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Dendritic Cells Containing Apoptotic Melanoma Cells Prime Human CD8$^+$ T Cells for Efficient Tumor Cell Lysis[1]

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ABSTRACT

Dendritic cells (DCs) phagocytose apoptotic influenza-infected monocytes and cross-present influenza antigen to CD8$^+$ T cells, generating a specific CTL response. We investigated whether apoptotic melanoma cells, presented by this mechanism, can lead to CTL responses to tumor-associated antigens and melanoma cells. Apoptotic HLA-A2$^-$ MEL-397 melanoma cells were internalized by HLA-A2$^+$ immature monocyte-derived DCs but failed to induce maturation of DCs. When exposed to interleukin 6, interleukin 1β, tumor necrosis factor α, and prostaglandin E_2, DCs containing apoptotic MEL-397 cell material matured normally [cross-presenting DCs (cp-DCs)]. Autologous CD8$^+$ CTL lines generated with cp-DCs produced tumor necrosis factor when stimulated with HLA-A2-binding immunodominant peptides from MelanA/MART1 and MAGE-3 (expressed by MEL-397 cells) but not tyrosinase (absent in MEL-397). T2 target cells loaded with the respective peptides were lysed by these cell lines, although to a lesser extent than by CTL lines generated in the presence of mature DCs and peptides from melanoma-associated antigens. In contrast, lines generated with cp-DCs lysed HLA-A2$^+$ MEL-526 melanoma cells or allogenic HLA-A2$^+$ cp-DCs efficiently, whereas the CTL generated with DCs and peptides had little lytic activity. Mature DCs containing apoptotic tumor cells may thus represent an alternative approach for the therapy of malignant tumors.

INTRODUCTION

During the past few years, several melanoma-associated antigens have been identified that can be recognized by tumor-infiltrating lymphocytes and CTLs (1). For melanoma, a large number of tumor-associated antigens has been identified, and the HLA restriction of their immunodominant T cell epitopes has been defined (2, 3). Using a staining tool consisting of tetrameric MHC class I/immunodominant peptide complexes, antigen-specific CTLs are detectable in large numbers in lymph nodes of melanoma patients (4). CD8$^+$ T cell precursors specific for tumor associated antigens have also been reported to be present in the blood of healthy donors (5). Strategies to increase and activate this cellular population in patients appear promising for the immunological treatment of tumors.

DCs[3] play a pivotal role in the initiation of T cell-dependent immune responses (6) and can be obtained by culturing peripheral blood monocytes in the presence of GM-CSF and IL-4 (7, 8). Antigen loading can be performed by pulsing DCs with synthetic immunodominant peptides from identified antigens, as recently reported for prostate cancer (9), carcinoembryonic antigen expressing tumors (10), and cutaneous malignant melanoma (11). Disadvantages of this approach include the uncertainty regarding the longevity of antigen expression (12), the need to determine the patient's HLA haplotype, the unavailability of peptides for all HLA haplotypes, and the lack of CD4 helper cell-related epitopes for most antigens. In addition, the CTLs resulting from such protocols have a good *in vitro* capacity for killing peptide-pulsed target cells but only a modest capacity for killing tumor cells (13).

Other methods for antigen loading include whole tumor cell preparations, *i.e.*, tumor lysates. Exogenous antigen is not presented solely by MHC class II antigens but also can gain access to the antigen processing pathway for presentation by MHC class I molecules (cross-presentation; for review see (14). Recently, the capacity of DCs to take up apoptotic cell material using either the vitronectin receptor $\alpha v\beta 3$ (15) or $\alpha v\beta 5$ and CD36 (16) was demonstrated. Albert *et al.* (17) showed efficient presentation of influenza antigen by DCs that had phagocytosed infected monocytes that were apoptotic. They showed influenza antigen presentation to T cells in the context of MHC class I (cross-priming). Furthermore, it was also shown that MHC class II restricted presentation of antigen from apoptotic cells by DCs is efficient (18).

We performed this study to determine whether DCs may prime for tumor-specific CTL responses after uptake of apoptotic melanoma cells. To this end, we first studied the uptake of apoptotic melanoma cells by immature monocyte-derived DCs. DCs were then matured (cp-DCs) and cultured with autologous CD8$^+$ T cells and IL-2 to generate T cell lines. In parallel, we generated T cell lines using DCs loaded with immunodominant peptides from melanoma-associated antigens. We compared the ability of the CTL lines generated by both methods to release TNF in response to the immunodominant TAA peptides and to kill target cells loaded with peptide. We further compared their ability to kill melanoma cells and allogenic cp-DCs. We demonstrate the generation of TAA-specific T cell lines by DCs loaded with apoptotic melanoma cells. Furthermore, CTL lines generated by this method are more potent in killing melanoma cells than CTL lines generated by peptide-loaded DCs.

MATERIALS AND METHODS

Cell Lines, Culture Media, and Peptides. Two melanoma cell lines, MEL-397 (HLA: A1, A10, B8, and B62) and MEL-526 (HLA: A2, A3, B50, and B62), were kindly provided by Dr. M. T. Lotze (University of Pittsburgh, Pittsburgh, PA). MEL-397 expresses MelanA/MART1, MAGE-3, and gp-100, and MEL-526 expresses MelanA/MART1, tyrosinase, MAGE-3, and gp-100 (19). They were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FCS (Life Technologies, Inc., Bale, Switzerland). Both cell lines were subcultured every 4 days after treatment with trypsin-EDTA (Life Technologies, Inc.). The TAP-deficient, HLA-A2-positive cell line T2 (ATCC no. CRL-1992; kindly provided by Dr. P. Romero, Ludwig Institute for Cancer Research, Lausanne, Switzerland) was cultured in the same medium. The natural killer cell-sensitive line K562 (kindly provided by Dr. E. Roosnek, University of Geneva, Geneva, Switzerland) was also cultured in the medium described above. HLA-A2-restricted immunodominant peptides corresponding to residues 27–35 (AAGIGILTV) from the MelanA/MART1 tumor associated antigen (20), to residues 369–377 (YMNGTMSQV) from tyrosinase (21), and to residues 271–279 (FLWGPRALV) from the MAGE-3 tumor antigen (13) were

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[3] The abbreviations used are: DC, dendritic cell; cp-DC, cross-presenting DC; TNF, tumor necrosis factor; IL, interleukin; PGE_2, prostaglandin E_2; GM-CSF, granulocyte-macrophage colony-stimulating factor; mAb, monoclonal antibody; 7-AAD, 7-amino-actinomycin D; PBMC, peripheral blood mononuclear cell.

synthesized and purified by HPLC to >95% purity (Department of Biochemistry, University of Lausanne, Lausanne, Switzerland).

Antibodies and Reagents. The following mAbs were used. FITC-labeled murine CD80 (BB1), CD40 (5C3), HLA-DR (G46–6), and HLA-ABC (G46–2.6) mAbs were purchased from PharMingen (Hamburg, Germany), and CD8 (DK25) mAb was purchased from DAKO Diagnostika GmbH (Hamburg, Germany). PE-conjugated murine CD86 (IT2.2) mAb was from PharMingen; CD83 (Hb15a) mAb was from Immunotech (Marseilles, France); CD4 (MT310) and CD16 (DJ130c) mAbs were from DAKO Diagnostika GmbH; and CD14 (MO-P9), CD19 (Leu-12), and CD3 (SK7) mAbs were from Becton Dickinson (Heidelberg, Germany). Purified control IgG1-PE was purchased from DAKO Diagnostika GmbH, and IgG2b-PE, IgG1-FITC, and IgG2b-FITC mAbs were from PharMingen.

Flow Cytometric Analysis. Cultured cells were washed; suspended at 3×10^5/ml in 50 μl of cold PBS, 0.1% sodium azide, 10 mg/ml BSA, and 200 μg/ml mouse IgG (Sigma); and incubated for 10 min on ice. Subsequent staining with labeled mAb or appropriate isotypic controls was performed for 30 min. Cells were then washed and resuspended in 300 μl of cold PBS, 1% human serum albumin containing 10 μg/ml 7-AAD (Sigma). Stained cells were analyzed for three-color immunofluorescence with a FACScalibur cell analyzer (Becton Dickinson, Mountain View, CA). Cell debris was eliminated from the analysis using a gate on forward and side scatter. A life gate was set using 7-AAD. At least 10^4 cells were analyzed for each sample. Results were processed using Cellquest software (Becton Dickinson).

DC Generation from Buffy Coats. Buffy coats of HLA-A2$^+$ healthy donors were obtained according to institutional guidelines. PBMCs were prepared by density centrifugation using Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden). PBMCs were resuspended (15×10^6 cells/well) in 6-well plates (Nunc, Roskilde, Denmark) and incubated for 1 h at 37°C. Nonadherent cells were removed and the remaining cells were fed with 3 ml of X-VIVO 15 medium (Bio-Whittaker, Walkersville, MD) containing 1% of heat-inactivated autologous plasma, 10^3 IU of GM-CSF/ml, and 10^3 IU of IL-4/ml (Stratagen, Hannover, Germany). Cells were refed with 0.5 ml of fresh medium containing 10^3 IU of GM-CSF and 10^3 IU of IL-4 per ml on days 2, 4, and 6. On day 7, the nonadherent cells were transferred to a new well with fresh medium. DC maturation was induced with a cocktail of cytokines as recently published (8). The following cytokines were added: IL-4, 1000 units/ml; IL-1β, 10 ng/ml; IL-6, 1000 units/ml (all from Stratagene); GM-CSF, 1000 units/ml (Leukomax, Novartis, Basel, Switzerland, kindly provided by Dr. P-Y. Dietrich, University of Geneva); PGE_2, 1 μg/ml (Prostin, Amersham Pharmacia Biotech); and TNF-α, 10 ng/ml (kindly provided by Dr. J-M. Dayer, University of Geneva). Cells were harvested after 2 days and used for flow cytometric analysis and/or culture with T cells. To have fresh autologous DCs at each time point of restimulation of cultured CD8$^+$ T cells, PBMCs were frozen in 10% DMSO (Fluka, Buchs, Switzerland) and 90% human serum albumin (Blutspendedienst SRK, Bern, Switzerland).

Induction of Apoptosis in MEL-397 Cells and Uptake of Apoptotic Cell Material by DCs. To induce apoptosis, MEL-397 cells were irradiated with UV-B (Philips UV, Philips, the Netherlands). After irradiation, MEL-397 cells were kept for 8 h in culture to allow apoptosis to occur. Apoptosis was measured using an annexin-V kit (PharMingen) and 7-AAD staining (22). The UV-B dose necessary to induce apoptosis in 70% of the melanoma cells 8 h after irradiation was calculated to be 6 J/cm^2. The uptake of apoptotic MEL-397 cell material by immature DCs was measured as described recently (16). Briefly, MEL-397 cells were labeled with PKH-26 dye (Sigma-Aldrich, Steinheim, Germany). Apoptotic cell material was then incubated with immature (day 7) DCs stained with PKH-67 (Sigma) at a 1:1 ratio for various periods of time. Flow cytometric analysis of samples from these cultures was performed using FL-1 (green fluorescence, PKH-67) for detection of DCs and FL-2 (red, PKH-26) for the detection of tumor cells.

Bioassay for TNF Quantification. A subclone (WEHI 1.14) of the TNF-sensitive WEHI 164 clone was used as described (23) with the following modifications. Fifty μl of graded dilutions from culture supernatant were added to 50 μl (2×10^4) WEHI 1.14 cells in flat-bottomed 96-well plates (Nunc) in duplicates and incubated for 24 h at 37°C. Twenty μl of MTS (333 μg/ml) (Promega, Madison, WI) and 1 μl of the electron coupling reagent phenazine methosulfate (25 μM; Sigma) were subsequently added to each well. After 2 h of incubation, the resulting intensity of the coloration was measured at 490 nm in a Thermomax microplate reader (Molecular Devices, Menlo Park, CA) and analyzed using Softmax software from the same company. Recombinant human TNF-α (kindly provided by Dr. J-M. Dayer, University of Geneva) was used as a standard. The sensitivity of this assay was 0.1 pg/ml. This assay does not distinguish TNF-α from lymphotoxin. Thus, the resulting activity is referred to as TNF.

RNase Protection Assay. Total RNA was extracted from DCs or MEL-397 melanoma cells using TRizol reagent (Life Technologies, Inc.). Multiprobe template set hCK2 (containing DNA templates for IL-12 p35, IL-12 p40, IL-10, IL-1α, IL-1β, IL-1RA, IL-6, IFN-γ, L32, and GAPDH) was purchased from PharMingen. The pSP64-hTNF plasmid (kindly provided by Dr. V. Jongeneel, Ludwig Institute of Cancer Research) was linearized with *Eco*RI and used to synthesize with SP6 RNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany), an antisense riboprobe encoding for human TNF-α. hCK2 DNA templates were used to synthesize the [α-^{32}P]UTP (800 Ci/mmol, 5 mCi/ml, Hartmann Analytic, Braunschweig, Germany)-labeled riboprobes in the presence of rNTPs using T7 RNA polymerase (Promega). Hybridization with 2.5 μg of each target RNA was performed overnight at 56°C followed by digestion with RNases A and T1 at 37°C for 30 min. The samples were then treated with a proteinase K/SDS mixture, extracted with phenol:chloroform:isoamylalcohol (25:24:1), and precipitated in the presence of carrier *Escherichia coli* MRE 600 tRNA (Roche Molecular Biochemicals). The samples were loaded onto an acrylamide-urea sequencing gel next to the labeled control probes, which had been treated with RNases A and T1 or left untreated, and run at 50 W for 1.5 h. The gel was absorbed to filter paper, dried under vacuum, and exposed on Kodak X-AR film with intensifying screens at –80°C.

The relative amounts of proinflammatory cytokine encoding mRNA were measured by scanning densitometry (Molecular Dynamics, Sunnyvale, CA) on subexposed autoradiograms, and further normalized using both housekeeping gene values (L32 and GAPDH).

T Cell Isolation and Cultures. PBMCs from allogenic buffy coats were allowed to adhere to plastic for 1 h at 37°C and then passed through a nylon wool column (Biotest, Dreieich, Germany). T cells (1×10^5 cells/well) were cultured in the presence of graded numbers of irradiated (3000 rad, ^{137}Cs source) DCs. Cells were cultured for 5 days in round-bottomed 96-well plates in 200 μl of RPMI 1640 supplemented with L-glutamine and penicillin and containing 5% heat-inactivated human AB$^+$ serum (Blood Transfusion Center, Annemasse, France). Proliferation was assessed by tritiated thymidine incorporation in the last 8–12 h of culture. All conditions were set up in triplicate.

Autologous CD8$^+$ T cells were isolated from nonadherent PBMCs using magnetic bead-conjugated mouse anti-human CD8$^+$ mAb (Miltenyi, Bergisch Gladbach, Germany), a MACS column for positive selection (VS), and a vario-MACS magnet according to the manufacturer's instructions. The purified cells contained 96–99% CD8$^+$ cells as assessed by flow cytometry.

Autologous CD8$^+$ cells (1.5×10^6) were added to 5×10^4 mature autologous DCs or cp-DCs and cultured in 1.5 ml/well X-VIVO 15 medium supplemented with 1% autologous plasma in 24-well plates. IL-2 was added in a concentration of 40 IU/ml at days 1, 4, and 7. The three peptides described above were added at 10^{-5} M when indicated. Nine days later, CD8$^+$ cells were restimulated under identical conditions using fresh generated autologous DCs. CTL lines were used after two cycles of stimulation for TNF assays and after three or four cycles of restimulation for CTL assays.

CTL Assay. MEL-526 cells, cp-DCs or T2 cells (10^6) were labeled with 60–100 μCi of $Na^{51}CrO_4$ (Amersham Pharmacia Biotech, Buckinghamshire, England) in 200 μl of serum-free RPMI for 1 h at 37°C. When T2 cells were used, a 1×10^{-5} M concentration of peptide was added 1 h prior to the labeling. After three washes, labeled cells were incubated with graded numbers of effector cells in round-bottomed 96-well plates. After 4 h, the supernatants were harvested and counted in an Isomedic gamma counter (Wallac, Turku, Finland). The mean of triplicate samples was calculated, and the percentage of specific ^{51}Cr release was determined according to the following equation: % specific ^{51}Cr release = 100 × (experimental ^{51}Cr release – spontaneous release)/(maximum ^{51}Cr release – spontaneous release).

Statistical Analysis. Statistical analysis was done with Wilcoxon's signed rank test.

RESULTS

Apoptotic MEL-397 Cells Are Efficiently Taken up by Immature DCs. MEL-397 cells were labeled with PKH-26 and UV-B irradiated (6 J/cm^2) to induce apoptosis. Cells were kept for 8 h in culture to permit apoptosis to occur and then added to an equal number of PKH-67 labeled immature DCs. The labeling with these two dyes allowed the simultaneous detection of DCs and MEL-397 cells by flow cytometry. MEL-397 cells were also labeled with 7-AAD before flow cytometry to detect apoptotic cells. The percentage of 7-AAD$^+$ MEL-397 cells was around 70% 8 h after irradiation but increased to 98% (SD: 2.2%) after 48 h, as measured in five independent experiments. The uptake of PKH-26$^+$ tumor cells by the DCs resulted in a double-positive fraction (Fig. 1). Very few double-positive cells were detected immediately after adding apoptotic MEL-397 cells to DCs. Most of the DCs and MEL-397 cells stained double-positive after 18 h. Additional incubation for a total of 48 h only slightly increased the percentage of double-positive cells. Internalization of MEL-397 cells by DCs was confirmed microscopically (not shown). No double-positive cells were observed in cultures kept on ice. Together, these results indicate that immature DCs can efficiently internalize cell material from apoptotic MEL-397 cells, presumably by phagocytosis as shown previously with other cells (16).

Apoptotic Tumor Cells Do Not Induce DC Maturation. We next studied the effect of apoptotic melanoma cell material on immature DCs. Steady state mRNA levels encoding for proinflammatory cytokines (TNF-α, IL-1α, IL-1β, IL-1RA, and IL-6) were measured by RNase protection assay 1 and 2 h after culturing immature DCs with or without apoptotic MEL-397 cells (Fig. 2*a*). The relative amount of proinflammatory cytokine mRNA induced by apoptotic cells was lower than by stimulation of immature DCs with exogenous TNF-α. To see whether the small induction of TNF-α mRNA induced by apoptotic MEL-397 cells was accompanied by release of protein, we measured bioactive TNF in the 48 h supernatant of these cultures. Induction of TNF protein induced by apoptotic MEL-397 cells was measurable and significant (Fig. 2*b*).

We further investigated the modulation of relevant surface markers known to be up-regulated upon DC maturation. Two days after adding apoptotic MEL-397 cells to immature DCs, the surface expression of CD83, CD86, CD80, CD40, and MHC class I and II was not altered (three experiments; data not shown).

We next tested the effect of apoptotic cell material on the capacity of DCs to stimulate allogenic T cells. DCs cultured with apoptotic MEL-397 cells for 2 days induced T cell proliferation comparable to that of immature control DCs (data not shown).

Together, these data suggest that the presence or the uptake of apoptotic MEL-397 cells does not induce features of DC maturation, with the exception of moderate induction of some inflammatory cytokines.

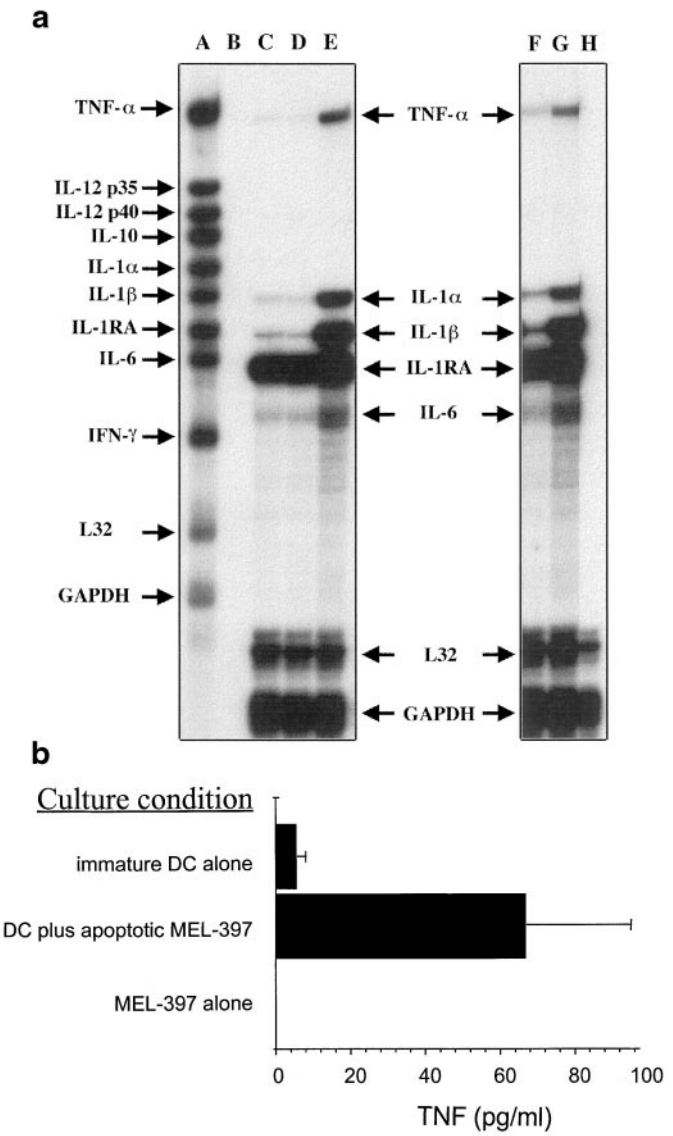

Fig. 2. Apoptotic cell material induces a minor induction of inflammatory cytokines in immature DCs. *a*, RNase protection assay for proinflammatory cytokines in immature DCs incubated with TNF-α for 1 h (*E*), with apoptotic MEL-397 cells for 1 (*F*) or 2 h (*G*), or immature DCs incubated alone for 1 (*C*) or 2 h (*D*). hCK2 multiprobe template set was left untreated (*A*), or treated with RNases A and T1 (*B*). Apoptotic MEL-397 cells were also cultured for 1 h in the presence of TNF-α (*H*). One representative experiment is shown. *b*, bioactive TNF was determined in the 48 h supernatant of immature DCs incubated for 2 days with apoptotic MEL-397 cells. One representative experiment of four is shown. The data represent mean of triplicates ± SD.

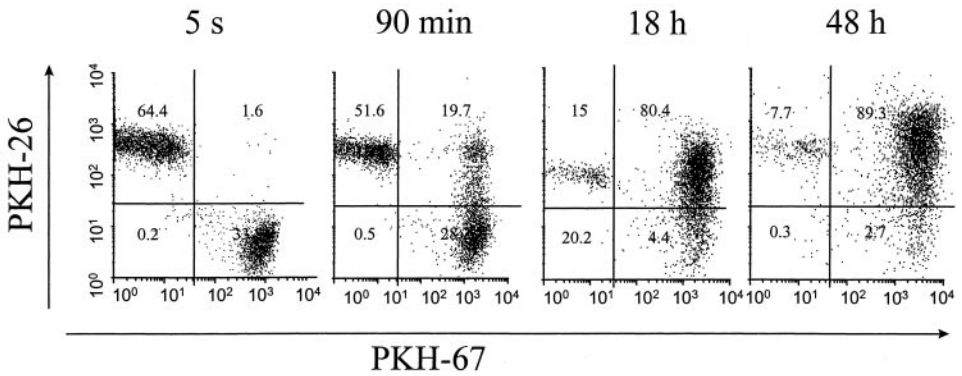

Fig. 1. Immature DCs take up apoptotic MEL-397 cells. Mel-397 cells were labeled with PKH-26 prior to UV-B irradiation and kept for 8 h in culture to let apoptosis occur. Immature DCs were stained with PKH-67. After the incubation of DCs with MEL-397 material, flow cytometry was performed at the indicated time points. One representative experiment of three with similar results is shown. No uptake of apoptotic MEL-397 cells was detectable when the incubation was performed on ice.

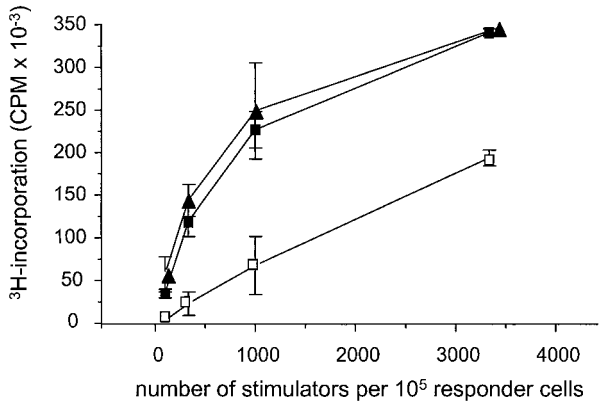

Fig. 3. The increase of allostimulatory capacity of immature DCs by maturation factors is not inhibited by apoptotic MEL-397 cells. Immature DCs containing (■) or not containing (▲) apoptotic MEL-397 cells were exposed to the maturation inducing cytokines TNF-α, IL-1β, IL-6, and PGE_2 for 2 days and subsequently used to stimulate allogenic T cell proliferation. T cell proliferation was measured by [^{3}H]thymidine incorporation after 5 days. Immature DCs not containing apoptotic cells served as control (□). The data represent mean of triplicates ± SD. One representative experiment of five with similar results is shown. In all experiments, the proliferation of T cells without DCs was below 600 cpm.

Characterization of cp-DCs. Because apoptotic MEL-397 cells did not induce maturation in DCs, we attempted to achieve this by adding a mixture of maturation factors. We exposed DCs containing or not containing apoptotic MEL-397 cells for 2 days to IL-1β, TNF-α, IL-6, and PGE_2, a mixture previously shown to induce full maturation in DCs (8). DCs incubated with apoptotic MEL-397 cells and subsequently matured are herein referred to as cp-DCs. In three independent experiments, we found no significant difference in the expression of maturation markers, costimulatory molecules, and MHC molecules in cp-DCs as compared to control mature DCs. Mean values were as follows: for CD83, 83.3% (SD, 12.5%) in cp-DCs *versus* 82.7% (SD, 10%) in normal DCs; for CD80, 69.7% (SD, 1.5%) *versus* 73% (SD, 4.6%); for CD86, 90.3% (SD, 6.7%) *versus* 94.5% (SD, 2.3%); for CD40, 54% (SD, 26%) *versus* 70% (SD, 19.3%); for MHC class I, 96% (SD, 2.8%) *versus* 94.2% (SD, 7.4%); and for MHC class II, 97.5% (SD, 1.3%) *versus* 98.6% (SD, 1%).

Furthermore, DCs matured alone or after the uptake of apoptotic MEL-397 cells had a similar capacity to stimulate the proliferation of allogenic T cells, as shown in Fig. 3.

cp-DCs Induce $CD8^+$ Cells That Respond Specifically to Peptides from Melanoma-associated Antigens. We next asked whether cp-DCs also stimulate the growth of autologous $CD8^+$ T cells. We cultured HLA-$A2^+$ cp-DCs with autologous $CD8^+$ cells from healthy donors plus IL-2. In addition, for control purpose, mature DCs and autologous $CD8^+$ T cells were cultured with IL-2 in the presence or absence of a 10^{-5} M concentration of the HLA-A2-restricted immunodominant peptides from the melanoma antigens MelanA/MART1, MAGE-3, or tyrosinase. The latter method has previously been used to generate specific T cell lines (5). Although neither $CD8^+$ T cells alone nor $CD8^+$ T cells cultured in the presence of apoptotic MEL-397 expanded at any time point, the $CD8^+$ cells in the presence of DCs proliferated, regardless of whether the culture contained apoptotic MEL-397 cells or peptides or none of these antigens (data not shown). After three or four cycles of restimulation under condition of the primary culture, the resulting cell lines were 97.5–99% $CD8^+$ cells. No $CD16^+$ or $CD19^+$ cells were identified.

To assess antigen-dependent responses, we incubated the T cell lines after two restimulations with immunodominant peptides from MelanA/MART1, MAGE-3, or tyrosinase. It is important to mention that MEL-397 cells used to prepare cp-DCs express MelanA/MART1 and MAGE-3 but not tyrosinase (19). After 48 h, we measured TNF in the supernatant. As shown in Fig. 4, T cells generated in the presence of peptide plus DCs only produced TNF when the same peptide used for primary culture was added for restimulation but not when unrelated peptides or no peptide was added. The $CD8^+$ cell lines generated in the presence of cp-DCs produced TNF only in presence of MelanA/MART1 and MAGE-3, whereas no TNF was released in the presence of tyrosinase peptide or in the absence of peptide. $CD8^+$ cells primed with DCs in the absence of exogenous peptide or apoptotic MEL-397 cells did not produce TNF. Together, these results demonstrate that $CD8^+$ T cell lines generated with either cp-DCs or DCs plus peptide from healthy individuals release TNF in an antigen specific manner.

cp-DCs Prime Autologous $CD8^+$ T Cells for Specific Killing of Target Cells Pulsed with Peptides Derived from Melanoma-associated Antigens. To see whether the T cell lines exhibit specific CTL activity, we used HLA-$A2^+$ T2 cells as target cells that were loaded with the peptides mentioned above. Because of TAP deficiency, T2 cells express at their surface empty MHC class I that can be loaded with HLA-A2-restricted peptides (24). Three cycles of restimulation with cp-DCs generated T cell lines with CTL activity toward MelanA/MART1 and MAGE-3 peptide-loaded T2 cells, but not T2 alone or T2 loaded with peptide from tyrosinase. At an E:T cell ratio of 60:1, the mean killing rates in five experiments performed with independent blood donors were as follows: MelanA/MART1 peptide-loaded T2 cells, 33.4% (SE, 7.4%); MAGE-3 peptide-loaded targets, 36% (SE, 8.9%); tyrosinase-loaded targets, 6.7% (SE, 0.6%); and unpulsed targets, 12.7% (SE, 4.1%; Fig. 5*a*). The killing of MelanA/MART1 and MAGE-3 peptide-loaded T2 cells was significantly higher compared to tyrosinase-loaded and unpulsed T2 cells ($P \leq 0.01$, Wilcoxon's test). When the natural killer-sensitive cell line K562 was used as target cell, no killing was observed (data not shown). CTL lines generated in the presence of peptide from MelanA/MART1, MAGE-3, or tyrosinase and autologous mature DCs killed T2 cells pulsed with the relevant peptide but not those pulsed with irrelevant peptide (Fig. 5) or no peptide (data not shown). In addition, these T cell lines killed peptide-loaded target cells more efficiently than CTLs primed with cp-DCs in three independent experiments (Fig. 5, *c* and *d*). T cell lines generated with DCs in the absence of exogenous antigen did not kill peptide-loaded or unloaded T2 cells (Fig. 5, *c* and *d*). These results demonstrate as a novel finding that priming of $CD8^+$ T cells with cp-DCs from normal donors can generate CTL lines

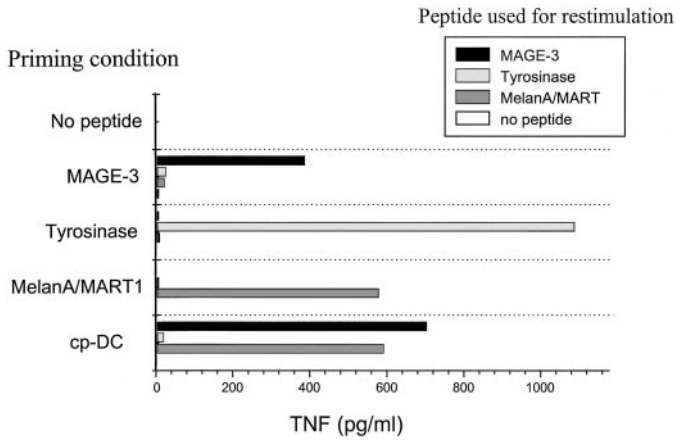

Fig. 4. Antigen-specific TNF production by primed $CD8^+$ CTL lines. After two cycles of restimulation under different priming conditions, the amount of TNF produced by $CD8^+$ T cells incubated with or without 10^{-5} M HLA-A2-binding peptide from melanoma-associated antigens was measured. One experiment of four with similar results is shown. The data are given as mean of duplicates.

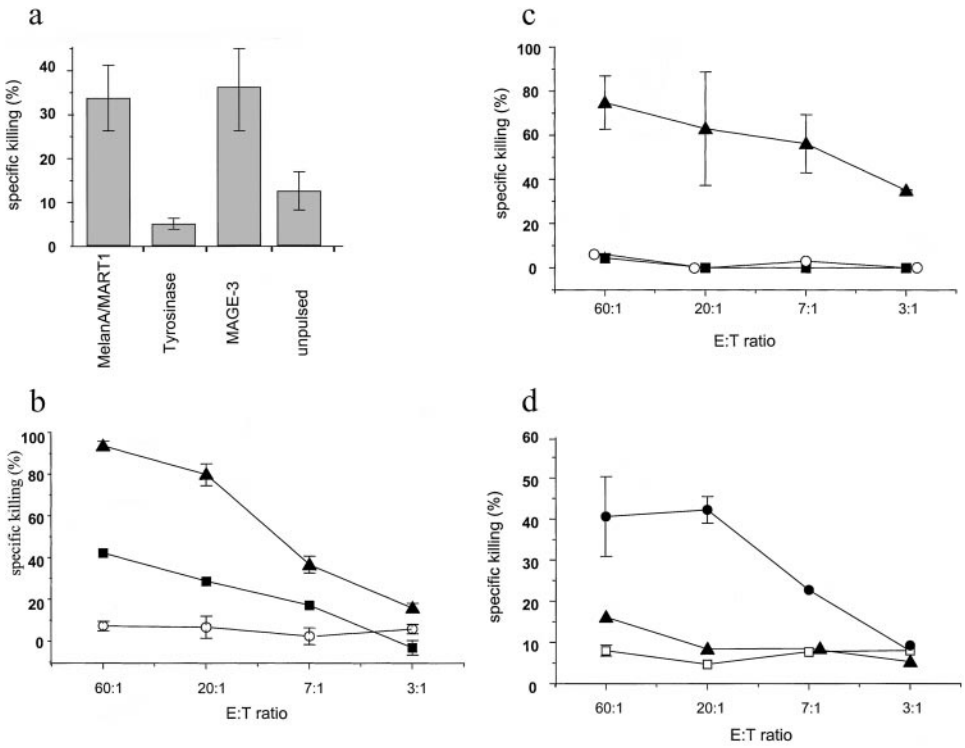

Fig. 5. Specific killing of T2 cells. CTL activity of $CD8^+$ T cell lines after three cycles of restimulation. *a*, T2 target cells were loaded with the indicated peptide, ^{51}Cr labeled, and used as target cells in standard 4 h ^{51}Cr release assays. CTL lines generated with cp-DCs were used as effector cells. The figure shows the medium specific lysis at a E:T ratio of 60:1 for five independent experiments ± SD. *b*, T2 target cells loaded with MelanA/MART1 peptide incubated with CTL lines generated with DCs and MelanA/MART1 peptide (▲), with CTL lines generated with DCs and tyrosinase peptide (○) or with cross-primed CTLs (■). *c*, tyrosinase peptide-loaded T2 cells incubated with CTL lines generated with DCs and tyrosinase peptide (▲), CTL lines generated with DCs but without exogenous peptide (○) and with cross-primed CTLs (■). *d*, MAGE-3 peptide-loaded T2 cells incubated with CTL lines generated with DCs and MAGE-3 peptide (●), with CTL lines generated with DCs but without exogenous peptide (□), and with cross-primed CTLs (▲). The killing of unloaded T2 cells was below 6% for all experiments. Three experiments were performed with similar results. Data are given as mean of triplicates ± SD.

specific for peptides from melanoma-associated antigens expressed by the MEL-397 cells. The results also confirm that peptide from MelanA/MART1, MAGE-3, and tyrosinase can be used to generate CTL lines that lyse peptide-loaded target cells.

cp-DCs Prime Autologous $CD8^+$ T Cells for Killing of HLA-A2-positive MEL 526 Cells. To evaluate the ability of the CTL lines to kill melanoma cells *in vitro*, we used MEL-526 cells as targets. This cell line expresses MelanA/MART1, MAGE-3, and gp-100 as MEL-397 cells plus tyrosinase but is HLA-A2 positive (19). HLA-A2-negative MEL-397 cells served as negative control. Again, we compared the killing by the cell lines generated by the two methods (three or four restimulation cycles). In nine experiments from nine independent donors, cross-primed CTL lines were able to lyse MEL-526 cells but not MEL-397 cells. Fig. 6 shows a representative experiment. The average percentage of MEL-526 cell lysis was 31.7% (SE, 8.2%) at an E:T cell ratio of 60:1. The average lysis of MEL-397 cells at the 60:1 E:T cell ratio was 6.4% (SE, 2.5%). The difference in the lysis of MEL-526 and MEL-397 cells was statistically significant ($P < 0.01$, Wilcoxon's test). CTL lines generated with DCs and peptide from MelanA/MART1 or MAGE-3 were significantly less efficient in killing MEL-526 cells (6.9 ± 2.3%) ($P < 0.01$, Wilcoxon' s test). The T cell lines generated with DCs alone lysed neither MEL-526 nor MEL-397 cells.

To give additional evidence that CTLs primed with cp-DCs can recognize melanoma-derived antigens in a MHC class I-restricted manner, allogenic HLA-A2-positive and -negative cp-DCs were used as target cells to test CTL lines (three cycles of restimulation). CTLs generated in the presence of cp-DCs killed $HLA\text{-}A2^+$ cp-DCs (Fig. 7*a*) but not $HLA\text{-}A2^+$ DCs without apoptotic MEL-397 cells (Fig. 7*b*). Furthermore, $HLA\text{-}A2^-$ DCs, containing MEL-397 cells or not, were not significantly lysed (data not shown). As observed with MEL target cells, CTLs generated in the presence of DCs plus peptide were not capable of exhibiting significant killing toward cp-DCs (Fig. 7*a*). These results confirm the higher killing capacity of $CD8^+$ T cell lines generated with cp-DCs than with peptide-loaded DCs when antigen is processed and presented naturally in target cells. Furthermore, these results suggest that cp-DCs are capable of inducing CTLs against antigen presented in a MHC class I-restricted manner.

DISCUSSION

We confirmed the uptake of apoptotic cell material by immature DCs with MEL-397 cells and the absence of DC maturation after uptake of these cells. The slight induction of proinflammatory cytokines by apoptotic MEL-397 cell material was, however, neither associated with increased expression of costimulatory molecules nor an enhanced capacity of stimulating allogenic T cells. This finding was recently reported for other transformed human cell lines (25) and for mouse fibroblasts (26). In our experiments, we show that DCs containing apoptotic MEL-397 cells could be matured with a mixture of IL-1β, TNF-α, IL-6, and PGE_2. Maturation in cp-DCs is supported by the surface marker profile and the enhanced stimulation of allogenic T cell proliferation.

Exogenous antigen can be presented by MHC class I molecules, a process termed cross-presentation (14). It was suggested by Bevan (27) that damaged cells could gain access to cross-presentation pathways. In addition, it has been demonstrated that particulate antigens prime more efficiently than soluble molecules for class I-restricted CTL responses (28). It was only recently that the highly efficient presentation of viral antigen after the phagocytosis of influenza-infected, apoptotic macrophages was demonstrated (17). These authors (17) induced CTL recall responses using cp-DCs. Presentation of antigen (ovalbumin) from apoptotic cells in the context of MHC class II has been demonstrated in a mouse model (18). To study whether the uptake of apoptotic cells by DCs can be used for *in vitro* priming of T cells against melanoma we cultured autologous $CD8^+$ cells with $HLA\text{-}A2^+$ DCs that were first allowed to take up apoptotic MEL-397 melanoma cells and then matured with a mixture of maturation factors (cp-DCs). $CD8^+$ cells cultured in the presence of peptide (10^{-5} M) and DCs served as control. $CD4^+$ T cells were

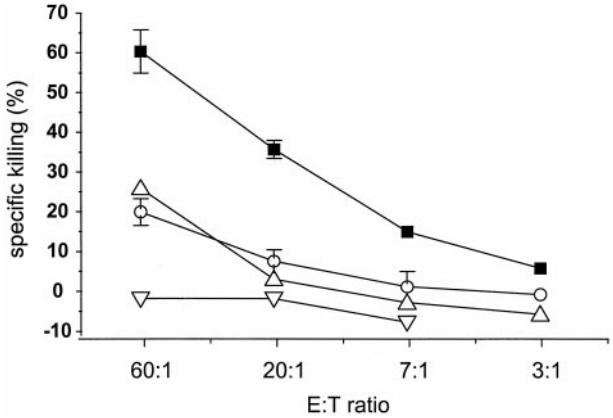

Fig. 6. CTL lines generated with cp-DCs kill a HLA-A2$^+$ melanoma cell line. Shown is CTL activity toward HLA-A2$^+$ MEL-526 target cells incubated with CD8$^+$ T cell lines generated with DCs and MelanA/MART1 peptide (○), CTL lines generated with DCs and MAGE-3 peptide (△), CTL lines generated without peptide (▽), and cross-primed CTL lines (■). The killing of HLA-A2$^-$ MEL-397 cells by the cross-primed CTL line was 18% (SD, 4.3%). Data are given as mean of triplicates ± SD.

eliminated to prevent any suppressor activity and overgrowth by this subset. T cell help was substituted by IL-2. Under other experimental conditions, help from CD4$^+$ T cells may be required for the successful induction of CD8-dependent immunity to exogenous antigen (29). As our mature DCs and cp-DCs expressed high levels of accessory molecules, including CD40, helper factors other than IL-2 from CD4$^+$ T cells were not required.

To investigate whether the cell lines generated by repetitive stimulation can recognize immunodominant peptide epitopes from melanoma-associated antigens expressed by MEL-397 (MelanA/MART1 and MAGE-3), we stimulated the T cell lines with these peptides and measured the TNF response as the first readout. It was not necessary to add exogenous antigen-presenting cells to observe responses by the CD8$^+$ T cell lines. This is not surprising, as activated CD8$^+$ cells were recently described to present peptide efficiently to each other (30). Both cell lines primed with cp-DCs and with DCs plus antigenic peptide exhibited specific responses. It is impressive that T cell lines generated by cp-DCs recognized single peptide epitopes from MelanA/Mart1 or MAGE-3 expressed by MEL-397 cells. From these experiments, it can be concluded that cp-DCs can prime for responses to MelanA/MART1 and MAGE-3. These data, together with the CTL experiments with cp-DCs as target cells, give evidence that DCs can cross-present melanoma antigens to CD8$^+$ T cells. Furthermore, these results show that many healthy individuals have circulating precursors for these antigens in the blood. Their frequency should be $>1.5 \times 10^{-6}$ as we used 1.5×10^6 cells per condition in the starting cultures. This is in the order of magnitude reported for MAGE-3 specific T cells measured by ELISPOT assay (6). Whether the specific cells within our T cell lines derive from naïve or memory cells remains to be established.

The differential killing capacity of CTL lines generated with the two different priming strategies when using peptide-loaded T2 target cells as opposed to MEL-526 cells and cp-DCs was striking. We did not measure the density of immunogenic peptide on the surface of DCs and the different target cells. It is very likely, however, that it was higher on T2 cells loaded with peptide and DCs to which peptide was added than on cp-DCs and MEL-526 cells. It has previously been shown with murine cells that priming with high levels of peptide selects for low-affinity/low-avidity T cells whereas low levels of peptide on antigen-presenting cells select for high-affinity/high-avidity T cells (31, 32). This may explain our results. The high density of peptide used for priming may have selected for low-affinity T cells that killed efficiently only when target cells with high peptide density (*i.e.*, peptide-loaded T2 cells) are used but not when naturally processed and presented antigen is displayed in low density on target cells, such as MEL-526 cells and cp-DCs. Conversely, the low peptide density on cp-DCs may have selected for high-affinity T cells that killed MEL-526 cells and cp-DCs efficiently. CTL lines generated with cp-DCs may have been less efficient to kill T2 cells loaded with a single antigenic peptide than CTL lines generated with DCs and peptide because the former may contain cells specific for several antigens from MEL-397 cells, whereas the latter contain cells with a single relevant specificity. Cross-primed CD8$^+$ T cell lines exhibited clear CTL activity (an average of 25% lysis above negative control) only at high E:T ratios (60: 1). This may be linked to the low density of antigen expressed on target cells (unlike in T2 cells loaded with peptide) and the relative low abundance of high avidity T cells in the effector population. Despite the differential killing activity of T cell lines generated by the two methods, the antigen presenting capacity of both peptide-exposed DCs and cp-DCs was high, because with both methods, lines with antigen specificity could be generated. Our cytotoxicity results therefore suggest that vaccination with mature DCs expressing naturally processed and presented antigen, such as cp-DCs, may be more efficient than vaccination with peptide-pulsed mature DCs. Our findings may thus contribute to the optimization of vaccination strategies using DCs. Transfection of RNA from tumor cells may represent an alternative approach to generate a low and natural density of T cell epitopes on DCs (33, 34).

Another potential advantage of cp-DCs is that determination of

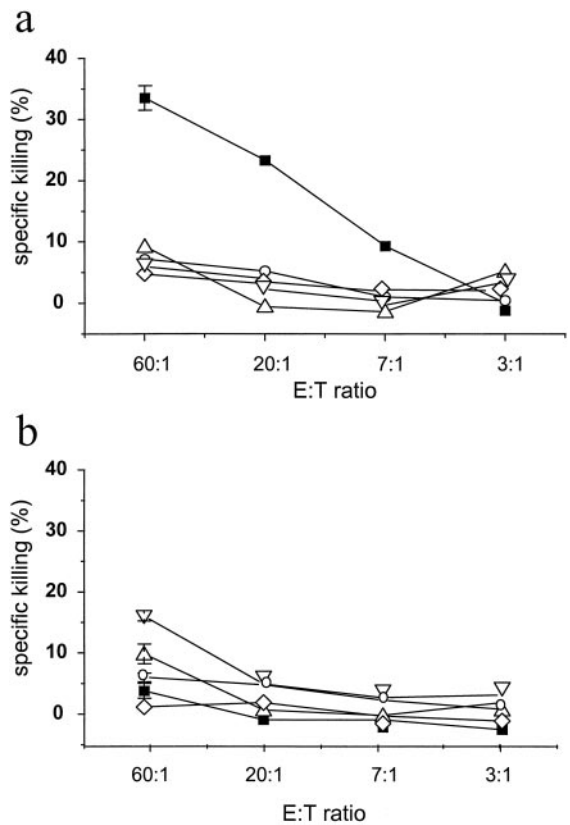

Fig. 7. CTL lines generated with cp-DCs kill allogenic HLA-A2$^+$ cp-DCs. CTL lines generated by three restimulations using cross-primed DCs (■), DCs without peptide (○), DCs and MelanA/MART1 peptide (◇), DCs and tyrosinase peptide (△), or DCs and MAGE-3 peptide (▽) were tested in their lytic activity toward allogenic HLA-A2$^+$ cp-DCs containing MEL-397 cells (*a*) as target cells or HLA-A2$^+$ DCs without MEL-397 cells (*b*). Two experiments were performed with similar results. Data are given as mean of triplicates ± SD.

MHC class I haplotypes is not a prerequisite (as in the peptide approach) because melanoma-associated antigens may also be recognized in the context of haplotypes other than HLA-A2. Furthermore, presentation of apoptotic melanoma cells by DCs has the potential benefit that presentation via MHC class II may generate helper epitopes that support the development of specific CTLs that might be important for antitumoral immunity (35). The induction of autoimmunity could be a potential disadvantage of cp-DCs, as with all whole cell preparations of tumor cells and RNA derived thereof (discussed in Ref. 36).

In conclusion, we have demonstrated that DCs containing apoptotic melanoma cells can efficiently prime autologous $CD8^+$ T cells *in vitro* to give rise to $CD8^+$ T cell lines specific for epitopes from the melanoma-associated antigens MelanA/MART1 and MAGE-3 and that these cell lines killed melanoma target cells more efficiently than cell lines generated with DCs and peptides.

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Dendritic Cells Containing Apoptotic Melanoma Cells Prime Human CD8 $^{+}$ T Cells for Efficient Tumor Cell Lysis

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