Therapy of Murine Tumors with Tumor Peptide–pulsed Dendritic Cells: Dependence on T Cells, B7 Costimulation, and T Helper Cell 1–associated Cytokines

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Summary

Antigen presentation by host dendritic cells (DC) is critical for the initiation of adaptive immune responses. We have previously demonstrated in immunogenic murine tumor models that bone marrow (BM)-derived DC pulsed ex vivo with synthetic tumor-associated peptides, naturally expressed by tumor cells, serve as effective antitumor vaccines, protecting animals against an otherwise lethal tumor challenge (Mayordomo, J. I., T. Zorina, W. J. Storkus, C. Celluzzi, L. D. Falo, C. J. Melief, T. Ildstad, W. M. Kast, A. B. DeLeo, and M. T. Lotze. 1995. *Nature Med.* 1:1297–1302). However, T cell–defined epitopes have not been identified for most human cancers. To explore the utility of this approach in the treatment of tumors expressing as yet uncharacterized epitopes, syngeneic granulocyte/macrophage colony–stimulating factor–stimulated and BM-derived DC, pulsed with unfractionated acid-eluted tumor peptides (Storkus, W. J., H. J. Zeh III, R. D. Salter, and M. T. Lotze. 1993. *J. Immunother.* 14:94–103) were used to treat mice bearing spontaneous, established tumors. The adoptive transfer of 5×10^5 tumor peptide–pulsed DC dramatically suppressed the growth of weakly immunogenic tumors in day 4 to day 8 established MCA205 (H-2^b) and TS/A (H-2^d) tumor models, when applied in three biweekly intravenous injections. Using the immunogenic C3 (H-2^b) tumor model in B6 mice, tumor peptide–pulsed DC therapy resulted in the erradication of established d14 tumors and long-term survival in 100% of treated animals. The DC-driven antitumor immune response was primarily cell mediated since the transfer of spleen cells, but not sera, from immunized mice efficiently protected sublethally irradiated naive mice against a subsequent tumor challenge. Furthermore, depletion of either CD4$^+$ or CD8$^+$ T cells from tumor-bearing mice before therapy totally suppressed the therapeutic efficacy of DC pulsed with tumor-derived peptides. Costimulation of the host cell–mediated antitumor immunity was critical since inoculation of the chimeric fusion protein CTLA4-Ig virtually abrogated the therapeutic effects of peptide-pulsed DC in vivo. The analysis of the cytokine pattern in the draining lymph nodes and spleens of tumor-bearing mice immunized with DC pulsed with tumor-eluted peptides revealed a marked upregulation of interleukin (IL) 4 and interferon (IFN) γ production, as compared with mice immunized with DC alone or DC pulsed with irrelevant peptides. DC-induced antitumor effects were completely blocked by coadministration of neutralizing monoclonal antibody directed against T helper cell 1–associated cytokines (such as IL-12, tumor necrosis factor α, IFN-γ), and eventually, but not initially, blocked by anti–mIL-4 mAb. Based on these results, we believe that DC pulsed with acid-eluted peptides derived from autologous tumors represents a novel approach to the treatment of established, weakly immunogenic tumors, and serves as a basis for designing clinical trials in cancer patients.

R ecent advances in the understanding of antigen presentation, antigen recognition requirements, and T cell activation have provided us with a mechanistic frame-

work allowing the modeling of novel immunotherapies for the treatment of cancer. Conceptually, an effective therapy capable of eliciting a cellular immune response should contain a series of MHC-presented tumor peptide epitopes capable of being recognized by T cells, provide adequate T cell

costimulation for effector cell activation, and be targeted to primary and secondary lymphoid organs rich in naive T cells.

Several reports have previously demonstrated that tumor-specific CTL can be generated that recognize peptide epitopes presented on the tumor cell surface in the context of MHC class I molecules (1–4). Tumors, however, have evolved various mechanisms to escape an immune response (1). Class I or antigen loss variants, secretion of immuno-suppressive agents, or lack of costimulation leading to antigen-specific T cell clonal anergy (5) have all been identified. To overcome these states of immunotolerance, cytokine/ costimulatory molecule–engineered tumor vaccines were generated and shown to be capable of eliciting long-term antitumor immune responses in many cases (6). Effective cytokine gene (i.e., GM-CSF, IL-4, IL-12)–transduced tumor vaccines have been characterized to induce in vivo initial influences of APC, including dendritic cells (DC)[1], with subsequent involvement of lymphocytes (7, 8). For CD4$^+$ T cell priming T cell priming, these APC are likely to ingest tumor Ags for processing and presentation in MHC class II complexes. Presentation of MHC class I–restricted tumor antigens to CD8$^+$ T lymphocytes was classically assumed to be presented by the cell naturally expressing the antigens, such as the tumor cell itself. However, recent evidence suggests that tumor cells are poor APC, and professional APC may be essential for induction of not only CD4$^+$ T cells but also for a CD8$^+$ T cell–mediated antitumor immune response. Huang et al. (9) reported that the in vivo priming of MHC class I–restricted responses involves a transfer of tumor antigens to a host bone marrow–derived DC (BM-DC) and subsequent presentation to CD8$^+$ T cell effectors. Moreover, a correlation between the number of presumptive DC infiltrating a cancer and longer patient survival or reduced frequency of metastatic disease have been observed for a variety of cancer types (10). First described by Steinman and Cohn in 1973 (11), DC have been known to be highly specialized antigen-presenting cells and to be the principal activators of resting, naive T cells in vitro and in vivo (12–15), capable of efficiently transporting antigens from the periphery to lymphoid organs. DC pulsed with ovalbumin (16) or viral peptides (17), lymphoma-derived surface Ig (18), or tumor-derived synthetic epitopes (1a,18a) and then injected as a vaccine have been reproducibly shown to immunize naive animals against a subsequent viral or tumor challenge. Defined tumor-derived T cell epitopes are, however, currently available for study in only a limited number of human tumor types (i.e., melanoma, ovarian, and breast carcinoma [1]).

To investigate the potential utility of DC-based vaccines for the treatment of cancer patients whose tumors express as yet uncharacterized T cell epitopes, we have evaluated the therapeutic efficacy of unfractionated, MHC class I–eluted tumor peptides (19) loaded onto BM-DC as cancer vaccines in murine tumor systems. We show in three different tumor models in two strains of mice that DC pulsed with

tumor peptides induced an MHC class I–restricted, T cell–mediated antitumor immune response that (a) is capable of markedly suppressing or slowing the growth of established tumors, (b) is completely dependent on costimulatory signals, Th1-associated cytokines, and requires both CD4$^+$ and CD8$^+$ T cells, (c) is Th0-like when characterized for the pattern of cytokines elaborated in response to tumor, and (d) confers significantly superior protection when transferred to naive mice if the donor lymphocytes were obtained from mice vaccinated with tumor peptide–pulsed BM-DC coadministered with low doses of rmIL-12.

Materials and Methods

BM-DC Isolation and Peptide Pulsing. Culture of BM-DC has been previously described (1a, 20). Briefly, lymphocyte-depleted mouse bone marrow suspensions, supplemented with rmGM-CSF + rmIL-4 (1,000 IU/ml each, DC media) for 5–8 d leads to the generation of large numbers ($\sim$10^7/mouse) of functional DC as defined by morphology, ultrastructure, phenotype, and strong mixed lymphocyte reaction–stimulating activity. The phenotype of BM-DC typically included high-frequency expression (60–95%) of CD45, CD44, CD11b, CD18, CD80, and CD86, as well as MHC class I and class II antigens. Before use in vaccines, DC were pulsed overnight with crude, acid-eluted tumor peptides from autologous tumors or unfractionated, acid-eluted peptides derived from syngeneic spleens, according to the procedure described below. Peptide-pulsed DC were extensively washed and then irradiated (3,000 rads) before i.v. injection in the tail vein of tumor-bearing animals. In the weakly immunogenic MCA205 and TS/A tumor models, animals were injected three to four times, starting at day 4 or day 8 after tumor establishment and subsequently, every 4 d, with 3–5 $\times$ 10^5 DC pulsed with peptides. Alternatively, in the immunogenic C3 tumor model, animals were injected on days 14, 21, and 28 after initial intradermal (i.d.) tumor inoculation.

Progressively growing tumors (MCA205 and C3 in C57BL/6 mice [B6] [H-2^b] or TS/A in BALB/c [H-2^d]) were surgically removed at day 20–25 ($\sim$150–250 mm^2 in size), and a single-cell suspension was obtained after brief enyzmatic digestion (60 min) with DNase I (0.1 mg/ml; #776785; Boehringer Mannheim Corp., Indianapolis, IN), collagenase (1 mg/ml, 173 U/mg) in AIMV media (GIBCO-BRL, Gaithersburg, MD). Viable cells ($\sim$1–5 $\times$ 10^9/cells) were washed three times in HBSS (Gibco Laboratories), and the cell pellet was treated with mild acid buffer to allow for rapid isolation of tumor-associated peptides, as previously described (19). Briefly, 10 ml of citrate–phosphate buffer, pH = 3.3, was added at room temperature, and cell pellets were immediately resuspended by pipetting and centrifuged for 5 min at 1,000 g. The cell-free supernatant was harvested, and peptides in acid-extracted supernatants were concentrated on activated SepPak C18 cartridges (Millipore Corp., Bedford, MA). The bound material was eluted with 2–3 ml of 60% acetontrile in water and lyophilized to near complete dryness (i.e., 20–50 μl). The peptides were then reconstituted in 1 ml HBSS (GIBCO-BRL) and stored frozen at $-20°$C until used in DC-pulsing experiments. 1 million BM-DC were pulsed with peptides derived from 10^8 to 10^9 tumor cell equivalents in a total volume of 1–2 ml of DC media overnight at 37°C, 5% CO$_2$.

Tumor Cell Lines and Mouse Strains. Female 6–8-wk-old C57BL/6 (B6) and BALB/c (H-2^d) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). MCA205 (H-2^b),

kindly provided by S. A. Rosenberg (National Cancer Institute [NCI], Bethesda, MD) is a methylcholanthrene-induced fibrosarcoma established in B6 mice. TS/A (H-2^d) is a tumor cell line established by P. Nanni (University of Bologna, Bologna, Italy) from the first in vivo transplant of a moderately differentiated mammary adenocarcinoma spontaneously arising in a 20-mo-old female BALB/c mouse (21) and kindly provided by G. Forni (Immunogenetic and Histocompatibility Center, Turin, Italy). The meth A sarcoma is a methylcholanthrene-induced tumor established in H-2^d mice (22). C3 is a tumor cell line obtained by transfecting C57BL/6 mouse embryonal fibroblasts with a plasmid containing the entire genome of the human papilloma virus type 16 (1a). All tumor cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Whitaker Bioproducts, Walkersville, MD), referred to henceforth as CM (culture medium).

In Vivo Tumor Therapy Models. Twice the minimal tumorigenic dose of tumor cells (2 × 10^5 MCA205, 10^5 TS/A, or 2 × 10^6 C3) were injected i.d. in the right flank of mice. Animals bearing day 4– to 8–established MCA205 or TS/A tumors were treated with three to four biweekly (every 4 d) i.v. injections of 3–5 × 10^5 syngenic BM-DC prepared as described above. Animals bearing day 14–established C3 tumors were treated weekly with syngeneic BM-DC (3–5 × 10^5) pulsed with peptide. The mean tumor size was monitored biweekly. All experiments were performed two to three times using individual treatment groups of five mice. Mice were killed when tumors became ulcerated or when they reached a size >250 mm^2.

Depletion Studies with Blocking Antibodies. CTLA4-Ig, a soluble fusion protein composed of the extracellular domain of the human CTLA4 receptor and the Fc portion of a human IgG1C$_r$ chain has been previously described (23) and was kindly provided by G. Davis (Repligen Corp., Cambridge, MA). 50 µg of CTLA4-IgG1 was admixed with DC-MCA205 or DC-TS/A in vitro, and the mixture was then administered in the tail vein of tumor-bearing animals (three to four injections every 4 d). Mice treated with human IgG (Gamimmune N, Miles Laboratories, Inc., Elkhart, IN) were used as controls. Hamster mAbs to mIFN-γ (H22) and mTNF-α (TN3 19.12) were prepared as previously reported (24). The C17.8 (kindly provided by M. Wysocka [25], The Wistar Institute, Philadelphia, PA) is an anti-mp40-IL-12–neutralizing mAb (rat IgG2a), which was administered i.p. (1 mg/mouse) 1 h before the first two DC injections. The 11B11 (26; rat IgG2a) (hybridoma kindly provided by G. Shearer, NCI) is a neutralizing anti-mIL-4 mAb administered by i.p. injections of 300 µg/mouse 4 h before and then 24, 48, and 72 h after each of the first two DC injections. Anti-CD4 (clone GK1.5, rat IgG2b) (27) and anti-CD8 (clone 2.43, rat IgG2b) (28) mAb (kindly provided by M. Brunda, Hoffman-La Roche, Nutley, NJ) were injected i.p. on days 2 and 10 (1 mg/mouse/injection) after tumor inoculation. Depletion of CD4$^+$ and CD8$^+$ T cells was monitored by flow cytometry. Normal rat IgG was administered in negative control groups of animals.

In Vitro Cytokine Release Assays. Retroperitoneal lymph nodes (ipsi- and contralateral from the tumor) and spleens of two mice per group, in two different experiments, were collected after three to four therapeutic applications (days 16–20). Cells were harvested after mechanical disruption of tissue in a culture dish, counted, and resuspended in CM + 50 IU/ml rhIL-2 (Chiron, Emeryville, CA) at a final concentration of 1.5 × 10^6/ml. 200 µl of cells/well were cocultured with 10^5 irradiated MCA205 (5,000 rads) in 96-well flat-bottomed plates. Plates were incubated for

24 h (for C57BL/6 mice) or 36 h (for BALB/c mice) at 37°C, 5% CO$_2$. The supernatants (100 µl) were harvested and assessed in commercial ELISAs for production of mIFN-γ and mIL-4 (Genzyme Corp., Cambridge, MA). The lower limit of sensitivity for each assay was 47 and 30 pg/ml, respectively.

Cytotoxicity Assays. YAC-1 (kindly provided by W. Chambers, University of Pittsburgh, Pittsburgh, PA), or the TAP–deficient T lymphoma RMA-S (kindly provided by H. G. Ljunngren, Massachusetts Institute of Technology, Cambridge, MA) were pulsed with 10 µl of splenic or MCA205-derived peptides and labeled in CM containing Na$_2$^{51}CrO$_4$ (150 µCi/million cells) for 1–2 h at 37°C, washed twice, and resuspended in CM at 2 × 10^5 cells/ml. MCA205 target cells were labeled in the same way. Equal volumes (100 µl) of target (admixed 1:10 with YAC-1 cold target provided to diminish nonspecific LAK activity) and effector cells (i.e., splenic cells after 5-d in vitro restimulation at effector-to-^{51}Cr–labeled-target ratios of 40:1 and 10:1) were plated in triplicate round-bottomed microtiter wells for 4 h at 37°C, 5% CO$_2$. After a 4-h incubation, 100 µl of supernatant was collected, and the percentage of specific ^{51}Cr release was calculated using the following formula: percent ^{51}Cr release = 100 × (cpm experiment − cpm spontaneous release)/(cpm maximum release − cpm spontaneous release), where spontaneous release was that obtained from target cells incubated with medium alone, and maximum release was obtained from target cells incubated in 5% Triton X-100 (Sigma Chemical Co., St. Louis, MO). The specificity of tumor-directed cytotoxicity was evaluated by the addition of mAb specific for H-2K^b (28-13-35; American Type Culture Collection, Rockville, MD), H-2D^b (28-11-55; ATCC), and anti-mCD3 (29B; GIBCO-BRL).

Immunohistochemistry. Mice were killed by asphyxiation with CO$_2$ gas. Tissue from tumor sites was removed and placed in a cryomold filled with OCT-embedding medium (Miles Laboratories Inc.). Tissues were snap-frozen by placing the mold on dry ice. Thin sections (5–7 µm) were cut by cryostat and placed on slides. Slides were stored at −80°C or were immediately stained. Routine hematoxylin–eosin staining was performed on all tissues. Cryostat sections were fixed in cold acetone, hydrated in PBS, and incubated in protein-blocking solution (Immunon; Shandon-Lipshaw, Pittsburgh, PA) for 8 min. They were then incubated overnight at 40°C with rat mAbs directed against mouse CD4 (TIB207; ATCC) and CD8 (TIB210; ATCC). Isotype-matched rat IgG was used as a negative control. Positive tissue controls consisted of frozen sections of spleens from BALB/c and B6 mice. After buffer washes, endogeneous peroxidase activity was quenched with 0.6% H$_2$O$_2$ in methanol. Species-absorbed biotinylated mouse anti–rat F(ab')$_2$ (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was applied to sections incubated with unlabeled primary Ab followed by treatment with streptavidin–peroxidase (Boehringer Mannheim Corp.) and 3'-amino-9-ethylcarbazole (Biomeda Corp., Foster City, CA). Sections were counterstained with hematoxylin and mounted in CrystalMount (Biomeda Corp.).

Statistical Analyses. Fisher's exact method was performed to interpret the significance of differences between experimental groups (presented as mean ± SEM). Significance at 95% confidence limits are presented for individual experiments.

Results

DC Pulsed with Acid-eluted Peptides Derived from Syngeneic Tumors Markedly Inhibits Tumor Progression in Mice Bearing Weakly Immunogenic Tumors. Twice the minimal tumori-

genic dose of tumor cells MCA205 were injected i.d. in the flank of syngeneic C57BL/6 mice on day 0. 4–8 d later, animals bearing established tumors (~10–15 mm^2 to 35 mm^2 in size, respectively) were treated three to four times with biweekly i.v. administration of 3–5 × 10^5 irradiated BM-DC prepulsed with either unfractionated MCA205 tumor– or unfractionated B6 spleen–derived acid-eluted peptides. As depicted in Fig. 1 A, the mean tumor size in the day 4 established tumor group injected with DC pulsed with the relevant MCA205-derived peptides is significantly smaller than the control groups (albumin, DC alone, or DC pulsed with splenic peptides) at all therapeutic time points. Whereas no animals were tumor free after three immunizations, in most cases, the tumor growth was suppressed within 7 d after the first immunization. Indeed, the tumor size remained static for >6 wk. Whereas control animals were sacrificed at day 20, animals receiving DC–tumor peptide therapy survived for 8–10 wk. Most of these antitumor effects can be attributed to the first two DC immunizations (data not shown). Tumor growth was also markedly reduced when the first therapeutic injection of DC–MCA205 was started later (day 8) (Figs. 1 A and 2 B). Essentially identical results were achieved in the TS/A (H-2^d) established tumor model (poorly immunogenic mammary adenocarcinoma) in BALB/c mice (Fig. 1 B). In a more immunogenic model (i.e., C3), tumors established for 14 d were successfully treated with DC pulsed with unfractionated acid-eluted peptides administered on days 14, 21, and 28. All DC–tumor peptide–treated animals exhibited complete and sustained tumor regressions (Fig. 1 C).

Coadministration of Low Doses of Systemic rmIL-12 Modulates DC-mediated Antitumor Effects by Enhancing the Memory Response. Macatonia et al. (29) recently reported that DC produce IL-12 and direct the development of Th1 cells from naive CD4$^+$ T cells in vitro. This finding prompted us to evaluate whether concomitant systemic administration of low doses of rmIL-12 (100 ng provided i.p. every 4 d) would augment the efficacy of DC–tumor peptide therapy in our in vivo models using poorly immunogenic tumors. As shown Fig. 2, A and B, despite a beneficial therapeutic effect of rmIL-12 alone in day 4 (but not day 8) tumor models, the coadministration of IL-12 and DC–MCA205 did not result in a statistically significant improvement over groups treated with DC–MCA205 alone, although the tumor growth curves were reproducibly slower for the DC–MCA205 + rmIL-12 group in three independent experiments. However, DC-MCA205 + rmIL-12 is significantly superior to administration of IL-12 alone ± DC or DC pulsed with irrelevant splenic peptides for the treatment of established day 8 tumors (Fig. 2 B).

To verify that DC–MCA205–induced antitumor effects stem from the activation of the host immune system and to further evaluate the effects of combined IL-12 administration on the in vivo priming of T cells, adoptive transfer experiments were performed. Sera and/or spleens were obtained from day 16–18 treatment groups and injected into sublethally irradiated naive littermates. As shown in Fig. 2 C, neither pooled sera from mice immunized with DC
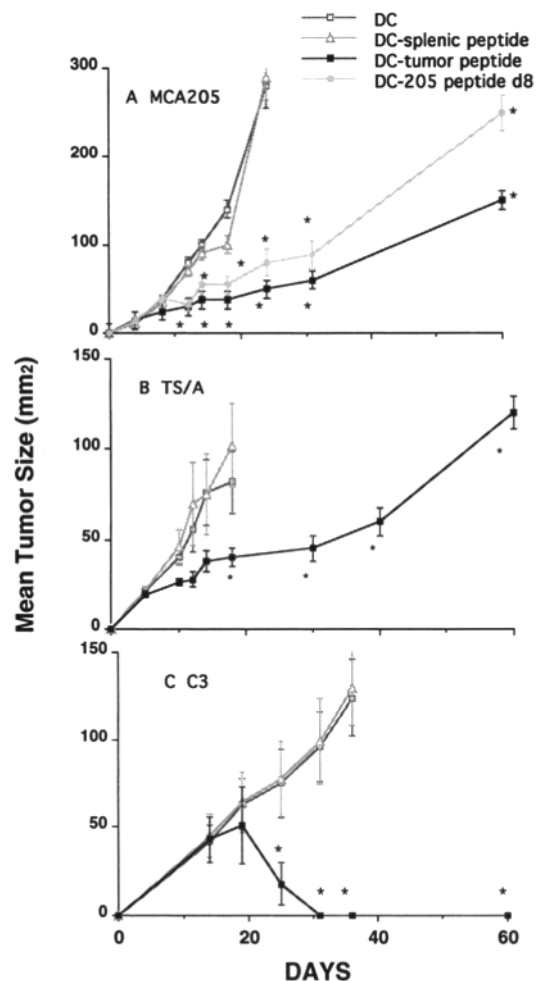

Figure 1. BM-DC pulsed with unfractionated, acid-eluted peptides derived from tumors but not from syngeneic spleens induce potent antitumor effects in vivo in mice bearing day 4–8 weakly immunogenic fibrosarcoma MCA205 or mammary adenocarcinoma TS/A, or day 14 immunogenic C3 tumors. 2 × 10^5 tumor cells MCA205 (A), 10^5 TS/A (B), or 2 × 10^6 C3 (C) were injected i.d. in one flank of C57BL/6 or BALB/c mice, respectively. Tumor-bearing mice were given three to four i.v. immunizations with BM-DC, beginning on day 4 or day 8 and repeated every 4 d (MCA205, TS/A) or every 7 d beginning on day 14 (C3). Before their in vivo injection, the BM-DC were pulsed with acid-eluted tumor peptides (DC-MCA205, DC-TS/A, DC-C3) or splenic peptides (DC-spleen B6 or BALB/c) overnight, or were not pulsed with peptides (DC). DC preparations were irradiated (3,000 rads) and washed to remove unbound peptides before i.v. injection of 3–5 × 10^5 DC/mouse. Mean tumor size ± SE of five mice per group, pooled from three different experiments, are depicted over time. Significant results at 95% (Fisher's exact method) are indicated (*).

alone or DC pulsed with splenic peptides (control groups), nor sera pooled from mice immunized with DC-MCA205 on day 4 or day 8 (from mice depicted in Fig. 1) were capable of transferring significant protective antitumor immunity. All mice were killed at day 15 with primary tumors >250 mm^2 and demonstrable metastatic disease. Conversely, the transfer of 10^7 pooled, cryopreserved splenocytes derived from DC–MCA205 day 4 or 8 treatment groups was reproducibly capable of significantly slowing tumor growth in these naive syngeneic mice (Fig. 2 D)
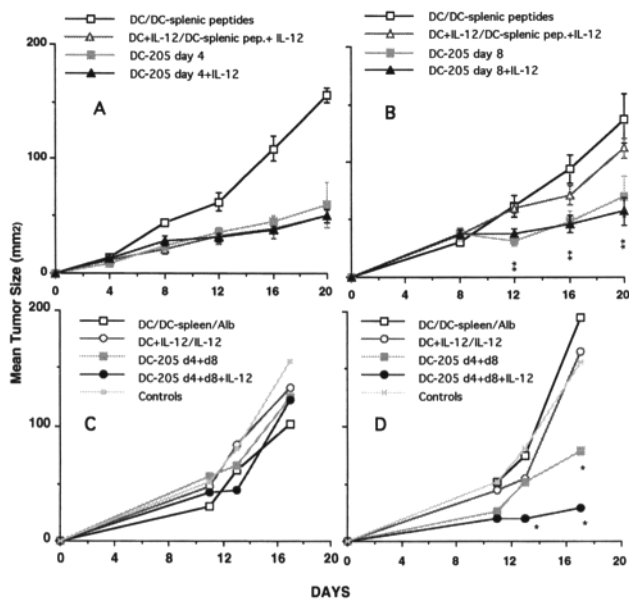
Figure 2. Coadministration of low doses of rmIL-12 does not significantly enhance the DC-MCA205–mediated antitumor response but augments the efficacy of adoptive transfer of immune cells. 50 ng of rmIL-12 was administered i.p. on the day of the DC injections and 1 d after DC injections, starting on day 4 (A) or day 8 (B). Mean tumor size ± SE of five mice per group, pooled from three different experiments, are depicted over time. Significant results at 95% (Fisher's exact method) are indicated (*) and support the therapeutic advantage of DC-MCA205 or DC-MCA205 + IL-12 over DC + IL-12 or IL-12 alone only in the case of the day 8 model. (C) DC-MCA205–mediated antitumor effects are not dependent on humoral immunity. Sublethally irradiated (500 rads) syngeneic mice (day −4) were injected with 200 µl of pooled sera (day −2, day −1) from immunized mice (DC alone ± rmIL-12, DC–spleen ± rmIL-12, or with DC-MCA205 day 4 or day 8 ± rmIL-12, and bled after three to four immunizations), and subjected to a lethal tumor challenge on day 0. Tumor growth was monitored. No significant benefit from the adoptive transfer of sera was observed. (D) DC-MCA205–mediated antitumor response is transferred by splenocytes derived from immunized mice. Sublethally irradiated (500 rads) syngeneic mice (day −4) were injected with 10^7 spleen cells from pooled cryopreserved spleens (day −1) from immunized mice (DC alone ± rmIL-12, DC–spleen ± rmIL-12, or with DC-MCA205 day 4 or day 8 ± rmIL-12, and killed after three to four immunizations) and subjected to a lethal tumor challenge on day 0. Established tumor growth was monitored. Significant results at 95% (Fisher's exact method) are indicated (*).

compared with control groups. Interestingly, the survival was even more prolonged in the groups receiving splenocytes derived from tumor-bearing mice immunized with DC-MCA205 day 4/8 + rmIL-12 compared with those mice immunized with DC-MCA205 on day 4 or 8 alone. There was no additional protective advantage of cotransferring sera along with splenocytes in these groups (data not shown). We conclude that DC pulsed with acid-eluted tumor peptides promote efficient cellular-mediated antitumor immunity and that coadministration of low doses of rmIL-12 may favor the priming of tumor-specific T cells. Analogous results were achieved in the TS/A model in BALB/c mice (data not shown). To test the specificity of the immune response elicited by the DC-TS/A, 10^5 TS/A and 10^5 meth A sarcoma cells were injected s.c. in opposite

flanks of sublethally irradiated BALB/c mice. Whereas the growth of TS/A was inhibited by the adoptive transfer of cells derived from mice immunized with DC-TS/A, there was no impact of this treatment on the growth of the "irrelevant" meth A sarcoma (data not shown).

These in vivo results were reflected in in vitro studies quantifying the specific cytolytic activity of in vivo primed CTL present in the spleens of mice immunized three times with DC–MCA205 or DC–spleen ± rmIL-12. Splenocytes were restimulated for 5 d in vitro with irradiated MCA205 tumors. After this restimulation, the splenocytes from mice immunized with DC–MCA205 and DC–MCA205 + rmIL-12 displayed significantly elevated cytolytic reactivity against MCA205 (Fig. 3, A and B) that was inhibited 50–60% by anti-H-2K^b and H-2D^b antibodies or by anti-CD3 (data not shown). These effector cells did not lyse the murine LAK-sensitive target RMA-S. Splenocytes derived from mice receiving DC–MCA205 + rmIL-12 typically displayed greater cytolytic reactivity than splenocytes from DC-MCA205 alone (Fig. 3 A).
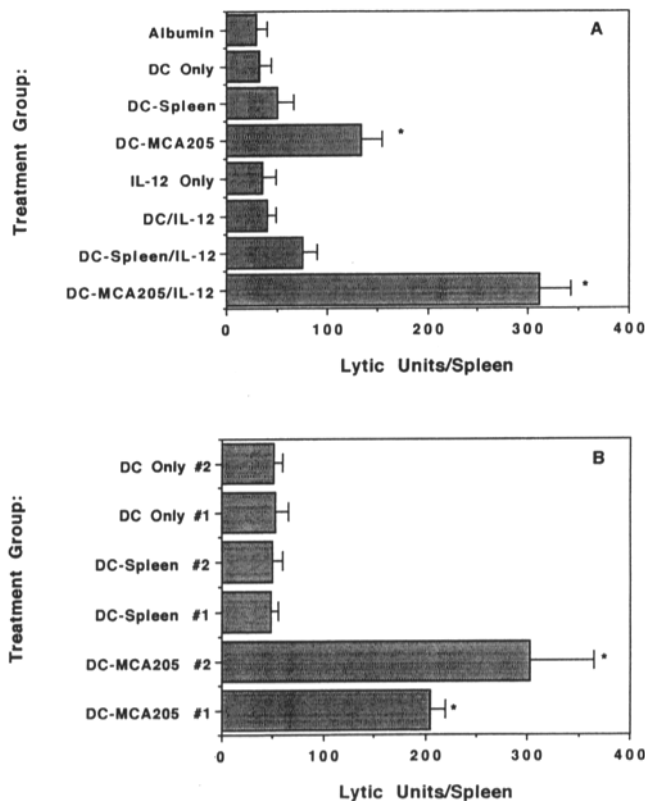

Figure 3. MCA205-specific CTL are generated in the spleens of mice immunized three to four times with DC pulsed with MCA205-derived tumor peptides (DC–MCA205) but not with splenic peptides (DC–spleen) or control DC (DC). Two spleens per group were harvested at day 16–18, with restimulation in vitro for 4–5 d with irradiated MCA205 tumor cells, before use as effector cells in 4-h ^{51}Cr release assays. Targets included ^{51}Cr-labeled MCA205 and the NK/LAK target cell line RMA-S (data not shown). The results of two representative experiments (A and B) are shown. Results are reported in lytic units per spleen against the MCA205 target cell as outlined in Materials and Methods. Significant results at 95% (Fisher's exact method) are indicated (*).

Table 1. *Dendritic Cells Pulsed with Acid-eluted Tumor Peptides Induce a Th0-associated Immune Response in the Draining Lymph Nodes and Spleens in B6 Mice*

Groups	Mean Tumor size (mm$_2$) D23	Nodes		Spleens	
		mIL-4*	mIFN-γ*	mIL-4*	mIFN-γ*
DC or DC-spleen	145 ± 19	5 ± 1	0 ± 0	6 ± 1	643 ± 460
DC or DC-spleen + IL-12	70 ± 20	10 ± 5	17 ± 12	6 ± 1	194 ± 90
DC-MCA205	65 ± 10‡	162 ± 78‡	379 ± 226‡	79 ± 21‡	1,515 ± 143
DC-MCA205 + IL-12	48 ± 5§	24 ± 9§	1,229 ± 702§	38 ± 5§	6,666 ± 760§

Retroperitoneal lymph nodes (ipsi- and contralateral from the tumor) and spleens of two mice per group, in two different experiments, were collected after three to four therapeutic applications (days 16–20). Cells were harvested and cocultured with 10^5 irradiated MCA205 (5,000 rads) in 96-well flat-bottomed plates. Plates were incubated for 24 h at 37°C, 5% CO_2. The supernatants were harvested and assessed in commercial ELISAs for production of mIFN-γ and mIL-4. Mice bearing day 4 MCA205 tumors were treated with either DC, DC pulsed with normal splenic acid-eluted peptides, or DC pulsed with acid-eluted MCA205 peptides. Identical treatment groups were also provided systemic rmIL-12 as indicated in Materials and Methods. Due to the lack of statistical difference between groups treated with DC alone and DC pulsed with acid-eluted splenic peptides (*DC-spleen*), and the same groups provided systemic rIL-12, we have combined their data and report mean and SE in this table.
*pg/ml/2 × 10^6 cells/24 h in Elisa ± SE.
‡Significant at 95% (Fisher's exact method) versus DC/DC spleen.
§Significant at 95% (Fisher's exact method) versus DC/DC spleen + IL-12.

Immunizations with DC Pulsed with Acid-eluted Peptides Enhance Cytokine Release from the Tumor-draining Lymph Nodes and in the Spleen in Response to Tumor. Since cellular immunity appeared to be critical in mediating antitumor effects, we analyzed the pattern of cytokines released in an in vitro recall response to MCA205 tumor by cells derived from the retroperitoneal draining lymph nodes and from the spleens of DC-treated tumor-bearing mice (Table 1). Freshly isolated mononuclear cells were cultured for 24 h in vitro in the presence of MCA205 tumor. The culture supernatant was then analyzed by ELISA for levels of mIL-4 and mIFN-γ production. As expected, a Th1-associated response was observed, with a significant enhancement of mIFN-γ production in the DC–MCA205 treatment groups. Interestingly, levels of the Th2-associated cytokine IL-4 were also dramatically elevated in the same groups. In vivo administration of IL-12 did not significantly alter these in vitro results. Similar results were observed for lymphoid cells derived from TS/A tumor-bearing BALB/c mice (Table 2).

Semiquantitative reverse transcription PCR performed on snap-frozen specimens suggested that DC–MCA205 therapy results in upregulation of messages encoding mIL-4, mIL-5, mIL-10, mIFN-γ, and mp40-IL-12 in situ in the nodes of treated mice (data not shown). Interestingly, no discernible qualitative differences in cytokine production were noted between the ipsilateral nodes and their contralateral counterparts. In contrast to the modulation of cytokine messages in the lymphoid organs of DC–MCA205–treated animals, there was no marked difference in cytokines produced in situ within tumor lesions in mice derived from any of the treatment groups.

Table 2. *Dendritic Cells Pulsed with Acid-eluted Tumor Peptides Induce a Th0-associated Immune Response in the Draining Lymph Nodes and Spleens in BALB/c Mice*

Groups	Mean Tumor size (mm$_2$) D23	Nodes		Spleens	
		mIL-4*	mIFN-γ*	mIL-4*	mIFN-γ*
DC or DC-spleen	124 ± 45	32 ± 15	182 ± 106	224 ± 25	5,485 ± 1,502
DC or DC-spleen + IL-12	79 ± 20	31 ± 23	651 ± 432	237 ± 55	7,563 ± 468
DC-TS/A	27 ± 10‡	268 ± 104‡	5,000 ± 120‡	412 ± 80‡	15,000 ± 376‡
DC-TS/A + IL-12	0	316‡	9,000‡	273	15,000‡

Retroperitoneal lymph nodes (ipsi- and contralateral from the tumor) and spleens of two mice per group, in two different experiments, were collected after three to four therapeutic applications (days 16–20). Cells were harvested and cocultured with 10^5 irradiated MCA205 (5,000 rads) in 96-well flat-bottomed plates. Plates were incubated for 36 h at 37°C, 5% CO_2. The supernatants were harvested and assessed in commericial ELISAs for production of mIFN-γ and mIL-4. Mice bearing day 5 TS/A tumors were treated with DC-based therapies as outlined and reported in Table 1.
*pg/ml/2 × 10^6 cells/36 h in Elisa ± SE.
‡Significant at 95% (Fisher's exact method) versus DC/DC spleen ± IL-12.

Costimulatory Pathways and Th1-associated Cytokines Are Involved in the DC-mediated Antitumor Effects. Our BM-DC cultures (5–8 d in IL-4 + GM-CSF) express high levels of the costimulatory molecules CD80 and CD86 (1a). Since costimulation of T cell–mediated antitumor immunity is critical for tumor rejection in vivo (30; Zitvogel, L., P. D. Robbins, W. J. Storkus, M. J. Maeurer, R. L. Campbell, M. R. Clarke, R. D. Schreiber, C. G. Davis, H. Tahara, and M. T. Lotze, manuscript submitted for publication), we investigated the role of these costimulatory molecules in the DC-MCA205–mediated antitumor effects by admixing and coinjecting DC–MCA205 with the chimeric fusion protein CTLA4-Ig i.v., which functionally blocks

Figure 4. Induction of an effective immune response by tumor peptide–pulsed DC requires CD4+ and CD8+ T cells, costimulation by B7, and IL-12, as well as its elicited cytokines, IFN-γ and TNF-α. For the four panels, the mean tumor size ± SE (five mice/group) over time is shown. The experiment depicted is representative of two experiments performed. Significant results compared with DC pulsed with MCA205 peptides (*solid squares*) at 95% (Fisher's exact method) are indicated (*). The open squares represent DC pulsed with splenic peptides. (*A*) Costimulatory molecules (i.e., CD80 and CD86 expressed on immature BM-DC-MCA205) are critical for promotion of an efficient therapy-induced antitumor immune response: DC-based therapy is completely abrogated by systemic applications of CTLA4-Ig. 50 μg of the chimeric fusion protein CTLA4-IgG1 was admixed with DC–MCA205 in vitro and the mixture administered i.v. on day 4, 8, 12, and 16. (*B*) DC-MCA205–mediated antitumor effects are partially or completely abrogated by in vivo depletion of the Th1-associated cytokines mIFN-γ and mTNF-α, and by anti–mIL-12. 1 mg of neutralizing C17.8 (rat IgG2a anti-mp40-IL-12) or 350 μg of anti–TNF-α + anti–IFN-γ mAb were injected i.p. 1 h before the first and second immunization with DC–MCA205 and on days 3, 10, and 17, respectively. (*C*) DC–MCA205–induced antitumor effects are partially inhibited by in vivo depletion of the Th2-associated cytokine mIL-4. 300 μg of 11B11 (rat IgG2a, anti–mIL-4) was administered i.p. every day starting 4 h before the first DC immunization for 8 d. (*D*) DC-MCA205–induced antitumor effects are completely inhibited by in vivo depletion of either the CD4+ or CD8+ T cell subsets. 1 mg/mouse of neutralizing anti-CD4 or anti-CD8 mAb or both was injected i.p. into tumor-bearing mice 2 d before the first immunization with DC–MCA205 and repeated 10 d later.

CD28/CTLA4-mediated T cell costimulation, on days 4, 8, 12, and 16. As depicted in Fig. 4 *A*, CTLA4-Ig administration markedly reduced the antitumor therapeutic impact of DC–MCA205 treatment, such that it was no longer statistically significant versus control groups.

To assess the relevance of our in vitro cytokine data (Tables 1 and 2) in the therapy-induced antitumor immune response in vivo, we treated the animals with neutralizing antibodies reactive with Th1-derived cytokines (Fig. 4 *B*). The coadministration of mAb anti–TNF-α and anti-mIFN-γ, or anti-mIL-12, completely abrogated DC-MCA205–mediated antitumor effects. However, when the first injection of blocking anti–mIL-12 antibodies was injected after the first immunization with DC–MCA205 was performed, the inhibition of therapeutic effects was not significant, stressing the importance of the first vaccination for priming antitumor CTL. Further immunizations may not be critical to amplify the recruitment of additional prime T cells, but perhaps to maintain or expand memory T cells, which are not critically dependent on antigen-specific costimulation. Since high levels of mIL-4 production were measured in the draining lymph nodes and the spleens of the mice treated with and responding to three to four DC–MCA205 or DC-TS/A peptide immunizations, we investigated whether this Th2-associated cytokine affected the observed antitumor therapeutic response in vivo. The 11B11 (anti–mIL-4) mAb was injected before the first DC–MCA205 injection and on a daily basis thereafter for 8 d. Although tumor volumes were greater in 11B11-treated mice, the difference did not appear significant at day 10 after tumor inoculation, compared with the control group receiving DC–MCA205 therapy and injected with normal rat serum (Fig. 4 *C*). However, by day 16, the difference started to appear significant at 95% (Fisher's exact method). This suggests that therapy-induced IL-4 may not play a dominant role in the priming of a specific antitumor immune response by the DC–MCA205, but that it may be implicated in maintaining and/or amplifying the induced antitumor immune responses (Tables 1 and 2). As previously shown (31), IL-4 can synergize with IL-12 in enhancing the proliferation of antigen-specific Th1 clones. In our models, IL-4 may cooperate with coinduced Th1–associated cytokines in promoting antitumor immunity.

CD8+ T Cells as well as CD4+ T Cells Are Required for the Priming and/or the Effector Phase of the DC-mediated Antitumor Immune Response In Vivo. We have previously demonstrated in our adoptive transfer experiments (Fig. 2 *D*) that DC–MCA205 induced cell-mediated immune response, protecting naive animals from a subsequent MCA205 challenge. Further, DC, arguably the best APC for priming native CD4+ T cells in vivo (11–15), elicited potent antitumor CTL responses (Figs. 3, *A* and *B*) in our in vitro studies. We next implemented in vivo depletion studies with neutralizing anti-CD4 and/or anti-CD8 antibodies, performed early after tumor inoculation, in order to assess the relative contribution of CD4+ and CD8+ T cells in the observed therapeutic effects. Both T cell subsets are implicated in DC–MCA205–induced antitumor immunity since
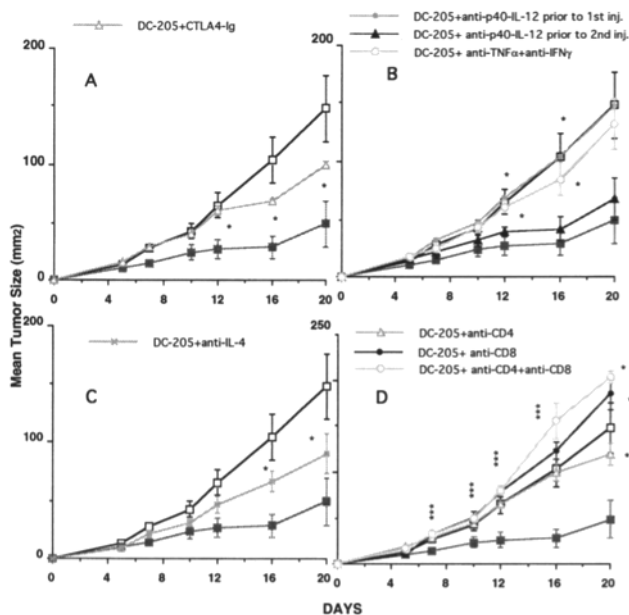
both anti-CD4 or anti-CD8 antibodies abrogated the anti-tumor therapeutic response (Fig. 4 D). The inhibition was significantly greater (Fisher's exact method, at 95%) by day 16, when both anti-CD4 and anti-CD8 antibodies were added, compared with that achieved with either one alone, suggesting a cooperativity between both subsets of T cells in the DC–MCA205–mediated antitumor effects.

Immunohistochemistry analysis documented a marked perilesional and intratumoral infiltration of CD8+ T cells (and to a lesser extent of CD4+ T cells) at day 16 in the groups treated with DC–MCA205 (data not shown), but not in those treated with DC pulsed with irrelevant peptides.

Discussion

Since Coley's first attempts to design therapeutically relevant tumoricidal cancer vaccines (31a), significant insight into those parameters critical to the induction and maintenance of an antitumor immune response has been gained. The current consensus is that (a) T cells can recognize tumor peptides derived from cellular antigens (1 and 1a, 2–4, 32), (b) efficient priming of antitumor immune responses results from collaboration of APC and tumor-specific CD4+ and CD8+ T cells, and (c) DC are the most potent APC for priming antigen-specific T cells in vivo and in vitro (11–15). Strategically located at sites of antigen exposure, DC efficiently internalize and process/present soluble antigen. This is particularly true for DC progenitors, such as those generated in bone marrow cultures containing GM-CSF (33–35).

The concept of using tumor antigen–pulsed APC as a means to elicit antitumor immunity is not novel (1 and 1a, 36). DC are ubiquitous in tissue and in blood, but their ex vivo expansion in large numbers has been difficult, until the recent descriptions of methods to generate cultured DC (20, 35, 37–40). Previous studies have, indeed, demonstrated the capacity of vaccines to elicit protective antitumor immune responses in naive animals using various forms of tumor antigens and diverse types of normal and transformed APC (18, 18a, 41, 42). The novelty of the present study is the use of unfractionated, acid-eluted tumor peptides derived from tumors grown in vivo, rather than synthetic peptides, in conjunction with DC grown in IL-4 + GM-CSF (1a) to serve as a therapy for established tumors. This protocol may effectively translate into human clinical trials designed to treat patients with diverse tumor histologies.

Although some genes encoding specific tumor-associated antigens recognized by CD8+ T cells have been recently cloned and the relevant antigenic peptides have been identified (1a, 2–4), the vast majority of the histologic subtypes of cancers express as yet undefined tumor epitopes. Further, an optimal host antitumor T cell response may require coordinate oligoclonal effector populations for the recognition of a broad spectrum of tumor epitopes rather than responses restricted to a single tumor-associated determinant (1a). Given such complexities, we have evaluated whether

unfractionated tumor-eluted peptides (containing multiple presumptive T cell epitopes) derived from autologous tumors of different histology and mouse strains can elicit efficient antitumor immunity when presented in vivo by syngeneic BM-DC. We have demonstrated that DC loaded with acid-eluted tumor peptides can drive a Th0-associated immune response in vivo, leading to the suppression of growth of poorly immunogenic tumors.

Although our method for generating tumor-derived epitopes effectively extracts MHC class I–bound peptides from the tumor cells (19), it is likely that MHC class II–bound tumor peptides or cell-associated proteins may also be eluted. Indeed, each of the tumor cell lines evaluated expressed low to moderate levels of MHC class II when grown in vivo (data not shown). DC priming of class II–restricted antitumor immune responses is circumstantially supported by our ability to significantly reduce the therapeutic efficacy of DC–MCA205 in tumor-bearing animals by depletion of CD4+ T cells (Fig. 4 D). We are currently evaluating the specific impact of affinity-purified MHC class I– and class II–derived tumor peptides in each of our tumor models. Of interest, acid-elution of peptides (which does not result in significant cellular lysis) yielded a superior immunogenic source of DC-presented epitopes than peptides extracted from cells by repeated cycles of freeze–thaw lysis (data not shown). This latter method presumably results in an unsuitably complex mixture of peptides that may be further complicated by the release of intracellular proteases. Such peptide complexity would be likely to dilute the effective loading of a sufficient threshold density of relevant T cell epitopes into MHC molecules expressed by DC.

Preliminary studies stress that DC pulsed with specific peptides injected i.v. do not traffic to the tumor itself (36). Rather, DC injected i.v. localize within the reticuloendothelial system (liver, spleen, and lymph nodes). Indeed, the tumors expressed the same cytokine pattern (IFN-γ and IL-10 mRNA in reverse transcription PCR, data not shown), in effective treatment groups and in control groups. Conversely, in tumor-bearing mice treated with three to four i.v. injections of DC pulsed with acid-eluted tumor peptides, the ipsi- and the contralateral lymph nodes displayed similarly elevated production of both Th1- and Th2-associated cytokines compared with controls, supporting the systemic effects of the DC-based therapy. This result may support a molecular dialogue between DC–T–B cells within lymph nodes involving DC-synthesized cytokines and costimulatory molecules, resulting in the induction of an antitumor immune response.

In vitro data suggest that DC produce IL-12 and direct a Th1 immune response in naive CD4+ T cells (29). Further costimulation of T cells with B7-1, IL-6, and IL-12 (all expressed by DC) is sufficient to generate antigen-specific CTL from naive splenocytes in a mixed lymphocyte tumor culture (43). We were able to demonstrate a specific class I–restricted cytolytic activity from the spleens of mice vaccinated with DC pulsed with relevant tumor peptides (Fig. 3), suggesting that a CTL response was elicited by the transferred DC in vivo.

The requirements for IL-12 and the B7/CD28 pathway in the therapeutic effects of DC vaccines were supported by our ability to neutralize the effectiveness of therapy by injection of CTLA4-Ig and neutralizing Ab anti–mp40–IL-12 in vivo. IL-12 is a potent inducer of IFN-γ and TNF-α production by both NK cells and T cells (44). IFN-γ and TNF-α are Th1-associated cytokines critically involved in the development of cell-mediated immune response (45). The neutralization of both cytokines totally abrogates the DC-induced antitumor response. These two cytokines simply account for the role of IL-12 in facilitating the observed immunoreactivity. Interestingly, when the anti–mIL-12 Abs were administered after the first immunization with DC pulsed with the relevant peptides, rather than before the first immunization, the DC-induced antitumor effects were not significantly affected, suggesting that as few as 5×10^5 DC/mouse can prime a sufficient number of naive T cells to affect tumor growth and that subsequent immunizations may play a role in amplifying or maintaining these "memory" antitumor T cells.

Increases in IL-4 production from draining lymph nodes and spleens were noted in groups of mice receiving three to four in vivo immunizations with DC pulsed with tumor peptides compared with DC pulsed with irrelevant peptides. This observation was not correlated with the size of the tumor but rather with the clinical response to treatment with DC pulsed with the relevant tumor peptides. Multiple vaccinations of antigen resulted in the progressive development of a Th2-associated response in addition to the Th1-associated immune response elicited during antigen priming. Indeed, the 11B11 neutralizing anti–mIL-4 Abs were not able to significantly block the initiation of a DC peptide–induced antitumor immune response when injected by day 4. The potential immunologic role of a late humoral response cannot be ruled out, however, since the 11B11 reagent eventually significantly inhibited the DC-MCA205–mediated antitumor immune response by day 16.

The antitumor therapeutic efficacy of peptide-pulsed DC was comparable, regardless of the tumor model and the mouse strain examined (C57BL/6 [H-2^b] or BALB/c [H-2^d]), and stresses the importance of both CD4$^+$ and CD8$^+$ T cells in the immunity induced by DC pulsed with class I–eluted tumor peptides. Mechanistically, we propose that BM-DC pulsed with tumor peptides migrate to lymph nodes and secrete or induce the secretion of IL-12, thereby driving a CD4$^+$ Th1-associated immune response. Such responses lead to the generation of IFN-γ and TNF-α, as well as, presumably, GM-CSF, IL-3, and other cytokines released by activated Th cells, which in turn may activate other APC in a positive feedback paracrine loop (upregulation of IL-12 and CD80/CD86 after CD40-gp39 ligation

[46]). DC may present class I–eluted peptides directly to CD8$^+$ T cells, which may also be activated after help provided by the DC-primed CD4$^+$ T cells. An enhanced CD8$^+$, and to a lesser extent, CD4$^+$, T cell infiltrate was reproducibly found in the peritumoral area as well as inside the tumor lesion in animals treated with DC pulsed with tumor peptides.

Of note, whereas in vivo antibody neutralization studies clearly support the requirement of IL-12 in the effectiveness of DC–tumor peptide–based therapy, no additional statistically significant advantage was observed with systemic coadministration of rIL-12, suggesting that DC-based therapies elicit sufficient IL-12 for clinical impact. Alternatively, the dose of systemic rIL-12 that we used may be inappropriate for the observation of any additional therapeutic benefit, a hypothesis that we are currently evaluating. Noguchi et al. (47) studied the influence of IL-12 on p53 peptide vaccination against established meth A sarcoma. They could successfully treat mice bearing established day 7 tumors by immunizing with a specific synthetic tumor peptide plus adjuvant only when IL-12 was coadministered at very low doses (4 ng/mouse/wk) allowing for induction of a specific CTL response. At higher doses, IL-12 suppressed the generation of specific antitumor CTL. Interestingly, in our models, while not reflected in enhanced therapeutic efficacy in situ, an enhanced tumor-specific CTL response could be elicited (Fig. 3 A), and the immunity transferred to naive animals was more potent when lymphocytes were derived from mice immunized with DC-MCA205 + IL-12 compared with DC-MCA205 alone. Thus, systemic administration of IL-12 appears to positively influence the quality or nature of the resultant cellular immune response. Notably, IL-12 (at the dose used) alone is an efficient antitumor agent in mice bearing early day 4 MCA205 (Fig. 2 A), whereas in later day 8 tumor models, it has very little effect unless coadministered with DC-MCA205 (Fig. 2 B).

In summary, 5×10^5 DC pulsed with acid-eluted tumor peptides significantly impair established tumor growth. The number of DC and the dose of peptides required for optimal therapeutic index remain to be defined. It is possible that the immunogenicity of peptide-pulsed DC may be further augmented by ex vivo culture of DC with cytokines in addition to IL-4 + GM-CSF or upon the engineering of cytokine genes into autologous DC, resulting in the paracrine delivery of cytokines at the site of DC–T–B cell clusters in lymphoid tissues. Systemic administration of cytokines such as IL-2 may also allow for further dissemination and expansion of tumor-specific T cells elicited by these DC vaccines.

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