressing CEA (Fig. 2D). Moreover, the difference in lysis was significant compared with other vaccinated groups at all E/T ratios tested (P < 0.03) except for the group immunized with peptide-2–pulsed dendritic cells plus 3H1-pulsed dendritic cells (P > 0.06). Tumor cell lysis was antigen-specific,
because the use of MC-38 parental tumor cells lacking CEA expression as target resulted in background lysis (Fig. 2E and F).

Next, we determined the effector cell phenotype responsible for specific killing of target cells. In these experiments, splenocytes obtained from mice immunized with peptide-pulsed dendritic cells or peptide-pulsed dendritic cells plus 3H1-pulsed dendritic cells, and ELISPOT assays were done in the presence of T2 cells pulsed with peptide-1 (○, †) or peptide-2 (●, ‡; E); or T2 cells pulsed with peptide-4 (○, ●; or peptide-5 (○, ●, ‡; F). The numbers of IFN-γ spots developed in the presence of unpulsed T2 cells were deduced from values obtained in the presence of peptide-pulsed T2 cells. G, proliferation of splenic T cells in response to CEA-pulsed or immunizing peptide–pulsed dendritic cells was measured by ([3H]thymidine incorporation. H, CEA-pulsed dendritic cell–stimulated culture supernatants were collected from T-cell proliferation experiments and analyzed to detect the presence of IFN-γ and IL-2 by quantitative ELISA. A to H, representative of three independent experiments.

Figure 4 Continued. E to F, the development of IFN-γ spot in the presence of titrated peptide pulsed on T2 cells. Splenocytes were harvested from mice immunized with peptide-pulsed dendritic cells or peptide-pulsed dendritic cells plus 3H1-pulsed dendritic cells, and ELISPOT assays were done in the presence of T2 cells pulsed with peptide-1 (○, †) or peptide-2 (●, ‡; E); or T2 cells pulsed with peptide-4 (○, ●; or peptide-5 (○, ●, ‡; F). The numbers of IFN-γ spots developed in the presence of unpulsed T2 cells were deduced from values obtained in the presence of peptide-pulsed T2 cells. G, proliferation of splenic T cells in response to CEA-pulsed or immunizing peptide–pulsed dendritic cells was measured by ([3H]thymidine incorporation. H, CEA-pulsed dendritic cell–stimulated culture supernatants were collected from T-cell proliferation experiments and analyzed to detect the presence of IFN-γ and IL-2 by quantitative ELISA. A to H, representative of three independent experiments.

The functional avidity of T cells was evaluated by the detection of IFN-γ spot in response to coculture with T2 cells pulsed with diminishing amounts of immunizing agonist peptide or the corresponding original peptide. The avidity of CD8 T cells producing IFN-γ spot in response to the original peptide (peptide-1 or peptide-4) was significantly lower even at high concentration of peptide when splenocytes obtained from mice immunized with peptide-2–pulsed dendritic cells or peptide-5–pulsed dendritic cells were present in immunized mice, and the number of IFN-γ–secreting cells increased significantly in groups of mice immunized with peptide-2–pulsed dendritic cells plus 3H1-pulsed dendritic cells or peptide-5–pulsed dendritic cells plus 3H1-pulsed dendritic cells (Fig. 4C). The use of peptide-pulsed dendritic cells or CEA-pulsed dendritic cells as stimulant resulted in significant increase of IFN-γ–secreting cells (Fig. 4B) and the numbers increased further in groups of mice treated with combined vaccines (Fig. 4D). CEA-specific, IFN-γ–secreting immune cells were significantly higher in mice immunized with peptide-5–pulsed dendritic cells or mice immunized with peptide-5–pulsed dendritic cells plus 3H1-pulsed dendritic cells compared with other vaccinated groups (P < 0.01).
were considered for analysis, respectively (Fig. 4E and F). However, the avidity of CD8 T cells producing higher number of IFN-γ spot in response to low concentration of agonist peptide (1 μg/mL) was obtained from mice immunized with peptide-2–pulsed dendritic cells (Fig. 4E) or peptide-5–pulsed dendritic cells (Fig. 4F). The response was better in the peptide-2–pulsed dendritic cell group compared with mice immunized with peptide-5–pulsed dendritic cells. The IFN-γ production in response to the immunizing agonist peptide increased further in groups of mice coimmunized with 3H1-pulsed dendritic cells (Fig. 4E and F). These data suggest that inclusion of CD4 help might have increased the overall avidity of CD8 T cells for peptide, which resulted in better protection against CEA-expressing tumors.

Taken together, ELISPOT and cytotoxicity assay data clearly suggest that peptide-pulsed dendritic cell vaccination induced CEA-specific, HLA-A2–restricted CTL responses in vivo, and the CTL activities were augmented by anti-idiotype 3H1. The lytic activities correlated with survival of immunized mice.

Splenocytes were also analyzed for in vitro proliferation in the presence of CEA-pulsed dendritic cells or immunized peptide-pulsed dendritic cells as stimulants (Fig. 4G), and culture supernatants were used for cytokine analysis quantitatively (Fig. 4H). Although peptide-specific proliferation by splenic lymphocytes did not vary considerably among different groups, CEA-specific proliferation was significantly higher in the group of mice immunized with peptide-2–pulsed dendritic cells plus 3H1-pulsed dendritic cells (P < 0.04) compared with other vaccinated groups (Fig. 4G). The analyses of cytokine production data using CEA-pulsed dendritic cells stimulated culture supernatants suggest that higher levels of IFN-γ and IL-2 were produced (P < 0.001) by immune splenocytes obtained from mice immunized with peptide-5–pulsed dendritic cells plus 3H1-pulsed dendritic cells (Fig. 4H), which correlated with increased survival. Flow cytometric analysis of intracellular staining suggested that both CD4 and CD8 T cells were positive for cytokine secretion (data not shown). Irrespective of vaccination, the production of Th2-associated cytokines IL-4 and ...
IL-10 were significantly lower compared with Th1-associated cytokines (data not shown).

Effects of in vivo depletion of CD4+ and CD8+ T cells in antitumor therapy mediated by peptide-5–pulsed dendritic cells plus 3H1-pulsed dendritic cells. Next, we characterized the subpopulation of cells responsible in the in vivo antitumor therapy after vaccination with peptide-5–pulsed dendritic cells plus 3H1-pulsed dendritic cells. Mice were depleted of CD4+ and/or CD8+ T cells or NK cells by mAbs as described. Antibodies were administered during and after the vaccination regimen to ensure continued depletion of the relevant cell populations. Successful depletion of >90% was confirmed by flow cytometry (Fig. 5A). Figure 5B shows individual tumor measurements on day 35 after tumor challenge. Tumor grew rapidly in the group of mice depleted of CD8+ T cells and tumor growth was even faster in mice depleted of both CD4+ and CD8+ T cells (P < 0.02). All those mice were dead within 5 to 6 weeks of tumor challenge (Fig. 5C), and the difference in survival was significant compared with nondepleted group of mice or mice depleted with control antibody (P = 0.004 and 0.003, respectively). The tumor growth was relatively slower in mice depleted of CD4+ T cells or NK cells alone (Fig. 5B). Although tumor-free survival was reduced in mice depleted of CD4+ T cells or NK cells, the difference in survival was not significant compared with nondepleted group of mice (P = 0.34 and 0.41, respectively).

A similar trend was observed in the group of mice treated with peptide-2–pulsed dendritic cells plus 3H1-pulsed dendritic cells (data not shown).

Memory response induced in mice that have been cured of MC-38-CEA-A2Kb tumors. The MC-38-CEA-A2Kb tumor growth was completely eradicated in 67.5% of the mice, which received vaccination with peptide-5–pulsed dendritic cells plus 3H1-pulsed dendritic cells and survived for more than 90 days without any relapse of tumor (Fig. 1C). To test whether immunologic memory was developed in these tumor-free mice, they were subsequently rechallenged with the same tumor cells used for the first challenge or with other syngeneic tumor cells. Mice cured of MC-38-CEA-A2Kb tumors rejected subsequent challenges with the same tumor or nontransfected parental MC-38 cells and remained tumor-free until the end of the experiment, whereas mice could not reject syngeneic unrelated tumor EL4 (Fig. 6A). These results indicate that the tumor protection was mediated by persistent antitumor immunity specific for antigens relevant to MC-38-CEA-A2Kb tumor. Interestingly, tumor-free mice also had protective immunity against subsequent challenge with the parental MC-38 cells, suggesting that mice rejecting CEA-transfected colon carcinoma developed immunity to other antigens expressed on MC-38-CEA-A2Kb cells and "shared" with the nontransfected parental MC-38 cells, resulting in long-lasting memory against these tumors.

Figure 6. Immunized mice that had eradicated MC-38-CEA-A2Kb tumor growth developed tumor-specific, long-lived memory responses. Group of mice bearing 7-d established tumors were immunized with peptide-5–pulsed dendritic cells plus 3H1-pulsed dendritic cells, and 67.5% of these mice eradicated the tumor growth completely and survived for more than 90 d. These mice were used for rechallenge experiments and adoptive transfer experiments. Surviving mice were rechallenged either with MC-38-CEA-A2Kb, nontransfected parental MC-38, or syngeneic unrelated EL4 tumor cells s.c. 13 wk after first tumor inoculation (A). A set of age-matched naive CEA-A2Kb-Tg mice were also challenged either with MC-38-CEA-A2Kb, MC-38, or EL4 tumor cells and used as controls (B). To perform the adoptive transfer experiment, antigen-specific lymphocytes were generated from tumor-free mice at 13 wk of post–tumor challenge as described, and CD4+ and CD8+ T cells were purified by magnetic-activated cell sorting. The purity of T cells was confirmed by flow cytometry (C). Stimulated cells (4 × 10^6) were infused i.v. into naive mice; on the next day, mice were challenged with MC-38-CEA-A2Kb tumor cells (5 × 10^5) s.c. Tumor growth (D) and survival (E) were recorded over time. A to E, representative of two independent experiments.
Induction of immunity to shared tumor antigens has also been observed in other studies during tumor rejection after immunization with target antigen (37, 38). All control CEA-A2Kb-Tg mice that were previously untreated died of progressive tumor growth, irrespective of challenge with MC-38-CEA-A2Kb, MC-38, or EL4 cells (Fig. 6D).

Finally, adoptive transfer experiment was done to determine whether this immunologic memory could be transferred into naïve CEA-A2Kb-Tg mice. Recipient mice were challenged with MC-38-CEA-A2Kb tumor cells 1 day after the adoptive transfer of in vitro stimulated splenocytes. As shown in Fig. 6D and E, adoptive transfer of total splenocytes (>85% cells were positive for CD4 and CD8 T cells) from tumor-free mice resulted in tumor protection in 100% of the mice, and these mice remained tumor-free until the end of the experiment (P < 0.006). On the other hand, tumor developed rapidly in control group of mice adoptively transfused with total splenocytes from naïve CEA-A2Kb-Tg mice (Fig. 6D), and all of these mice died within 6 weeks of tumor challenge (Fig. 6E). Adoptive transfer with purified CD4+ or CD8+ T cells (Fig. 6C) resulted in partial protection over the control group of mice (Fig. 6D and E). These data suggest that both tumor antigen–specific memory CD4+ and CD8+ T cells could play an important role in the control of tumor growth in this murine model of colon carcinoma.

Discussion

CTLs are an important effector arm in the antitumor immune response, and the induction of potent CTL responses has been one of the major goals in developing immunotherapeutic strategies for cancer (39). Accumulating evidence, however, suggests that CD4+ T-cell response also plays a key role in tumor immunity (8, 11). CD4+ T cells provide important functions for the induction, expansion, and persistence of CD8+ CTLs (40). Secretion of effector cytokines such as IFN-γ by CD4+ T cells sensitizes tumor cells to CTL lysis via up-regulation of MHC class I molecules, stimulates the innate arm of the immune system at the tumor site, and, as recently suggested, inhibits local angiogenesis (41). The importance of the CD4+ T-cell response in tumor immunity was highlighted in murine studies showing that CD4+ T cells can eradicate tumor in the absence of CD8+ T cells (42, 43) or constitute the dominant effector arm in the antitumor response (10). Therefore, an optimal antitumor immune response may require the concomitant activations of both CD4+ and CD8+ T cells.

We have previously reported that mice immunized with 3H1-pulsed dendritic cells induced CEA-specific CD4+ and CD8+ T-cell responses and were protected from CEA-expressing tumor growth in the prophylactic setting (21, 23, 44). We have also identified a peptide from 3H1 (designated LCD-2) that induced CTLs recognizing CEA-positive colon carcinoma cells in a MHC class I–restricted fashion (23). These data suggest that dendritic cells pulsed with 3H1 lead to epitope presentation through both MHC class I and class II. In the current study, we wanted to combine 3H1 with appropriate CTL epitopes of CEA in an attempt to develop an effective vaccine against CEA-expressing colon carcinoma in a therapeutic setting. It is our hypothesis that the combination of vaccines that will elicit both tumor-specific CTL and T-helper responses will further augment antitumor immune responses.

In clinical practice, most candidates for cancer therapy are patients with existing tumor burdens. Therefore, we must direct our questions of therapeutic efficacy to the elimination of well-established tumors in experimental animal models. In this study, we selected to work with 7-day well-established MC-38-CEA-A2Kb tumors (4–5 mm in diameter) with well-developed vasculatures. Our results show that to generate antitumor immunity in CEA-A2Kb-Tg mice, the application of multiple immunizations is required. In these experiments, dendritic cells were used as a means to deliver and present antigen to naïve T cells and mice were immunized with peptide-pulsed dendritic cells (2 × 10^5–3 × 10^6 cells) and/or 3H1-pulsed dendritic cells (2 × 10^5–3 × 10^6 cells). In a pilot experiment, mice were immunized either with peptide-pulsed dendritic cells (4 × 10^5 cells), 3H1-pulsed dendritic cells (4 × 10^5 cells), or peptide-pulsed dendritic cells (2 × 10^5 cells) plus 3H1-pulsed dendritic cells (2 × 10^5 cells) using the same vaccination protocol (Fig. 1A) in a therapeutic setting. Our data suggested that there was no noticeable difference in immune responses or survival of immunized mice, which received twice the amount of peptide-pulsed dendritic cells or 3H1–pulsed dendritic cells compared with other experiments (data not shown). Within this range, we did not detect any statistically significant relationship between increased dendritic cell dose and immune response or tumor protection, suggesting that antigen-presenting cell dose may not be the limiting factor. In a phase I dendritic cell dose escalation trial, patients received increasing doses of CEA-derived peptide-pulsed dendritic cells ranging from 10^5 to 10^6 cells per injection and the immune or clinical responses did not depend on dendritic cell doses used (45). We have found a clear beneficial effect in mice immunized with agonist peptide for CEA_{691}^(_YMYGMLGV, peptide 2) or mice immunized with agonist peptide for CAP-1 (YLSGADLN, peptide 5) along with 3H1. This trend toward greater protection was correlated with greater in vitro cytotoxic activity, larger numbers of IFN-γ-producing cells in ELISPOT assays, higher CEA-specific T-cell proliferation, and increased levels of type 1 cytokine secretion. Anti-idiotypic antibody 3H1 might have stimulated CD4+ T cells to provide the critical help to CD8+ T cells, and, as a consequence, stronger antitumor responses were observed. It is obvious that CD8+ T cells are the major effector cells in this model because antigen-specific CTL activity was detected in vitro and in vivo depletion of CD8+ T cells abrogated the tumor protective immunity. Recent data suggest that CD4+ T cells not only provide help to CD8+ T-cell activation but also enhance the ability of effector cells to infiltrate tumors and are responsible for maintaining the pool of antigen-specific activated CD8+ T cells conferring antitumor protection (10, 46).

Recently, Zhou et al. (47) reported that CEA-A2Kb-Tg mice immunized with CAP1-6D-DNA minigene vaccine failed to induce a CAP1-6D-specific CTL response. As the authors suggested, CAP1-6D-DNA minigene vaccine itself may be insufficient to induce such response. We have shown several lines of evidence that dendritic cell–based vaccination resulted in induction of CAP1-6D–specific CTL responses and combined vaccination strategy augmented CTL responses, which resulted in the in vivo tumor protection. Our results are in accordance with two recent publications suggesting the development of CTL response specific for CAP-1 in HLA-A2-Tg and CEA-A2Kb-Tg mice (48, 49), and it has already been documented that CAP1-6D is more antigenic than CAP-1 (36).

Presumably, the development of long-lived immunologic memory was not only dependent on the CEA antigen but likely also on other unidentified antigens of MC-38-CEA-A2Kb tumors. Immune responses to additional undefined shared tumor antigens should augment antitumor immunity induced using defined antigens. Similarly, it may also be possible to induce immunotherapeutic
responses by immunization of tumor-bearing hosts with dendritic cells pulsed with a potent peptide antigen and subsequent immunization with their own killed tumor cells that have been transfected with the antigen gene. This approach may be particularly significant for patients with tumors whose tumor rejection antigens have not yet been defined.

Nonetheless, the ability to transfer immunity by the infusion of splenocytes indicates the presence of antigen-specific T cells in tumor-protected mice. The adoptive transfer experiment suggests that both CD4+ and CD8+ T cells are required to achieve tumor protection. It is possible that the cytokines produced by the transferred CD4+ T cells is required for the maintenance of transferred CD8+ T cells and/or activation of host CD4+ or CD8+ T cells. Several recent studies have documented that CD4+ T-cell help is critical for the maintenance of CD8+ T-cell population (13, 14).

From our in vivo depletion experiments and adoptive transfer experiments, we cannot rule out the possible involvement of NK cells in antitumor immunity. Although expression of MHC class I molecules on antigen-presenting cells negatively regulates NK cell effectors (50), it is still unclear what initiates the activation of NK cells in vivo. MC-38-CEA-A2Kb tumor cells are highly positive for surface expression of MHC class I molecules and therefore it is speculated that in vivo tumor regression was not due to direct cytotoxic effect of NK cells in our model system. Because IL-12 is a known stimulator of NK cells (51), we postulate that the IL-12 generated from bone marrow–derived dendritic cells (44) might have activated NK cells in vivo and stimulated them to secrete IFN-γ (51). IFN-γ might have up-regulated surface MHC expression on tumor cells and rendered them more susceptible for CD8+ T-cell–mediated lysis. Additional experiments are needed to further evaluate those possibilities. Finally, for translation of this study to clinical application, it is important to note that we did not detect signs of autoimmune disease despite the fact that some vaccinated mice were kept for a total of 6 months to conduct experiments related to memory response.

Taken together, the results presented in this work show that the immune responses induced by combined vaccines consisting of peptide-2–pulsed dendritic cells plus 3H1-pulsed dendritic cells or peptide-5–pulsed dendritic cells plus 3H1-pulsed dendritic cells were effective in increasing the survival with complete tumor eradication in 50% and 67.5% of the mice, respectively, in the therapeutic settings. To the best of our knowledge, this is the first study documenting the positive outcome of vaccine strategy consisting of CTL epitope of CEA and an anti-idiotype antibody mimicking CEA in a clinically relevant established tumor-bearing mouse model transgenic for both CEA and HLA-A2. In future studies, we will perform dendritic cell–based vaccination experiments combining two different CD8 T-cell epitopes (peptide-2 and peptide-5) along with 3H1 to reduce the tumor burden in 100% mice, if possible. Nevertheless, we realize that to obtain a better antitumor effect, additional immunologic manipulations, such as CTLA-4 blockade or the removal of T regulatory cells, may be necessary. We believe that our findings hold promise for better immunotherapeutic approaches in the treatment of cancer patients with CEA+ tumors.

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References


Therapy of Established Tumors in a Novel Murine Model Transgenic for Human Carcinoembryonic Antigen and HLA-A2 with a Combination of Anti-idiotype Vaccine and CTL Peptides of Carcinoembryonic Antigen


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