Phases of Apoptosis of Melanoma Cells, but not of Normal Melanocytes, Differently Affect Maturation of Myeloid Dendritic Cells

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ABSTRACT

In this study, we investigated whether maturation of monocyte-derived myeloid dendritic cells (DCs) is differentially affected by the uptake of dying human melanoma cells in distinct phases of apoptosis. Maturation of monocyte-derived DCs, as documented by phenotype analysis and T-cell immunostimulatory activity, was inhibited by phagocytosis of dying melanoma cells containing a large fraction of cells in early apoptosis (Annexin-V+ and propidium iodide+) but promoted by the same tumors when in late apoptosis/secondary necrosis (Annexin-V+ and propidium iodide+) or when dying by primary necrosis. These opposite effects on DC maturation were observed after the uptake of early or late apoptotic cells from most vertical growth phase primary tumors and all metastases but not after the uptake of dying cells from a radial growth phase primary tumor or normal adult melanocytes. Inhibition of DC maturation by early apoptotic melanoma cells correlated with expression of interleukin-10 in neoplastic cells and was prevented by preincubating the tumor cells with a neutralizing antibody to interleukin-10 before tumor uptake by DCs. Cross-presentation of the melanoma-associated antigen gp100209–217 to peptide-specific CTLs by HLA-A*0201+ DCs was achieved 48–72 h after phagocytosis of HLA-A*0201+ melanoma cells in apoptosis, or primary necrosis, but only when tumor necrosis factor-α was added to DCs 4 h after the initiation of tumor phagocytosis. These results suggest that phases of apoptosis and neoplastic transformation affect maturation of myeloid DCs that take up dying cells of the melanocyte lineage. However, neoplastic cells in late apoptosis, or even in primary necrosis, induce only a partial DC differentiation not sufficient to achieve cross-presentation of tumor antigens to CTLs unless further DC maturation is promoted by additional signals. These results suggest a novel mechanism of tumor escape that may prevent the development of antitumor immunity through the maturation block induced in DCs by neoplastic cells in the early phase of apoptosis.

INTRODUCTION

The main function of DCs1 is the uptake and processing of soluble or cellular antigens for presentation to T cells (1, 2). DCs can take up soluble antigens such as proteins and immune complexes, internalize exosomes from living cells (3), or phagocytose dying cells. Recognition and uptake of cells dying by apoptosis (4) is regulated by specific receptors including αvβ5, CD36, and the recently identified PS receptor (5, 6). In addition, DCs can phagocytose dying cells by primary necrosis through interaction of the CD91 receptor with heat shock proteins exposed on the surface of the dying cell (7, 8). The uptake of dying cells impacts on the maturation of DCs. Following the hypothesis put forward by Matzinger (9) that the immune system could be activated by internal injury representing a threat to the organism, it was found that maturation of DCs could be promoted by endocytosis of dying normal cells, such as fibroblasts in primary necrosis (10). Even the uptake of dying neoplastic cells can promote DC maturation but only when DCs are exposed to tumor cells dying of necrosis and not of apoptosis (11). This confirmed that professional APCs can adopt distinct differentiation choices depending on the type of cell death affecting the cells that they encounter. As proposed recently by Binder et al. (8), the uptake of apoptotic cells through CD36 and PS receptor may lead to antinflammatory signals, whereas interaction with necrotic cells, through CD91, will lead to activation of innate and adaptive immune mechanisms. A consequence of this model for the initiation of TA-specific immune responses is that TA immunogenicity in vivo may be modulated by the type of neoplastic cell death, in agreement with evidence obtained in murine models (12). On the other hand, as recently argued by Steinman et al. (13), the uptake by DCs of apoptotic cells may not be immunologically “null” but may represent an important mechanism for establishing peripheral tolerance to self; and it is possible that neoplastic cells dying of apoptosis may exploit a similar mechanism to induce tolerance and inhibit the development of TA-specific immunity.

However, additional factors not yet investigated are likely to contribute to the outcome of the DC-tumor interaction. For example, it is not known whether the phase of neoplastic cell apoptosis and neoplastic transformation, or even tumor progression, may impact on the maturation of DCs that phagocytose dying cells. On one hand, several phases have been characterized in programmed cell death (4, 14) from the early step of PS exposure on the cell surface to the later events, defined as secondary necrosis, and characterized by cell membrane damage revealed by using propidium iodide staining (14). Because necrotic tumor cells can promote maturation whereas apoptotic cells cannot, it is possible that the effects of dying tumor cells on DC maturation and function may depend not only on the type of cell death (apoptosis versus necrosis) but even on the phase of tumor cell apoptosis (early apoptosis versus secondary necrosis). On the other hand, neoplastic transformation and tumor progression can be associated with acquisition of immunosuppressive activity. Some of these mechanisms depend on the production of soluble factors such as VEGF, IL-10, and TGF-β1, which can impair DC maturation or function (15–18).

In this study, we investigated whether the uptake of dying cells of the melanocyte lineage can affect myeloid DC maturation, depending on the interplay between the phase of apoptosis and neoplastic transformation/tumor progression. The results suggest that tumor cells in an early phase of apoptosis inhibit DC maturation, whereas cells in late apoptosis or even primary necrosis deliver a partial maturation signal that is not sufficient to achieve TA cross-presentation to CTLs.

MATERIALS AND METHODS

Normal and Neoplastic Cells. Cell lines from VGP primary tumors and metastatic melanomas were established from surgical specimens and maintained in culture as described (19). The WM 35 cell line (20), a RGP primary tumor obtained from Dr. M. Herlyn (The Wistar Institute, Philadelphia, PA), and normal adult human melanocytes from epitherm (Melanopack; Clonetics...
Fig. 1. Induction of melanoma cell apoptosis by serum removal followed by UV-irradiation. Meta-static melanoma cells were seeded in serum-free medium for 24 h and then treated with UV-irradiation. Apoptosis was evaluated in normolirradiated control cultures kept in serum-free medium for 48 h (A), or at 24 h (B) or 48 h (C) after UV-irradiation. In each dot plot, the percentage of Annexin-V⁻/PI⁺ (cells in early apoptosis), Annexin-V⁻/PI⁻ (cells not undergoing apoptosis), Annexin-V⁺/PI⁻ (cells in late apoptosis/secondary necrosis), or Annexin-V⁻/PI⁻ (top right quadrant) is reported.

Corps., San Diego, CA) were cultured as described (20, 21). PHA blasts were obtained from peripheral blood lymphocytes of healthy donors, cultured for 6 days in RPMI 1640 (BioWhittaker Inc., Verviers, Belgium), and supplemented with 1% heat-inactivated pooled human serum in the presence of 1% PHA (Life Technologies, Inc., San Giuliano Milanese, Italy). Fibroblasts were obtained from fragments of normal skin isolated in surgical specimens of primary melanomas and cultured in RPMI 1640 and 10% FCS (BioWhittaker). All of the normal and neoplastic cell cultures were routinely checked for the absence of Mycoplasma contamination by using PCR (Mycoplasma Plus PCR Primer Set; Stratagene, La Jolla, CA).

Induction of Primary Necrosis and Apoptosis in Normal and Neoplastic Cells. Primary necrosis of melanoma cells was induced by freezing cells in liquid N₂ and thawing at room temperature. Apoptosis of normal melanocytes was induced with an overnight culture on plates precoated with 50 μg/ml poly-L-lysine (Sigma-Aldrich, Milan, Italy) in medium without serum, as described (22). Apoptosis of fibroblasts and PHA blasts was induced by using γ-irradiation (50 Gy) from a 137Cs source. Apoptosis of melanoma cells was induced with a culture in RPMI 1640 without FCS for 24–48 h, followed by irradiation with a UV lamp (Philips Electronics, Milan, Italy) calibrated to provide 20 mJ/cm²/s for 20–30 s. Quantification of apoptotic cells was performed with two color flow cytometry analyses using the Annexin-V–FITC Apoptosis Detection Kit I (PharMingen, Becton Dickinson, Milan, Italy; Ref. 21).

Materials and Methods.

Tumor Cell Lysates. Cells were lysed by using four to five cycles of freezing in liquid N₂ and thawing at room temperature. Dead cells and subcellular particles were removed by using centrifugation (10 min at 600 rpm). Protein concentration of the tumor cell lysates was determined by the Bradford method using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). Aliquots (1 ml) of the cell lysates containing 1 mg/ml protein were biotinylated by using the enhanced chemiluminescence protein biotinylation module (Amersham Life Science, Arlington Heights, IL).

Dendritic Cells. Peripheral blood mononuclear cells from healthy donors were isolated by using centrifugation of blood samples on Ficoll gradients (Hypaque; Amersham Pharmacia Biotech AB, Uppsala, Sweden), resuspended in RPMI 1640–10% FCS, and allowed to adhere for 2 h at 37°C in 150-cm² culture flasks (Corning). The adherent fraction (>90% CD14⁻) was used to generate immature myeloid DCs by using culture with hHuGM-CSF (50 ng/ml Mielogen; Schering-Plough, Milan, Italy) and IL-4 (20 ng/ml; Peprotech, Rocky Hill, NJ), as described by Sallusto and Lanzavecchia (23).

Antibodies and Flow Cytometry. The following panel of mAbs was used: anti-CD1a (T6-RD1; Beckman Coulter, Hialeah, FL), anti-CD80 (Becton Dickinson), anti-CD83 (PharMingen, Becton Dickinson), anti-CD86 (Ansell, Bayport, MN), anti-CD14 (Leu M3; Becton Dickinson), anti-CD40 (Caltag, Burlingame, CA), and anti-HLA-DR (Becton Dickinson). In some instances a secondary FITC-labeled F(ab')₂ goat antimouse immunoglobulin (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) was used. Negative

Fig. 2. Immature DCs phagocytose apoptotic and necrotic melanoma cells and melanoma cell lysates. A, day 5, immature DCs were cocultured for 36 h at either 4°C (2 and 4) or 37°C (1 and 5) with PKH2-GL-labeled metastatic melanoma cells in early apoptosis (2 and 3) or primary necrosis (4 and 5) and analyzed by flow cytometry after staining with anti-CD1a. CD1a⁺ cells were gated (1) and analyzed for PKH2-GL-associated green fluorescence. At 37°C, DCs that internalized apoptotic or necrotic melanoma cells were identified as single positive cells because the membranes of live DCs prevented staining of internalized apoptotic or necrotic cells by ethidium bromide (3 and 5, bottom right quadrant). At 4°C, DCs bound but did not internalize apoptotic or necrotic tumor cells and thus appeared as double-positive cells (2 and 4, top right quadrant). B, day 5, immature DCs were cocultured for 4 h at 4°C (1 and 3) or 37°C (2 and 4) with (3 and 4) or without (1 and 2) biotinylated melanoma cell lysates, detected as described in “Materials and Methods.” The insets show a higher magnification of a representative area in each panel.
controls consisted of FITC-conjugated IgG1, phycoerythrin-conjugated IgG1, and IgG2a mAbs specific for irrelevant antigens (Becton Dickinson). Intracytoplasmatic analysis for cytokine expression was performed by using flow cytometry in saponin-permeabilized cells as described (24) using mAbs to human IL-10, VEGF (PeproTech), or TGF-β1 (Serotec Ltd., Kidlington, United Kingdom). All of the flow cytometry analyses were performed by using a FACScalibur instrument (Becton Dickinson).

**Phagocytosis Assay and DC Maturation.** Phagocytosis of apoptotic or necrotic cells by day 5, immature DCs was verified by using flow cytometry (25). Melanoma cells were labeled with PKH2-GL aliphatic green fluoro-

![Fig. 3. Dendritic cell maturation is inhibited by the uptake of metastatic melanoma cells in early apoptosis but promoted by the same tumor cells in late apoptosis/secondary necrosis.](image)

**Table 1. DC maturation after the uptake of metastatic melanoma cells in primary necrosis or in early apoptosis**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Day of DC culture</th>
<th>Tumor Type of tumor cell death</th>
<th>TNF-α</th>
<th>CD80</th>
<th>CD86</th>
<th>CD40</th>
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<td>98 (222)</td>
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<tr>
<td>7</td>
<td>+</td>
<td>Me12657 Primary necrosis</td>
<td></td>
<td>96</td>
<td>89</td>
<td>90</td>
<td>99 (437)</td>
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<tr>
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<td>83</td>
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</tr>
<tr>
<td>2</td>
<td>5</td>
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<td>99</td>
<td>99</td>
<td>86</td>
<td>100 (296)</td>
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<td>7</td>
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<td>64</td>
<td>47</td>
<td>40</td>
<td>96 (177)</td>
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<tr>
<td>7</td>
<td>+</td>
<td>Me15392 Primary necrosis</td>
<td></td>
<td>83</td>
<td>75</td>
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<td>98 (289)</td>
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<td></td>
<td>68</td>
<td>61</td>
<td>61</td>
<td>98 (334)</td>
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* Day 5, immature myeloid DCs were cocultured for 48 h with or without metastatic melanoma cells in either primary necrosis or early apoptosis.

* Primary necrosis was induced by freezing melanoma cells in liquid N2, followed by thawing at room temperature. Tumor apoptosis was induced by using serum deprivation followed by UV-irradiation. Phase of apoptosis of neoplastic cells was monitored as described in the legend to Fig. 1, before coculture with DCs. “Early” apoptosis was defined as the condition where the largest fraction of apoptotic cells showed an Annexin-V−, PI− phenotype.

* TNF-α (20 ng/ml) was added to DC cultures at day 5.

* Expression of CD80, CD86, CD40, and HLA-DR antigens was evaluated on DCs by using flow cytometry.

* Results are expressed as a percentage of positive cells (in parentheses: mean fluorescence intensity on a 4-decade log scale). Bold values represent significant phenotypic changes in comparison with phenotype, at day 7, of DCs cultured alone (Kurtosis statistics, P < 0.01).
Early apoptotic melanoma blocks DC maturation

4. T-cell immunostimulatory activity of DCs is affected by the uptake of early or late apoptotic melanoma cells, necrotic melanoma, or tumor cell lysates. A, a 5-day T-cell proliferation assay was carried out with allogeneic DCs as stimulators. Day 5 immature DCs were cultured for 48 h alone (○), with early (□) or late (△) apoptotic melanoma cells, melanoma lysates (▲), or melanoma cells in primary necrosis induced by freezing and thawing (○). At day 7 all DC cultures were fixed and added to allogeneic T cells. Results are expressed as cpm × 10⁻³. Early and late phase of apoptosis of melanoma cells was defined as in the legend to Fig. 3. T-cell response at DC:lymphocyte ratios of 1:20 and 1:40, when cultured with DCs exposed to melanoma cells in late apoptosis, primary necrosis, or to melanoma cell lysates, is significantly higher than proliferation to DCs kept alone or exposed to early apoptotic tumor cells (p < 0.01, ANOVA followed by SNK test). B, phenotype of DCs used for T-cell proliferation. DCs cultured alone (empty histograms) or after the uptake of early apoptotic (cross-hatched histograms) or late apoptotic melanoma (horizontally hatched histograms), tumor cell lysate (diagonally hatched histograms), or neoplastic cells in primary necrosis induced by freezing/thawing (black histograms).

RESULTS

UV-induced Neoplastic Cell Apoptosis and the Uptake of Dying Cells by DCs. Apoptosis of melanoma cell lines was induced by serum starvation followed by UV-irradiation. Fig. 1B shows that 24 h after UV-irradiation a large fraction of melanoma cells was in early apoptosis, as determined by using staining for Annexin-V, but not for PI, whereas a smaller fraction stained with both Annexin-V and PI. At 48 h after UV-irradiation (Fig. 1, panel C) most cells progressed to late apoptosis/secondary necrosis, as determined by using double staining for Annexin-V and PI. By varying (24–48 h) the length of time of culture without FCS and the interval (18–48 h) between UV-irradiation and analysis of apoptosis, phases of early and late apoptosis were identified in all melanoma cell lines tested (data not shown).

The phagocytic capacity of day 5, monocyte-derived, immature DCs was assessed by evaluating their ability to engulf melanoma cells in early phase of apoptosis, induced by serum starvation and UV-irradiation, or in primary necrosis, obtained by using freezing and thawing. As shown by using cytofluorimetric analysis, in a 36-h coculture experiment 80% of the CD1a⁺ DCs efficiently picked up apoptotic cells at 37°C (Fig. 2A, panel 3), a process that was inhibited at 4°C (Fig. 2A, panel 2). Similar results were obtained after the uptake of tumor cells in primary necrosis (Fig. 2A, panels 4 and 5). Immature DCs were also able to phagocytose melanoma cell lysates at 37°C, as shown by diffuse cytoplasmic staining for the tumor cell lysate (Fig. 2B, panel 4), but not at 4°C (Fig. 2B, panel 2).

DC Maturation Is Inhibited by the Uptake of Early Apoptotic Melanoma Cells, but Promoted by the Same Tumor Cells in Late Apoptosis/Secondary Necrosis or in Primary Necrosis. DC maturation was monitored by evaluating the level of expression of several markers (CD80, CD83, CD86, CD40, and HLA-DR antigens) before and after the uptake of metastatic melanoma cells in early or late phase of apoptosis. DCs not exposed to apoptotic melanoma cells progressed in their maturation stage from day 5 (Fig. 3A) to day 7 (Fig. 3B), as indicated by increasing fluorescence intensity for all phenotypic markers. The uptake of melanoma cells in early apoptosis (Fig. 3C) blocked DC maturation to the day 5 level, whereas engulfment of tumor cells in late apoptosis/secondary necrosis (Fig. 3D) promoted further DC maturation beyond the levels seen in day 7 DCs cultured alone (compare Fig. 3D with Fig. 3B). Coculture of day 5 DCs with metastatic melanoma cells in primary necrosis (induced by using freezing and thawing) promoted DC maturation as effectively as chromatin (Sigma-Aldrich) and then induced to undergo apoptosis (by using UV-irradiation) or primary necrosis (by using freezing/thawing). PKH2-GL-labeled apoptotic or necrotic cells were then incubated with DCs at a ratio of 1:1 at 37°C. After 36 h, the cell cultures were stained with anti-CD1a mAb. Cells that expressed CD1a were gated by using flow cytometry and analyzed for PKH2-GL-associated green fluorescence. Immediately before flow cytometry analysis, ethidium bromide (Sigma-Aldrich) at 0.5 μg/ml was added. The membranes of live DCs prevented staining of internalized apoptotic or necrotic cells by ethidium bromide. This enabled us to discriminate between DCs that had engulfed apoptotic or necrotic melanoma cells, appearing as PKH2-GL⁺, ethidium bromide⁻ events in FL-1, versus FL-2 plots from DCs that bound but did not internalize melanoma cells, appearing as double-positive PKH2-GL⁺, ethidium bromide⁺ events. Phagocytosis by DCs of tumor cell lysates was assessed by pulsing 1 × 10⁵ immature DCs in 100 μl of RPMI 1640 with 50 μl of the biotinylated cell lysates (containing 1 mg protein/ml) for 4 h at 37°C or 37°C. Tumor cell lysate-pulsed DCs were then washed twice with PBS, spun onto poly-L-lysine-coated coverslips, treated with horseradish peroxidase-labeled streptavidin (Dako, Milan, Italy) for 30 min, and finally visualized with the use of red 3-aminoe-9-ethylcarbazole (Sigma-Aldrich). In some experiments dying melanoma cells were preincubated for 2 h with 10 μg/ml neutralizing mAb to VEGF (R&D Systems, Minneapolis, MN) or IL-10 (R&D Systems). Neoplastic cells were then cocultured with immature DCs for 48 h.

T-Cell Proliferation. Day 5, immature DCs (1 × 10⁵) were cocultured for 48 h with early and late apoptotic cells, cells in primary necrosis (5 × 10⁵), or with a dose of cell lysate equivalent to 5 × 10⁵ cells. DCs were then fixed with 1% paraformaldehyde for 30 min on ice, washed extensively, and added at DC:T-cell ratios from 1:20 to 1:160 to allogeneic T cells (2 × 10⁵/well) in 96-well flat-bottomed plates (Costar Corp., Cambridge, MA). After 4 days, T cells were pulsed for 18 h with 1 μCi of [³⁵S]thymidine/well (NEN, Boston, MA) and proliferation was evaluated as described (21).

Cross-Presentation of MAAs by DCs to CTLs. CTL lines directed to tumor peptides Melan-A/Mart-1, 12-26 and gp100 209-217 were obtained from peripheral blood lymphocytes of HLA-A*0201 melanoma patients as described using peptide-loaded T2 cells as APCs (19). To test cross-presentation of MAAs by DCs, day 5, immature DCs (1 × 10⁵) from a HLA A*0201 donor were cocultured for 24, 48, or 72 h with 3 × 10⁶ apoptotic (by using UV-irradiation) or necrotic (by using freezing/thawing) melanoma cells from a Melan-A/Mart-1, gp100⁺ melanoma cell line isolated from a HLA-A201-negative patient. Aliquots of DCs were also pulsed with 10 μg/ml synthetic peptides Melan-A/Mart-1, 12-26 and gp100 209-217, or tyrosinase-368-377 (28). In some DC-tumor combinations, TNF-α (Knoll AG, Ludwigshafen, Germany) at 20 ng/ml was added to DCs 4 h after initiation of tumor uptake. Lysis of antigen-pulsed DCs (1 × 10⁵/well) by the peptide-specific T-cell lines was tested in a 6-h [⁵¹Cr] release assay at an E:T ratio of 20:1 as described (19). HLA-A2 restriction of DC lysis by peptide-specific CTL lines was verified by preincubating peptide-pulsed or tumor-loaded DCs with 10 μg/ml anti-HLA-A2-specific mAb CR11-351 (29).
TNF-α (Table 1, experiment 1). In addition, the maturation block induced in DCs by coculture with early apoptotic melanoma cells was only partially reduced, but not abolished, by TNF-α (Table 1, experiment 2). The uptake of melanoma cell lysates promoted DC maturation to a similar extent as seen after the uptake of necrotic melanoma cells, whereas coculture of DCs with live tumor cells did not affect DC maturation (data not shown).

The immunostimulatory activity of DCs exposed to dying melanoma cells was tested in a 5-day allogeneic T-cell proliferation experiment. The lowest T-cell immunostimulatory activity was exerted by DCs that had been cultured alone or with early apoptotic melanoma cells (Fig. 4A). In agreement with the promotion of DC maturation seen by using phenotypic analysis (Fig. 4B), a more potent T-cell response was induced by DCs cocultured with melanoma cells in late apoptosis/secondary necrosis (by UV-irradiation), melanoma cell lysates, and melanoma cells in primary necrosis induced by using freezing and thawing (Fig. 4A).

A Role of Tumor-derived IL-10 in the Inhibitory Effect of Early Apoptotic Melanoma Cells on DC Maturation. The cell lines from 13 primary and metastatic melanomas, previously characterized for their impact on DC maturation, were analyzed by using intracellular staining and flow cytometry for expression of IL-10, VEGF, and TGF-β1 proteins, factors that can impact on DC maturation (15–18). TGF-β1 was either not expressed or very weakly expressed in most cell lines (representative data on cell lines from 4 primary and 4 metastatic tumors are shown in Fig. 5). VEGF was found in most cell lines although at different levels. Interestingly, IL-10 signal was very weak (Fig. 5) in two cell lines from primary tumors (WM35 and Mel10538) that did not inhibit DC maturation (as shown in Table 2) and its expression was abolished in apoptotic cells from WM35 and Mel10538. In all of the tumors that could inhibit DC maturation, IL-10 was still expressed in late apoptosis/secondary necrosis, as well as in primary necrosis (data not shown). To evaluate the possible role of melanoma-derived IL-10 in inhibition of DC maturation, early apoptotic tumor cells were co-cultivated with a neutralizing mAb to IL-10.
before they were added to DCs. As shown in Fig. 6, most of the inhibitory effect on DC maturation exerted by early apoptotic melanoma cells was prevented by a neutralizing anti-IL10 mAb but not by an anti-VEGF mAb. In contrast with a recent report (30), control experiments indicated that maturation of DCs was not affected when these cells were cultured from days 5–7 without apoptotic tumor cells but in the presence of 0.1 to 15 μg/ml of the anti-IL-10 mAb (Fig. 6; data not shown).

Cross-Presentation of TA by DCs, after the Uptake of Melanoma Cells in Early Apoptosis or Primary Necrosis, Requires Additional Maturation Signals. Two CTL lines directed to gp100209–217 or Melan-A/Mart-127–35 peptides were used to test cross-presentation, by HLA-A*0201+ DCs, of TA after the uptake of melanoma cells in early apoptosis (induced by using serum starvation and UV-irradiation) or in primary necrosis (by using freezing/thawing). To this end, a gp100+, Melan-A/Mart-1+ melanoma from an HLA-A*0201-negative patient was used to ensure that CTL recognition of DCs was not attributable to transfer of preformed HLA-A*0201-peptide complexes through melanoma exosomes or membrane fragments (3, 31). As shown in Fig. 7, when DCs were cocultured for at least 48–72 h with melanoma cells in early apoptosis or primary necrosis, HLA-A2-restricted lysis of DCs was achieved by the gp100209–217-specific CTL indicating efficient cross-presentation of gp100209–217 peptide. However, this was observed only when an exogenous maturation signal (TNF-α) was delivered to DCs 4 h after initiation of tumor uptake (Fig. 7). By contrast, cross-presentation of Melan-A/Mart-1 epitope, which is not produced by the immunoproteasome of DCs (32), was never obtained by either immature or TNF-α-matured DCs although the Melan-A/Mart-1-specific CTL line recognized the same DCs when loaded with the Melan-A/Mart-1 synthetic peptide (Fig. 7). Cross-presentation of gp100209–217 epitope was also achieved after the uptake of melanoma cells in late apoptosis, but even in this instance, only after DC maturation with TNF-α (data not shown).

Taken together, these data suggest that both apoptotic and necrotic melanoma cells are effective sources of TA that can be transferred to immature DCs. However, tumor cells in early apoptosis inhibit DC maturation, whereas cells in late apoptosis or even in primary necrosis deliver to DCs only a partial maturation signal, documented by DC phenotype and T-cell immunostimulatory activity but not sufficient for achieving efficient antigen cross-presentation to CTLs unless a further strong maturation signal (e.g., TNF-α) is provided.

DISCUSSION

The results of this study suggest that phases of tumor cell apoptosis and necrotic transformation contribute to the modulation of DC maturation after the uptake of dying cells of the melanocyte lineage.
IL-10 gene expression and IL-10 protein synthesis in human melanoma has been subjected to considerable investigation. The evidence indicates that IL-10 is an autocrine growth factor for melanoma cells (33) and a marker of tumor progression, because IL-10 protein can be found in vivo in a higher proportion of metastatic lesions in comparison with primary tumors whereas immunoreactivity for IL-10 is not found in normal skin and melanocytic nevi (34). Furthermore, circulating levels of IL-10 are elevated in metastatic disease in comparison with stage I and II patients and healthy subjects (16). In agreement with the relationship between tumor progression and IL-10 expression, we found that intracellular expression of IL-10 protein was weak in a RGP tumor and in 1 of 5 VGP melanomas and abolished in early apoptosis in these 2 tumors. This correlated with a lack of inhibitory activity by these cell lines on DC maturation. By contrast, IL-10 was expressed at higher levels in the metastatic tumors and not affected by early apoptosis. In addition, the experiments with neutralizing antibodies to cytokines known to have an inhibitory activity on DCs suggested that IL-10 expressed in melanoma cells can play a role in the inhibition of DC maturation after the uptake of early apoptotic cells. This is in agreement with data (35) indicating that IL-10 can block the differentiation to DCs of human monocytes cultured in GM-CSF and IL-13. Similar results have been obtained in the murine system, where addition of EBV-encoded viral IL-10 to bone marrow DC progenitors, or even transduction of such progenitors with vIL-10, inhibited DC maturation and reduced the ability of APCs to stimulate proliferation of T cells (36). Additional evidence indicates that IL-10-treated human DCs can induce a state of anergy in MAA-specific T cells (17), thus providing support to the notion that IL-10 production by tumor cells may contribute to convert DCs from immunostimulatory to tolerogenic APCs, a process that may inhibit the development of TA-specific immunity.

The experiments aimed at verifying cross-presentation of MAAs to antigen-specific T cells after the uptake of dying melanoma cells indicated the requirement for an exogenous maturation signal, represented by TNF-α, added to DCs 4 h after the initiation of tumor uptake. In addition to affecting the expression of several surface markers and costimulatory molecules, exposure of immature DCs to TNF-α can induce a switch to low endocytic/high immunostimulatory phenotype, activate expression of proinflammatory factors such as IL-12, IL-15, and Rantes, and promotes the expression of chemokine receptors that contribute to DC migration from peripheral tissues to lymphoid organs (37–39). The maturation of DCs by signals such as TNF-α impacts even on the production of MHC-peptide complexes. Under the influence of maturation signals, exogenous antigens sequestered in lysosomes are processed to MHC-class II peptides and exported at the cell surface (40). The requirement for a maturation signal to allow epitope cross-presentation by immature DCs after the uptake of apoptotic cells has been shown recently by Sauter et al. (11). These authors have reported that T-cell recognition of influenza virus epitopes can be achieved when immature DCs that are uptaking apoptotic cells infected with the virus are exposed to supernatants from necrotic cells. Although we adopted a different experimental approach, based on DC uptake of necrotic melanoma cells and not on exposure of DCs to the necrotic cell supernatant, we found that the maturation induced in APCs in these conditions was not sufficient to achieve cross-presentation of MAA as the gp100_209–217 epitope. Thus, whereas the phenotype of DCs after the uptake of necrotic melanoma cells indicated that maturation had occurred to a similar extent as that seen after exposure to TNF-α, nevertheless, this process was not associated with the ability to cross-present tumor-derived epitopes unless TNF-α was provided.

Several studies have addressed the issue of activation of tumor-specific T cells by DCs loaded with killed tumor cells. Such antigen-
specific T cells, directed to melanoma or prostate antigens, have been isolated in some instances after being cultured with immature myeloid DCs loaded with dying tumor cells without the need for exogenous maturation signal (41, 42). However, one cannot exclude the possibility that factors released by T cells during coculture with DCs may contribute to promote maturation of the APCs. Furthermore, when lymphocytes from tumor patients have been used, the induction of antigen-specific T cells by tumor-loaded DCs may indicate reactivation of in vivo primed memory T cells, a process that may not require the same level of APC maturation as the activation of naive T cells (43). In agreement with this possibility, cross-priming of sorted CD45RA⁺/CD27⁺ naive T cells directed to MAAs has been achieved by DCs or DCs matured with TNF-α after the uptake of tumor phagocytosis. Lysis of DCs after tumor uptake was tested in the absence (−) or presence (+) of an HLA-A2, -A68-specific mAb (CR11-351). Aliquots of immature DCs or DCs matured with TNF-α were also loaded with gp100209-217 or Melan-A/Mart-127-35 peptides. Results expressed as percentage of lysis at an E:T ratio of 20:1 in a 6-h 51Cr release assay.

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**Fig. 7. Cross-presentation of MAAs to CTLs, after the uptake of apoptotic or necrotic melanoma cells, requires an exogenous maturation signal.**

--Day 5, immature DCs from a HLA-A*0201⁺ donor were cocultured for 24, 48, or 72 h with early apoptotic (at 24 h after UV-irradiation) or necrotic (induced by freezing/thawing) cells from a HLA-A*0201⁺, gp100⁺, Melan-A/Mart-1⁺ metastatic melanoma and then tested for lysis by CTLs directed to gp100209-217 or Melan-A/Mart-127-35. TNF-α at 20 ng/ml was added (+) or not (−) to DCs 4 h after the initiation of tumor phagocytosis. Lysis of DCs after tumor uptake was tested in the absence (−) or presence (+) of an HLA-A2, -A68-specific mAb (CR11-351). Aliquots of immature DCs or DCs matured with TNF-α were also loaded with gp100209-217 or Melan-A/Mart-127-35 peptides. Results expressed as percentage of lysis at an E:T ratio of 20:1 in a 6-h 51Cr release assay.

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<table>
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<tr>
<th>Experimental Conditions</th>
<th>Lysis of DCs by CTL Lines to:</th>
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<tbody>
<tr>
<td>bTNF-α Tumor mAb Peptides</td>
<td>GP100209-217</td>
</tr>
<tr>
<td>- -</td>
<td>24</td>
</tr>
<tr>
<td>- Apoptotic</td>
<td>24</td>
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<tr>
<td>- Necrotic</td>
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<td>+ -</td>
<td>24</td>
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<tr>
<td>+ Apoptotic</td>
<td>24</td>
</tr>
<tr>
<td>+ Necrotic</td>
<td>24</td>
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