

# Efficient Responses in a Murine Renal Tumor Model by Electroloading Dendritic Cells With Whole-Tumor Lysate

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**Summary:** Electroporation of dendritic cells (DCs) with tumor lysate elicited greater antitumor responses in vitro and in vivo, using less lysate than standard coinubation. Electroloaded DCs had normal surface marker expression and matured into competent antigen-presenting cells. In a renal carcinoma (RENCA) model, mice were pretreated with lysate-loaded DCs before tumor challenge. Mice that received DCs electroloaded with RENCA lysate had significantly smaller tumors ( $9 \pm 6 \text{ mm}^2$ ) than mice given DCs coinubated with the same lysate ( $23 \pm 5 \text{ mm}^2$ ). To evaluate a metastatic therapeutic tumor model, mice were first injected with Lewis lung carcinoma (LLC) and then given 2 doses of cryopreserved LLC lysate-loaded DCs. Mice treated with electroloaded DCs had a 50% reduction in lung metastases compared with control mice that received no DCs or DCs loaded with liver lysate. In contrast, DCs coinubated with LLC lysate were indistinguishable from controls. Tumor lysate-electroloaded but not-coinubated DCs also primed syngeneic mouse splenocytes in vitro to produce interferon- $\gamma$  and, specifically, lyse tumor cells. The electroloaded DCs elicited specific T-cell responses with less lysate than the amount reported in standard coinubation procedures. This approach may be particularly useful when small amounts of tumor material are available.

**Key Words:** dendritic cells, whole-tumor lysate, mouse tumor models, electroporation, immunotherapy

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Dendritic cells (DCs) have enormous potential as tools for vaccination and cancer immunotherapy because of their essential role in the establishment of immune responses. They are considered to be among the most professional antigen-presenting cells (APCs) because they are capable of stimulating naive and memory T cells, natural killer (NK) cells, and B cells and of producing important immunomodulatory cytokines, such as interleukin (IL)-2 and IL-12, which are essential for cell-mediated killing.<sup>1,2</sup>

Numerous studies have demonstrated the feasibility of manipulating DCs with tumor antigens for immunotherapy protocols. The most common method involves simply

coinubating the DCs with antigen, a process known as “pulsing,” which takes advantage of the ability of DCs to take up extracellular material through macropinocytosis.<sup>3</sup> Several recent studies have shown that DCs pulsed with tumor antigenic complementary DNA (cDNA),<sup>4</sup> peptide,<sup>5–8</sup> or messenger RNA (mRNA)<sup>9,10</sup> can lead to specific T-cell responses against target cells expressing the antigen to which they were raised. Other methods of DC manipulation include viral vectors,<sup>11–14</sup> electroporation,<sup>4,9</sup> DC fusion to the tumor cells<sup>15,16</sup> and molecularly engineered fusion proteins that are taken up via receptor-mediated endocytosis.<sup>17–20</sup> Unfortunately, there is no “gold standard” in DC processing protocols. The method of choice usually depends on the selection, source, and availability of antigenic material used for the particular study. Regardless of the method used for modifying DCs, the fundamental readout remains the same: the elicitation of specific T-cell responses and tumor killing in vitro and/or in vivo.

The selection of antigenic material is probably one of the most critical aspects of designing an effective DC-based immunotherapy protocol. The use of whole-tumor lysate offers several important advantages over the single antigen approach. First, it eliminates the requirement to identify and characterize the specific antigen. The entire antigenic repertoire of the cancer cells is prepared as a lysate and simultaneously incorporated into the DCs, which efficiently handle the processing and presentation of antigens without the actual need to identify the antigens. For some cancers in which there is a paucity of well-characterized tumor-associated antigens, whole-tumor lysate might be the most practical approach. Second, the use of lysate generally offers a broader repertoire of tumor-associated antigens, which has been hypothesized to reduce the likelihood for tumor escape.<sup>21</sup> Because different antigens would be expected to be presented in class I and class II major histocompatibility complex (MHC) pathways, the whole-tumor lysate approach might also be expected to favor simultaneous CD4 and CD8 T-cell stimulation, which is believed to be optimal for tumor killing.<sup>15</sup> This stands in contrast to the single peptide approach, which is restricted to a given human leukocyte antigen (HLA) type and the induction of only cytotoxic T-cell responses. Indeed, whole-tumor lysates may more appropriately reflect the physiologic process by which a growing tumor induces an immune response in vivo.<sup>21</sup> Many studies have indeed shown the feasibility of manipulating DCs with whole-tumor lysate, most commonly by standard coinubation and/or pulsing, for treating many different types of cancers, including

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solid carcinomas,<sup>21–24</sup> myeloma,<sup>25</sup> glioma,<sup>26</sup> and melanoma.<sup>8,14</sup> These studies demonstrate that DCs coincubated with whole-tumor lysate can elicit specific antitumor T-cell responses.

In this study, we used electroporation (EP) to achieve the safe, consistent, and efficient loading of DCs with whole-tumor lysate. The electroloaded DCs elicited enhanced specific T-cell responses *in vitro* and antitumor immune responses *in vivo* with considerably less lysate than the amount reported to achieve an effective immune response in standard coincubation protocols. This approach may be particularly useful when small or limiting amounts of starting tumor material are available. The electroporation-based cell loading system designed by MaxCyte can effectively load DCs with tumor antigens in a scalable and closed and/or sterile environment that is suitable for clinical applications.<sup>27</sup>

## MATERIALS AND METHODS

### Isolation of Murine Dendritic Cells

C57BL/6 and Balb/C male mice (6 weeks of age) were obtained from The Jackson Laboratories (Bar Harbor, ME). Mice were cared for under an approved institutional animal care and use committee (IACUC) protocol.

To prepare DCs, mice were killed and the long bone marrows were extracted. Red blood cells were lysed with ACK cell lysing solution (Fisher). The washed bone marrow cells were then seeded in T175 tissue culture flasks at a final cell concentration of  $5 \times 10^6$  cells/mL in AIM-V (BioWhittaker) media supplemented with 0.2% human serum albumin (Sigma), 2 mM L-glutamine (Hyclone), 25 ng/mL recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF; R&D Systems), and 12.5 ng/mL recombinant murine IL-4 (R&D Systems). On days 2 and 3 and days 6 and 7, the cells were fed new AIM-V media containing these same supplements for a total of 2 complete media changes. The suspension cells were collected after 9 days in culture. A small aliquot of the cells was analyzed for surface marker expression by flow cytometry. The DCs were cryopreserved in fetal bovine serum (FBS) containing 10% dimethyl sulfoxide (DMSO) according to established protocols until used.

### Preparation of Whole-Tumor Lysate

Whole-tumor cell lysate was initially prepared from the *in vitro* culture of the following tumor cell lines: renal carcinoma (RENCA), murine melanoma (B16-F10), and Lewis lung carcinoma (LLC). Each of these tumor cell lines was generously provided by Kirk Volker (EntreMed) and cultured as described previously.<sup>28</sup> For lysate preparation, the trypsinized tumor cells were washed once in phosphate-buffered saline (PBS) and resuspended in PBS at a final concentration of  $1 \times 10^8$  cells/mL. Cells were then subjected to 4 successive cycles of rapid freezing in a dry ice/alcohol bath and thawing in a 37°C water bath. The lysed cells were then centrifuged for 10 minutes at 4°C, and the supernatants were transferred to new tubes. Total protein concentrations were quantitated using a commercially available protein assay kit (Pierce). Lysates were stored at –80°C until use.

To eliminate the potential for tissue culture contaminants (eg, FBS, trypsin) in our lysate preparations, subsequent

experiments used whole-tumor lysates that were prepared with established tumors freshly removed from animals. The tumors were dissected free of as much normal tissue as possible, finely minced, diluted in PBS, and subjected to 4 freeze/thaw cycles as described previously. Control liver lysates were prepared at the same time. Total protein concentrations were quantitated as described previously, and equivalent amounts of total lysate protein were used for all experiments.

### Flow Cytometry

Bone marrow–derived DCs were routinely analyzed by flow cytometry using monoclonal antibodies to the following surface antigens: CD3, CD14, CD19, MHC class II (I-A/I-E), CD80, CD83, and CD86. All antibodies were purchased from BD-Pharmingen and were directly conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE). After 9 days in culture, bone marrow–derived cells were negative for CD19, had <5% CD3<sup>+</sup> and CD14<sup>+</sup> cells, and were >90% viable, as determined by propidium iodide exclusion. Approximately 75% to 80% of the cells were MHC class II, CD80/86<sup>+</sup>, and CD83<sup>–</sup> immature DCs (Fig. 1).

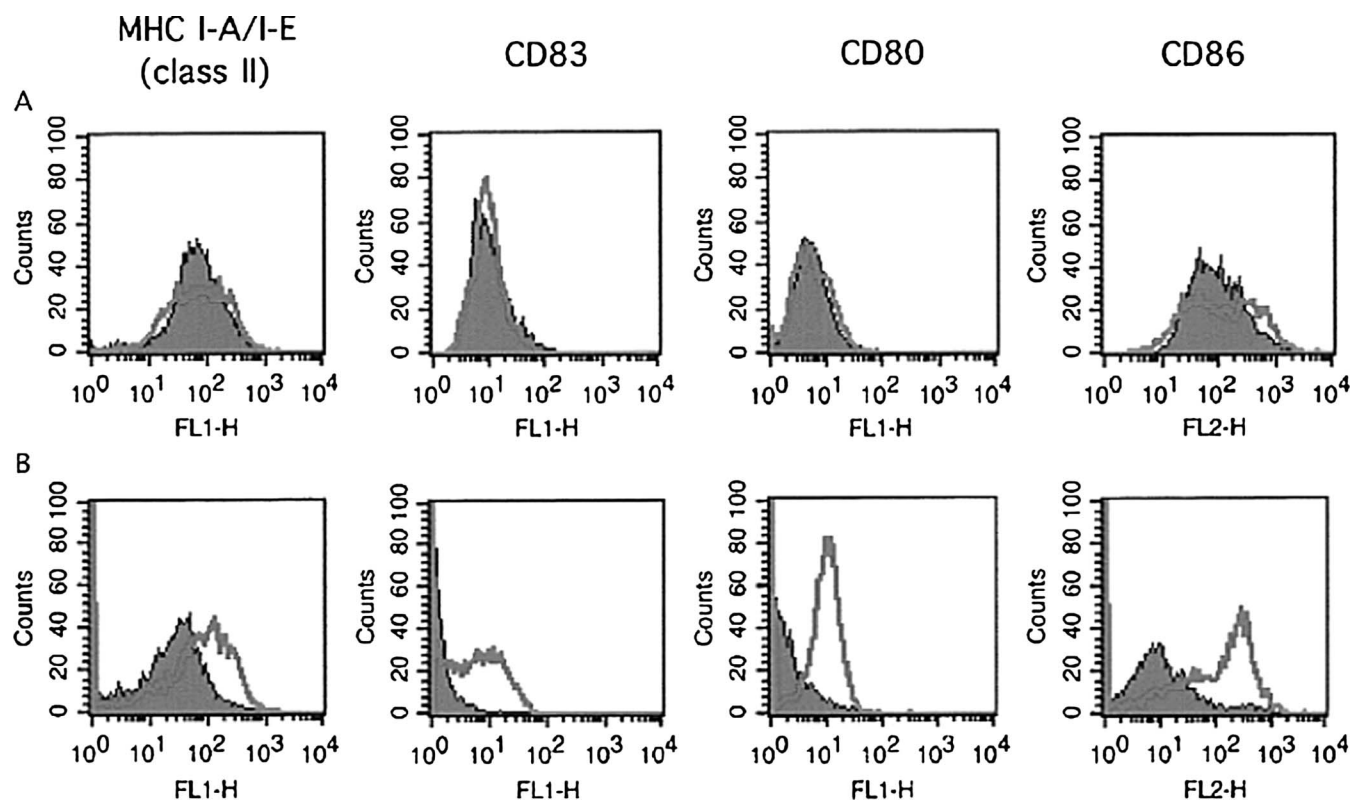
Flow cytometry was also used to quantify the amount of macromolecules that had been loaded into DCs. At various times after coculturing or electroporating DCs in the presence of 0.5 mg/mL FITC-dextran (molecular weight [MW] = 250 kDa; Sigma), cells were washed extensively in PBS and analyzed by flow cytometry. Nonloaded cells were used for control gating purposes. Cell loading was calculated as the percentage of cells that had taken up the FITC-dextran. Cell viability was determined by propidium iodide exclusion.

### Electroloading of Dendritic Cells

DCs were collected, washed once with PBS, and prepared for electroporation as previously described.<sup>28</sup> Whole-tumor lysate was added to the DCs at a cell ratio that ranged from 1 to 100 DCs for each tumor cell equivalent (TC). This inversely correlates to a total protein concentration of 5000 µg/mL (1 DC/1 TC) down to 50 µg/mL (100 DCs/1 TC) total protein. Most experiments used a cell ratio of 10 DCs/1 TC (500 µg/mL protein) in a scalable cell volume. DCs were electroloaded using a MaxCyte GT electroporation-based system and 25- to 100-µL cuvettes<sup>27,28</sup> or simply coincubated with an equivalent amount of lysate in the absence of electroporation (standard pulsing). After 30 minutes at 37°C, the loaded (unwashed) DCs were plated in 6-well tissue culture plates containing complete AIM-V medium, supplemented with 25 ng/mL murine granulocyte-macrophage colony-stimulating factor (mGM-CSF), murine tumor necrosis factor-α (mTNFα), and murine interferon-γ (mIFNγ) (each from R&D Systems); 12.5 ng/mL murine interleukin (mIL)-4 (R&D Systems); 1 µg/mL prostaglandin E<sub>2</sub> (PGE<sub>2</sub>; Sigma); and 10 µg/mL lipopolysaccharide (LPS; Sigma) overnight for maturation.

### Mouse Tumor Models

For the RENCA tumor challenge model, syngeneic (Balb/C) mice were first injected subcutaneously with  $1 \times 10^6$  DCs that had been loaded (electroloaded or coincubated) with RENCA lysate. As controls, some mice were given no DCs at all and others were given  $1 \times 10^6$  DCs that had been electroloaded



**FIGURE 1.** Flow cytometric analysis of immature and mature DCs. The DCs were immunostained with fluorescently conjugated antibodies and analyzed by flow cytometry as described in the Materials and Methods section. A, Electroporation did not alter MHC class II, CD83, CD80, or CD86 surface expression. Immature DCs that were not electroporated (gray solid curve) were compared with immature DCs that had been electroloaded (clear open curve). B, Maturation of DC cultures with cytokines for 24 hours upregulated MHC class II, CD80, CD86, and CD83 expression. The gray solid lines represent immature DCs, and the clear open lines represent matured DCs. Note that the fluorescence (FL1 and FL2) voltages were not the same between the 2 experiments; thus, the mean fluorescence intensity of the immature DCs in B is shifted to the left by approximately 1 log as compared with that in A.

with an equivalent amount of whole-liver lysate (0.5 mg/mL). Ten days later, mice were injected subcutaneously at a different site with  $5 \times 10^5$  live intact RENCA cells. Primary tumor areas were measured twice a week using mechanical calipers. Tumor volumes were calculated by the formula  $v = \pi ab^2/6$ , where  $a$  is the longest diameter and  $b$  is the next longest diameter perpendicular to  $a$ .<sup>29,30</sup>

For the LLC therapeutic model, syngeneic (C57BL/6) mice were first injected intravenously (tail vein) with  $1 \times 10^5$  live intact LLC cells (day 0). Then, on days 3 and 6, mice were injected intravenously with 2 separate yet equivalent doses of  $1 \times 10^6$  cryopreserved, lysate-loaded, and matured DCs. On day 17, mice were killed and their lungs were dissected, washed, and weighed. Lung weight was used as an indicator of lung metastases; control mice that were not given any tumor cells were used as baseline lung weight controls.

### In Vitro Cytotoxicity Assay

C57BL/6 and Balb/C bone marrow-derived DCs were loaded with B16 melanoma or RENCA lysate by electroporation or coincubation (ratio of 10 DCs/1 TC). As a control, DCs were electroloaded with an equivalent amount of whole-liver lysate. Loaded DCs were matured overnight and coincubated with syngeneic naive splenocytes at a ratio of 1

DC/10 splenocytes in the presence of 25 ng/mL mIL-2 and mIL-7 each (R&D Systems). As an additional control, splenocytes were cultured in media alone (splenocytes only). Splenocytes were restimulated weekly with fresh DCs and cytokines for a total of 4 *in vitro* stimulations. The primed splenocytes were then collected, washed, and coincubated with  $^{51}\text{Cr}$ -labeled B16 melanoma, LLC, or RENCA cells at various tumor/effector ratios. After 4 hours at 37°C, the supernatants were collected and analyzed using a scintillation counter. Results were normalized by subtracting spontaneous  $^{51}\text{Cr}$  release from tumor cells and presented as a percentage of maximal cell killing (using 2% Triton X-100 to obtain maximal  $^{51}\text{Cr}$  release).

### Statistics

The Student paired *t* test (Microsoft Excel) was used to determine significance between experimental groups. Results were considered to be significant for  $P < 0.05$  values.

## RESULTS

### Evaluation of Dendritic Cell Surface Marker Expression

DCs were routinely immunostained with fluorescently conjugated antibodies and analyzed for surface marker expression by flow cytometry. Control cells, analyzed in the

absence of any antibody, were normalized between  $10^0$  and  $10^1$  in fluorescence intensity. As shown in Figure 1A and B, immature DCs were identified by their high expression of class II MHC (I-A/I-E), low to moderate expression of CD80 and CD86, and negative CD83 expression. The DC cultures were also negative for CD3, CD19, and CD14 antigens (data not shown) to reduce the potential for contaminating T cells, B cells, and monocytes, respectively. The analysis of surface marker antigens was evaluated in immature DCs before and after electroporation. The expression of MHC class II, CD83, CD80, and CD86 was unaltered after the DCs were electroloaded in the presence of tumor lysate (see Fig. 1A). Thus, the cell loading protocols used in this study did not significantly alter the normal repertoire of surface antigens, particularly those involved in antigen presentation and costimulatory signaling pathways.

As shown in Figure 1B, the overnight treatment of DC cultures with cytokine maturation cocktail resulted in the expected upregulation of CD83, CD80, and CD86 surface expression by at least 1 log for each marker. MHC class II expression was also modestly upregulated on DC maturation (see Fig. 1B). The cell culture supernatants of immature and mature DCs were collected and analyzed by enzyme-linked immunosorbent assay (ELISA) for human interleukin (hIL)-12 secretion. On maturation, 100 to 200 pg hIL-12 per  $1e6$  DCs was detected, whereas no hIL-12 expression was detected in the immature DC cultures. We conclude that the immature and mature DCs used in our studies displayed the predicted profile of surface antigen expression and cytokine secretion.<sup>31</sup> The viabilities of the immature, mature, and electroloaded DCs were also indistinguishable at >90%, as determined by propidium iodide exclusion (data not shown).

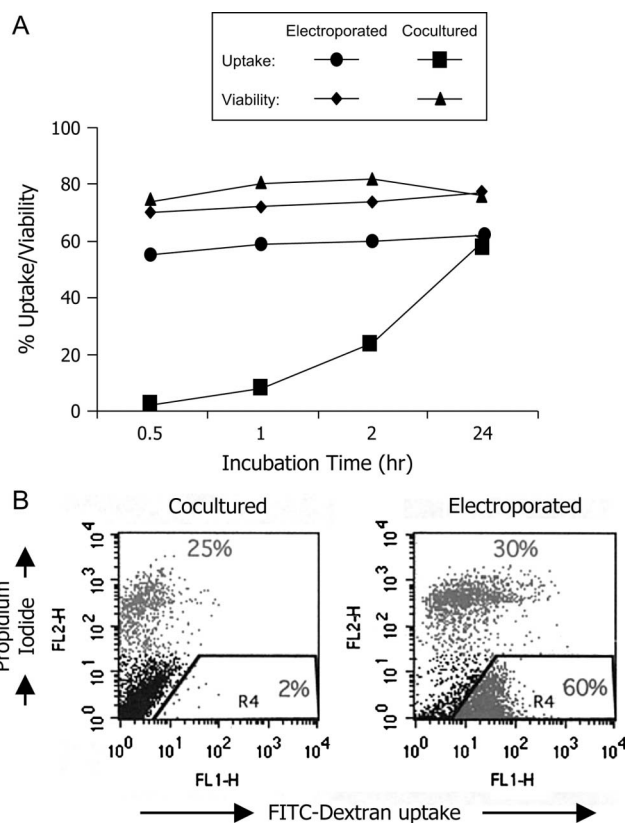
### Efficient Electroloading of Dendritic Cells With Macromolecules

We first evaluated the loading of DCs with a large antigenically inert macromolecule, FITC-conjugated dextran (MW = 250 kDa). Immature DCs from C57BL6 mice were coincubated with 0.5 mg/mL FITC-dextran. Some of these DCs were electroloaded as described in the Materials and Methods section, whereas others served as coincubation controls (standard coincubation or “pulsing”). After various periods, the coincubated and electroloaded DCs were washed and analyzed by flow cytometry for FITC-dextran uptake as well as cell viability, with the latter being determined by propidium iodide staining. As shown in Figure 2A, the electroloaded DCs had virtually instantaneous loading of the FITC-dextran (55%–60%) and cell viability was indistinguishable from that of the coincubated DC controls (70%–80%). The recovery time after electroporation could be as short as 30 minutes; at that point, none of the coincubated DCs had taken up the FITC-dextran (see Fig. 2B). Lengthening the incubation time after electroporation had no effect on cell viability or loading. No significant uptake was noted in the DCs coincubated with FITC-dextran until increasing the time to at least 2 hours. At this point, only approximately 25% to 30% of the DCs had taken up the FITC-dextran as compared with the consistent 60% to 65% seen with the electroloaded DCs. Furthermore, it took 24 hours for the coincubation

results to resemble the consistent levels seen using electroloaded DCs. These results demonstrate that electroporation-mediated loading of DCs was instantaneous and safe.

### Dendritic Cells Electroloaded With Whole-Tumor Lysate Elicited Greater Naive Splenocyte Responses

We next compared the efficacy of electroloaded and coincubated DCs to elicit naive splenocyte priming in vitro. C57BL6 bone marrow–derived DCs were electroloaded with B16 melanoma lysate or coincubated as previously described. As a control, DCs were electroloaded with an equivalent amount of syngeneic whole-liver lysate. The loaded DCs were matured overnight and cocultured with syngeneic naive splenocytes at a ratio of 1 DC/10 splenocytes. As an additional



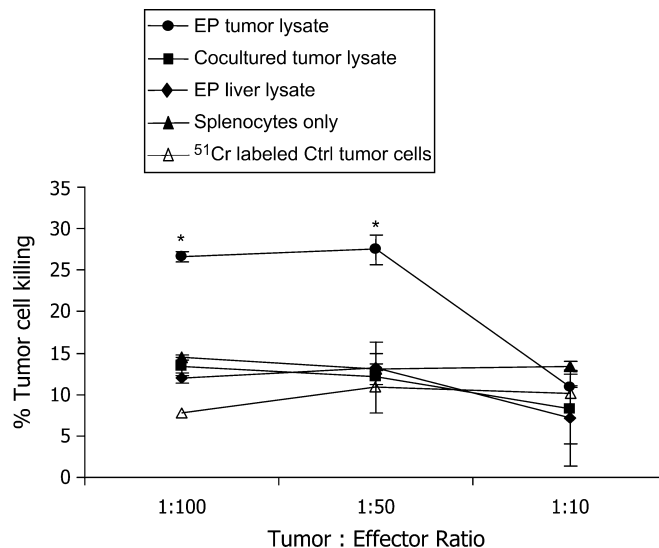
**FIGURE 2.** Efficient loading of DCs with macromolecules. The DCs were mixed with 0.5 mg/mL FITC-dextran (250 kDa) and then electroloaded and washed (circles) or left in standard culture without washing (squares). After various lengths of time in culture, the DCs were washed twice (electroloaded and coincubated) with PBS and analyzed by flow cytometry. Cell viability was determined using propidium iodide exclusion. A, Time course of FITC-dextran loading shows instantaneous uptake using electroporation. In contrast, overnight coincubation was required to obtain similar FITC-dextran uptake. Identical viability results were observed for electroloaded and coincubated DCs at all time points tested. B, Flow cytometric analysis 30 minutes after EP is shown. The percentage of loaded cells (FL1) is shown in region R4, whereas the percentage of dead cells (FL2) is indicated at the top of the panels.

control, splenocytes were cultured in media alone (splenocytes only). Splenocytes were restimulated weekly for a total of 4 in vitro stimulations as described in the Materials and Methods section. DCs used for these restimulations were cryopreserved so that consistent doses could be administered and the feasibility of using frozen lysate-loaded DCs could be demonstrated. The primed splenocytes were then collected, washed, and mixed with  $^{51}\text{Cr}$ -labeled B16 tumor cells at various tumor/effector ratios. As shown in Figure 3, only the DCs that had been electroloaded with B16 lysate elicited tumor cell killing to levels significantly greater than those of controls (EP tumor lysate;  $P < 0.01$ ). In contrast, results using DCs that had been coincubated with B16 lysate were indistinguishable from those using liver lysate and splenocyte only (no DC priming) controls (see Fig. 3). These results were specific, in that the same splenocytes that were primed using B16 lysate-loaded DCs failed to kill  $^{51}\text{Cr}$ -labeled irrelevant (LLC) tumor cells (see Fig. 3;  $^{51}\text{Cr}$ -labeled control tumor cells). Similar results were obtained when we repeated the experiment using BalbC DCs electroporated with RENCA lysate, syngeneic splenocytes, and  $^{51}\text{Cr}$ -labeled RENCA cells (data not shown). The results from several different tumor models and animal strains demonstrate the practicality of tailoring this approach to multiple tumor models, provided that a sufficient source of tumor material is available for preparation of the lysates.

We also compared the conditioned culture media from these in vitro primed splenocytes for interferon- $\gamma$  (IFN $\gamma$ ) secretion. As shown in Figure 4, DCs electroloaded with RENCA tumor lysate caused significantly greater amounts of IFN $\gamma$  to be secreted into the supernatants of the DC/splenocyte cocultures (EP tumor lysate;  $P < 0.05$ ). In contrast, no significant increase was detected using DCs that had been electroporated in the absence of any tumor lysate or those coincubated with tumor lysate (see Fig. 4). Furthermore, no appreciable increase in IFN $\gamma$  levels was observed when these in vitro primed splenocytes were cultured in the absence of any DCs or restimulated with DCs electroloaded with an irrelevant tumor lysate (see Fig. 4, second DC EP control tumor lysate). As a positive control for this assay, we treated the splenocytes with 10  $\mu\text{g}/\text{mL}$  phytohemagglutinin (PHA) for 24 hours and could detect 10 to 20 ng/mL IFN $\gamma$  in these cultures (data not shown).

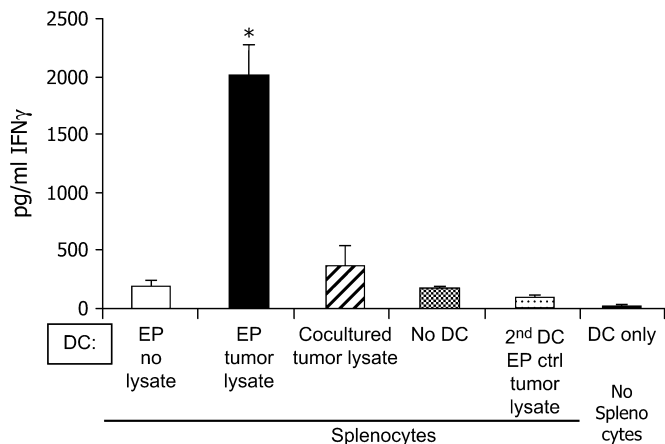
### In Vivo Efficacy of Whole-Tumor Lysate Electroloaded Dendritic Cells in a Tumor Challenge Model

To explore the efficacy of lysate-loaded DCs in vivo, BalbC-derived DCs were coincubated or electroloaded with whole RENCA tumor lysate at a cell ratio of 10 DCs/1 TC as previously described. The loaded DCs were then allowed to mature overnight. Roughly,  $1 \times 10^6$  DCs were injected in syngeneic BalbC mice subcutaneously. Ten days later, the mice were challenged subcutaneously with  $5 \times 10^5$  RENCA tumor cells at a different site. The size of the tumors was measured starting at 9 days after tumor challenge. As shown in Figure 5, the DCs that were electroloaded with RENCA lysate resulted in significantly decreased tumor volumes ( $n = 3$ ;  $P < 0.05$ ). This significant decrease was noted between days 14 and 18 after RENCA injection. Controls included mice injected with no DCs at all, DCs electroloaded in the absence of lysate, and



**FIGURE 3.** DCs electroloaded with B16 melanoma lysate elicited specific tumor cell killing in naive splenocytes in vitro. C57BL6 mouse bone marrow-derived DCs were loaded with B16 melanoma lysate by electroporation or coincubation (10 DCs/1 TC). As a control, DCs were electroloaded with an equivalent amount of whole-liver lysate (EP liver lysate). Loaded DCs were matured overnight and coincubated with syngeneic naive splenocytes at a ratio of 1 DC/10 splenocytes. As an additional control, splenocytes were cultured in media alone (splenocytes only). Splenocytes were restimulated weekly for a total of 4 in vitro stimulations by adding the same amount of modified DCs to the cultures. The primed splenocytes were then washed, counted, and coincubated with  $^{51}\text{Cr}$ -labeled B16 melanoma cells or  $^{51}\text{Cr}$ -labeled irrelevant LLC tumor cells at various tumor/effector ratios. After 4 hours at  $37^\circ\text{C}$ , the supernatants were collected and analyzed using a scintillation counter. Results were normalized by subtracting spontaneous  $^{51}\text{Cr}$  release from tumor cells and presented as a percentage of maximal cell killing (using 2% Triton X-100 to obtain maximal  $^{51}\text{Cr}$  release). Only the DCs that had been electroloaded with B16 tumor lysate elicited specific tumor cell killing to levels significantly greater than those of controls ( $*P < 0.01$ ). In contrast, splenocytes primed with B16 lysate-electroloaded DCs did not kill  $^{51}\text{Cr}$ -labeled irrelevant (LLC) tumor cells (open triangles).

DCs electroloaded with irrelevant (liver) lysate. Although the trend of all mouse groups was a steady increase in overall tumor volume, only the electroloaded DCs had any significant reduction and/or delay in primary tumor formation. In contrast, no beneficial effect was observed using DCs that had been simply coincubated with RENCA lysate (see Fig. 5). The fact that whole-liver lysate-electroloaded DCs had no effect strongly supports the hypothesis that the reduced tumor burden is not attributable to nonspecific responses to altered host antigens. Furthermore, we performed subsequent experiments using dissected RENCA tumors as the starting material for tumor lysates rather than cultured RENCA cells to reduce the risk of contamination of lysates with tissue culture reagents. These studies demonstrated an identical reduction of primary tumor volumes only with the DCs electroloaded with RENCA lysate (data not shown). These results demonstrate that even

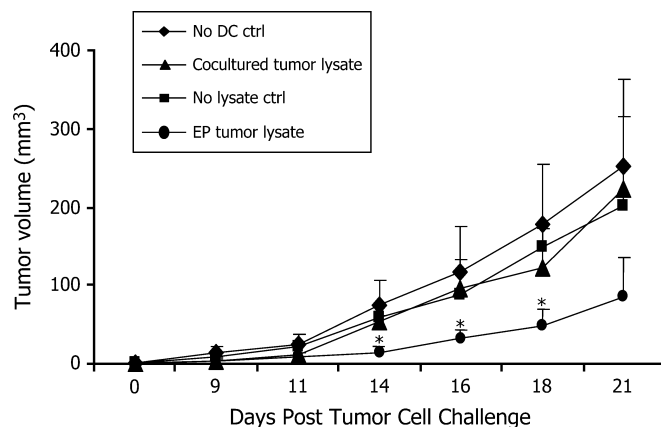


**FIGURE 4.** DCs electroloaded with whole-tumor cell lysate generated greater naive T-cell activation in vitro than standard cocultivation. Balb/C mouse-derived DCs were cocultured or electroloaded with RENCA lysate and then matured as previously described. The matured DCs (5e4) were then incubated with 5e5 syngeneic splenocytes (at a ratio of 1 DC/10 splenocytes). Splenocytes were restimulated with an additional 5e4 cryopreserved modified DCs 7 days later. Twenty-four to 48 hours after restimulation, tissue culture supernatant was collected and analyzed by ELISA for IFN $\gamma$  production. Naive splenocytes primed with lysate-electroloaded DCs produced significant amounts of IFN $\gamma$  (EP tumor lysate;  $P < 0.05$ ). In contrast, DCs that had been cocultured with tumor lysate failed to stimulate significant IFN $\gamma$  production (cocultured tumor lysate), as did DCs electroporated in the absence of any tumor lysate (EP, no lysate). No appreciable levels of IFN $\gamma$  were detected using splenocytes or DCs alone or if restimulation used DCs electroloaded with an irrelevant tumor lysate.

only 1 dose of lysate-electroloaded DCs was capable of mediating significant protection against the primary tumor burden.

### Dendritic Cells Electroloaded With Tumor Lysate Reduced Metastases in a Therapeutic Model

In this LLC model, we first administered, by intravenous injection of tail veins, 5e5 LLC cells into syngeneic C57BL6 mice. Isolated C57BL6 DCs were again cocultured or electroloaded with LLC whole-tumor cell lysate and matured as previously described. As a control, DCs were electroloaded with whole-liver lysate. The modified and matured DCs were cryopreserved until used. On days 3 and 6 after LLC injection, a dose of 1e6 DCs was administered intravenously (8–10 mice per group). One group of mice was not given any DCs at all (no DC control). On day 15 after LLC injection, mice were killed and lungs were dissected and weighed as an indicator of metastases. As shown in Figure 6, only the DCs electroloaded with LLC lysate significantly reduced LLC lung metastases, as indicated by a significant decrease in lung weights ( $n = 2$ ;  $P < 0.01$ ). In contrast, no effect at all was observed using DCs cocultured with LLC lysate or DCs electroporated in the absence of LLC lysate (see Fig. 6). It was unlikely that nonspecific alterations in lung weights (eg, lung edema) could



**FIGURE 5.** DCs electroloaded with RENCA lysate delayed primary tumor burden. Balb/C mouse bone marrow-derived DCs were cocultured or electroloaded with RENCA tumor lysate at a cell ratio of 10 DCs/1 TC and then matured as previously described. Then, approximately 1e6 mock or treated DCs were injected in syngeneic Balb/C mice subcutaneously. Ten days later, the mice were challenged with 5e5 RENCA tumor cells at a different site. The size of the tumors was measured starting at 9 days after tumor challenge. Significantly smaller tumor volumes were detected at days 14, 16, and 18 in those mice that received tumor lysate-electroloaded DCs ( $*P < 0.05$  as compared with no DCs and no lysate controls).

be caused by introducing DCs and/or electroporated cells, because the lung weights were extremely similar among the “no DC,” “EP liver lysate,” and “coculture LLC lysate” treatment groups (see Fig. 6).

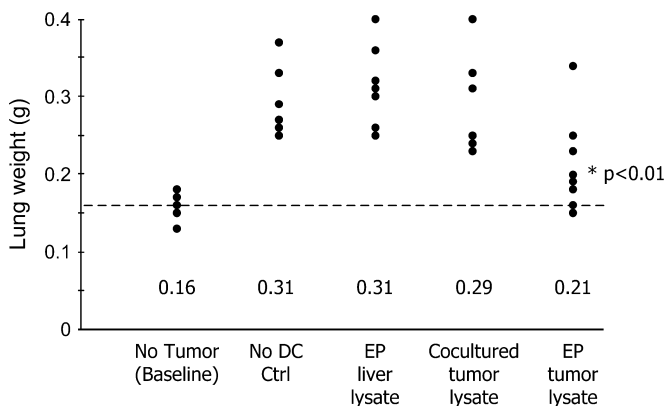
### Dendritic Cells Electroloaded With Tumor Lysate Enhanced Tumor Killing by Memory T Cells In Vitro

We investigated whether lysate-electroloaded DCs could effectively stimulate primed or memory splenocytes to elicit enhanced tumor cell killing. At the end of the LLC therapeutic experiment, splenocytes from those mice given DCs electroloaded with LLC lysate were isolated, cryopreserved, and later used for in vitro restimulations. C57BL6-derived DCs were cocultured or electroloaded with LLC lysate as before. Again, as a control, DCs were electroloaded with an equivalent amount of whole-liver lysate. The loaded DCs were matured overnight and cocultured with syngeneic in vivo primed splenocytes exactly as described previously for the naive splenocyte experiment (see Fig. 3). As an additional control, splenocytes were cultured in media alone (splenocytes only). Splenocytes were restimulated exactly as before and then cocultured with  $^{51}\text{Cr}$ -labeled LLC cells at various tumor/effector ratios. As shown in Figure 7, the DCs that had been electroloaded with LLC lysate elicited tumor cell killing to levels significantly greater than those of controls ( $P < 0.001$  at a tumor/effector cell ratio of 1:50 or 1:100). Furthermore, those DCs that had been cocultured with LLC lysate could also elicit specific tumor cell killing, albeit to a far lesser extent than those DCs that had been electroloaded with LLC lysate ( $P < 0.005$  as compared with the EP LLC lysate group). As

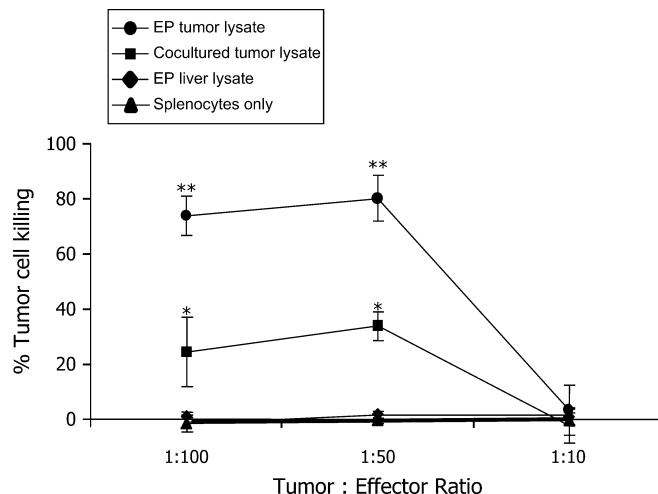
before, splenocytes cultured in the absence of any DCs or those cocultured with DCs that had been electroloaded with control liver lysate failed to elicit any specific tumor cell killing whatsoever.

## DISCUSSION

We performed side-by-side comparisons between DCs coincubated and DCs electroloaded with whole-tumor lysate and demonstrated that the electroporated DCs are capable of eliciting significantly greater antitumor responses in vitro and in vivo. Using IFN $\gamma$  production as well as specific tumor cell-killing assays, we showed that the electroloaded DCs were capable of priming naive and memory splenocytes in vitro better than DCs that had been coincubated with antigen. In a tumor challenge and a therapeutic metastatic tumor model, those DCs electroloaded with whole-tumor lysate consistently blocked significant tumor growth in vivo. These results were observed for RENCA, B16 melanoma, and LLC, each considered to be poorly immunogenic and highly aggressive, with control mice routinely succumbing to disease within several weeks. Our consistent results demonstrated the suitability of applying the electroporation of whole-tumor lysate to different



**FIGURE 6.** DCs electroloaded with LLC lysate reduced lung metastases.  $5 \times 10^5$  LLC cells were first administered intravenously (tail vein) into C57BL6 mice. Isolated C57BL6 DCs were coincubated or electroloaded with LLC whole-tumor cell lysate and matured as previously described. As a control, DCs were electroporated in the absence of any lysate (no lysate). The DCs were then cryopreserved in 2 separate doses each. On day 3 after LLC injection,  $1 \times 10^6$  DCs were administered by tail vein injection (8 mice per group). One group of mice was not given any DCs at all (no DC control). After an additional 3 days (day 6), a second dose of identically loaded DCs ( $1 \times 10^6$ ) was injected again. On day 15 after LLC injection, mice were killed and lungs were dissected and weighed. The no tumor control group reflects normal lung weights of mice that were not challenged with any LLC (the dotted line indicates the mean baseline). Administration of DCs that had been electroloaded with LLC lysate caused a significant reduction in LLC lung metastases, as indicated by a significant decrease in lung weights ( $n = 2$ ;  $P < 0.01$ ). Each dot on the graph represents 1 mouse from a group of at least 8 mice. In contrast, the mice given no DCs, DCs electroloaded with control liver lysate, or DC coincubated with LLC lysate each had a large and indistinguishable extent of tumor metastases.



**FIGURE 7.** DCs electroloaded with LLC lysate elicited specific tumor cell killing in primed splenocytes in vitro. Splenocytes were isolated from C57BL6 mice that had previously received 2 intravenous administrations of  $1 \times 10^6$  syngeneic DCs electroloaded with LLC lysate (in vivo priming). These splenocytes were then cocultured in vitro with lysate-loaded DCs at a ratio of 1 DC/10 splenocytes. Splenocytes were restimulated once a week for a total of 3 restimulations with lysate-loaded DCs. As controls, splenocytes were cultured in the absence of any DCs (splenocytes only) or mixed with irrelevant lysate-loaded DCs (liver lysate control). DCs that had been coincubated with LLC lysate were compared with those that had been electroloaded with LLC lysate. The primed splenocytes were then washed and incubated with  $^{51}\text{Cr}$ -labeled intact LLC cells for a standard cell-mediated killing assay. Results were normalized as previously described. The DCs electroloaded with LLC lysate elicited 70% to 80% splenocyte-mediated specific tumor cell killing ( $**P < 0.005$ ). In contrast, the DCs coincubated with LLC lysate gave rise to 20% to 30% specific splenocyte-mediated tumor cell killing ( $*P < 0.02$ ). Splenocytes that had been cultured with no DCs or DCs that had been electroloaded with control liver lysate yielded no specific tumor cell killing responses.

types of cancer, provided that the starting tumor lysate material was syngeneic to the target tumor cells.

The amount of tumor lysate relative to the number of DCs used in our study was at least 10 to 30 times less than that used in several studies that coincubated DCs with tumor lysate. Whereas we most commonly used a ratio of 10 DCs/1 TC, several published preclinical and clinical studies selected ratios of 1:1<sup>23,32</sup> or 1 DC/3 TCs.<sup>21,33</sup> In another report, a ratio of 1 DC/100 TCs was chosen.<sup>34</sup> The ratios used in those studies reflect increased amounts of lysate equivalents relative to the number of DCs. Furthermore, because we could electroporate cells in a completely scalable cell volume, ranging to as little as 20  $\mu\text{L}$ , we were able to concentrate the cells in as small a processing volume as desired. These features enabled us to use small amounts of tumor lysate, and they would seem to offer considerable advantages to those clinical studies that involve limited starting tumor material. For example, at the 10:1 ratio that we used, the total amount of protein in the lysate was approximately 0.5 mg/mL, or less than 10  $\mu\text{g}$  protein for  $1 \times 10^6$  DCs. In contrast, Thuman et al,<sup>35</sup> using DCs coincubated

with FITC-labeled ovalbumin (MW = 45 kDa), demonstrated that at least 1 mg/mL protein needed to be added for 24 hours to achieve any significant uptake and that between 1 and 5 mg/mL protein had to be used per  $1 \times 10^6$  DCs. In summary, we found that significantly less starting lysate was required for electroloading DCs to achieve enhanced antitumor responses in each of our assays. We could only detect specific antitumor responses using DCs coincubated with lysate when we used memory splenocytes, and those results still remained significantly less than the results obtained using DCs electroloaded with lysate (see Fig. 7). Just as importantly, the comparison of tumor cell killing when we used naive (see Fig. 3) or memory (see Fig. 7) splenocytes strongly supports our hypothesis that the antitumor effects we observed *in vivo* were indeed mediated by CD8<sup>+</sup> cytotoxic T cells primed by lysate-electroloaded DCs. Our tumor cell killing results are consistent with the findings of others who, using similar cell restimulation protocols, reported approximately 25% to 30% and 70% to 80% specific tumor cell lysis using normal<sup>24,34</sup> and memory<sup>25,34</sup> T cells, respectively.

The involvement of CD8<sup>+</sup> T cells in our experiments is also supported by the hypothesis that electroporation, by delivering protein antigens directly into the cytoplasm, may facilitate class I presentation of processed antigens to these effector cells.<sup>36</sup> In contrast, antigenic uptake by macropinocytosis has been associated with class II MHC presentation.<sup>3</sup> In our antigen electroloading protocol, we actually reasoned that class I- and class II-restricted antigen presentation could likely be occurring simultaneously. After the DCs had been electroporated, the antigen was not washed away and the DCs were allowed to mature in the continued presence of any tumor lysate material. During this overnight incubation, the electroloaded DCs were placed in exactly the same culturing conditions as those that were coincubated with lysate without any electroporation. Indeed, it has been well established by cell depletion studies that CD4<sup>+</sup> and CD8<sup>+</sup> T cells are both required for tumor rejection.<sup>21,37</sup>

Electroporation can be used to mediate the safe, efficient, and instantaneous loading of DCs with protein antigens. The electroloaded DCs can be readily matured or applied to any suitable immunotherapy protocol within 30 minutes after cell processing. Our observation that at least 24 hours was required to see significant antigen uptake using standard DC coincubation is consistent with the findings of others.<sup>35</sup> Electroporation as a general cell loading method offers several other significant advantages. There are no toxic or costly materials associated with electroporation, and it does not seem to be an antigen-specific process. Several recent studies have taken the alternative approach of generating fusion proteins consisting of a single tumor-associated antigen and known ligands of DC surface receptors (eg, GM-CSF, mannose)<sup>17–20</sup> or viral (eg, HIV-Tat, Vp22)<sup>14,38,39</sup> transporter molecules. In contrast, with electroporation, the cells are simply mixed with the desired molecules or lysate and processed in a suitable cell handling volume. Indeed, we found that the processed DCs retained normal surface expression of costimulatory molecules (see Fig. 1A). The electroloaded DCs could also be cryopreserved and thereby administered as consistent functional doses during our restimulations.

The use of whole-tumor lysate as antigenic material offered considerable advantages. It eliminated the requirement to identify and characterize the specific antigens. We also believe that the use of lysate could offer a broader repertoire of tumor-associated antigens, and thereby reduce the likelihood for tumor escape.<sup>21</sup> The use of lysate did raise several concerns about the specificity of the T-cell responses, however. Because we initially prepared lysates from tumor cells cultured *in vitro*, we first needed to rule out the possibility that tissue culture reagents, such as trypsin or serum, could potentially be contaminating the lysate preparations and eliciting nonspecific T-cell responses. To address this, we injected mice with live tumor cells and allowed them to develop tumors. We then prepared identical tumor lysates by freeze/thawing the dissected tumors and normalizing by total protein. Results for each of the tumor models described in this study were identical using tumor lysates prepared by either method. Although we were not as concerned about identifying the specific tumor antigen(s) that was mediating antitumor responses, we also needed to rule out the possibility that nontumor host antigens contained within the lysate could be having an unforeseen priming effect. We therefore generated syngeneic whole-liver lysates and used them in all studies at total protein levels equivalent to those used for the tumor lysate. The mice given DCs that had been electroloaded with whole-liver lysate were always indistinguishable from the control mice given no DCs. Furthermore, no protective effect was ever observed using unprocessed DCs (no tumor lysate added) or DCs electroloaded with whole-liver lysate. We therefore conclude that although the specific tumor antigen(s) mediating the T-cell priming was not known, the tumor protection seen in our studies was definitely DC dependent as well as tumor lysate dependent.

Taken together, our results highlight the feasibility of using electroporation as an efficient means to load DCs with tumor antigens instantly. Electroporation offers considerable advantages over standard DC pulsing protocols, specifically the elicitation of greater T-cell responses while using considerably less tumor lysate. Although we used small cuvettes in this study, the MaxCyte GT system can be configured as a closed sterile unit and scaled up to larger volumes.<sup>27</sup> The entire process is thus flexible to meet the needs of clinical immunotherapy protocols.

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