Combined Intratumoral Injection of Bone Marrow-derived Dendritic Cells and Systemic Chemotherapy to Treat Pre-existing Murine Tumors

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INTRODUCTION

Dendritic cells (DCs) are attractive candidates for innovative cancer immunotherapy by virtue of their potential to function as professional antigen-presenting cells for initiating cellular immune responses. In this study, we evaluated a possible synergy of conventional chemotherapy together with intratumoral injection of syngeneic bone marrow-derived DCs for the treatment of preexisting tumors. Using murine CT26 colon adenocarcinoma cells (parental or modified to express β-galactosidase as a model tumor antigen) to produce s.c. tumors in syngeneic BALB/c mice, the data demonstrate that direct injections of DCs at the tumor site result in partial eradication of established tumors. Strikingly, the addition of systemic chemotherapy (cylophosphamide) combined with local intratumoral injection of DCs led to complete tumor regression in the treated animals. The tumor-free mice were able to resist a repeat challenge with the same tumor, suggesting that the animals had acquired long term antitumor immunity. Supporting evidence for the paradigm of systemic chemotherapy and intratumoral administration of DCs was obtained using melanoma B16 syngeneic tumor treated with Adriamycin plus DCs. These novel findings raise the possibility of using this potent strategy of combined intratumoral injections of DCs and systemic chemotherapy for cancer treatment.

MATERIALS AND METHODS

Mice. Female BALB/c (H-2b) and C57Bl/6 mice, 6–8 weeks of age, were purchased from The Jackson Laboratory (Bar Harbor, ME). The animals were housed under specific pathogen-free conditions according to NIH guidelines.

Cell Lines. CT26WT (H-2b) is a clone of the N-nitroso-N-methylurethane-induced BALB/c undifferentiated colon adenocarcinoma cell line. CT26.CL25 is a clone of CT26.WT that has been transduced with the Escherichia coli βgal gene (both cell lines provided by N. P. Restifo, National Cancer Institute, Bethesda, MD; Ref. 22). B16 is a C57Bl/6-derived melanoma (American Type Culture Collection, Rockville, MD). C3, a tumor cell line, was generated by transfecting C57Bl/6 mouse embryonal fibroblasts with plasmids containing the entire genome of the human papilloma virus type 16 (gift of M. Vieboom, University of Leiden, Leiden, the Netherlands; Ref. 23). The SVBalb (H-2b) fibroblast cell line (provided by Dr. L. Gooding, Emory University, Atlanta, GA), syngeneic to BALB/c, was used as an in vitro stimulator of cells in the CTL assay.

CT26.WT cells were cultured in complete RPMI-1640 medium with 10% fetal bovine serum, 2 mM L-glutamine, 100 µg/ml streptomycin, and 100 units/ml penicillin. CT26.CL25 cells were maintained in the same medium plus 400 µg/ml G418 (Life Technologies, Inc., Gaithersburg, MD). SVBalb and B16 cells were grown in DMEM containing 10% FCS and antibiotics. C3 cells were cultured in Iscove’s modified Dulbecco medium supplemented with 8% FCS, penicillin, and 2-mercaptoethanol.

Chemotherapy Agents. Both CTX and MMC were from Sigma Chemical Co. (St. Louis, MO), and 5-FU was from SoloPak Laboratories (Elk Grove Village, IL). ADR was from Genisia Laboratories, Ltd. (Irvine, CA).

DCs. Primary DCs were obtained from mouse bone marrow precursors as described previously (18, 25). Briefly, murine bone marrow cells were harvested from femurs and tibias and then plated in complete RPMI 1640 containing recombinant murine GM-CSF (500 units/ml; Sigma Chemical Co.) and recombinant murine IL-4 (20 ng/ml; Genzyme, Farmington, MA). On day 4 of culture, cells were fed with fresh medium and harvested after an additional 4 days of culture.
2, nonadherent granulocytes were gently removed, and fresh medium with GM-CSF and IL-4 was added. On day 4, loosely adherent cells were dislodged and replated. On days 5-7 of culture, immature DCs, as nonadherent proliferating aggregates, were collected. Functional analysis of the bone marrow-derived DCs was performed, and the maturation status and percentage of DCs were verified by flow cytometry (18), with three surface markers (CD11b, CD44, and class II antigen IAβ) showing the purity of DCs to be ≥72%.

**Combined Intratumoral DCs and Chemotherapy.** Preliminary studies were carried out using four antigen-tumor agents (5-FU, CTX, ADR, and MMC) to control the growth of the model tumors (CT26.WT, CT26.CL25, B16, and C3) with different doses and schedules to determine the drug dose for inhibition of tumor growth in vivo without major systemic toxicity. Mitomycin and 5-FU were not found to be useful for the tumors evaluated and were not assessed further.

For the CT26.CL25 or CT26.WT tumor models, 10^5 cells/mouse in 100 μl of HBSS were implanted into BALB/c mice in the lower right flank. On day 11, the average tumor sizes of CT26.CL25 and CT26.WT were 27.8 ± 3.8 and 22.0 ± 2.1 mm^2, respectively. The mice then received i.p. injections of CTX (150 mg/kg body weight); injections were repeated 2 days later with the same dose. Four injections of DCs (each injection was 10^6 cells/mouse in 30 μl of HBSS) were given into the tumor on days 12, 13, 14, and 18. The experimental groups included: group 1, untreated controls; group 2, intratumoral injections of DC alone; group 3, CTX treatment alone; group 4, combined CTX and intratumoral DC injections; and group 5, combined CTX and DCs that were injected s.c. in the left flank, a site distant to the tumor. For all animal experiments, the tumor size was measured biweekly using a caliper and expressed as the product of the maximal perpendicular diameters (mm^2).

**Results.**

**Overview of the DC + Chemotherapy Models.** To determine whether direct administration of syngeneic DCs to tumors in the context of concomitant chemotherapy suppresses growth of preexisting tumors, four well-characterized tumor models (colon CT26.CL25 and CT26.WT, melanoma B16, and sarcoma C3) were evaluated. The starting point of treatment for this study was an average tumor size ranging from 21 to 28 mm^2. As described below, intratumoral injections of immature DCs alone or chemotherapy alone to CT26.CL25, CT26.WT, and B16 tumors had minimal effects. In contrast, when equivalent numbers of DCs were delivered into mice with sarcoma C3 tumors (which grow slower than B16 and CT26 tumors and express high levels of human papilloma virus), complete eradication of all preestablished C3 tumors was observed in the absence of chemotherapy (not shown). In this context, the C3 tumors in BALB/c mice were not evaluated further.

The selected antitumor agent for the combination treatment of CT26.WT and CT26.CL25 cells was CTX, because it was the most effective drug among the four drugs tested (CTX, 5-FU, MMC, and ADR) for delaying CT26 colon tumor growth in vivo (not shown). In contrast, for the B16 tumors, the most effective chemotherapy was ADR (not shown).

**Combination Treatment with Chemotherapy and Intratumor Administration of DCs.** To test the hypothesis that systemic administration of chemotherapeutic agents together with intratumoral administration of DCs will control tumor growth, treatment with chemotherapy was initiated when the tumor was of appropriate size, and local administration of DCs were started 1 day after the first dose of chemotherapy.

For the CT26.CL25 colon carcinoma tumors in BALB/c mice, the tumors grew rapidly in the control (untreated) mice, as well as in the mice treated with DCs alone (Fig. 1A and B). Although treatment with systemic CTX alone (Fig. 1A) or systemic CTX together with DCs in the opposite flank (Fig. 1B) resulted in partial suppression of tumor growth, the combination of systemic CTX + DCs administered directly to the tumor resulted in complete suppression of tumor growth (P < 0.03, all observations of CTX + DCs compared with other groups; Fig. 1A and B). Consistent with the complete suppression of tumor growth in the CTX + DC treated mice, the CTX + DC treated mice had 100% survival (Fig. 1C). In contrast, there was...
reduced survival for all of the control groups (\(P < 0.01\), all observations of CTX + DCs compared with other groups; Fig. 2C).

Finally, assessment of survival in the B16 tumors in C57Bl/6 mice showed that the combination of DC + ADR markedly enhanced survival compared with ADR alone, or DC alone compared with the control (untreated) mice (\(P < 0.001\), all observations of ADR + DCs compared with other groups; Fig. 3).

Persistent Antitumor Memory after Combined Chemotherapy and Intratumor Administration of DCs. To further understand the mechanism responsible for complete regression of preestablished s.c. tumors and to evaluate the memory of antitumor immunity in vivo after combined chemotherapy and administration of DCs, the follow-

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**Fig. 1.** Combined intratumoral injection of bone marrow-derived DCs and systemic CTX treatment on the growth of established CT26.CL25 syngeneic colon carcinoma tumors. CT26.CL25 cells \(10^5\) were injected s.c. in the lower right flank of BALB/c mice. On days 11 and 13, mice with established tumors (on day 11, average tumor size was \(27.8\pm3.8\) mm\(^2\)) were treated with i.p. CTX (150 mg/kg each administration) and received intratumor administration of syngeneic DCs on days 12, 13, 14, and 18 \(4\times10^6\) cells in 50 \(\mu\)l of each administration). The experimental groups included: group 1, controls with intratumor HBSS injections \(50\) \(\mu\)l, \(n=5\); group 2, intratumoral injections of DCs alone \(n=5\); group 3, i.p. CTX alone \(n=5\); group 4, i.p. CTX and intratumoral DCs \(n=5\); and group 5, i.p. CTX and DCs injected s.c. in the left flank, a site distal to the tumor \(n=5\). A, tumor size for groups 1, 3, and 4. The data are presented as mean tumor size (mm\(^2\)) for each group; bars, SE. B, tumor size for groups 2 and 5. C, survival of mice recorded as the percentage of surviving animals on a given day. Surviving mice had no sign of tumor when the experiment was terminated. Experiments were repeated at least twice with similar results.

**Fig. 2.** Combined intratumoral injection of bone marrow-derived DCs and systemic CTX treatment on the growth of established CT26.WT syngeneic colon carcinoma tumors. The experiments carried out were identical to that described for CT26.CL25 tumors in Fig. 1, except tumor cells were the parental wild-type tumors, whereas the CT26.CL25 tumor cells in Fig. 1 were modified to express \(\beta\) gal as a model antigen. On day 11, average tumor size for CT26.WT was \(22.0\pm2.1\) mm\(^2\). Data are presented for controls \(n=5\); DCs alone \(n=5\); CTX alone \(n=5\); CTX + DCs \(n=5\); and CTX + DCs in the flank opposite from the tumor \(n=5\). A, tumor size for groups 1, 3, and 4. B, tumor size for groups 2 and 5. C, survival, all groups. See Fig. 1 legend for details.
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treated tumor-free mice also had protective immunity against subse-

CT26 tumor

persistent antitumor immunity specific for antigens relevant to the

(Fig. 5), further indicating that the tumor protection was mediated by

and CT26.CL25 tumor cells. The results show that these mice resisted

Surviving mice had no sign of tumor when the experiment was terminated.

Fig. 3. Combined intratumoral injection of bone marrow-derived DCs and systemic ADR treatment on tumor growth of established B16 tumors. Naïve C57Bl/6 mice were injected s.c. with 2 × 10^7 B16 cells in the lower right flank. On days 9, 10, and 11, mice with established tumors (on day 9, average tumor size was 25.7 ± 4.6 mm^2) received ADR (i.p. 3 mg/kg each administration) and intratumoral DCs on days 10, 11, 12, and 16 (10^6 cells in 50 μl of HBSS each administration). The experimental groups included: group 1, control with intratumor HBSS injections (□); group 2, DCs alone (○); group 3, ADR alone (•); and group 4, ADR and intratumoral DCs (■). Each group included five mice. Shown is survival, recorded as the percentage of surviving animals on a given day.

Fig. 4. CTLs in long-term CT26.CL25 tumor-free mice that survived after treatment with CTX combined with intratumoral administration of DCs. CTLs were measured with a ^51Cr release assay at different ratios of effector to targets. Splenocytes isolated from CT26.CL25 tumor-free mice were stimulated for 5 days in vitro with syngeneic fibroblasts (SVBalf) pulsed with βgal peptide and then measured for specific cell lysis against syngeneic target cells (○, SVBalf; □, CT26.CL25; ■, SVBalf pulsed with βgal peptide). Viability were evaluated using the CT26.CL25 model: the generation of antitumor CTLs; a second lethal challenge of the same tumor to the surviving mice; and adoptive transfer of the tumor-specific CTLs. The CT26.CL25 tumor cells not only carry the artificial βgal gene as a model tumor antigen but also its parent antigens (29). Splenocytes from CT26.CL25 tumor-free mice were harvested and stimulated in vitro with βgal peptide and then measured for specific cell lysis against a panel of target cells in a standard ^51Cr release assay. Significant lysis of βgal-expressing CT26.CL25 target cells or βgal peptide-pulsed SVBalf target cells by the splenocytes of the surviving mice was observed, indicating that the cellular immune response contained CTLs specific against βgal (Fig. 4). Moreover, the CT26.CL25 tumor-free mice that survived after the combined CTX + DC therapy were s.c. rechallenged bilaterally with CT26.WT and CT26.CL25 tumor cells. The results show that these mice resisted lethal doses of not only CT26.CL25s but also CT26.WT tumor cells (Fig. 5), further indicating that the tumor protection was mediated by persistent antitumor immunity specific for antigens relevant to the CT26 tumor per se. It is of interest that the CT26.CL25 CTX + DC-treated tumor-free mice also had protective immunity against subsequent challenge with the nontransfected parental tumor line (CT26.WT), suggesting that the intratumor-injected DCs presented in vivo both βgal and parent antigens, resulting in long-lasting immunity against these tumors. Furthermore, CT26.WT tumor-free mice treated with the combination therapy survived a second challenge of CT26.WT and CT26.CL25 (not shown), demonstrating that the observed effect of established immunological memory is not only dependent on the βgal antigen but likely also on other unidentified antigens of CT26 tumors.

Consistent with the concept that the CTX + DC therapy induced specific antitumor immunity, the βgal-specific CTLs generated from CTX + DC-treated tumor-free mice were transferred to mice and the mice rechallenged with CT26.CL25 tumor cells. Mice receiving CTLs from DC- and CTX-treated mice, but not from CTL-negative mice, were protected from a subsequent lethal tumor challenge with the CT26.CL25 cells (Fig. 6).

To exclude the possibility that locally released toxic factors and/or changes in the microenvironment as a result of exogenous DCs added in large amounts might have caused tumor regression, bilateral tumors (CT26.CL25) were inoculated in both lower flanks. The right tumor was considered as primary tumor and was treated by DC injection in combination with systemic chemotherapy (CTX), whereas the secondary tumor on the left side was untouched. Both tumors stopped growing, regressed, and disappeared simultaneously (Fig. 7), indicating that the antitumor suppression was systemic rather than local. This...
observation may have significant clinical implications because it suggests that treatment of a single tumor with the intratumoral DC injection, and systemic chemotherapy might be sufficient to generate effective therapeutic immunity against metastatic cancer.

**DISCUSSION**

A number of studies have demonstrated that antitumor immunity can be achieved by generating DCs in vitro from proliferating hematopoietic precursors, loading the DCs ex vivo with tumor-specific antigens and reinfusing the DCs back to the recipient host (11–18). In contrast, the present study demonstrates the effectiveness of an antitumor strategy that is similar but eliminates the step of loading the DCs ex vivo with tumor-specific antigens. We achieved this by combining intratumor administration of DCs with systemic administration of chemotherapy. Using a model of murine flank tumors syngeneic to BALB/c and C57Bl/6, the data demonstrate that the combination of systemic chemotherapy plus direct intratumor administration of syngeneic DCs results in complete suppression of tumor growth. Importantly, the combined therapy induced the generation of tumor-specific cytotoxic T cells that were able to protect animals from tumor challenge, and that the chemotherapy + DC-treated animals were able to resist repeat challenge with the same tumor, suggesting that the mice had acquired long-term antitumor immunity. These observations suggest that it is not necessary to identify specific tumor antigens for immunotherapy using DCs. Instead, conventional cancer therapies, such as chemotherapy, may cause sufficient death of tumor cells by providing antigens to DCs in the milieu of the tumor.

**Immunotherapy with DCs.** The state of maturation of DCs can be controlled in vitro by the culture conditions used to produce DCs. Immature DCs are produced by culturing DC precursors in the presence of GM-CSF and IL-4, whereas further maturation of DCs can be achieved by longer culture or subsequent addition of cytokines such as tumor necrosis factor-α or CD40 ligand (1, 2, 30). Because immature DCs have phagocytic activity and perform effective capture of exogenous antigens (1, 2), the DCs used in the present study were generated by 6-day exposure to GM-CSF and IL-4. The hypothesis underlying the use of these immature DCs is that the DCs will reside in the tumor for a short period of time, where they take up and efficiently process antigens from dying and apoptotic cells (19, 21). Once antigens have been captured, antigen-bearing DCs rapidly undergo a functional maturation process and become very potent antigen-presenting cells, with down-regulated processing capacity and increased surface expression of MHC and costimulatory molecules (1, 2). There is increasing evidence that the tumor antigens endocytosed by bone marrow-derived DCs are introduced not only into the MHC class II but also class I processing pathway to cross-prime naive T cells for developing potent immunity (3, 19, 31, 32). In addition, there is evidence that antigens can be transferred from antigen-bearing DCs to naive DCs in vivo and that antigen-pulsed DCs act synergistically with these DCs in the production of primary T-cell proliferation to antigens (33).

In the context of the central role of DCs in presenting MHC-restricted tumor antigens, a variety of strategies have been developed to use DCs in immunotherapy against tumors (11–18, 34, 35). Although the molecular identification of the tumor-specific antigens would greatly expand the use of DC-based tumor vaccine strategies, most tumor-specific antigens remain unknown and may vary between different individuals. Antigen identification, a fundamental requirement for directly pulsing defined peptides and proteins with DCs for tumor vaccination, can be bypassed by approaches that arm the DCs with the full antigenic spectrum of the tumor by using the tumor cell itself as a source of antigens. Strategies to accomplish this include pulsing DCs with tumor-derived lysates or tumor mRNA, or by fusing tumors with DCs in vitro, or coculturing tumor cells with DCs in vitro (11–18). These strategies can induce CTLs directed specifically against the tumor but require the in vitro step of exposing the DCs to the tumor. One alternative approach is to attract functional DCs to the tumor site, resulting in an immune response attributable to the short-term contact of DCs and tumor cells in vivo (35). Another is the strategy used in the present study, i.e., to take the DCs generated in vitro and directly administer the DCs into the tumor, bringing the DCs and tumor into close contact. Although there are data that blood and tumor-draining lymph node DCs are impaired in cancer patients because of a systemic effect of the tumor on DCs (36), functional DCs prepared from bone marrow or peripheral blood-derived precursors appear to escape this impairment (37). In experimental animal models, reports from other groups have showed that mice given i.v. injections of 10⁶ DCs develop a weak protective cellular immunity (15), whereas the administration of 5 × 10⁵ bone marrow-derived DCs in combination with a vaccine results in eradication of tumors (38).

A number of mechanisms have been proposed to explain the failure in cancer patients of chemotherapy alone as a sole or follow-up treatment after surgery. A major limitation is acquired drug resistance, which may occur because of drug-targeted gene amplification and mutation in some tumor cells which escape the cytotoxic effect of the drug and lead to tumor recurrence (39). Similarly, there are many ways in which tumors can escape recognition and clearance by the immune system. The reasons that the host is unable to trigger the recruitment and activation of DCs at the tumor site of rapidly growing tumors may include secretion of immunosuppressive factors such as tumor necrosis factor-β, fas ligand, and IL-10 to inhibit the host’s immune system, and lack of enough systemic functional DCs to achieve a local critical mass in the tumor-bearing host (40). In this context, the injection of in vitro generated DCs directly into the tumor may be able to break an operational state of tumor tolerance and trigger tumor regression, resulting in the development of effective endogenous immunity against cancer. The positive results from the present study show that the apparent limitation of DC function in tumor-bearing hosts can be overcome if sufficient autologous DCs at the tumor site are provided in the context of systemic chemotherapy. The results in the present study have clinical implications. Because chemotherapy is effective in controlling the rate of tumor growth and
causing tumor cells to undergo apoptosis, local DC-based therapy may be synergistic with chemotherapy in enhancing antitumor host responses. On the basis of the fact that complete tumor regression and long-lasting tumor immunity was observed in the present study, we suggest that the same strategy could be applied to treat other tumors by DCs combined with effective drugs. However, before undertaking such treatments, the routes and doses of administration of DCs and drugs need to be optimized. We predict that intratumoral DC injections combined with other efficient cancer treatments such as radiotherapy, antiangiogenesis agents, or prodrug strategies may be made more effective by virtue of increasing local toxic effects against tumors with minimal systemic immunosuppression. However, we do not have direct evidence showing how, after chemotherapy, the increased dying tumor cells release tumor antigens to be taken up by DCs in vivo. This putative mechanism remains hypothetical and will be researched in additional studies.

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REFERENCES

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