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Dendritic Cells Charged with Apoptotic Tumor Cells Induce Long-Lived Protective CD4$^+$ and CD8$^+$ T Cell Immunity against B16 Melanoma[1]

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Dendritic cells (DCs) are potent APCs and attractive vectors for cancer immunotherapy. Using the B16 melanoma, a poorly immunogenic experimental tumor that expresses low levels of MHC class I products, we investigated whether DCs loaded ex vivo with apoptotic tumor cells could elicit combined CD4$^+$ and CD8$^+$ T cell dependent, long term immunity following injection into mice. The bone marrow-derived DCs underwent maturation during overnight coculture with apoptotic melanoma cells. Following injection, DCs migrated to the draining lymph nodes comparably to control DCs at a level corresponding to ~0.5% of the injected inoculum. Mice vaccinated with tumor-loaded DCs were protected against an intracutaneous challenge with B16, with 80% of the mice remaining tumor-free 12 wk after challenge. CD4$^+$ and CD8$^+$ T cells were efficiently primed in vaccinated animals, as evidenced by IFN-γ secretion after in vitro stimulation with DCs loaded with apoptotic B16 or DCs pulsed with the naturally expressed melanoma Ag, tyrosinase-related protein 2. In addition, B16 melanoma cells were recognized by immune CD8$^+$ T cells in vitro, and cytolytic activity against tyrosinase-related protein $2_{180-188}$-pulsed target cells was observed in vivo. When either CD4$^+$ or CD8$^+$ T cells were depleted at the time of challenge, the protection was completely abrogated. Mice receiving a tumor challenge 10 wk after vaccination were also protected, consistent with the induction of tumor-specific memory. Therefore, DCs loaded with cells undergoing apoptotic death can prime melanoma-specific helper and CTLs and provide long term protection against a poorly immunogenic tumor in mice. *The Journal of Immunology,* **2003, 171: 5940–5947.**

Melanoma is the cancer with the fastest increasing incidence (1), and survival of patients with distant metastases is generally <1 year. With increased understanding of the requirements for initiating immunity, dendritic cells (DCs)[3] have become attractive vectors for immunotherapy of this and other cancers (2–9). DCs are proving to be effective in initiating and expanding immune responses in humans (10, 11), providing a rationale for their use as adjuvants for active immunization against melanoma (12–16). Different strategies have been developed to load DCs with tumor Ags, including MHC-restricted synthetic peptides derived from the Ag (12–17), tumor RNA (18, 19), tumor lysates (20), tumor-derived exosomes (21), and dying tumor cells (22–26); these have been assessed in preclinical models and, in some cases, clinical trials (12–26). Tumor cells (dying cells or Ab-coated tumor cells) are of interest as an antigenic source, because the DCs would have the potential to present a broad range of tumor-associated Ags and on both MHC class I and II products regardless of the MHC haplotype of the patient (27–30).

To test whether combined CD4$^+$ and CD8$^+$ long term immunity could be elicited by DCs loaded with tumor cells, we have conducted preclinical analyses to immunize mice with DCs previously exposed to apoptotic melanoma cells ex vivo. There are already some examples, using DCs bearing defined tumor Ag or tumor cells engineered to secrete GM-CSF, where T cell-based resistance to melanoma has been achieved in animals (31–33), but there is no precedent for the induction of combined MHC I- and II-restricted immunity in vivo with DCs loaded with tumor cells. We will show that DCs, after phagocytosis of apoptotic tumor cells, elicit long-lived combined CD4$^+$ and CD8$^+$ immunity against the MHC class I low B16 murine melanoma.

Materials and Methods

Animals and cell lines

Six- to 8-wk-old male C57BL/6 (HH-2^b, CD45.2$^+$) mice were obtained from the Facultad de Ciencias Veterinarias, University of La Plata (Buenos Aires, Argentina) and from Taconic Farms (Germantown, NY), and CD45.1$^+$ B6.SJL-Ptprc were obtained from Taconic Farms. Mice were maintained in pathogen-free conditions, and studies were performed in accordance with local ethical guidelines. B16 (HH-2^b) is a poorly immunogenic murine melanoma of spontaneous origin (34). The B16-F1 line (a gift from Dr. A. Vecchi, Istituto Mario Negri, Milan, Italy) was maintained in culture in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated FCS (Natocor, Córdoba, Argentina), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies,

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[3] Abbreviations used in this paper: DC, dendritic cell; Apo, apoptotic B16 cells; DC-Apo, DCs cocultured with Apo; DC+Apo, DCs and Apo without coculture; DC-Nec, DCs cocultured with Nec; DC-Spleen, DCs cocultured with apoptotic splenocytes; Nec, necrotic B16 cells; PI, propidium iodide; TRP-2, tyrosinase-related protein-2.

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Gaithersburg, MD). The EL-4 thymoma (American Type Culture Collection, Manassas, VA) was used as a control tumor. Cell lines were periodically tested to be mycoplasm-free.

Abs and reagents

We purchased anti-CD16/32, FITC-conjugated anti-CD86, I-A^b, rat IgG, mouse IgG, PE-anti-CD11c, biotinylated anti-CD45.2, and allophycocyanin-streptavidin (all from BD Biosciences, Mountain View, CA); anti-CD8 and CD4 MACS microbeads (Miltenyi Biotec, Auburn, CA); CFSE (Molecular Probes, Eugene, OR); and ACK buffer (BioSource International, Camarillo, CA).

Induction and detection of apoptosis and necrosis

After reaching 70–80% confluence, tumor cells were harvested with 0.5 mM EDTA, washed with PBS (HyClone, Logan, UT), and resuspended for irradiation (70 Gy, linear accelerator; Siemens, New York, NY). Irradiated cells were washed twice, cultured for 48 h, and harvested by pipetting for coculture with DCs or for detection of apoptosis using the annexin V-FITC apoptosis detection kit (BD PharMingen, San Diego, CA) on a FACSVantage SE (BD Biosciences). UV-irradiated (100 J/m^2, UV Stratalinker 2400; Stratagene, La Jolla, CA) syngeneic splenocytes were used as control apoptotic cells. Necrosis of B16-F1 cells was induced by four rapid cycles of freeze/thaw, as evidenced by combined annexin V/propidium iodide (PI) staining.

Generation of bone marrow-derived DCs and culture with apoptotic cells

As previously described (35), bone marrow cells were harvested from femurs and tibias of normal C57BL/6 mice and washed with PBS. Cells were resuspended in RPMI 1640 (Sigma-Aldrich) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 μg/ml gentamicin (Life Technologies), 50 μM 2-ME (Sigma-Aldrich), and 10% supernatant from CHO cells transfected with the plasmid pCDNA3 containing the recombinant mouse GM-CSF gene (the pCDNA3-rmGM-CSF plasmid was provided by Dr. O. Burrone, International Center for Genetic Engineering and Biotechnology, Trieste, Italy) and cultured in 100-mm diameter plates. On day 3 the cells were refed with fresh medium. On day 5 of culture immature DCs were harvested and used for in vitro phenotypic analysis and for coculture with apoptotic cells (Apo). For coculture, 2×10^5 DCs were incubated with Apo at a 1/1 ratio for 24 h, washed twice with PBS, and used for vaccination. Coculture with necrotic cells or syngeneic splenocytes was performed at a 1/1 ratio.

Phagocytosis of irradiated B16 melanoma cells

After irradiation, apoptotic B16 cells were dyed red using PKH26-GL (Sigma-Aldrich) and cocultured with immature DCs that were dyed green with PKH67-GL (Sigma-Aldrich) at a 1/1 ratio. After 24 h, flow cytometric analysis was performed, and phagocytosis of Apo was defined by the percentage of double-positive cells. Alternatively, phagocytosis was observed by confocal microscopy (LSM 510; Zeiss, New York, NY).

Surface phenotype and in vivo migration of DCs

The surface phenotype of bone marrow-derived DCs was analyzed before and after coculture with apoptotic tumor cells. DCs were double-stained with PE-conjugated anti-CD11c and FITC-conjugated anti-CD86 or anti-MHC II (I-A^b). B6 bone marrow-derived DCs (CD45.2^+) with and without previous coculture with apoptotic B16 were injected s.c. into CD45.1^+ recipient mice, and draining lymph nodes were removed 24 h later. Single-cell suspensions were prepared with 400 U/ml collagenase (Roche, Indianapolis, IN) for 25 min. Cells were then incubated with PE-conjugated anti-CD11c, allophycocyanin-conjugated anti-CD45.2, and FITC-conjugated anti-CD86 or MHC II. Flow cytometric data were analyzed with FlowJo (Tree Star software, San Carlos, CA).

Vaccination of mice

Mice were injected s.c. with 2×10^5 DCs cocultured with Apo (DC-Apo), DCs alone, Apo alone, mixtures of DCs plus Apo without previous coculture (DC + Apo), DCs cocultured with necrotic cells (DC-Nec), necrotic cells alone (Nec), DCs cocultured with apoptotic syngeneic splenocytes (DC-Spleen), or vehicle (PBS) weekly for 4 wk. One week after the last injection, mice received a tumor challenge with 1.3×10^4 live B16 cells. Animals were monitored for tumor growth every other day by palpation, and diameters were measured using a Vernier caliper. Tumor-bearing animals were sacrificed when the tumors displayed severe ulceration or reached a size of 250 mm^2. For evaluation of immunological memory, mice were challenged 10 wk after the last vaccination. In the case of tumor-free animals, experiments were terminated 12 wk after tumor challenge, and autopsies were performed. All experiments included 10–13 mice/group.

In vivo depletion of $CD4^+$ and $CD8^+$ T lymphocytes

Mice immunized as described were injected with murine ascites containing 1 mg of rat monoclonal anti-CD4 (clone GK 1.5) or anti-CD8 (clone 53-6.72; hybridomas were provided by Dr. A. Vecchi). Three consecutive injections were performed i.v. on the day before tumor challenge, followed by two i.p. injections on the day of and the day after tumor challenge. Control experiments were performed by injecting the same amount of standard rat IgG (Sigma-Aldrich). Depletion of $CD4^+$ and $CD8^+$ T cells from spleen was monitored using anti-CD4 and anti-CD8 mAb.

IFN-γ production by $CD4^+$ and $CD8^+$ T cells

Four weeks after vaccination, $CD4^+$ and $CD8^+$ T cells were isolated from spleen of vaccinated or control mice using MACS microbeads. Purified $CD4^+$ or $CD8^+$ T cells were subsequently cocultured in 96-well, flat-bottom microtiter plates (3×10^5 cells/well) in the presence of B16 cells, DC-Apo, or DCs that had been pretreated with tyrosinase-related protein 2 $(TRP2)_{180-188}$ peptide (SVYDFFVWL; a gift from Dr. A. Kalergis, Rockefeller University, New York, NY) or OVA (SIINFEKL) as a control peptide (1 h 37°C, 1 μM) for 48 h in Click's medium containing 0.75% mouse serum. The titers of IFN-γ in the culture supernatants were determined by ELISA (OptEIA kit; BD PharMingen) according to the manufacturer's protocol.

In vivo cytotoxicity assay

C57BL/6 splenocytes were divided into two populations and labeled with either a high concentration (5 μM) or a low concentration (0.5 μM) of CFSE for 10 min at 37°C, then washed twice with PBS containing 0.1% BSA. $CFSE^{high}$ cells were pulsed with 1 μM $TRP2_{180-188}$ peptide, while $CFSE^{low}$ remained nonpulsed. After washing, $CFSE^{high}$ cells were mixed with equal numbers of $CFSE^{low}$ cells, and 10^7 or 5×10^6 of this cell suspension was injected i.v. into DC-Apo- or DC-vaccinated mice. Spleens and lymph nodes from recipient mice were taken 10 h later for flow cytometric analysis. In vivo killing was indicated by loss of $CFSE^{high}$ peptide-pulsed cells relative to $CFSE^{low}$ nonpulsed cells. The percentage of specific lysis was calculated according to the formula: (1 − (ratio unprimed/ratio primed) × 100), where ratio unprimed = % $CFSE^{low}$/$CFSE^{high}$ cells remaining in control mice, and ratio primed = % $CFSE^{low}$/$CFSE^{high}$ cells remaining in vaccinated mice.

Statistical analysis

Statistical analyses were performed using the Wilcoxon rank test.

Results

Apoptotic B16 melanoma cells are efficiently phagocytosed by DCs

Whole tumor cells are an advantageous source of Ags for DC vaccination because they provide a wide range of tumor Ags that can be processed onto both MHC class I and class II products by DCs. To induce apoptosis, B16 cells were irradiated (70 Gy) and cultured for 48 h. The tumor cells then failed to grow in culture and could not induce tumors in mice at high doses (10^6 cells). Apoptosis was documented by annexin V/PI staining (Fig. 1). After 48 h, cultured unirradiated cells contained 10–12% early apoptotic cells characterized by annexin V^+/PI^- staining (Fig. 1*A*, *lower right*). In contrast, 70–80% of irradiated cultured tumor cells had undergone early apoptotic death (Fig. 1*B*). Twelve to 19% of the cells displayed annexin V^+/PI^+ staining, indicating secondary necrosis in both unirradiated and irradiated cultures (Fig. 1, *A* and *B*, *upper right*). Eighty percent of freeze/thawed necrotic cells displayed annexin V^+/PI^+ staining (data not shown). Then PKH67 green-labeled DCs (Fig. 1*C*) were cultured with PKH26 red-labeled, apoptotic tumor cells (Fig. 1*D*) for 24 h, and phagocytosis was documented by the percentage of double-positive cells. At 37°C, >40% of immature DCs phagocytosed apoptotic material (Fig. 1*E*), and this was confirmed by confocal fluorescence microscopy and electron microscopy (data not shown). Freeze/

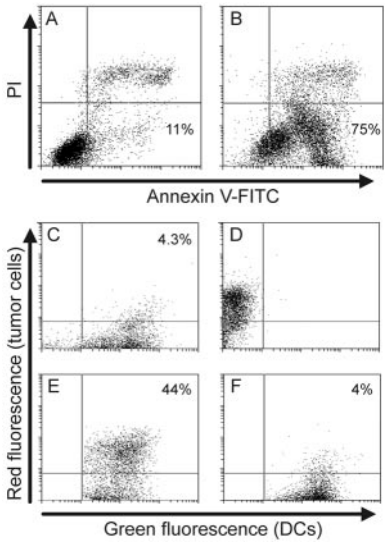

FIGURE 1. Apoptosis of irradiated B16 melanoma cells and phagocytosis by DCs. B16-F1 melanoma cells were cultured for 48 h, either untreated (*A*) or after irradiation (*B*), and stained with annexin V-FITC and PI. Early apoptotic cells were defined as annexin V-FITC$^+$, but PI$^-$, while necrotic cells were double-positive. To demonstrate phagocytosis (*C–F*), DCs were labeled green with PKH67-GL fluorescent cell linker (*C*), and apoptotic cells were labeled red with PKH26-GL (*D*); these were cultured together at a 1/1 ratio for 24 h at 37°C (*E*) or 4°C (*F*). The frequency of double-positive phagocytic DCs (gated as green profiles) is shown in the *upper right* quadrants.

thawed necrotic cells were also phagocytosed by DCs to a comparable extent as apoptotic cells (not shown). To rule out the possibility of nonspecific tumor cell binding, coculture of apoptotic tumor and DCs was performed at 4°C; the percentage of double-positive cells was only 4% (Fig. 1*F*). DCs also efficiently phagocytosed apoptotic syngeneic splenocytes (data not shown).

DCs loaded with apoptotic B16 cells migrate to lymph nodes and up-regulate CD86 and MCH II expression

To induce a primary immune response, DCs have to undergo a maturation process that involves up-regulation of MHC and costimulatory molecules, and the DCs must also migrate to lymph nodes to select infrequent naive T cell clones for activation. To assess these attributes in our system, we cultured irradiated B16 cells for 24 h with DCs and double-stained with PE-anti-CD11c and either FITC-anti-CD86 or FITC-anti-MHC II. While the expression of CD11c remained similar to that of DCs that were not cultured with tumor cells, a large fraction of the tumor-exposed DCs up-regulated CD86 as well as MHC II (Fig. 2*A*). We then analyzed the migratory capacity of these cells. CD45.2$^+$ DCs that had been pulsed with apoptotic B16 cells or left unpulsed were injected s.c. into CD45.1$^+$ recipient mice. After 24 h the draining lymph nodes were obtained, and the surface phenotype of the injected CD45.2$^+$ cells was analyzed. CD11c$^+$ cells from both tumor-pulsed and unpulsed DCs cultures were found in the draining nodes, and both showed an up-regulation of CD86 and MHC II molecules (Fig. 2*B*). The number of injected CD45.2$^+$ cells in the lymph nodes corresponded to ~0.5% of the administered inoculum, consistent with prior data (36). Therefore, a s.c. injection of DCs, with or without exposure to apoptotic tumor, leads to the residence of comparable, but small, numbers of mature DCs in the draining lymph nodes, setting the stage for testing their immunizing capacity against B16 melanoma challenge.

DCs loaded with apoptotic B16 cells induce specific resistance to B16 tumors including long term memory

To evaluate whether DCs cocultured with apoptotic B16 cells (DC-Apo) were able to elicit resistance to challenge with tumor, mice were vaccinated s.c. weekly for 4 wk with 2×10^5 DC-Apo and challenged with 1.3×10^4 B16 cells 1 wk later. The efficiency of vaccination by DC-Apo was compared with that in several other types of DCs controls: DCs plus Apo without previous coculture (DC+Apo), DCs alone, apoptotic tumor cells alone (Apo), DCs cocultured with necrotic cells (DC-Nec), necrotic cells alone (Nec), and vehicle (PBS; Fig. 3*A*). Also, to rule out the possibility of a nonspecific activation by DCs phagocytosing apoptotic cells, a group of mice that received DCs cocultured with syngeneic apoptotic splenocytes (DC-Spleen) was included. All mice were monitored every other day after tumor challenge, and the tumor volumes were measured.

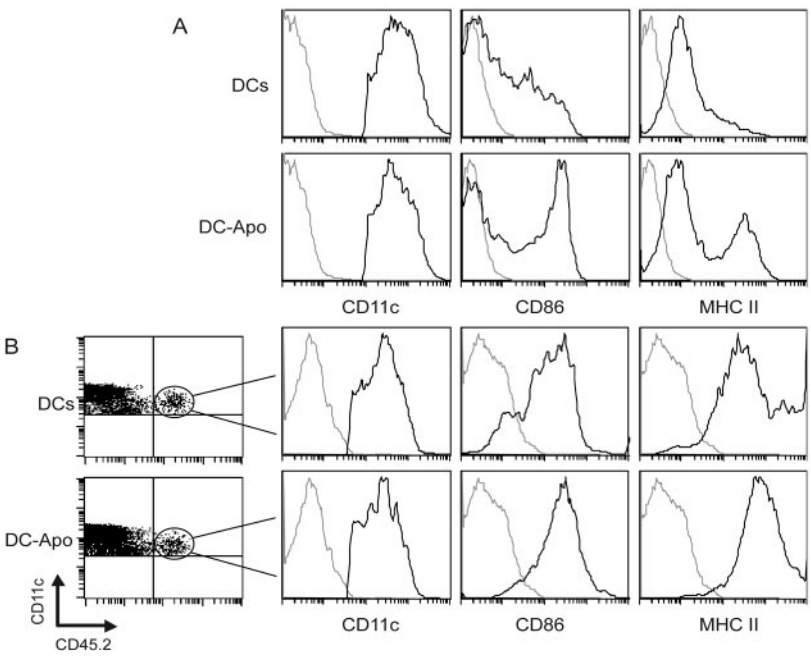

FIGURE 2. Phenotype of DCs before and after coculture with apoptotic B16 and migration into the lymph nodes. *A*, Surface phenotype of DCs without (DCs) and with (DC-Apo) coculture with apoptotic B16 cells. The DCs and DC-Apo were stained with PE-conjugated anti-CD11c, followed by FITC-conjugated anti-CD86 or MHC II (I-A^b). CD86 and MHC II expression was evaluated on CD11c$^+$-gated cells. *B*, CD45.2$^+$ DCs or CD45.2$^+$ DC-Apo were injected s.c into CD45.1$^+$ mice. Twenty-four hours later, draining lymph nodes were harvested, and single-cell suspensions were obtained. Cells were then incubated with allophycocyanin-conjugated anti-CD45.2, PE-conjugated anti-CD11c, followed by FITC-conjugated anti-CD86 or MHC II. The expression of CD86 and MHC II was analyzed on CD45.2$^+$, CD11c$^+$-gated cells, which were not found in uninjected CD45.1$^+$ mice. Gray lines show staining with isotype-matched control Abs. One representative experiment of three is shown.

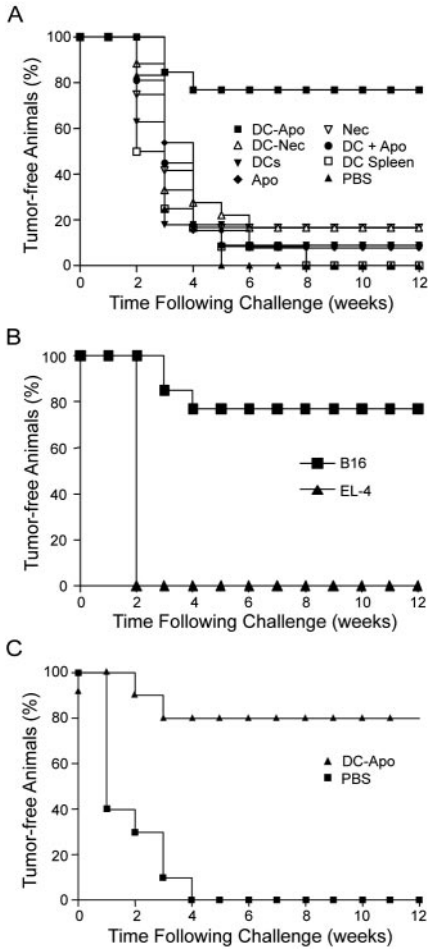

FIGURE 3. Protection against tumor development after vaccination with DCs loaded with apoptotic B16 melanoma. *A,* Mice (10–13/group) were vaccinated with DCs that had phagocytosed apoptotic tumor cells for 24 h (DC-Apo), DCs alone, apoptotic tumor cells alone (Apo), a mixture of DCs and tumor cells (DC+Apo), DCs that had phagocytosed apoptotic spleen cells (DC-Spleen), PBS, DCs that had phagocytosed necrotic B16 melanoma cells (DC-Nec), or necrotic tumor cells alone (Nec) weekly for 4 wk and challenged with 1.3×10^4 B16 cells 1 wk later. *B,* Mice (10/group) were vaccinated with DC-Apo and challenged with either B16 or EL-4 cells 1 wk after immunization. *C,* Mice (10/group) were vaccinated with DC-Apo or PBS weekly for 4 wk and challenged 10 wk later with 1.3×10^4 B16 cells. In *A–C,* mice were monitored every other day for tumor growth and were scored positive when tumors were palpable. One representative experiment of three is shown.

Eighty percent of the mice vaccinated with DC-Apo remained tumor-free 12 wk after challenge relative to 0% in the PBS controls (Fig. 3*A*). None of the control groups showed protection relative to the nonvaccinated animals. When mice were injected with Apo only or with DC+Apo without previous coculture, only 8% ($p < 0.01$) remained tumor-free at wk 12, indicating that DCs are necessary to induce strong protection and that they need to be previously cocultured with Apo. In mice injected with DCs only, 9% ($p < 0.01$) of the mice were tumor-free after 12 wk, ruling out the possibility that tumor rejection was solely due to NK cell expansion, which can be induced by DCs (37–39) or to immunity in response to culture medium proteins presented by DCs. It has been shown that DCs cultured in the presence of FCS are capable of inducing $CD4^+$ T cell help (40, 41) and to prime CTLs more efficiently than DCs cultured in FCS-free conditions (40). Therefore, the presence of FCS-derived peptides loaded onto the DCs might have contributed to the observed antitumor response. When mice were vaccinated with DCs loaded with syngeneic apoptotic splenocytes, 0% remained tumor-free ($p < 0.01$), indicating that the induction of immunity was not due to a nonspecific activation of DCs after phagocytosis of dying cells. In contrast to DC-Apo, only 17% ($p < 0.01$) of the mice injected with DC-Nec remained tumor-free, demonstrating that Apo are a better source of Ag for DCs priming. In summary, only mice vaccinated with DC-Apo showed strong protection against the poorly immunogenic B16 melanoma (Table I).

To analyze the specificity of this antitumor response, mice vaccinated with DCs loaded with apoptotic B16 cells were challenged with the EL-4 lymphoma, but all the mice developed tumor within 2 wk (Fig. 3*B*). This indicates that vaccination with DCs loaded with apoptotic B16 cells induces a strong protection against B16 challenge, but does not protect against a nonrelated syngeneic tumor.

To test whether vaccinated mice could acquire immunological memory, mice were vaccinated with DC-Apo and challenged with B16 10 wk later. Strong protection was again achieved after vaccination with DC-Apo. Twelve weeks after tumor challenge, 80% of the mice remained tumor-free vs 0% of control mice ($p < 0.01$; Fig. 3*C*). These data indicate that DC-Apo vaccination imparts considerable memory to B16.

Both $CD4^+$ and $CD8^+$ T cells are required for the antitumor response

We studied the role of $CD4^+$ and $CD8^+$ T cells in the observed resistance to B16, which, as mentioned, has not previously been shown to elicit combined $CD4^+$ and $CD8^+$ immunity in mice. Mice vaccinated with DC-Apo received anti-CD4, anti-CD8, or control rat IgG on the day before tumor challenge, followed by two injections on days 0 and 1 after tumor challenge. In vivo depletion of either $CD4^+$ or $CD8^+$ T cells by Ab treatment completely abrogated the protection from tumor challenge (Fig. 4). Since these depletions were performed after DC-Apo vaccination, the results suggest that both $CD4^+$ and $CD8^+$ T cells play a crucial role in resistance to B16 melanoma following vaccination with DC-Apo.

DCs charged with apoptotic B16 efficiently prime both $CD4^+$ and $CD8^+$ T cells

To verify that both $CD4^+$ and $CD8^+$ T cells could be primed to produce IFN-γ after immunization with DC-Apo, $CD4^+$ and $CD8^+$ T cells were purified by positive selection from spleens

Table I. *Tumor-free survival after vaccination with DCs loaded with apoptotic B16 melanoma*[a]

Expt. No.	DC-Apo	DC	Apo	PBS
1	10/13 (77%)	1/11 (9%)	1/12 (8%)	0/12 (0%)
2	8/10 (80%)	1/11 (9%)	1/12 (8%)	0/10 (0%)
3	8/10 (80%)	1/10 (10%)	1/10 (10%)	0/10 (0%)
	DC + Apo	DC-Nec	Nec	DC-Spleen
1	1/12 (8%)	2/13 (15%)	1/8 (12%)	0/12 (0%)
2	1/11 (9%)	2/11 (18%)	2/11 (18%)	0/12 (0%)

[a] C57BL/6 mice were vaccinated s.c. with 2×10^5 DCs loaded with apoptotic B16 cells (DC-Apo), DCs alone, apoptotic tumor cells alone (Apo), a mixture of DCs and tumor cells (DC + Apo), DCs that had phagocytosed apoptotic spleen cells (DC-Spleen), PBS, DCs that had phagocytosed necrotic B16 melanoma cells (DC-Nec), or necrotic tumor cells alone (Nec) weekly for 4 wk and challenged with 1.3×10^4 B16 cells 1 wk later. Results are presented as the number of tumor-free survivors over the total number of animals 12 wk following tumor challenge.

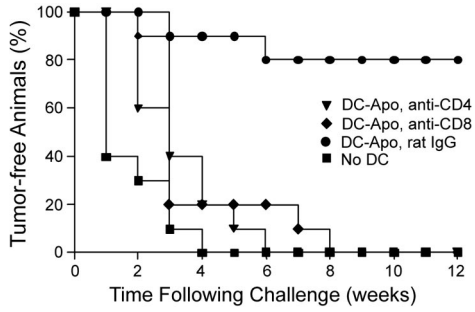

FIGURE 4. In vivo depletion of either $CD4^+$ or $CD8^+$ T cells ablates protective antitumor immunity. Mice (10/group) were vaccinated on days 0, 7, 14, and 21 with DC-Apo or PBS. Vaccinated animals were divided into groups treated with anti-CD4, anti-CD8, or normal rat IgG on days 27, 28, and 29. Then mice were challenged with 1.3×10^4 B16 cells on day 28. Mice were monitored every other day and were scored positive when the tumors were palpable. One representative experiment of two is shown.

obtained from mice injected with DC-Apo or control mice 4 wk after immunization. The T cell fractions were stimulated in vitro for 48 h with intact B16 cells or with DCs that had been pretreated with TRP-2 peptide or OVA-derived control peptide, and the medium was then assessed for the production of IFN-γ (Fig. 5). TRP-2 is a tissue differentiation Ag expressed by normal melanocytes and melanoma cells in both humans and mice (31, 42–45) and was used as a model tumor Ag. As shown in Fig. 5, only $CD8^+$ T cells from DC-Apo-vaccinated mice were able to respond to intact B16 cells. However, both subsets responded to DC-TRP-2

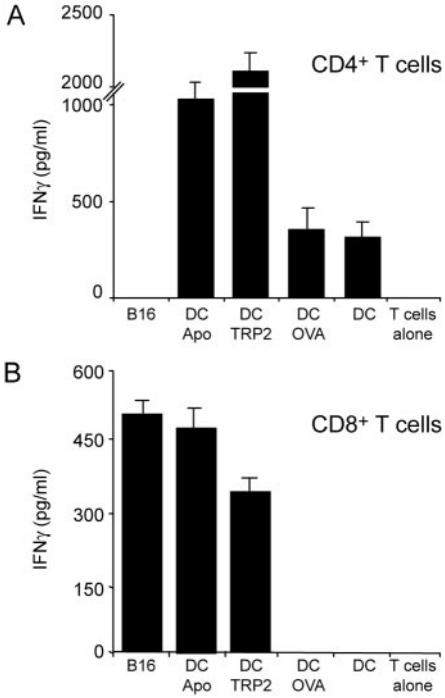

FIGURE 5. $CD4^+$ or $CD8^+$ T cells are primed by DC-Apo to secrete IFN-γ. $CD4^+$ (*A*) or $CD8^+$ (*B*) T cells were purified using MACS beads from spleens obtained from DC-Apo-vaccinated or control mice 4 wk after immunization. These cells were stimulated with intact B16 cells, DC-Apo, DC pulsed with TRP-2 peptide, or DC pulsed with OVA peptide, as described in *Materials and Methods*. After 48 h culture supernatants were harvested, and IFN-γ release was measured by ELISA. No IFN-γ was produced by the PBS control mice (not shown). The mean ± SD of triplicate determinations are shown.

and DC-Apo, with the $CD4^+$ T cell responses being more vigorous (Fig. 5). As controls, $CD8^+$ T cells from mice vaccinated with DC-Apo made <30 pg/ml without challenge or following challenge with DCs only or DC-OVA peptide (Fig. 5*B*). $CD4^+$ T cells from mice injected with DC-Apo also did not release IFN-γ in the absence of DCs, but did form small amounts of cytokine following culture with DCs only or with DCs loaded with the control peptide (Fig. 5*A*). Although these assays were performed using mouse serum, the DCs used as APCs had been cultured in 10% FCS, and the presence of FCS-derived peptides could explain the control $CD4^+$ T cell response observed against DC-OVA or DCs alone (40, 41). T cells from control nonvaccinated mice made <30 pg/ml of IFN-γ with or without challenge (data not shown). Our data indicate that IFN-γ-producing, TRP-2-specific, $CD4^+$ and $CD8^+$ T cells are primed when mice are vaccinated with DCs loaded with B16 melanoma.

In vivo cytotoxic effector activity induced after vaccination with DC-Apo

To evaluate whether Ag-specific cytolytic activity could be detected in vivo following vaccination with DC-Apo, an in vivo cytotoxicity assay was performed. Syngeneic splenocytes were labeled with a high concentration of CFSE and loaded with the TRP-2 peptide. These were coinjected i.v. with equal numbers of non-TRP-2-loaded splenocytes, which were labeled with a lower concentration of CFSE. The mice had been vaccinated with DC-Apo or DCs 4 wk earlier, and the spleens were harvested 10 h later after injection of $CFSE^{high}$ and $CFSE^{low}$ cells. The specific cytolytic activity was measured by the disappearance of TRP-2-loaded $CFSE^{high}$ target cells. No cytolytic activity was observed in mice injected with DCs, but in mice injected with DC-Apo, the specific cytolytic activity was 75% when 10^7 cells were transferred and went up to 92% when 5×10^6 cells were transferred (Fig. 6). We did not determine whether $CD4^+$ or $CD8^+$ T cells were responsible for this lytic activity, but nevertheless, our data indicate that vaccination with DC-Apo leads to the production of effector T

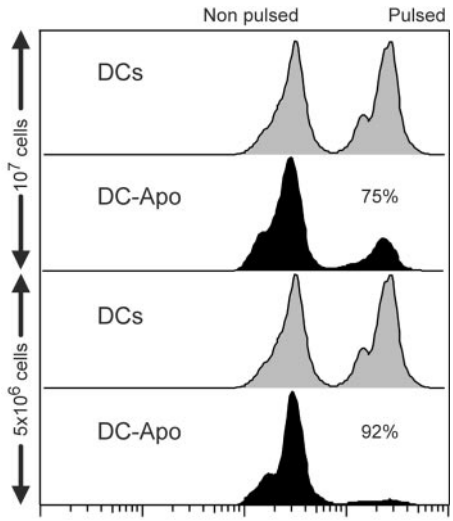

FIGURE 6. In vivo cytolytic activity. $CFSE^{high}$ TRP-$2_{180-188}$-pulsed splenocytes and equal numbers of $CFSE^{low}$ nonpulsed splenocytes were coinjected i.v. into mice vaccinated with DC-Apo or DCs 4 wk after immunization. Ten hours later spleens were harvested, and in vivo cytolysis of transferred cells was measured by the selective disappearance of the TRP-2-loaded $CFSE^{high}$ cells. Specific lysis was calculated as described in *Materials and Methods*.

cells that both secrete IFN-γ (Fig. 5) and have cytolytic activity (Fig. 6).

Discussion

DCs-based immunotherapy is being developed as a novel approach to vaccination against cancer. DCs have the potential to be loaded with a spectrum of tumor Ags and to prime both $CD8^+$ and $CD4^+$ T cells. However, the immune response to DCs loaded with different sources of tumor Ag requires investigation. In this study we demonstrate that B16 melanoma cells undergoing apoptotic death as a result of irradiation and culture are an effective source of Ag for DCs-based vaccines, including the induction of both $CD8^+$- and $CD4^+$-dependent, long-lived antitumor responses. The B16 melanoma model has been a valuable model for the study of tumor immunity, but previous reports using apoptotic/necrotic cells to load DCs (46, 47) have not documented combined $CD4^+$ and $CD8^+$ dependent long term immunity.

In addition to being a source of tumor Ags for MHC class I and II products, dying cells may influence the maturation state of the DCs and thereby the quantity and quality of the ensuing immune response. There are conflicting reports about whether apoptotic or necrotic cells are capable of inducing DC maturation. Sauter et al. (48) found that incubation of DCs with necrotic, but not apoptotic, tumor cell lines induce maturation, while other reports concluded that incubation with apoptotic cells is sufficient (25, 26, 46, 49). Our results show that after coculture with gamma irradiation induced apoptotic B16 melanoma cells, a high proportion of DCs phagocytose the apoptotic cells and up-regulate the expression of CD86 and MHC class II molecules (Fig. 2*A*). However, it is possible that the presence of necrotic cells (<20%) in our apoptotic cell preparation was relevant to the observed DCs maturation.

We also characterized the DCs that migrate to the draining lymph nodes following s.c. injection, comparing DCs that had been pulsed or not pulsed with apoptotic cells. The migrants uniformly expressed high levels of CD86 and MHC II molecules (Fig. 2*B*) comparable to fully mature and immunogenic DCs. However, when we enumerated the migrating DCs only ~0.5% of the original inoculum was found in the draining lymph nodes 1 day after injection.

Our current vaccine model uses four weekly injections of DC-Apo, and we have used this to prevent rather than treat B16 melanomas. Eighty percent of the mice remained tumor-free 12 wk following challenge with B16 cells (Fig. 3*A*). No protection was observed when DC-Apo-vaccinated mice were challenged with the syngeneic EL-4 lymphoma (Fig. 3*B*), suggesting that the immune response was directed against B16 melanoma Ag. Moreover, vaccination with DC-Apo induced immunological memory, since, again, 80% of mice remained tumor-free when they received the tumor challenge 10 wk after immunization (Fig. 3*C*). It will be important to use DCs in the therapeutic setting, but at the tumor cells doses we have used, large (~1-cm diameter) tumors are evident by 14 days, too quickly to study therapy. However, a critical limitation in the current use of DC-Apo, as evident in Fig. 2*B*, is the low number of migrating DCs 1 day after injecting 200,000 cells in each of the four paws, since a total of only ~4,000 DCs were found in the draining lymph nodes. This suggests that with current methods the injected DCs are functioning far below their potential. This will need to be addressed to achieve strong T cell immune responses and to approach therapeutic models.

When either $CD4^+$ or $CD8^+$ T cell subsets were depleted by Ab treatment of vaccinated mice, the immune protection was completely abrogated, indicating that both T cell subsets were necessary for effective in vivo vaccination with DC-Apo (Fig. 4). In vitro T cell assays also indicated that both $CD4^+$ and $CD8^+$ T cells were primed to produce IFN-γ after restimulation with DCs loaded with apoptotic B16 cells or DCs loaded with the TRP-2 melanoma Ag (Fig. 5). In addition, the $CD8^+$ cells were able to produce IFN-γ upon coculture with B16 melanoma cells, and the immunized mice were able to kill TRP-2-coated splenocytes in vivo (Fig. 6). However, NK cells as well as other innate pathways, such as phagocytes, NKT cells, and complement components, could also contribute to the resistance that takes place in the presence of tumor-specific T cells that are elicited by DC-Apo**.** In the results reported by Hung et al. (33), the immune response induced by vaccination with B16 cells engineered to secrete GM-CSF required $CD4^+$ T cells at the effector phase of the antitumor response. In contrast, their data showed that depletion of $CD8^+$ T cells only partially reverted the protective response, suggesting that vaccination with B16:GM-CSF-secreting cells did not induce effective $CD8^+$ immunity. Similarly, Shibagaki et al. (32) showed that vaccination with B16:GM-CSF-secreting cells was relatively inefficient with regard to CTL and memory induction. Our data indicate that DC-Apo have an advantage in eliciting combined helper and cytotoxic T cell immunity.

Although the $CD4^+$ helper cells did not recognize B16 melanoma cells directly, because the latter do not express MHC class II, the immune $CD4^+$ T cells did recognize DCs loaded with tumor (Fig. 5). Ossendorp et al. (50) have previously shown that optimal CTL induction requires cognate $CD4^+$ T cell help even for MHC class II-negative tumors. In that study the authors used a peptide vaccination strategy to show that depletion of $CD4^+$ T cells during either the induction or effector phase of the immune response abrogates the survival of preimmunized mice. Likewise, Mumberg et al. (51) showed that adoptively transferred $CD4^+$ T cells eliminate MHC class II-negative tumor cells in SCID mice. IFN-γ was required, but it did not induce, MHC class II expression on the tumor, suggesting that tumor Ag were being cross-presented by MHC class II^+ host cells. Our data together with others (31, 33, 50) indicate that tumor-specific $CD4^+$ T cells are required upon tumor challenge for the effective antitumor response in immunocompetent mice. Such T cells may have several functions: to help $CD8^+$ T cell function in the draining lymph node, to contribute to CTL infiltration into the tumor bed (52), to maintain in situ CTL effector functions (53), to release factors that more directly provide resistance against the tumor, i.e., IFN-γ (54–56), and/or to recruit other effector cells (33).

We found that the TRP-$2_{180-188}$ peptide was recognized by both $CD4^+$ and $CD8^+$ T cells. This peptide has been identified as a relevant MHC class I (K^b) epitope (43, 44), but Wang et al. (31) have reported that immunization with DCs loaded with TRP-$2_{180-188}$ peptide fused to a cell-penetrating peptide induce a $CD4^+$ T cell response as well, and that this is required for antitumor immunity, as evidenced by $CD4^+$ T cell depletion and the use of $CD4^+$ knockout mice. Peptides are known to bind to MHC class II molecules via a nine amino acid core sequence (57–59), and this 9-aa core also is recognized by the TCR (60). Therefore, the strong CD4 T cell response to TRP-2 that we observed is not totally surprising.

When DCs are loaded with TRP-2 peptide only (using peptide itself or a cell-penetrating fusion TRP-2 peptide, TRP-2 protein transduction) and used to vaccinate mice against B16 melanoma, the survival was found to be lower than that observed with B16:GM-CSF-secreting cells, presumably because TRP-2 is but one of many Ags in B16 cells (31–33). We also suspect that our use of DC-Apo immunizes $CD4^+$ or $CD8^+$ T cells to many more B16 melanoma Ags than TRP-2, but these remain to be identified.

A perplexing finding in our study was that DCs loaded with apoptotic cells, but not necrotic cells, were immunogenic, even though both sources of dying cells were phagocytosed well by the

DCs. Shimizu et al. (61), using lysates or peptide-pulsed DCs vaccines, reported that $CD4^+$ T cell protection required the addition of IL-2 and the addition of the immunogenic protein keyhole limpet hemocyanin to the immunizing protocol. Kotera et al. (46) have reported that DCs pulsed with highly enriched apoptotic or necrotic (lysates) B16 cells have equivalent antitumor activity, although their experiments were only followed for 18 days after challenge, and the immunization conditions only allowed for a delay in tumor growth. However, there are other examples showing that DCs loaded with apoptotic cells are more efficient than DCs loaded with necrotic cells in activating T cells (24, 27, 62, 63). In the results reported by Albert et al. (27), Ag acquired from apoptotic cells were efficiently cross-presented by DCs, while DCs carrying Ag derived from necrotic cells were inefficient at inducing CTLs. Strome et al. (62) found that DCs loaded with irradiated tumor cells were effective stimulators of OVA-specific CTL clones in vitro, whereas DCs loaded with freeze-thawed tumor cells failed to stimulate IFN-γ secretion. Furthermore, they reported an inhibitory effect of freeze-thawed tumor cells on the in vitro CTL activity. Similar results were observed when they used human melanoma cell lines and gp100-specific CTL clones. Then the authors assessed the two Ag-loading strategies in vivo, and again only DCs loaded with irradiated tumor cells were effective in preventing the outgrowth of the OVA-expressing E.G7 thymoma. Schnurr et al. (63) have found that DCs bearing apoptotic pancreatic tumor cells were better suited than tumor-lysate-loaded DCs to activate both $CD8^+$ and $CD4^+$ T cells in vitro. Using a squamous cell carcinoma model, Hoffmann et al. (24) have reported that apoptotic cell-loaded DCs likewise induce stronger CTL responses compared with cell lysate-loaded DCs. Perhaps necrotic cell lysates are adequate for loading DCs with tumor-associated Ags only when the latter are more resistant to proteolysis or when the lysates provide additional stimulatory stimuli for the DCs.

Many studies have been conducted using dying tumor cells as a source of Ags (22–26, 46, 62, 63). However, none of these has shown in vivo induction of combined $CD4^+$ and $CD8^+$ antitumor immunity. In the present study we found that DCs phagocytosing apoptotic tumor cells elicit long-lived combined $CD4^+$ and $CD8^+$ immunity against the poorly immunogenic B16 melanoma. DC-Apo have been shown by Berard et al. (22) to be able to prime naive human T cells in vitro to melanoma Ags. These findings together with our preclinical model set the stage for comparable studies in humans with DC-Apo as adjuvants for active immunization against cancer.

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