

Innate Direct Anticancer Effector Function of Human Immature Dendritic Cells. I. Involvement of an Apoptosis-Inducing Pathway¹

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Dendritic cells (DCs) mediate cross-priming of tumor-specific T cells by acquiring tumor Ags from dead cancer cells. The process of cross-priming would be most economical and efficient if DCs also induce death of cancer cells. In this study, we demonstrate that normal human *in vitro* generated immature DCs consistently and efficiently induce apoptosis in cancer cell lines, freshly isolated noncultured cancer cells, and normal proliferating endothelial cells, but not in most normal cells. In addition, *in vivo* generated noncultured peripheral blood immature DCs mediate similar tumoricidal activity as their *in vitro* counterpart, indicating that this DC activity might be biologically relevant. In contrast to immature DCs, freshly isolated monocytes (myeloid DC precursors) and *in vitro* generated mature DCs are not cytotoxic or are less cytotoxic, respectively, suggesting that DC-mediated killing of cancer cells is developmentally regulated. Comparable cytotoxic activity is mediated by untreated DCs, paraformaldehyde-fixed DCs, and soluble products of DCs, and is destructible by proteases, indicating that both cell membrane-bound and secreted proteins mediate this DC function. Overall, our data demonstrate that human immature DCs are capable of inducing apoptosis in cancer cells and thus to both directly mediate anticancer activity and initiate processing of cellular tumor Ags. *The Journal of Immunology*, 2002, 168: 1823–1830.

Bone marrow-derived dendritic cells (DCs)³ are professional APCs that are optimally suited to mediate the cross-priming of effector T cells reactive against specific proteins and glycolipids (1–3). In the cancer setting, DC cross-priming has been intensively scrutinized with the ultimate goal of promoting enhanced protective or therapeutic immunity. The major sequential steps of this T cell priming process have been well defined (1–10). Thus, DCs first capture and digest dead cancer cells, then process their proteins into peptide epitopes, and finally present these epitopes in the context of the cell surface expressed MHC molecules to TCRs on specific CD4⁺ and CD8⁺ T cells. The consequent generation of antitumor effector T cells is invigorated and regulated by costimulatory molecule and cytokine signals contributed by DCs (4–10). These observations have led to the belief that, although DCs provide critical help to the evolution of clinically relevant, tumor-reactive T cells, their role is largely

that of Ag scavengers, processors, and presenters, but not that of antitumor effector cells.

It has been generally presumed that the majority of tumor debris acquired by cross-presenting DCs is the result of either spontaneous tumor necrosis or specific T cell- or nonspecific NK cell-mediated tumor cell death. A far more efficient process can be envisioned that involves direct DC-mediated killing of tumor cells, with the subsequent ingestion of resulting dead cancer cells and associated tumor Ags. Indeed, it has been shown that human activated DCs acquire the ability to directly kill rare tumor cell lines (11, 12). However, it is unlikely that activated DCs are the physiological mediators of the presumed activity, because they are induced by cytokines produced in an immune response (1–3) and therefore could not be involved in the initiation of cross-priming. More likely, physiological mediators of the antitumor cytotoxicity might be immature DCs, as they are produced by normal hematopoiesis, reside in the periphery (1–3), and are able to both populate cancer tissues (13) and uptake cellular debris (5–9). This hypothesis has remained untested.

In this study, we determined that both *in vitro* and *in vivo* generated human blood-derived immature DCs are potent and promiscuous anticancer cytotoxic cells capable of inducing efficient and selective apoptotic death in a variety of human cancer cell lines and freshly isolated tumor cells, but not in normal cells.

Materials and Methods

Reagents

The following Abs were applied in this study: FITC-conjugated anti-human CD3 (IgG1) mouse mAb, PE-conjugated anti-human CD56 (IgG1) mouse mAb, FITC anti-human CD56 (IgG2b) mouse mAb, FITC anti-human CD16 (IgG1) mouse mAb, PE anti-human CD14 (IgG2a) mouse mAb, FITC anti-human CD14 (IgG2a) mouse mAb, FITC anti-human CD19 (IgG1) mouse mAb (all from BD Biosciences, San Jose, CA); FITC anti-human CD80 (IgM) mouse mAb and PE anti-human CD86 (IgG1) mouse mAb (Ansell, Bayport, MN); FITC anti-human CD11c (IgG1)

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Received for publication July 16, 2001. Accepted for publication December 5, 2001.

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¹ This work was supported by National Institutes of Health-National Institute of Dental and Craniofacial Research Oral Cancer Center of Discovery Grant 1-PO DE13059-01 and National Institutes of Health Grant RO1 CA 82016. B.M.J. is a visiting research fellow from the Institute for Oncology and Radiology (Pasterova, Belgrade, Yugoslavia) and was supported by National Institutes of Health Oral Cancer Center Grant 1-PO DE13059.

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³ Abbreviations used in this paper: DC, dendritic cell; PBMNL, peripheral blood mononuclear leukocyte; SCCHN, squamous cell carcinoma of the head and neck; fmk, fluoromethyl ketone; Z-VAD-Fmk, Z-Val-Ala-Asp(OMe)-fmk; NuMA, nuclear matrix protein; PI, propidium iodide; BrdU, 5-bromo-2'-deoxyuridine; CD40L, CD40 ligand; DiOC₆(3), 3,3'-dihexyloxycarbocyanine iodide.

mouse mAb and PE anti-human CD33 (IgG2b) mouse mAb (Caltag Laboratories, Burlingame, CA); FITC anti-human CD40 (IgG1) mouse mAb (Ancell); PE anti-human CD83 (IgG2b) mouse mAb (Immunotech, Marseille, France); FITC anti-human HLA-ABC class I (IgG2a) mouse mAb (Serotec, Burlington, Ontario, Canada); PE anti-human HLA-DR (IgG2a) mouse mAb and biotin-conjugated anti-human IL-3R α (IgG2a) mouse mAb (BD Biosciences); FITC anti-human TCR $\alpha\beta$ (IgG1) mouse mAb, FITC H130 anti-human CD45 (IgG1) mouse mAb, PE H130 human DC45 (IgG1) mouse mAb, and conjugated isotype control mAbs (Caltag Laboratories); anti-human CD3 (IgG1) mouse mAb, anti-human CD5 (IgG1) mouse mAb, anti-human CD19 (IgG1) mouse mAb, anti-human CD56 (IgG2a) mouse mAb, anti-human CD16 (IgG1) mouse mAb, and anti-glycophorin A (IgG1) mouse mAb (DAKO, Carpinteria, CA).

The following cytokines and ligands were used: recombinant human GM-CSF and recombinant human IL-4 (both kindly provided by Schering-Plough Research Institute, Kenilworth, NJ), and recombinant human trimeric CD40 ligand (CD40L; kindly provided by Immunex, Seattle, WA).

The following inhibitors of apoptosis were used: caspase-8 inhibitor IETD-fluoromethyl ketone (fmk), caspase-3 inhibitor DEVD-CHO, caspase-9 inhibitor Ac-LEHD-CHO (Calbiochem, San Diego, CA), pan-caspase inhibitor Z-Val-Ala-Asp(OMe)-fmk (Z-VAD-fmk; Enzyme Systems Products, Livermore, CA), and adenine nucleotide translocase inhibitor bongkrekic acid (Biomol, Plymouth Meeting, PA).

Isolation of monocytes

Monocytes (CD5⁻CD3⁻TCR $\alpha\beta$ ⁻CD19⁻CD56⁻CD16⁻CD14⁺) were purified from normal human peripheral blood mononuclear leukocytes (PBMNL) to 95% purity using a modification of the previously described negative immunoselection technique with Ab-coated magnetic beads (14).

Isolation of DCs

DCs were purified from PBMNL as 90% pure populations of CD3⁻TCR $\alpha\beta$ ⁻CD14⁻CD19⁻CD16⁻CD56⁻CD11b⁻CD4⁺HLA-DR⁺ cells by the use of a MACS Blood Dendritic Cell Isolation kit (Miltenyi Biotec, Auburn, CA), as described in the manufacturer's protocol. To obtain highly purified (i.e., 95%) populations of DCs, the enriched DC populations were simultaneously labeled with FITC-conjugated anti-TCR $\alpha\beta$, anti-CD14, anti-CD19, anti-CD56, and PE-conjugated anti-HLA-DR mAbs and sorted as TCR $\alpha\beta$ ⁻CD14⁻CD19⁻CD56⁻HLA-DR⁺ cells using a FACStar^{plus} cell sorter (all mAbs and the cell sorter were from BD Biosciences).

DC cultures

Immature DCs were generated using a slight modification of the previously described technique (15). Briefly, freshly purified monocytes were resuspended (12.5×10^6 cells/2.5 ml) in AIM-V medium containing 2.5% FCS (both from Life Technologies, Long Island, NY), placed into T25 tissue culture flasks (Falcon, BD Labware, Franklin Lakes, NJ) and incubated at 37°C for 2 h. Following this incubation, the nonadherent cells were removed and the adherent cells were washed carefully five times with warm (37°C) RPMI 1640 (Life Technologies) medium. After the last washing, the adherent cells were supplemented with AIM-V 2.5% FCS medium containing 1000 U of IL-4 and 1000 U of GM-CSF and cultured for 5–7 days.

Mature DCs were generated from day 5 immature DCs by an additional 2-day incubation in the presence of IL-4, GM-CSF (1000 U/ml), and CD40L (2 μ g/ml) (16). Following the above-described two-step purification of monocytes, including negative selection on immunomagnetic beads and adherence, the isolated adherent DC precursors (monocytes) as well as monocyte-derived DCs were consistently 98% pure populations of CD14⁺ and CD3⁻CD14⁻CD19⁻CD56⁻CD16⁻MHC-ABC⁺MHC-DR⁺ cells, respectively.

Cell lines and freshly isolated tumor cells

All cell lines were of human origin and mycoplasma free. Normal cell lines included skin keratinocytes (NHEK-Ad), dermal fibroblasts (NHDF-Ad), epidermal melanocytes (HMEM-Neo), mammary epithelial cells (Clonetics, BioWhittaker, Walkersville, MD), HUVEC (Cell Systems, Kirkland, CA), and T cell blasts (PBMNL stimulated for 7 days with 10 μ g/ml Con A). Leukemia cell lines used in this study were K562 myeloid leukemia, Daudi Burkitt's B cell lymphoma, MOLT-4 acute lymphoblastic leukemia, and Jurkat T cell leukemia (American Type Culture Collection (ATCC), Manassas, VA). Solid tumor-derived cell lines included gliomas P303, P319 (University of Pittsburgh Cancer Institute (UPCI), Pittsburgh, PA), and SNB19 (National Institutes of Health, Bethesda, MD); melanomas FemX (ATCC), Pmel 136.34, and Pmel 255.1 (UPCI); breast carcinomas BT-20, MCF-7, and SK-BR-3 (ATCC); lung squamous cell carcinomas LC

226, LC 358 and LC 596 (UPCI); lung small cell carcinomas LC H69 and LC H345 (UPCI); HR gastric carcinoma (UPCI); colon carcinomas LS-174 and LS-180 (NeoRx, Seattle, WA); SK-OV-3, Caov-3, and OVCAR-3 ovarian carcinomas (ATCC); renal cell carcinoma (UPCI); and squamous cell carcinomas of the head and neck (SCCHN) PCI-1, PCI-4A, PCI-4B, PCI-6A, PCI-6B, PCI-13, PCI-15A, PCI-15B, PCI-22A, PCI-22B, PCI-30, and PCI-37A (UPCI). The cell lines were grown under standard cell culture conditions, using the optimal culture conditions, as previously described (17). Fresh viable tumor cells were isolated directly from surgical specimens of SCCHN primary tumors by collagenase digestion and purification with annexin V magnetic beads (MACS; Miltenyi Biotec).

Cytotoxicity assays

⁵¹Cr release and [³H]thymidine release assays. ⁵¹Cr release and [³H]thymidine release assays were performed as previously described (17).

MTT assay. MTT assay, previously used to measure antitumor cell-mediated cytotoxicity (18), was modified for adherent effector cells. In addition to the previously used controls background of medium alone and total viability/spontaneous death of untreated target cells, we used a new control, background of effector cells. Experimental wells contained medium and both DCs and tumor cells. The percentage of cytotoxicity was calculated using the following formula: % cytotoxicity = (TS – BG0) – (E – BGEC)/(TS – BG0) \times 100, where BG0 is background of medium alone, TS is total viability/spontaneous death of untreated target cells, BGEC is background of effector cells, and E is experimental well.

NuMA release assay. Nuclear matrix protein (NuMA) release assay was used to measure decay of the essential cellular protein and thus to demonstrate cell death using the NMP 41/7 ELISA kit (Oncogene Research Products, Boston, MA), according to the manufacturer's specifications. DCs and tumor cells were coincubated for 72 h. Spontaneous release of NuMA from target cells alone, spontaneous release of NuMA from effector cells alone, and total release of NuMA from target cells induced by irradiation with ultraviolet C rays represented control samples. Release of NuMA following coincubation of DCs and tumor cells at various DC:tumor (E:T) ratios was experimental sample. Cytotoxicity was calculated according to the following formula: % cytotoxicity = E – (S1 + S2)/(T – S1) \times 100, where S1 is spontaneous release of NuMA from target cells alone, S2 is spontaneous release of NuMA from effector cells alone, T is target cells induced by irradiation with ultraviolet C rays, and E is experimental sample.

Annexin V assays. Annexin V assays were used to detect target cells showing externalization of the phosphatidylserine on the outer leaflet of cell membrane, which represents an early feature of apoptosis (19). Target cells were exposed for 4 h to DCs or DC culture-conditioned medium. After removal of the effectors, target cells were maintained in culture for an additional 20 h to allow for apoptosis to occur. Tumor cells were harvested by gentle trypsinization and were then stained with 1 μ g/ml FITC-conjugated Annexin V (Molecular Probes, Eugene, OR) or 10 μ g/ml propidium iodide (PI) and PE- or FITC-conjugated anti-CD45 mAb, respectively. Cells were analyzed by flow cytometry as previously outlined (17).

DiOC₆(3) assessment. 3,3'-Dihexyloxycarbocyanine iodide (DiOC₆(3)) assessment of changes of the mitochondrial transmembrane potential, which characterize apoptosis, was performed as previously described (20). Following the induction of killing, as described above for the annexin V assay, but before flow cytometry analysis, target cells were stained with 40 nM DiOC₆(3) solution for 15 min at 37°C and then with PE-conjugated anti-CD45 mAb.

TUNEL assays. TUNEL assays were performed by staining nuclear DNA in situ with the death detection kit (Roche Diagnostic Systems, Indianapolis, IN), followed by flow cytometry analysis, as per the manufacturer's instructions. Tumor cell death was induced by DCs as described above for the annexin V assay, and the resulting cells were first stained with PE-conjugated anti-CD45 mAb and then fixed with 4% paraformaldehyde, labeled with TUNEL, and analyzed by flow cytometry (17).

Internucleosomal DNA fragmentation. The internucleosomal DNA fragmentation in target cells exposed to DCs was assessed by a modified DNA laddering assay. Nuclear DNA was labeled in viable target cells by 5-bromo-2'-deoxyuridine (BrdU; Boehringer Mannheim-Roche, Indianapolis, IN). Apoptosis and internucleosomal DNA fragmentation in the BrdU-labeled target cells were induced by their coincubation with DCs. E:T ratios and the time of the coincubation were as indicated. After the coculture, the cells were harvested and their DNA was extracted and purified using the manufacturer's procedure for the TACS DNA Laddering Apoptosis Detection kit (R&D Systems, Minneapolis, MN). Samples of purified DNA (0.75 μ g) were then loaded on 1% agarose gel, and horizontal gel electrophoresis was performed for 4 h at room temperature. DNA was

then transferred from the gel onto uncharged nylon membrane (Millipore, Bedford, MA) and BrdU-labeled DNA was detected by standard Western blotting procedures using sequential incubations of the membrane with primary anti-BrdU mAb (Boehringer Mannheim-Roche), biotin-conjugated secondary goat anti-mouse Ig polyclonal Ab (Jackson Immuno-Research Laboratories, West Grove, PA), streptavidin-HRP, HRP substrate, and ECL reagent (Trevigen, Gaithersburg, MD).

Flow cytometry

All analyses were performed using a FACScan (BD Biosciences) flow cytometer. Phenotypes of monocytes and DCs were determined by direct two-color staining with fluorochrome-conjugated mAbs specific for the lineage markers of T cells, B cells, NK cells, monocytes, and DCs, as previously described (21).

Statistical analysis

$LU_{20}/10^7$ effector cells were determined using the formula $10^7/(T \times X_{20})$, where T is the number of target cells and X_{20} is the estimated E:T ratio at which 20% of the target cells were killed. Statistical analyses of results were performed using the Wilcoxon's signed-rank pair tests. To determine whether the data provide evidence for differences in profiles of percent killing as a function of E:T ratio, multivariate permutation methods were used. Differences were considered significant when the value of p was < 0.05 .

Results

Killing of cancer cells by DCs

First, we examined whether *in vitro* generated, monocyte-derived DCs could mediate death of cancer cells and, if they could do so, whether death was the result of cell necrosis or apoptosis. We produced highly pure populations (95%) of $CD3^-CD14^-CD19^-CD56^-$ immature and mature DCs from purified $CD14^+$ blood monocytes by *in vitro* culture and stimulation with GM-CSF plus IL-4 and/or GM-CSF plus IL-4 plus CD40L, respectively. The two DC populations displayed predictably distinct morphology (data not shown), distinct phenotypes (i.e., immature DCs were $CD83^-$ and $CD80^{low}CD86^{low}CD40^{low}$ MHC class I^{int}MHC class II^{int}, while mature DCs were $CD83^+$ and $CD80^{high}CD86^{high}CD40^{high}$ MHC class I^{high}MHC class II^{high}) (Fig. 1), and distinct functions (i.e., immature DCs exhibited high phagocytic and intermediate MLR-inducing activities, while mature DCs had low phagocytic and high MLR-inducing activities) (Ref. 3 and data not shown).

Initially, immature DCs were tested for killing ability against PCI-13 SCCHN cell targets using a variety of apoptosis-specific assays (Fig. 2). In all the assays applied, DCs mediated significant anticancer cytotoxic activity, effective at low E:T cell ratios (i.e., 0.1:1–3:1). Thus, DCs were capable of inducing in cancer cells substantial fragmentation and release of [³H]thymidine-labeled DNA (16–24%) within 1 h (Fig. 2A), the formation of typical DNA ladders over 2–8 h of coculture (Fig. 2B), and a significant increase of TUNEL incorporation into nuclear DNA following 24 h of the cellular interaction (Fig. 2C). These results unequivocally demonstrated that DCs effectively induced apoptosis-specific internucleosomal fragmentation of DNA into 200-bp fragments and their oligomers in the nuclei of cancer cells. Next, we demonstrated that DCs induced ~30% of cancer cells to become reactive with annexin V (Fig. 2D) but only 5% of cancer cells to be stained with PI (data not shown), after 4 or 24 h of incubation. Therefore, this interaction provoked apoptosis-specific phosphatidylserine externalization on the tumor cell membrane. DCs also induced a large proportion of cancer cells to become nonreactive with MTT (Fig. 2E), DiOC₆(3) (Fig. 2F), or rhodamine 123 (data not shown) within 3–24 h of their cocultivation. These results showed that DCs effectively promoted apoptosis-specific mitochondrial damage and dysfunction in cancer cells by affecting mitochondrial dehydrogenases (assessed in MTT assay) and/or mitochondrial transmembrane potential (18), as measured in

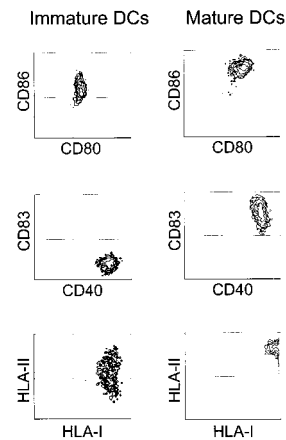


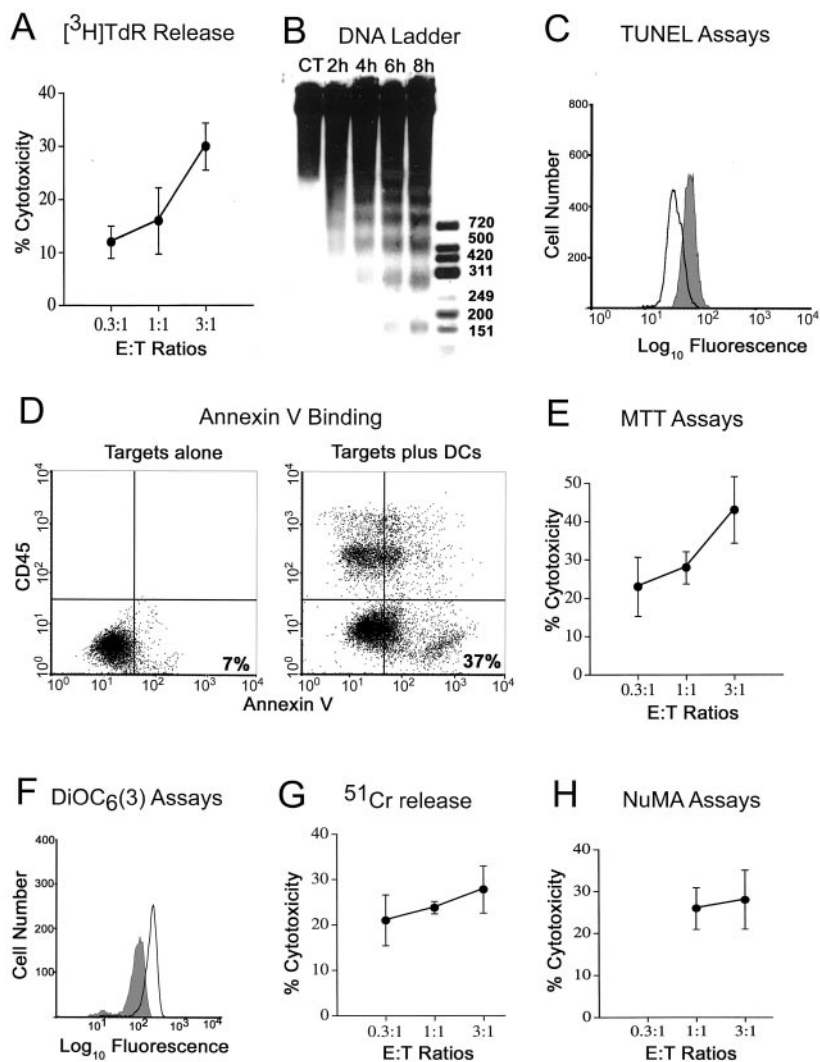
FIGURE 1. Phenotypes of immature and mature DCs used in this study. Pure populations of immature and mature DCs (95% $CD3^-CD14^-CD19^-CD56^-$) were generated from purified (95% $CD14^+$) normal blood monocytes. Immature DCs were produced by 7-day GM-CSF plus IL-4 stimulation of monocytes. Mature DCs were generated from monocytes by their 5-day stimulation with GM-CSF plus IL-4 followed by 2-day induction with GM-CSF plus IL-4 plus CD40L. At the end of these cultures, immature and mature DCs were harvested, stained with fluorochrome-conjugated mAbs against the indicated cell surface markers, and analyzed using two-color flow cytometry, as described in *Materials and Methods*. The data are two-color contour maps for FITC (FL1) and PE (FL2) fluorescence.

DiOC₆(3) and rhodamine 123 assays. DCs also lysed 20–30% of cancer cells in either 24- or 72-h ⁵¹Cr release (Fig. 2G) or NuMA release (Fig. 2H) assays, respectively. These results indicated that DCs mediated disruption of cancer cell membrane and disintegration of nuclear proteins, respectively, at later time points of the interaction. Notably, no killing of tumor target cells was detected in assays measuring necrosis, such as 4-h ⁵¹Cr release (17) or PI uptake (data not shown). Therefore, a substantial proportion of cancer cells that are exposed to DCs appears to be killed, and this killing is mediated by apoptotic mechanism(s).

Signaling pathways and role of caspases in DC-induced apoptosis of cancer cells

The cell stress-induced mitochondrial pathway and the death receptor-transduced pathway are main signaling mechanisms of apoptosis (22, 23). The former involves an early activation of the caspase-9 (22), while the latter involves an early activation of the caspase-8 (23). Each of two pathways is able to activate the caspase-3 and other common effector caspases. In addition, they can activate and amplify each other. To assess the potential role of two apoptotic signaling pathways and activation of caspases in DC-mediated killing of cancer cells, we examined the use of relevant caspases and involvement of mitochondria by applying the specific inhibitors (Table I). We found that pharmacological inhibitors of the caspase-8 (IETD-fmk), caspase-9 (Ac-LEHD-CHO), mitochondrial permeability transition pore (bongkrekic acid), caspase-3 (DEVD-CHO), and pan-caspases (Z-VAD-fmk) inhibited a notable proportion of DC-mediated killing of cancer cells (49, 41, 52, 57, and 67%, respectively). These data show that the two main apoptotic pathways and activation of caspases have critical roles in DC induction of apoptosis in cancer cells. The findings also indicate that in the course of DC-mediated killing of cancer cells either of two pathways is primarily and independently activated or one of the pathways is primarily activated while the other is secondarily activated.

FIGURE 2. DCs efficiently kill cancer cells by inducing apoptosis. Various cytotoxicity assays were performed with immature DCs as effectors and PCI-13 SCCHN cells as targets. *A*, One-hour [3 H]thymidine ([3 H]TdR) release assays. *B*, Two- to 8-h DNA laddering assays. *C*, Twenty-four-hour TUNEL assays. *D*, Twenty-four-hour annexin V assays. *E*, Three-hour MTT assays. *F*, Twenty-four-hour DiOC₆(3) assays. *G*, Twenty-four-hour 51 Cr release assays. *H*, Seventy-two-hour NuMA protein release assays. Each presented result is from one representative experiment of at least three performed. *A*, *E*, *G*, and *H*, Data are mean percentages of killing \pm SD. *B–D* and *F*, Results obtained in the cytotoxicity assays performed in a 3:1 E:T ratio. *B*, Data are blots of electrophoresed DNA of target cells, selectively prelabeled with BrdU and revealed by anti-BrdU mAb and ECL. CT, Target cells incubated alone for 8 h; 2, 4, 6, and 8 h, time periods of coincubation of target cells with DCs. *C* and *F*, The data were obtained by FACS analysis of CD45⁺ cells, following double-labeling of effector and target cells with fluorochrome-conjugated anti-CD45 mAb and a corresponding apoptosis marker. The data are overlays of single-color histograms of log₁₀ fluorescence intensity obtained with apoptosis markers using untreated (open histogram) or DC-treated (filled histogram) cancer cells. *D*, Dot-plots of two-color flow cytometry of untreated (Targets alone) or DC-treated (Targets plus DCs) cancer cells.



Kinetics of DC-mediated tumor cell death

To determine the time dependency of killing of cancer cells by DCs, we performed kinetic experiments. In MTT assays, we observed substantial killing of cancer cells by 30 min and an increase of the activity for up to 3 h of coincubation (Fig. 3). After 3 h, no further increase was evident. Similarly, in DNA laddering assays, the bands of 200-bp DNA fragments and their oligomers were readily visible after 2 h, reaching peak levels after 8 h of coincu-

bation (Fig. 2*B*). Therefore, the killing of tumor cells mediated by DCs was very rapid.

Ability of *in vivo* generated immature DCs to kill cancer cells

To evaluate the *in vivo* relevance of our *in vitro* findings, we examined whether freshly isolated, noncultured, normal blood donor DCs could kill cancer cells. The DCs directly obtained from PBMNL had a typical immature DC phenotype. Thus, they

Table I. DCs induce apoptosis in cancer cells via death receptor and mitochondrial signaling pathways^a

| Treatment | % Cytotoxicity | | | LU ₂₀ /10 ⁷ DCs |
|-------------------------------|----------------|---------------|-----------------|---------------------------------------|
| | 3:1 E:T ratio | 1:1 E:T ratio | 0.3:1 E:T ratio | |
| DMSO | 20.6 | 9.3 | 5.0 | 727.9 |
| IETD-fmk (100 μ M) | 13.0 | 3.0 | 2.7 | 373.7 |
| Ac-LEHD-CHO (50 μ M) | 13.9 | 5.8 | 0.9 | 427.9 |
| Bongkreikic acid (50 μ M) | 12.7 | 2.3 | 0.0 | 346.7 |
| DEVd-CHO (50 μ M) | 10.9 | 3.7 | 0.0 | 311.5 |
| Z-VAD-fmk (100 μ M) | 8.3 | 3.7 | 1.2 | 243.5 |

^a PCI-13 cell targets were labeled with [3 H]thymidine and preincubated with the indicated reagents at 37°C for 1 h. After that, target cells were mixed with *in vitro* generated immature DCs in three different E:T ratios and 3-h [3 H]thymidine release cytotoxicity assays were performed. The presented results are from a representative experiment of six similar experiments performed. The data are mean percentages of cytotoxicity obtained in triplicate and LU₂₀/10⁷ DCs. DMSO concentration in both control and experimental wells was 0.5%. IETD-fmk, Caspase-8 inhibitor; Ac-LEHD-CHO, caspase-9 inhibitor; bongkreikic acid, mitochondrial permeability transition pore inhibitor; DEVd-CHO, caspase-3 inhibitor; and Z-VAD-fmk, pan-caspase inhibitor.

were CD3⁻TCR $\alpha\beta$ ⁻CD14⁻CD19⁻CD16⁻CD56⁻CD11b⁻CD4⁺HLA-DR⁺ (Fig. 4A and data not shown). They expressed low levels of CD80, CD86, and CD40, and moderate levels of MHC class I and class II molecules, but did not express the CD83 marker of mature DCs (Fig. 4A). Assessments of the anticancer cytotoxic activity of these DCs in 3- (Fig. 4B) and 24-h (data not shown) MTT assays revealed that they were also able to induce death in cancer cell targets. Therefore, *in vivo* and *in vitro* generated immature DCs were similar in their ability to mediate death of cancer cells. These observations suggest that this DC effector function might be physiologically relevant.

Susceptibility of cell targets to killing by DCs

To assess the range of target cells that are susceptible to killing by DCs, we next tested cytotoxicity of *in vitro* generated immature DCs against several normal and cancer cell lines and freshly isolated cancer cells, using 3-h MTT, 1-h [³H]thymidine release, and 24-h ⁵¹Cr release assays. Six normal cell lines, 36 malignant cell lines, and four different samples of fresh SCCHN cells, directly obtained from surgical specimens of primary tumors of head and neck cancer patients, were evaluated in total (Fig. 5). Normal proliferating endothelial cells, 35 of 36 tested leukemia and cancer cell lines, and four of four freshly isolated cancer cell samples were efficiently killed by DCs, in marked contrast to normal fibroblasts, keratinocytes, melanocytes, mammary epithelial cells, and autologous T cell blasts, which were not harmed by immature DCs. The only cancer cell line found to be relatively resistant to killing by DCs was PCI-30 SCCHN. These data demonstrate that immature DCs are promiscuous anticancer cytotoxic cells, which selectively kill cancer cells and spare normal cells.

Tumoricidal capacity of immature DCs vs monocytes and mature DCs

In contrast to immature DCs, their direct precursors, freshly isolated blood monocytes, did not show cytotoxic activity against cancer cells in our experiments (data not shown). Therefore, the anticancer killing ability appears to be acquired by immature DCs. Next, we tested the anticancer cytotoxic activity of mature DCs and compared it with that of immature DCs. In all three cytotoxicity assays used, including the 1-h [³H]thymidine release, 24-h MTT, and 24-h ⁵¹Cr release assays, mature DCs exhibited significant cytotoxic reactivity against cancer cells (Table II). However, the levels of killing by mature DCs were 4- to 4.9-fold lower than those observed for donor-matched immature DCs. This indicates

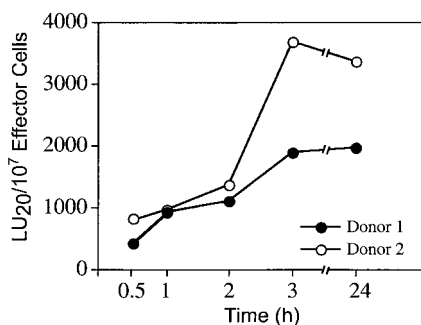


FIGURE 3. Immature DCs rapidly induce apoptosis in tumor cell lines. Kinetics of killing PCI-13 SCCHN cells by immature DCs were assessed using MTT assays. DCs were obtained *in vitro* from two different normal donors (Donor 1 and Donor 2) by GM-CSF plus IL-4 induction of monocytes, and simultaneously tested. Data are LU₂₀/10⁷ DCs, based on mean percentages of killing obtained in four E:T ratios (3:1, 1:1, 0.3:1, and 0.1:1), each performed in triplicate.

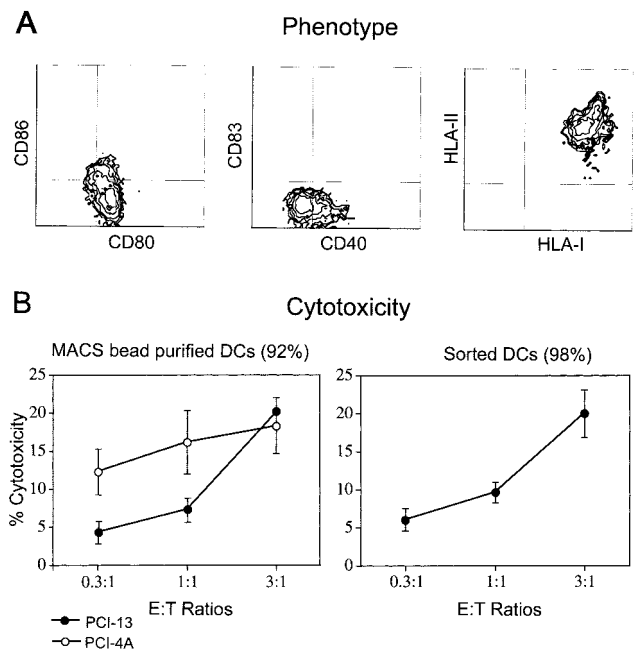


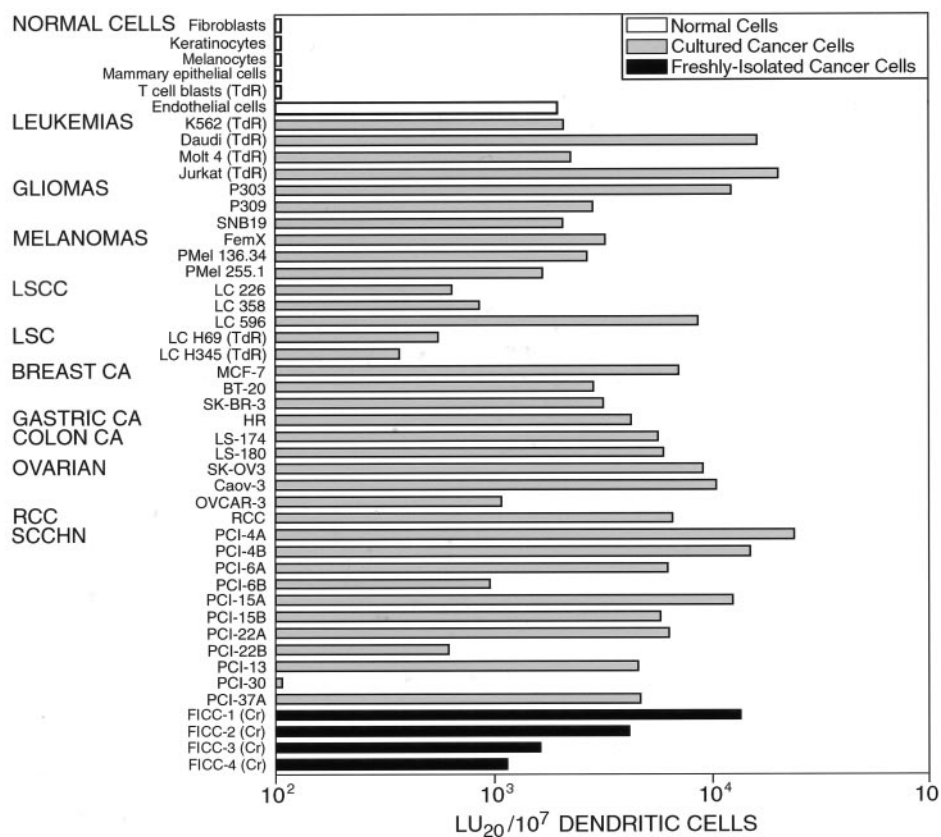
FIGURE 4. Freshly isolated normal donor blood DCs are cytotoxic for cancer cells. DCs were isolated and purified from normal PBMNL by MACS beads and/or cell sorting. These freshly isolated DCs were tested for phenotype using flow cytometry (A) and anticancer cytotoxic activity using 3-h MTT assays (B). A, Two-color contour plots produced by flow cytometry analysis. B, Mean percentages of cytotoxicity ± SD. In the parentheses are percentages of HLA-DR⁺CD3⁻CD56⁻CD14⁻CD19⁻ cells in purified populations of blood DCs used in the experiments shown. The presented results are from a representative experiment of five performed.

that maturation of DCs, known to be accompanied by their multiple phenotypic and functional changes, including that of Ag-presenting activities, is also accompanied by a significant decrease of their tumoricidal function.

Killing of cancer cells by DCs is mediated by both cell membrane-bound and secreted proteins

To examine the mechanisms underlying the cytotoxic activity of DCs against cancer cells, we evaluated the cytotoxic activity associated with paraformaldehyde-fixed DCs and DC culture-conditioned medium. It has been previously shown that cell fixation eliminates secretory activity and preserves cell membrane-bound cytotoxic ligands (17), while conditioned cell culture media may contain secreted soluble forms of cytotoxic ligands (24). We observed that both fixed DCs and DC culture-conditioned medium were cytotoxic for cancer cells (Table III). However, these cytotoxic activities were lower than those of control unmanipulated DCs. Similarly, inhibition of cellular secretion by elimination of extracellular Ca²⁺, using the Ca²⁺ chelation with EGTA or EDTA, only partially suppressed DC-mediated killing of cancer cells (data not shown). In contrast, the apoptotic activity of DC supernatants was efficiently inhibited by the proteases papain or trypsin (Fig. 6). The inhibition showed a dose dependency and was complete in the presence of ≥250 μg of the proteases per 1 ml of DC supernatant, whereas it gradually decreased at lower concentrations of the enzymes. These data suggest that the mediators of immature DC-associated tumoricidal activity are both cell membrane-bound and secreted proteins.

FIGURE 5. Immature DCs efficiently and promiscuously kill tumor cells but not normal cells. The susceptibility of various normal cells, cancer cell lines, and freshly isolated cancer cells (FICC) to killing by immature DCs was tested in 3-h MTT, 1-h [^3H]thymidine (TdR) release, and 24-h ^{51}Cr (Cr) release assays. The data are $\text{LU}_{20}/10^7$ DCs. LSCC, Lung squamous cell carcinomas; LSC, lung small cell carcinoma; RCC, renal cell carcinoma; CA, carcinomas. SCCHN cell lines labeled with A or B were originated from primary or metastatic lesions, respectively. The cytotoxicity against the targets was tested two to three times and similar results were obtained. The data presented are from one representative experiment.



DC-mediated cytotoxicity in normal individuals

To determine whether the observed anticancer apoptosis-inducing activity is a general function of DCs, we analyzed the killing ability of *in vitro* generated, monocyte-derived, immature DCs obtained from the peripheral blood of 25 different normal blood donors. We found that DCs isolated from all individuals evaluated were cytotoxic against cancer cell targets (Fig. 7). These data show that tumoricidal activity is an essential function of normal human immature DCs. We also observed a significant individual variability in the tested population of effectors obtained from different donors. This suggests that DC-mediated killing is genetically and/or environmentally determined.

Discussion

Our data provide novel information that human nonactivated immature DCs not only uptake death cancer cells and process tumor Ags at the periphery (1–9) but also selectively induce apoptotic death of cancer cells without damaging normal cells. In particular, our findings suggest that DCs induce rapid and marked internucleosomal DNA fragmentation, cell surface expression of phosphatidylserine, mitochondrial damage, and activation of caspases,

followed by a later-stage disintegration of the cell membrane and degradation of nuclear proteins in tumor cells. The rapid induction of both mitochondrial and DNA damages as well as the observation that DCs kill target cells in MTT assays before development of internucleosomal DNA fragments suggest an ordered progression of tumor cell destruction. These data are consistent with a mechanism in which DCs either simultaneously or subsequently induce mitochondrial damage and DNA fragmentation. In addition, our data show that both the caspase-8- and caspase-9-related apoptosis signaling pathways are simultaneously induced in tumor cells by DCs, indicating that signal transductions via TNF family death receptors and/or cell stress (22, 23) are involved in apoptotic killing of tumor cells by DCs. In a companion manuscript (25), we

Table II. Immature and mature DCs differ in their effectiveness to mediate apoptosis against cancer cells^a

| Effector Cells | $\text{LU}_{20}/10^7$ Effector Cells | | |
|----------------|---------------------------------------|----------|-------------------------------|
| | 1-h [^3H]thymidine release | 24-h MTT | 24-h ^{51}Cr release |
| Immature DCs | 281.3 | 6827.9 | 3056.9 |
| Mature DCs | 57.1 | 1697.9 | 650.6 |

^a Targets were PCI-4B SCCHN cells. All differences between the results obtained with two types of DCs were statistically significant ($p < 0.001$). The presented data are from a representative experiment of three similar experiments performed.

Table III. Fixed DCs and DC culture conditioned media are cytotoxic for human cancer cells^a

| Effectors | % Killing ($\text{LU}_{20}/10^7$ effectors) | | |
|----------------|--|---------------|---------------|
| | PCI-13 | PCI-4A | PCI-4B |
| Untreated DCs | 32.6 (1374.9) | 52.7 (6219.0) | 41.6 (2789.0) |
| Fixed DCs | 13.1 (168.3) | 36.4 (1802.4) | 25.4 (1278.0) |
| DC supernatant | 27.8 | 44.8 | 29.7 |

^a Immature DCs, either untreated or following fixation with 1% paraformaldehyde, were tested for cytotoxicity using MTT assays, as previously described (17). DC conditioned media were collected from cultures of immature DCs ($1 \times 10^6/\text{ml}$) following their incubation for 48 h. The assays were performed in triplicates. Data are mean percentages of killing obtained using a 3:1 E:T ratio and a 1:4 dilution of the conditioned culture media. SD of the results were $<10\%$ of the means. In parentheses are $\text{LU}_{20}/10^7$ DCs, calculated on the basis of mean percentages of killing obtained in four E:T ratios (3:1, 1:1, 0.1:1, and 0.01:1). The differences between killing mediated by untreated and fixed DCs were statistically significant ($p < 0.005$). The presented results are from a representative experiment of two similar experiments performed.

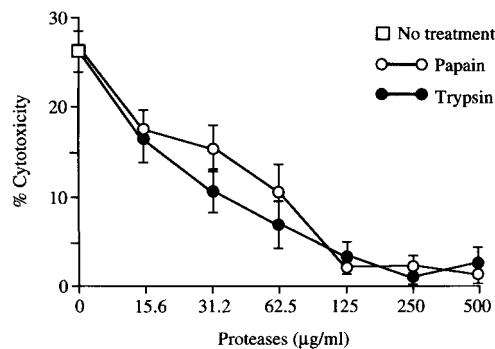


FIGURE 6. Tumoricidal activity of DC supernatants was inhibited by proteases. DC supernatants were obtained by 48-h culture of in vitro generated day-5 immature DCs in serum-free AIM-V medium supplemented with GM-CSF plus IL-4. A part of the supernatants was coincubated with trypsin or papain at indicated various concentrations for 60 min at 37°C. Following this enzymatic treatment, the supernatants were mixed with FCS at a final concentration of 25% and incubated for 30 min at 37°C, to neutralize the excess of proteases. Cytotoxic activity of the supernatants was then assessed against PCI-13 cell targets using 24-h MTT assays. The presented results are from a representative experiment of four similar experiments performed. The data are mean percentages of cytotoxicity \pm SD of triplicates.

delineate the receptor-ligand interactions responsible for this DC-mediated apoptotic death of tumor cells.

Previous studies have indicated that certain subsets of rat DCs (26) and activated human DCs might be cytotoxic for rare tumor cell targets (11, 12). From these studies it was not clear whether the killing of cancer cells mediated by DCs represented an infrequent in vitro artifact, a rudimentary activity, or a major activity of DCs. Furthermore, the mechanisms and biological significance of such activity were not clear. Our current data and those presented in a companion manuscript (25) support the conclusion that human nonactivated immature DCs are cytotoxic in vitro in low E:T ratios for the large majority of cancer cell lines and freshly isolated cancer cells, but not for normal cells. In addition, our data demonstrate that anticancer activity of DCs is a constitutive function of these effector cells in normal individuals. Therefore, cytotoxic activity mediated by DCs is an efficient, largely promiscuous (i.e., with regard to tumor target range), selective, and essential anticancer effector function. Our finding that DCs also kill proliferating "normal" endothelial cells may suggest that DCs are also capable of

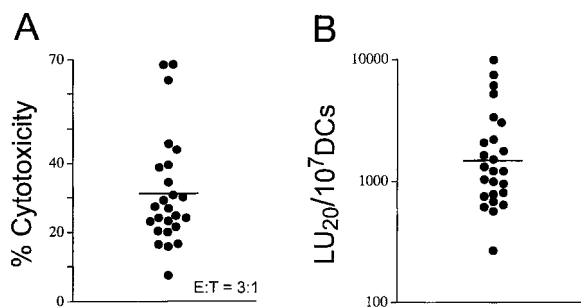


FIGURE 7. Anticancer cytotoxicity is a general function of DCs in normal individuals. The anticancer cytotoxic activity of cultured, monocyte-derived, immature DCs obtained from 25 different normal individuals was tested against PCI-13 target cells in 3-h MTT assays. The data are reported in both mean percentages of cytotoxicity (E:T ratio, 3:1) (A) and LU (LU₂₀/10⁷ DCs) (B) for each individual tested. Each dot represents the data from a given individual and the horizontal bars represent the overall mean values for all donors evaluated.

mediating antitumor activity by destroying newly forming tumor blood vessels.

The finding that immature DCs are more effective at mediating tumor cell apoptosis than mature DCs is intriguing. Coupled with the known ability of immature, but not mature, DCs to readily ingest apoptotic bodies, this provides a logical and economical paradigm for effective cross-presentation of tumor-derived epitopes important for the induction of effective antitumor cellular immunity (5–9). The observed DC-mediated killing is consistent with an innate immune effector function of these cells and may represent a missing link between innate and adaptive anticancer immunity.

Considering the in vivo distribution of DCs in normal tissues as well as their increased numbers in cancer tissues of patients exhibiting better clinical prognoses (1–3, 13), our findings strongly indicate that DC-mediated anticancer cytotoxic activity might be critically and directly involved in elimination of newly generated cancer cells and in control of tumor progression in vivo. Furthermore, this mechanism may prove to be important in the periodic restimulation of memory CD4 and CD8 antitumor T cell responses in patients who evolve micrometastatic disease but who remain clinically tumor free.

Acknowledgments

We thank Dr. Daniel E. Johnson for critical reading and comments on the manuscript.

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