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Antigen-Processing Machinery in Human Dendritic Cells: Up-Regulation by Maturation and Down-Regulation by Tumor Cells[1]

Theresa L. Whiteside,[2]*† Joanna Stanson,* Michael R. Shurin,*† and Soldano Ferrone‡

It has been known for some time that functional properties of dendritic cells (DC), and in particular their ability to process and present Ags to T cells, can be modulated by cytokine-induced maturation and by interactions with tumor cells. However, the molecular basis for these functional changes is unknown. We have investigated whether changes in expression of Ag-processing machinery (APM) components in DC are associated with alterations in their ability to present tumor-derived Ags to T cells. Using a panel of mAbs specific for individual APM components and a quantitative flow cytometry-based method, the level of APM components was measured in DC generated from peripheral blood monocytes of 12 normal donors and of 8 patients with cancer. Immature DC had significantly lower ($p < 0.01$) expression of MB1, LMP-7, LMP-10, TAP-1, and tapasin than mature DC. However, maturation in the presence of a cytokine mixture up-regulated expression of these components in DC obtained from normal donors and patients with cancer. Immature DC incubated with tumor cells had significantly lower ($p < 0.001$) expression of MB1, LMP-2, LMP-7, LMP-10, and endoplasmic reticulum p75 than controls. These changes were associated with a decreased ability of DC to present tumor-derived Ags to T cells, as measured in ELISPOT assays and with apoptosis of T cells in DC-T cell cultures. Thus, tumor cells have a significant suppressive effect on DC; however, ex vivo maturation of DC derived from patients with cancer in a polarizing cytokine mix restores normal expression of APM components and Ag-processing capabilities. ***The Journal of Immunology,*** **2004, 173: 1526–1534.**

Dendritic cells (DC)[3] are potent APCs that play a key role in host immune response against infectious agents and tumors (1, 2). Among the various functions that DC perform, Ag uptake and processing are perhaps the most intensely investigated. The processing of exogenous internalized or endogenous Ags is accomplished through a complex series of intracytoplasmic events involving Ag-processing machinery (APM). The latter is composed of multiple molecular species organized to efficiently handle the Ags that enter the processing pathway (3, 4). The HLA class I Ag-processing pathway starts with cellular proteins marked for ubiquitination and subsequently degraded by the proteasome. The multicatalytic proteasome complex, particularly the IFN-γ-inducible subunits, LMP-2, LMP-7, and LMP-10 (5), is involved in the generation of antigenic peptide fragments. These fragments are then translocated across the endoplasmic reticulum (ER) membrane via the ATP-dependent heterodimeric transporter associated with Ag-processing subunits TAP-1 and TAP-2. Within the ER, the HLA class I H chain is synthesized and associates with β_2-microglobulin (β_2m) with the assistance of the chaperone proteins (calnexin, calreticulin, and ERp57), which monitor the proper folding of HLA class I molecules. The HLA class I-β_2m complex then associates with tapasin, which allows the dimeric complex to interact with TAP and ensures proper peptide loading into HLA-I-β_2m complex. Then, the trimeric HLA class I-β_2m-peptide complex is transported to the plasma membrane (3).

It has been recognized for some time that functional changes, in particular alterations in the ability to process and present Ags to T cells, are associated with DC maturation (1, 2) and with exposure of DC to tumor cells (6–8). To date, little is known about the molecular mechanisms underlying these functional changes, and specifically about APM component expression in DC (9–11). Therefore, taking advantage of a panel of recently developed mAbs to the individual APM components (12) and of the quantitative flow cytometry-based method, we measured in this study the expression of the various components in immature DC (iDC) as well as ex vivo-matured DC (mDC) obtained from peripheral blood monocytes of normal donors (ND) and of patients with cancer. In addition, we evaluated the effects of tumor cells on expression of APM components in DC and their functional potential.

Our data establish for the first time a molecular basis for functional changes that occur during DC maturation. These experiments are also relevant to current DC-based vaccine clinical trials in patients with cancer. The use of autologous ex vivo-activated DC for immunotherapy of cancer is based on the rationale that cross-presentation of tumor-associated Ags (TAA) by DC leads to the generation in vivo of effective TAA-specific immune responses. However, endogenous DC in tumor-bearing animals (6, 7) as well as in patients with cancer (8, 13) have been observed to contain various maturational and functional defects. The abnormalities in dendropoiesis reported for DCs in patients with cancer

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[3] Abbreviations used in this paper: DC, dendritic cell; iDC, immature DC; mDC, mature DC; APM, Ag-processing machinery; HNC, head and neck cancer; TAA, tumor-associated Ag; ER, endoplasmic reticulum; β_2m, β_2-microglobulin; MESF, molecular equivalents of soluble fluorochrome; IVS, in vitro sensitization; poly(IC), polyinosinic-polycytidylic acid.

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Table I. *mAbs specific for APM components and HLA Ags and used for their quantitation in human DC*

mAb	Specificity	Reference
SY-4	Delta	Unpublished
SJJ-3	MB-1	Unpublished
SY-1	LMP-2	Unpublished
SY-3	LMP-7	Unpublished
TO-6	LMP-10	Unpublished
TO-1	TAP-1	Unpublished
TO-5	Calnexin	24
TO-11	Calreticulin	24
TO-2	ERp57	24
TO-3	Tapasin	24
HC-10	HLA class I	25
L368	β_2m	26
LGH-612.14	HLA-DR, -DQ, -DP	27

Table II. *Reproducibility and sensitivity of intracytoplasmic staining for APM components in microwave-treated or saponin-permeabilized DC*[a]

	% Positive Cells			
		Microwave		
APM Components	0.25% saponin	Lab 1	Lab 2	Range
IgG control	2	1	1	1–2
Delta	79	80	69	39–95
MB-1	37	18	12	6–59
LMP-2	56	79	69	26–84
LMP-7	11	4	3	2–23
TAP-1	17	9	6	3–33
ERp57	16	11	7	3–33
Tapasin	27	58	42	10–64

[a] Comparisons were performed in two laboratories, using the same DC preparations. DC were permeabilized by microwave or saponin treatments and stained with the mAbs specific for the individual APM components. The percentage of stained cells was determined by flow cytometry. Data are mean percentages of stained cells from three cell preparations.

(14) and evidence that coincubation of DCs with tumor cells induces DC apoptosis (15) suggest that the fate of these APCs may be impaired in the tumor microenvironment. Collectively, these observations point to a dysfunction of DCs in tumor-bearing hosts and indicate that the adoptive transfer of ex vivo-activated DC might correct the existing deficiencies in TAA-specific immune responses and thus be of therapeutic benefit. Our findings show the critical importance of APM for the functional integrity of TAA-specific immune responses and for ex vivo generation of DCs for therapeutic transfers to patients with cancer.

Materials and Methods

Preparation of DC

Buffy coats obtained from platelet donors at the Central Blood Bank of Pittsburgh (Pittsburgh, PA) were used as a source of normal monocytes for DC generation. Heparinized peripheral blood was obtained from consented patients with head and neck cancer (HNC), melanoma, or cutaneous T cell lymphoma for DC generation. Peripheral blood or buffy coats were centrifuged on Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO) gradients. Monocytes were obtained by adherence to plastic as previously described (16) and incubated in AIM V medium (Invitrogen Life Technologies, Carlsbad, CA) containing GM-CSF (1000 U/ml) and IL-4 (10 ng/ml). Cells were harvested on day 6, examined for viability, and analyzed for expression of CD3, CD14, CD19, CD40, CD80, CD83, CD86, and HLA-DR Ags by flow cytometry. For maturation, DC were incubated in medium supplemented with IL-1β, IL-6, and TNF-α, all at 10 ng/ml, for 24 h at 37°C. In some experiments, this cytokine mixture also contained PGE_2 (10 ng/ml), IFN-α (1000 U/ml), IFN-γ (1000 U/ml), and polyinosinic-polycytidylic acid (poly(IC); 20 μg/ml; Sigma-Aldrich). DC maturation was monitored by measuring the expression of CD40, CD80, CD83, and CCR7 by flow cytometry. iDC and mDC were harvested and used for staining with the anti-APM component-specific mAbs.

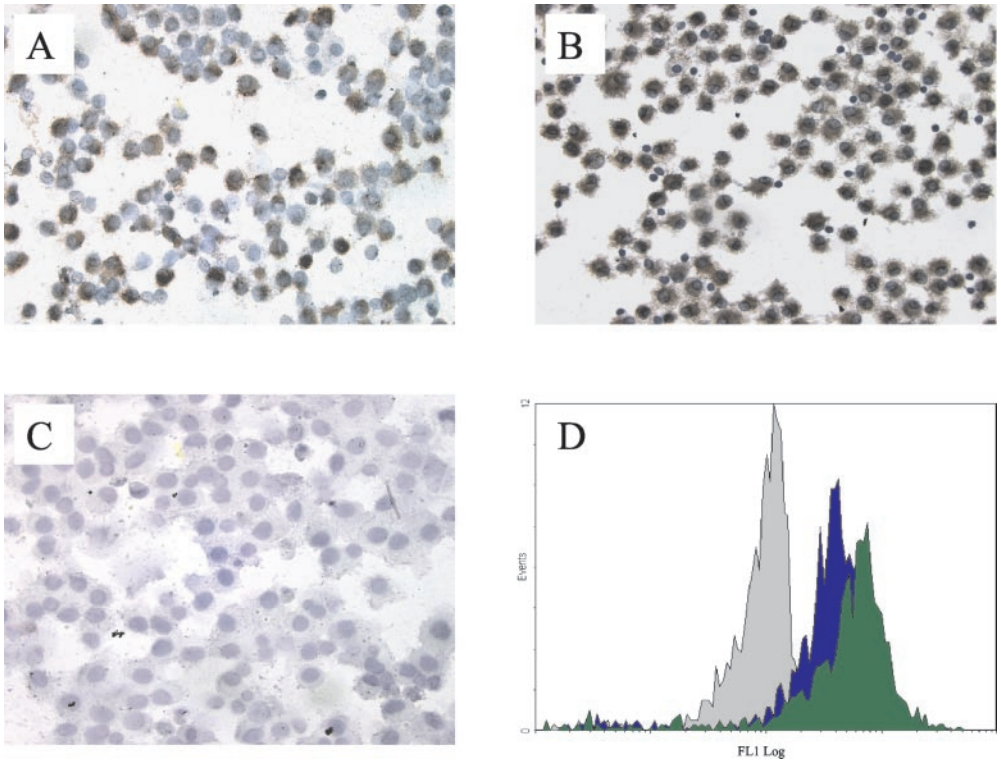


FIGURE 1. Up-regulation of Delta expression in human mDC. Immunocytochemistry for expression of the APM component, Delta, in cytocentrifuge preparations of iDC and mDC is shown in *A* and *B*, respectively. ×400. *C*, Control DC stained with isotype control IgG. *D*, Flow cytometry histograms for Delta: isotype control (neutral); iDC (blue); and mDC (green). FL, Fluorescence.

Table III. *Expression of APM components in iDC and mDC*[a]

APM Components	No. of Donors	iDC	mDC
MB-1	6	54 (13–87)[b]	90 (26–99)
LMP-2	6	85 (59–95)	92 (82–99)
LMP-7	6	5 (2–16)	29 (3–94)
LMP-10	6	34 (6–37)	60 (13–92)
TAP-1	6	20 (8–76)	44 (9–97)
ERp57	5	8 (1–14)	27 (1–89)
Tapasin	5	46 (15–86)	70 (21–98)

[a] Permeabilized DC were stained with the mAb specific for the individual APM components. The percentage of stained cells was determined by flow cytometry. Data are mean percentages of stained cells.
[b] Numbers in parentheses, range.

Antibodies

mAbs to individual APM components were generated and characterized according to the methodology we previously described (12). The designations and specificity of the mAbs used for staining of DC and their sources are listed in Table I. mAb W6/32 was produced by Dr. A. DeLeo (University of Pittsburgh, Pittsburgh, PA) using a hybridoma obtained from American Type Culture Collection (Manassas, VA). Labeled mAbs for staining of surface Ags on lymphocytes (CD3, CD19, monocytes (CD14) or DC (anti-HLA-DR, -CD80, -CD83, -CD86, -CD11c, -CD40, -CD25) by flow cytometry and anti-CCCR7 mAbs were purchased from Beckman Coulter (La Brea, CA). FITC-conjugated goat anti-mouse IgG Abs were purchased from Caltag Laboratories (Burlingame, CA). Anti-IFN-γ Abs (1-D1K and 7-B6-1) for use in ELISPOT assays were purchased from MABTECH (Mariemont, OH).

Cell lines

The squamous cell carcinoma of the head and neck cell lines PCI-4B and PCI-38, were established in our laboratory from a lymph node metastasis and from a primary tumor, respectively, as previously described (17). The melanoma cell line Mel 526 was obtained from Dr. W. J. Storkus (University of Pittsburgh). The cell lines were cultured to confluence in RPMI 1640 supplemented with 10% (v/v) FCS and antibiotics (all from Invitrogen Life Technologies).

Cytokines

IL-1β, IL-4, IL-6, and TNF-α were purchased from R&D Systems (Minneapolis, MN); IFN-α from Schering (Kenilworth, NJ); IFN-γ from Intermune (Brisbane, CA). GM-CSF was purchased from Immunex (Seattle, WA).

Table IV. *Expression of APM components and HLA Ags by iDC and mDC generated from a representative ND*[a]

	% Positive Cells	
	iDC	mDC
IgG + GAMI (control)	5	10
MB-1	59	80
Delta	95	96
LMP-2	80	88
LMP-7	10	30
LMP-10	60	68
TAP-1	32	47
ERp57	20	27
Calnexin	87	99
Calreticulin	79	99
Tapasin	54	65
HLA class I	100	100
β_2m	100	100
HLA-DR, -DQ, -DP	100	99

[a] DC were stained with the mAbs specific for the individual APM components and for HLA Ags. The percentage of stained cells was determined by flow cytometry. Data are mean percentages of stained cells in DC of a representative ND among eight studied.

Table V. *Quantitative comparison between the level of APM component expression in iDC and mDC generated from a representative normal donor*[a]

	MESF U $\times 10^4$	
	iDC	mDC
IgG + GAMI (control)	50	55
MB-1	100	120
Delta	520	620
LMP-2	180	370
LMP-7	80	120
LMP-10	170	430
TAP-1	90	130
ERp57	100	160
Calnexin	260	310
Calreticulin	430	360
Tapasin	110	140
HLA class I	430	610
β_2m	290	750
HLA-DR, -DQ, -DP	260	370

[a] DC of a representative ND (see Table IV) were stained with the mAbs specific for the individual APM components and for HLA Ags. The staining intensity is expressed in MESF units as determined by quantitative flow cytometry.

Staining methods

Intracellular staining of DC with the APM component-specific mAbs was performed as previously described (16) with the following modifications. Cells were washed in PBS containing 1% (w/v) BSA and placed in tubes ($2–5 \times 10^5$ cells/tube). Cells were fixed with 2% (w/v) paraformaldehyde for 20 min at room temperature, washed extensively in PBS-BSA, resuspended in 10 ml of PBS-BSA buffer, transferred into Pyrex flasks (Corning Glass, Corning, NY), and subjected to the microwave treatment for 60 s at low power (<700 W). Cells were then chilled on ice for 10 min, washed, and permeabilized, using 0.1% (w/v) saponin in the PBS-BSA buffer and incubated with the primary Abs (at 10–25 μg/ml saponin-containing buffer) for 30 min at room temperature. After being washed with three changes of the saponin buffer, cells were incubated with optimally pretitered goat anti-mouse IgG Abs for 30 min at room temperature. Cells were then washed in saponin buffer, fixed with 1% (w/v) paraformaldehyde, and analyzed in a Coulter EPICS XL-MCLDC flow cytometer (Beckman Coulter, Miami, FL).

For immunocytochemistry, cells were cytocentrifuged onto positively charged glass slides in a Shandon cytocentrifuge (Shandon, Pittsburgh, PA), fixed in acetone-methanol (1:1), and air dried. Next, the cytospun cells were rehydrated in PBS buffer and blocked with the protein-blocking solution (Shandon). Slides were then incubated with the primary Abs for 1 h at room temperature. After extensive washing, slides were incubated with LSAB-2 reagents (Dako, Carpinteria, CA) according to the directions provided by the manufacturer. Color was developed with diaminobenzidine chromogen (Vector Laboratories, Burlingame, CA), and the slides were counterstained with Gills hematoxylin (Vector Laboratories).

Flow cytometry

Stained cells were examined in an EPICS XL-MCLDC instrument. Two approaches were used: 1) to determine the percentages of stained cells, gate was set on DC, and the number of stained cells vs that of total cells in the gate was determined for each mAb; 2) to quantitate the expression level of each protein, molecular equivalents of soluble fluorochrome (MESF) units were determined, using a standard curve generated by the use of a mixture of four standard beads of known fluorescence intensity and unlabeled blank beads contained in the Quantum Kit (Bangs Laboratories, Fishers, IN). Mean fluorescence intensity of each sample was transformed into MESF units, using the calibration curve calculated by the Quick Cal program supplied by Bangs Laboratories. The fluorescence from the beads was calibrated in terms of MESF each time the experiment was performed.

Coincubation of DC with tumor cell lines

Human iDC or mDC generated from PBMC of ND were cocultured for 24 h at 37°C with tumor cells in a Transwell plate (Costar, Cambridge, MA), plating DC in the bottom compartment and tumor cells in the upper compartment at a 1:1 ratio. Control cultures contained iDC or mDC alone.

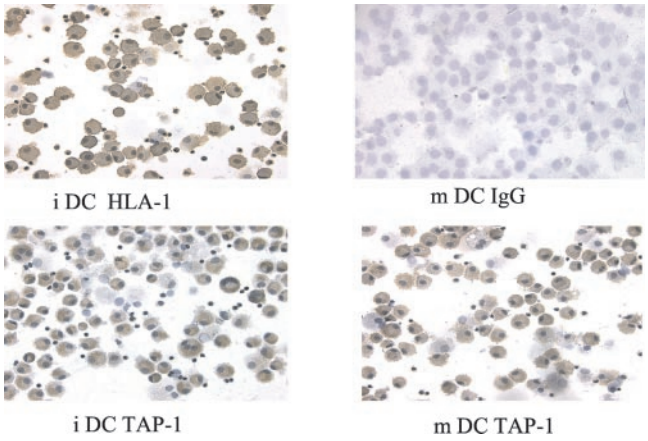


FIGURE 2. Immunocytochemistry for expression of TAP-1 in iDC and mDC. IHC for HLA class I (HLA-1) Ag expression serves as a positive control and for IgG isotype as a negative control. ×400.

At the end of a 24-h incubation at 37°C, DC were harvested and used for experiments.

Melanoma Ag (MA)-specific cytolytic T cell generation

The DC coincubated with tumor cells or control DC were pulsed with a Mel526 cell lysate (20 μg protein per ml) for 12–18 h at 37°C, matured in the presence of a cytokine mixture, as described above, and then coincubated with autologous PBMC for 7 or 14 days at 37°C to generate MA-specific T cells by in vitro sensitization (IVS).

ELISPOT assays

The IVS-generated T cells (responders) were harvested and immediately plated in wells of ELISPOT plates (Millipore; Fisher Scientific, Hampton, NH) at a concentration of 1×10^5 cells/well. Mel526 cells (stimulators) were added (1×10^3 cells/well), and the plates were incubated for 24 h at 37°C. The ELISPOT assay for IFN-γ was performed as previously described (18).

In some experiments, the Mel526-specific CTL line originally obtained from Dr. S. Rosenberg (Surgery Branch, National Cancer Institute, Bethesda, MD) and maintained in our laboratory (18) was used as responder cells to measure the ability of different DC (HLA-$A2^+$) pulsed with Mel526 cell lysates or $gp100_{209-217}$ peptide to induce IFN-γ production. The specificity of this T cell line for Mel526 cells was previously tested by us in ELISPOT assays (18).

Preparation of cell lysate

Mel526 cells were harvested using trypsin buffer. After two washes with cold 1× PBS, the cell pellet was then resuspended in lysis buffer (20 mM HEPES buffer, 50 mM NaCl, 10 mM KCl, 1 mM EDTA, 200 mM sucrose, pH 7. 4) at a concentration of 5×10^6 cells/250 μl of buffer. Mel526 cells were then subjected to repeated (3×) freezing at −80°C and thawing at 37°C. After the last thaw, cells were sonicated for 10 s/ml of lysate and centrifuged at 14,000 rpm for 20 min at 4°C. Supernatant was then collected, and the protein concentration was determined, using a Bio-Rad protein assay (Hercules, CA). The cell lysate was then aliquoted into vials and stored at −80°C.

Table VI. *Up-regulation of APM component expression in mDC correlates with expression of maturation surface markers on these cells*[a]

	Surface Markers on DC (%)			APM Expression (MESF U × 10^4)		
	CD80	CD83	CCR7	LMP-2	TAP-1	IgG cont
iDC	20	35	6	124	172	32
mDC_2	70	84	30	148	204	33
mDC_4	95	92	56	164	353	26

[a] Maturation of DC was performed in the presence of IL-6 + IL-1β + TNF-α + PGE_2 (mDC2) or IL-6 + IL-1β + TNF-α + IFN-γ + IFN-α + poly(IC) (mDC4) and was followed by staining for APM components and maturation markers and by flow cytometry as described in *Materials and Methods.* Results are from a representative experiment of three performed.

Statistical analysis

Results were analyzed using a paired Student *t* test. $p < 0.05$ was considered as significant. The permutation test was used to determine significant differences between triplicate control wells vs experimental wells in ELISPOT assays.

Results

Purity and phenotype of iDC and mDC

iDC generated from monocytes obtained from ND or patients with cancer were evaluated for purity by manual cell counts and by flow cytometry. These preparations routinely contained >80% of cells with DC morphology and had the following phenotype (percent range for DC obtained from 12 ND): HLA-DR^+ (80–99%); $CD86^+$ (80–99%); CD80 (20–25%); $CD83^+$ (1–15%); $CD11c^+$ (80–99%); $CD40^+$ (50–80%); $CD14^+$ (0–5%); $CD3^+$ (0–10%); $CD19^+$ (0–5%). On maturation, the phenotype of DC was: HLA-DR^+ (99–100%); $CD86^+$ (90–99%); $CD80^+$ (80–95%); $CD83^+$ (77–96%); CD40 (70–90%); CD25 (20–45%); and $CCR7^+$ (30–60%). The DC preparations generated from patients with cancer had similar phenotypic characteristics. To be considered as mDC,

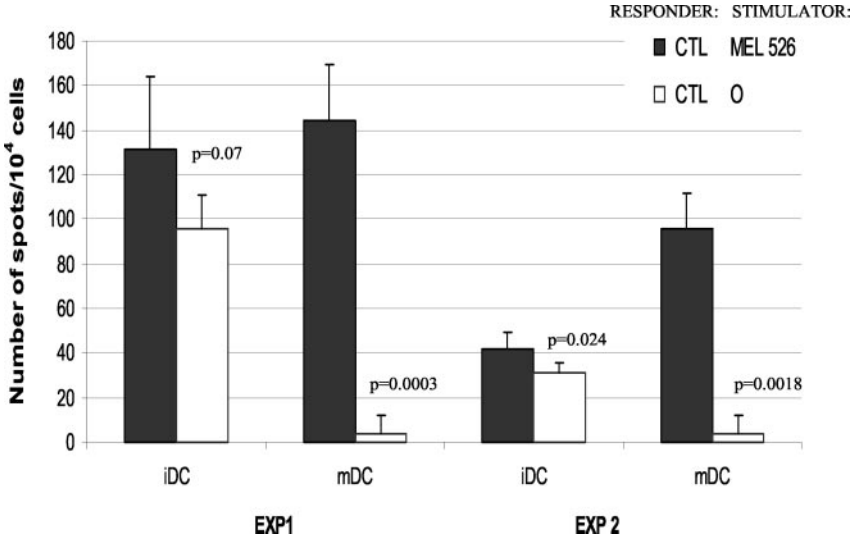


FIGURE 3. Higher effector cell function in ELISPOT assays of TAA-specific CTL generated with mDC than of CTL generated with iDC. Results of IFN-γ ELISPOT assays performed with the T cells generated in IVS cultures by priming with iDC or mDC. T lymphocytes in IVS cultures were stimulated with autologous iDC or mDC pulsed with a Mel526 cell lysate as a source of TAA. On day 7, T cells were harvested and tested in ELISPOT assays for IFN-γ production. Mel526 cells served as stimulators. Mean spots formed in triplicate wells are shown ± SD (bars). *p* values indicate differences between spot numbers in experimental vs control wells. Data from two representative experiments of four performed are shown. The number of background spots was high in the control wells, when iDC were used for Ag presentation to T cells.

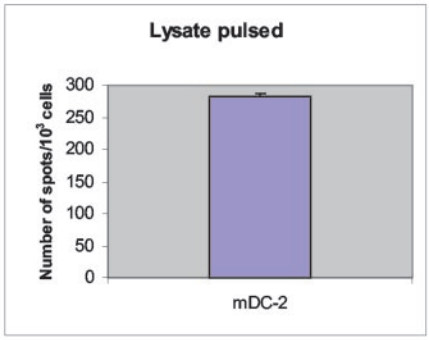
Expression of APM components in iDC or mDC pulsed with antigens or peptides

Exp. A — Lysate pulsed

	MESFX10^4 iDC	MESF X10^4 mDC
DELTA	133	289
LMP-2	118	133
LMP-10	111	140
TAP-1	164	188
Calnexin	587	680
Tapasin	118	148
IgG	32	37

Exp. B — gp-100$_{209\text{-}217}$ peptide pulsed

	MESF X10^4 iDC	MESF X10^4 mDC
DELTA	79	75
LMP-2	38	36
LMP-10	40	73
TAP-1	32	32
Calnexin	107	131
Tapasin	71	69
IgG	11	11



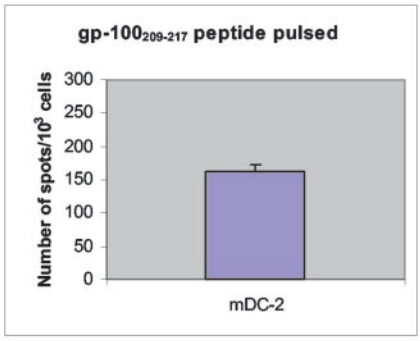


FIGURE 4. Association of up-regulation of APM component expression in melanoma cell lysate-pulsed and matured DC with efficient Ag presentation to T cells. *A,* iDC were pulsed with the melanoma cell lysate and matured in the presence of cytokines (IL-6, IL1β, TNF-α). DC were tested for expression of APM components by quantitative flow cytometry. mDC were also used as stimulators of Mel526-specific CTL line in ELISPOT assays. *B,* iDC pulsed with gp100$_{209–217}$ peptide and then matured in the cytokine mixture showed a modest up-regulation of APM component expression and gave a relatively low number of spots in ELISPOT assays. Background spots (unpulsed DC + CTL) were subtracted from experimental results. The results are from two separate experiments, using DC generated from HLA-A2$^+$ ND. Each experiment was performed twice with similar results.

the cells had to express >70% CD80, >70% CD83, and >30% CCR7.

Verification of the permeabilization and staining procedures

The usual procedure for permeabilization of mononuclear cells involves incubation of cells in the presence of saponin. However, in recent studies (12), microwave treatment has been shown to be the most effective method for staining lymphoid and melanoma cell lines with APM component-specific mAbs. Therefore, in preliminary experiments, we first compared the saponin-based and the microwave methods to ensure that the microwave treatment of DC performed under the conditions described in *Materials and Methods* was optimal for mAb access to subcellular compartments without interfering with the integrity of the APM components and/or DC morphology. The comparisons were performed independently in two laboratories (by T.L.W. and M.S.). The results presented in Table II show that the microwave treatment was better than saponin permeabilization for staining of DC with APM component-specific mAbs, given that the percentage of stained cells was consistently higher in microwave-treated DC than in saponin-permeabilized DC in the three experiments performed. Also, the procedure performed in parallel in the two laboratories was reproducible, because it yielded similar expression results in terms of the percentage of stained cells. In addition, immunocytochemistry on cytocentrifuge smears showed that the morphology of DC as well as staining intensity and percentages of stained DC were not compromised by the microwave treatment (Fig. 1).

APM component expression in iDC and mDC of ND

DC were generated from monocytes obtained from eight ND and stained with mAbs specific for APM components, HLA class I Ags, β_2m, and HLA class II Ags. Table III shows that expression of most of the APM components was up-regulated in DC matured in the presence of cytokines. Table III lists only those APM components that were substantially and consistently increased in the percent of stained cells in mDC preparations relative to iDC preparations. Two additional points are noteworthy. A considerable variability was observed among ND in the expression of APM components by DC, as indicated by a broad range of the percent stained cells (Table III). Furthermore, HLA class I Ags, β_2m, and HLA class II Ags were expressed in almost 100% of both iDC and mDC.

In additional experiments, a quantitative flow cytometry assay was used to measure the level of APM component expression in DC. In this assay, fluoresceinated beads served to obtain a standard curve expressing fluorescence intensity in terms of MESF units in every assay. Measurements using the MESF units can minimize day-to-day and instrument-to-instrument variability, thus providing a stable and standardized method for use in clinical laboratories. With this strategy, substantial increases in the percentage of stained cells (Table IV), in the level of expression as measured by staining intensity (Table V), or in both were consistently observed in mDC compared with iDC. Importantly, HLA class I Ag, β_2m, and HLA class II Ag expression was higher in mDC than in iDC (Tables IV and V). In addition, immunocytochemistry performed

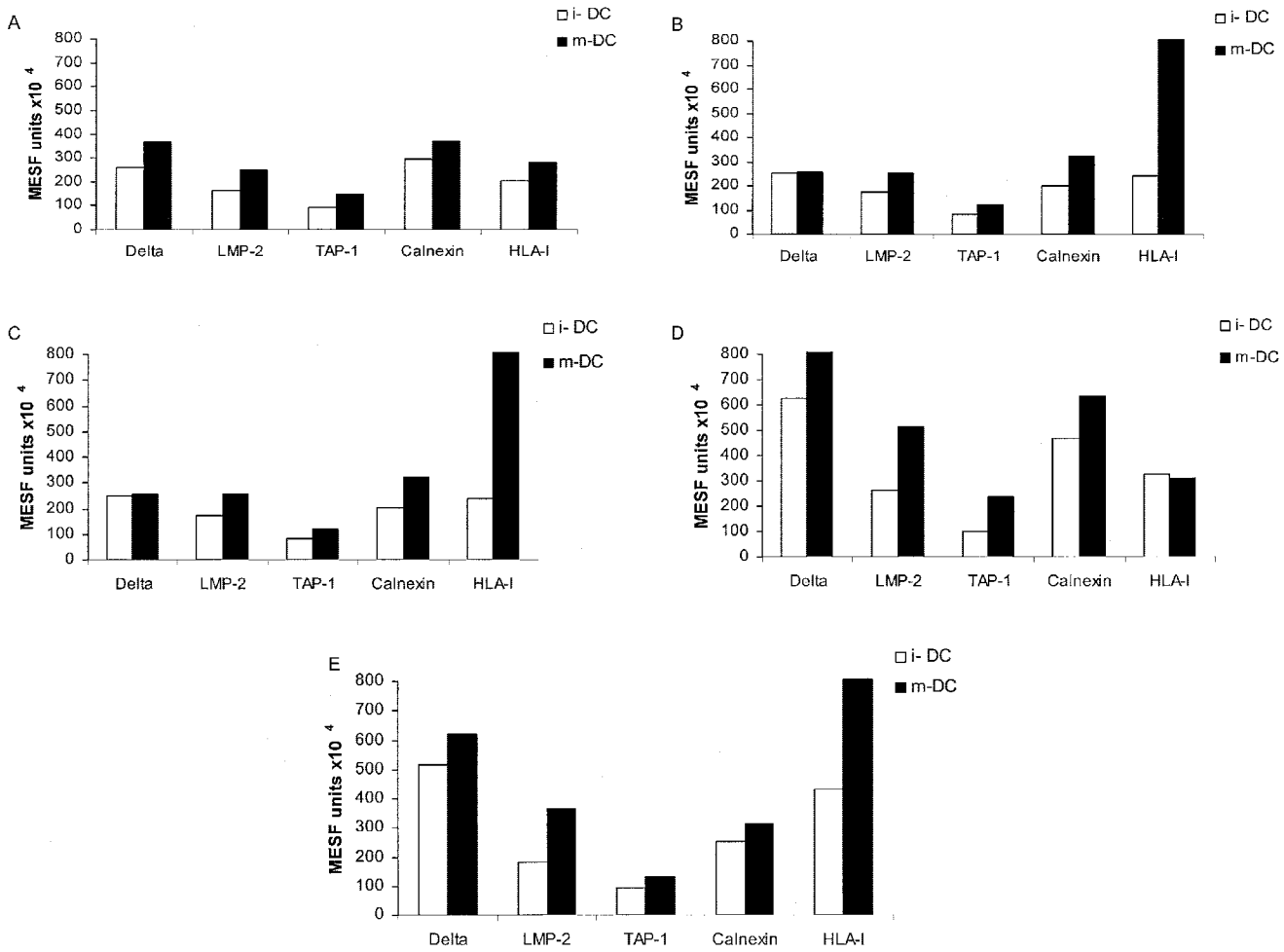


FIGURE 5. APM component expression (in MESF units) in iDC and mDC generated from PBMC of four patients with cancer. APM component expression in iDC from cancer patients is not significantly different from that in iDC of ND. However, up-regulation of individual APM component expression in mDC of patients is less marked. *A*, HNC; *B* and *C*, metastatic melanoma; *D*, cutaneous T cell lymphoma; *E*, ND.

on smears of iDC and mDC confirmed that the expression of APM components was higher in mDC than in iDC, as illustrated for TAP-1 in Fig. 2.

By using different cytokine mixtures for DC maturation, it was possible to show that up-regulation of APM component expression was related to the surface expression of maturation markers CD80, CD83, CD40, and CCR7 on DC. The highest up-regulation of APM component expression was observed in mDC incubated in the presence of a mixture containing IL-6, IL1β, TNF-α, IFN-γ, IFN-α, and poly(IC). These mDC preparations (mDC4) contained >90% $CD80^+$, > 90% $CD83^+$, and >50% $CCR7^+$ cells (Table VI).

We next tested whether up-regulated APM component expression in mDC was associated with an increased ability to present Ags to T cells, i.e., to generate MA-specific CTL. To this end, iDC were pulsed with a Mel526 cell lysate and either matured or not matured in the presence of cytokines. These iDC or mDC were then coincubated with autologous PBMC for 7 or 14 days. Next, IFN-γ ELISPOT assays were performed with effector T cells generated by IVS as responders and Mel526 cells as stimulators. T cells coincubated with mDC consistently gave a significantly higher number of spots than T cells coincubated with iDC (Fig. 3). In addition, effector T cells generated with iDC spontaneously released IFN-γ, giving a very high background in ELISPOT assays, whereas T cells primed with mDC gave only few background spots (Fig. 3). Thus, priming only with mDC resulted in the generation of MA-specific effector cells. These results are compatible with the hypothesis that the DC maturation process, which includes up-regulation of the APM components, is of key importance for subsequent productive interactions of mDC with T cells.

In additional experiments, we showed that up-regulated expression of APM components in mDC correlated with a greater ability of these APC to stimulate MA-specific CTL in ELISPOT assays. As shown in Fig. 4, DC maturation in the presence of the MA requiring processing (i.e., the melanoma cell lysate) was accompanied by up-regulation of APM component expression in mDC. These mDC were used as stimulators in ELISPOT assays, and they effectively induced IFN-γ production in MA-specific CTL. In contrast, pulsing of iDC with the $gp100_{209-217}$ peptide followed by maturation resulted in only modest up-regulation of APM component expression, and these DC mediated a lower response than DC pulsed with the melanoma cell lysate in ELISPOT assays (Fig. 4). In aggregate, these data suggest that up-regulated APM component expression correlates with mDC abilities to prime precursor T cells as well as present MA epitopes to MA-specific CTL.

APM component expression in iDC and mDC obtained from patients with cancer

We next asked whether APM component expression in iDC and mDC generated from the patients' PBMC was comparable with

Table VII. *APM component down-regulation in human DC coincubated with tumor cell lines*[a]

APM Component	% Positive Cells											
	Coincubation with PCI-4B cells						Coincubation with PCI-38 cells					
	Donor 1		Donor 2		Donor 3		Donor 4		Donor 5		Donor 6	
	+Tu	−Tu	+Tu	−Tu	+Tu	−Tu	+Tu	−Tu	+Tu	−Tu	+Tu	−Tu
MB-1	2	60	48	61	40	92	24	74	48	60	34	92
LMP-2	68	75	43	61	53	82	13	49	58	75	63	82
LMP-7	29	74	25	35	24	38	12	26	42	74	15	38
LMP-10	49	73	68	80	10	38	45	59	46	73	17	38
ERp57	65	57	36	75	25	75	ND	60	42	58	25	75

[a] Monocyte-derived DC were cultured in the presence of IL-4 and GM-CSF and then coincubated with tumor cells in wells of Transwell plates as described in *Materials and Methods.* after coincubation for 24 h at 37°C, the DC were harvested and stained with mAbs specific for the individual APM components. The percentage of stained cells was determined by flow cytometry.

that seen in iDC and mDC generated from ND's PBMC. Comparison of the fluorescence intensity (in MESF units) for all individual APM components showed similar expression in iDC obtained from patients with cancer and ND (Fig. 5). Changes in APM component expression during maturation of DC obtained from patients with cancer were also measured and found to be up-regulated in mDC vs iDC. The largest maturation-related increases were observed in the expression of Delta, LMP-2, TAP-1, calnexin, and HLA class I Ags (Fig. 5, *A–D*). However, these increases were modest compared with those seen with DC of ND (Fig. 5*E*). Furthermore, in some patients with metastatic melanoma, monocyte-derived DC failed to up-regulate APM component expression on exposure of iDC to the cytokine maturation mixture for 18–24 h. When pulsed with a $gp100_{209-217}$ peptide and used as stimulators in ELISPOT assays, these DC gave 1 log fewer spots than similarly matured and pulsed DC obtained from ND (data not shown).

APM component down-regulation in DC incubated with tumor cells

To study the effects of tumor cells on APM component expression in DC, iDC obtained from 6 ND were coincubated with HNC tumor cell lines (PCI-4B and PCI-38) in Transwell plates. At the end of a 24-h incubation at 37°C, DC were tested for APM component expression. As shown in Table VII, percentages of DC expressing MB1, LMP-2, LMP-7, LMP-10, and ERp57 proteins were consistently decreased on coincubation with tumor cells. In contrast, no significant change was detected in the percentage of DC expressing Delta, calnexin, calreticulin, and tapasin. HLA class I and class II Ag expression was also reduced in iDC incubated with tumor cells (data not shown).

To compare effects of tumor cell supernatants on down-regulation of APM component expression in iDC vs mDC, the Transwell experiments were performed under different conditions: 1) after maturation with cytokines; 2) during maturation; and 3) before maturation. These experiments showed that tumor cell supernatants consistently down-regulated early APM component expression in iDC but not in DC undergoing maturation in the presence of cytokines and in mDC.

Functional defects in DC after coincubation with tumor cells

Additional experiments tested whether APM component down-regulation observed in DC coincubated with tumor cells was associated with an impairment of their Ag-processing capability. To this end, iDC were incubated with a tumor cell supernatant for 18 h, pulsed with a Mel526 cell lysate, and matured in the presence of cytokines. Control iDC, which had not been incubated with tumor cell supernatant, were pulsed with the same Mel526 cell lysate, incubated in medium alone, and matured. The mDC were then cocultured with autologous PBMC for 7 or 14 days. T cells generated in the control or experimental cocultures were tested in ELISPOT assays for IFN-γ production in the presence of Mel526 cells as stimulators. As shown in Fig. 6, when DC exposed to tumor cell supernatants were used for IVS, they generated T cells which were unresponsive to Mel526 cells. In contrast, DC not treated with tumor cell supernatants induced T cells that responded to Mel526 targets by IFN-γ production, giving a high number of spots. This response was HLA class I-restricted, because it was inhibited by mAb W6/32 (Fig. 6).

In addition, at the termination of the IVS cultures on day 14, the number and viability of T lymphocytes were determined. Table VIII shows that the number of viable T lymphocytes recovered from cocultures with DC pretreated with tumor cell supernatants was significantly decreased compared with the number of T cells recovered from control cocultures. Thus, DC pretreated with tumor cell supernatants failed to support expansion and/or survival of T lymphocytes in these cocultures.

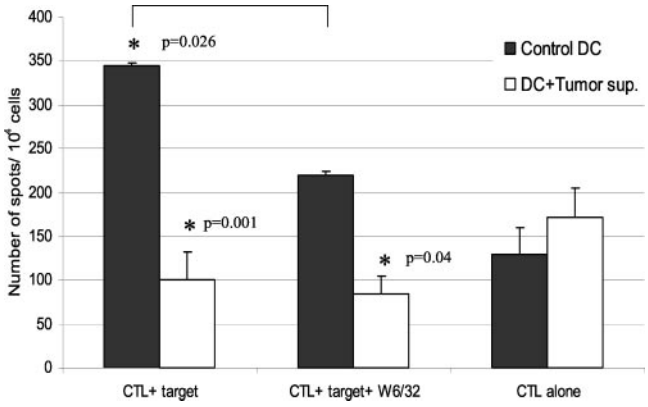


FIGURE 6. Inhibitory effects of coincubation of DC with tumor cells in a Transwell system. iDC generated from ND's PBMC were incubated with PCI-13 tumor cells plated in Transwells for 24 h, pulsed with a Mel562 cell lysate, and matured with cytokines (IL-1β, IL-6, TNF-α). Control iDC were incubated in medium before pulsing with the Mel526 cell lysate and maturation. Cocultures with autologous PBMC were then established with these mDC. T cell were harvested on day 14 and tested by ELISPOT assay for IFN-γ production after stimulation by Mel562 cells. Histograms show mean numbers of spots in experimental (DC coincubated with tumor) vs control wells (DC incubated with medium). Inhibition of spot formation with the W6/32 mAb indicates that T cells generated in the IVS cultures were MHC class I restricted. Data are mean numbers of spots in triplicate wells ± SD. *p* values refer to differences in spot numbers between experimental and control wells. sup, Supernatant.

Table VIII. *Decreases in T lymphocyte number in 14-day IVS cultures containing DC pretreated with tumor cell supernatants*[a]

Treatment of DC with Tumor Supernatants	Expt. 1	Expt. 2	Expt. 3	Expt. 4
No	7.0	3.6	3.2	1.6
Yes	3.2	0.8	1.0	1.0

[a] The IVS cocultures of DC with autologous PBMC were set up as described in *Materials and Methods.* Total numbers $\times 10^6$ viable T cells recovered from each culture are shown. $p = 0.019$ for the difference between treated and untreated DC for the four experiments.

Discussion

We have measured for the first time levels of all the APM components in DC and have demonstrated up-regulation in expression of some of these components in mDC. Our findings are in agreement with the scanty information in the literature about expression of some of the APM components (9, 10) and HLA class I molecules (10) in DC. Because DC are responsible for driving and orchestrating the quality as well as the magnitude of TA-specific immune responses, APM component expression is likely to play a critical role in this process. As in other cell types, the Ag processing/presentation pathway in DC consists of a series of molecules, each responsible for a distinct functional step and each necessary for implementing an effective interaction with a targeted T cell (3, 4). Whereas iDC are responsible for Ag uptake as well as processing, mDC present Ags to T cells (1, 2). In this article, we demonstrate not only that iDC and mDC express different levels of APM components but also that maturation-associated up-regulation of the APM components translates into more effective DC interaction with autologous T cells as evidenced by their increased recognition of tumor cells.

Although adoptive transfers of DC in the form of vaccines are being used with increasing frequency in patients with cancer, to the best of our knowledge, no information is available about expression of individual APM components in DC used for therapy. Although it has been acknowledged that the maturation step in a mix of cytokines is important for the quality of DC-driven immune responses (19), the molecular changes associated with these functional phenomena have remained unknown. We have demonstrated that DC maturation allows for a better expression of APM components in these cells. Thus, mDC were shown to express higher levels of APM components and in a greater proportion of cells than iDC. In parallel in vitro experiments, these mDC were shown to generate superior TAA-specific T cell responses, as measured in ELISPOT assays.

The second DC-related issue in the therapeutic use of autologous antitumor vaccines concerns the quality of DC generated ex vivo from monocytes obtained from patients with cancer. In view of reports in the literature describing defective dendropoiesis or tumor-induced apoptosis of DC in patients with cancer (14, 15), this is a legitimate concern. For this reason, we compared expression of APM components in iDC and mDC generated ex vivo from monocytes of patients with various malignancies and found that it is suppressed relative to that seen in DC of ND. APM component expression was generally not up-regulated during maturation in cytokines to the levels observed in mDC obtained from ND. In some patients with melanoma, it was not up-regulated at all, suggesting a need for a stronger maturation signal. This is an important observation, which focuses attention on the composition of the cytokine mix used for ex vivo DC maturation. As recently shown by Kalinski and colleagues (19), ex vivo polarization of DC in appropriately selected mix of cytokines determines the quality of T cell responses induced ex vivo and, presumably, in vivo. The addition of IFN-α, IFN-γ, and poly(IC) to the conventional maturation mixture (20) appears to induce higher expression of APM components in DC of patients with cancer.

Finally, we investigated ex vivo effects of tumor cells on APM component expression in human DC and on the functional potential of the DC to generate TAA-specific effector T cells. Dysfunction of DC, including defects in Ag processing/presentation, might provide tumor cells with a mechanism of escape from the host's immune system. For example, down-regulation of proteins responsible for processing and/or presentation of TAA could impair the generation of TAA-specific immune responses (21, 22). Although various tumor cells probably have the ability to process Ags, it has now been accepted that DC are largely responsible for processing and cross-presentation to T cells of TA internalized in the form of apoptotic/necrotic tumor cells or Ag-Ab complexes (23).

Using tumor cell lines established by us and previously shown to exert inhibitory ex vivo effects on human DC (PCI-4B and PCI-38; our unpublished data), we demonstrated that in a Transwell system, these tumor cells down-regulated expression of several APM components in iDC. In addition, functions of these DC coincubated with tumor cells were impaired. When DC coincubated with tumor cells were used for ex vivo generation of TA-specific T cells, the cultures contained a significantly lower frequency of such T cells than the control cultures. These results show that human tumor cells or their supernatants have profound effects on the Ag-processing function of DC and on their ability to prime T cells. It is likely that similar mechanisms operate in vivo. Therefore, adoptive transfers of DC to patients with cancer should be combined with cytokines able to up-regulate expression of APM components in DC and to protect these APC from tumor-induced dysfunction.

The data reported here provide important insights into the crucial significance of the Ag-processing pathway in human DC for the generation and maintenance of TAA-specific immune responses. They also indicate that tumors can exert strongly inhibitory effects on DC and suggest that in the context of biotherapies with DC, it is necessary to focus attention on the quality of Ag-processing functions in adoptively transferred DC. Lastly, the association between APM component down-regulation and functional defects of DC suggests that these components could serve as molecular markers to monitor the quality of DC preparations used in active specific immunotherapy of patients with cancer. If additional studies in a large number of patients show an association between APM component down-regulation in DC and defects in the induction of TAA-specific immune responses, monitoring of APM component expression in DC preparations may be used for evaluation of their functional quality.

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