Dead or Alive: Immunogenicity of Human Melanoma Cells When Presented by Dendritic Cells

Munitta Shaif-Muthana, Catherine McIntyre, Karen Sisley, Ian Rennie, and Anna Murray

Institute for Cancer Studies, Division of Oncology and Cellular Pathology, University of Sheffield Medical School, Sheffield, S10 2RX [M. S.-M.; C. M., A. M.], and Department of Ophthalmology and Orthoptics, Royal Hallamshire Hospital, Sheffield, S10 2RX [K. S., I. R.]; United Kingdom

ABSTRACT

Uveal melanoma is an aggressive malignancy with a poor prognosis despite current therapeutic intervention. These tumors have been shown to be antigenic because they express a number of melanoma-associated antigens and are therefore attractive targets for immunotherapy. Here, we investigated the immunogenicity of uveal melanoma cells that have undergone apoptosis and compared this with their necrotic or live counterparts. The fate of the tumor antigens in these cells largely depends on their ability to be processed and phagocyted by dendritic cells (DCs). Flow cytometric analysis shows that human DCs form conjugates more efficiently with dead uveal melanoma cells, and consequently these are effective stimuli of lymphocyte proliferation. However, only DCs pulsed with apoptotic cells were able to induce proliferation of CD8+ cytotoxic T cells and stimulate antigen-specific T cells. This study demonstrates for the first time that DCs derived from melanoma patients process and present antigens derived from both HLA-matched or HLA-mismatched human apoptotic tumor cells stimulating both CD4+ and CD8+ T cells. This approach may be important to the development of DC-based immunotherapies for melanoma.

INTRODUCTION

The recent identification of a variety of tumor antigens and their peptide products, which are recognized by HLA class I-restricted CTLs,3 has led to new approaches in the development of cancer immunotherapies (1). Following the pioneering work of van der Bruggen et al. (2), definitive evidence for a number of cutaneous melanoma antigens recognized by antimalanoma CTLs have been identified. Apart from the melanoma tumor-specific antigens, members of the MAGE family (3), the melanocytic lineage-specific tyrosinase (4), and the differentiation antigens GP100 (5), Melan-A/MART-1, and gp75 (6, 7) have also been described. Uveal melanomas similar to cutaneous melanomas have been shown to be immunogenic. We have shown that although uveal melanoma cells do not express the MAGE genes, they express higher-than-normal levels of tyrosinase, MART-1, and GP100 (8). We also reported that uveal melanoma patients do indeed have precursor lymphocytes that can recognize and kill target cells presenting these peptides (9), thus encouraging the use of immunotherapy in uveal melanoma patients.

In the past, clinical responses of melanoma patients vaccinated with peptides derived from the tumor antigens, either alone or with adjuvant, have been rare (10–12). Overwhelming evidence suggests that successful activation of CTLs with antitumor immunity is critically dependent on presentation of the tumor antigen by its natural adjuvant, the DC (13–15). DCs expressing high levels of MHC class I and II and costimulatory molecules have demonstrated high efficiency and potency in presenting tumor peptides to enhance cellular immunity both in vitro (16, 17) and in vivo (18). The use of peptide-pulsed DCs, though clearly effective, requires prior knowledge of patient HLA types and the sequences of the relevant peptide epitopes. To overcome this limitation, tumor cells themselves may be used as immunogens. We know that tumor cells express tumor antigens, which can be recognized by T cells (19); however, advanced tumors generally are not immunogenic, at least in part because they do not express costimulatory molecules (20). Recent studies have demonstrated that human DCs can acquire antigen from apoptotic cells and stimulate antigen-specific, class I-restricted T cells (21). Processing of the phagocyted apoptotic cells yields antigens that access the cytosol and are subsequently presented on MHC class I molecules of APCs via a TAP-dependent pathway, enabling their recognition by antigen-specific CTLs (22). This raises the possibility that antimalanoma-specific CTL responses may be primed against tumor antigens derived from melanoma cells induced to undergo apoptosis.

Tumor-cell damage induced by current cancer treatment modalities largely results in tumor-cell apoptosis (23), and this could be enhanced by the application of DC-based immunotherapies. Herein, we have investigated the significance of human DCs exposed to irradiated uveal melanoma cells that have undergone apoptosis as a source of tumor antigen and compared this with their necrotic and live HLA counterparts in their ability to stimulate an immune response. Phagocytic uptake of the melanoma cells by the DCs was determined flow cytometrically and by electron and fluorescent microscopy. The immunogenicity of the melanoma cells cocultured with DCs was demonstrated by their ability to induce lymphocyte proliferation and tumor-specific CTLs in vitro.

MATERIALS AND METHODS

Cultured Cell Lines. The cutaneous melanoma cell lines FM3 (HLA-A2, A3+, B44+) and FM79 (HLA-A2+, A25+, A32+, B44+) were a kind gift from J. Jeuthen (Copenhagen, Denmark). The 174 CEM-T2 (T2) cell line (HLA-A2+, B44-) was a generous gift from J. Bartholomew, (Paterson Institute, Manchester United Kingdom). K562 is an erythroleukemia cell line that is HLA class I-negative and sensitive to natural killer cell-mediated lysis, supplied by Imperial Cancer Research Fund, London, United Kingdom. IM61 (HLA-A2+, A3+, B44+) is a LCL line provided by A. Rickinson (Birmingham, United Kingdom). All of the above cell lines were cultured in RPMI 1640 medium supplemented with 10% FCS. The ocular melanoma lines Mel 157d (HLA-A1+, A2+, B7+) and Mel 257 (HLA-A1+, A2+, B7+) were both derived from intraocular melanoma obtained from patients attending the Department of Ophthalmology and Orthoptics, Royal Hallamshire Hospital. The cell lines were established by mingling of the tumor mass and subsequent culture in RPMI 1640 (Life Technologies, Inc.) supplemented with 20% FCS (Life Technologies, Inc.), 10 ng/ml epidermal growth factor, 2 mM L-glutamine, 2 mg/ml glucose, 100 units/ml penicillin, 100 mg/ml streptomycin, and 2.5 mg/ml fungizone. The molecular HLA types of all of the cell lines were determined by the Tissue Typing Department of the Blood Transfusion Service, Sheffield, or by the Oxford Transplant Center.

Generation of DCs from Peripheral Blood. The collection of blood for this study was approved by South Sheffield Research Ethics Committee. DCs were prepared according to protocols published previously (24, 25). In brief, peripheral blood was obtained from HLA-A2+ healthy donors into heparin,
and PBMCs were isolated by standard gradient centrifugation in J prep (Nucleom, Bucks, United Kingdom). The plasma layer was collected, heat-inactivated at 56°C, and stored at 4°C. To generate DCs, PBMCs were resuspended in RPMI 1640 plus 10% FCS and allowed to adhere to tissue flasks. After 2 h at 37°C, the nonadherent cells were removed and frozen at 1 × 10^7 cells/ml for future use as a source of T cells. The adherent cells were subsequently cultured for 7 days with 800 units/ml granulocyte macrophage colony-stimulating factor (Roche, Welwyn, United Kingdom) and 500 units/ml IL-4 (Peprotech, London, United Kingdom). Blood samples were also collected from patients with uveal melanoma who were undergoing treatment at the Royal Hallamshire Hospital. Sixty ml were obtained, of which 50 ml were used for DC generation as described above, and 10 ml were sent for HLA-typing at the Blood Transfusion Service.

Flow cytometric analysis (FACSScan, Becton Dickinson) indicated that these cells expressed high levels of MHC class I, MHC class II, CD1a, CD40, and CD54 and moderate levels of the costimulatory molecules CD80 and CD86.

Induction of uveal melanoma cells apoptosis was achieved using a cesium source (Gammacell 3000; Elan, Nordion International, Inc.). Tumor cells (2 × 10^6) were seeded into T25 tissue culture flasks and 24 h later exposed to 100 cGy. Apoptosis was determined flow cytometrically by Annexin-V-FITC and PI staining (Apopotarget, Biosource International). As described previously, early apoptosis is defined as Annexin V+/PI− staining (21). A daily time course following irradiation showed these cells to achieve maximum apoptosis at day 14 (>40%), these cells remained negative for trypan blue staining. Necrosis was induced by heating the cells at 50°C for 30 min (26), as evidenced microscopically by 100% staining with trypan blue. NR live tumor cells were obtained by mitomycin-C treatment (Sigma, Dorset, United Kingdom; Ref. 27).

Phagocytosis of Apoptotic Cells. HLA-A2+ uveal melanoma cells (Mel 157d) were dyed red using PKH26 (Sigma, Bioscience) and induced to undergo apoptosis by irradiation. These were cocultured with HLA-A2+ DCs stained for MHC class II (FITC-conjugated) and CD54 (Serotec, Oxford, United Kingdom) at a ratio of 1:1 (28). The DCs were also cocultured with PKH26-labeled necrotic and NR Mel 157d tumor cells at the same ratio. To determine phagocytosis, FACSscan analysis was performed at various time points. To discriminate DC-tumor cell conjugation from phagocytosis, double positive populations were sorted using a FACSBlend (Becton Dickinson) and further examined by electron microscopy (Philips) and fluorescent microscopy (Lablux 12; Leitz).

Proliferation Assay. Proliferation assays were performed to test the immunostimulatory ability of the DCs cocultured with different preparations of melanoma cells. DCs (2 × 10^4/ml) from HLA-A2+ donors were irradiated (3000 rad) and serially diluted into 96-well microtiter plates (CoStar) with HLA-A2 apototic, necrotic, or NR Mel 157d tumor cells for a minimum of 1 h. Subsequently, the cryopreserved autologous nonadherent cells were thawed, washed, and added at 1 × 10^5 cells/well in a final volume of 200 µl/well. Cells were incubated for 5 days and then pulsed with 5 µCi of [3H]thymidine for 18 h. Cells were harvested and counted, and proliferation was determined by [3H]thymidine incorporation using a Top Count scintillation counter (Canberra Packard). Where T cell subsets were used as responder lymphocytes, the nonadherent cells were negatively enriched for CD3, CD4, and CD8 cells by immunomagnetic separation (Dynal, Wirral, United Kingdom) according to the manufacturer’s instructions; cells were routinely found to have >98% purity (FACSscan).

Induction of CTLs. Nonadherent cells were enriched for CD8+ cells using anti-CD4 dynabeads (Dynal). These effector cells (2 × 10^5) were then cocultured with 2 × 10^5 autologous DCs and 2 × 10^4 apoptotic, necrotic, or NR 157d tumor cells/well of a 24-well plate, in 2 ml of Aim-V (Life Technologies, Paisley, United Kingdom) containing 10% autologous human plasma. Effector cells were restimulated every 7 days for a total of three cycles. These cells were harvested and added at 10^6 to wells containing irradiated autologous PBMC (2 × 10^5) and the appropriate treated tumor cells (2 × 10^5). On day 28, CTLs were tested for their lytic activity in a standard 51Cr release cytotoxicity assay. Briefly, 10^6 tumor targets were labeled with 5.55 MBq of Na^24CrO_4 for 1 h at 37°C. To the target T2 an equal volume of MA2.1 antibody was added. This is an anti-HLA-A2 monoclonal antibody known to enhance the association of peptide with HLA-A2+ target cells and increase the sensitivity of lysis (29).

The hybridoma cell line producing this antibody was purchased from the American Type Culture Collection. Targets were incubated for an additional h in the presence or absence of 10 µg/ml tyrosinase peptide (YMNGTMSQV). The peptide was synthesized by Alto Bioscience (Birmingham, United Kingdom) and shown to be at least 80% pure using high-performance liquid chromatography and mass spectrometry. Various amounts of effector cells were added to 2 × 10^5 target cells in the presence of 40% excess cold K562 to block nonspecific lysis, in U-bottomed, 96-well microtiter plates. After a 4-h incubation, 50 µl of supernatant was harvested, and its radioactive content was measured. The percentage specific lysis was calculated as follows:

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\text{% Lysis} = \left(\frac{\text{[cpm test]} - \text{[cpm spontaneous]}}{\text{[cpm maximum]} - \text{[cpm spontaneous]}}\right) \times 100
\]

RESULTS

DCs Efficiently Phagocytose Apoptotic Melanoma Cells. Although uveal melanoma cells, like cutaneous melanoma, are relatively radio-resistant (30), it was demonstrated recently that higher single doses of radiation were more effective than fractionated doses at killing the tumor cells (31, 32). Following a method suggested by Logani et al. (33), significant apoptosis of the uveal melanoma cells was achieved. In brief, the Mel 157d tumor cells were plated onto T25 tissue culture flasks; some flasks were exposed to a high single dose of radiation and the remainder served as controls. Apoptosis was confirmed by Annexin V+/PI− staining. A time course revealed that control flasks displayed hardly any apoptosis, whereas the irradiated tumor cells achieved maximal apoptosis at day 14 after irradiation (Fig. 1A). The upper left-hand quadrants of Fig. 1B and C are the Annexin V+/PI− populations on day 14; <6% apoptosis was observed in control cultures and >60% cell death by apoptosis in the irradiated cultures, respectively.

Previous studies have indicated that immature DCs are capable of phagocytosing monocytes induced to apoptose by flu infection (21). We have further demonstrated that DCs can also engulf irradiated melanoma cells undergoing apoptosis. In brief, the melanoma cell line (Mel 157d) was dyed red using PKH-26 and treated with γ radiation to induce apoptosis, heated to 50°C for 30 min to induce necrosis, or mitomycin-C treated to yield NR tumor cells. Day-7 DCs were stained for MHC class II, for which the tumor cells were negative (Fig. 2B, i and ii), and subsequently cocultured with the treated melanoma cells. After 4 h at 37°C, the cocultures were analyzed by FACSscan, enabling the quantification of phagocytosis as demonstrated by double positives (Fig. 2B, iv, vi, and viii). Thirty percent of the irradiated cells,
24% of the necrotic cells, and <11% of the NR tumor cell cocultures displayed double positives. Increasing the ratio of tumor cells:DCs by 2:1 or 4:1, respectively, did not increase the percentage of double positives (data not shown). As a control, the assay was carried out at 4°C, where the low temperature inhibited uptake (Fig. 2, iii, v, and vii). The double positives were sorted using FACSVantage to discriminate between DCs that actually engulfed the tumor cells and those which conjugated to, but did not internalize them. Hence, sorted cells were further analyzed to visually confirm the uptake shown by the FACS. Electron and fluorescent microscopy demonstrated that only intact apoptotic cells were internalized by DCs (Fig. 3). Data not shown clearly indicated that necrotic and NR tumor cells were not phagocytosed, therefore implying that DCs preferentially acquire only the apoptotic material from the irradiated cultures. This is in agreement with other published data (26). Furthermore, uptake of apoptotic cells occurred as early as 1 h after coculture with no difference observed after 24 h (data not shown).

**DCs Pulsed with Apoptotic and Necrotic Melanoma Cells Induced Lymphocyte Proliferation.** To address whether DCs pulsed with melanoma cells have immunostimulatory activity, proliferation assays were performed. HLA-A2+ DCs were used as stimulator cells, and these were cocultured with the treated melanoma cells for a minimum of 1 h, followed by the addition of autologous responder lymphocytes. In all experiments, both apoptotic and necrotic tumor cell sources, when cocultured with DCs, were immunostimulatory even at low responder:stimulator ratios. By contrast, NR tumor cells were unable to stimulate a comparable response (Fig. 4A). It is clear from the controls that the tumor cells were compulsory for proliferation to occur, because the culture of responders and stimulators together or individually yielded little thymidine incorporation. However, the addition of increased numbers of apoptotic, necrotic, or NR live tumor cells to the cocultures did not improve their immunogenicity (data not shown). To further investigate which lymphocytes were involved in the marked proliferative responses, the nonadherent cells were purified into respective CD3, CD4, and CD8 subsets by...
immunomagnetic separation. In cultures containing both apoptotic and necrotic cells, proliferation of CD3 T cells was the most marked. More importantly, proliferation of the CD4 and CD8 subsets was evident only when apoptotic cells were used (Fig. 4B). The increased proliferation demonstrated with necrotic cells using whole lymphocytes or CD3 cells was markedly reduced when CD4$^+$ or CD8$^+$ cells were depleted, indicating that the presence of both these cells is necessary for necrotic cells to be immunostimulatory (Fig. 4C).

**Induction of CTLs Using Apoptotic Cells as a Source of Tumor Antigen.** The fate of antigens contained in the apoptotic cells depends largely on which scavenger cells pick up the antigen. DCs have been shown to induce antigen-specific CTLs when pulsed with apoptotic cells (21, 28). Indeed, we confirm these observations and demonstrate that DCs pulsed with apoptotic melanoma cells yielded potent CTLs (Fig. 5). In **vitro** cultures of DCs, autologous CD8$^+$-enriched lymphocytes, and allogeneic apoptotic tumor cells were established as described in “Materials and Methods.” After three restimulations, CTLs were tested for their cytolytic activity against HLA-matched and -mismatched target cells and an HLA-A2$^+$-restricted tyrosinase peptide known to be naturally processed and presented by tyrosinase-positive melanoma cells. The bulk CTL populations efficiently lysed HLA-A2$^+$ T2 target cells pulsed with tyrosinase peptide, whereas only background lysis was observed in the absence of peptide. In addition, the CTLs displayed strong lytic activity against the uveal melanoma cell line Mel 157d that was used to generate the apoptotic cells originally. In all experiments, killing was enhanced when this cell line was pulsed with tyrosinase. Furthermore the CTLs recognized and lysed the cutaneous melanoma cell line FM3 (HLA-A2$^+$), which is tyrosinase-positive, but not FM79 (HLA-A2$^+$, tyrosinase-positive), indicating that killing is HLA-A2 restricted. Of note, all of the targets were tested for tyrosinase antigen expression by reverse transcription-PCR and immunohistochemical staining (data not shown). In effect, this demonstrates that DCs can present antigen derived from phagocytosed apoptotic cells and stimulate peptide-specific CTLs. These CTLs efficiently lysed peptide-loaded targets as well as the native epitope derived from tyrosinase endogenously processed and presented by HLA-A2$^+$ melanoma targets. The result reproduced in Fig. 5A is representative of six experiments with six donors, each resulting in the same potent response.

We next compared the immunogenicity of apoptotic cells to necrotic and NR tumor cells and consistently found that apoptotic cells were far superior at inducing CTL with cytoltyc activity for tumor cell targets even at low E:T ratios (Fig. 5B). In contrast to the proliferation data, necrotic cells yielded CTLs, which displayed far less lytic activity (Fig. 5C). Antigen from necrotic cells does not gain access to MHC class I (21), therefore excluding that immunogenicity was attributable to soluble antigens released by the dying cells. This is in agreement with other studies (21, 34). Similarly, if the DCs were cocultured with NR tumor cells, the CTLs displayed no significant lytic activity (Fig. 5D).

**DCs Derived from Uveal Melanoma Patients Present Antigen from Apoptotic Cells and Stimulate Proliferative and Cytolytic Responses.** To evaluate whether melanoma patient DCs could elicit a similar immune response as shown for the healthy donors, we next demonstrated that DCs from four HLA-A2$^+$ uveal melanoma patients pulsed with allogeneic apoptotic cells were able to stimulate the proliferation of autologous lymphocytes (Fig. 6). Proliferation in the presence of the apoptotic cells was 8-fold higher than that induced by the responders plus stimulators. Similarly, the proliferation induced by responders and apoptotic cells was only minimal, indicating the importance of APCs as stimulators. Likewise, we have also succeeded in generating CTLs from a number of uveal melanoma patients of various HLA types. In the instance where the patient is HLA-A2$^+$ (patient A, see Fig. 7), the CTLs efficiently lysed the appropriate melanoma targets that were HLA-A2$^+$ and tyrosinase-positive (Mel 157d and, to a lesser extent, FM3). As anticipated, only low levels of killing was evidenced for the lymphoblastoid cell line (IM61), which has a nonmelanoma origin, and the HLA-mismatched cutaneous target, FM79. In a similar manner, CTLs produced from patient B displayed potent lytic activity against both Mel 157d and FM3 at ratios as low as 12:1. Patient B underwent surgical enucleation, after which the ocular melanoma cell line Mel 257 was established and used as a target. Significant killing was detected even at ratios of <6:1; implying that these cells share tumor antigens in common with the 157D cell line. More remarkable is that these CTLs appear to have a higher affinity for autologous targets, although they were raised against antigens derived from allogeneic melanoma cells.

Furthermore, we assessed whether CTLs could be generated from patients of other HLA types. Indeed, we found that DCs from patients who are not A2$^+$ acquire tumor antigen from HLA-mismatched apoptotic cells and stimulate autologous T cells. Patient C expresses HLA-A3, B7, and B44. CTLs generated from this patient specifically lysed the targets FM3 and FM79 only. Both these targets have in common with the CTLs the B44 allele. This suggests that DCs acquire...
antigen from phagocytosed HLA-mismatched apoptotic cells and stimulate MHC class I-restricted CTLs, a phenomenon known as cross-priming.

DISCUSSION

Apoptosis is a major cause of cell death in health and disease (23). Cells undergoing apoptosis are characterized by distinct morphological changes including membrane blebbing, nuclear and cellular shrinkage, and chromatin condensation (35, 36). These changes ultimately lead to their swift recognition and engulfment by scavenger cells. This phagocytic process serves as a mechanism by which the clearance of intact dying cells prevents secondary necrosis of apoptotic cells and the leakage of toxic molecules into the microenvironment (37). Recent data suggests that cells undergoing apoptosis may trigger a specific immune response (38), however the fate of the antigen contained in the apoptotic cells largely depends on the type of scavenger cells involved in its processing and presentation (34). DCs have been shown to efficiently phagocytose apoptotic cells (26, 28) and elicit antigen-specific immune responses (21).

In this report, we demonstrate that uveal melanoma cells, although thought of as traditionally radio-resistant, can be induced to undergo apoptosis by irradiation. We also show that DCs can phagocytose uveal melanoma cells that have undergone apoptosis far more effectively than live tumor cells or cells that were killed by nonapoptotic methods. The immunogenicity of the melanoma cells either dead or alive was determined in proliferative or cytolytic assays. Our data indicate that DCs pulsed with killed melanoma cells elicit greater lymphocyte proliferation than do DCs pulsed with live tumor cells. This suggests that dead melanoma cells provide a heterogeneous mixture of antigens that DCs can internalize and present via both class I and II. However, when the lymphocytes were separated into their corresponding subsets, discrepancies were evident with regard to the type of tumor cell death. In cultures containing necrotic tumor cells, proliferation of T cells required the presence of both CD4+ and CD8+ cells because the separated T cells failed to proliferate. This is not altogether surprising considering both helper (CD4+) and cytolytic (CD8+) T cells contribute to antitumor immunity (39). On the other hand, apoptotic cell-containing cultures synergistically stimulated helper and lytic T cell subsets, verifying that tumor antigens were presented via both classes of MHC molecules. The marked proliferation of CD4+ T cells could be attributable to the high expression of class II antigens on DCs, which potentiates their ability to induce CD4+ T cells. In addition to class II usage, we ascertained whether antigen from the melanoma cells (killed or living) accessed the class I pathway of DCs and stimulated CTLs. When CTL responses were measured to melanoma cells, two striking observations were made: (a) CD8+ T cells developed without the addition of CD4+ helper cells or the presence of exogenous cytokines. This is reminiscent of human alloreactive responses in which CD4+ helper cells are not required for the generation of CD8+ CTLs if DCs are the APCs (40, 41). There are several examples of CTL development in the apparent absence of CD4+ help; e.g., studies have shown that the absence of helper cells

![Fig. 5. DCs pulsed with apoptotic cells are potent inducers of antigen-specific T cells. DCs were cocultured with autologous CD8+ enriched T cells and allogeneic Mel 157d apoptotic cells. After three restimulations, CTLs (effectors) were tested for their lytic activity to chromium-labeled target cells (A). E/T ratio; T: target. Ratios, 100:1, 50:1, and 25:1. Targets included T2 cells loaded with tyrosinase (T2+) or unpulsed T2 cells (T2), Mel 157d cells pulsed with (157d+) or without (157d−) tyrosinase, and cutaneous melanoma cells (FM3 and FM79). In an independent experiment, the cytolytic activity of CTLs from a single donor stimulated with DCs pulsed with apoptotic (B), necrotic (C), or NR tumor cells (D) were also compared. Each experiment reproduced in the figure was repeated at least three times with similar results. Cytotoxicity against a number of targets are shown.](Image 65x104 to 275x226)

![Fig. 6. Proliferation of lymphocytes derived from uveal melanoma patients. Responders were stimulated for 5 days with autologous DCs that were pulsed or unpulsed with HLA-matched allogeneic apoptotic cells. For the control wells we used responder: stimulator ratios of 10:1, the apoptotic cells (1 × 10⁶) were added to 1 × 10⁵ responders (R+S), and to equal numbers of stimulators (S+Apopt). Proliferation as measured by cpm of thymidine uptake is shown. One representative experiment of four is shown.](Image 207x459 to 560x741)
PBMC used for restimulation; and (b) cells to CTLs; presumably the sequestered apoptotic material is demonstrated that although macrophages efficiently phagocytose dying rather than macrophages is supported by recent studies which demonstrate that macrophages can gain access to the cytosol of DCs for subsequent presentation to T cells. The generated CTLs displayed reactivity with HLA-matched targets including tyrosinase-pulsed T2 cells and melanoma cells from both uveal and cutaneous origin naturally expressing this antigen, indicating that tyrosinase is an immunodominant antigen in this system, although other unknown shared tumor antigens are probably processed.

Recent reports have suggested a reduced immunogenicity of apoptotic cells compared with their viable counterparts (34, 45) or necrotic cells (46–48). Certainly, we do not propose that these apoptotic cells are independently immunogenic, and we show that in the presence of just apoptotic cells, or for that matter necrotic or live tumor cells, no T cell stimulation transpires. The immunogenicity of the tumor cells appears to be highly dependent on the APCs involved in the processing and presentation of the antigenic material contained within it. Additional studies have demonstrated that phagocytosis of apoptotic cells by APCs suppresses the induction of inflammatory responses normally observed with necrotic cells (49, 50). These studies use macrophages that vary in their ability to provide the required costimulatory signals to T lymphocytes (27), and antigen presentation without the correct costimuli will normally induce a tolerogenic effect (51). Our findings probably do not conflict with these latter reports, inasmuch as we used DCs as APCs because of their ability to elicit immune responses; moreover, they express the costimulatory signals required for T cell activation. The use of DCs rather than macrophages is supported by recent studies which demonstrate that although macrophages efficiently phagocytose dying cells, they were unable to cross-present antigens from within those cells to CTLs; presumably the sequestered apoptotic material is degraded (21, 28). Similarly Ronchetti et al. showed that in a mouse model immunization with DCs, but not with macrophages pulsed with apoptotic tumor cells, primes tumor-specific CTLs and confers protection against a tumor challenge. In contrast to our study, Gallucci et al. demonstrate that in their hands necrotic fibroblasts cocultured with DCs stimulate proliferation of a CD4+ T cell clone, whereas living or apoptotic fibroblasts do not. Indeed, our studies support the fact that necrotic cells induce lymphocyte proliferation, however, in our system apoptotic cells were superior stimuli. Furthermore, although they show priming of naive T cells by measuring delayed-type hypersensitivity to mice immunized with either apoptotic, necrotic, or live fibroblasts, they do not present data relating to CD8+ cell proliferation or cytolytic activity. However it is possible that a different process is being studied in our system, because the DC origin, its state of activation, and the antigenic source we use are different. Similarly, discrepancies could be attributable to differences in our methods, e.g., to induce apoptosis we use irradiation as opposed to drug treatment to instigate necrosis, and cells were heat treated rather than being frozen and thawed. Thus in our system, apoptosis and not necrosis of melanoma cells is required for the generation and packaging of antigenic material for delivery to DCs and subsequent presentation to CD8+ CTLs.

In addition, we found that uveal melanoma patient-derived DCs are indiscriminate of the tumor source because they can acquire and cross-present antigen from allogeneic HLA-matched or HLA-mismatched tumor cells. Although we used uveal melanoma as a model in this system, our observations may be applied to other tumor systems, and on the basis of these data the following scenario could be proposed: (a) cross-priming could be an effective antigen-loading strategy in DC vaccines, e.g., melanoma-specific CTL responses may be inducible by pulsing patient-derived DCs with apoptotic melanoma cells. Moreover, if tumor is removed from the patients, this can serve as an autologous source of apoptotic cells capable of stimulating immunity against unique antigens expressed by the tumor that may be an important component of an effective immune response; (b) current cancer therapies often result in tumor cell apoptosis. If these are followed by immunization with cultured DCs, this could augment the primary treatments; and (c) by adapting this system, DCs could also be used to generate large numbers of CD8+ CTLs for adoptive transfer into immunosuppressed patients. Immunotherapy with CD8+ CTLs has been shown to amplify the immune response (42); bone marrow transplant recipients given CMV-specific CTLs by adoptive transfer do not develop the disease (52). There is concern that DCs may not become activated after phagocytosis of apoptotic cells, therefore inducing T-cell tolerance (53). Other studies have shown that apoptotic cells can, in fact, trigger DC activation (54). Moreover, from a therapeutic perspective, this could be avoided by using adjuvants found in vaccine formulations (55) because these lead to effective APC activation and have the potential to convert T-cell tolerance to T-cell priming. The novel approaches described represent the potential role of DCs as adjuvants for cancer immunotherapy.

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