Local Administration of Dendritic Cells Inhibits Established Breast Tumor Growth: Implications for Apoptosis-inducing Agents

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ABSTRACT

Dendritic cells (DCs) can efficiently acquire foreign antigen(s) from apoptotic cells and induce MHC class I-restricted, antigen-specific CTLs. An accumulation of DCs within solid tumor masses in situ has been associated indirectly with a more favorable prognosis. Therefore, DCs may offer an efficient means for triggering immune responses within tumors, particularly in those masses containing significant apoptosis. We examined whether delivery of DCs could, alone, impact on the progressive growth of a tumor with a relatively high apoptotic index. We detected significant early apoptosis within the mass of a s.c. growing murine MT-901 breast carcinoma. DCs could efficiently engulf MT-901 tumor apoptotic cells in vitro. Intratumoral injections of syngeneic but not allogeneic DCs resulted in significant inhibition of MT-901 tumor growth. Histological examination of the tumor revealed intense mononuclear cell infiltration during and after DC injections. Tumor growth inhibition was relatively radiosensitive and dependent on host-derived CD8+ T cells. The baseline level of tumor apoptosis could be increased substantially by tumor necrosis factor α administration, leading to a greater DC-mediated antitumor effect. The antitumor effect could also be enhanced by first pulsing DCs with the foreign helper protein, keyhole limpet hemocyanin, prior to intratumoral delivery and combining it with the systemic administration of interleukin 2. Splenocytes from treated animals showed heightened levels of specific CTL activity and production of cytokines. The level of in situ tumor apoptosis appears to play a critical role in DC-mediated antitumor effects. The potential implication of these findings in DC-based tumor therapy strategies is discussed.

INTRODUCTION

DCs are potent antigen-presenting cells that can both elicit primary and boost secondary immune responses (1–3). Since their original identification by Steinman (1), much attention is now being focused on the role of DCs in eliciting antitumor immunity and in potential therapeutic applications. In this regard, DCs pulsed with defined tumor-associated peptides or proteins have been shown to elicit potent antitumor T-cell responses both in vitro and in vivo (3–5). We have reported that murine DCs can efficiently present antigens associated with whole tumor cell lysates to naive and primed T cells in vitro and can elicit antitumor immunity resulting in tumor regression in vivo (6, 7). Moreover, initial clinical trials involving DC-based immunization of patients with tumors of hematological (8) or solid tumor (9, 10) origin have shown promise by generating antitumor T-cell reactivity as well as, in some cases, by resulting in partial and complete tumor responses. There is also indirect evidence that suggests the infiltration of solid tumor masses with greater numbers of DCs in situ is associated with better prognosis (reviewed in Ref. 11). Whether or not this observation directly reflects the induction of an immune response of beneficial consequence in these patients remains to be determined.

It has been shown recently that immature DCs can efficiently acquire antigen from apoptotic cells and induce MHC class I-restricted, antigen-specific CD8+ CTLs (12). This finding adds additional support to the concept that DCs may play the predominant role in “cross-priming” events for the elicitation of an immune response in vivo (12, 13). Albert et al. (14) have shown further that the process of phagocytosis of apoptotic cells requires cell surface expression of α, β, and CD36 molecules by the immature DCs. Our recent studies have demonstrated that bone marrow-derived DCs in early culture are highly active at engulfing high-molecular-weight dextran particles in vitro (15). Because of these findings, it is conceivable that DCs may offer an efficient means for triggering immune responses in situ within tumors, particularly in those masses containing a significant baseline level of apoptotic cells.

Our preclinical and clinical therapeutic studies have involved the administration of DCs primed with whole tumor lysates (6, 7, 16, 17). In breast cancer, as an example, this approach is difficult, because only rarely has it been possible to isolate enough viable tumor cells from an individual to produce the vaccine. Thus, we have focused our efforts on designing alternative strategies to overcome this potential limitation in DC-based tumor vaccine development. In the present study, we evaluated the effect of i.t. injections of bone marrow-derived DCs on the s.c. growth of the murine MT-901 breast tumor, which we show has a prominent baseline level of early apoptosis. We demonstrate that DCs can efficiently uptake apoptotic MT-901 tumor cells and that local injections of DCs alone can result in regression of this breast tumor in vivo, which is dependent on host CD8+ T-cell immunity. Of further importance, the in vivo administration of a tumor apoptosis-inducing agent, TNF-α, can enhance the therapeutic efficacy of DCs delivered locally at the site of established tumor.

MATERIALS AND METHODS

Animals. Six- to 8-week-old female BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed at the Animal Maintenance Facility of the University of Michigan Medical Center. The animals were used for experiments at 8–10 weeks of age.

Medium and Cytokines. CM consisted of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mM nonessential amino acids, 1 μM sodium pyruvate, 100 μg/ml streptomycin, 100 units/ml penicillin, 50 μg/ml gentamicin, and 0.5 μg/ml fungizone, all from Life Technologies, Inc. (Grand Island, NY) and 5 × 10^-3 M 2-mercaptoethanol from Sigma Chemical Co. (St. Louis, MO). Recombinant cytokines were used at the following concentrations, diluted in CM: recombinant murine GM-CSF, 10 ng/ml (specific activity, ≈ 5 × 10^6 units/mg) from Immunex Corp. (Seattle, WA); recombinant murine IL-4, 10 ng/ml (specific activity, 2.8 × 10^8 units/mg) from Schering-Plough Research Institute (Kenilworth, NJ); and recombinant...
human IL-2, (specific activity, 18 × 10^6 IU/mg) from Chiron Corp. (Emeryville, CA). Recombinant human TNF-α (specific activity, 8.2 × 10^6 units/mg) from Knoll AG (Ludwigshafen, Germany) was administered to tumor-bearing mice at a single dose of 6 μg i.v.

**Tumors.** MT-7 is a cultured murine tumor cell line derived from a dimethylbenzanthracene-induced mammary carcinoma in the BALB/c strain (18). A subline, denoted MT-901, was derived from an early in vivo passage of cultured MT-7 tumor injected s.c. This tumor is weakly immunogenic and expresses MHC class I (but not MHC class II) molecules. Tumors were maintained in vitro followed by one in vivo passage by s.c. injection in syngeneic mice prior to use. Tumor cell suspensions were prepared from solid tumors by enzymatic digestion in 40 ml of RPMI 1640 containing 8000 units of collagenase (type III; Sigma) for 18–24 h at 37°C, 65 rpm. The digest was then filtered over 100-nylon mesh (Nytex; TETKO Inc., Briarcliff Manor, NY) and washed three times with brief incubations in HBSS (Life Technologies, Inc.). Renca is an immunogenic murine renal cell carcinoma of spontaneous origin in the BALB/c strain (19, 20).

**Detection of Apoptotic Cells.** Suspensions of MT-901 tumor were prepared at day 8 after the s.c. injection of 5 × 10^4 viable cells in BALB/c mice and were analyzed for cells undergoing apoptosis using a standard FACS assay (R&D Systems, Inc., Minneapolis, MN), which detects binding of annexin V-flourescein and exclusion of propidium iodide (annexin V/PI assay; Refs. 12, 21, 22). Tumors of mice receiving systemic TNF-α were examined at 24 h after treatment.

In additional studies, DCs that had engulfed apoptotic tumor cells were examined by transmission electron microscopy. DCs were fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 3 h at 4°C. After being washed twice in buffer, the samples were postfixed in 2% OsO4 in buffer for 1 h at room temperature. The cell pellets were washed 2 times in buffer and dehydrated in increasing concentrations of alcohol for 10 min each to final dehydration in two washes of propylene oxide. The samples were infiltrated with increasing concentrations of epoxy resin-propylene oxide and finally embedded in pure epon. Thin sections were obtained on an American Optical Ultracut ultramicrotome, stained with uranyl acetate and lead citrate, and viewed on a Philips 400T electron microscope.

**Splenocytes.** Spleen cells obtained from naive BALB/c mice were treated with ammonium chloride-potassium lysin buffer (0.83% ammonium chloride, 0.1% KHCO₃, and 0.004% EDTA) for 1 min to deplete erythrocytes and were washed twice with HBSS. They were then enumerated and resuspended in HBSS for injection.

**Generation of Bone Marrow-derived DCs.** Erythrocyte-depleted mouse bone marrow cells from flushed marrow cavities of femurs and tibias were cultured in CM supplemented with 10 ng/ml GM-CSF and 10 ng/ml IL-4 at 1 × 10^6 cells/ml as described previously (15, 23). On day 3, DCs were harvested by gentle pipetting and were resuspended at 5 × 10^6 cells/ml in CM. Three ml of the DC suspension were overlayed onto three ml of a 14.5% (by weight) metrizamide (Sigma)-CM solution in a 15-ml centrifuge tube. The resulting gradient was centrifuged at 2000 rpm, brake off, 4°C, for 15 min. The low-density interface containing the DCs was collected by gentle pipette aspiration. The DCs were washed twice with HBSS, enumerated, and resuspended in HBSS for injection.

**Antigen Pulsing of DCs.** In some experiments, DCs were pulsed with KHL (M, 350,000/400,000 subunits, endotoxin-free; Calbiochem-Navabiochem Corp., San Diego, CA) at 50 μg/ml for 18 h.

**In Vivo Treatment of s.c. Tumor.** BALB/c mice received 5 × 10^6 viable MT-901 tumor cells s.c. on day 0. In some experiments, groups of mice also first received total body irradiation with 500 rad before tumor injection. Except where specifically indicated, all of the mice were then injected on days 3, 10, 17, and 21 with 1 × 10^6 DCs (or normal splenocytes) in two 25-μl i.t. injections. A control group received HBSS alone. In other experiments, mice with more established MT-901 tumor received DCs i.t. on days 6, 14, and 20 in combination with i.v. TNF-α on days 5 and 13. Control mice received DC, TNF-α, or HBSS alone. The size of the tumor was assessed in a blinded, coded fashion at least twice weekly and recorded as tumor area (in mm²) by measuring the largest perpendicular diameters with calipers, as described previously (24). Data are reported as the average tumor area ± SE.

**Allogeneic DC Injection.** Normal BALB/c mice were injected s.c. in the right flank with 1 × 10^6 MT-901 tumor cells. At day 14 after injection, the mice received i.t. injections with either 2 × 10^6 unpulsed allogeneic DCs in 50 μl (derived from C57BL/6), 2 × 10^6 unpulsed syngeneic DCs (derived from BALB/c), or HBSS. The tumor size was measured as described above.

**Depletion of CD8⁺ T cells and Treatment of s.c. Tumor.** BALB/c mice were depleted of CD8⁺ T cells by 200-μl i.v. injection of anti-CD8 (2.43, rat IgG2b) monoclonal ascites antibody (American Type Culture Collection, Rockville, MD) on days 0, 7, 14, and 21, as described previously (25). Control mice received rat IgG (Sigma) for isotype control of antibody function. The efficacy of depletion was analyzed by FACS and determined to be 99–100% effective (Ref. 25; data not shown). On day 0, all of the mice received 3 × 10^6 viable MT-901 tumor cells s.c. Mice receiving either anti-CD8 or rat IgG antibody were then also injected on days 3, 10, 17, and 21 with 1 × 10^6 DCs in two 25-μl i.t. injections. Control groups received HBSS injections. The size of the tumor was assessed in a blinded, coded fashion at least twice weekly and recorded as tumor area (in mm²) by measuring the largest perpendicular diameters with calipers, as described previously (24). Data are reported as the average tumor area ± SE.

**Treatment of s.c. Tumor with KHL-pulsed DCs.** Normal BALB/c mice received 5 × 10^6 viable MT-901 tumor cells s.c. on day 0. The mice were then injected on days 7, 10, 13, 17, and 20 with 2 × 10^6 DCs in a 50-μl i.t. injection. Control groups of mice received either unpulsed DCs (2 × 10^6/50 μl), normal splenocytes (2 × 10^6/50 μl), or HBSS alone. IL-2 was given i.p. twice daily at 60,000 IU in 0.5 ml of HBSS for 2 days after each treatment. The size of the tumor was assessed, and survival was followed as recorded as the percentage of surviving animals over time (in days).

At day 120 after tumor injection, the mice that had experienced complete tumor elimination were rechallenged with 1 × 10^6 viable MT-901 tumor cells in the left flank and 5 × 10^6 Renca tumor cells in the right flank. Tumor size was then monitored as described above.

**Cytotoxicity and Cytokine Assays.** At day 40 after tumor injection, mice that had experienced complete tumor regression were killed to harvest the spleen. Erythrocyte-depleted splenocytes (5 × 10^6 cells/ml) were cultured in vitro with UVB-irradiated MT-901 tumor cells (2.5 × 10^6 cells/ml) in a 150-cm² flask for 5 days. On day 1, recombinant human IL-2 was added at 120 IU/ml. On day 5, the cells were collected, and dead cells were removed by density gradient. The resulting viable cells were then tested for specific cytotoxicity in a standard 4-h [3]Cr-release assay, as described previously (7). Percentage specific cytotoxicity was calculated as 100 × [(experimental release − spontaneous release)/(maximal release − spontaneous release)]. LUs were then calculated as number of effector cells/l × 10^6 cells to achieve 20% lysis (LUs/10^6 cells).

Aliquots of splenocytes (2 × 10^6 cells/ml) were also cultured for 48 h in vitro with 4 × 10^6 UVB-irradiated MT-901 or Renca tumor cells in 24-well culture plates. Culture supernatants were collected for measurements of murine IFN-γ and GM-CSF release by standard ELISA (PharMingen).

**Histological Analysis.** Tumor and the surrounding rim of normal skin and underlying connective tissue, at days 12, 19, and 24 after tumor injection, were excised under strict sterile control and treated mice and were submitted for histological processing. The paraffin-embedded tissues were sectioned at 4 μm and stained with H&E. Slides of sectioned tissues were prepared and evaluated by a pathologist (B. J. N.).

**RESULTS**

**Measurement of Tumor Apoptosis.** We first assessed the level of baseline apoptosis in a series of s.c. growing murine tumors, including chemically induced sarcomas and a breast carcinoma. We quantitated the level of apoptosis of the dispersed solid masses by FACS analysis using the annexin V/PI assay, as described previously (12, 21, 22). In our initial screening studies, a substantial proportion (32% and 41%) of two separately harvested MT-901 breast tumors represented cells undergoing early apoptosis after s.c. injection of an initial suspension of viable single cells (Fig. 1). In contrast, the sarcomas demonstrated a relatively low baseline level of apoptosis (~4–8%) and were resistant to treatment by DCs alone administered i.t. (data not shown). These sarcomas nonetheless were inherently weakly immunogenic, similar to that of the MT-901 breast tumor, and likewise could elicit antigenic immunity in vivo, particularly when lysates were prepared. 

229
DCs inhibit breast tumor growth in situ

Fig. 1. Detection of cells undergoing apoptosis within 8-day s.c. MT-901 tumors. Tumor cell suspensions were made and analyzed by FACS for annexin V-FITC (ANN) and propidium iodide (PI)-stained cells as described in "Materials and Methods." The two histograms shown represent tumors obtained from two separate mice. The upper right hand quadrant, ANN\(^+\)/PI\(^-\); the lower right hand quadrant, ANN\(^+\)/PI\(^-\) staining cells; the latter is indicative of cells in the early phases of apoptosis.

Fig. 2. Bone marrow-derived DCs efficiently engulf apoptotic MT-901 breast tumor cells. Transmission electron microscopy demonstrates the presence of an intact apoptotic tumor cell within the DC at 15 h.

and pulsed onto bone marrow-derived DCs and used as the immunogen (7, 16).

**i.t. Injections of DCs.** In our earlier studies (7), immunization of mice with DCs alone failed to impact significantly on the growth of MT-901 mammary tumor located at either distant s.c. or distant pulmonary sites. To overcome the potential requirement for large numbers of DCs to first effectively traffic to and then persist within a solid tumor mass for a sufficient period to phagocytose apoptotic cells, we examined the effect of local delivery by direct i.t. injections of bone marrow-derived DCs alone. As shown in Fig. 2, bone marrow-derived DCs were highly efficient at engulfing whole, apoptotic MT-901 tumor cells in vitro. Mice with palpable, s.c. MT-901 mammary tumor received four courses of DCs i.t. on days 3, 10, 17, and 21 after tumor injection. As shown in Fig. 3, significant tumor growth inhibition was achieved. By day 21, tumor size in the DC-treated group averaged about 40 mm\(^2\) compared with those of >120 mm\(^2\) in untreated, control mice (P < 0.01). In a series of separate experiments, cohorts of mice that experienced complete tumor regression after DC treatment (with an overall cure rate of 20% based on 5 of 25 mice rendered completely disease-free) were rechallenged s.c. between 6 weeks and 10 months with a lethal dose (2 mice rendered completely disease-free) were rechallenged s.c. after DC treatment (with an overall cure rate of 20% based on 5 of 25

**Immunological Assessment of the Antitumor Effect of i.t.-administered DCs.** Histological examination of skin samples was then performed on all of the tumors removed after a second (day 12), third (day 19), and fourth (day 24) i.t. administration of DCs. Fig. 6 depicts the results at day 24. At low- and high-power views, tumors from control, HBSS-injected mice were extremely large with central necrosis and extensive surrounding cohesive clusters of viable malignant cells (i.e., with enlarged hyperchromatic and pleomorphic nuclei with irregular nuclear membrane and nucleoli) intermingled by polymorphonuclear leukocytes, but only rare lymphocytes (Fig. 6, A and D). At day 24, tumors removed from total body-irradiated mice that were injected with DCs i.t. exhibited prominent collections of large, viable malignant cells with focal areas of necrosis and polymorphonuclear leukocytes but with minimal mononuclear cell infiltration (Fig. 6, B and E). Tumors removed from nonirradiated mice that were injected with DCs i.t. showed early evidence of only a few viable tumor cells identified with minimal necrosis but moderate peritumoral lymphocytic infiltration. After the fourth DC injection (at day 24), only rare viable tumor cells could be identified among the extensive mononuclear cell infiltrate that generally had replaced the normal upper and deep dermis (Fig. 6, C and F). Focally, small gland formation was observed among the MT-901 tumor cells in which surrounding lymphocytes were seen in close proximity. No such evidence of differentiation by these tumor cells was seen in any of the above two control groups.

To define further the nature of the host-derived lymphoid component, we selectively depleted CD8\(^+\) T cells in mice by the systemic administration of specific monoclonal antibody, as described previously (7, 25). Similar to our earlier findings reported with whole tumor lysate-pulsed DC immunizations, which demonstrated a predominant role of CD8\(^+\) T cells (7), removal of this immune cell subset significantly reduced the capacity of DCs injected i.t. to inhibit the growth of the MT-901 mammary tumor (Fig. 7). In additional studies (not shown), sublethal (500 rad) total body irradiation of mice before tumor injection and DC administration was also found to eliminate the antitumor effect of DC injections; all of the treated tumors continued
to grow unabated similar to those in the control mice receiving HBSS alone, which corroborated our histological findings (Fig. 6).

Enhancement of Tumor Apoptosis and DC Antitumor Effect by TNF-α. We next evaluated whether increasing the level of apoptosis within the MT-901 tumor in vivo could augment the antitumor efficacy of DC administered i.t., particularly against a larger tumor mass. A single i.v. injection of 6 μg of TNF-α could increase the level of apoptosis in the MT-901 tumor to >60% of the mass (Fig. 8). Mice with well-established s.c. MT-901 tumor were then treated on day 6 with DCs alone i.t. after prior systemic administration of TNF-α. As shown in Fig. 9, greater tumor growth inhibition was achieved by the combination compared with either treatment alone; 50% of mice receiving the combination therapy were rendered tumor-free.

Enhancement of DC Antitumor Effect by Foreign Helper Protein Pulsing and IL-2 Administration. We have demonstrated previously that KLH, a strongly immunogenic carrier protein, could augment the efficacy of tumor lysate- or peptide-pulsed DC immunization in mediating successful immune priming against murine tumors; this effect could be further enhanced by the systemic administration of IL-2 (27). Fig. 10 shows the results of a representative experiment; the upper and lower panels show tumor size measurements and overall survival, respectively. i.t. injections of DCs when combined with the systemic administration of IL-2 could result in substantial MT-901 tumor growth inhibition; 60% of the treated mice underwent complete tumor regression (P < 0.05). This antitumor effect could be enhanced further by pulsing DCs with KLH prior to i.t. injection and IL-2 administration (P < 0.05), which resulted in all of the treated animals experiencing complete tumor eradication and prolonged disease-free survival. All of the animals cured of established tumor were then challenged s.c. with 1 × 10^6 viable MT-901 tumor cells (i.e., twice the dose level as that of the initial tumor challenge) in the left flank and 5 × 10^5 viable Renca cells in the right flank to evaluate the level and specificity of protective immunity. All of the mice were fully protected against outgrowth of the MT-901 tumor but experienced progressive growth of the irrelevant Renca tumor on the contralateral side (data not shown).

Splenocytes harvested from mice that had experienced complete tumor eradication were examined for their functional reactivity after...
As shown in Table 1, CTLs with heightened activity against MT-901 tumor target cells could be generated from splenocytes of animals treated with KLH-pulsed DCs plus IL-2 (333 LUs) compared with DCs plus IL-2 (17 LUs) and to splenocytes from control, naive mice (<1 LU). No lysis by CTLs was detected against the irrelevant, Renca tumor target (all <1 LU). The splenocytes were also examined for the production of cytokines, namely GM-CSF and IFN-γ (Fig. 11). Splenocytes isolated from MT-901 tumor-cured mice treated i.t. with KLH-pulsed DCs followed by IL-2 also produced greater amounts of GM-CSF (~2,000 pg) and IFN-γ (>11,000 pg) when specifically stimulated in vitro with MT-901 tumor cells. Splenocytes isolated from MT-901 tumor-cured mice treated i.t. with unpulsed DCs followed by IL-2 also produced the two cytokines, but at significantly lower amounts. As a control for tumor specificity, low-to-negligible cytokine production was observed by stimulation of the splenocytes by the irrelevant, control Renca tumor.

DISCUSSION

The presence of increased DC numbers within solid tumor masses has been correlated in some studies with improved prognosis (reviewed in Ref. 11). The data reported herein demonstrate that i.t. injections of DCs harvested from early (3-day) cultures of bone marrow cells in the presence of GM-CSF and IL-4 can mediate tumor growth inhibition. Similar to in vivo immunization studies using antigen-pulsed DCs (7), this tumor regression was dependent on host-derived CD8+ T cells and was also relatively radiosensitive. In preliminary experiments, we have also noted that the tumor growth inhibition elicited by the local administration of DCs alone but not splenocytes alone could elicit the regression of an established MT-901 breast tumor nodule distant (contralateral left flank) from the injected lesion, which again argues that the therapeutic efficacy of i.t. injections of DCs is immune mediated and is systemic in nature.

Syngeneic but not allogeneic DCs could mediate tumor regression when delivered i.t., although both sources of DCs could engulf MT-901 apoptotic tumor cells in vitro. Thus, the phagocytic activity of DCs to efficiently remove apoptotic tumor cells within the mass was in itself not sufficient to reduce tumor growth in vivo. The lack of antitumor effect by allogeneic DCs in our study is seemingly at odds with the published work of others (27, 28). In those latter studies, fusions between tumor cells and allogeneic DCs could elicit tumor
regression in vivo and could lead to the generation of MHC-restricted, tumor-specific CTLs in vitro. It is conceivable that heterokaryons expressing both tumor cell- and DC-derived MHC molecules after electrofusion or chemical fusion in vitro could explain the difference in results between these studies and ours.

We demonstrated that KLH, a strongly immunogenic carrier protein to elicit T-cell help, could enhance the antitumor effect of i.t. delivered DCs when combined with the systemic administration of IL-2. These data confirm published studies of others (29) as well as our own (27), which showed that KLH could augment by a CD4\(^+\) T cell-dependent mechanism the efficacy of tumor lysate- or peptide-pulsed DCs immunization in mediating both successful immune priming toward and therapeutic rejection of tumors in vivo (27). Splenocytes from mice treated i.t. with KLH-pulsed DCs followed by IL-2 administration displayed heightened levels of CTL activity as well as IFN-\(\gamma\) and GM-CSF secretion in a tumor-specific fashion (Table 1; Fig. 11). These findings are of particular interest because both cytolytic and noncytolytic, tumor-specific tumor-infiltrating lymphocytes have been shown to mediate potent antitumor effects in vivo upon adoptive transfer (30, 31).

Recent evidence has shown that immature DCs can readily acquire antigen(s) by uptake of apoptotic cells, which in turn can elicit MHC class I-restricted CTLs (12, 14). Such a process may play an important physiological role in vivo in the acquisition of foreign antigens in vivo, including those derived from tumors, virally infected and normal tissues, as well as organ transplants. Moreover, it has been shown that
necrotic, but not apoptotic, cells can trigger maturation of DCs in vitro (32). Thus, it is conceivable that the balance between the levels of apoptotic versus necrotic cells within a tumor mass may influence the capacity of DCs to trigger an effective immune response in situ, which may lead to a good versus a poor prognosis. We had reported previously that bone marrow-derived DCs, at a relatively immature stage, could efficiently engulf dextran particles (15), including those of $M_r$ 500,000. In this regard, we also showed in Fig. 2 that DCs were readily capable of efficiently engulfing intact, apoptotic MT-901 breast tumor cells.

In our current study, the MT-901 mammary tumor was found to have a prominent baseline level of cells undergoing early apoptosis within the mass. Moreover, the data of Figs. 8 and 9 demonstrated that the administration of TNF-α could mediate increased tumor apoptosis as well as enhance the antitumor effect elicited by the local delivery of DCs. We had demonstrated previously the antitumor effects of recombinant TNF-α in a variety of murine tumor models when administered alone (33, 34), or combined with chemotherapy (35) or IL-2 (36). We also showed that TNF-α mediated the antitumor effect in vivo by a combination of apoptotic, vascular, and immune T-cell mechanisms (33–36).

Future studies will determine whether or not other interventions that can selectively increase tumor apoptosis in situ and/or enhance elicited host T-cell immunity will result in more effective tumor regression by locally (or perhaps systemically) introduced ex vivo generated DCs alone or DCs generated directly in situ by the in vivo use of recombinant FLT-3L and CD40L (37). These efforts will be particularly important for tumors with relatively low apoptotic cell indices, which are also resistant to DC therapy alone. As examples, the systemic administration of a trimeric form of TNF-related apoptosis-inducing ligand (TRAIL) has been shown to elicit apoptosis and actively suppress certain human and murine tumors in vivo without demonstrable toxic side effects to normal tissues (38–40). In addition, we reported previously that the systemic administration of IL-2 could augment the antitumor effects of tumor lysate-pulsed DC vaccines (16), thus, arguing for its use in the setting of tumor apoptosis-inducing agents and local DC administration. We have also shown previously in murine tumor models that the administration of several distinct chemotherapeutic agents (e.g., cyclophosphamide, 5-fluoruracil, 1,3-bis(2-chloroethyl)-1-nitrosourea, and doxorubicin) can augment the antitumor efficacy of both TNF-α (35) and IL-2 (41). Taxol (paclitaxel) can mediate tumor apoptosis directly (42, 43) and cisplatin can substantially augment the level of tumor apoptosis induced by i.t. injections of the adenovirus-p53 vector (44) in both murine and human tumors. Local delivery of a recombinant adenovirus vector encoding a wild-type p53 cDNA (Adv5-p53) resulted in enhanced tumor apoptosis in a variety of murine and human tumors experimentally (44–46) as well as more recently in Phase I clinical trials in patients with advanced non-small cell lung cancer (47) and recurrent head and neck squamous cell carcinoma (45). In laboratory studies, the administration of cisplatin (or VP-16) before local delivery of Adv5-p53 resulted in enhanced tumor apoptosis in vitro and in vivo as well as in enhanced antitumor effects in vivo (44). Indeed, in preliminary studies, we have now observed that the weakly immunogenic sarcoma MCA-207 could be rendered sensitive to therapy by i.t. injections of DCs after the induction of apoptosis within the mass by the delivery of Adv5-p53.5

Although we have focused on one type of DC, additional comparisons are needed. It has been suggested that the state of maturation of DCs may be important for their optimal use in immunization strategies (48, 49). Strategies that have resulted in DC maturation include the use of CD40L (37, 50–52), lipopolysaccharide (52), monocyte conditioned medium (53), and, in our own published work and that of others, TNF-α (54, 55). Also of importance to the use of DCs in our models is the discovery of DC subsets or subpopulations, which differ in their capacity to elicit antigen-specific Th1/Tc1 versus Th2/Tc2 immune responses (56, 57). In this regard, distinct roles of antigen-specific Th1/Tc1 and Th2/Tc2 cells may predominate during eradication of established murine tumors in vivo (58, 59). Moreover, it remains to be determined whether site-directed injections of immature DCs alone into apoptotic tumor-involved lymph nodes will lead to a more efficient means of eliciting both a local and systemic immune responses.

### Table 1

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<th>Treatment*</th>
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| Fig. 11. i.t. delivery of KLH-pulsed DCs plus the systemic administration of IL-2 enhance specific T-cell production of cytokines. Mice were treated as described in “Materials and Methods.” After tumor eradication, aliquots of splenocytes (2 × 10^6 cells/ml) were cultured for 48-h in vitro with 4 × 10^6 UVB-irradiated MT-901 or irrelevant Renca tumor cells in 24-well culture plates. Culture supernatants were collected for measurement of murine GM-CSF (upper panel) and IFN-γ (lower panel) release by standard ELISA (pg/ml; mean ± SE of triplicate samples).
response compared with that with tumor lysate or peptide(s)-pulsed DCs injected into uninvolved lymph nodes (9).


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