Tumoricidal Activity of Monocyte-Derived Dendritic Cells: Evidence for a Caspase-8-Dependent, Fas-Associated Death Domain-Independent Mechanism[1]

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Monocyte-derived dendritic cells (DC) were found to be cytotoxic for several tumor cell lines including Jurkat cells, which were killed through a calcium-independent pathway. K562 cells were resistant, excluding a NK cell-like activity. DC-mediated apoptosis did not involve classical death receptors because it was not reversed by blocking TNF/TNFR, CD95/CD95 ligand, or TNF-related apoptosis-inducing ligand/TNF-related apoptosis-inducing ligand receptor interactions. Fas-associated death domain-deficient, but not caspase-8 deficient, Jurkat cells were killed by DC. Indeed, caspase-8 cleavage was demonstrated in Jurkat cells cocultured with DC, and the use of specific caspase inhibitors confirmed that apoptosis triggered by DC was caspase-8 dependent. Furthermore, the involvement of Bcl-2 family members in the control of DC-mediated apoptosis was demonstrated by Bid cleavage in Jurkat cells cocultured with DC and resistance of Jurkat cells overexpressing Bcl-2 to DC-mediated cytotoxicity. Overall, these data indicate that monocyte-derived DC exert a caspase-8-dependent, Fas associated death domain-independent tumoricidal activity, a finding that could be relevant to their therapeutic use in cancer. *The Journal of Immunology*, **2001, 167: 3565–3569.**

Endowed with a large array of specialized properties, dendritic cells (DC)[3] are an essential link between the innate and adaptative immune responses. Besides their potent Ag-presenting function, DC were more recently found to act as killer cells in several in vitro expriments. In mice, Süss and Shortman (1) have demonstrated that a subset of spleen DC expressing CD8α are able to kill $CD4^+$ T lymphocytes through the expression of CD95 ligand (CD95L). A CD95L-mediated killing was also described in murine bone marrow-derived DC (2). Two different types of cytotoxicity were discovered in the rat splenic DC population. Indeed, cultured splenic DC exhibit a Ca^{2+}-dependent NK-like cytotoxicity, whereas a subset of freshly extracted splenic DC display an intrinsic killing property through a Ca^{2+}-independent mechanism (3, 4). In human DC, TNF-related apoptosis-inducing ligand (TRAIL) was detected on the surface of IFN-γ- or IFN-α-stimulated $CD11c^+$ blood DC subset, which enables them to kill TRAIL-sensitive target cells (5). Type I IFNs, in combination with GM-CSF, were also shown to promote monocyte differentiation into TRAIL-expressing DC (6). Functional TRAIL production was found in GM-CSF/IL-4 monocyte-derived DC after IFN-β treatment or measles virus infection (7, 8). All these data suggest an important role for DC in the killing of tumor cells.

Human DC generated from peripheral blood monocytes in GM-CSF and IL-4 constitute a promising source of vaccines in antitumor immunotherapy. Recently, several clinical trials are investigating the effect of monocyte-derived DC in induction of efficient cell-mediated immune response against cancer (9, 10). In this context, we were interested in analyzing the cytotoxic potential of human monocyte-derived DC against a range of tumor cell lines.

Materials and Methods

Culture medium and reagents

Culture medium consisted of RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with 2 mM L-glutamine, 20 μg/ml gentamicin, 50 μM 2-ME, 1% nonessential amino-acids, and 10% FCS (BioWhittaker). The rIL-4 was kindly provided by Schering-Plough (Kenilworth, NJ), and rGM-CSF was obtained from Novartis (Basel, Switzerland). Agonistic anti-human CD95 mAb (clone CH11) was purchased from Immunotech (Marseille, France), recombinant human TRAIL from R&D Systems (Abingdon, U.K.), neutralizing monoclonal anti-human TNF-α from BioSource International (Nivelles, Belgium), antagonistic anti-human CD95 mAb (clone ZB4) from Immunotech, and recombinant human TRAILR2-Fc chimera from R&D Systems. The caspase inhibitors benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) and z-Ile-Glu-Thr-Asp-fluoromethylketone (zIETD-fmk) were purchased from Enzyme Systems Products (Livermore, CA). EGTA and $MgCl_2$ were purchased from Sigma (Bornem, Belgique). LPS from *Escherishia coli* (0128:B12) was also purchased from Sigma. Recombinant human IFN-α was purchased from Schering-Plough, recombinant human IFN-β1a was kindly provided by G. J. van Daal (Serono, Benelux, Den Haag, The Netherlands), and recombinant human IFN-γ was purchased from R&D Systems.

Generation of monocyte-derived DC

DC were generated from the adherent fraction of PBMC cultured for 7 days in GM-CSF (800 U/ml) and IL-4 (500 U/ml) as described by Romani et al. (11). As we have previously reported (12), the DC-enriched fraction obtained according to this protocol routinely contains >95% DC. In some experiments, DC were stimulated for 24 h with either LPS (1 μg/ml), or

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[3] Abbreviations used in this paper: DC, dendritic cell; TRAIL, TNF-related apoptosis-inducing ligand; CD95L, CD95 ligand; FADD, Fas-associated death domain; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; zIETD-fmk, z-Ile-Glu-Thr-Asp-fluoromethylketone; $DIOC_6$, 3,3′-dihexylocarbocyanine iodide; PI, propidium iodide; $\Delta\Psi_m$, mitochondrial transmembrane potential; RIP, receptor-interacting protein; tBid, 15-kDa truncated form of Bid.

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Table I. *Cytotoxic activity of human monocyte-derived DC*[a]

Target Cell	No. Donors Tested	Cytotoxicity (%)
Jurkat (T cell lymphoma)	25	39 ± 2.8***
Molt-4 (T cell lymphoma)	18	46 ± 2.6***
HCT-15 (colorectal adenocarcinoma)	8	27 ± 3.2**
MCF-7 (breast adenocarcinoma)	8	37 ± 3.1**
U87 (glioblastoma)	5	30 ± 1.7*
A498 (renal adenocarcinoma)	5	25 ± 2.3*
786.O (renal adenocarcinoma)	5	31 ± 4.2*
Caki.2 (renal adenocarcinoma)	5	22 ± 2.8*
Daudi (Burkitt's lymphoma)	8	12 ± 1.0*
Cem (T cell lymphoma)	13	4 ± 1.6
K562	14	9 ± 2.1

[a] DC were cocultured with different [^{3}H]thymidine-labeled tumor cell lines. After 18 h, intact nuclei were harvested and radioactivity was measured. Data are expressed as percentages of cytotoxicity (mean ± SEM) at 10:1 DC:target cell ratio.

*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$, as compared with background levels in absence of DC.

IFNs (IFN-α, 1000 U/ml; IFN-β, 1000 U/ml; IFN-γ, 100 U/ml). After being washed, DC were used in a JAM test assay.

Cell lines

The Jurkat and Molt-4 cell lines were obtained from the Institut Pasteur (Lille, France). The CEM cell line was obtained from Dr. T. Velu (Université Libre de Bruxelles, Bruxelles, Belgium). The HCT-15, MCF-7, U87, A498, 786.O, and Caki.2 cell lines were provided by Dr. R. Kiss (Université Libre de Bruxelles). The Daudi and K562 cell lines were purchased from American Type Culture Collection (Manassas, VA). The Fas-associated death domain (FADD)-deficient Jurkat cell line (I2.1), the caspase-8 deficient Jurkat cell line (I9.2), and the parental cell line (JA.3) were kindly provided by Dr. J. Blenis (Harvard Medical School, Boston, MA) (13). The Bcl-2-overexpressing Jurkat (JB2) cell line was obtained from Dr. S. Nagata (Osaka University Medical School, Suita, Japan) (14).

JAM test

Target cells were labeled with 5 μCi/ml of [^{3}H]thymidine by overnight incubation at 37°C. Labeled target cells were harvested, washed, and seeded in 96-well U-buttom plates at a density of 10,000 cells/well. Effector cells were washed and added to the target cells. After 18 h, intact nuclei (unfragmented high m.w. DNA) were harvested using a micro 96 harvester, and radioactivity was measured on a microplate beta counter (Packard Instrument, Meriden, CT). Data were expressed as the percentage of cytotoxicity calculated by the following formula: [1 − (cpm with effector/cpm without effector)] × 100.

Apoptotic death detection: 3,3′-dihexylocarbocyanine iodide ($DIOC_6$)/propidium iodide (PI) staining

$DIOC_6$/PI double staining was performed to detect apoptotic cells by flow cytometry. Cells were incubated for 15 min at 37°C with 25 nM $DIOC_6$ (Molecular Probes, Leiden, The Netherlands) in culture medium to evaluate mitochondrial transmembrane potential ($\Delta\Psi_m$). As $\Delta\Psi_m$ decreases with cell commitment to apoptosis, $DIOC_6$ stained living cells but not apoptotic cells. PI (1 μg/ml) was added before FACS analysis (15).

Western blot analysis

Immunoblots were performed according to standard protocols. Cells were washed twice with cold PBS and were lysed in 2× sample buffer. Total cell extracts were resolved by 15% SDS-PAGE, transferred to nitrocellulose membranes (Millipore, Bedford, MA), and incubated with a 1/2000 dilution of anti-caspase-8 (Cell Signaling Technology, Hertfordshire, Hitchin, U.K.) and a 1/1000 dilution of anti-Bid (R&D Systems) in 5% BSA or in 2% milk, 1× TBS, and 0.1% Tween 20 at 4°C with gentle shaking overnight, respectively. After three washes, incubation in a 1/2000 dilution of HRP-conjugated anti-mouse IgG (Amersham, Little Chalfont, U.K.) was performed in 5% milk for 1 h at room temperature, followed by five additional washes. Bound Abs were detected using an enzymatic chemiluminescence kit (Amersham).

Statistical analysis

The one-tailed Mann Whitney *U* test was used to determine statistical significance.

Results

Monocyte-derived DC exhibit a tumoricidal activity

To examine whether human monocyte-derived DC affect the viability of tumor cells, we cocultured immature DC with a panel of tumor cell lines at an E:T ratio of 10:1 and measured the percentage of cytotoxicity of target cells using the JAM test. We observed that DC exhibited significant cytotoxic activity against 9 of 11 tumor lines tested. As shown in Table I, Molt-4, Jurkat, HCT-15, MCF-7, U87, A498, 786.O, and Caki.2 cell lines were susceptible to DC-mediated apoptosis, as well as Daudi cells to a lesser extent. In contrast, the CEM and K562 cell lines were found to be resistant. The cytotoxic activity of DC toward tumor target was also detected using a chromium-release assay (data not shown). May-Grünwald Giemsa staining of DC/Jurkat cells cocultures revealed that Jurkat cells displayed morphological features of apoptosis. Indeed, as compared with Jurkat cells cultured alone (Fig. 1*A*, *left*), Jurkat cells cultured in the presence of DC exhibited either an early apoptotic state characterized by an intact nuclear membrane and a fragmented nucleus or a late apoptotic state (Fig. 1*A*, *right*). Double stainings with $DIOC_6$ and PI were also performed on DC/Jurkat cell cocultures to measure the percentage of $DIOC_6^-PI^-$ Jurkat cells. Indeed, $DIOC_6^-PI^-$ Jurkat cells correspond to cells that have undergone a decrease of their $\Delta\psi_m$ during the apoptotic process before the loss of plasma membrane integrity. As shown in Fig. 1*B*, 32% of Jurkat cells were $DIOC_6^-PI^-$ when cocultured with DC, in comparison to 3.7% when cultured alone.

The cytotoxic activity was dependent on the number of DC and was not calcium dependent because it was not affected by the addition of EGTA/Mg^{2+} calcium chelator in the DC/Jurkat cell cocultures (Fig. 2).

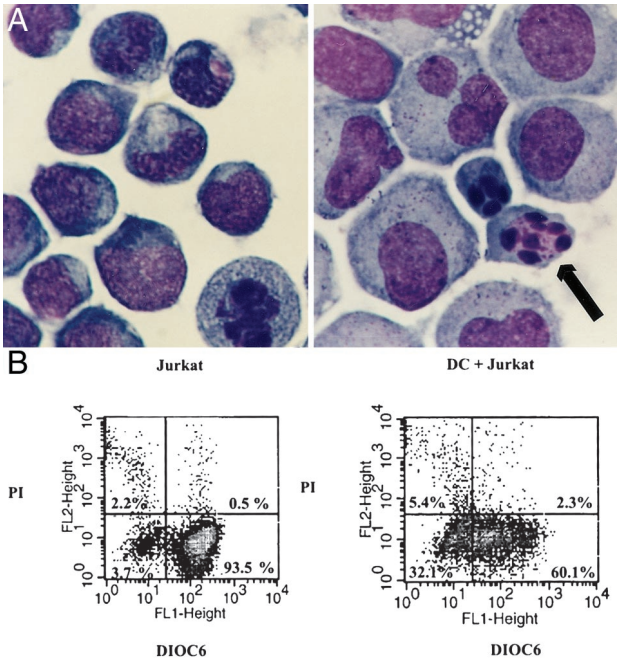

FIGURE 1. DC induce apoptosis in Jurkat cells. *A*, Morphology of Jurkat cells alone (*left*) or cocultured with DC at a DC:Jurkat ratio of 10:1 (*right*). May-Grünwald Giemsa staining of cytospin preparations. Magnification, ×1000. *B*, Jurkat cells were cocultured with DC at a DC:target cell ratio of 10:1. After 18 h, apoptosis was evaluated by flow cytometry analysis. Jurkat cells were gated in forward/side scatter and then analyzed for the $DIOC_6$/PI double staining. Data are representative of one of four independent experiments.

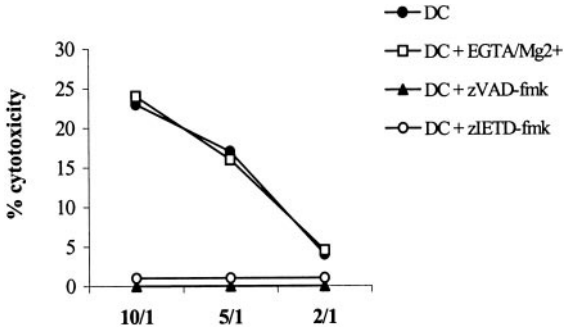

FIGURE 2. Tumoricidal activity of monocyte-derived DC is Ca^{2+} independent and caspase dependent. Jurkat cells were labeled with [^{3}H]thymidine and cocultured with DC at different E:T ratios in the presence or absence of either zVAD-fmk (20 μM), zIETD-fmk (20 μM), or EGTA/Mg^{2+} (8 and 6 mM, respectively). Following incubation, the percentage of cytotoxicity was determined using the JAM test as described in *Materials and Methods*. Data represent one representative of three experiments.

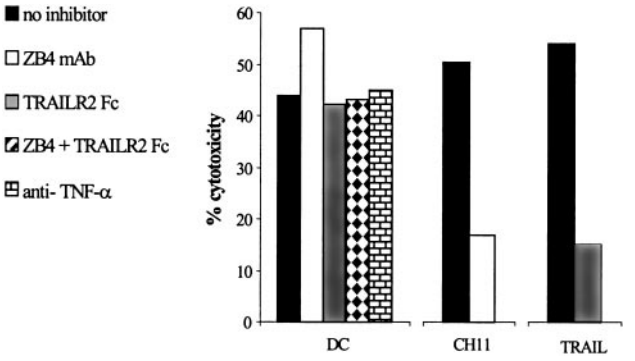

FIGURE 3. Tumoricidal activity of monocyte-derived DC is TNF-α, CD95L, and TRAIL independent. Jurkat cells were labeled with [^{3}H]thymidine and cocultured with DC at a DC:Jurkat cell ratio of 10:1 in the presence or absence of either anti-TNF-α (10 μg/ml), ZB4 anti-CD95 mAb (500 ng/ml), TRAILR2-Fc (20 μg/ml), or a combination of the latter two. As a control, CH11 agonistic anti-CD95 mAb (1 μg/ml) or TRAIL (100 ng/ml) were applied in the presence or absence of ZB4 and TRAILR2-Fc, respectively. Results of the JAM test, expressed as the percentage of cytotoxicity, are from one representative of five experiments.

Activation of DC using IFNs (IFN-α, IFN-β, and IFN-γ) or bacterial LPS did not affect the cytotoxic activity against Jurkat cells. Indeed, the percentages of cytotoxicity from three independent experiments of LPS- or IFN-activated DC vs unactivated DC with Jurkat cell cocultures were similar (Table II).

The cytotoxic activity of monocyte-derived DC is not mediated by TNF-α, CD95L, or TRAIL

Although DC are known to secrete TNF-α, this cytokine is clearly not involved in the cytotoxic activity reported here because the Jurkat cells we used were resistant to rTNF-α (data not shown), and the addition of a blocking anti-TNF-α Ab did not inhibit DC-mediated apoptosis (Fig. 3). Moreover, DC-induced apoptotic death did not appear to be mediated by a soluble molecule, because no cytotoxic activity against Jurkat cells was detected in the supernatant of a DC culture or in the supernatant of a DC/Jurkat cell coculture (data not shown). We then considered the possible role of CD95L and TRAIL in the apoptosis-inducing activity of DC. In these experiments, anti-human CD95 mAb (ZB4) and the fusion protein TRAILR2-Fc were added individually or in combination to the coculture of DC with their targets. As shown in Fig. 3, neither ZB4 nor TRAILR2-Fc inhibited the apoptosis induced by DC. As control, we verified that these reagents inhibited apoptosis induced by the agonistic anti-CD95 mAb (CH11) or TRAIL, respectively. In the next experiments, we found that FADD-deficient Jurkat cells were sensitive to DC-mediated apoptosis, excluding a role for all classical death-inducing ligands depending on FADD recruitment (Fig. 4).

Table II. *Cytotoxic activity of IFN- or LPS-stimulated DC*[a]

Culture	Cytotoxicity (%)		
	Expt. 1	Expt. 2	Expt. 3
DC	60	37	34
DC IFN-α	65	34	34
DC IFN-β	66	38	−
DC IFN-γ	63	37	−
DC	35	32	27
DC LPS	38	29	26

[a] [^{3}H]Thymidine-labeled Jurkat cells were cocultured either with unstimulated DC, IFN-stimulated DC, or LPS-stimulated DC. After 18 h, the percentage of cytotoxicity was determined using the JAM test as described in *Materials and Methods.*

Role of caspase-8 and Bcl-2 in DC-mediated apoptosis

We further considered the intracellular signaling pathway of apoptosis triggered by DC toward tumor cells. To determine whether caspase activation is involved in DC-induced killing, DC were cocultured with Jurkat cells in presence or absence of the broad-range caspase inhibitor zVAD-fmk. As shown in Fig. 3, zVAD-fmk completely prevented the induction of apoptosis by DC. This inhibitory effect of zVAD-fmk, observed in 10 independent experiments, was statistically significant ($p < 0.01$). We analyzed the effect of a caspase-8 inhibitor (zIETD-fmk) in the same cocultures because caspase-8 is a major initiator that can activate downstream effector caspases (16). As shown in Fig. 3, zIETD-fmk also abolished the cytotoxic activity of DC against Jurkat cells. The role of caspase-8 in DC-induced apoptosis was confirmed using caspase-8-deficient Jurkat cells. Indeed, DC could not exert their killing activity when caspase-8-deficient cells were used as targets instead of the parental Jurkat cell line (Fig. 4). Moreover, Western blot analysis confirmed that DC/Jurkat cell interaction resulted in the cleavage of caspase-8. Indeed, as shown in Fig. 5, the cleaved forms of caspase-8 were readily detected in Jurkat cells after 2 h of incubation with DC, and the levels increased after 4 h and were maintained until 6 h of the coculture. In parallel, no cleaved forms of caspase-8 were found in DC or Jurkat cells cultured alone. As

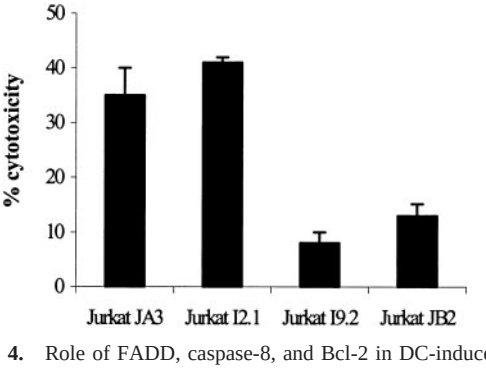

FIGURE 4. Role of FADD, caspase-8, and Bcl-2 in DC-induced cytotoxicity. [^{3}H]Thymidine-labeled Jurkat JA3, Jurkat I2.1, Jurkat I9.2 (caspase 8 deficient), and Jurkat JB2 cells overexpressing Bcl-2 were cocultured with DC at a DC:target ratio of 10:1. Results of the JAM test are given as the percentage of cytotoxicity (mean ± SEM) of four independent experiments.

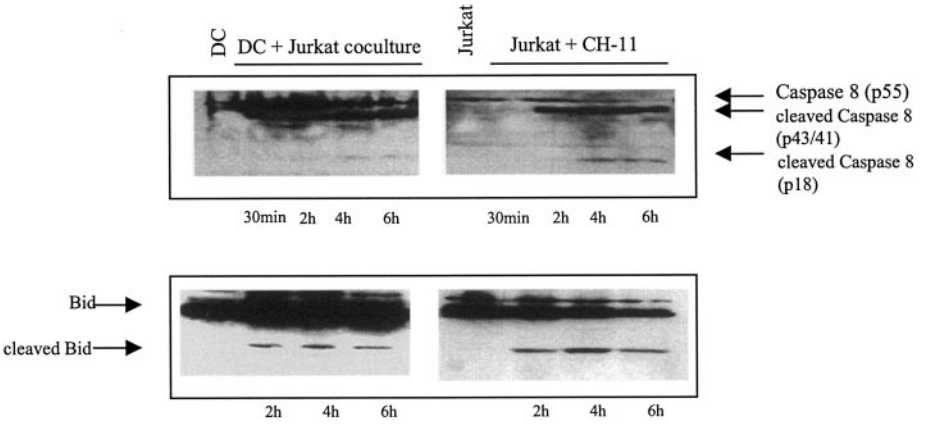

FIGURE 5. DC-induced apoptosis results in cleavage of caspase-8 and Bid. Jurkat cells were incubated with either anti-CD95 mAb (CH11 1 μg/ml) or with DC (DC:Jurkat cell ratio of 10^5:10^4 per 200 μl) for different times. Cells extracts were analyzed for the presence of the cleavage forms of caspase-8 (p43/41 and p18) and Bid (15 kDa) by immunoblotting as described in *Materials and Methods*.

a control, cleaved forms of caspase-8 were detected following anti-CD95 (CH11) stimulation (Fig. 5).

In a final set of experiments, we analyzed the involvement of Bcl-2 family members in the regulation of DC-mediated apoptosis. First, Bcl-2-overexpressing Jurkat cells were compared with parental Jurkat cells for their sensitivity to DC killing. As shown in Fig. 4, Bcl-2-overexpressing Jurkat cells were protected from DC-induced cytotoxicity as compared with parental Jurkat cells. This observation led us to investigate the possibility that DC-mediated apoptosis could trigger the activation of Bid, a proapototic molecule of the Bcl-2 family acting at the mitochondrial level (17). Indeed, Bid was previously found to be cleaved by caspase-8 to generate the 15-kDa truncated form of Bid (tBid). Translocation of tBid from the cytosol to the mitochondria was demonstrated to be critical for cytochrome *c* release, which in turn activates downstream caspases (18, 19). Cleavage of Bid was analyzed at different incubation times of DC/Jurkat cell cocultures. As shown in Fig. 5, tBid was detectable at 2 h, peaked at 4 h, and decreased at 6 h. No tBid was apparent in DC or Jurkat cells cultured alone. In parallel, anti-CD95-stimulated Jurkat cells were used as control for Bid cleavage. As shown in Fig. 5, tBid was generated in Jurkat cells in response to anti-CD95 (CH11) triggering.

Discussion

We report here that human DC generated by culturing peripheral blood monocytes in the presence of GM-CSF and IL-4 have potent cytotoxic activity in vitro on a wide spectrum of human tumor cell lines of different tissues origin.

The tumoricidal activity was not mediated by the CD95L/CD95, TRAIL/TRAILR, or TNF/TNFR systems and was clearly distinct from the cytotoxic activity previously found in human DC. Indeed, the present-described activity is an intrinsic property of immature monocyte-derived DC because it did not require any stimulation. Our findings extend data from previous studies in which human DC acquired a cytotoxic potential after measles virus infection or IFN stimulation (5–8). In both situations, tumor killing was partially mediated by TRAIL expression on DC. Interestingly, immature monocyte-derived DC might exert antitumor activity not only through the cytotoxic pathway described here but also via a TNF-α-dependent inhibition of tumor growth, as reported by Chapoval et al. (20).

The fact that K562 were resistant to DC-induced killing excluded the possibility that DC exert their cytotoxic effect through a NK cell-like activity. Moreover, the killing mechanism didn't seem to involve granule exocytosis because DC-induced cytotoxixity did not require Ca^{2+}. Interestingly, similar data were obtained in a rat splenic DC subset, which exhibits a killing property through a Ca^{2+}-independent mechanism that does not involve CD95L, TRAIL, or TNF (4). In our experiments, we found that FADD-deficient Jurkat cells were sensitive to DC-mediated apoptosis, excluding a role for all classical death-inducing ligands depending on FADD recruitment.

To get insight into the mechanism that could be responsible for DC tumoricidal activity, we have investigated the role of key molecules involved in the apoptosis pathway. First, we demonstrated that DC-induced apoptosis in Jurkat cells was dependent on caspase-8 activation. We next found that Bcl-2-overexpressing Jurkat cells were protected from DC-mediated cytotoxicity, suggesting that a DC-induced apoptotic signal would be tightly controlled at the mitochondrial level by a balance between antiapoptotic or proapoptotic molecules of the Bcl-2 family. This prompted us to investigate the possibility that apoptosis triggered by DC would involve activation of Bid, a proapoptotic Bcl-2 family member. We found that DC were able to mediate Bid cleavage into Jurkat cells. From these data, we conclude that monocyte-derived DC trigger the activation of caspase-8 into target, which in turn cleaves Bid, inducing mitochondrial changes leading to apoptosis.

We conclude that monocyte-derived human DC exhibit a novel caspase-8-dependent, FADD-independent tumoricidal activity. This finding represents additional evidence for the existence of alternative death pathways including the recently described receptor-interacting protein-dependent CD95-induced pathway (21). Together with the evidence of their tumoristatic activity (20), our observations could be relevant to the therapeutic use of DC as antitumor vaccines. Indeed, our observations suggest that monocyte-derived DC directly injected into tumors could first induce apoptosis in cancer cells and then process tumor-derived Ags from these apoptotic cells and further induce tumor-specific T cell responses (22, 23)

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