Murine Dendritic Cells Pulsed with an Anti-Idiotypic Antibody Induce Antigen-specific Protective Antitumor Immunity

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ABSTRACT

In this study, using the carcinoembryonic antigen (CEA)-expressing C15 murine colon carcinoma system in syngeneic C57BL/6 mice, we have evaluated the efficacy of bone marrow-derived dendritic cells (DCs) pulsed with the murine anti-idiotype antibody 3H1 as a tumor vaccine. Anti-idiotype 3H1 mimics a distinct and specific epitope of CEA and can generate anti-CEA immunity in mice, rabbits, monkeys, and humans when used with a conventional immune adjuvant. Our goal was to determine whether the use of DC as direct antigen-presenting cells would improve the potency of 3H1 as vaccine. Bone marrow-DC pulsed with 3H1 and injected into naïve mice induced both humoral and cellular anti-3H1, as well as anti-CEA immunity. Specific killing of C15 cells in in vitro antibody-dependent cellular cytotoxicity has been observed by immune sera. Immune-splenic lymphocytes when stimulated in vitro with 3H1 or CEA, showed increased proliferative CD4+ Th1 type T-cell response and secreted significantly high levels of Th1 cytokines [IFN-γ, interleukin (IL)-2] and low levels of Th2 cytokines (IL-4, IL-10). This vaccine also induced MHC class I antigen-restricted CD8+ T-cell responses. The up-regulation of activation markers CD69 and CD25 on CD8+ CTLs correlated with antigen-specific strong CTL responses in vitro. The immunity induced in mice resulted in a complete rejection of CEAexpressing C15 tumor cells in 100% of experimental mice, whereas no protection was observed when 3H1-pulsed DC-vaccinated mice were challenged with CEA-negative MC-38 cells. The tumor rejection in 3H1-pulsed DC-treated mice was associated with the induction of a memory response that helped those mice to survive a second challenge with a lethal dose of C15 cells.

INTRODUCTION

DCs4 form a network of sentinel cells in the periphery to facilitate antigen delivery to secondary lymphoid organs (1). Transport of antigen by DC is probably of key importance to initiate an immune response, thus permitting establishment of immunological memory (1–4). In addition, because of their excellent T-cell-stimulatory properties, small numbers of DCs are sufficient to induce an immune response in vivo (4). In different settings, these specialized antigen-presenting cells can induce both the generation and proliferation of specific CTLs and Th cells via antigen presentation by MHC class I and class II molecules, respectively. In addition to activating naïve T cells in the extrafollicular areas of secondary lymphoid organs, DCs may directly modulate B cell growth and differentiation (5). The role of DCs in humoral responses has been documented in vitro (6) and in vivo (7–10). Notably, DCs incubated in vitro with antigen can induce, upon reinjection into mice, a protective humoral response (10). Because of these properties, much attention has been directed toward the use of DCs in vaccine strategies for the treatment of cancer. In this regard, DCs pulsed with tumor-associated antigens in various forms, including whole cell lysate (11–14), peptides (15, 16), proteins (17, 18), RNA (19), DNA (20, 21), or DCs fused with tumor cells (22), have been studied for antitumor effects in experimental tumor models. Whole tumor cells have also been used for DC priming (23). In all of these approaches, there is a requirement for in vitro manipulation of the DCs to acquire and present tumor-specific antigens.

A prerequisite for successful vaccination is the establishment of a long-lasting protective immune response, and in this regard, immunotherapy is an attractive approach to cancer therapy. The aim of immunotherapy is to induce or increase the ability of tumor-reactive T cells to mediate antitumor immune responses in vivo. One area of active immunotherapy involves the use of anti-Id antibodies. This idea is based on Jerne’s network concept (24). According to this hypothesis, antibody of the type Ab2β represents essentially the internal image of the target antigen, and this idiotypic antibody can be used as a surrogate TAA. We have generated a murine monoclonal Ab2β designated 3H1 (25), which mimics a distinct and specific epitope of the TAA. CEA. CEA is present at high density on tumor cells of >95% of colorectal, 70% of lung adenocarcinoma, and 50% of breast cancer patients (26–28). We have shown previously that 3H1 can induce anti-CEA antibody in small animals (25), nonhuman primates (29), and in humans (30–32). In these studies, although induction of anti-CEA humoral immunity by the surrogate antigen could be demonstrated, a definitive CTL response could not be shown. The objective of the current study was to assess the ability of bone marrow-generated DCs pulsed with 3H1 to induce long-term antitumor immunity.

MATERIALS AND METHODS

Animals

Female (6–8 weeks old) C57BL/6 (H-2b) mice were used in all experiments and were purchased from Harlan Laboratories (Indianapolis, IN). Mice were housed in a specific pathogen-free environment and treated in accordance with the guidelines established by the Animal Care and Use Committee of the University of Cincinnati Medical Center.

Generation, Culture, and Flow Cytometry of BmDCs

BmDCs were generated as previously described (33) with some modifications. Briefly, bone marrow was collected from tibias and femurs of female C57BL/6 mice, passed through a nylon mesh to remove small pieces of bone and debris, resuspended in complete medium (CM: RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin (all from Life Technologies, Inc., Grand Island, NY, and 50 μg 2-mercaptoethanol (Sigma, St. Louis, MO)), and cultured in tissue culture dishes (Becton Dickinson, Franklin Lakes, NJ) for 2 h. Nonadherent cells were collected and aliquots of 3 × 10⁶ cells were placed in a 60-mm dish (Becton Dickinson) containing 5 ml of CM with 10 ng/ml rm granulocyte macrophage colony-stimulating factor and 5 ng/ml rmIL-4 (PharMingen, San Diego, CA). Cells were cultured for 9 days. On day 9 of culture, most of the nonadherent cells had acquired typical DC morphology,
and these cells were used as the source of DCs in subsequent experiments. *Escherichia coli*-lipopolysaccharide (0.2 µg/ml; Sigma) was added for the final 2 days of cell culture.

Expression of surface molecules was quantitated by flow cytometry using the following antibodies against MHC molecules: H2K (FITC-conjugated), I-A/I-E (FITC-conjugated); CD11c (FITC-conjugated); CD40 (PE-conjugated); CD54 (FITC-conjugated); CD80 (FITC-conjugated); CD86 (PE-conjugated); and CD14 (PE-conjugated), all obtained from PharMingen. Species and isotype-matched mAb were used as controls. For flow cytometry, aliquots of $2 \times 10^6$ BmDCs were incubated with the mAbs for 30 min at 4°C. The cells were washed twice in fluorescence-activated cell sorting buffer (PBS + 0.1% fetal bovine serum (v/v) + 0.02% NaN₃) and subsequently analyzed in a flow cytometer (EPICS XL/MCL, Beckman-Coulter Inc., Hialeah, FL). Data were analyzed using WinList software (version 5.0). Live cells were selected for analysis using forward versus side scatter gating.

**Anti-Id Vaccine and Peptide**

Generation, purification, and characterization of anti-Id antibody 3H1 designated as CeaVac have been described earlier (25). One control anti-Id antibody, IAT7 (34), which mimics a melanoma-associated antigen, ganglioside Ga₄, was also used in this study. Peptides were designed based on the amino acid sequence homology of 3H1 and CEA and were synthesized as described previously (35). The purity of the peptides was >90% by high-performance liquid chromatography analysis.

**Antigen Pulsing of DCs**

For antigen pulsing, bone marrow-derived day 8 DCs were incubated with anti-Id mAb, 3H1, or isotype-matched control anti-Id mAb IAT7 for overnight at 37°C, in presence of 120 µg/ml antigen. The nonadherent cells were then collected and were mainly DCs (referred to as antigen-pulsed DCs). At this time point supernatants from unpulsed or antigen-pulsed DC cultures were harvested for measurement of IL-12p70 production by standard ELISA kit (R&D Systems, Minneapolis, MN).

**Immunization Protocol**

Before immunization, unpulsed or antigen-pulsed 9-day-cultured BmDCs were washed three times in PBS. A total of $2 \times 10^6$ DCs in 0.1 ml of PBS were injected s.c. in the lower right flank of syngeneic C57BL/6 mice. Each mouse received three immunizations every other week. One group of mice received immunizations with 3H1-pulsed DCs; the other group received immunizations with unpulsed DCs, and a mock vaccination with PBS was also performed in another group of mice for comparison. All mice were bled 7–10 days after each immunization and sera were stored at −20°C.

**Determination of Antigen-specific Antibody Levels**

RIA for detection of Ab3 was performed as described previously (36). Briefly, microtiter plates coated with 3H1 were incubated with diluted mouse sera. After washings, the antigen-antibody reaction was tagged using $^{125}$I-labeled 3H1. Assays were performed in triplicate. Sera obtained from mice immunized with IAT7-pulsed DCs were used as control.

ELISA for detection of anti-CEA antibodies was performed as described previously (36). Assays were performed at least in triplicate for each sample. In select experiments, alkaline phosphatase-conjugated anti-IgG1, anti-IgG2a, anti-IgG2b, anti-IgA, or anti-IgM (1:1500; Southern Biotechnology, Birmingham, AL) antibodies were used for subclass determination.

Idiotype and epitope analysis of Ab3. Experiments were performed as described previously (36). The serum from each mouse was checked for the ability to inhibit the binding of $^{125}$I-labeled 8019 (Ab1) to 3H1 bound to microtiter plates. Also, the inhibition of 8019 binding to CEA by murine sera was tested by RIA. Pooled serum from normal mice was used as control in these experiments.

**ADCC**

The ability of Ab3 to lyse CEA-positive tumor cells in conjunction with effector cells was tested by standard ADCC as described previously (36). Briefly, spleen cells were isolated from immunized mice for use as effector cells. Assays were performed in triplicate wells. Preimmune sera as well as sera from mice immunized with IAT7-pulsed DCs were used as controls. To calculate lysis specifically attributable to ADCC, the percentage of lysis attributable to effector cells in the absence of the antibody was subtracted from each value obtained.

**T-Cell Proliferation Assays**

Spleens were harvested from mice 7–10 days after the last immunization or harvested after 5 weeks of posttumor challenge. These cells (2 $\times 10^6$/well) were cultured in absence and presence of different stimulants (0.5–1 µg/well). In select experiments, peptides were used as stimulants at a concentration of 5 µg/well. T-cell proliferation was measured by $[^3]$Hthyminde incorporation as described previously (36). Assays were performed in triplicate wells. In parallel experiments, cell-free supernatants were harvested after 48 and 72 h of culture for quantitation of IL-2, IL-4, IL-10, and IFN-γ, respectively.

**Cytokine Analysis**

Supernatant from T cell cultures were analyzed for the presence of IL-2, IL-4, IL-10, and IFN-γ by ELISA using commercially available kits (R&D Systems). All samples were tested in triplicate. The lower limit of detection for cytokines was 5 pg/ml each.

**Antigen-specific CTLs**

Splenocytes were harvested and pooled from 3 mice/group 7–10 days after the last immunization or after 5 weeks of posttumor challenge. These cells (2 $\times 10^6$/ml) were restimulated by culturing with 3H1 (25 µg/ml) and 20 units/ml rhIL-2 (Sigma). After 5 days of culture, the in vitro restimulated viable splenocytes were examined for specific cell lysis using standard 6 or 18-h $^{3}$Cr release assays (15–17) against a variety of target cell lines. Assays were performed in triplicate wells. The spontaneous release of all assays was <25% of the maximum release.

Antibody-blocking experiments with anti-CD8 ($53–6.7$) or anti-CD4 (GK1.5) mAb were performed to establish the effector cell phenotype responsible for specific killing of C15 cells and were carried out by preincubating effector cells for 60 min at 37°C. Antibody-blocking experiments with anti-H-2Kb/HLA-DR or anti-I-A$^b$ mAb were performed to demonstrate MHC class I antigen restriction of CTL response against C15 cells and were carried out by preincubating labeled target cells for 30 min at 37°C. Isotype-matched mAbs were used as control. Effector or labeled target cells were preincubated with 5 µg/ml antibody before the addition of untreated labeled target or effector cells respectively. All antibodies used in blocking experiments were obtained from PharMingen.

In select experiments, purified CD8$^+$ and CD4$^+$ T cells were obtained from antigen-specific CTL culture by magnetic bead separation of T cells using either anti-CD8 (53–6.7) or anti-CD4 (GK1.5) magnetic beads, according to the manufacturer’s specifications (Mini MACS; Miltenyi Biotec, Auburn, CA). All samples were passed through the column twice and were 95% pure as determined by flow cytometry. These purified CD8$^+$ and CD4$^+$ T cells were used as effector cells in CTL assay.

**Flow Cytometry of CTL Culture**

Mice immunized with 3H1-pulsed DCs or IAT7-pulsed DCs and challenged with C15 tumor cells were sacrificed after 5 weeks of posttumor inoculation, and splenocytes were harvested and restimulated by culture with 3H1 and rhIL-2 as described above. After 5 days of culture, viable splenocytes were used for staining. Antibodies (PharMingen) used to phenotype the cells were anti-CD28-PE (hamster antimouse), anti-CD4-PE (rat antihuman), anti-CD8-PE (rat antihuman), anti-CD25-FITC (rat antihuman), and anti-CD69-FITC (hamster antihuman). For two-color immunofluorescence, cells were preincubating labeled target cells for 30 min at 37°C. Isotype-matched mAbs were used as control. Effector or labeled target cells were preincubated with 5 µg/ml antibody before the addition of untreated labeled target or effector cells respectively. All antibodies used in blocking experiments were obtained from PharMingen.

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**Mice Immunized with Anti-Id Antibody-Pulsed DC and Tumor Immunity**

A murine tumor model expressing human CEA was developed by Dr. F. James Primus at Vanderbilt University (Nashville, TN). Murine colorectal......
canceroma cells, MC-38, were transduced with human CEA (37). The transduced cell line, C15, constitutively express CEA in culture. When the stably transfected CEA-transduced cells (1 × 10^6 cells/mouse) or nontransduced cells (5 × 10^5 cells/mouse) are injected s.c. into syngeneic C57BL/6 (H-2^b) mice, tumors develop in 100% of the mice within 10–15 days. Experiments included 10 mice/group.

Cell Lines

EL-4 is a T thymoma derived from the C57BL/6 mouse. YAC-1 cells are sensitive to natural killer cells. MC-38 is a chemically induced murine colon carcinoma cell line, and the CEA-expressing (clone C15-4.3) MC-38 transfectant has been described previously (37).

Survival of Mice Immunized with 3H1-pulsed DCs and Control Vaccines

Mice were immunized with 3H1-pulsed DCs or control vaccines (1A7-pulsed DCs or unpulsed DCs) or PBS as described above. Each group of immunized mice was additionally divided into two equal groups for tumor challenge. Tumor challenge was performed s.c. with 1 × 10^6 of CEA-positive C15 cells or 5 × 10^5 of CEA-negative MC-38 cells in the lower left flank. Growth of tumor and survival were monitored daily. Tumor size was assessed once a week and recorded as a tumor area (in mm^2) by measuring the largest perpendicular diameters with vernier calipers. All experiments were performed two to three times using individual groups of 12–24 mice. Mice were sacrificed when tumors became ulcerated or when they reached a size >250 mm^2.

Secondary Tumor Challenge in Tumor-free Mice

To determine whether the tumor-specific CTLs induced by BmDCs via s.c. injections acquiring tumor antigens in vivo were responsible for rejection of s.c. tumors, 3H1-specific CTLs were generated from tumor-free mice as described above and then i.v. infused into naive mice (3 × 10^6 cells/mouse). One day later, these mice were s.c. challenged with 1 × 10^6 C15 cells and monitored for tumor growth and survival.

Statistical Analysis

Student’s t test was applied to perform statistical analysis using SigmaStat software (Jandel, San Rafael, CA). P < 0.05 was considered to indicate statistical significance.

RESULTS

Cell Surface Phenotype of and IL-12 Production by BmDCs.

The culture conditions resulted in the generation of BmDCs with typical morphology and fine membrane projections. DCs were stained and analyzed by flow cytometry for surface expression of MHC molecules, costimulatory molecules, and antigens associated with maturation. BmDCs expressed high levels of the DC-associated marker CD11c, the costimulatory molecules CD80, CD86, CD40, and CD54, and MHC class I/II (Fig. 1). To assess the percentage of contaminating macrophages in the culture conditions, the expression of the macrophage-related surface molecule CD14 was determined. The percentage of cells expressing CD14 was <5%, indicating low numbers of contaminating macrophages in the BmDC cultures.

We have evaluated the production of a Th1-polarizing cytokine, IL-12, by 1 × 10^6 DCs pulsed for overnight with anti-Id antibody as a source of antigen. As shown by a representative experiment (of three performed) in Table 1, DCs cultured with anti-Id antibody, 3H1, produced higher amounts of IL-12p70 as compared with unpulsed DCs.

Antigen-pulsed DCs Can Induce Antigen-specific Humoral Response in Vivo.

To evaluate the ability of DCs to induce an in vivo humoral response, we have measured the titer of anti-anti-Id antibodies in the sera of immunized mice. The animals were bled 7–10 days after each immunization, as indicated in the figure legend 2. Ab3 produced in the sera of these mice was estimated by a RIA. Data in Fig. 2 show that levels of Ab3 in 3H1-pulsed DC-immunized mice sera are low but detectable after first immunization. However, signif-

Fig. 1. Surface phenotype of BmDCs generated under culture conditions as described in “Materials and Methods.” Cells were analyzed by flow cytometry. — shows the fluorescence-activated cell sorting profiles after staining with isotype control mAb. The data are representative of three different experiments.
significant Ab3 was generated after three immunizations. For control, sera from mice immunized with 1A7-pulsed DCs were used for 3H1 binding assay. Radioactive 3H1 bound by control sera were equal to or less than the preimmune sera (data not shown).

Anti-CEA antibody developed by mice immunized with 3H1-pulsed DCs was determined by ELISA. Ab3 antibody induced by Ab2β is polyclonal, and a subgroup of the Ab3 has a similar binding site (paratope) as Ab1. This type of Ab3 is also known as Ab1’ to indicate that it might differ in its other idiotypes from Ab1. Ab1’ recognizes the cognate TAA and is the only antibody in the Ab3 population that is likely to have the antitumor property. As shown in Fig. 3A, the level of Ab1’ was detectable after first immunization in sera obtained from mice immunized with 3H1-pulsed DCs. Significant level of Ab1’ appeared after the third immunization. Sera from mice after each immunization with 1A7-pulsed DCs were used as control in this assay. Preimmune serum (1:20 dilution) had an absorbance of 0.20 at 405 nm.

The selective activation of cloned murine CD4⁺ cells representing Th1 and Th2 cells depends on unique cofactors produced by specialized antigen-presenting cell populations (38). Because the regulation of isotype switching in vivo is dependent upon the activation of different types of T-helper cells (39), we investigated the class distribution of the specific anti-CEA antibodies. Fig. 3B summarizes the isotypes of the CEA-specific antibodies from mice tested in two independent experiments. Specific IgG2a antibodies mostly were detected in this group of 3H1-pulsed DC-immunized mice, suggesting that this isotype may be relevant for the in vivo resistance to tumor growth. Enhanced production of IgG1 reflects a Th2 response in mice, whereas predominant IgG2a production indicates a Th1 response (40). Murine IgG2a is more effective than IgG1 at mediating antibody-dependent cellular cytotoxicity (41). Indeed, among the antibodies, murine IgG2a have been reported to be more effective than other isotypes in the suppression of tumor growth in a number of animal models (42, 43).

Ab3 induced in mice by 3H1-pulsed DC vaccine was analyzed by the inhibition of binding of Ab1 (8019) to Ab2 (3H1) by the mouse serum as described in “Material and Methods.” As presented in Table 2, >40% inhibition of radiolabeled Ab1 binding to Ab2 was observed by the Ab3 sera at a 1:20 dilution. We additionally analyzed the binding inhibition of radiolabeled 8019 to CEA in presence of mouse Ab3 serum. Sera from 5 of 5 mice inhibited this binding, and the inhibition was significant at 20-fold dilution of the serum. These results suggested that the Ab3 induced in mice immunized with 3H1-pulsed DCs share the same Id as Ab1 and may also contain Ab1’ antibodies.

ADCC by Immune Sera. To determine whether the Ab1’ generated by 3H1-pulsed DC immunization is cytolytic for CEA-positive tumor cells such as C15, ADCC was determined in vitro. As shown in Fig. 4, significant ADCC was observed in mice immunized with 3H1-pulsed DCs, whereas ADCC with preimmune sera or sera from mice immunized with unpulsed DCs or control 1A7-pulsed DCs was negligible. MC-38 cells lacking CEA expression as targets resulted in a background lysis, suggesting that tumor cell lysis was dependent on antigen expression by target cells.

Proliferation of T Cells in Presence of Proteins and Peptides. Assuming that antigen-pulsed DC prime naïve T cells toward antigen-specific T cells and that these cells predominantly proliferate when
exposed to antigen, we assessed the proliferation of spleen cells from immunized mice in presence of different stimulators. For positive control, 1.0 μg of ConA was used in these experiments. Spleens from at least 3 mice in each group, selected at random, were pooled for use in this assay. Significant proliferation was observed in presence of 3H1 or CEA from spleen cells isolated from mice immunized with 3H1-pulsed DCs (Fig. 5A). Spleen cells from 1A7-pulsed DCs, unpulsed DCs, or PBS-vaccinated mice showed negligible stimulation with either 3H1 or CEA. The proliferation of T cells from mice after three immunizations with 3H1-pulsed DCs, in the presence of 1A7 as the stimulant in the assay, was significantly lower than the value obtained with 3H1 (P < 0.005) as the stimulant. However, spleen cells harvested from 1A7-pulsed DC-immunized mice and placed in secondary culture in vitro in presence of 1A7 resulted in 1A7-specific proliferative response.

To analyze the subsets of T-cell populations that become responsive by 3H1-DC vaccination, CD4+ and CD8+ T cells were separated from immunized mice splenocytes, and their proliferation was analyzed in presence of different stimulants. From our previous study (35), we have also considered two peptides for analysis, one from 3H1 (LCD-2) and the other from CEA (CEA-B), containing common sequence LIDG. Besides LCD-2 and CEA-B, two control peptides, HFW-1 and CAP-1, were also included for analysis. As illustrated in Fig. 5B, the bulk splenocyte populations and purified CD4+ T cells responded to 3H1 and peptides (LCD-2, CEA-B), whereas CD8+ T-cell populations were unresponsive. No population of lymphocytes proliferated in presence of anti-Id antibody 1A7 or control peptides HFW-1 and CAP-1 (Fig. 5B). It may be possible that purified CD8+ T cells were relatively unresponsive in vitro as CD4+ T-cell help was not provided.

Cytokine Production by Activated T Cells. Because cellular immunity appears to be critical in mediating antitumor effects, we analyzed the pattern of cytokines released in vitro by stimulated CD4+ T cells of immunized mice. Th1 subset of CD4+ T-helper cells secretes IL-2 and IFN-γ, whereas Th2 subset secretes IL-4, IL-5, and IL-10. To determine whether the stimulated CD4+ cells constitute mainly Th1 or Th2 helper cells, freshly isolated splenocytes were cultured in vitro in presence of 3H1, and the culture supernatant was then analyzed by ELISA for levels of mIL-2, mIFN-γ, mIL-4, and mIL-10 production. A Th1-associated response was observed, with a significant enhancement of mIL-2 and mIFN-γ production in the group of mice immunized with 3H1-pulsed DCs (Table 3). The levels of the Th2-associated cytokines IL-4 and IL-10 obtained from the cultures were much lower than that of the Th1-associated cytokines.

Induction of CEA-specific Cytolytic T-Cell Response in Mice Immunized with 3H1-pulsed BmDCs. To assess whether DCs pulsed with soluble 3H1 protein are capable of inducing cytolytic response in vivo, spleen cells from immunized mice were harvested 7–10 days after the last immunization and restimulated in vitro in presence of 3H1 and rHL-2 for 5 days, and these cells were used as effector cells. Effector cells obtained from mice immunized with 3H1-pulsed DCs lysed the CEA-expressing murine colon carcinoma cells, C15 significantly (Fig. 6A). Tumor cell lysis was antigen specific because the use of MC-38 parental tumor cells lacking CEA expression as targets resulted in a background lysis. The lysis of C15 tumor cells was significantly greater (P < 0.005) than that obtained with MC-38 targets at all E:T cell ratios tested. The use of nonspecific EL-4 thymoma cells lacking CEA expression as targets also resulted in insignificant lympholytic activity.

Table 3. Th1-associated immune response in splenocytes from immunized mice

<table>
<thead>
<tr>
<th>Stimulated</th>
<th>mIL-2</th>
<th>mIFN-γ</th>
<th>mIL-4</th>
<th>mIL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>3H1-DCs</td>
<td>980 ± 32</td>
<td>5340 ± 80</td>
<td>234 ± 16</td>
<td>104 ± 12</td>
</tr>
<tr>
<td>1A7-DCs</td>
<td>204 ± 12</td>
<td>480 ± 15</td>
<td>170 ± 12</td>
<td>96 ± 8</td>
</tr>
</tbody>
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*Spontaneous (2 × 107/well) obtained from different groups of immunized mice were cocultured in presence of 3H1 (1.0 μg) in 96-well flat-bottomed microtiter plates in a final volume of 200 μL. Cell-free supernatants were harvested after 48 h (for quantitation of IL-2, IL-4, IL-10) and 72 h (for quantitation of IFN-γ). Cytokine levels were measured using standard ELISA kits. Data represent means ± SE of triplicate cultures. One representative experiment of two is shown.
in a background lysis. The nonspecific killing activity was measured by lysis of natural killer-sensitive YAC-1 cells. However, the lysis of C15 cells with spleen cells from all 3H1-DC immune mice was significantly greater (P < 0.005) than that obtained with control targets. There was no difference in lysis between the control targets (P > 0.1). Similar results were obtained when additional immunized mice were studied. DCs pulsed with unrelated anti-Id antibody, 1A7, did not induce CEA-specific cytolytic response in vivo. Only background lysis was observed with splenocytes obtained from this group of immunized mice (Fig. 6B). Similar results were obtained from other control animals immunized either with DCs alone (Fig. 6C) or mock vaccinated with PBS (Fig. 6D). Note, however, that immunization of mice with DCs generated some nonspecific lytic activity, which is typically seen with BmDCs as evidenced by previous studies (16, 44).

Splenocytes from 3H1-DC-vaccinated mice were also stimulated in vitro culture with either 3H1 or CEA or 1A7 and tested 5 days later for lytic activity on CEA transfected C15 or parental MC-38 cells. Splenocytes stimulated in vitro with either 3H1 or CEA-lysed C15 cells specifically (Fig. 6E) but not MC-38 cells (Fig. 6F). However, splenocytes stimulated in vitro with control anti-Id antibody, 1A7, did not display any significant in vitro cytotoxicity (Fig. 6, E and F). The lytic activity against C15 cells was significantly higher with CTLs derived from both splenocytes stimulated with either 3H1 (P < 0.005) or CEA (P < 0.003) than that obtained with control target cells at all E:T cell ratios tested.

Moreover, that DCs exposed to 3H1 can induce antigen-specific CTL response was shown by the experiment in which DCs were used as targets. 3H1-DC-vaccinated mice splenocytes were restimulated in vitro in presence of 3H1 and rhIL-2. Five days later, stimulated cells were tested for their ability to lyse the DC target cells pulsed with either 3H1 or CEA or 1A7 as indicated in the panel. Each experiment included 6 mice and was repeated at least twice.
were lysed if pulsed with 3H1 (P < 0.04) or CEA (P < 0.009). This result indicates that DCs can process the soluble protein for antigen-specific MHC class I presentation.

**DCs Pulsed with 3H1 Induces Specific MHC Class I Antigen-restricted CD8+ T-Cell-mediated Cytotoxicity.** To determine whether the antitumor activity was associated with the presence of tumor-specific CD8+ CTLs and/or CD4+ helper T lymphocytes, anti-CD8 or anti-CD4 mAb was incorporated as blocking reagents in cytotoxicity assay. The specific killing of C15 cells was significantly inhibited by preincubation of effector cells with anti-CD8 mAb (Fig. 7A), whereas the antibody against CD4 was relatively less effective. This inhibition ranged from 80 to 92% at all E:T cell ratios tested compared with the isotype-matched control antibody (P < 0.001). The inhibition of CTL activity with anti-CD4 mAb ranged from 8 to 30% at all E:T cell ratios tested (P > 0.1). This result indicates that antitumor response observed was primarily mediated by CD8+ CTLs.

Antibody-blocking experiments with anti-MHC class I or anti-MHC class II mAb were also performed to determine the MHC class I antigen-restricted CTL specificity for C15 target cells. Inhibition of CTL activity was obtained by treatment with anti-H-2Kb/H-2Db mAb (Fig. 7B). This inhibition ranged from 78 to 83% at all E:T cell ratios tested compared with isotype-matched control antibody (P < 0.02). In contrast, the inhibition of CTL activity with anti-I-Aβ mAb ranged from 0 to 25% (P > 0.1), which indicated MHC class I-restricted tumor cell lysis.

Additional experiments were performed to establish that the CD8+ effector cell population was responsible for specific killing of C15 cells. Immunized mice splenocytes were restimulated in vitro in presence of 3H1 and rHL-2. On day 5, CD8+ and CD4+ T cells were purified from this stimulated culture using microbeads and used separately for cytotoxicity assay. The assay was performed at lower E:T cell ratios because of scarcity of purified effector cells. As shown in Fig. 7C, CD4+ T cells did not display any significant in vitro cytotoxicity, whereas CD8+ T cells had significant antigen-specific lytic activity (P < 0.001), indicating that CD8+ CTLs were primarily responsible for antigen-specific lysis induced by BmDCs.

**Vaccination with 3H1-pulsed DCs Protects Mice against a Challenge with CEA-transfected Tumor Cells.** To determine whether the immune response induced by protein-pulsed DCs can induce resistance against a challenge with live tumor cells 2 weeks after the last immunization, each group of immunized mice were divided into two equal subgroups, and one group was challenged with CEA-expressing C15 cells, whereas the second group was challenged with CEA-negative MC-38 cells. The group of mice immunized with 3H1-pulsed DCs were completely protected from C15 tumor growth (Fig. 8) and from death (Fig. 9A). Tumors in control mice (immunized with unpulsed DCs or PBS) grew progressively (Fig. 8) and were lethal (Fig. 9A). Mice immunized with DCs pulsed with 1A7 were not protected (Figs. 8 and 9A), suggesting that s.c.-injected DCs do not induce tumor immunity by antigen-independent mechanisms in this model. It is also unlikely that protection was the result of carryover of free 3H1 because the 3H1-pulsed DCs were extensively washed before injection. Also, 3H1 alone does not induce any immune responses in mice (unpublished data). Furthermore, mice immunized with 3H1-pulsed DCs were not protected from challenge with non-transfected parent MC-38 cells (Fig. 9B), indicating that protective immunity was antigen specific, depending on CEA expression by the tumor target.

**Th1-associated Cytokines and CD8+ T Cells Are Involved in the DC-mediated Antitumor Effects.** To additionally understand the mechanism responsible for complete protection of s.c. tumors, tumor-protected mice were selected at random after 5 weeks of posttumor challenge, and cellular immune responses were measured by (a) T-cell proliferation assay, (b) in vitro cytokine release pattern, (c) cytotoxicity assay, (d) s.c. challenge with MC-38 cells, and (e) splenocytes and lymph nodes restimulation in vitro with antigen-presenting cells.
unpulsed or control protein-pulsed DCs to elicit a detectable level of protective immunity in mice inoculated with either C15 or MC-38 tumor cells (Figs. 9, A and B).

The role of CD8$^+$ T cells in antitumor immunity was additionally suggested by up-regulation of the activation markers such as CD69 (46), CD25, and CD28 on T cells. The data depicted in Fig. 11A indicate that 1.4% of CD8$^+$ and 1.5% of CD4$^+$ cells expressed CD69, whereas 4.2% of CD8$^+$ and 1.5% of CD4$^+$ cells expressed CD25 when effector cells obtained from tumor-bearing 1A7-DC-immunized control mice were used for analysis. On the other hand, expression of both CD69 and CD25 on CD8$^+$ cells was significantly high when effector cells obtained from tumor-protected mice were used for analysis. A total of 33.3% of CD8$^+$ and 2.6% of CD4$^+$ cells expressed CD69, whereas 42.7% of CD8$^+$ and 2.4% of CD4$^+$ cells expressed CD25 (Fig. 11A). The expression of CD28 was negligible in tumor-bearing control mice, whereas 93.0% cells expressed CD28 when effector cells of tumor-protected mice were used for analysis (Fig. 11B). These results, therefore, are consistent with the idea that CD8$^+$ T cells are the primary effector arm in CTL response and that CD4$^+$ T cells are necessary for the induction of an antitumor CD8$^+$ T-cell-dependent response but may not participate as an effector population directly.

Immunized Mice that Had Rejected Tumor-generated Antitumor Memory Response. Immunized mice that had rejected C15 tumor cells were additionally divided into two subgroups. One group was challenged with C15 cells, whereas the other group was challenged with nontransfected parent MC-38 cells. The results show that these mice resisted lethal doses of C15 cells (Fig. 12A), indicating that the tumor protection was mediated by persistent antitumor immunity specific for antigens relevant to C15 tumor. It is of interest that the tumor-free mice also had protective immunity against subsequent
challenge with the nontransfected parental tumor line MC-38 (Fig. 12B), suggesting that mice rejecting CEA-transfected carcinoma developed immunity to other antigens expressed on C15 and shared with the nontransfected parent carcinoma cell line MC-38, resulting in long-lasting memory against these tumors. Induction of immunity to shared tumor antigens has also been observed in other tumor model during tumor rejection after particular antigen immunization (47, 48). Of note, in different sets of experiments, 3H1-DC-immunized mice that had rejected C15 tumor cells remained tumor free, and after 8 months of first tumor inoculation, these mice were additionally challenged with either C15 or MC-38 cells and followed for tumor growth and survival. Interestingly, they also resisted the second tumor challenge (data not shown).

To additionally evaluate the effects of in vivo priming of T cells, adoptive transfer experiments were performed. 3H1-pulsed DC-immunized mice that had rejected C15 tumors were sacrificed after 5 weeks of posttumor challenge, and splenocytes were restimulated in vitro in presence of 3H1 and rhIL-2 for 5 days. The resulting stimulated cells were composed primarily of CD8+ T cells (>75%), and the remaining cells were mostly CD4+ (10%) as determined by flow cytometry (data not shown). These cells were transferred to naïve mice, and the mice were challenged with C15 tumor cells. Naïve mice without transfer of stimulated cells and challenged with C15 cells were used as control. As shown in Fig. 13, mice receiving the in vitro-stimulated immune splenocytes were protected from a subsequent lethal tumor challenge, but no protection was observed in control mice. The detail mechanisms of this protection is under study.

Fig. 11. Expression of activation markers by CTLs obtained from tumor-protected mice. Splenocytes harvested from groups of mice after 5 weeks of posttumor challenge were restimulated with 3H1 and rhIL-2 for 5 days. At the end of the culture, cells were analyzed for the expression of CD4, CD8, CD69, and CD25 by two-color flow cytometry (A). Expression of CD28 by total lymphocyte populations was also analyzed (B). Middle panel represents CTLs obtained from tumor-bearing control mice, and right panel represents CTLs obtained from tumor-free mice. A representative experiment of three is shown.

Fig. 12. Vaccination with 3H1-pulsed DCs and challenge with C15 tumor cells induces long-lasting protective immunity to subsequent tumor challenge. Surviving mice that had been immunized with 3H1-pulsed DCs and challenged as described (Fig. 8) were rechallenged with either CEA-transfected C15 tumor cells (A) or parental MC-38 tumor cells (B) s.c. 13 weeks after first tumor inoculation. A set of age-matched syngeneic mice was also challenged with either C15 or MC-38 cells and used as controls. Survival is recorded as described (Fig. 9). Each group contained 6 mice. Experiments were repeated twice, and a representative experiment is shown.

Fig. 13. Adoptive transfer of immune splenocytes to prevent the growth of a subsequent tumor challenge. 3H1-specific lymphocytes were generated from C15 tumor-free mice as described in Fig. 8. Stimulated cells (3 × 10^6) were infused i.v. into naïve mice. One day later, the mice were challenged s.c. with 1 × 10^6 C15 cells and considered as the experimental group. Naïve mice without transfer of stimulated cells and challenged with C15 cells were used as control. The mice were followed for tumor growth as in Fig. 8. The number of tumor-bearing animals compared with the total number of mice in each group is indicated. A representative experiment of two is shown.

DISCUSSION

In this study, we have shown that BmDCs pulsed with 3H1 induce a protective immune response against a challenge with tumor cells expressing CEA antigen. The tumor protection is antigen specific because tumor development was not prevented when the immunized mice were challenged with the same tumor cells that were CEA negative. DCs can express a number of Fc receptors, which bind the constant chain of immunoglobulins and mediate the endocytosis of immune complexes (49). Several studies have shown that DCs pulsed
with soluble proteins can present peptide epitopes derived from these exogenous antigens to MHC class I molecules and induce an antigen-specific CTL response. It has been reported (18) that DCs use macropinocytosis to capture soluble antigens that are then presented on MHC class I molecules. These exogenous antigens access the cytosol of DCs and are processed for presentation via the same pathway described for conventional MHC class I-restricted cytosolic antigens. 3H1 protein is likely to be internalized and degraded to peptides by DCs. The degraded peptides bound to the MHC molecules are presented to T cells by DCs. T cells with appropriate receptors are expanded and expected to constitute the anti-3H1 cytotoxic, helper, and memory cells. T cells induced by immunizations with 3H1-pulsed DCs will recognize CEA-positive tumor cells only when amino acid sequence of 3H1 has linear homology to CEA. We have identified a number of peptides in 3H1 having linear sequence homology to CEA (35).

The role for DCs in the activation of T cells is well established, it is less clear to what extent DCs regulate other cells. It is known that DC are essential for the development of antibody responses (5–10, 50), but this has been thought to be simply a secondary consequence of the requirement for DCs to activate naïve helper T cells. Several findings suggest that DCs may directly regulate B cell maturation (5). Our results demonstrate that a syngeneic DC that has been pulsed in vitro with native protein induces a strong specific B cell response in vivo in mice after immunizations. Ab3 induced in mice by 3H1-pulsed DC vaccine inhibited the Ab1:Ab2 binding in this system, and binding of Ab1 to CEA was also inhibited (Table 2). These results suggested that the relevant Ab1′ had been induced by 3H1 immunizations. The development of CEA-specific IgG2a antibody correlates with ADCC invoked in mice by immunizations with 3H1-pulsed DCs. ADCC may be an additional important mechanism for tumor protection in this model. Our results are in accordance with previous studies showing that murine IgG2a is effective for prevention of tumor growth (43).

The proliferative response of splenocytes in presence of 3H1 and CEA reflects that mice immunized with 3H1-pulsed DCs induced anti-3H1, as well as anti-CEA immune responses. The phenotyping of proliferating splenocytes indicated that they are predominantly CD4+ T cells. Specific stimulation of the subsets of T cells in the presence of anti-Id antibodies, and 3H1-derived peptides confirmed this observation (Fig. 5). The cytokine profile in cocultures of mononuclear cells with 3H1 indicated the induction of a Th1 immune response with high levels of IL-2 and IFN-γ and low levels of IL-4 and IL-10. The enhanced IFN-γ production correlates with CEA-specific IgG2a titers observed and is likely to be mediated by IL-12 produced by DCs (51, 52). As s.c. vaccination leads to a rapid accumulation of DCs in lymph nodes, we speculate that BmDCs pulsed with antigen migrate to lymph nodes and secrete or induce the secretion of IL-12, thereby driving a CD4+ Th1-associated immune response.

CD8+ CTLs are an important effector arm in antitumor immunity. To investigate the in vivo priming of a CD8-mediated T-cell response after prophylactic vaccination with soluble protein-loaded DCs, antigen-specific CTL response was observed in vitro. The effector cells obtained from mice immunized with 3H1-pulsed DCs could lyse the CEA-expressing murine carcinoma cells, C15, but not the CEA-negative parent tumor cells, MC-38. This is not surprising because a number of peptides from 3H1 show considerable amino acid sequence homology to CEA (35). HLA motif analysis of these cross-reacting peptides show low but positive scores (53) with MHC class I Kb molecules. The CTL activity was mostly mediated by CD8+ T cells and MHC class I restricted as was evidenced by blocking experiments (Fig. 7). DCs may present class I peptides directly to CD8+ T cells, which may also be activated after help provided by the DC-primed CD4+ T cells. Cytokines such as IL-2 and IFN-γ may allow for dissemination and expansion of tumor-specific T cells elicited by this DC vaccine. Therefore, the use of 3H1-pulsed DCs may be advantageous because it would contribute MHC class II-restricted epitopes, as well as additional MHC class I restricted epitopes.

The immunity induced in mice immunized with 3H1-pulsed DC vaccine resulted in complete protection in 100% of experimental mice against live tumor challenge expressing CEA (Figs. 8 and 9A). Our data correlates with the results performed by Mayordomo et al. (15), which indicate that BmDCs grown in medium containing granulocyte macrophage colony-stimulating factor and IL-4 are capable of generating a complete protective antitumor immune response. To additionally evaluate the role of CD4+ and CD8+ T cells in mice, which were protected from tumor growth, cellular immune responses were measured after 5 weeks of posttumor challenge. CD4+ T-helper cell response was detected by proliferative T-cell response (Fig. 10A) and cytokine release pattern in tumor-protected mice. Interestingly, the amount of Th1-biasing cytokines such as IL-2 and IFN-γ production remained almost unchanged before and after tumor challenge in mice vaccinated with 3H1-pulsed DCs. The increase in the expression of the activation markers such as CD69 and CD25 on CD8+ T cells but not on CD4+ T cells correlated with antigen-specific CTL responses in vitro (Fig. 11). The increase over control in the expression of CD25 and CD69 indicates that T-cell activation took place in secondary lymphoid tissues after vaccination and tumor cell challenge. The marked increase in the expression of CD28 on T cells is particularly significant because ligation of CD28 with B7.1 and B7.2 initiates T-cell responses and the production of armed effector T cells. Importantly, the up-regulation of these activation markers correlated completely with the increase in tumor-protective immunity induced by 3H1-based DC vaccine.

In this model, DC vaccinations have also induced antitumor memory responses. The presented results show that anti-Id antibody-pulsed DCs induce long-lived CTL memory responses that are capable of rapidly responding to and protecting against a subsequent tumor challenge (Figs. 12 and 13).

In summary, our results have demonstrated that anti-Id 3H1-pulsed DCs can induce both humoral and cellular immune responses in mice with protective CEA-specific antitumor immunity. We have shown convincing evidence that CEA-specific CTL response can be generated after immunization with 3H1-pulsed DCs in the wild-type C57BL/6 mice in the prophylactic setting. The use of protein-pulsed DCs may be advantageous because it can activate both MHC class I-restricted CD4+ T cells, as well as MHC class I-restricted CD8+ T cells. The implications of these findings for the use of 3H1-pulsed DCs as vaccine in CEA-positive human cancer patients or in CEA-transgenic mice need to be investigated. Currently, we are engaged to evaluate the potency of this vaccine in a therapeutic model with established tumor, which is transgenic for human CEA. We will also determine the role of CD8+ and or CD4+ T cells directly in this model after selective depletion of particular T cells.

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