Combined Dendritic Cell Cryotherapy of Tumor Induces Systemic Antimetastatic Immunity

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Abstract

Purpose: Cryotherapy of localized prostate, renal, and hepatic primary tumors and metastases is considered a minimally invasive treatment demonstrating a low complication rate in comparison with conventional surgery. The main drawback of cryotherapy is that it has no systemic effect on distant metastases. We investigated whether intratumoral injections of dendritic cells following cryotherapy of local tumors (cryoimmunotherapy) provides an improved approach to cancer treatment, combining local tumor destruction and systemic anticancer immunity.

Experimental Designs: The 3LL murine Lewis lung carcinoma clone D122 and the ovalbumin-transfected B16 melanoma clone MO5 served as models for spontaneous metastasis. The antimetastatic effect of cryoimmunotherapy was assessed in the lung carcinoma model by monitoring mouse survival, lung weight, and induction of tumor-specific CTLs. The mechanism of cryoimmunotherapy was elucidated in the melanoma model using adoptive transfer of T cell receptor transgenic OT-I CTLs into the tumor-bearing mice, and analysis of Th1/Th2 responses by intracellular cytokine staining in CD4 and CD8 cells.

Results: Cryoimmunotherapy caused robust and tumor-specific CTL responses, increased Th1 responses, significantly prolonged survival and dramatically reduced lung metastasis. Although intratumor administration of dendritic cells alone increased the proliferation rate of CD8 cells, only cryoimmunotherapy resulted in the generation of effector memory cells. Furthermore, cryoimmunotherapy protected mice that had survived primary MO5 tumors from rechallenge with parental tumors.

Conclusions: These results present cryoimmunotherapy as a novel approach for systemic treatment of cancer. We envisage that cryotherapy of tumors combined with subsequent *in situ* immunotherapy by autologous unmodified immature dendritic cells can be applied in practice.

Minimally invasive therapies are an alternative approach to surgical intervention in the treatment of malignant diseases. Cryoablation, i.e., tissue destruction by repeated deep freezing and thawing, is under the larger category of thermal therapy and, during the past decade, it has become an acceptable clinical tool for the management of dermatologic tumors, hepatocellular carcinoma, renal and prostate tumors, and hepatic colorectal metastases (1, 2). Compared with surgical excision, the main advantages are the potential for less invasiveness resulting in reduced mortality and morbidity, and the ability to perform ablative procedures on outpatients, which decreases the treatment cost. In the case of hepatic colorectal metastases, the use of cryosurgery improves the percentages of resectability (2). A comparative study on domestic pigs showed that the cryoablation of renal parenchyma is beneficial over other necrosis-inducing ablations such as microwave thermoablation, radiofrequency energy, and chemoablation by ethanol, hypertonic saline, and acetic acid gels, in terms of reproducibility, consistency in size and shape, and the ability to monitor by ultrasound (3).

The mechanisms of cryoablation are multifactorial, yet they culminate in necrotic cell death secondary to (*a*) direct cellular damage by ice crystals, and (*b*) vascular and endothelial injury with eventual ischemia (4). The freezing-stimulated change in the immune response is another proposed mechanism of cryosurgery. Although few animal models and clinical case studies describe a "cryoimmunologic response" and even spontaneous regression of the metastases following cryoablation (5), the majority of studies prove no change in the immune status (4). Moreover, others suggest suppressed immunity, increased tumor growth, and metastasis after cryoablation of primary tumors (6). The main drawback of cryotherapy, therefore, is that apart from its local effect on tumors, it does not elicit a systemic antitumor response to preclude metastasis.

Immunotherapy, which aims at eliciting or enhancing the body's own capacity to mount an effective antitumor response, is another approach to cancer treatment. Dendritic cells are professional antigen-presenting cells, which are crucially

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important in antigen capture, processing, and presentation to the effector arm of the immune system. The use of dendritic cells as adjuvant in order to explore immune-mediated resistance to cancer has been supported by many animal experiments as well as initial human trials (7). Mature dendritic cells direct T cell differentiation into effector or memory cells, inducing natural killer cell activation and B cell differentiation into antibody-forming cells (8). Necrotic cell death stimulates dendritic cell maturation (9), therefore, immunotherapies that preferentially promote or increase tumor cell death by necrosis are more likely to induce antigen-specific tumor immunity and rejection. The density of dendritic cells present within tumors correlates with prognosis and their migration from the vicinity of the tumor to the draining lymph nodes is essential for the induction of immunity (10).

In the current study, we investigated the therapeutic value and immune consequences of combined cryotherapy followed by intratumor injection of immature dendritic cells (cryoimmunotherapy) in nonimmunogenic highly metastatic preclinical models. The rationale for cryoimmunotherapy stems from the fact that routine cryotherapy releases, following necrosis, large amounts of tumor antigens and inflammatory signals that are necessary for dendritic cell maturation. We aimed at elucidating whether cryoimmunotherapy is superior to cryo- and immunotherapy alone in terms of antimetastatic effects, survival, and creation of functional antitumor memory.

Materials and Methods

Mice and cell lines. Eight to 12, 12-week-old OT-I T cell receptor (Vα2/Vβ5) transgenic mice, encoding a T cell receptor specific for the ovalbumin epitope (SIINFEKL-H-2K^b; ref. 11) and C57BL/6 mice were bred in the Weizmann Institute. All mouse experiments were approved and conducted under Institutional Animal Care and Use Committee guidelines and according to the Helsinki Declaration.

3LL Lewis lung carcinoma and B16 melanoma clones, of C57BL/6 origin, were maintained in DMEM supplemented with 10% FCS, L-glutamine, sodium pyruvate, nonessential amino acids, and penicillin/streptomycin. Clone D122-3LL is a low K^b-expressor, highly metastatic, and poorly immunogenic. K^{b}39.5 is a K^b-transfected D122 clone. MO5, ovalbumin-transfected B16 and K^{b}39.5 cells were maintained in 500 μg/mL G-418.

Tumor growth and spontaneous metastasis. For the D122 spontaneous metastasis model, 8 to 11 mice per group were inoculated at the intrafootpad with 2×10^5 cells per mouse. When the tumor reached 8 mm in diameter, the tumor-bearing leg was amputated below the knee (12). Mice were monitored daily and sacrificed when moribund or 23 to 30 days post-amputation. Lung metastatic load was assessed by weighing. Survival was defined as the day when mice were sacrificed.

For the MO5 model, 10^6 cells per mouse were inoculated intrafootpad. Ten days later, when the tumor reached 3 to 4 mm in diameter, the mice were treated (as described below) and injected with OT-1 CD8 T cells. Mice that rejected the MO5 tumor (about 50% of total) were rechallenged, at day 60 post-rejection, with 10^6 B16-F1 cells s.c. in the upper back. Tumor take and growth were monitored over 90 days.

Generation of dendritic cells from mouse bone marrow. The procedure was described by Lutz at al. (13) and used with minor modifications. C57BL/6 BM cells (4×10^6) were cultured in 100 mm bacteriologic plates (Falcon) in 10 mL dendritic cell medium supplemented with 200 units/mL recombinant murine granulocyte macrophage colony-stimulating factor (Prospec, Israel). On day 3, another 10 mL dendritic cell medium containing 200 units/mL granulocyte macrophage colony-stimulating factor were added to the plates. On day 6, half of the culture supernatant was replaced with fresh dendritic cell medium containing 200 units/mL granulocyte macrophage colony-stimulating factor. On day 8, nonadherent cells were collected, resuspended in 10 mL fresh dendritic cell medium containing 100 units/mL granulocyte macrophage colony-stimulating factor and seeded in 100 mm tissue culture plates (Falcon). On day 9, nonadherent cells with the typical characteristics of immature dendritic cells (>95% CD11c$^+$, CD80^{low}, CD86^{low}, MHC II^{low}) were harvested, washed, and resuspended in PBS at 4×10^7 cells/mL before injection.

Treatment modes. Mice were treated when tumors reached 3 to 4 mm in diameter. In the single immunotherapy, the animals were injected intratumorally with 10^6 immature dendritic cells in a volume of 50 μL. In the combined treatment, local cryotherapy was followed by intratumoral injection of 10^6 immature dendritic cells. Anesthetized mice (ketamine-HCL, 100 mg/kg, i.p.) underwent cryotherapy by applying mild pressure for 10 seconds with frozen tweezers (which were kept in liquid nitrogen) to the tumor-bearing foot. The ice ball reached the size of 5 mm. To ensure complete thawing of the treated area before vaccination, mice were immunized after 1 hour. For the D122 spontaneous metastasis model, mice were treated twice at weekly intervals. For the MO5 model, mice were treated once followed by adoptive transfer of OT-I CD8 T cells. All control groups received PBS.

In vitro *cytotoxicity assay.* D122 carcinoma-bearing C57BL/6 mice were treated *in situ* twice with weekly intervals as follows: cryotherapy, intratumor injection of 10^6 immature dendritic cells, combination of either *in situ* or i.v. dendritic cell injection following cryotherapy. The control group received intratumor injections of PBS. Spleens were harvested from mice 10 days after the last treatment/immunization. Splenocytes were restimulated *in vitro* for 5 days on mitomycin C (80 μg/mL) treated and irradiated (5,000 rad) K^{b}39.5 tumor monolayers in RPMI-HEPES medium containing 10% FCS, 1 mmol/L glutamine, combined antibiotics, 1 mmol/L sodium pyruvate, 10 mmol/L HEPES (pH 7.4), 5×10^{-5} mol/L β-mercaptoethanol, and 1% nonessential amino acids. Viable lymphocytes (effector cells) were separated by lympholyte-M (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) centrifugation, resuspended in RPMI-HEPES medium, and admixed at different ratios with 5×10^3 [^{35}S]-L-methionine labeled target cells. CTL assays were done in U-shaped microtiter wells at 37°C, 5% CO_2, for 5 hours (14). Cultures were terminated by centrifugation at $50 \times g$ for 10 minutes at 4°C. A total of 50 μL of the supernatants was mixed with scintillation fluid and measured in a β counter (BD Biosciences, San Jose, CA). Percentage of specific lysis was calculated as follows:

$$\%\ \text{lysis} = (\text{cpm in experimental well} - \text{cpm spontaneous release})/(\text{cpm maximal release} - \text{cpm spontaneous release}) \times 100.$$

Spontaneous release was determined by incubation of 100 μL labeled target cells with 100 μL of medium. Maximal release was determined by lysis of target cells in 100 μL 0.1 N NaOH.

Adoptive transfer of OT-I CD8 T cells and flow cytometry. CD8$^+$ T cells from OT-I spleens were purified (>95%) by magnetic cell sorting (Mitenyi, Germany), according to the manufacturer's instructions. CD8$^+$ T cells (2×10^6) were transferred by i.v. injection into tumor-bearing C57BL/6 recipients, previously treated as described above.

In some experiments, cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR), according to the manufacturer's recommendations. Animals were sacrificed on the indicated days after cell transfer or on day 60 following tumor rejections. Cells from draining lymph nodes and spleens were stained with anti-CD8-APC, anti-Vα2-PE, anti-CD44-FITC, anti-CD62L-PE, anti-CD69-FITC, or isotype control monoclonal antibody (BD PharMingen, San Diego, CA) and analyzed by FACSort and CellQuest

software (BD). For the CFSE experiments, fluorescence levels were detected on $CD8^+V\alpha2^+$ gated populations.

Intracellular cytokine staining. The frequency of IFN-γ or IL-4-producing cells in spleens of treated MO5 tumor bearing or naïve C57BL/6 mice (three per group) was detected, 8 days following treatment, using the Cytofix/Cytoperm Plus Kit with GolgiPlug (BD). Cells (1×10^6) were cultured for 6 hours in 50 ng/mL of phorbol 12-myristate 13-acetate (Sigma, St. Louis, MO) and 750 ng/mL ionomycin (Sigma). Brefeldin A (10 μg/mL, GolgiPlug) was added for the last 4 hours to enable intracellular proteins to accumulate. Then, the cells were harvested, suspended in staining buffer (PBS with 1% FCS and 0.1% sodium azide) and blocked with purified anti-FcγRII/III monoclonal antibody (2.4G2, eBioscience, San Diego, CA) for 15 minutes at 4°C, and surfaces stained with antigen-presenting cell–conjugated anti-CD4 or CD8 (e-Bioscience) for 30 minutes. at 4°C. The cells were washed with staining buffer, fixed with 4% paraformaldehyde for 20 minutes at 4°C and washed twice. Then permeabilized in PBS containing 5% FCS, 0.1% saponin and 0.1% sodium azide for 15 minutes at 4°C and incubated in the presence of FITC conjugated anti-mouse IFN-γ (XMG1.2), IL-4 (BVD6-24G2), or isotype control (all eBioscience). Fifty $\times 10^3$ events were collected for each sample. The data were analyzed using Cell Quest software (BD).

Statistical analysis. The data obtained in the CTL assays were analyzed by one-way ANOVA and significance was assessed by the Tukey-Kramer test. Statistical analysis of the metastatic loads was done with unpaired Welch *t* test. Dunnett multiple comparison test and log-rank test were applied for survival analysis. Fisher exact test was applied to analyze the results of mouse survival following rechallenge with secondary tumors.

Results

To study the influence of the proposed treatment on systemic antitumor immunity, we examined the induction of CTL responses in D122 tumor-bearing mice following cryo-, immune-, or cryoimmunotherapy, as described in Materials and Methods. The results showed induction of antitumor CTL responses in mice that were injected intratumorally with dendritic cells (Fig. 1A). The effect of local immunotherapy was significant at effector-to-target ratios of 100:1 and 50:1 (P = 0.001) compared with untreated or cryotreated mice, respectively. The highest amount of tumor-specific CTL activity was obtained when dendritic cells were injected intratumorally following *in situ* cryotherapy. Compared with immunotherapy, the combined treatment induced significantly higher lysis (P = 0.005) at all effector-to-target ratios. CTLs were tumor-specific; they lysed D122 cells, but not irrelevant B16 melanoma cells. Cryotherapy alone or combined with i.v. administration of dendritic cells was ineffective in generating antitumor CTLs (Fig. 1A).

To examine whether cryoimmunotherapy-induced antitumor immunity has a protective antimetastatic impact, we used a spontaneous metastasis tumor model. Mice were inoculated intrafootpad with D122 cells and the resulting primary lesions were treated *in situ* by cryo-, immune-, or cryoimmunotherapy. To examine the importance of locally performed immunotherapy, as compared with distal administration of

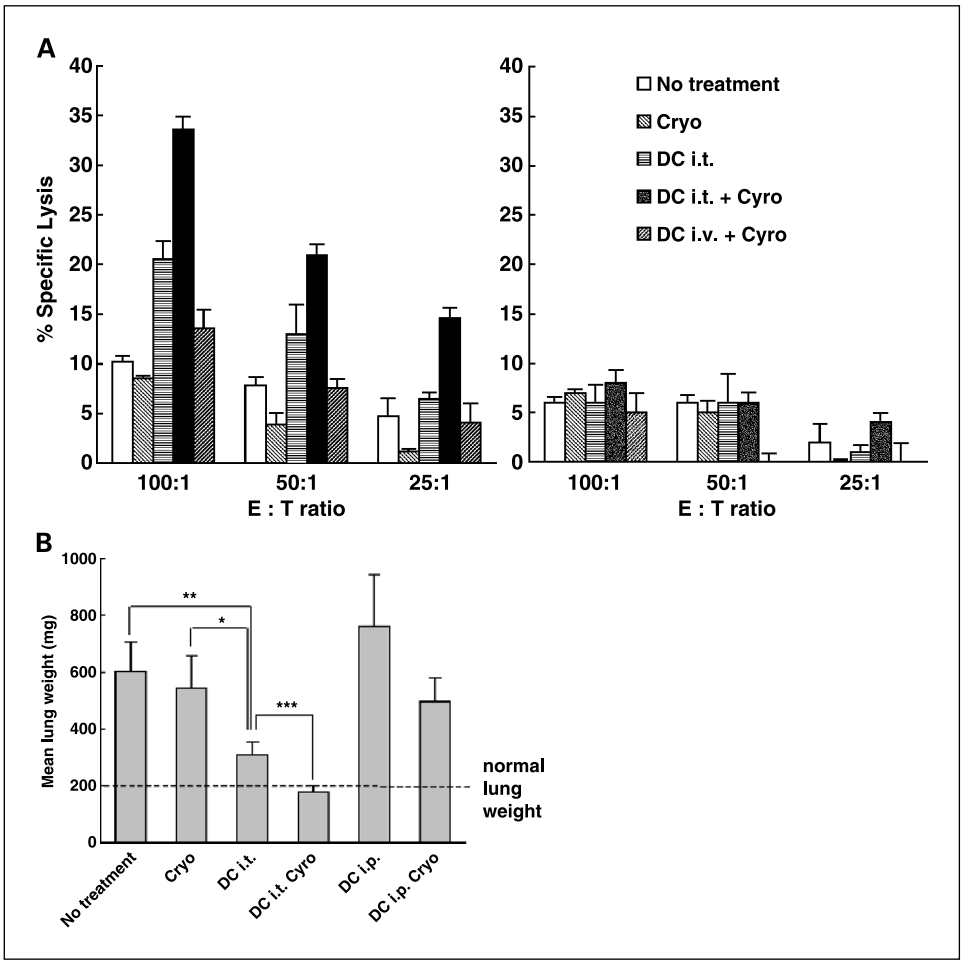

Fig. 1. Induction of antitumor systemic immunity by cryoimmunotherapy. *A*, tumor-specific CTL activity in tumor-bearing mice following *in situ* cryoimmunotherapy. D122 carcinoma-bearing C57BL/6 mice were treated *in situ* twice with weekly intervals as follows: cryotherapy (Cryo), intratumor injection of 10^6 immature dendritic cells (DC i.t.), combination of either *in situ* (DC i.t. + Cryo) or i.v. (DC i.v. + Cryo) dendritic cell injection following cryotherapy. Ten days after the last treatment, splenocytes were restimulated on irradiated and mitomycin C-treated 39.5 cells for 5 days, and reacted with 5,000 ^{35}S-methionine-labeled D122 (specific targets, left) or B16 melanoma (nonspecific targets, right) cells for 5 hours at 37°C, as described in Materials and Methods. The effector-to-target ratios of 100:1, 50:1, 25:1 are shown. Spontaneous release did not exceed 20% of maximal release. Results are representative of three independent experiments. *B*, protection from D122 metastasis by *in situ* cryoimmunotherapy. C57BL/6 (8 mice per group) were inoculated intrafootpad with 2×10^5 D122 cells. Two weeks later, the tumors, 3 to 4 mm in diameter, were treated as designated. When tumors reached diameters of 8 mm, tumor-bearing feet were surgically removed and 23 to 30 days later, in accordance with the death of the control group, mice were sacrificed and lung weight representing metastatic load was determined. The results are presented as mean lung weight (mg) ± SD. *Dotted line*, normal lung weight. Statistical significance was determined by unpaired Welch *t* test: *, P = 0.0034; **, P = 0.0019; ***, $P < 0.0001$.

dendritic cells, we injected dendritic cells i.p. with or without cryotherapy of the primary lesions. Intratumor injections of dendritic cells significantly reduced metastatic spread compared with untreated ($P = 0.0019$) or cryotreated ($P = 0.0034$) mice (Fig. 1B). In concordance with the CTL results, combined cryoimmunotherapy of primary lesions was superior to immunotherapy alone ($P < 0.0001$). No antimetastatic effect was observed in mice, in which dendritic cells were given i.p.

Alternatively, tumor-bearing feet of the mice were amputated and their post-surgical survival was monitored. Only mice that were treated *in situ* by cryoimmunotherapy had statistically significant increase of survival ($P = 0.005$) in comparison with untreated mice (Fig. 2). Five out of 10 mice from the group that underwent local cryoimmunotherapy were alive at day 32 following amputations, whereas no other mice survived at that time point. Thus, cryoimmunotherapy of primary lesions led to induction of tumor-specific CTLs and an antimetastatic effect, as a result.

We then sought to elucidate the mechanism of cryoimmunotherapy-induced antitumor immunity, using an adoptive transfer model. OT-I mouse-derived naïve CD8 T cells specific to K^b-SIINFEKL ($ovalbumin_{257-265}$) were transferred into C57BL/6 mice bearing B16-MO5 melanomas expressing ovalbumin. The treatment effect on kinetics of CTL proliferation was assessed by prelabeling of the cells with CFSE. Upon cell division, CFSE is distributed equally between daughter cells, allowing the resolution of up to eight cycles of cell division by flow cytometry. As shown in Fig. 3, mice treated with cryotherapy alone showed a modest degree of OT-I cell proliferation that was indistinguishable from that seen in untreated mice. In contrast, intratumor dendritic cell administration, alone or following cryotherapy, triggered vigorous CD8

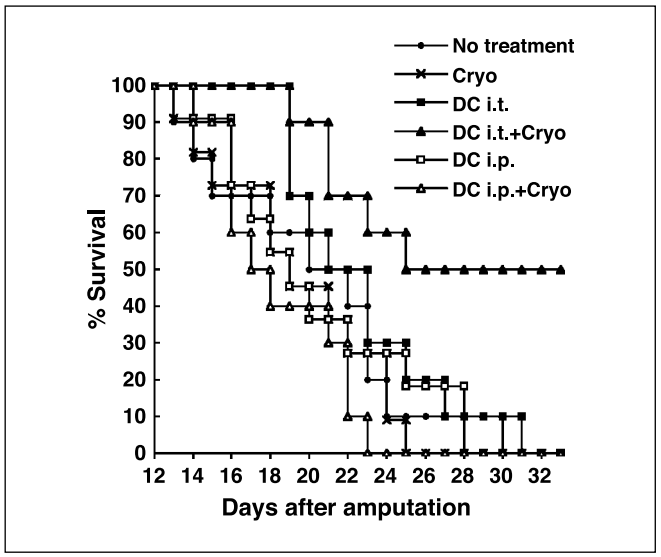

Fig. 2. Local cryoimmunotherapy results in prolonged animal survival following surgical removal of the primary tumors. Survival of C57BL/6 mice (10-11 mice per group) was monitored over time after surgical removal of the intrafootpad-implanted primary D122 tumors, which were treated as designated. Percentage of survival of the treated groups are plotted against the days after the amputation of tumor-bearing feet. Statistical significance was determined by the log-rank test analyzing survival of mice treated by DC i.t + Cryo in relation to other groups as follows: untreated ($P = 0.0092$), Cryo ($P = 0.0042$), DC i.t. ($P = 0.0219$), DC i.p. ($P = 0.0071$), DC i.p. + Cryo ($P = 0.0011$).

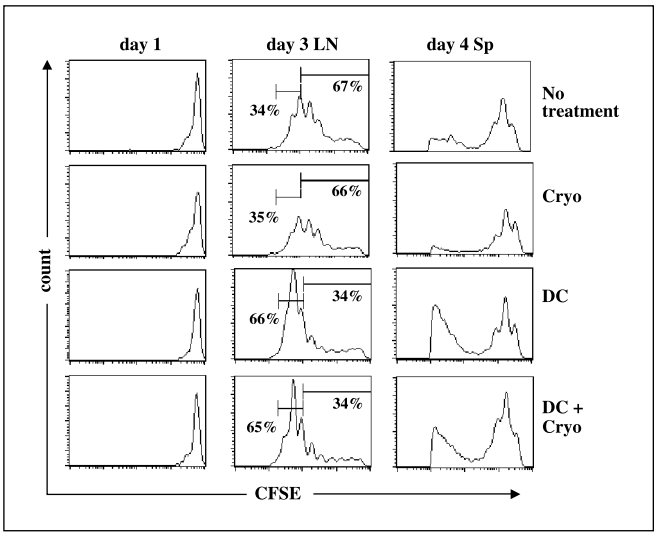

Fig. 3. Cell division history of adoptively transferred OT-I mouse-derived CD8 T cells. The extent of cell division, based on CFSE fluorescence, in the OT-I-derived naïve CD8 lymphocytes was examined 1, 3 and 4 days post-transfer to C57BL/6 tumor-bearing mice following single cryo- or immunotherapy or combined cryoimmunotherapy, as described. Histograms displaying CFSE fluorescence are gated on CD8+ lymphocytes. Results are shown for a representative mouse from groups of three, and similar results were obtained in three independent experiments.

T cell proliferation in draining lymph nodes and spleens of tumor-bearing mice, resulting in increase of the proportion of cells that divided for seven to eight times to 65% and 66%, as compared with only 34% and 35% in nontreated or cryotreated mice, respectively (Fig. 3). To examine whether the extensive proliferation of these cells is accompanied by changes in their activation status, we evaluated expression patterns of activation-related cell-surface molecules. Expressions of the early activation marker CD69, as well as of adhesion molecules CD44 and CD62L were found to be comparable in all tested groups.[3] These findings indicate that cryoimmunotherapy does not increase the percentage of activated CD8 lymphocytes, but rather affects their proliferation to the same extent as *in situ* immunotherapy alone.

We then addressed the fate of adoptively transferred CD8 T cells either in mice that were treated *in situ* with dendritic cells alone or in combination with cryotherapy. To this end, the phenotypes of memory CD8 T cells were studied in mice on day 60 following adoptive transfer–induced tumor rejections. At this time point, memory CD8 T cells express high levels of CD44 (15), whereas CD62L expression may vary from low/intermediate for the effector memory subset (Tm_e), to high for the central memory population (Tm_c; ref. 16). Intratumor injections of dendritic cells (± cryotherapy) increased the subset of $CD44^+CD8^+$, as compared with cryotreated or untreated mice (Fig. 4A). The percentage of $CD8^+CD44^+$ T cells derived from naïve mice was comparable with that of cryotreated or untreated tumor-bearing mice.[3] Sixty days following tumor rejections, transferred CD8 T cells in untreated or cryotreated mice mostly (77%) expressed high levels of CD62L, reflecting a naïve ($CD44^{low}$, $CD62L^{high}$) cell

[3] Unpublished data.

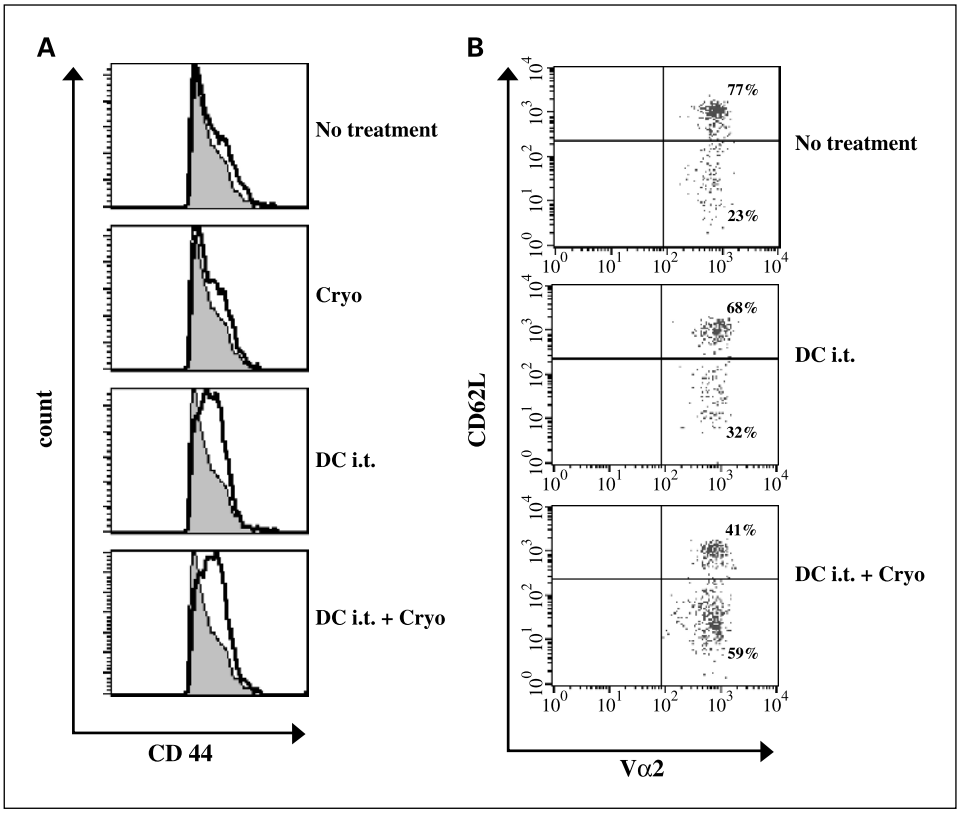

Fig. 4. CD44 and CD62L expression on the transferred CD8 Tcells 60 days following tumor rejection. OT-I-derived naïve CD8 lymphocytes were transferred into C57BL/6 MO5 melanoma-bearing mice that underwent intralesional cryotherapy, immunotherapy, or combined cryoimmunotherapy, as described. The control group was adoptively transferred only with no additional treatment. The adoptive transfer resulted in tumor rejection in 50% to 60% of mice. Sixty days following tumor rejection, the draining lymph node-derived lymphocytes were stained with anti-CD8, anti-Vα2, anti-CD44 (*A*) or anti-CD62L (*B*) antibodies and analyzed by flow cytometry. The cells were gated on $CD8^+V\alpha2^+$ lymphocytes. Results are shown for a representative mouse from groups of three, and similar results were obtained in three independent experiments.

population. In contrast, $CD8^+$ T cells derived from the mice following cryoimmunotherapy were mainly (59%) CD62L-negative, supporting the existence of an effector memory ($CD44^{high}$, $CD62L^{low}$) cell population (Fig. 4). To examine the functional value of the antitumor memory, the mice that had survived primary B16-MO5 tumors following adoptive transfer and indicated treatments were rechallenged with secondary B16-F1 (ovalbumin-negative) tumors and monitored for survival. As shown in Table 1, only cryoimmunotherapy induces statistically significant protection (six out of seven mice) from the secondary tumors for >90 days ($P = 0.010$ and $P = 0.029$, compared with nontreated and cryotreated mice, respectively). Immunotherapy alone was much less potent (three out of eight, $P > 0.5$, compared with either nontreated or cryotreated mice) and cryotherapy alone was almost ineffective (one out of seven). Hence, cryoimmunotherapy induced an effector memory CD8 T subset, which was probably important for protection against the secondary tumors.

Because type 1 and type 2 CD8 and CD4 T cell responses may influence antitumor reactions, we next evaluated the cytokine-releasing profiles of $CD4^+$ and $CD8^+$ T cells following cryo-, immuno-, or combined cryoimmunotherapy of MO5-bearing mice. Type 1 (IFN-γ) and type 2 (IL-4) cytokine profiles were examined by intracellular staining and flow cytometry. The levels of $CD8^+$ T cells producing IFN-γ were 2- to 3-fold higher in immuno- or combined cryoimmunotherapy-treated mice relative to nontreated or cryotherapy treated recipients.[3] This was observed in draining lymph nodes on days 1 and 8 post-treatment. No staining for IL-4 was found in $CD8^+$ T cells. The proportion of $CD4^+$ T cells predominantly producing IFN-γ (Th1 type) was markedly higher in mice treated by cryoimmunotherapy as compared with other groups (Fig. 5). The percentage of $CD4^+$ T cells producing IL-4 (Th2 type) was increased in dendritic cell–treated mice and was similar to that in mice following combined treatment. This suggests that IFN-γ-producing Th1 rather than Th2 responses were induced by *in situ* cryoimmunotherapy.

Table 1. Protection against parental tumor rechallenge in primary tumor–rejecting mice

Treatment	Number of rechallenged mice	Number of surviving mice (>90 days)
No treatment	8	1 (12·5%)
Cryotherapy	7	1 (14·3%)
Immunotherapy	8	3 (37·5%)
Cryoimmunotherapy	7	6 (85·7%)

NOTE: C57BL/6 mice (15-16 per group) were inoculated at the intrafoot-pad with 10^6 MO5 cells per mouse. Ten days later, when the tumor reached 3 to 4 mm in diameter, the mice were treated and injected with OT-I CD8 T cells, as described in Materials and Methods. Mice that rejected the MO5 tumor were rechallenged, at day 60 post-rejection, with 10^6 parental B16-F1 cells s.c. in the upper back. Mouse survival was monitored for >90 days following rechallenge. $P = 0.010$ for cryoimmunotherapy/no treatment; $P = 0.029$ for cryoimmunotherapy/cryotherapy; $P = 0.569$ for immunotherapy/no treatment or immunotherapy/cryotherapy, according to Fisher exact test.

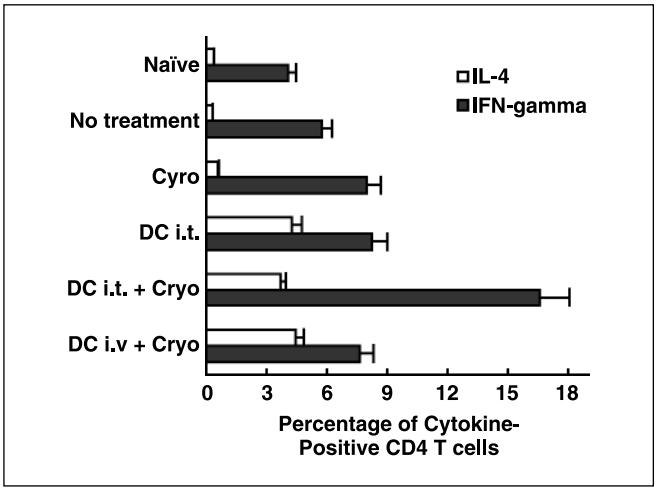

Fig. 5. Intracellular IFN-γ or IL-4 expression by CD4 T cells following stimulation with PMA and ionomycin. Eight days post-treatment, splenocytes were stimulated with PMA and ionomycin in the presence of brefeldin A as described in Materials and Methods. The cells were gated on CD4 T cells that expressed a given cytokine. *Columns*, means; *bars* ±SD (n = 3/group).

Discussion

Cryosurgery has reemerged as an *in situ* treatment for various, primarily urologic malignancies (17). At present, local destruction of tumor masses by freezing and thawing provides a conventional estimate for the clinical use of cryotherapy, whereas the antimetastatic effect is considered negligible (4). Numerous studies revealed reduced immune responses and elevated metastasis following cryosurgery (4, 18). There is evidence that by combining immunoadjuvants with cryosurgery, one may augment the host systemic antitumor immune response. A short list of studies addressing this issue includes intralesional injections of bacille Calmette-Guérin in animal models and in clinical settings (19, 20), as well as systemic administration of protein-bound polysaccharide in a murine model (18).

This is the first study to address the influence of *in situ* administration of immature dendritic cells following cryotherapy of primary tumors on systemic antitumor immunity. Our findings that show induction of tumor-specific CTL, of Th1-type helper cells, strong antimetastatic effects, and prolonged survival of cryoimmunotherapy-treated mice (Figs. 1 and 2), support the assumption that *in situ* injection of immature unloaded dendritic cells following cryosurgery has several advantages over cryosurgery alone or the use of *ex vivo* matured, antigen-loaded dendritic cells.

First, the destruction of the large tumor mass by cryotherapy leaves only minimal tumor burden to be dealt with by the immune system. Antitumor immune responses alone commonly failed to overcome billions of rapidly dividing tumor cells. Thus, removal of the primary tumor may markedly enhance the chances of successful destruction of residual cells or distant metastases. Second, as the dendritic cells are injected straight into the tumor, it is expected that the large majority will take up antigen for presentation to lymphocytes. This is of importance, because most studies present data showing that only a small proportion of dendritic cells injected by other routes arrived at the target organ (21). Furthermore, direct intratumor injection of genetically modified dendritic cells or coadministration of dendritic cells and adenoviral vector expressing CD40 ligand reduces the incidence and growth of tumors in preclinical models (22, 23). In agreement with these data, intratumor administration of dendritic cells raised tumor-specific CTL and Th1-type CD4 cells, and decreased metastatic burden in a spontaneous metastasis model, whereas dendritic cells injected i.v. or i.p. failed to do so (Fig. 1). Finally, the microenvironment of the freeze-thawed tumor-containing inflammatory cytokines is an excellent milieu for the maturation of dendritic cells into cells which will process and present a wide spectrum of tumor cell antigens in the context of type 1 cellular responses. Indeed, combined cryoimmunotherapy induced the highest levels of IFN-γ-producing $CD4^+$ cells (Fig. 5) as well as CTLs. The capacity of dendritic cells to expand cancer-directed immune responses is well-documented, whereas the mode of dendritic cell maturation and loading methods often determine the fate of the immune response toward either stimulation or tolerance (24, 25). Immature rather then mature dendritic cells are particularly efficient at antigen ingestion and processing (25). There is evidence that dying cells are captured 5-fold better by immature compared with mature dendritic cells (26). Only fully mature dendritic cells, which express high levels of MHC class I and class II and costimulatory molecules, as well as produce large amounts of the proinflammatory cytokines and IL-2, induce T-cell immunity (27). Furthermore, the mode of tumor cell death may determine whether T cell activation or T cell tolerance occurs. Current evidence suggests that necrotic rather then apoptotic cell death provides the necessary signals for the full activation of dendritic cells, which promotes T cell responses (26). Indeed, our findings indicate that compared with immunotherapy alone, cryotherapy-induced necrotic tumor cell death prior to dendritic cell administration markedly enhances systemic antitumor immunity.

CD8 T cells play an essential role in immunity against cancer, and are the most specific and functionally active effector cells. A predominant role of CD8 T cells in tumor growth inhibition upon intratumor administration of dendritic cells has been reported (28). Using adoptive transfer of purified CD8 T cells specific to K^b-SIINFEKL into melanoma-bearing mice, we elucidated the kinetics of CD8 T cell proliferation following priming. *In situ* administration of dendritic cells triggered robust proliferation of the transferred T cells, as compared with cryotherapy alone (Fig. 3). The extremely high ($K_d = 10^{-14}$) affinity of the transgenic T cell receptor to its complex could account for the lack of additional enhancement of the proliferation rate in mice that underwent cryoimmunotherapy. T cell clones with low-affinity T cell receptors are expanded by prolonged exposure to high doses of antigens (29). Therefore, one may assume that such polyclonal T cell responses occur upon cryoimmunotherapy and contribute to the observed antitumor immunity. In this regard, cryoimmunotherapy protected mice that had survived primary MO5 melanomas from rechallenge with parental B16-F1 clone (Table 1). Given that B16-F1 cells do not expresses ovalbumin and, therefore, are not a direct target for OT-I T cells, epitope spread, and polyclonal T cell responses seem to underlie the observed protection. Finally, the data in Fig. 4 shows the existence of effector memory CD8 T cells in mice after cryoimmunotherapy. These cells are preferably $CD44^{high}CD62L^{low}$, produce high levels of effector cytokines, and contain perforin (15, 16).

Recent evidence has shown that cancer patient–derived memory T cell subsets can efficiently recognize and reject autologous breast tumors (30). This is of importance because induction of functional memory could provide an additional estimate of clinical use of cryoimmunotherapy.

In conclusion, this study presents a novel approach for systemic treatment of cancer. We envisage that cryotherapy of tumors combined with subsequent *in situ* immunotherapy by autologous unmodified immature dendritic cells can be applied in practice. It was shown in animal models that intratumor delivery of granulocyte macrophage colony-stimulating factor promotes tumor rejection (31). The use of immunomodulators that recruit and activate dendritic cells might potentially substitute for direct intratumor injection of *ex vivo*-generated cells. Our future studies will address this issue.

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