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*J Immunol* 1998; 161:5516-5524; ;
http://www.jimmunol.org/content/161/10/5516

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The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Eliciting T Cell Immunity Against Poorly Immunogenic Tumors by Immunization with Dendritic Cell-Tumor Fusion Vaccines

Jianli Wang,* Scott Saffold,* Xuetao Cao,† John Krauss,* and Wei Chen2*†

Dendritic cells (DCs) are the most effective APCs and are being studied as natural adjuvants or Ag delivery vehicles to elicit T cell-mediated antitumor immunity. This study examined whether inoculation of DCs fused with poorly immunogenic tumor cells elicited tumor-reactive T cells for adoptive immunotherapy. DCs derived from bone marrow of C57BL/6 (B6) mice were fused with syngeneic B16 melanoma or RMA-S lymphoma cells by polyethylene glycol. The B16/DC and RMA-S/DC fusion hybrids expressed MHC class I, class II Ags, costimulatory molecules, as well as DC-specific and tumor-derived surface markers. The tumor/DC hybrids were capable of processing and presenting tumor-derived Ags, and immunization of B6 mice with irradiated B16/DC or RMA-S/DC vaccine elicited tumor-specific CTL activities. Vaccination of B6 mice with irradiated B16/DC fusion preparations induced partial host protective immunity against B16 tumor challenge. Reduced tumor incidence and prolonged survival time were observed. Adoptive transfer of T cells derived from B16/DC vaccine-primed lymph node into B16 tumor-bearing mice greatly reduced the number of established pulmonary metastases with or without in vivo administration of IL-2. Moreover, adoptive transfer of RMA-S/DC vaccine-primed, cultured lymph node T cells eradicated disseminated FBL-3 tumor. The results demonstrate that tumor/DC fusion products are effective cellular vaccines for eliciting T cell-mediated antitumor immunity. The Journal of Immunology, 1998, 161: 5516–5524.

Malignant transformed cells from humans and rodents express tumor-specific or tumor-associated proteins that can function as target Ags for the host immune system (1–4). Vaccination of tumor-bearing host with tumor Ags to eliciting T cell-mediated immunity to eradicate established tumor and treating malignancy by adoptive transfer of in vitro cultured tumor-reactive T cells have been the two major approaches to achieve the goals of cellular immunotherapy of cancer (4, 5). However, most human tumors are poorly immunogenic and are capable of evading the host immune system. Accumulating evidence has demonstrated that tumor cells may down-regulate the expression of signals that are essential for the activation of host T cells. The mechanisms include defective expression of MHC Ags, absence of costimulatory or adhesion molecules, and alteration of Ag-processing or transport, resulting in an inability to present tumor-associated Ags. Strategies to augment the host immune response to tumor have included introduction of genes encoding MHC Ags (6–8), costimulatory molecule (9–11), or cytokines (12–14) into tumor/DC hybrids were capable of processing and presenting tumor-derived Ags, and immunization of B6 mice with irradiated B16/DC or RMA-S/DC vaccine elicited tumor-specific CTL activities. Vaccination of B6 mice with irradiated B16/DC fusion preparations induced partial host protective immunity against B16 tumor challenge. Reduced tumor incidence and prolonged survival time were observed. Adoptive transfer of T cells derived from B16/DC vaccine-primed lymph nodes into B16 tumor-bearing mice greatly reduced the number of established pulmonary metastases with or without in vivo administration of IL-2. Moreover, adoptive transfer of RMA-S/DC vaccine-primed, cultured lymph node T cells eradicated disseminated FBL-3 tumor. The results demonstrate that tumor/DC fusion products are effective cellular vaccines for eliciting T cell-mediated antitumor immunity. The Journal of Immunology, 1998, 161: 5516–5524.

Received for publication February 13, 1998. Accepted for publication July 8, 1998.

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*Center for Surgery Research and †Departments of Hematology/Medical Oncology and Immunology, The Cleveland Clinic Foundation, Cleveland, OH 44195; and ‡ Department of Immunology, Second Military Medical University, Shanghai Peoples Republic of China

5 Abbreviations used in this paper: DCs, dendritic cells; F-, M-, and R-MuLV, Friend, Moloney, and Rauscher murine leukemia virus; LN, lymph node; CM, complete medium; BM, bone marrow; GM-CSF, granulocyte-macrophage CSF; PEG, polyethylene glycol; PE, phycoerythrin; CY, cyclophosphamide; HAT, hypoxanthine-aminopterin-thymidine.
may be superior to the use of a single dominant epitope to induce antitumor immunity more effectively.

In this study we examined whether fusion of DCs with syngeneic poorly immunogenic tumors could generate hybrid cellular vaccines to effectively elicit host T cell-mediated antitumor immunity. It is hypothesized that the fusion of tumor cells with DCs would generate a hybrid vaccine with potent Ag-processing and-presenting capabilities and expression of multiple tumor Ags, thereby inducing more effective antitumor immunity. A recent study by Gong et al. demonstrated that fusion of a relatively immunogenic mouse tumor, MC38 carcinoma, with syngeneic DCs as a vaccine induced T cell protective immunity against tumor challenge and immune rejection of established tumor (26). In this study, B16 (B16.F10) melanoma and RMA-S lymphoma cell lines were selected for the fusion experiments. B16 tumor is poorly immunogenic and lacks the expression of MHC and costimulatory molecules. Immunization with irradiated B16 tumor cells fails to induce systemic immunity or elicit functional tumor-reactive T cells. RMA-S is a Rauscher MuLV (R-MuLV)-induced T cell lymphoma of B6 mouse origin that is genetically defective of TAP and thus does not process endogenous Ags (27). Our results demonstrate that fusion of DCs with syngeneic tumor generated hybrid cells that express both DC-derived accessory molecules and tumor-derived Ags. The tumor/DC fusion hybrids were capable of processing and presenting tumor-associated Ags to elicit tumor-reactive CTLs. Vaccination of B6 mice with B16/DC fusion preparations induced partial protective immunity against tumor challenge. In addition, immunization with B16/DC or RMA-S/DC vaccine could prime lymph node (LN) T cells for adoptive immunotherapy. The transfer of such vaccine-primed, ex vivo expanded LN T cells into tumor-bearing mice specifically reduced the number of established B16 pulmonary metastases and effectively eradicated disseminated FBL-3 tumor.

Materials and Methods

Mice

Six- to eight-week-old C57BL/6 (denoted B6, H-2b) mice and BALB/c (H-2d) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were maintained in specific pathogen-free conditions and were used at the age of 8–10 wk.

Tumor cell lines

B16.F10, a spontaneous melanoma cell line of B6 mouse origin, was a gift from Dr. Steven A. Rosenberg’s laboratory (National Cancer Institute, Bethesda, MD). A B16 tumor line expressing the murine costimulatory molecule B7.1 (B16.B7.1) was provided by Dr. Suzanne Ostrand-Rosenberg (University of Maryland, Baltimore, MD). RMA-S is a genetically TAP-defective subline of RMA, a R-MuLV-induced T cell lymphoma of B6 mouse origin, and thus does not process endogenous Ags (27). BFL-3 is a F-MuLV-induced leukemia of B6 origin that expresses tumor-associated Ags cross-reactive with syngeneic leukemia and lymphoma induced by F-MuLV and R-MuLV tumors (28). EL-4 is a dimethylbenzanthrene-induced T cell lymphoma of B6 origin. MCA 207 is a fibrosarcoma of B6 mouse origin induced by 3-methylcholanthrene. LSTRA is a Moloney MuLV-induced lymphoma of BALB/c mouse origin. All tumor cell lines were maintained in culture in complete medium (CM) consisting of RPMI 1640 supplemented with 10% heat-inactivated FCS, 0.1 mM nonessential amino acid, 1 mM sodium pyruvate, 2 mM fresh L-glutamine, 100 U/ml penicillin, 50 μg/ml gentamicin, 0.5 μg/ml fungizone (all from Life Technologies, Grand Island, NY), and 5 × 10^{-7} M 2-ME (Sigma, St. Louis, MO).

Preparation of DCs

The procedure used for generation of DCs from bone marrow (BM) culture was described previously with some modification (29). In brief, the BM cells prepared from femurs and tibias of normal B6 mice were depleted of RBC with ammonium chloride. The BM cells were treated with a mixture of mAbs (PharMingen, San Diego, CA) against I-A^b, B-220/CD45R, Lyt-2, and L3T4 plus low-tox-M rabbit complement (Accurate Chemical and Scientific Corp., Westbury, NY). The lymphocyte-depleted BM cells were then incubated in CM supplemented with recombinant mouse GM-CSF (10 ng/ml; PharMingen) and IL-4 (10 ng/ml; Sigma) in 24-well plates at 5 × 10^5 cells/well. On day 5, one-half of the media in each well was replaced with fresh CM containing mouse GM-CSF (10 ng/ml) and IL-4 (10 ng/ml). On day 6, the nonadherent DCs were harvested by gentle pipetting and were used in the studies. The average cell yield of DCs derived from the BM culture was 25 × 10^3 cells/mouse, and ~90% of the cells expressed the N418 Ag.

Fusion protocol

DCs derived from BM culture were fused with tumor cells at a 3:1 (DC: tumor) ratio using polyethylene glycol (PEG; m.w. 1450)/DMSO solution (Sigma). In brief, tumor cells were cultured in CM supplemented with 20% FCS and 1× OPI solution (oxaaloacetate, pyruvate, and insulin; Sigma) for 4–6 h before fusion. Tumor cells and DCs were then mixed and washed with serum-free medium. After removing the medium, 1 ml of PEG was added to the cell pellet while resuspending the cells by stirring for 2 min. An additional 10 ml of serum-free medium was added to the cell suspension over the next 3 min with continued stirring. The cells were centrifuged at 400 × g for 5 min. The cells were resuspended with 20% FCS CM and cultured for 24 h before staining or being used as targets or vaccines. Fusion preparations of DCs with B16 or RMA-S were termed B16/DC and RMA-S/DC, respectively.

Phenotype staining

B16, RMA-S, DCs, and their fusion hybrids were analyzed by staining with FITC- or PE-conjugated mAbs (PharMingen) against MHC Ags (D^b, K^b), adhesion and costimulatory molecules (B7.1, ICAM-1), and lymphocyte Ags (Thy-1.2, SmIg) at 4°C for 45 min. DCs were identified by labeling with mAb against CD11c (N418) (30). B16, B16/DC or B6/B16 fused cells were stained with mAb against AKV Env gp85 protein (M562, provided by Dr. Masaru Taniguchi, Ciba University, Tokyo, Japan) as a B16 tumor-specific marker (31). RMA-S and RMA-S/DC fused cells were stained with Thy-1.2 or mAb against the R-MuLV-encoded Gag p12 protein (584, provided by Dr. Bruce Chesbro, National Institute of Allergy and Infectious Diseases, Hamilton, MO) as RMA-S tumor-derived marker (32). The method for labeling cells with TRITC (rhodamine) was similar as previously described (33). Briefly, cells were resuspended in RPMI 1640 at 1 × 10^6 cells/ml and incubated with TRITC (0.5 μg/ml) in 37°C for 45 min. The labeled cells were washed three times and used for fusion studies. The phenotypes of fresh and cultured LN T cells were determined by FACs analysis following staining with FITC- or PE-conjugated mAbs against Thy-1.2, Lyt-2, and L3T4 (PharMingen). All cells were washed twice with HBSS and fixed with 0.2% paraformaldehyde. Fluorescence intensity and cell positive percentage were measured on a FACSScan flow microfluorometer (Becton Dickinson, Sunnyvale, CA).

T cell lines

FBL-3-specific CD8^+ CTLs were generated from B6 mice immune to FBL-3 as described previously (28). B16 tumor-reactive CTLs were generated from spleens of B6 mice immunized with B16/DC fusion preparation. Briefly, B6 mice were s.c. immunized twice in a 2-wk interval on the flanks with 2 × 10^5 (10^5 side) irradiated (15,000 rad) B16 or B16/DC suspended in 0.1 ml of HBSS. One week after final immunization, spleens from each group of mice were harvested. Splenocytes were stained with 4 × 10^5 from normal B6 mice or mice vaccinated with irradiated B16 or B16/DC were cultured with 10^5 irradiated (15,000 rad) B16.B7.1 tumor cells in wells of 24-well plates in CM. On days 2 and 4, one-half of the medium was changed with CM containing 5 U/ml human rIL-2 (Chiron, Emeryville, CA). After 5–7 days, lymphocytes were harvested and used as effectors in a cytotoxicity assay. FMR tumor-reactive CTLs were generated from spleens of B6 mice immunized with irradiated RMA-S/DC fusion preparation or RMA-S tumor cells in a similar fashion.

Proliferative assay

For MLR, graded doses (10^4–10^6) of irradiated (3000 rad) DCs or B6 splenocytes were placed in 96-well plates as stimulators and co-cultivated with BALB/c splenocytes (10^5/well) as responders. The plates were incubated in a humidified atmosphere under 5% CO_2 tension at 37°C for 96 h and were pulsed for 16 h with 1 μCi of [^3]H]thymidine/well before harvest. All determinations were conducted in triplicate, and thymidine incorporation was determined. Stimulation indexes were calculated by dividing the counts per minute (mean) obtained from each group by the counts per minute (mean) from irradiated BALB/c spleen cells alone.
The methods for assessing the ability of DCs to process and present FBL-3 tumor proteins to FBL-3 specific CD4\(^+\) T cells were similar as described previously (34). Briefly, graded doses (10\(^3\)-10\(^7\) cells/well) of irradiated DCs or B6 spleen cells were incubated with varying concentrations (1:50,000 to 1:640,000 dilution) of purified F-MuLV proteins in wells of 96-well plates as stimulators. Resting FBL-3 env gp70-specific CD4\(^+\) Th clone (C8) was used as the responder (2 \(\times\) 10\(^4\) cells/well). The plates were incubated at 37°C for 96 h and were pulsed for 16 h with 1 \(\mu\)Ci of [\(^{3}H\)]thymidine/well before harvesting. All determinations were conducted in triplicate, and thymidine incorporation was determined. Stimulation indexes were calculated by dividing the sample cpm by the mean cpm of C8 clone incubated with irradiated DCs or B6 spleen cells without tumor Ags.

Cytotoxicity assay

Target cells were incubated at 37°C with 250 \(\mu\)Ci of [\(^{51}Cr\)] (New England Nuclear, Boston, MA) in 1 ml of 20% FCS RPMI 1640 medium for 45 min. Labeled targets were washed three times with HBSS and resuspended in 20% FCS RPMI at 10\(^5\) cells/ml. [\(^{51}Cr\)]-labeled target cells (10\(^5\) cells in 100 \(\mu\)l) were placed into each well of 96-well plates, and 100 \(\mu\)l/well of each dilution of T cells as effectors was added. Plates were incubated at 37°C for 4 h. The supernatant from each well was harvested, and the amount of [\(^{51}Cr\)] released was counted in a gamma counter. The percentage of specific lysis was calculated by dividing the sample cpm by the mean cpm of C8 clone incubated with irradiated DCs or B6 spleen cells without tumor Ags.

In vitro activation of LN T cells

B6 mice were s.c. immunized twice in a 2-wk interval on the flanks with 2 \(\times\) 10\(^6\) (10\(^5\)/side) irradiated (15,000 rad) tumor, tumor/DC fusion preparation, or tumor mixed with DCs (1/1) suspended in 0.1 ml of HBSS. One week after the final immunization, inguinal LNs from each group of mice were harvested. LN cells from each group of mice were activated and expanded in culture using anti-CD3 plus IL-2 as described previously (35). In brief, LN cells (3-4 \(\times\) 10\(^6\) cells/well) were activated on 24-well plates coated with anti-CD3 mAb (145-2C11) and incubated at 37°C for 2 days. The activated cells were suspended at 1-2 \(\times\) 10\(^5\) cells/ml in CM containing IL-2 (4 U/ml) and incubated in gas-permeable culture bags (Baxter Healthcare, Deerfield, IL) for an additional 3 days. The derived LN T cells were harvested and used as effectors cells for adoptive immunotherapy.

Tumor challenge

B6 mice were s.c. immunized twice in a 2-wk interval with 10\(^6\) irradiated (15,000 rad) B16, B16 mixed with DCs (1/1), or B6/DC fusion preparation (adherent cells from BM culture) for an additional 3 days. The derived LN T cells were expanded in culture using anti-CD3 plus IL-2 as described previously (35). In brief, LN cells (3-4 \(\times\) 10\(^6\) cells/well) were activated on 24-well plates coated with anti-CD3 mAb (145-2C11) and incubated at 37°C for 2 days. The activated cells were suspended at 1-2 \(\times\) 10\(^5\) cells/ml in CM containing IL-2 (4 U/ml) and incubated in gas-permeable culture bags (Baxter Healthcare, Deerfield, IL) for an additional 3 days. The derived LN T cells were harvested and used as effectors cells for adoptive immunotherapy.

Adaptive immunotherapy models

For therapy of B16 pulmonary metastases, B6 mice were injected i.v. with 10\(^7\) live B16 tumor cells in 1 ml of PBS to initiate pulmonary metastases. Three days after tumor inoculation, mice were randomly divided into several groups to receive treatments by i.v. injection of 5 \(\times\) 10\(^5\) cultured LN T cells suspended in 1 ml of PBS. On day 21 after tumor inoculation, mice from each group were killed, and lungs were inflated with Fekete’s solution. Lung metastases were counted. In some experiments, tumor-bearing mice were i.p. administrated IL-2 (15,000 U, twice/day for 5 days) following the adoptive transfer of cultured LN T cells. For therapy of FBL-3 tumor, B6 mice were inoculated i.p. with 5 \(\times\) 10\(^5\) viable FBL-3 tumor cells on day 0. By day 5, the tumor was disseminated, and mice were treated with cyclophosphamide (CY) at a dose of 180 mg/kg followed in 6 h by i.p. injection of cultured LN T cells (5 \(\times\) 10\(^5\) cells/mouse) suspended in 0.5 ml of PBS. The tumor growth and the survival time of each group of mice were monitored and recorded on a regular basis.

Statistical analysis

The significant differences in the numbers of pulmonary metastases and the survival time between groups were determined by the Mann-Whitney rank sum test. Two-sided \(p<0.05\) was considered statistically significant.

RESULTS

Fusion of DCs with syngeneic tumors

DCs from B6 mice were prepared and enriched from BM culture supplemented with recombinant mouse GM-CSF and IL-4. The purity and phenotype of the BM-derived DCs were identified by labeling with a panel of Abs against mouse DCs or lymphoid cell surface markers. The results (Fig. 1) showed that DCs derived from BM culture were positive for D\(^b\), K\(^b\), IA\(\alpha\), B7.1, ICAM-1, and CD11c, but were negative for Thy-1.2, SmIg, B6 tumor marker (MuLV AKV Env gp85), and RMA-S tumor marker (R-MuLV Gag p12), as expected. The purity of DCs (N418\(^+\) cells) was >90%. The results shown in Fig. 1 confirmed that B16 tumor expressed very low amounts of D\(^b\) and K\(^b\), but was negative for IA\(\alpha\), B7.1, ICAM-1, CD11c, and CD11b, and were negative for Thy-1.2, SmIg, B6 tumor marker (MuLV AKV Env gp85), and RMA-S tumor marker (R-MuLV Gag p12), as expected. The purity of DCs (N418\(^+\) cells) was >90%. The results shown in Fig. 1 confirmed that B16 tumor expressed very low amounts of D\(^b\) and K\(^b\), but was negative for IA\(\alpha\), B7.1, ICAM-1, CD11c, and CD11b.

Functional studies demonstrated that DCs enriched from BM culture as stimulators (10\(^3\)-10\(^7\) cells/well) induced strong proliferative responses of allogeneic T cells in MLR, with stimulation indexes 12.6- to 46.6-fold greater than that induced by the same number of DCs to process tumor-associated proteins was confirmed in experiments demonstrating that DCs (10\(^3\)-10\(^7\) cells/well) pulsed with varying concentrations of FBL-3 Env gp70 proteins as stimulators induced strong proliferative responses of FBL-3-specific CD4\(^+\) Th cells. DCs pulsed with FBL-3 tumor proteins induced T cell proliferative responses (stimulation index) 13.8- to 75.8-fold greater than that induced by the same number of DCs as stimulators (10\(^3\)-10\(^7\) cells/well) induced strong proliferative responses of allogeneic T cells in MLR, with stimulation indexes 12.6- to 46.6-fold greater than that induced by the same number of DCs.
than those induced by the same cell number of Ag-pulsed B6 spleen cells (data not shown).

Fusion of DCs with syngeneic tumor B16 or RMA-S cells by PEG was conducted at a DC:tumor ratio of 3:1. Fusion efficiency was determined by staining the fusion preparations of B16 fused with B16 or B16 mixed with DCs (1:1) used as controls. Phenotype staining results showed that the nonadherent cells from B16/DC overnight culture consisted mostly of unfused DCs (90.2%), some B16 cells (5.8%), and a small number of tumor-DC fusions (3.1%). B16 labeled with TRITC (rhodamine) were fused with DCs by PEG. The fusion preparations were cultured overnight and stained with mAbs against IAb or CD11c. The representative fluorescence photomicrographs showed B16 labeled with rhodamine (a), DCs stained with anti-CD11c (b), B16 and DC mixture stained with anti-CD11c (c) or IAb (e), and B16/DC fusion preparations stained with anti-CD11c (d) or IAb (f). Original magnification, ×400.

Tumor/DC fusion hybrids can present tumor-associated Ags and elicit tumor-reactive CTLs

To assess the ability of tumor/DC fusion hybrids to process and present intracellular proteins derived from tumor cells, RMA-S/DC-fused cells were used. RMA-S is genetically defective of TAP and thus does not process endogenous Ags. Our previous study has
identified the immunodominant CTL epitope of FMR-MuLV-induced tumors in B6 mice as a D\textsuperscript{b}-restricted nonamer peptide (p85–93) located in the leader sequence of the gPr80 gag protein encoded by the FMR-MuLV \textit{gag} gene. CD8\textsuperscript{+} CTLs specific against FMR-MuLV-induced tumors in B6 mice can effectively lyse FBL-3, MBL-2, and RMA, but fail to recognize and lyse RMA-S (28). To determine whether RMA-S/DC-fused cells can process and present tumor-associated proteins, the RMA-S/DC fusion preparation (containing 15–25% fused cells) was labeled with \textsuperscript{51}Cr and used as a target in the CTL assays. The results (Fig. 3 \textit{A}) showed that CD8\textsuperscript{+} CTLs specific for FBL-3 tumor lysed FBL-3 and RMA-S/DC, but did not lyse RMA-S, or RMA-S mixed with DCs as targets. To determine whether vaccination with RMA-S/DC can elicit tumor-reactive T cells, B6 mice were immunized with irradiated RMA-S or RMA-S/DC fusion preparation. Spleens from each group of mice were harvested and stimulated in vitro with irradiated FBL-3 tumor. T cells derived from the mixed tumor-lymphocyte cultures were tested for cytolytic activity against FBL-3, EL-4, RMA-S, and LSTRA as targets in a standard 4-h \textsuperscript{51}Cr release assay.

In similar experiments, B6 mice were immunized with irradiated B16, or B16/DC fusion preparation (adherent cells from overnight culture). Spleens from each group of mice were harvested and stimulated in vitro with irradiated B16.B7.1 tumor. T cells derived from the mixed tumor-lymphocyte cultures were tested for cytotoxicity against EL-4, MCA207, LSTRA, B16, and B16.B7.1. The results showed that cultured lymphocytes from mice vaccinated with B16 had no detectable cytotoxicity against EL-4, MCA207, LSTRA, or B16 (Fig. 4 \textit{A}). By contrast, cultured lymphocytes from mice vaccinated with B16/DC specifically lysed B16, but not EL-4, MCA 207, or LSTRA (Fig. 4 \textit{B}). A higher level of cytolytic activity against B16.B7.1 was observed.

\textbf{Immunizations with B16/DC induce partial host protective immunity against B16 tumor challenge}

To determine whether B16/DC-fused cells could induce host protective immunity against B16 tumor, groups of naive B6 mice were s.c. immunized twice with irradiated B16, B16/DC, B16 mixed with DCs, or HBSS as control. Ten days after the final immunization, mice were s.c. injected with varying doses of B16 tumor and monitored for tumor growth and survival time. The results (Fig. 5) showed that mice immunized with B16 or HBSS were all
Adoptive transfer of T cells primed with tumor/DC fusion vaccines can mediate effective therapy of established tumors

In a variety of animal tumor models, studies have shown that LNs draining tumor or the vaccination site are an excellent source for obtaining in vivo primed T cells for adoptive immunotherapy (35, 36). LNs draining the vaccination site contain a much higher frequency of Ag-primed T cells than spleen or peripheral blood (37). B6 mice were s.c. immunized on the flanks with irradiated B16, B16 mixed with DCs, B16/DC, or HBSS as a control. One week after the final immunization, mice were challenged by s.c. injection of 10^4 B16 tumor cells. Tumor growth and survival time in each group of mice were recorded. Tumor incidence was considered positive when the average diameters of the tumor exceeded 3 mm. The data presented were pooled from two separated experiments.

FIGURE 5. Immunization of B6 mice with B16/DC fusion vaccine elicits host resistance against B16 tumor challenge. B6 mice were s.c. immunized twice in a 2-wk interval with 2 × 10^6 irradiated B16, B16 mixed with DCs, B16/DC, or HBSS as a control. One week after the final immunization, mice were challenged by s.c. injection of 10^4 B16 tumor cells. Tumor growth and survival time in each group of mice were recorded. Tumor incidence was considered positive when the average diameters of the tumor exceeded 3 mm. The data presented were pooled from two separated experiments.

dose IL-2 expanded the cell number 2- to 3-fold in 5 days. The LN T cells were greatly enriched in the 5-day short term culture from initially 35–40% Thy-1.2^+ cells to a purity of 99.8% Thy-1.2^+ T cells (65% CD8^+, 30% CD4^+).

To evaluate the therapeutic effects of LN T cells primed with B16/DC fusion vaccine, B6 mice bearing 3-day pulmonary metastases were treated by adoptive transfer of cultured LN T cells derived from different vaccination groups. Mice were sacrificed on day 21 after tumor inoculation, and pulmonary metastatic nodules of each mouse were counted. The results (Fig. 6A) showed that without therapy all mice developed >250 lung metastases on day 21. Adoptive transfer of LN T cells derived from normal B6 mice or mice vaccinated with irradiated B16 or with B16 mixed with DCs had no therapeutic effect on established pulmonary metastases (p > 0.05). All tumor-bearing mice in these treatment groups developed >200 lung metastases on day 21. Adoptive transfer of LN T cells derived from normal B6 mice or mice vaccinated with irradiated B16 or with B16 mixed with DCs plus in vivo administration of IL-2 had no significant reduction of pulmonary metastases was achieved in mice treated with LN T cells derived from mice vaccinated with B16/DC. The mean number of lung metastatic nodules per mouse (33.1 ± 12.2) was significantly less than that in the other treatment groups (p < 0.01). In a separate experiment, B6 mice bearing 3-day pulmonary metastases were treated by adoptive transfer of cultured draining LN T cells plus i.p. administration of IL-2. The results (Fig. 6B) showed that mice that received no therapy or that were treated with IL-2 alone developed >250 lung metastases on day 21. Adoptive transfer of cultured LN T cells from mice vaccinated with B16 mixed with DCs plus in vivo administration of IL-2 had no significant therapeutic effect on established pulmonary metastases (p > 0.05). However, a dramatic reduction of pulmonary metastases was achieved in mice treated with LN T cells derived from mice vaccinated with B16/DC. The mean number of lung metastatic nodules per mouse (4.8 ± 3.5) was significantly less than that in the other treatment groups (p < 0.01). The specificity of therapeutic efficacy of the cultured draining LN T cells derived from mice vaccinated with B16/DC was validated by adoptive transfer of cultured LN T cells from mice vaccinated with irradiated B16, B16/DC, or B16 mixed with DCs into B6 mice with disseminated FBL-3 leukemia. No significant therapeutic effect was observed in mice treated with CY plus adoptive transfer of cultured LN T cells from mice vaccinated with irradiated B16, B16/DC, or B16 mixed with DCs compared with that in mice treated with CY alone (p > 0.05; data not shown).

Table 1. LN cell yield of each vaccination groups

<table>
<thead>
<tr>
<th>Source of LN</th>
<th>Fresh Cell Yield (×10^6/LN)</th>
<th>Anti-CD3 + IL-2 (×10^6/LN)</th>
</tr>
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<tr>
<td>Expt. 1</td>
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<tr>
<td>HBSS</td>
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</table>

*B6 mice were s.c. immunized twice in a 2-wk interval with 2 × 10^6 irradiated tumor (B16, RMA-S, or FBL-3), tumor plus DC (1:1), tumor/DC fusion preparation, or HBSS as controls. At 7 days after the final immunization, inguinal LNs from each group of mice were harvested and counted. LN cells from each vaccination group (5–10 mice/group) were pooled and expanded in a 5-day culture using anti-CD3 plus IL-2. Derived LN T cells were harvested, counted, and used as effector cells for adoptive immunotherapy.
Cultured draining LN T cells derived from mice vaccinated with irradiated FBL-3, RMA-S, RMA-S mixed with DCs, or RMA-S/DC were similarly tested for adoptive immunotherapy of mice with established FBL-3 tumor. Without therapy, FBL-3 tumor-bearing mice died of disseminated leukemia in 10 days. CY treatment reduced the tumor burden, potentially ablated host suppressor T cells, and prolonged survival, but all mice died by day 25. Treatment with CY plus adoptive transfer of cultured draining LN T cells from mice vaccinated with irradiated FBL-3 was effective and cured 100% of the mice (>60 days). However, no significant therapeutic effect was observed in mice treated with CY plus cultured LN T cells from mice vaccinated with irradiated RMA-S or RMA-S mixed with DCs compared with that in mice treated with CY alone (p > 0.05; Fig. 7A). By contrast, treatment with CY plus adoptive transfer of cultured LN T cells from mice vaccinated with irradiated RMA-S/DC was effective and cured 80–100% of FBL-3 tumor-bearing mice (Fig. 7, B and C). No significant therapeutic effects were observed in mice treated with CY plus cultured LN T cells primed with irradiated RMA-S mixed with DCs (Fig. 7B) or cultured LN T cells primed with B16/DC (Fig. 7C) compared with those in mice treated with CY alone (p > 0.05). These results demonstrated that vaccination of B6 mice with B16/DC or RMA-S/DC elicited T cells with therapeutic effectiveness against established B16 and FBL-3 tumor.

Discussion

Insufficient tumor Ag presentation by either tumor cells or host professional APC could be a major cause of the absence of an immune response in tumor-bearing host (16). Fusion of tumor cells with APC may be an effective strategy to induce host T cell-mediated antitumor responses (38). A recent study by Gong et al. showed that vaccination with MC38 carcinoma and DC fusion cells elicited T cell protective immunity against tumor challenge and induced immune rejection of established tumor (26). Our results confirmed, in two poorly immunogenic murine tumor models, that fusion of DCs with tumor cells resulted in the generation of hybrid cells that expressed MHC class I Ags, class II Ags, co-stimulatory (B7.1) molecules, as well as DCs and tumor-derived surface markers. Furthermore, our results demonstrated that vaccination of B6 mice with B16/DC or RMA-S/DC elicited T cells with therapeutic effectiveness against established B16 and FBL-3 tumor.

FIGURE 6. Adoptive transfer of LN T cells primed with B16/DC greatly reduces pulmonary metastases. B6 mice were given i.v. injection of 10⁵ B16 tumor cells to establish pulmonary metastases. Three days after tumor inoculation, mice were left untreated or were treated by i.v. injection of 5 × 10⁷ cultured LN T cells from normal B6 mice or mice vaccinated with irradiated B16, B16 mixed with DCs (1:1), or B16/DC fusion preparation. On day 21 after tumor inoculation, mice were sacrificed, and pulmonary metastatic nodules of each mouse were counted. Each point in the figure represents the number of lung metastasis of one mouse. The data presented in A were pooled from two separate experiments. The data in B were from one representative experiment in which mice were i.p. administered IL-2 (30,000 U/day for 5 days) following adoptive transfer of vaccine-primed, cultured LN T cells.

FIGURE 7. Adoptive transfer of RMA-S/DC vaccine-primed, ex vivo expanded LN T cells effectively eradicates established FBL-3 tumor. Tumor vaccine-primed, ex vivo expanded LN T cells used in adoptive immunotherapy were generated as described in Table I. B6 mice were inoculated i.p. with 5 × 10⁶ viable FBL-3 tumor cells. After 5 days, mice were left untreated or were treated by i.p. injection of 180 mg/kg CY or CY plus vaccine-primed, ex vivo expanded LN T cells (5 × 10⁷/mouse). The data presented in A–C are from three independent experiments.
tumor/DC fusion hybrids were capable of presenting tumor-associated Ags and eliciting tumor-specific CTLs. In vivo, vaccination of mice with tumor/DC fusion cells induced a detectable protective immunity against tumor challenge. Most significantly, adoptive transfer of activated draining LN T cells primed by vaccination with tumor/DC fusions was therapeutically effective against established tumors.

In the past several years the methods for isolation and in vitro culture of DCs from mouse and human have been well developed. DC precursors obtained from blood, bone marrow, or lymphoid organs can mature and grow in the presence of GM-CSF, IL-4, and/or TNF-α to large numbers for prolonged periods of time, with maintenance of Ag processing and presenting functions (29, 39–45). Clinically, DCs can be generated from blood or BM of cancer patients, and tumor cells can be obtained from biopsy samples or surgically removed tumor mass. Thus, immunization with tumor/DC fused cells as vaccines is potentially applicable for human cancer treatment. Compared with other currently used strategies for developing DC-based vaccines such as loading DCs with tumor antigenic proteins or peptides, fusion of DCs with tumor cells have several advantages. First, the hybrid cells have the potential for allowing a nature processing antigenic tumor protein to be present in MHC-peptide complexes on the cell surface. Second, vaccination with tumor/DC hybrids may allow relatively stable, persistent Ag production and presentation in vivo in contrast to peptide-loaded DCs. Third, the fusion of tumor cell with DCs expressing the entire repertoire of tumor Ags should allow immunization of host with multiple tumor Ags without knowing the identity of the tumor Ags. An optimal antitumor response may require the involvement of polyclonal effector subpopulations directed to a broad range of tumor epitopes rather than a response restricted to a single tumor determinant. Immunization with multiple tumor Ags should be more effective than that with a single epitope. Vaccination with DCs pulsed with unfractonated peptides eluted from tumor cells (46) or tumor-derived mRNA (47) may also offer the advantage of providing multiple Ags to the immune system. However, immunization with tumor/DC fusion preparations represents a much simpler and straightforward strategy to elicit T cell-mediated antitumor immunity.

Host T cell-mediated antitumor immunity is known to involve both CD4+ and CD8+ subsets in MHC class I- and class II-restricted responses, respectively (48). Our results showed that fusion of DCs with poorly immunogenic tumors could generate hybrid cells that expressed tumor-derived Ags and were positive for MHC class I, MHC class II, as well as costimulatory molecules essential for the activation of T cells. Fused RMA-S/DC cells were sensitive to lysis by CTL, thus confirming the capability of the tumor/DC fused cells to process and present tumor-associated proteins to T cells. Studies by Öhle et al. (49) reported that fusion of RMA-S with an allogeneic fibroblast line (A9, K+/D2β2m-1) generated RMA-S/A9 fusion hybrids that were capable of processing and presenting influenza virus nucleoprotein to Dd-restricted influenza-specific CTLs after infection with recombinant influenza virus. Our results showed that FMR-MuLV gag-specific CTLs recognized and lysed RMA-S/DC fused cells, but not RMA-S or RMA-S mixed with DCs. The capability of the tumor/DC fused cells to process and present tumor-associated proteins to T cells was also supported by the finding that vaccination of B6 mice with RMA-S/DC or B16/DC fusion preparations elicited detectable specific CTL activities against FBL-3 or B16 tumor cells, respectively. The CTL generated lysed B16.B17.1 more effectively than B16 cells. It is possible that B7 expression enhanced the sensitivity of target cells to lysis. There is also a possibility that tumor/DC fusion hybrids could express a broader range of antigenic epitopes and allow the activation of polyclonal effector subpopulations of T cells. Recently, such a mechanism was demonstrated using genetic modification of tumor cells with the B7 gene to elicit CTLs against otherwise silent subdominant tumor Ags (50). Recent studies have identified at least two antigenic peptides derived from mouse tyrosinase-related protein-2 (51) and mouse gp100 (N. P. Restifo, unpublished observation) as CTL epitopes of B16 tumor cells. Although not defined, B16 cells may carry MHC class II-restricted T cell epitopes that are capable of inducing CD4+ T cell-mediated antitumor immunity. In a recent study our results demonstrated that vaccination of B6 mice with B16/DC fused cells elicited both tumor-reactive CD4+ and CD8+ T lymphocytes and that both T cell subsets were essential for the therapeutic antitumor effect.

Efficient separation of the tumor/DC hybrids from parental tumor cells in the fusion preparations remains a technical challenge. Without selection, the unfused parental tumor cells almost invariably overgrow the fusion hybrid in several days. A potential approach is drug selection of fused cells. In Gong’s report, the fused cells were selected in hypoxanthine-aminopterin-thymidine (HAT)-containing medium, as the parental MC38/MUC1 and MC38 tumor cells were HAT sensitive but the fused cells were not (26). However, this method may not be applicable to most tumor cell lines because it would require establishment of an autologous HAT-sensitive tumor line from individual patients for fusion. In our study immunization with preparations containing about 20% fused cells proved effective in eliciting tumor-reactive T cells and host protective antitumor immunity. Apparently, the contaminating unfused cells do not impede the immunogenicity of the tumor/DC fused cells, so that selection may not be a clinically relevant problem. The host protective antitumor immunity induced by vaccination with tumor/DC fusion could be improved by repeated administration and alternative immunization strategies to elicit more effective responses. Indeed, vaccination with purified B16/DC fused cells separated by using an anti-Ia mAb-mediated MiniMACS separation column (Miltenyi Biotec, GmbH; Germany) as vaccine has been shown to induce much stronger host protective immunity against high dose B16 tumor challenge (10⁶ cells/mouse) with a greater percentage of tumor-free mice (see Footnote 4). In this study we used BM DCs from 6-day culture in GM-CSF- plus IL-4-supplemented medium. It is known that such relatively immature DCs have more active Ag processing capability but are less effective in T cell activation than the mature DCs. Whether fusion of tumor cells with mature DCs (5- to 7-day GM-CSF plus IL-4 culture followed by treatment with TNF-α) could generate more potent tumor/DC vaccine is yet to be determined.

One of the major reasons to use tumor/DC fused cells for vaccination is that the tumor/DC fusion hybrids can process and present endogenous tumor-associated proteins to elicit MHC class I-restricted tumor-reactive CTLs. The most recent study by Ashley et al. demonstrated that vaccination with DCs pulsed with unfractonated tumor extracts could induce specific CTLs and potent antitumor immunity (52). The relative potency of Ag-pulsed DCs compared with tumor/DC fusion cells in eliciting anti-tumor immunity remains unclear.

Acknowledgment

We thank Drs. Suyu Shu and Greg E. Plautz for their critical review of this manuscript.

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IMMUNOTHERAPY WITH TUMOR-DUCTION VACCINES

References


tected tumor cells present endogenous antigen and are potent inducers of tumor-
