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Cutting Edge: Bystander Apoptosis Triggers Dendritic Cell Maturation and Antigen-Presenting Function[1]

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Physiologic cell death via apoptosis occurs without inflammation or autoimmunity. Here, we investigated the outcome of the interaction of apoptotic cells with dendritic cells (DCs), which are potent professional APCs. DCs internalized apoptotic cells and processed them for presentation to both MHC class I- and class II-restricted T cells with an efficiency that was dependent upon the number of apoptotic cells. The latter event was accompanied by the autocrine/paracrine secretion of IL-1β and TNF-α, with eventual DC maturation. High numbers of apoptotic cells, mimicking a failure of their in vivo clearance, are therefore sufficient to trigger DC maturation and the presentation of intracellular Ags from apoptotic cells, even in the absence of exogenous "danger" signals. ***The Journal of Immunology,*** **1998, 161: 4467–4471.**

Immature dendritic cells (DCs)[3] express low levels of MHC class I, class II, and costimulatory molecules and poorly initiate immune responses (1, 2). They satisfy the requirements for tolerance induction via cross-presentation of self Ags, because they capture Ags at the periphery, process them into the class I and class II pathways, and traffic to draining lymph nodes (3). There, "tolerogenic" DCs should initiate the proliferation of both naive $CD4^+$ and $CD8^+$ T cells (3). Infectious agents induce proinflammatory cytokine secretion and DC maturation; DCs lose the ability to take up Ags while up-regulating MHC molecules and adhesion and costimulatory signals, consequently becoming able to trigger a productive activation of Ag-specific T cells. Th cells further modulate the Ag-presenting function of DCs via a CD40/CD40 ligand interaction, favoring T cell responses aimed to control and possibly to eradicate the infectious noxa (4, 5).

Single cells are deleted from living tissues via apoptosis. Little is known regarding the interaction between DCs and apoptotic cells in peripheral tissues. Apoptotic cells contain relevant Ags targeted in autoimmune diseases, which are selectively cleaved, phosphorylated, and clustered in membrane blebs (6–8); a defective clearance of dead cells by scavenger macrophages contributes to chronic inflammation and autoimmunity in systemic lupus erythematosus patients (9). Macrophages and DCs internalize apoptotic cells and present Ags derived from the processing of these cells to class I-restricted T lymphocytes (10, 11). When massive apoptosis was triggered in the presence of strong "danger" signals such as viral infection in vitro, DCs primed virus specific cytotoxic T cells (11). In vivo, the apoptosis of islet β cells that was induced by CTLs dramatically enhanced the cross-presentation of tissue-restricted Ags (3).

Physiologic apoptosis occurs asynchronously and in the likely absence of maturative stimuli for DCs. A censorship on the Ag-presenting function of DCs or on DC maturation would prevent the generation of immune responses toward self Ags contained into apoptotic cells. Conversely, DCs undergoing maturation after the internalization of apoptotic cells may be implicated in cross-priming phenomena (3) during virus-specific and autoimmune responses. In this study, we report that high numbers of bystander apoptotic cells trigger DC maturation and function in vitro. The feedback regulation of these events, which occurs via the selective release of maturative factors, may be crucial in determining whether the recognition of cells undergoing apoptosis productively activates or silences autoreactive T cells.

Materials and Methods

Cells

The $H\text{-}2^b$ T cell lymphoma line RMA and the melanoma cell line B16F1 were grown in RPMI 1640 medium (Life Technologies, Grand Island, NY) containing 10% FCS (HyClone, Logan, UT) (tissue culture medium (TCM)). The immature DC line D1 (12) was cultured in Iscove's modified Dulbecco's medium supplemented with 30% NIH-3T3 supernatant containing 10 to 20 ng/ml mouse granulocyte-macrophage CSF. The IL-2-dependent CTLL-2 cell line was purchased from the American Type Culture Collection (Manassas, VA). Class I-restricted B3Z and class II-restricted B097–10 T cell hybridomas, which recognize epitopes between residues 257–264 and 327–339 of the OVA Ag, respectively, were grown

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[3] Abbreviations used in this paper: DC, dendritic cell; TCM, tissue culture medium; BFA, brefeldin A; CCD, cytochalasin D.

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in TCM. OVA-RMA cells express a nonsecreted truncated form of OVA which is devoid of the leader signal (residues 49–386).

Apoptosis induction and detection

Cells were irradiated with a UV lamp for 20 seconds in TCM before a 16-h incubation at 37°C. Most cells (consistently >95%) underwent programmed cell death via apoptosis. The actual induction of apoptosis was routinely verified (10, 13); nuclear apoptotic blebbing and incipient chromatin condensation were assessed by fluorescence microscopy after permeabilization and staining with propidium iodide, and cells with sub G_1 hypodiploid DNA content were identified by flow cytometry. The exposure of anionic phospholipids on the outer membrane was confirmed by confocal imaging after staining with the phosphatidylserine-binding protein annexin V (Bender MedSystems, Prodotti Gianni, Milan, Italy). Staining with FITC-annexin V yielded a typical "patchy" profile corresponding to membrane apoptotic blebs (13). Where indicated, cells (10×10^6/100 μl) were resuspended in a hypotonic buffer (10 mM sodium phosphate, pH 7.4) and killed by necrosis upon three to five cycles of rapid freezing/thawing. Necrosis induction was confirmed based on morphologic and cytometric evidence.

Ag presentation

A total of 5×10^4 B097–10 or B3Z hybridoma cells were incubated with a serial dilution of D1 cells that had been pulsed with apoptotic RMA or OVA-RMA cells or left unpulsed. In selected experiments, D1 cells were treated with 1 μg/ml of brefeldin A (BFA) or 10 μg/ml of cytochalasin D (CCD) for 1 h at 37°C. IL-2 secretion by hybridoma cells was assessed by evaluating the growth of the IL-2-dependent CTLL-2 cell line (14).

Confocal microscopy and flow cytometry

The phagocytosis of apoptotic cells was quantified by flow cytometry of biotin-labeled apoptotic cells. Constant numbers of D1 cells (200,000 per sample) were incubated for 60 min with increasing numbers of apoptotic cells (1,000–500,000 per sample). Effective internalization was confirmed by an ethidium bromide exclusion test (10). D1 cell phenotype was assessed by flow cytometry (12, 15). Phagocytosis of apoptotic cells was also visualized by confocal microscopy (10); D1 cells incubated with increasing numbers of apoptotic OVA-RMA cells were fixed in 4% paraformaldehyde or chased further for 60 min. Internalized apoptotic cells were revealed with an FITC-labeled anti-CD3 mAb. DCs were counterstained using phycoerythrin-labeled phalloidin.

Cytokines

D1 cells were cultured with increasing numbers of apoptotic OVA-RMA cells for 20 h in medium devoid of granulocyte-macrophage CSF. All media used were endotoxin-free, as assayed by the *Limulus* amebocyte lysate test (Whittaker Bioproducts, PBI International, Milan, Italy). Supernatants were cleared by centrifugation and stored at −30°C. IL-1β, TNF-α, IL-10, IL-12, and IFN-γ cytokine concentrations were detected using Endogen mouse ELISAs (Woburn, MA).

Results and Discussion

The D1 cells (12) that we used to analyze the interaction between DCs and bystander apoptotic cells comprise a homogeneous population of spleen-derived DCs which are capable of internalizing soluble Ags by macropinocytosis and particulate Ags, such as bacteria, via phagocytosis (15). Murine RMA lymphoma cells, which express a leaderless nonsecreted mutant of the OVA Ag (OVA-RMA cells), were committed to apoptosis by UV irradiation (10). Figure 1 shows that D1 cells efficiently phagocytosed OVA-RMA cells undergoing apoptosis; the phagocytosis of biotin-labeled apoptotic cells, which was assessed by flow cytometry after the addition of FITC-labeled streptavidin, was inhibited at 4°C or by CCD which disrupts the actin-based cytoskeleton. When D1 cells were challenged with increasing numbers of apoptotic cells, a linear increase in phagocytosis was observed. Similar results were obtained by confocal microscopy imaging (data not shown).

DCs efficiently present microbial particulate Ags internalized by phagocytosis (16–18). Therefore, we investigated whether Ags from internalized apoptotic cells were also processed and presented. Figure 2, *A* and *B*, shows that D1 cells that internalized either low or high numbers of OVA-RMA apoptotic cells activated OVA-specific class I- and class II-restricted hybridoma T cells, as assessed measuring specific IL-2 secretion. The cross-priming of naive T cells strictly requires a cognate presentation of Ags to $CD4^+$ Th cells by a bone marrow-derived APC (19). It is tempting to speculate that death by apoptosis provides the cell-associated debris that, upon processing by professional APCs, determines the cross-priming of $CD8^+$ cells toward cell-associated Ags (3, 19).

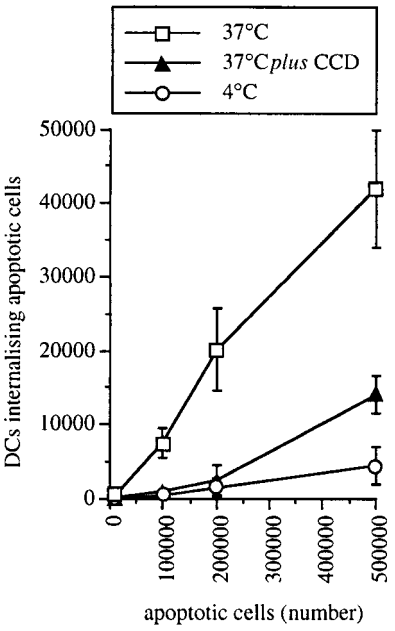

FIGURE 1. DCs internalize apoptotic OVA-RMA cells. Increasing actual numbers of biotin-labeled green fluorescent apoptotic OVA-RMA cells (*x*-axis) were coincubated with D1 cells (200,000 per sample) for 1 h at 4°C (○) or for 1 h at 37°C in the absence (□) or presence (▲) of CCD before FACS analysis. After the addition of FITC-labeled streptavidin, D1 cells that internalized apoptotic cells (*y*-axis) were identified as green fluorescent cells with physical characteristics and surface markers compatible with DCs. D1 cells that bound to apoptotic cells without internalizing them were identified with ethidium bromide (10) and excluded. The data shown (mean ± SE) represent results obtained in three independent experiments.

We subsequently investigated whether intracellular processing of internalized apoptotic OVA-RMA cells by DCs was necessary for T cell activation. CCD substantially inhibited T cell activation in vitro, suggesting that active phagocytosis and integrity of the actin-based cytoskeleton are required (Fig. 2*C*). BFA, which inhibits newly synthesized protein egress from the endoplasmic reticulum, interfered with both class I- and class II-restricted T hybridoma cell activation, implicating nascent MHC molecules in Ag presentation (Fig. 2*C*). D1 cells pulsed with living OVA-RMA cells or with OVA-RMA cells killed by necrosis did not activate T cell hybridomas (data not shown and Fig. 2*C*), suggesting that the mode of cell death, and possibly the reorganization of the plasma membrane typical of apoptosis (20), is relevant for the correct internalization, processing, and presentation of dying cells. Both early (i.e., cells irradiated and allowed to undergo apoptosis in the presence of DCs) and late (i.e. cells allowed to proceed into the apoptotic pathway until they are all permeable before incubation with DCs) apoptotic cells cross-activated T cells (data not shown).

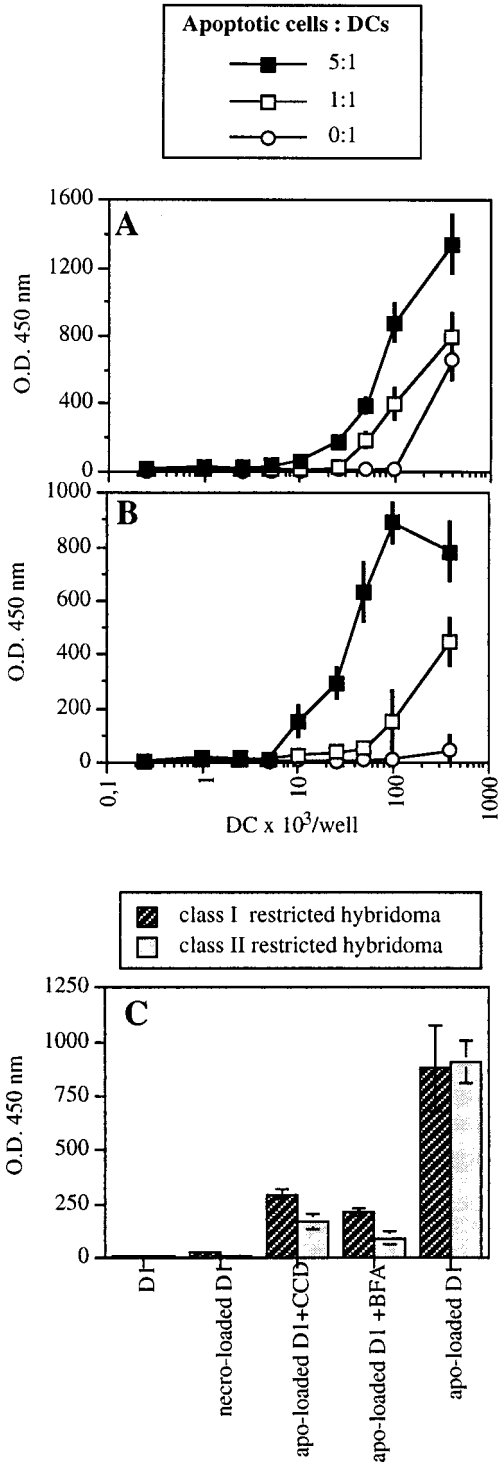

FIGURE 2. Internalized apoptotic cells are processed by DCs for presentation to class I- and class II-restricted hybridoma cells. D1 cells were challenged with increasing actual numbers of apoptotic cells. ■, a 5:1 ratio between apoptotic cells and D1 cells; □, a 1:1 ratio; and ○, D1 cells alone. D1 cells were then retrieved by centrifugation over density gradients and used to activate class I- (*A*) and class II- (*B*) restricted T hybridoma cells. T cell activation was assessed by measuring IL-2 secretion (*y*-axis, see *Materials and Methods*). Ag presentation required the egress of newly synthesized proteins from the endoplasmic reticulum, which was inhibited by BFA, and also the integrity of the actin-based cytoskeleton, which was inhibited by CCD (*C*). Furthermore, such presentation requires apoptosis induction, since D1 cells incubated with necrotic OVA-RMA cells did not activate either T cell hybridoma (*C*). The data shown (mean ± SE) are representative of five independent experiments.

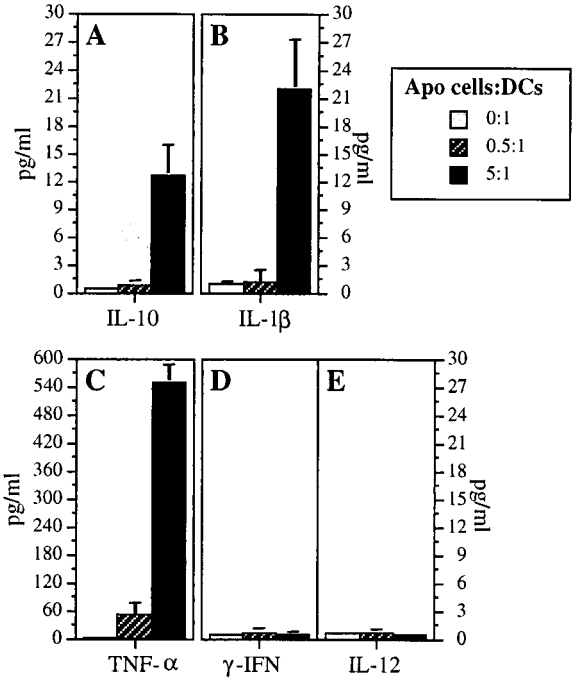

FIGURE 3. Apoptotic cells trigger the dose-dependent secretion of inflammatory cytokines by DCs. When challenged with a fivefold excess of apoptotic cells (5:1 apoptotic cell to DC ratio, filled columns), D1 cells secreted substantial amounts of IL-1β and TNF-α but not IL-10, IL-12, or IFN-γ; in contrast, only background cytokine levels were detected at a 0.5:1 apoptotic cell to DC ratio (hatched columns). The data shown (mean ± SE) represent results obtained in three independent experiments.

Therefore, apoptotic cells that escape immediate clearance by professional scavenger phagocytes in vivo and reach a late apoptotic stage may retain the ability to sustain immune (and possibly autoimmune) responses. Supernatants of apoptotic OVA-RMA cells did not endow D1 cells with the ability to activate T cell hybridomas (data not shown), ruling out a role of soluble Ag release from apoptotic cells.

Ag presentation has different outcomes in vivo according to DC maturation, which is regulated by different cytokines (1, 2). To determine the release of IL-1β, TNF-α, IL-10, IL-12, and IFN-γ, we challenged D1 cells with increasing amounts of apoptotic OVA-RMA cells. Figure 3 shows that D1 cells secreted substantial amounts of IL-1β and TNF-α but not IFN-γ when challenged with high numbers of apoptotic cells. It is of interest that the recognition of high numbers of apoptotic cells induced the release of minute amounts of IL-10 by D1 cells (~10 pg/ml). Recognition of high numbers of apoptotic cells by scavenger microglial cells triggered an almost 10-fold increase in IL-10 production (P.R. and A.A.M., unpublished observations). The molecular basis of the different response to the same stimulus is currently under investigation. The lack of IL-12 secretion by D1 cells recognizing apoptotic cells is not surprising, because it requires the engagement of MHC class II and CD40 molecules (4, 5); accordingly, D1 cells did not release IL-12 in vitro, even when challenged with microbial stimuli (15).

We subsequently verified whether the secretion of proinflammatory cytokines induced by high numbers of apoptotic cells was accompanied by D1 cell maturation; this apoptotic cell recognition induced the consistent up-regulation of the membrane expression of MHC class II (I-A), CD86, and CD40 molecules (Fig. 4). A similar effect was obtained by adding exogenous recombinant

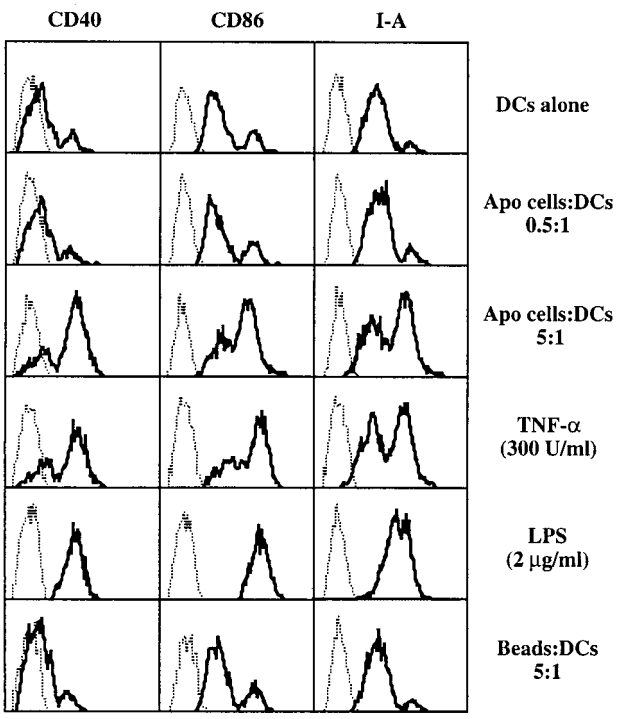

FIGURE 4. Bystander apoptosis triggers DC maturation. The D1 cell maturative state upon recognition of apoptotic cells was assessed by flow cytometry (see *Materials and Methods*) based on the relative surface expression of MHC class II molecules, CD86, or CD40 (solid lines). Background fluorescence as assessed with the second step reagent only is reported in each panel (dotted lines). High numbers of apoptotic cells (5:1 apoptotic cell to D1 cell ratio) delivered a maturative signal similar to that induced by LPS or TNF-α treatment. Maturation was not due to phagocytosis, because it was not induced by a 5:1 excess of inert phagocytic substrates (latex beads). The data shown are representative of six consistent, independent experiments.

TNF-α (Fig. 4). Low numbers of apoptotic cells, which induced the secretion of minimal amounts of TNF-α and not IL-1β, did not influence the maturation of D1 cells (Fig. 4). The phagocytosis of an excess of inert substrates was not sufficient per se to trigger D1 cell maturation (Fig. 4). Taken together, these results support the hypothesis that DCs control their own maturation by selectively releasing maturative factors when challenged with a relative excess of apoptotic cells.

In vivo, single dying cells are outnumbered by their living neighbors, who contribute to the clearance of these cells in combination with professional scavenger macrophages (20, 21). Immature DCs are interspersed in peripheral tissues among semiprofessional and professional phagocytes; these phagocytes will normally compete for the phagocytosis of apoptotic cells, possibly sequestering the majority of them from DCs. Massive apoptosis occurs in response to viral infections, ischemic events, or environmental insults (20). When challenged with an excess of apoptotic cells, which is possibly representative of these extreme situations, higher number of DCs effectively internalized apoptotic cells after 60 min (Fig. 1). Therefore, massive apoptosis may facilitate DC recruitment in the clearance of apoptotic cells, particularly in anatomical districts that are comparatively devoid of professional scavenger macrophages (22). Professional scavenger phagocytes, like macrophages, secrete a vast array of factors when engulfing apoptotic cells (23, 24) including IL-10, which selectively blocks DC maturation (2).

The asynchronous death of cells interspersed in solid tissues recruits phagocytes that sequester the Ag from DCs and interfere with the maturation state of DCs by means of soluble factors. In the presence of defects in the clearance of apoptotic cells, as described in animals bearing a genetic deficiency of C1q molecules (25), the persistence of uncleared corpses may deliver a danger signal and induce the simultaneous activation of class I- and class II-restricted T cells by mature DCs that are fully competent to initiate an autoimmune response. Of interest, both C1q-deficient mice and all patients in which C1q defects have been characterized develop full-blown systemic lupus erythematosus disease (discussed in Refs. 9 and 26).

At a 0.5:1 DC to apoptotic cell ratio, a condition in which D1 cells consistently activated class I- and class II-restricted T cell hybridomas, only background levels of IL-1β and TNF-α were detected (Fig. 3). The cross-presentation of intracellular Ags derived from dying cells by DCs in the absence of maturative factors is associated with a lack of up-regulation of MHC and costimulatory molecules and possibly with cross-tolerance induction (3).

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