Activation-induced Apoptosis in Human Macrophages: Developmental Regulation of a Novel Cell Death Pathway by Macrophage Colony-stimulating Factor and Interferon γ

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Summary

Activated macrophages (Mϕs) are important participants in host defense, but their uncontrolled activation leads rapidly to septic shock and death. One mechanism for regulating other dangerous cells in the immune system is programmed cell death, or apoptosis. Monocytes are known to undergo spontaneous apoptosis upon leaving the circulation unless provided with specific survival signals, but mature tissue Mϕs are more robust cells, and it was not clear that they could be similarly regulated by apoptosis. We now show that during differentiation monocytes rapidly lose their sensitivity to apoptosis triggered by passive cytokine withdrawal, but they may retain a novel pathway which initiates apoptosis after activation with specific stimuli (zymosan and phorbol esters). Sensitivity to activation-induced apoptosis was developmentally determined, being downregulated by the maturation-promoting cytokine macrophage colony-stimulating factor but stably upregulated by even transient exposure to the proinflammatory cytokine interferon γ (IFN-γ). Apoptosis began within 2–4 h of activation, occurred in >95% of susceptible cells, and in mixed cocultures selectively affected only those Mϕs with a history of IFN-γ priming. Consistent with a possible role for protein kinase C in the signaling pathway leading to cell death, the kinase inhibitor staurosporine was protective against both phorbol ester– and zymosan-induced apoptosis. Our studies describe a novel form of activation-induced Mϕ apoptosis which is developmentally regulated by two physiologically relevant cytokines. We speculate that apoptosis may serve to restrict the destructive potential of inflammatory Mϕs.

Tissue macrophages (Mϕs)[1], once regarded principally as scavengers and accessory cells, are now recognized as pivotal regulators of inflammation and immunity (1, 2). In addition, they are the principal effector cells against a broad class of facultative intracellular pathogens, including clinically important mycobacteria, fungi, and protozoa (3). To eliminate these highly resistant microorganisms, Mϕs must be "primed" by proinflammatory cytokines, in particular the T cell–derived lymphokine IFN-γ (4). Although IFN-γ–primed Mϕs are crucial for host defense (5, 6), they are also dangerous cells because of their production of free radicals, lytic enzymes, and inflammatory cytokines (7). These can cause extensive local damage, and are responsible for many of the systemic symptoms associated with acute and chronic inflammation (8). If uncontrolled, Mϕ activation leads to the clinical syndrome of septic shock and ultimately to the death of the host (8, 9).

These dangers suggest that activated Mϕs must be subject to strict regulatory control. One mechanism known to regulate other potentially dangerous cells in the immune system is programmed cell death, or apoptosis (reviewed in reference 10). This phenomenon has been most extensively studied in the lymphoid lineage, where autoreactive thymocytes undergo activation-induced apoptosis upon encountering "self" antigens in the thymus (11), but apoptosis also appears to play an important role in limiting the persistence of activated T cells (12, 13), B cells (14, 15), and granulocytes (16). Thus, apoptosis would seem a logical candidate to participate in Mϕ regulation as well.

Consistent with this hypothesis, studies by Mangan et al. (17, 18) have shown that the precursor cells for Mϕs, circulating monocytes, spontaneously undergo apoptosis unless given "permission" to survive by inflammatory mediators or growth factors. However, as circulating monocytes differentiate into mature Mϕs they become robust, long-lived cells,

[1] *Abbreviations used in this paper:* MCSF, macrophage colony-stimulating factor; Mϕs, macrophages; NO, nitric oxide; PKC, protein kinase C; rh, recombinant human; TdT, terminal deoxytransferase.

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and tissue Mϕs have been found to be resistant even to such apoptotic stimuli as antineoplastic agents and ionizing radiation (19). Thus, it was not clear that mature Mϕs were subject to apoptosis in the same fashion as fresh monocytes.

In this report we examine the regulation of apoptosis during the process of in vitro differentiation from monocyte to monocyte-derived Mϕs (henceforward referred to as Mϕs). We used a defined model using the maturation-promoting cytokine macrophage colony–stimulating factor (MCSF) and the inflammatory cytokine IFN-γ. We have previously shown that the interaction of these two agents during differentiation markedly affects the phenotype and function of the resultant Mϕs (20). In this study we asked (*a*) whether mature Mϕs, like fresh monocytes, remained dependent on permissive cytokines for continued survival; (*b*) if not, whether the mature Mϕs possessed an inducible apoptosis pathway; and (*c*) whether the cytokine environment present during differentiation influenced the susceptibility of Mϕs to apoptosis.

Materials and Methods

Cytokines and Reagents. Recombinant human MCSF (rhMCSF), sp act 1.65×10^6 U/mg protein by mouse bone marrow colony assay, was the gift of Genetics Institute, Inc. (Cambridge, MA). rhIFN-γ, 1.8×10^7 U/mg protein, was the gift of Genentech (South San Francisco, CA). Both cytokines contained <0.03 endotoxin U/ml by limulus amebocyte lysate assay (Associates of Cape Cod, Inc., Woods Hole, MA) at 10× the working dilutions used. Staurosporine (Sigma Chemical Co., St. Louis, MO) was prepared as a 10,000× stock in ethanol. All other reagents were obtained from Sigma Chemical Co. unless otherwise specified.

Isolation and Culture of Human Monocytes. PBMC were obtained by leukocytapheresis of volunteer donors under a protocol approved by our Institutional Review Board. Monocytes were isolated either by sequential centrifugation over Ficoll-Hypaque and hypertonic Percoll as previously described (20), or by counterflow centrifugal elutriation (modified from reference 21). These cells were either used immediately or cryopreserved in liquid nitrogen. Fresh and frozen monocytes behaved indistinguishably in our system and have been used interchangeably in our recent reports (20, 22, 23). Final monocyte purification was accomplished by adherence to serum-coated plastic as described (24). The adherent population was reproducibly >95% monocytes by Wright's stain morphology and CD11c expression.

For monocyte culture our minimal medium consisted of RPMI 1640 (GIBCO BRL, Gaithersburg, MD) with penicillin, streptomycin, and 2 mM glutamine. Except for cytokine withdrawal experiments, this basal medium was routinely supplemented with 10% bovine calf serum (Hyclone Laboratories, Inc., Logan, UT) and 200 U/ml rhMCSF. All media and reagents contained <0.03 U/ml endotoxin.

Derivation of the MCSF and IFN-γ Mϕ Phenotypes. To model monocyte differentiation in vitro we used our previously described system of long-term culture in rhMCSF (24). To derive the contrasting IFN-γ–primed phenotype, we added 100 U/ml IFN-γ plus rhMCSF (even with IFN-γ, Mϕs require MCSF for long-term survival). In this report we refer to these two phenotypes as MCSF-type and IFN-γ-type Mϕs. We have previously shown that essentially all of the starting monocytes survive for >7 d in our system (20), so the emergence of different phenotypes reflects alternative maturation pathways rather than selective survival of particular subpopulations.

Activation of Mϕs. Mϕs were activated with serum-opsonized zymosan (20 μg/ml, prepared as described by Pick [25]), FMLP (10 μM), LPS (100 ng/ml), antibody-coated microparticles (Advanced Magnetics, Inc., Cambridge, MA), PMA (50 ng/ml), thymeleatoxin (100 ng/ml; LC Laboratories, Woburn, MA), and the inactive phorbol ester 4α-phorbol 12,13 didecanoate (50 ng/ml). Each agent was screened for evidence of biologic effect in our cells, defined as either respiratory burst by nitroblue tetrazolium reduction (25), adherence and spreading on serum-coated substrate, or phagocytosis of the stimulus. Each agent triggered at least one of these responses, with the exception of the 4α-phorbol 12,13 didecanoate negative control.

Assessment of Mϕ Survival. Mϕs undergoing activation-induced apoptosis progressed rapidly to secondary necrosis (see Fig. 3), leaving only cellular debris by 24 h. In experiments where only end-point data were required survival was assessed by phase contrast microscopy and trypan blue dye exclusion. Where precise quantitation of surviving cells was important we measured the DNA content in adherent Mϕ monolayers by the dye-fluorescence method of Downs and Wilfinger (26). This technique had the important advantage that fusion of individual cells into multinucleated giant cells would not be falsely interpreted as cell death. The results from this assay were always confirmed qualitatively by phase contrast microscopy or trypan blue dye exclusion, with good agreement.

Assessment of Apoptosis. Internucleosomal DNA fragmentation was assessed using a whole-cell lysate technique as described by Zakeri et al. (27). Briefly, $2–3 \times 10^6$ cells were lysed (1% SDS, 100 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 7.4), treated with 0.2 mg/ml proteinase K for 1 h at 56°C followed by 500 U/ml DNAse-free RNAse (Sigma Chemical Co.) for 1 h at 37°C, then electrophoresed in a 1.5% agarose, Tris-borate-EDTA gel and stained with ethidium bromide. The THP-1 human macrophage–like cell line (American Type Culture Collection, Rockville, MD), induced to undergo apoptosis by exposure to 5 μg/ml actinomycin D or 200 nM staurosporine, was used as a positive control.

To detect DNA fragmentation in individual cells we used an in situ nick/end-labeling technique similar to that of Gavrieli et al. (28), based on incorporation of digoxigenin-labeled nucleotides at sites of DNA strand breaks by terminal deoxytransferase (TdT) (Apoptag; Oncor Inc., Gaithersburg, MD). Cytocentrifuge slides were prepared and processed as described in the manufacturer's protocol. Incorporated label was detected using fluorescein-conjugated antidigoxigenin antibody. Negative controls were processed without TdT; positive controls were pretreated for 5 min with 1,000 U/ml deoxyribonuclease I, which produced intense labeling of all nuclei.

Loss of DNA from apoptotic nuclei was quantitated by flow cytometry (29). Mϕs were activated with either zymosan or PMA as described. After 24 h to allow for loss of fragmented DNA, nuclei were released from the cells by treatment with 1% Triton X-100 in 0.1 M citric acid, stained with 10 μg/ml propidium iodide, and analyzed by flow cytometry.

For ultrastructural studies, monocytes were cultured for 4 d in rhMCSF + IFN-γ then exposed to 50 ng/ml PMA for 0, 2, or 4 h, harvested, and fixed in 2% glutaraldehyde for transmission electron microscopy.

Assays for Reactive Intermediates of Oxygen and Nitrogen. Hydrogen peroxide production was quantitated as the peroxidase-catalyzed extinction of scopoletin fluorescence measured against a standard curve of reagent H_2O_2, after the method of Nathan and Root (30). Superoxide production was determined spectrophotometrically as oxidation of ferricytochrome c, after the method of Pick (25). Nitric oxide (NO) production was assayed as nitrite formation by the diaminonaphthalene fluorescence method of Damiani and Burdini (31) as previously described (32); this assay had a de-

tection limit in our hands of 30 pmol nitrite. The complete lack of NO production in our system seen with this technique was confirmed using a sensitive bioassay (induction of guanidyl cyclase activity in cultured smooth muscle cells after coculture with activated Mϕs, generously performed for us by N. Marczin and J. Catravas using their published protocol [33]).

Scavengers and inhibitors of reactive oxygen species were used at the highest concentration determined in preliminary titration experiments to be nontoxic to the cells (individual agents and concentrations are given in Results). Each agent was added 1 h before the addition of PMA, except for *N*-acetylcysteine which was added 3 h before permit incorporation into the intracellular thiol pool.

PKH26 Labeling and Coculture. Stably apoptosis-sensitive Mϕs were derived by exposing monocytes to rhMCSF + IFN-γ for 24 h. These cultures were then washed and continued in rhMCSF alone for 3 d. Apoptosis-resistant Mϕs were derived by culturing monocytes in rhMCSF alone for 4 d. The MCSF-type cells were then detached from their culture dishes with 2 mM EDTA as previously described (22), labeled with the tracking dye PKH26 (Zynaxis Cell Science, Malvern, PA) according to the manufacturer's protocol, mixed 1:1 with IFN-γ-type cells (also harvested with EDTA), and the combined cell suspension replated and activated with PMA. After 24 h the cocultures were harvested and analyzed by flow cytometry. Preliminary experiments had documented that PKH26 labeling did not affect the survival or sensitivity to apoptosis of either cell type, and that the dye did not transfer from labeled to unlabeled cells. The cells recovered from cocultures were counted, and the absolute number of each cell type calculated based on the flow cytometry analysis.

BCL-2 Analysis. Mϕs were harvested with EDTA as described above. Expression of BCL-2 protein was assessed by flow cytometry using fluorescein-conjugated mAb 124 (34) from Dako Corp. (Carpinteria, CA), as described by Tamaru et al. (35). Peripheral blood lymphocytes were used as positive controls; isotype-matched irrelevant mAb was used as a negative control. Immunoblots were prepared by Laemmli SDS-PAGE followed by electrophoretic transfer to polyvinyl difluoride membranes (36). Blots were blocked (3% goat serum in 0.1 M NaCl, 0.1% Tween-20, 10 mM Tris, pH 7.5), incubated with mAb 124, and developed with a commercial peroxidase-based chemiluminescence system (ECL; Amersham Corp., Arlington Heights, IL).

Protein Kinase C Assays. Enzymatic activity consistent with protein kinase (PKC) was defined as Ca^{2+}/diolein dependent phosphorylation of a PKC-selective polypeptide substrate (37) based on residues 4–14 of myelin basic protein (MBP_{4-14}; LC Laboratories). After exposure to PMA for 15 min at 37°C, cells were separated into soluble and particulate fractions by sonication and ultracentrifugation (100,000 *g* × 15 min), and kinase activity measured as previously described (38). Activity associated with the particulate fraction was assayed without detergent extraction.

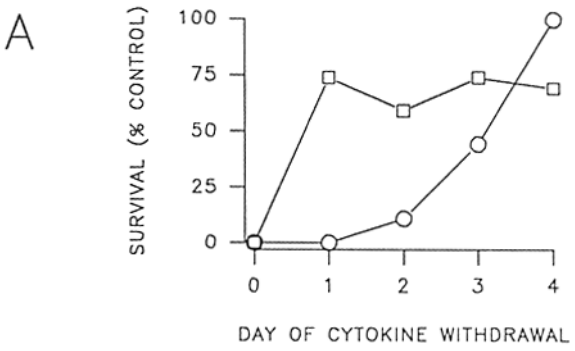
For immunoblots, nuclei were isolated by treatment of whole cells on ice with 1% Triton X-100 in 0.15 M NaCl, 10 mM sodium phosphate, pH 7.4, with inhibitors of proteases and phosphatases (39). After 10 s the detergent was diluted with ice-cold buffer to minimize extraction of PKC, and the nuclei pelleted and washed. Nuclear and nonnuclear fractions were transferred to Laemmli sample buffer and immunoblots performed using affinity-purified polyclonal antibody against the α isoform of PKC, prepared as described (40), followed by detection with ^{125}I-protein A.

Statistical Analysis. In most experiments apoptosis was a uniform, "all-or-none" phenomenon and differences were evident without statistical analysis. Pre- and postactivation samples were compared by paired *t* test. Titrations were compared by one-way analysis of variance. In all figures error bars reflect standard deviation.

Results

Differentiating Mϕs Become Resistant to Cytokine Withdrawal. As expected from previous reports (17), we found that fresh monocytes cultured in the absence of serum or activating factors rapidly lost viability (<20% surviving at 48 h) and displayed the light-microscopic features of apoptosis. However, as monocytes differentiated in response to rhMCSF they became increasingly indifferent to cytokine withdrawal (Fig. 1 *A*), such that by day 4 they no longer required serum or any exogenous growth factors for long-term survival. If IFN-γ was present (Fig. 1 *A*), monocytes became independent of cytokines even more rapidly (within 24 h). Mature Mϕs not only survived without growth factors but remained functionally active, as indicated by the respiratory burst shown in Fig. 1 *B*.

Activation of IFN-γ–type Mϕs Triggers Cell Death. We next activated MCSF-type and IFN-γ–type Mϕs with a panel of standard agents including LPS, FMLP, serum-opsonized zymosan, antibody-coated microparticles, and PMA. Both types of Mϕs responded to each agent with the expected evidence of activation (see Materials and Methods), and none of the stimuli affected viability in short-term assays (1–2 h). However, in the case of IFN-γ–type Mϕs, activation with two agents, PMA or opsonized zymosan, resulted in the uniform



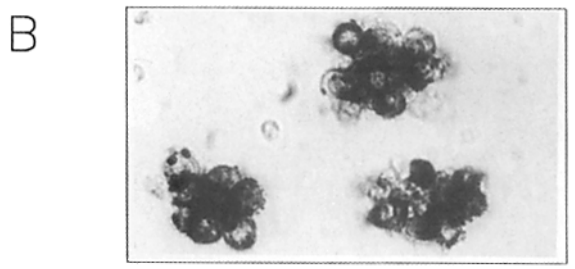


Figure 1. Differentiating Mϕs become resistant to cytokine withdrawal. (*A*) Fresh monocytes were exposed to rhMCSF alone (*circles*) or rhMCSF + IFN-γ (*squares*) for the times indicated, then washed and continued in minimal medium without serum or cytokines. Survival was quantitated on day 7 as the DNA content of the adherent cell monolayer, expressed as a percentage of controls (monocytes cultured with serum and cytokines). Each point is the mean of triplicate measurements; SD were all less than ±10%. Representative of three experiments. (*B*) Monocytes exposed to rhMCSF + IFN-γ for 24 h then continued for 4 d in minimal medium retained an active respiratory burst (shown as nitroblue tetrazolium reduction, dark reaction product).

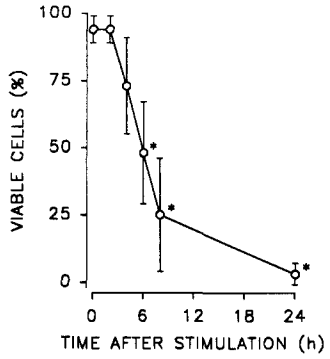


Figure 2. Activation of Mϕs primed with IFN-γ results in rapid cell death. Monocytes were cultured for 4 d in rhMCSF + IFN-γ, and viability was assessed by trypan blue dye exclusion after activation with PMA or zymosan (both agents gave similar results). Each point represents the average of data from at least three separate experiments. Points marked with an asterisk are significantly different from the baseline value by one-way analysis of variance ($p < 0.05$).

and progressive death of >95% of cells within 24 h of exposure (Fig. 2). Activation-induced cell death occurred only in IFN-γ–type Mϕs; MCSF-type Mϕs responded identically to the activating agents but did not go on to die (discussed further in Fig. 5, below). The lethal effect of activation on IFN-γ–type Mϕs was reproducible in >120 experiments with 14 donors, with no negative experiments observed to date.

Activation-induced Cell Death Occurs via Apoptosis. We next asked whether the death of IFN-γ–type Mϕs was associated with the oligonucleosomal DNA cleavage which accompanies apoptosis in many systems (41). As shown in Fig. 3 *A*, we found little evidence of oligonucleosomal fragmentation in dying Mϕs compared to our positive controls (THP-1 leukemia cells treated with actinomycin D or staurosporine). A modest oligonucleosomal pattern could be demonstrated if low molecular weight DNA was selectively enriched before electrophoresis (e.g., as described in reference 17), but we were unsure that this minor population constituted a biologically significant indicator of apoptosis (for this reason, data not shown).

However, it has recently been shown that low molecular weight DNA fragments may not be generated during apoptosis (27), despite the presence of extensive high molecular weight DNA fragmentation and other characteristic changes of apoptosis (42). We therefore turned to three additional techniques for detecting apoptosis: in situ labeling of DNA strand breaks by the enzyme TdT (28), quantitative loss of fragmented DNA from individual nuclei measured by flow cytometry (29), and the hallmark ultrastructural changes (43).

As shown in Fig. 3 *B*, TdT labeling revealed extensive and progressive DNA strand breaks beginning 1–2 h after activation, at a time when the cells still uniformly retained membrane integrity by trypan blue dye exclusion (Fig. 2). The onset of DNA fragmentation in individual cells was asynchronous, but by 4–5 h after activation 80–90% of cells were either TdT positive or had progressed to secondary necrosis. When nuclear DNA content was quantitated by propidium iodide staining (Fig. 3 *C*) a prominent hypodiploid peak was evident in the PMA-activated cells, consistent with loss of low molecular weight DNA and formation of micronuclei

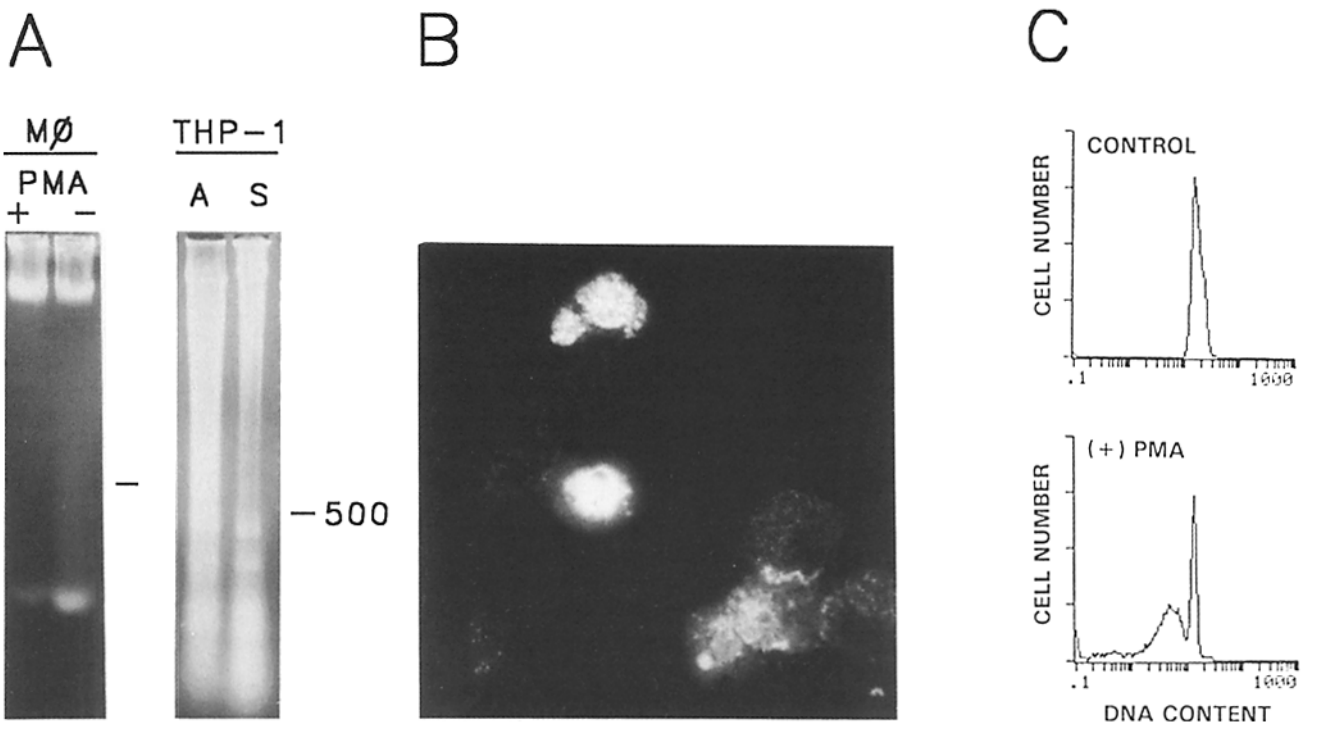


Figure 3. DNA fragmentation during Mϕ apoptosis. Mϕs cultured in IFN-γ were exposed to PMA for 5 h, then harvested for DNA analysis. (*A*) Agarose gel electrophoresis revealed little oligonucleosomal DNA fragmentation compared to positive controls cells (THP-1 treated with actinomycin D [lane *A*] or high-dose staurosporine [*S*]). Representative of eight experiments. (*B*) However, DNA strand breaks were readily detected in individual cells by nick/end labeling with TdT. Representative of four experiments. (*C*) Loss of fragmented DNA from apoptotic nuclei by propidium iodide staining and flow cytometry, visible as a hypodiploid peak in the PMA-treated cells. (Necrotic cells, killed by exposure to 10 mM HCl for 4 h, showed a single diploid peak similar to the untreated controls, not shown). Representative of three experiments using both PMA and zymosan.

(29). Finally, activated IFN-γ–type Mϕs displayed the characteristic ultrastructural changes of apoptosis (43) during the first 2–4 h after activation, as described in Fig. 4. Subsequently, these cells showed progressive edema and eventual lysis consistent with secondary necrosis, which was coincident with the onset of positive trypan blue staining (Fig. 2).

Taken together, these findings supported the conclusion that activation-induced cell death in IFN-γ–type Mϕs occurred via apoptosis. Since apoptosis was consistent and uniform, and since secondary necrosis followed rapidly, end-point viability assays such as those used in Figs. 1 and 2 appeared reasonable measurements of activation-induced cell death in our system, and these were used in subsequent experiments.

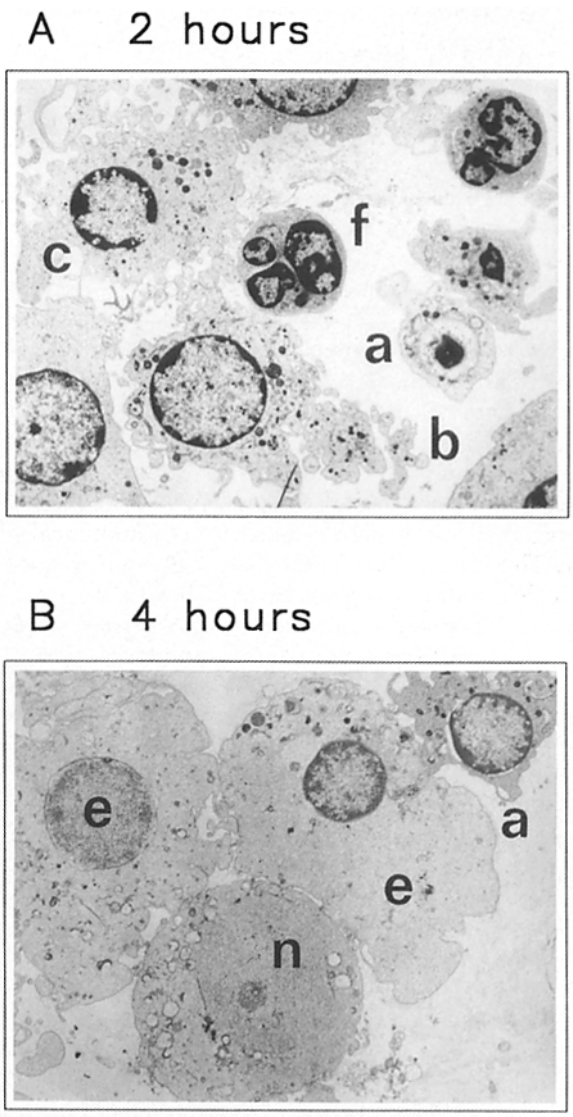


Figure 4. Ultrastructural changes of activation-induced apoptosis. Monocytes were cultured for 4 d in rhMCSF + IFN-γ, then exposed to PMA. (*A*) After 2 h, there was widespread evidence of membrane blebbing (*b*), loss of cytoplasmic volume, chromatin condensation (*c*) with micronucleation, nuclear fragmentation (*f*), and formation of apoptotic bodies (*a*). (*B*) By 4 h, many of the apoptotic cells (*a*) had begun to develop progressive cytoplasmic and nuclear edema (*e*), leading ultimately to secondary necrosis (*n*). ×2,750.

Activation-induced Apoptosis Is Developmentally Regulated. Since the IFN-γ and MCSF Mϕ subtypes both derived from the same starting population, we wished to determine the point at which differences in sensitivity to apoptosis emerged. We found that fresh monocytes were initially susceptible to activation-induced apoptosis, but during differentiation in rhMCSF they progressively acquired resistance (Fig. 5 *A*). However, if IFN-γ was present, differentiating monocytes remained sensitive to apoptosis indefinitely, despite the presence of rhMCSF. To exert fully this effect, IFN-γ had to be added early in differentiation; if its addition was delayed it

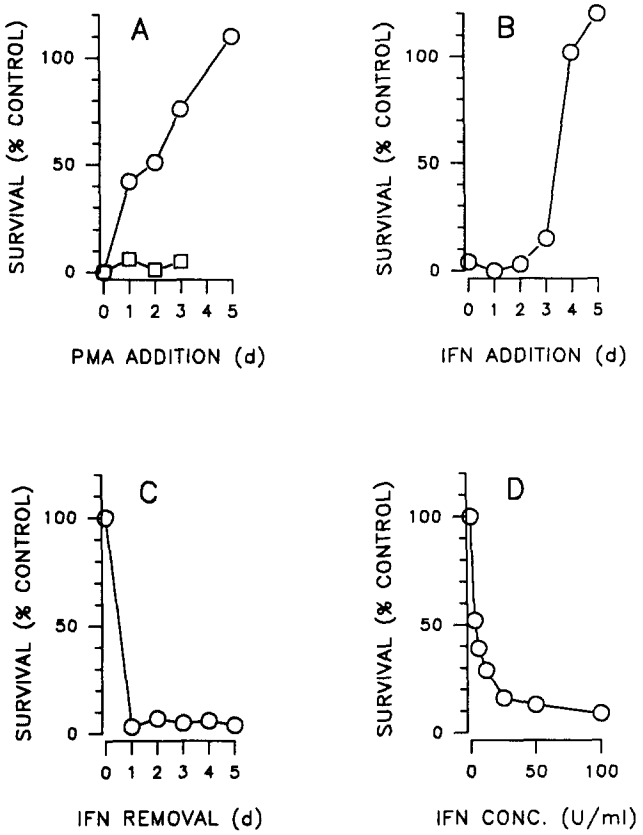


Figure 5. Developmental regulation of susceptibility to apoptosis. In all experiments, monocytes were cultured for 6 d in rhMCSF, with IFN-γ added at various times as described. PMA was added on day 5 unless otherwise indicated; survival was quantitated on day 6 by DNA content and expressed as percentage of controls without PMA. (*A*) Cultures were initiated in either rhMCSF alone (*circles*) or rhMCSF + IFN-γ (*squares*) and replicate cultures activated with PMA on each day. (IFN-γ–type Mϕs never acquired resistance to apoptosis and were not routinely assayed past day 3, but in other experiments they remained sensitive at least to day 10, the limit of our assays.) (*B*) IFN-γ was added on successive days and the cultures activated on day 5. Delayed addition of IFN-γ was progressively less effective at restoring cells to the fully apoptosis-sensitive state. (*C*) IFN-γ was added on day 0, then on successive days replicate cultures were washed and continued in rhMCSF alone until activation on day 5 (day 0 controls never received IFN-γ). Exposure to IFN-γ for the first 24 h of culture was sufficient to confer long-lasting susceptibility to apoptosis. (*D*) Dose–response relationship for IFN-γ–induced sensitivity to apoptosis. Each of the above graphs is representative of three to five similar experiments. Points are the mean of triplicate determinations; SD was $< \pm 10\%$ for all points and error bars have been omitted for clarity.

became progressively less effective in maintaining uniform sensitivity to apoptosis (Fig. 5 *B*). However, even a brief exposure to IFN-γ at the onset of differentiation was sufficient to confer sustained sensitivity to apoptosis, even if the IFN-γ was subsequently removed (Fig. 5 *C*). Thus, activation-induced apoptosis appeared to be a stable, developmentally regulated attribute of the IFN-γ phenotype, which was determined during a critical early period in differentiation.

Activation-induced Apoptosis Is Selective for IFN-γ–primed Mϕs. To test for selectivity we derived MCSF-type and IFN-γ–type Mϕs in parallel cultures, then combined the populations on day 4 and continued the cocultures in rhMCSF alone. (To emphasize the developmental nature of the effects under study, IFN-γ–type Mϕs were exposed to IFN-γ only for the first 24 h of culture as described above). The MCSF-type Mϕs were labeled with a stable tracking dye so that the subpopulations could be distinguished. As shown in Fig. 6, when mixed cocultures were activated with PMA there was a selective and complete elimination of only those Mϕs with a history of IFN-γ exposure. These experiments supported our hypothesis that sensitivity to activation-induced apoptosis was determined by the developmental history of the individual cell, rather than by the cytokine environment prevailing at the time of activation.

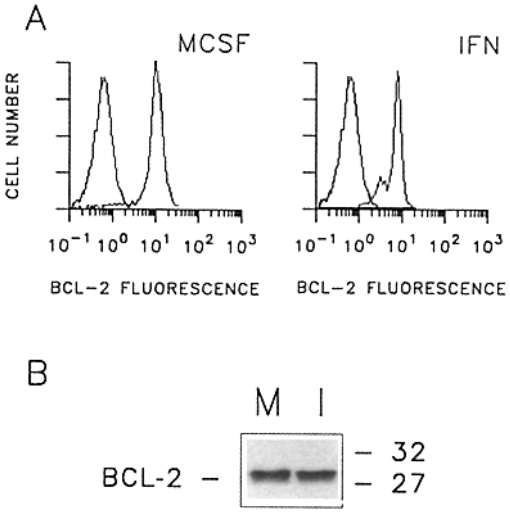


Figure 7. Comparable expression of BCL-2 in sensitive and resistant Mϕs. Mϕs were cultured for 4 d in rhMCSF or rhMCSF + IFN-γ and analyzed for BCL-2 protein by direct immunofluorescence (*A*) or immunoblot (*B*). Equal numbers of cells were loaded in each lane. Representative of three experiments.

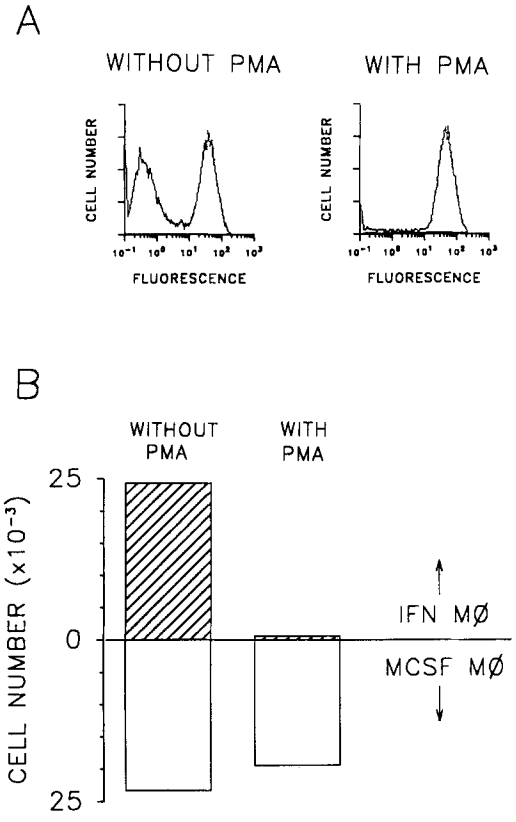


Figure 6. Selective elimination of apoptosis-sensitive cells in mixed cocultures. MCSF-type and IFN-γ–type Mϕs were derived in separate cultures. The MCSF-type cells were labeled with PKH26 tracking dye, mixed with equal numbers of IFN-γ–type cells, and the cocultures were activated with PMA. (*A*) Selective elimination of the IFN-γ–type (PKH26-negative) Mϕ population after activation, compared to control cultures without PMA. Representative of three experiments. (*B*) Quantitative results for the experiment shown in *A*, based on cell counts after coculture.

Apoptosis Occurs Despite BCL-2 Expression. We compared expression of the antiapoptosis BCL-2 protein in sensitive and resistant Mϕ subtypes. As shown in Fig. 7, both subtypes expressed similar amounts of BCL-2 protein, whether assessed at the single-cell level by flow cytometry, or by immunoblot analysis. This level of BCL-2 expression was comparable to that found in resting lymphocytes (not shown).

Apoptosis Is Not due to Accumulation of Nonspecific Toxic Factors. Activated Mϕs can secrete toxic substances, particularly reactive oxygen species and NO. We therefore asked whether scavengers of these by-products could protect against apoptosis. Each of the agents chosen had been successfully used in other settings to protect against free radical injury (25, 44–47). However, we found that none of them prevented activation-induced apoptosis in our system (Table 1). In addition, measured levels of hydrogen peroxide (a major reactive oxygen species secreted by Mϕs) in the culture medium remained within a nontoxic range throughout the time that the cells were undergoing apoptosis (maximum concentration <15 μM, compared to a measured LD_{50} for our cells of >100 μM, n = 3, assays as described in Materials and Methods). The concentration of superoxide anion was even lower than that of H_2O_2 (<5 μM).

NO also did not appear to play a major role in our system, since (*a*) the NO synthase inhibitor arginine methyl ester did not protect against apoptosis (Table 1); (*b*) pharmacologic generation of NO by 1 mM sodium nitroprusside did not initiate apoptosis (three experiments, not shown); and (*c*) as described in Materials and Methods, NO production was undetectable (<30 pmol/ml) in our system even after IFN-γ priming and PMA triggering. Taken together, these data sug-

Table 1. *Lack of Effect of Free Radical Scavengers and Inhibitors on Activation-induced Cell Death*

Agent	Concentration*	Survival‡ (−)PMA	Survival‡ (+)PMA	n§
Catalase	5,000 U/ml	+	−	5
Superoxide dismutase	300 U/ml	+	−	4
N-acetylcysteine	30 mM	+	−	8‖
Butyrated hydroxyanisole	400 μM	+	−	5
Pyrrolidine dithiocarbamate	60 μM	+	−	4
Dihydroxybenzene-disulfonic acid	10 mM	+	−	5
β-mercaptoethanol	10 mM	+	−	2
Arginine methyl ester	1 mM	+	−	4

* The highest concentration of inhibitor which was not toxic to the cells was used for all experiments.
‡ Apoptosis in these experiments was uniform and unambiguous, so survival is reported simply as + (>90% viable) or − (<10% viable) at 24 h.
§ Number of replicate experiments.
‖ In two/eight experiments with *N*-acetylcysteine a small number of cells in the PMA-challenged group still excluded trypan blue at 24 h. These cells were found on cytocentrifuge preparation to be apoptotic, and uniformly progressed to necrosis by 48 h.

gested that nonspecific toxicity from reactive intermediates of oxygen and nitrogen appeared unlikely to account for apoptosis in our system.

Role of PKC. PMA and zymosan have both been reported to activate PKC in Mϕs (48, 49). Preliminary experiments in our system had shown that 4α-phorbol 12,13 didecanoate (a phorbol ester which does not activate PKC) had no effect on apoptosis, while thymeleatoxin (which is highly selective for PKC [50]) triggered apoptosis indistinguishably from PMA (LD_{50} ≈10 nM, three experiments). We therefore asked whether there was functional evidence of PKC activation before apoptosis in our cells. As shown in Fig. 8 *A*, PMA caused translocation of enzymatic activity consistent with PKC from the soluble to the particulate fraction within 15 min of exposure. When we examined a representative isoform of PKC (PKC-α, the classical isoform most abundantly expressed in our cells) by immunoblot analysis, immunoreactive protein was also found to translocate to the particulate fraction following PMA stimulation. This translocation of PKC-α included its appearance in the nucleus (Fig. 8 *B*), the site of many early changes of apoptosis.

To test whether activation of PKC was functionally related to apoptosis, we asked whether pharmacologic inhibitors of PKC would block PMA-induced cell death. Because many PKC inhibitors are themselves lethal to living cells (51), we tested several compounds to identify the least toxic. Calphostin C, H7, and chelerythrine all proved highly toxic, but the broad-spectrum kinase inhibitor staurosporine was well tolerated in the range required to inhibit PKC (51). As shown in Fig. 8 *C*, a concentration of 10 nM staurosporine was fully protective against PMA-induced apoptosis. Staurosporine was also significantly protective against zymosan-induced apoptosis (Fig. 8 *D*), although complete protection was not achieved with this agonist. Taken together, our findings thus suggested a possible functional role for PKC in the signaling pathway leading to apoptosis in our cells.

Discussion

In this study we describe a novel pathway capable of inducing rapid and selective apoptosis of a specific subset of mature Mϕs after activation. The fact that differentiating monocytes became resistant to the form of apoptosis induced by passive cytokine withdrawal emphasizes the need for an inducible pathway if apoptosis is to play a role in regulating mature Mϕs. Not all activating agents led to apoptosis: simple stimuli such as purified LPS or FMLP were not lethal. However, opsonized zymosan, a complex particulate stimulus consisting of yeast cell walls, activated complement, and serum proteins, triggered prompt apoptosis, perhaps due to its closer resemblance to physiologic Mϕ targets.

The role for PKC implied by our observations was unexpected, since in many systems PKC has been found to suppress apoptosis rather than initiate it (reviewed in reference 52). Recently, however, several forms of apoptosis apparently mediated by PKC have been described (53–55). PKC is known to comprise a family of related isoenzymes with different functional properties (56), and the effects of PKC activation may change with the developmental stage of a particular cell type (57) (as witness the different responses of our two Mϕ subtypes). The brief series of kinase experiments included in this report are intended only to support a role for PKC in initiating apoptosis in our system. A more detailed characterization of PKC activity in these cells will be presented elsewhere (Munn, D. H., A. C. Beall, M. Mabie, and D. Throckmorton, manuscript in preparation).

Several questions regarding activation-induced Mϕ apoptosis remain unanswered. The role of BCL-2 is not yet clear.

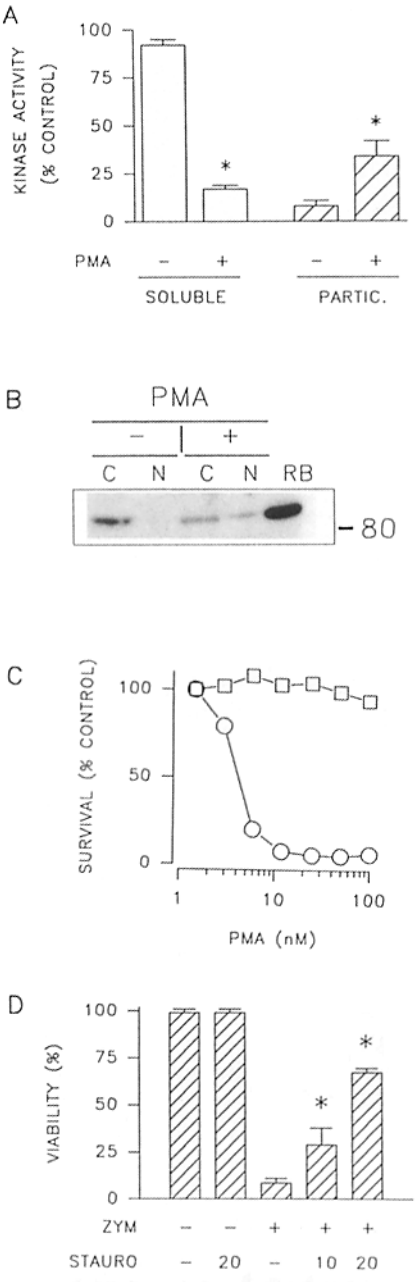


Figure 8. Evidence for PKC activation during apoptosis. Monocytes were cultured for 4 d in rhMCSF + IFN-γ then activated with PMA. (*A*) Translocation of Ca^{2+}/lipid-dependent kinase activity from soluble to particulate fraction after 15 min exposure to PMA. Each point represents the average (±SD) of three separate experiments, normalized to the total kinase activity (soluble + particulate) in the control cells for each experiment. Asterisks indicate significant ($p < 0.01$) difference compared to the control values by paired *t* test. (*B*) Translocation of immunoreactive PKC-α from cytosol to nucleus in response to PMA. (Translocation was also observed if cells were fractionated into soluble and particulate compartments, not shown.) Control: rat brain cytosol (*RB*). (*C*) Inhibition of PMA-induced apoptosis (*circles*) by the kinase inhibitor staurosporine, 20 nM, added 1 h before PMA exposure (*squares*). Survival measured by DNA content after 24 h. (*D*) Inhibition of zymosan-induced apoptosis by 10 and 20 nM staurosporine (asterisks indicate significant differences vs zymosan alone by one-way analysis of variance, $p < 0.01$). Survival measured by trypan blue dye exclusion after 8 h. All figures representative of at least three experiments with similar results.

Quantitative differences in BCL-2 expression apparently do not underlie the differences in our cells, but we have not yet tested for dominant regulators of BCL-2 function such as BAX or BCL-X (58, 59). A second question concerns the role of reactive oxygen and nitrogen species. IFN-γ-primed Mϕs produce reactive oxygen species after activation, and oxidative stress can trigger apoptosis (60). Although we were unable to inhibit apoptosis with a variety of free-radical scavengers, it may be that these agents did not function effectively at the intracellular level. At present we cannot definitively exclude a role for intracellular oxidative stress in Mϕ apoptosis. NO, which in mice can be produced by activated Mϕs, has also been reported to cause apoptosis (61). However, we, like others (see also reference 62), could not detect NO production in our human system despite the use of sensitive assays, nor did NO generated pharmacologically from sodium nitroprusside initiate apoptosis. Thus NO seemed unlikely to participate in our system.

Proposed Physiologic Significance. Conceptually, the phenomenon of activation-induced Mϕ death appears paradoxical: why should Mϕs die simply because they perform their intended function? We speculate that this arrangement may confer two important benefits. The first is to regulate the destructive potential of IFN-γ–primed Mϕs. Unlike lymphocytes, which have an elaborate regulatory network to ensure the death of unwanted cells, and granulocytes, which spontaneously undergo apoptosis within hours of entering the tissues, Mϕs have few known mechanisms to limit their activation. The form of apoptosis which we describe allows for normal activation in the short term, thus permitting the Mϕs to perform their antimicrobial function, but it strictly limits the subsequent persistence of the activated cells.

The second proposed benefit arises from the unusual nature of facultative intracellular pathogens. These highly resistant microorganisms have evolved the ability to preferentially survive and replicate within Mϕs after phagocytosis, where they are shielded from immune attack (3). Priming with IFN-γ is intended to help Mϕs destroy such pathogens as they are ingested (4–6), but any organisms which survive possess a variety of strategies to subvert their Mϕ host into an unintentional incubator (3, 63). The prompt death of Mϕs after they have attempted to kill such pathogens thus becomes logical if it is seen as denying surviving microorganisms their intended sanctuary site.

Taken together, our data suggest a model in which naive monocytes "decide" during terminal differentiation whether or not to remain subject to activation-induced apoptosis. This decision is based on the cytokine milieu encountered during differentiation, and thus reflects the intended function, and also the destructive potential, of the mature Mϕs.

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