Phagocytosis of Apoptotic Cells by Macrophages Induces Novel Signaling Events Leading to Cytokine-Independent Survival and Inhibition of Proliferation: Activation of Akt and Inhibition of Extracellular Signal-Regulated Kinases 1 and 2

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Phagocytosis of Apoptotic Cells by Macrophages Induces Novel Signaling Events Leading to Cytokine-Independent Survival and Inhibition of Proliferation: Activation of Akt and Inhibition of Extracellular Signal-Regulated Kinases 1 and 2

Suman M. Reddy,² K.-H. Kevin Hsiao,² Vivian Elizabeth Abernethy,³ Hanli Fan,³ Angelika Longacre,† Wilfred Lieberthal,* Joyce Rauch,‡ Jason S. Koh,† and Jerrold S. Levine³†

Recent evidence indicates that phagocytic clearance of apoptotic cells, initially thought to be a silent event, can modulate macrophage (Mφ) function. We show in this work that phagocytic uptake of apoptotic cells or bodies, in the absence of serum or soluble survival factors, inhibits apoptosis and maintains viability of primary cultures of murine peritoneal and bone marrow Mφ with a potency approaching that of serum-supplemented medium. Apoptotic uptake also profoundly inhibits the proliferation of bone marrow Mφ stimulated to proliferate by M-CSF. While inhibition of proliferation is an unusual property for survival factors, the combination of increased survival and decreased proliferation may aid the Mφ in its role as a scavenger during resolution of inflammation. The ability of apoptotic cells to promote survival and inhibit proliferation appears to be the result of simultaneous activation of Akt and inhibition of the mitogen-activated protein kinases extracellular signal-regulated kinase (ERK)1 and ERK2 (ERK1/2). While several activators of the innate immune system, or danger signals, also inhibit apoptosis and proliferation, danger signals and necrotic cells differ from apoptotic cells in that they activate, rather than inhibit, ERK1/2. These signaling differences may underlie the opposing tendencies of apoptotic cells and danger signals in promoting tolerance vs immunity. The Journal of Immunology, 2002, 169: 702–713.

A poptosis is an active energy-dependent process that almost always occurs without inflammatory injury to surrounding tissues (1). This remarkable feature of apoptosis occurs for several reasons. First, the cell membrane of cells undergoing apoptosis remains intact until relatively late (1). Second, apoptotic cells express unique surface markers that permit their rapid recognition and ingestion by phagocytes (2, 3). Hence, as long as phagocytic clearance of an apoptotic cell occurs before breakdown of its cell membrane, none of its cytosolic contents will be released into the extracellular space. In this way, despite the billions of cells that die each day by apoptosis, tissues are protected from an otherwise harmful exposure to the inflammatory contents of dying cells. Moreover, uptake of apoptotic cells actively inhibits the release of proinflammatory mediators such as TNF-α by macrophages (Mφ) (4, 5).

Most, if not all, cells undergo apoptosis if grown in the absence of so-called survival factors (6). Appreciation of this phenomenon has led to the concept that all cells possess a genetically built-in default pathway capable of initiating apoptotic death unless specifically inhibited by signals from the extracellular environment (7). Thus, to maintain viability, a cell must receive a more or less continuous stream of extracellular survival signals to keep its default pathway in a state of constant inactivation (6, 7). Therefore, inhibition of apoptosis by survival factors is an example of negative regulation, meaning that, in the absence of survival factors, cells will automatically undergo apoptosis.

In situations in which a relative deficiency of survival factors results in apoptotic death, it is crucial that phagocytic cells remain viable so that apoptotic cells can be cleared before losing membrane integrity and leaking their toxic intracellular contents. An important physiologic correlate of this occurs during the resolution phase of inflammation. During initiation of inflammation a wide variety of cytokines and chemokines are released, leading to the recruitment and maintenance of various effector cells of the immune system. As inflammation subsides, the concentration of these cytokines and chemokines diminishes, resulting in a relative deficiency of survival factors for the recruited cell populations still present in the area. Increased competition for a diminishing supply of survival factors leads to a failure to inhibit the default pathway

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4 Abbreviations used in this paper: Mφ, macrophage; BM, bone marrow derived; CM, conditioned medium; ERK, extracellular signal-regulated kinase; FN, fibronectin; MAPK, mitogen-activated protein kinase; P, peritoneal; PEC, peritoneal exudate cell; PI, propidium iodide; P38K, phosphatidylinositol 3-kinase; PS, phosphatidylerine; MEK1, mitogen-activated protein kinase/ERK kinase 1; TEM, transmission electron microscopy; BrdU, bromodeoxyuridine.
of apoptosis and, consequently, increased cell death. Rapid clearance of these apoptotic cells is essential to prevent leakage of their intracellular contents and generation of a new inflammatory focus.

Therefore, we hypothesized that professional phagocytic cells such as the MΦ should have a survival advantage over other cells in situations where there is a deficiency of survival factors. A possible mechanism for conferring such an advantage would be if the binding and/or uptake of apoptotic cells provided phagocytic cells with an independent survival signal that was capable of inhibiting the default pathway of apoptosis, even in the absence of other classic survival signals.

In this paper we demonstrate that uptake of apoptotic cells, in the absence of serum or other soluble survival factors, is able to inhibit apoptosis and maintain viability of primary cultures of murine MΦ, both peritoneal (P) and bone marrow derived (BM). Moreover, we show that apoptotic cells inhibit the proliferation of BM MΦ that have been stimulated with M-CSF. Inhibition of both apoptosis and proliferation is an unusual pattern, as most survival factors, especially cytokines, simultaneously inhibit apoptosis and stimulate proliferation (6, 7). However, concomitant inhibition of apoptosis and proliferation has been described in MΦ stimulated by danger signals, such as LPS, TNF-α, and bacterial DNA (8, 9).

We show that phagocytic uptake of apoptotic cells, like danger signals, mediates survival through activation of Akt. However, in contrast to danger signals, which activate the mitogen-activated protein kinase (MAPK) elements extracellular signal-regulated kinase (ERK)1 and ERK2 (9–12), apoptotic cells mediate their effect on proliferation through inhibition of ERK1 and ERK2. We further show that necrotic cells, like danger signals and unlike apoptotic cells, activate ERK1 and ERK2. Thus, uptake of apoptotic cells by MΦ induces novel signal transduction events, leading to promotion of survival and inhibition of proliferation. Both effects may have important consequences not only for resolution of inflammation but also for generation and maintenance of immune tolerance.

Materials and Methods

Reagents and cell culture

FBS, colchicine, bovine plasma fibronectin (FN), glucosamine, N-acetyl-

\( \text{glucosamine} \), and fraction V delipidated BSA were obtained from Sigma-Aldrich (St. Louis, MO). Dioleoyl phosphatidylserine (PS) and dioleoyl phosphatidylethanolamine were obtained from Avanti Polar Lipids (Alabaster, AL), LY294002 was obtained from Biomol (Plymouth Meeting, PA), H33342 dye was obtained from Calbiochem (San Diego, CA), RGD buffer (Arg-Gly-Asp-Sepharose matrix) was obtained from American Peptide Company (Sunnyvale, CA), and latex beads (1.15 μm) were obtained from Seradyn (Indianapolis, IN).

Peritoneal cells

Peritoneal exudate cells (PEC) were harvested by lavage from BALB/c or C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) 3 days after i.p. injection of 1.5 ml of 4.05% thioglycolate broth (13, 14). Cells were washed twice in RPMI 1640 and plated in 24-well tissue culture plates at 2 × 10^5 cells per well in RPMI 1640 plus 2.5% FBS, with 2 mM l-glutamine, 5 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin. After a 4-h incubation at 37°C, nonadherent cells were removed by washing with RPMI 1640. The remaining adherent cells, >98% MΦ as determined by morphologic examination and nonspecific esterase staining, were cultured in R.0 (R.2.5 minus FBS) or R.10 medium (R.0 plus 10% FBS), containing various concentrations of apoptotic cells or bodies.

BM MΦ

BM MΦ were obtained as described (13). Briefly, BM cells were expressed from the femur and tibia of BALB/c or C57BL/6 mice. After centrifugation at 500 × g, RBCs were lysed by resuspending the cell pellet in Tris/NH4Cl lysis buffer (9:1 mixture of 0.16 M NH4Cl (pH 7.2) and 0.17 M Tris base (pH 7.2)) at 37°C for 5 min. Cells were then washed three times in RPMI 1640 and plated in 24-well tissue culture plates at 1 × 10^5 cells per well in R.10 medium containing 15% L929 cell conditioned medium (CM) as a source of M-CSF. After 3 days of incubation, 50% of the medium was removed and replaced with fresh R.10 plus 15% L929 cell CM. After an additional 3 days, when BM MΦ were ~80% confluent, cells were used for survival studies.

Preparation of splenocytes and thymocytes

Splenocytes were harvested from the spleens of BALB/c or C57BL/6 mice (15). After lysing RBCs in Tris/NH4Cl lysis buffer, splenocytes were washed three times with RPMI 1640 and suspended in R.10 at 5 × 10^7 cells/ml. Apoptosis was induced by 600 rad of gamma irradiation from a 137Cs source followed by incubation at 37°C for 8 h. Thymocytes were harvested from the thymuses of BALB/c or C57BL/6 mice and suspended in R.10 at 5 × 10^7 cells/ml. Apoptosis was induced by addition of 5 × 10^{-4} M hydrocortisone followed by incubation at 37°C for 8 h. Before addition to MΦ cultures, apoptotic thymocytes or splenocytes were washed three times in RPMI 1640 and resuspended in R.0.

Documentation of splenocyte and thymocyte apoptosis

Viable cells were defined as propidium iodide (PI)-negative cells with faint nuclear Hoechst staining. Apoptotic cells were defined as PI-negative cells with bright nuclear Hoechst staining and decreased cell size. Postapoptotic cells (i.e., apoptotic cells that had lost cell membrane integrity) were defined as PI-positive cells with bright Hoechst staining and decreased cell size. By these criteria, ≥60% of cells were apoptotic, ~15% were viable, and ~25% were postapoptotic (15, 16). Necrotic cells, as defined by increased cell size in association with uptake of PI and faint Hoechst staining, comprised <0.1% of the final cell population (15, 16).

Preparation of apoptotic bodies

After induction of apoptosis, apoptotic splenocytes and thymocytes were pelleted by centrifugation at 500 × g for 10 min. The supernatant, containing apoptotic bodies, was removed and subjected to high-speed centrifugation at 13,000 × g for 30 min. The resulting sediment consisted of apoptotic bodies, as confirmed by transmission electron microscopy (TEM) (data not shown).

Jurkat T cells

The Jurkat human T cell leukemia line (no. TIB-152; American Type Culture Collection, Manassas, VA) was maintained in RPMI 1640 containing 10% FBS, 1 mM sodium pyruvate, 2 mM l-glutamine, 10 mM HEPES, and 100 U/ml penicillin-streptomycin. Apoptosis was induced by transferring cells to FBS-free medium containing 0.5% fatty acid-free BSA (Sigma-Aldrich) and 1 μg/ml staurosporine (Sigma-Aldrich), and incubating for 3 h. Necrosis was induced by one of two methods: heating to 65°C for 40 min or a single round of freeze-thaw cell lysis at ~70°C.

Induction of apoptosis and necrosis was confirmed by flow cytometry. Viable cells were defined as cells that were both PI and annexin V negative. Apoptotic cells were defined as PI-negative cells with annexin V staining and decreased cell size. Necrotic cells were defined as PI-positive cells lacking annexin V staining and of normal or increased cell size. Postapoptotic cells were defined as PI-positive cells with annexin V staining and decreased cell size. By these criteria, apoptotic Jurkat cell preparations contained ~85% apoptotic and ~15% postapoptotic cells. Both necrotic Jurkat T cell preparations contained >95% necrotic cells.

Preparation of phospholipids

A total of 99.95% delipidated fraction V BSA was dissolved at 10 mg/ml in calcium- and magnesium-free PBS, through which oxygen-free nitrogen had been bubbled for 20 min. Phospholipids were added to a final concentration of 0.5 mg/ml (1.1 nM) and stored under argon at ~70°C.

Thymidine incorporation

MΦ were cultured for 2 days in R.0, R.0 plus M-CSF, or R.0 plus various concentrations of apoptotic cells or bodies. A total of 2 μCi of [3H]thymidine (2 Ci/mmol; Du Pont NEN, Boston, MA) was added for the final 18 h. Cells were washed three times in RPMI 1640, then solubilized in 1 ml of 0.1 N NaOH. [3H]Thymidine cpm were measured by adding samples to scintillation fluid and counting using a beta counter (model 1600TR Tri-Carb liquid scintillation analyzer beta counter; Packard Instrument, Meriden, CT).

BrdU incorporation

MΦ were cultured identically as in the studies measuring [3H]thymidine incorporation. Cell proliferation was assessed using a colorimetric bromodeoxyuridine (BrdU) cell proliferation ELISA (Roche Diagnostic, Indianapolis, IN) according to the manufacturer’s specifications.

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**Antibodies**

Akt was detected with polyclonal rabbit sera recognizing either the active Ser473-phosphorylated form of Akt or total Akt irrespective of its state of phosphorylation (New England Biolabs, Beverly, MA). ERK1 and ERK2 (ERK1/2) were detected with polyclonal rabbit sera recognizing either the active Thr202- and Tyr204-phosphorylated form of ERK1/2 or total ERK1/2 irrespective of its state of phosphorylation. Secondary Ab was an alkaline phosphatase-labeled donkey anti-rabbit IgG (Promega, Madison, WI).

**Western blotting**

Møs were lysed in lysis buffer (20 mM Tris-HCl (pH 7.5), 140 mM NaCl, 50 mg/ml deoxycholate, 0.1% SDS, 1% Triton X-100, 10% glycerol, 1 mM Na3VO4, 1 mM DTT, 1 mM PMSF, 0.2% protease inhibitor mixture; Sigma-Aldrich). Following sonication (12 pulses), lysates were centrifuged at 14,000 × g for 10 min at 4°C, and the supernatants were stored at −70°C. Samples (20 μg each) were separated by SDS-PAGE, then transferred onto polyvinylidene difluoride membranes at 100 V for 1 h. Membranes were incubated in blocking buffer according to the manufacturer’s directions (Applied Biosystems, Bedford, MA), then exposed to primary Ab. Blots were washed three times with TBST buffer (100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20) and incubated with secondary Ab. Detection of secondary Ab was according to the manufacturer’s protocol (Chemiluminescent Immunoblot Detection Systems; Applied Biosystems).

**MTT assay**

Møs were cultured for 72 h in R.0, R.10, or R.0 plus various concentrations of apoptotic bodies and cells. The number of remaining viable Møs was determined using a modification of the MTT assay (17). After removing the growth medium, 165 μl of MTT dissolved in R.0 (1 mg/ml) was added to each well. After incubation at 37°C for 4 h, the MTT formazan was dissolved by adding 165 μl of 10% sodium dodecyl sulfate in 0.01 N HCl. Aliquots from each well were read using a microELISA plate reader with a test wavelength of 570 nm and a reference wavelength of 650 nm.

Data are presented as the percentage of increased viability above R.0 and normalized so that culture in R.10 represents 100%, using the following formula: % viability = (OD570 (apoptotic cells) − OD570 (R.0))/ (OD570 (R.10) − OD570 (R.0)) × 100%. For experiments using LY294002, data are presented as the percentage of viability of that seen with apoptotic bodies or cells alone in the absence of inhibitor, using the following formula: % viability = (OD570 (apoptotic cells + inhibitor)/ OD570 (apoptotic cells)) × 100%.

Normalization was necessary to facilitate comparison among results from separate experiments because the absolute number of Møs per well varied considerably from experiment to experiment, especially in the case of proliferative BM Møs. These formulas are consistent with those in our previous studies on Mø survival (14, 18).

**Statistics**

Quadruplicate wells were examined in each experiment, and the results were averaged. A minimum of three experiments was performed for each data point. Data are expressed as mean ± SEM of the averaged values obtained from each experiment. Statistical significance was determined by a two-tailed Student’s t test. All immunoblots are representative of at least three independent experiments.

**Results**

**Phagocytosis of Mø apoptotic debris contributes to the overall survival of cultured P Mø**

We have previously shown that primary cultures of murine P Mø undergo apoptosis upon withdrawal of FBS or growth factors (14, 18). After 72 h in FBS-free medium (R.0), the majority of Møs exhibits typical features of apoptosis, such as decreased cell size and rounding, nuclear condensation and fragmentation, DNA laddering, and detachment from the monolayer. Nonetheless, ~25% of Møs remain viable after 72 h, despite an absence of soluble survival factors (14, 18). While adhesion-mediated signaling events clearly play a role in residual Mø survival (14), we hypothesized that an additional survival activity might occur via phagocytosis of dying Mø by the remaining viable Møs. Because cells subjected to an apoptotic stimulus die individually in an asynchronous manner over hours or days (19), FBS-free culture should lead to a more or less continuous supply of apoptotic cells and bodies. Therefore, if phagocytosis contributes to residual Mø viability, then repeated washing of Mø monolayers should lead to decreased Mø survival.

After being plated in R.0, Møs were subjected to up to four sequential washes, occurring at 4, 24, 48, and/or 72 h after plating. For each wash, medium was removed, cells were rinsed with RPMI 1640, and fresh R.0 was added. After 96 h, Mø survival was evaluated by MTT assay (Fig. 1). Data are normalized so that survival of Mø in unwashed wells represents 100%. Mø survival decreased progressively with an increasing number of washes, plateauring at ~17% after four washes. The decrease in survival observed with each additional wash was statistically significant, including that between three and four washes (18.1 ± 1.2 vs 16.6 ± 0.8%; p < 0.01). The largest decrease in survival occurred with the first wash at 4 h. It should be noted that this initial wash at 4 h is part of our standard protocol for isolating P Møs and is used to remove nonadherent PEC (see Materials and Methods), nearly all of which would be expected to undergo apoptosis. The diminishing effect of each successive wash is consistent with a diminishing supply of apoptotic cells, as fewer and fewer Møs remain alive with progressive time.

To assess directly the relative roles of mechanical trauma and apoptotic debris in modulating Mø survival, we determined the effects of Mø CM and its components on Mø survival. CM was generated by incubating Mø cultures for 24 h in R.0 after an initial wash to remove nonadherent PEC. CM-derived Mø apoptotic debris was then separated from the supernatant by high-speed centri‐
fugation (13,000 × g). TEM of the ultracentrifuged pellet confirmed the presence of apoptotic Mø cells and bodies (data not shown). Separate Mø cultures were washed 4 h after plating to remove nonadherent PEC. One of the following media was then added: fresh R.0, whole CM, CM supernatant, or CM-derived apoptotic cells and bodies resuspended in fresh R.0. All Mø cultures underwent an equal number of washes, and survival was evaluated 72 h later (Fig. 2). Survival was greatest for those conditions containing apoptotic debris, either CM-derived apoptotic cells and bodies resuspended in fresh R.0 or whole CM (71.3 ± 11.2% and 58.8 ± 4.4%, respectively; p < 0.05 for both conditions vs fresh R.0). In contrast, survival was lowest for those conditions lacking apoptotic debris, either R.0 or CM supernatant (49.7 ± 2.8% and 49.4 ± 5.3%, respectively). Because the mechanical trauma of washing was equivalent for all conditions, these data indicate that the loss of survival activity induced by repetitive washing of Mø

![FIGURE 1](http://www.jimmunol.org/) Sequential washing of Mø cultures decreases cell survival. P Mø cultured in FBS-free medium (R.0) were washed at the indicated times and fresh R.0 was added. After 96 h, relative cell number was determined by MTT assay. The decrease in survival seen with each successive wash was statistically significant, including that between three and four washes (p < 0.01).
cultures resides at least partially in removal of apoptotic cells and bodies that have detached from the monolayer. The similarity between fresh R.0 and CM supernatant \((p > 0.95)\) implies that soluble mediators released by \(\text{M} \Phi\) into CM do not significantly contribute to the effect of washing on \(\text{M} \Phi\) survival.

**Apoptotic splenocytes and thymocytes enhance the survival of murine \(P\) and \(BM \text{M} \Phi\)**

We next tested directly the effect of apoptotic cells on \(\text{M} \Phi\) survival. To eliminate any confounding influence on \(\text{M} \Phi\) survival by interaction with foreign cells, we used syngeneic splenocytes and thymocytes as a source of apoptotic cells. Apoptosis was induced by two different means, either gamma irradiation (splenocytes) or exposure to hydrocortisone (thymocytes). In addition, because recognition and uptake of apoptotic cells by \(\text{M} \Phi\) is dependent upon their state of differentiation and/or activation (20, 21), we determined the effect of apoptotic cells on two separate \(\text{M} \Phi\) populations: terminally differentiated elicited \(P \text{M} \Phi\) and unactivated \(BM \text{M} \Phi\) induced to differentiate in vitro from \(BM\) precursor cells under the influence of \(M\)-CSF.

Addition of apoptotic splenocytes or thymocytes enhanced the survival of both \(P\) and \(BM \text{M} \Phi\) (Fig. 3). The survival activity of apoptotic splenocytes at the highest ratio used (20 apoptotic cells per \(\text{M} \Phi\)) was 69.1 ± 7.5% and 91.4 ± 12.7% of that seen with 10% FBS for \(P\) and \(BM \text{M} \Phi\), respectively. A significant effect of apoptotic splenocytes on the survival of \(P\) and \(BM \text{M} \Phi\) was seen at a ratio as low as one apoptotic cell per \(\text{M} \Phi\) \((p < 0.01,\) compared with R.0). The survival activity of apoptotic thymocytes was less than that of splenocytes. At the highest ratio used (20:1), the survival activity of apoptotic thymocytes was 37.2 ± 4.3% and 24.7 ± 5.8% of that seen with 10% FBS for \(P\) and \(BM \text{M} \Phi\), respectively. A significant effect of apoptotic thymocytes on the survival of \(P\) and \(BM \text{M} \Phi\) was seen at a ratio as low as one apoptotic cell per \(\text{M} \Phi\) \((p < 0.05,\) compared with R.0).

To rule out an artifactual effect of apoptotic cells on the MTT assay, apoptotic splenocytes or thymocytes were also added to blank wells in the absence of \(\text{M} \Phi\) and cultured for 72 h. The number of apoptotic cells was equivalent to that used at the highest

**FIGURE 2.** Decreased survival with sequential washing is attributable to removal of apoptotic bodies. \(P \text{M} \Phi\) were washed after 4 h of culture in R.0, and their medium was replaced with one of the following: fresh R.0, un-separated CM, supernatant portion of CM, or apoptotic debris portion of CM resuspended in fresh R.0. CM was generated by incubating separate \(\text{M} \Phi\) cultures for 24 h in R.0 after an initial wash to remove nonadherent cells. CM was separated into supernatant and apoptotic debris by ultracentrifugation at 13,000 \(x\) \(g\). Greater survival occurred for those conditions containing apoptotic debris, and lesser survival occurred for those lacking apoptotic debris \(p < 0.05\). *\(p < 0.05,\) for both CM un-separated and CM apoptotic bodies vs R.0.

**FIGURE 3.** Apoptotic splenocytes and thymocytes are survival factors for \(P\) and \(BM \text{M} \Phi\). \(\text{M} \Phi\) were cultured for 72 h in R.0 plus various numbers of apoptotic splenocytes (A) or thymocytes (B). Relative \(\text{M} \Phi\) survival was determined by MTT assay. Data are presented as the percentage of response of that for 10% FBS (P) or M-CSF (BM). Addition of zero apoptotic cells is identical to culturing \(\text{M} \Phi\) in R.0. By our normalization procedure, the percentage of survival in R.0 (zero apoptotic cells) is set equal to 0. Values of \(p < 0.05\) for all ratios of apoptotic splenocytes or thymocytes compared with R.0.

20:1 ratio. Although ~15% of cells were still viable at the time of plating, there were no viable cells after 72 h and MTT readings from these wells were no different from background, thereby confirming that the observed effect of apoptotic cells is attributable to increased \(\text{M} \Phi\) survival.

**Splenocyte, but not thymocyte, apoptotic bodies enhance the survival of murine \(P\) and \(BM \text{M} \Phi\)**

Apoptotic cells undergo a process of plasma membrane blebbing that leads to fragmentation of the cell into apoptotic bodies, which are membrane-enclosed vesicles containing nuclear fragments of condensed chromatin and cytosolic organelles such as mitochondria (1). Apoptotic bodies, like intact apoptotic cells, are rapidly ingested by phagocytes. We produced apoptotic bodies by a two-step centrifugation, an initial low-speed centrifugation to eliminate intact cells followed by a high-speed centrifugation to collect apoptotic bodies. TEM of the pellet confirmed the presence of splenocyte and thymocyte apoptotic bodies without intact cells (data not shown).

Addition of splenocyte apoptotic bodies enhanced the survival of both \(P\) and \(BM \text{M} \Phi\) (Fig. 4). Because the actual number of apoptotic bodies could not be directly measured, the data are expressed in terms of the number of cells from which the apoptotic bodies were derived. The survival activity of splenocyte apoptotic bodies at the highest ratio used (100:1) was 39.6 ± 4.9% and 69.6 ± 23.7% for \(P\) and \(BM \text{M} \Phi\), respectively. A significant effect of splenocyte apoptotic bodies on \(P\) and \(BM \text{M} \Phi\) survival was observed at an equivalent cell ratio as low as 6.25:1 \((p < 0.05)\). In
Addition of zero apoptotic cells corresponds to culturing M/Ph in the presence of M-CSF or apoptotic cells or bodies from splenocytes and thymocytes was near background and did not differ from that seen with R.0 (data not shown). These results are consistent with our previously reported finding that P MΦ are terminally differentiated and do not proliferate (14, 18). However, in contrast to P MΦ, BM MΦ proliferate in response to mitogens such as M-CSF. Data for BM MΦ are presented as the percentage of increased [3H]thymidine incorporation above R.0 and normalized so that culture with M-CSF represents 100%. As shown in Fig. 5, [3H]thymidine incorporation by BM MΦ in the presence of apoptotic cells or bodies was not significantly different from that in R.0. We conclude that apoptotic cells and bodies are not mitogenic for P or BM MΦ; therefore, apoptotic cells maintain MΦ viability solely through inhibition of apoptosis.

The absence of a proliferative effect by apoptotic cells and bodies on BM MΦ is somewhat surprising, as most survival factors stimulate proliferation at the same time they promote survival (6, 7). However, our result may make sense from a teleological point of view. If uptake of apoptotic cells confers a survival advantage to MΦ when there is a relative deficiency of survival factors, then a desired outcome would be ongoing clearance of apoptotic cells without the burden of an increased number of cells, phagocytic or otherwise. Therefore, we determined the effect of apoptotic cells and bodies on BM MΦ induced to proliferate by M-CSF. As shown, splenocyte apoptotic cells and bodies profoundly inhibited M-CSF-induced proliferation of BM MΦ (2.7 ± 1% and 5.5 ± 2.2% of proliferation seen with M-CSF alone, respectively). Thymocyte apoptotic cells and bodies also significantly inhibited M-CSF-induced proliferation of BM MΦ (64.7 ± 12.6% and 53.2 ± 7.3%, respectively), though to a lesser extent than splenocyte apoptotic material. We confirmed these results using BrdU incorporation. Apoptotic splenocytes and thymocytes reduced M-CSF-induced proliferation to 24.2 ± 23.4% and 9.1 ± 9.1% of that with M-CSF alone, respectively. We conclude that apoptotic cells and bodies differ from most survival factors in that they inhibit, rather than stimulate, proliferation.

marked contrast, the addition of thymocyte apoptotic bodies produced no significant effect on the survival of BM MΦ and even had a negative effect on the survival of P MΦ at several cell ratios. This difference between splenocyte and thymocyte apoptotic bodies persisted when apoptotic bodies from these two sources were normalized for protein content (data not shown). We cannot explain this difference between apoptotic bodies derived from splenocytes vs thymocytes. While, in most cases, phagocytes appear to recognize universal markers of apoptosis present on virtually all cells undergoing apoptosis, there is precedent for phagocytic discrimination based on the identity of the apoptotic cell (22).

**Apoptotic cells and bodies inhibit proliferation at the same time they promote survival**

The increased numbers of viable MΦ seen with addition of apoptotic cells or bodies is potentially the result of two independent processes: inhibition of apoptosis and/or stimulation of proliferation. We assessed the contribution of cell-coupled proliferation to increased P and BM MΦ viability by measuring [3H]thymidine incorporation as an index of DNA synthesis. MΦ were cultured for 48 h in R.0 alone, R.0 plus M-CSF, or R.0 plus apoptotic cells or bodies. [3H]Thymidine was added for the final 18 h.

**FIGURE 4.** Apoptotic bodies are survival factors for P and BM MΦ. MΦ were cultured for 72 h in R.0 plus apoptotic bodies derived from various cell equivalents of apoptotic splenocytes (A) or thymocytes (B). Relative MΦ survival was determined by MTT assay. Data are presented as the percentage of response of that for 10% FBS (P) or M-CSF (BM). Addition of zero apoptotic cells corresponds to culturing MΦ in R.0. By our normalization procedure, percent survival in R.0 (zero apoptotic cells) is equal to 0. Values of p < 0.05 for all ratios of apoptotic splenocyte bodies compared with R.0; p = NS for all ratios of apoptotic thymocyte bodies compared with R.0.

**FIGURE 5.** Apoptotic cells and bodies inhibit the proliferation of BM MΦ. BM MΦ were cultured for 48 h in the presence of R.0, M-CSF, apoptotic splenocytes or thymocytes (20 apoptotic cells per MΦ) alone or in the presence of M-CSF, or bodies derived from apoptotic splenocytes and thymocytes (100 apoptotic cell equivalents per MΦ) alone or in the presence of M-CSF. [3H]Thymidine incorporation was determined during the final 18 h of incubation. Data are presented as the percentage of change in [3H]thymidine incorporation compared with M-CSF. *, p < 0.01 for all apoptotic conditions in the presence of M-CSF compared with M-CSF alone; p = NS for all apoptotic conditions alone compared with R.0.
Inhibiting the phagocytic uptake of apoptotic cells with colchicine blocks the survival activity for Mφ

To confirm that apoptotic cells promote Mφ survival in a receptor-specific manner, we assessed the effect of apoptotic cells on Mφ survival in the presence and absence of colchicine, a microtubular inhibitor that prevents phagocytosis of apoptotic cells (23). P and BM Mφ were preincubated with colchicine (10^{-5} or 10^{-6} M) for 1 h before addition of apoptotic cells (20 apoptotic cells per Mφ). After 4 h, the colchicine and apoptotic cells were removed by washing. To prevent internalization of apoptotic cells that had bound to Mφ cell surface receptors and were not washed away, we added back colchicine to all appropriate wells. Mφ survival was determined 72 h later by MTT assay. Data are again presented as the percentage of survival above that for R.0, and normalized so that survival in 10% FBS is 100%. Light microscopic examination confirmed inhibition of apoptotic cell uptake by colchicine at both concentrations (data not shown).

The survival activity of apoptotic splenocytes and thymocytes for P Mφ was inhibited at both concentrations of colchicine (Fig. 6A). Loss of survival activity in the presence of colchicine is not the result of toxicity, as colchicine had no effect on the survival of P Mφ cultured in 10% FBS (122.9 ± 26.8% and 118.8 ± 26.3% for 10^{-5} and 10^{-6} M colchicine, respectively; p = NS vs 10% FBS). The results for BM Mφ were similar (Fig. 6B). At 10^{-5} M, colchicine significantly decreased the survival activity of both apoptotic splenocytes and thymocytes for BM Mφ, whereas, at 10^{-6} M, the effect of colchicine was significant only for apoptotic thymocytes. As in the case of P Mφ, colchicine was not toxic to BM Mφ (92.8 ± 20% and 78.9 ± 17.4% for 10^{-5} and 10^{-6} M colchicine, respectively; p = NS vs 10% FBS). Colchicine also did not affect survival of P and BM Mφ in R.0 (−11.3 ± 7.8% and −10.3 ± 10.4% for P Mφ, and −0.6 ± 5.5% and −3.1 ± 4.3% for BM Mφ, with 10^{-5} and 10^{-6} M colchicine, respectively; p = NS vs R.0 for P and BM Mφ at both concentrations of colchicine).

**FIGURE 6.** Inhibition of apoptotic uptake by colchicine blocks the survival activity of apoptotic splenocytes and thymocytes. P (A) and BM (B) Mφ were cultured for 4 h in R.0 plus apoptotic splenocytes or thymocytes (20 apoptotic cells per Mφ) in the presence or absence of colchicine (10^{-5} or 10^{-6} M). Monolayers were then washed and cell viability was assessed 72 h later by MTT assay. Data are presented as the percentage of response of that for 10% FBS (P) or M-CSF (BM). *p < 0.05 for colchicine (10^{-5} and 10^{-6} M) vs no colchicine for all combinations of P and BM Mφ and apoptotic splenocytes and thymocytes, with the sole exception of BM Mφ exposed to splenocytes and 10^{-6} M colchicine; p = NS for colchicine vs no colchicine for P and BM Mφ cultured in R.0 alone or 10% FBS (P) and M-CSF (BM).

Apoptotic cell-mediated survival occurs through PI3K and Akt

Activation of phosphatidylinositol 3-kinase (PI3K) and its downstream target Akt plays a critical role in survival factor signaling by most cytokines and in inhibition of apoptosis by adhesive interactions (14, 24, 25). Therefore, we investigated the role of PI3K and Akt in Mφ survival induced by apoptotic cells.

LY294002 is a potent and specific inhibitor of PI3K with an IC_{50} of ≈1 μM (14, 25). P and BM Mφ were cultured for 72 h in R.0 plus apoptotic cells or bodies and LY294002. Data are normalized so that Mφ survival in the absence of LY294002 is 100%. As shown in Fig. 7, LY294002 inhibited the survival activity of

**FIGURE 7.** PI3K is necessary for Mφ survival mediated by apoptotic cells and bodies. P (A) and BM (B) Mφ were cultured for 72 h in R.0 plus apoptotic splenocytes or thymocytes (20 apoptotic cells per Mφ) or splenocyte apoptotic bodies (100 apoptotic cell equivalents per Mφ) and the indicated concentrations of LY294002. Cell viability was assessed by the MTT assay. Data are presented as the percentage of response of that for apoptotic cells or bodies in the absence of LY294002. It should be noted that, in the absence of LY294002, <25% of P or BM Mφ remain viable after 72 h of culture in R.0. In the presence of LY294002, this percentage decreases further as a result of inhibition of basal PI3K activity.
spleenocyte apoptotic cells and bodies and thymocyte apoptotic cells for both P and BM Mφ in a dose-dependent manner. In all cases, the IC_{50} for inhibition of survival by LY294002 was very close to the IC_{50} for inhibition of PI3K activity.

We next showed that uptake of apoptotic cells induces activation of Akt. P and BM Mφ were deprived of FBS and/or M-CSF for 48 h and then given apoptotic cells. Total cellular Akt was detected with a polyclonal serum that recognizes Akt irrespective of its state of phosphorylation, whereas activated Akt was detected with a polyclonal serum that recognizes only the active Ser473-phosphorylated form of Akt. Uptake of a mixture of apoptotic spleenocytes and thymocytes increased the amount of active phosphorylated Akt in P and BM Mφ (Fig. 8A). Similar results were obtained when Mφ were exposed to unmixed populations of apoptotic spleenocytes or thymocytes (data not shown). An increase of activated Akt was evident 15 min after addition of apoptotic cells. Levels of activated Akt remained elevated up to 30 min (Fig. 8A). Both times likely underestimate the actual kinetics of signal transduction, because, as opposed to soluble ligands, contact between Mφ and apoptotic cells does not occur immediately but is dependent on settling of apoptotic cells to the bottom of the culture well. The degree of activation of Akt by apoptotic cell uptake was comparable to that induced by high-dose insulin (5 μM) (Fig. 8A). As would be expected given these short time periods, total Akt was unaffected by apoptotic cell uptake (Fig. 8A). Inhibition of apoptotic uptake by preculturing P and BM Mφ for 1 h with colchicine (10^{-5} M) led to near-maximal inhibition of Akt activation (Fig. 8B).

Finally, because we were adding up to 20 apoptotic cells per Mφ in these studies and Mφ were ingesting many of these apoptotic cells, it was crucial to assess total and activated Akt in the apoptotic cells themselves (Fig. 8A). While apoptotic thymocytes and splenocytes both contained abundant Akt, activated Akt could not be detected in either population, even upon prolonged exposure of blots. In some cases, despite equal protein loading, the relative amount of total Akt was less in apoptotic cells than in Mφ (Fig. 8A), reflecting either differences in expression of Akt between these two cell types or partial degradation of Akt protein as a result of apoptosis. We conclude that phagocytosis of apoptotic cells by Mφ leads to activation of Akt, most likely through PI3K.

**Inhibition of binding of apoptotic cells to the vitronectin receptor on BM Mφ blocks phosphorylation of Akt**

While colchicine inhibits the internalization of apoptotic cells by Mφ, it does not affect the interaction of apoptotic cells with Mφ surface receptors. We next determined whether inhibition of binding of apoptotic cells to Mφ surface receptors would affect activation of Akt. For reasons discussed below, these studies were of necessity limited to BM Mφ. Apoptotic uptake by unactivated BM Mφ occurs preferentially through an integrin-dependent mechanism (20, 21, 26). Thrombospondin, secreted by the BM Mφ, acts as a bridging protein that simultaneously binds the vitronectin receptor (α3β1 integrin) and CD36 (type B scavenger receptor) on the BM Mφ and an as-yet-uncharacterized thrombospondin-binding monely on the apoptotic cell (27). Peptides and proteins bearing the RGD integrin-recognition signal inhibit uptake of apoptotic cells by BM Mφ through competition for the α3β1 integrin (26, 27). We could not use RGD-containing peptides because they induced apoptosis of BM Mφ (data not shown), as has been reported in other cell types (28). Therefore, we used the RGD-containing protein FN (26).

BM Mφ were preincubated for 6 h with FN (100 μg/ml) and then exposed to apoptotic cells. Total and activated Akt were assessed 30 min after the addition of apoptotic cells (Fig. 9). Light microscopic examination showed that FN inhibited apoptotic cell uptake by ~75% (data not shown), in accord with published data (26). Addition of FN alone to BM Mφ led to an increase in activated Akt. This result is consistent with integrin-mediated activation of PI3K and Akt through binding of FN to the α3β1 integrin (29). The increase of activated Akt induced by a 6-h incubation with FN was less than that induced by a 15-min exposure to apoptotic cells alone. Importantly, preinculture with FN inhibited the increase in activated Akt produced by apoptotic cells so that the level of activated Akt was equivalent by densitometry to that seen with FN alone. We conclude that inhibition of the interaction of apoptotic

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**FIGURE 8.** Uptake of apoptotic cells by Mφ activates Akt. P and BM Mφ were cultured for 48 h in R.0 then preincubated in the absence (A) or presence (B) of colchicine (10^{-5} M) for 1 h before the addition of apoptotic spleenocytes and/or thymocytes (20 apoptotic cells per Mφ). Total and activated (Ser473-phosphorylated) Akt were determined by immunoblotting at the indicated times (A) or at 30 min after the addition of apoptotic cells (B).

**FIGURE 9.** Inhibition of the binding of apoptotic cells to the vitronectin receptor on BM Mφ blocks activation of Akt. BM Mφ were cultured for 48 h in R.0 and then preincubated for 6 h in the presence or absence of FN (100 μg/ml) before the addition of a mixture of apoptotic spleenocytes and thymocytes (20 apoptotic cells per Mφ). Total and activated (Ser473-phosphorylated) Akt were determined by immunoblotting 15 min after the addition of apoptotic cells.
cells with the vitronectin receptor on BM Mϕ prevents antiapoptotic signaling events in Mϕ.

We were unable to extend these studies to other Mϕ apoptotic cell receptors for two major reasons. First, Mϕ possess multiple distinct cell surface receptors that act in parallel to mediate the recognition and uptake of apoptotic cells (2, 3). Thus, even simultaneous inhibition of multiple receptors rarely diminishes apoptotic uptake by >75% (22). The success of FN in inhibiting activation of Akt is attributable to the predominant use by BM Mϕ of the αvβ3 integrin in mediating apoptotic cell uptake (20, 21, 26). Second, and more importantly, many of the known inhibitors of apoptotic uptake act by competing with apoptotic cells for binding to Mϕ receptors and may therefore themselves act as survival factors. Indeed, consistent with its activation of Akt, FN alone acted as a survival factor for BM Mϕ, having 10.7 ± 2.4% of the survival activity of M-CSF (*p* < 0.05, compared with R0).

As an additional example, PS, an anionic phospholipid that is expressed on the surface of apoptotic cells and predominantly mediates the uptake of apoptotic cells by P Mϕ (20, 30), independently promoted the survival of P Mϕ (39.2 ± 3.9% survival activity of FBS; *p* < 0.000001, compared with R0). PS also significantly promoted the survival of BM Mϕ (9 ± 1.6% survival activity of FBS; *p* < 0.000001, compared with R0), though to a much lower extent. This is consistent with the fact that P Mϕ preferentially, but not exclusively, recognize apoptotic cells via a PS receptor, whereas BM Mϕ preferentially, but not exclusively, recognize apoptotic cells via an integrin-dependent mechanism (20–22). The effect of the neutral phospholipid phosphatidylethanolamine was significantly less than that of PS for both P and BM Mϕ (23.8 ± 3.5% and –5 ± 2.3%, respectively; *p* < 0.0001, compared with PS). Finally, glucosamine and N-acetyl-d-glucosamine, which preferentially inhibit apoptotic uptake by BM and P Mϕ, respectively (21), each weakly promoted Mϕ survival (data not shown).

The survival activity of these inhibitors prevented their use as competitive antagonists of apoptotic cell uptake in both Mϕ survival studies and Akt assays. Nevertheless, their independent ability to promote the survival of Mϕ cultured under FBS-free conditions offers strong support to the hypothesis that apoptotic cells act as Mϕ survival factors via their interaction with apoptotic receptors on the Mϕ cell surface. Our data with colchicine imply that mere engagement of Mϕ apoptotic cell receptors may be insufficient to promote survival, and that signaling events related to receptor and/or apoptotic cell internalization are necessary to initiate survival signals.

**Apoptotic cell-mediated inhibition of proliferation occurs through inhibition of the MAPK pathway**

We next studied the mechanism by which apoptotic cells inhibited the proliferation of BM Mϕ stimulated by M-CSF. Recent studies in fibroblasts have shown that commitment to enter the cell cycle requires the temporally coordinated input of three signaling intermediates and/or pathways: mitogen-activated protein kinase/ERK kinase 1 (MEK1) (which lies directly upstream from and activates the MAPK family members ERK1/2), e-Myc, and PI3K (31). Because apoptotic cells activated Akt (cf Fig. 8A) and, presumably, PI3K (cf Fig. 6), inhibition of proliferation should involve either e-Myc or MEK1.

We focused on ERK1/2, the immediate downstream target of MEK1. Total cellular ERK1/2 was detected with a polyclonal serum that recognizes ERK1/2 irrespective of its state of phosphorylation, whereas activated ERK1/2 was detected with a polyclonal serum that recognizes only the active phosphorylated form of ERK1/2. Consistent with the failure of apoptotic cells to stimulate proliferation (cf Fig. 5), apoptotic cells did not activate ERK1/2 in P or BM Mϕ and even reduced basal ERK1/2 activity in BM Mϕ (Fig. 10A).

Because P Mϕ are nonproliferative, the remainder of these studies were performed exclusively with BM Mϕ. As expected, stimulation of BM Mϕ to enter the cell cycle with M-CSF led to activation of ERK1/2 (Fig. 10B). Strikingly, the addition of apoptotic cells to M-CSF-stimulated BM Mϕ almost completely inhibited ERK1/2 activation. Marked inhibition of ERK1/2 activation was seen, irrespective of whether apoptotic cells were added 30 min before or 15 min after stimulation with M-CSF. As expected, given these short time periods, total ERK1/2 was unaffected by apoptotic cell uptake. As in the case of Akt, activated ERK1/2 could not be detected in apoptotic cells (Fig. 10B). The relative amount of total ERK1/2 was less in apoptotic cells than in Mϕ, again reflecting either differences in expression of ERK1/2 between these two cell types or partial degradation of ERK1/2 protein as a result of apoptosis.

We next determined whether inhibition of apoptotic uptake by preincubating BM Mϕ for 6 h with FN (100 μg/ml) could prevent the decrease of ERK1/2 activation (Fig. 11). In the absence of FN, apoptotic cells inhibited M-CSF-induced ERK1/2 activation (Fig. 11, cf lanes 4 and 5). As assessed by densitometry, inhibition by apoptotic cells was ≥100%, because ERK1/2 activity in the presence of M-CSF and apoptotic cells (Fig. 11, lane 5) was less than constitutive ERK1/2 activity in resting Mϕ (Fig. 11, lane 2). These data are in accord with those of Fig. 10. Preincubating BM Mϕ with FN partially abrogated the inhibition of M-CSF-induced ERK1/2 activity produced by apoptotic cells (Fig. 11, cf lanes 7 and 8). As assessed by densitometry, the addition of FN restored M-CSF-induced ERK1/2 activity in the presence of apoptotic cells from 0 (Fig. 11, lane 5) to ~60% (Fig. 11, lane 8) of that seen with

**FIGURE 10.** Uptake of apoptotic cells by BM Mϕ inhibits ERK1/2 activation. P and BM Mϕ were cultured for 48 h in R0. A mixture of apoptotic splenocytes and thymocytes (20 apoptotic cells per Mϕ) were added either alone (A) or 30 min before or 15 min after stimulation with M-CSF (B). The circled element indicates which of M-CSF or apoptotic cells was added first. Total and activated ERK1/2 were determined by immunoblotting at the indicated times (A) or after a total of 45 min in the case of consecutive stimulation with M-CSF and/or apoptotic cells (B).
M-CSF alone (Fig. 11, lane 7). The degree to which FN abrogated the inhibitory effect of apoptotic cells on M-CSF-induced ERK1/2 activity (~60%) corresponds roughly to the degree to which FN inhibited apoptotic uptake by BM Mφ (~75%).

**Signaling events induced by apoptotic cells are distinct from those induced by necrotic cells and phagocytosis**

To determine whether activation of Akt and inhibition of ERK1/2 occur specifically in response to apoptotic cells and are not non-specific events in response to phagocytosis or cellular clearance, we tested the effect of uptake of latex particles (1.15 μm) and necrotic cells. For these studies, we used human Jurkat T cells. Apoptosis was induced by a 3-h exposure to the broad-spectrum kinase inhibitor staurosporine (1 μg/ml), while necrosis was induced by either heating to 65°C for 40 min or a single round of freeze-thaw lysis at −70°C. The degree to which FN abrogated the inhibitory effect of apoptotic cells on M-CSF-induced ERK1/2 activity (~60%) corresponds roughly to the degree to which FN inhibited apoptotic uptake by BM Mφ (~75%).

**Inhibition of the binding of apoptotic cells to the vitronectin receptor on BM Mφ blocks inhibition of MAPK activity.** BM Mφ were cultured for 48 h in R.0 and then preincubated for 6 h in the presence or absence of FN (100 μg/ml) before the addition of a mixture of apoptotic splenocytes and thymocytes (20 apoptotic cells per Mφ). The circled element indicates that stimulation with M-CSF preceded addition of apoptotic cells by 15 min. Total and activated ERK1/2 were determined by immunoblotting 30 min after addition of apoptotic cells.

**Phagocytic clearance of necrotic cells and latex beads by Mφ activates Akt.** BM Mφ were incubated for 48 h in R.0 before the addition of apoptotic or necrotic Jurkat T cells (one cell per Mφ) or latex beads (1.15 μm). Apoptosis was induced by a 3-h treatment with staurosporine (1 μg/ml), while necrosis was induced by a single round of freeze-thaw lysis at −70°C. Total and activated (Ser473-phosphorylated) Akt were determined by immunoblotting at 30 min after addition of cells or latex beads.

**Discussion**

Phagocytic clearance of apoptotic cells plays a critical role in the resolution of inflammation (3). As inflammation subsides, large numbers of recruited inflammatory cells remain amid a diminishing supply of growth and survival factors. This deficiency of survival factors results in a failure to suppress the default pathway of apoptosis (6, 7) so that the recruited inflammatory cells, which are no longer needed, die by apoptosis. Rapid and efficient clearance of these apoptotic cells is essential to prevent leakage of toxic intracellular contents that may perpetuate tissue injury and inflammation. Mφ are the major cells responsible for clearance of apoptotic cells (2, 3), yet Mφ are no different from other cells in their dependence upon survival factors to maintain viability (9, 14, 18). We reasoned that Mφ must have a selective advantage over other cells if they are to continue their essential role of clearance and not succumb to the deficiency of survival factors.

In this paper, we show that Mφ do indeed have a survival advantage when there is a deficiency of survival factors, and that this advantage is conferred directly by the uptake of apoptotic cells. Thus, in the complete absence of serum or other soluble survival factors, the addition of apoptotic cells to Mφ inhibited apoptosis and maintained Mφ viability with a potency approaching that of 10% FBS. Uptake of apoptotic cells acted as a survival factor for two distinct Mφ populations—elicited terminally differentiated P Mφ and unactivated proliferating BM Mφ—suggesting that the role of apoptotic clearance as a survival factor may generalize to other phagocytic cells. In addition, the survival activity of apoptotic cell uptake was independent of the source of apoptotic cells or the method of induction of apoptosis, as gamma irradiated splenocytes and hydrocortisone-treated thymocytes both potently promoted Mφ survival. This result is not surprising, given the highly conserved features of apoptotic cells across multiple species. In fact, apoptotic human Jurkat T cells were as effective as murine splenocytes or thymocytes in promoting murine Mφ survival.

While splenocyte apoptotic bodies also acted as potent Mφ survival factors, thymocyte apoptotic bodies were ineffective. This difference may reflect an overall greater potency of apoptotic splenocytes over thymocytes as survival factors, whether as intact
cells (cf. Fig. 3) or as apoptotic bodies (cf. Fig. 4). Although we did not investigate the basis for this difference, subtle discrimination of apoptotic cells by a given phagocytic cell type is not without precedent. For example, the anti-CD14 mAb MEM-18 inhibited M/H9278 uptake of apoptotic Jurkat T cells but not that of apoptotic neutrophils (22).

Promotion of M/H9278 survival by phagocytic uptake of apoptotic cells most likely occurs through activation of Akt by PI3K. The addition of apoptotic cells to P and BM M/H9278 led to an increase in the amount of Ser 473-phosphorylated Akt, the active form of this enzyme. Inhibiting the uptake of apoptotic cells with colchicine blocked activation of Akt and enhancement of M/H9278 survival in both P and BM M/H9278. Similarly, FN, a known inhibitor of apoptotic uptake by BM Mφ (26, 27), prevented activation of Akt. Moreover, the PI3K inhibitor LY294002 (25) inhibited the survival activity of phagocytic uptake of apoptotic cells and bodies in both P and BM Mφ. In all cases, the IC₅₀ for inhibition of survival matched the IC₅₀ for inhibition of PI3K activity (14, 25).

Our studies do not elucidate the signaling pathways by which phagocytosis of apoptotic cells activates PI3K and Akt. The fact that colchicine inhibited activation of Akt suggests that mere engagement of M/H9278 apoptotic receptors may be insufficient. Colchicine blocks the internalization of apoptotic cells but not their interaction with receptors on the M/H9278 cell surface (23). Therefore, events associated with receptor and/or apoptotic cell internalization may be required. A similar requirement for internalization seems to underlie the ability of bacterial DNA to promote Mφ survival (9, 32, 33).

As opposed to most other survival factors, which promote proliferation at the same time as they inhibit apoptosis, uptake of apoptotic cells by BM Mφ led to near complete inhibition of M-CSF-induced proliferation. Most soluble Mφ survival factors, such as M-CSF or GM-CSF, stimulate Mφ proliferation (6, 7). Similarly, adhesive interactions between the cell and extracellular matrix promote survival (29) and are necessary for cell proliferation (34). Thus, the pattern of increased survival and decreased proliferation is relatively unusual for a survival factor. However, from a

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<td>M-CSF-induced ERK1/2</td>
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*↑, Induction; ↓, inhibition; −↑, no effect or slight induction; −↓, no effect or slight inhibition; −, no effect.
teleological point of view, inhibition of proliferation by uptake of apoptotic cells may be a beneficial event in that it limits the number of cells, phagocytic or otherwise, under conditions in which there is a deficiency of survival factors.

The combination of signaling events by which uptake of apoptotic cells simultaneously promotes survival and inhibits proliferation has a particularly elegant basis. Recent studies in fibroblasts have shown that continuous stimulation by growth factors throughout G1 phase is not an absolute requirement for entry into the cell cycle (31, 35, 36). Rather, fibroblasts can be induced to enter the cell cycle with two short pulses of mitogen, the first occurring near the onset of G1 and the second occurring ~8 h later and several hours before the completion of G1 (31, 35). Surprisingly, of the multiple signaling pathways initiated by mitogens during these two narrow windows of time, the temporally coordinated combination of just three is sufficient to drive cells through G1 and into S phase. Activation of MEK1 (which lies directly upstream from and activates ERK1/2) and induction of c-Myc are sufficient during the first window (31, 35), whereas activation of PI3K is sufficient during the second (35, 36). Although growth factors activate PI3K during both windows of time, activation of PI3K is entirely dispensable during the second window (31, 35) whereas activation of PI3K during the first window blocks proliferation (36). While the precise role of PI3K during the first window has yet to be established, it seems likely that one of its functions is promotion of survival via Akt.

This model of cell cycle progression provides an explanation for the effects of apoptotic uptake on Mϕ survival and proliferation. Inhibition of ERK1/2 eliminates one of the two signaling events required for progression through the first window of G1, thereby inhibiting proliferation at the very onset of G1. Although apoptotic uptake activates PI3K, Mϕ do not progress to the second window of G1, in which PI3K can propel cells through the final steps of G1. While PI3K is ineffective at inducing cell cycle entry because of inhibition of ERK1/2, it can still promote cell survival through activation of Akt. Thus, by simultaneously activating PI3K and inhibiting ERK1/2, apoptotic uptake leads to enhanced Mϕ survival with suppression of proliferation.

It should be noted that inhibition of ERK1/2 is an unusual mechanism by which extracellular agents block proliferation. While mechanisms of antiproliferation vary widely and ultimately entail modulation of components and regulators of the cell cycle machinery (35, 37), the majority of antiproliferative agents, including members of the TGF-β family, activate rather than inhibit ERK1/2 (9–12, 38–40). Indeed, for many agents it is the duration and intensity of ERK1/2 activation that seem to determine the effect on cell proliferation, with transient low-magnitude activation favoring proliferation and sustained high-magnitude stimulation favoring antiproliferation (38, 39, 41). The two most prominent groups of antiproliferative agents that inhibit ERK1/2 are 1) agents that raise intracellular cAMP (42, 43) and 2) members of the IFN family, especially IFN-α (44, 45). Elevated intracellular cAMP is unlikely to explain the effect of apoptotic uptake because cAMP has been shown to enhance, rather than inhibit, ERK activity in BM Mϕ (46), as opposed to other cell types. In the case of IFN-α, inhibition of ERK1/2 has been examined only in transformed cell lines, with the proposed mechanism involving inhibition of protein kinase C (45). Taken together, our data suggest that uptake of apoptotic cells by Mϕ induces novel signaling pathways that lead to inhibition of both apoptosis and proliferation through simultaneous activation of PI3K and inhibition of ERK1/2. A number of microbial products, or danger signals (e.g., LPS, TNF-α, and bacterial DNA), which activate the innate immune system, have also been shown to inhibit apoptosis and proliferation of Mϕ. Importantly, while apoptotic cells and danger signals both activate PI3K, they have opposite effects on ERK1/2. Indeed, apoptotic and necrotic cells had opposite effects on ERK1/2 activity (Table I). It is tempting to speculate that the different signaling events induced by apoptotic cells vs necrotic cells or danger signals may underlie the divergent outcomes generated by Ag presented in the context of apoptotic cells or danger signals, namely tolerance or immunity.

References


