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Differential Effects of Apoptotic Versus Lysed Cells on Macrophage Production of Cytokines: Role of Proteases

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Granulocytes undergoing apoptosis are recognized and removed by phagocytes before their lysis. The release of their formidable arsenal of proteases and other toxic intracellular contents into tissues can create significant damage, prolonging the inflammatory response. Binding and/or uptake of apoptotic cells by macrophages inhibits release of proinflammatory cytokines by mechanisms that involve anti-inflammatory mediators, including TGF-β. To model the direct effects of necrotic cells on macrophage cytokine production, we added lysed or apoptotic neutrophils and lymphocytes to mouse and human macrophages in the absence of serum to avoid complement activation. The results confirmed the ability of lysed neutrophils, but not lymphocytes, to significantly stimulate production of macrophage-inflammatory protein 2 or IL-8, TNF-α, and IL-10. Concomitantly, induction of TGF-β1 by lysed neutrophils was significantly lower than that observed for apoptotic cells. The addition of selected serine protease inhibitors and anti-human elastase Abs markedly reduced the proinflammatory effects, the lysed neutrophils then behaving as an anti-inflammatory stimulus similar to intact apoptotic cells. Separation of lysed neutrophils into membrane and soluble fractions showed that the neutrophil membranes behaved like apoptotic cells. Thus, the cytokine response seen when macrophages were exposed to lysed neutrophils was largely due to liberated proteases. Therefore, we suggest that anti-inflammatory signals can be given by PtdSer-containing cell membranes, whether from early apoptotic, late apoptotic, or lysed cells, but can be overcome by proteases liberated during lysis. Therefore, the outcome of an inflammatory reaction and the potential immunogenicity of Ags within the damaged cell will be determined by which signals predominate.


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2 Address correspondence and reprint requests to Dr. Valerie A. Fadok, National Jewish Medical and Research Center, DS09, 1400 Jackson Street, Denver, CO 80206. E-mail address: fadok@njc.org
3 Abbreviations used in this paper: HMDM, human monocyte-derived macrophages; MIP2, macrophage-inflammatory protein 2; BMDM, bone marrow-derived macrophage(s); AEBSF, 4-(2-aminoethyl) benzenesulfonylfluoride; TMB, tetramethylbenzidine.

Materials and Methods

Cell culture

HMDM were cultured as described previously (3, 12). In brief, mononuclear cells were isolated from the blood of normal donors, and plated at 4
LYSED NEUTROPHILS STIMULATE MACROPHAGE TNF-α, CHEMOKINE, AND IL-10

In the presence of serum, neutrophils are activated by other neutrophils to produce TGF-β, TNF-α, and IL-8. This phenomenon is known as the “lysed neutrophil effect.” The present study investigated the role of lymphotoxin, a cytokine produced by lymphocytes, in the activation of neutrophils.

**Materials and Methods**

**Induction of apoptosis**

Human neutrophils and the human T cell line Jurkat were used as apoptotic targets. Neutrophils were induced to undergo apoptosis by exposure to UV irradiation for 5 min followed by culture for 3 h. As described previously, Jurkat T cells were incubated with 10 ng/ml of TNF-α and BMDM with 75 μg/ml of pepstatin, and 15 μg/ml of neutrophil elastase and 5 U/ml purified human cathepsin G (both neutrophils were incubated with the macrophages in the presence or absence of lymphocytes). The percent apoptosis was assessed by staining the macrophage monolayers with annexin V and PI, and by flow cytometry, using FITC-conjugated annexin V. For neutrophils, the average percent apoptosis was 74.3 ± 5.4% (SEM); for Jurkats, 75.3 ± 4.2% (SEM); trypan blue positivity was <4% for either population; propidium iodide positivity was 6%. Results of annexin V positivity were not significantly different from apoptosis as assessed by morphology (data not shown).

**Lysis**

Human neutrophils and Jurkat T cells were rapidly frozen as cell pellets on dry ice, then stored at −80°C. They were thawed at room temperature without washing, and suspended in X-Vivo without serum before use. The average percent lysis, assessed by trypan blue positivity, for neutrophils was 82.6 ± 2.4% (SEM) and for Jurkat T cells 87.6 ± 1.4% (SEM). Staining with FITC-conjugated annexin V and propidium iodide revealed that 100% of each population were positive for both. One cycle of freeze/thaw was used to give intact but trypan blue-positive bodies. For preparation of membrane and cytosol fractions, the cells were subjected to five freeze/thaw cycles, homogenized in X-Vivo medium, and centrifuged at 100,000 × g for 2 h. The membrane pellets or membrane-free supernatants were added to macrophages as cell equivalents to compare them to the intact but leaky lysed cells. Membrane and cytosolic fractions were also made from apoptotic and viable granulocytes by homogenizing them in X-Vivo, and by ultracentrifugation.

**Macrophage cytokine production**

Macrophages were cultivated in 24-well plates and each condition was run in duplicate. Five million apoptotic or necrotic (lysed) cells were added per well of macrophages for 1 h, then washed. Fresh X-Vivo medium without serum was added, and supernatants were collected 24 h later. replicate wells were used to assess uptake of the phagocytic targets. For neutrophils, the monolayers were stained for myeloperoxidase as previously described and shown; only those macrophages that had engulfed neutrophils, the monolayers were stained for myeloperoxidase as previously described and shown; only those macrophages that had engulfed neutrophils were scored as positive for staining (3). Uptake of apoptotic neutrophils was associated with intracellular staining of discrete intracellular bodies, whereas uptake of necrotic (lysed) cells was associated with paler staining intracellular bodies and with diffuse staining of the macrophages. Uptake of lymphocytes was assessed by staining the macrophage monolayers with a modified Wright’s Giemsa stain. For some experiments, the lyzed neutrophils were incubated with the macrophages in the presence or absence of 100 μg/ml PMN (Sigma, St. Louis, MO), 50 μM 4-(2-aminoethyl)benzenesulfonylfluoride (AEBSF) (Calbiochem, La Jolla, CA), 5 μg/ml aprotonin, 0.25 μg/ml leupeptin, 1 μg/ml E-64, 40 μg/ml bestatin, 0.7 μg/ml pepstatin, and 15 μg/ml calpain 1 inhibitor (Boehringer Mannheim, Indianapolis, IN), anti-human neutrophil elastase, anti-human cathepsin G, and isotype controls for 24 h before collection of supernatants for evaluation of cytokine concentrations. Preliminary dose responses were performed to insure a lack of toxicity to the macrophage monolayers. Viability at the concentrations used was verified by trypan blue concentration (<4% positive cells) and by preservation of the stimulatory response to zymosan (assessed by measurement of macrophage-inflammatory protein 2 (MIP2), TNF-α, IL-10, and TGF-β).

Anti-human elastase and anti-human cathepsin G Abs were purified sheep IgG purchased from Cortex Biochem (San Leandro, CA), and were used at 50 μg/ml. Zymosan (Sigma) was used as a stimulus for cytokine production; HMDM were stimulated with 25 μg/ml and BMDM with 75 μg/ml. In some experiments, 0.01 U/ml purified human neutrophil elastase and 5 U/ml purified human cathepsin G (both obtained from Calbiochem) were added to determine their direct effects on macrophage cytokine production. The concentrations of these cytokines were determined by lack of effects on macrophage viability and peak of cytokine production. Supernatants from duplicated wells were pooled, centrifuged to remove cellular debris, and stored at −70°C until analyzed. As a control, apoptotic or lysed cells were cultured for the same time periods in X-Vivo medium without macrophages to determine whether they produced cytokines. Neither apoptotic neutrophils nor apoptotic Jurkat T cells produced significant levels of any of the cytokines tested.

**Analysis of cytokines**

Cytokines assayed included TGF-β1, TNF-α, IL-10, and either MIP2 for mouse or IL-8 for human cells. Matched Ab pairs for the ELISAs were purchased from R&D Systems (Minneapolis, MN). For TGF-β1, supernatants were activated with HCl before analysis. Cytokine levels were detected following incubation with the biotinylated secondary Abs by incubation with avidin-conjugated HRP, then tetramethylbenzidine and H2O2 as substrate. The plates were read in a Bio-Tek EL309 ELISA reader (Biotek Instruments, Winooska, VT), and results were analyzed using the log/log curve fit option from Delta Soft 3 (BioMetals, Princeton, NJ).

**Measurement of elastase**

Freshly isolated neutrophils were suspended at 10 million per ml in X-Vivo, then immediately centrifuged, separated into pellet and supernatant, and frozen at −70°C. Early and late apoptotic neutrophils were prepared as described above except that they were cultured at 10 million per ml in X-Vivo medium, then centrifuged. The samples were frozen as pellets, and the supernatant was derived from culture. Lysed neutrophils were prepared by suspending neutrophils at 10 million per ml in X-Vivo and freezing at −70°C. On the day of the analysis, the pellets and supernatants were thawed. The necrotic cell preparations were thawed, then centrifuged to separate the material into pellet and supernatant. All cell pellets were solubilized in lysis buffer containing 50 mM Tris (pH 7.4), 1% Triton X-100, 0.25% deoxycholate, 150 mM NaCl, and 1 mM EGTA. For elastase analysis, 20 μl of solubilized pellet or supernatant was added in triplicate to 96-well ELISA plates, followed by 55 μl elastase reaction buffer (0.1 M HEPES, 0.5 M NaCl, 10% DMSO pH 7.5). Then, 150 μl of 0.2 M Elastase substrate 1 (methoxy succinyl-ala-ala-pro-val-nitroanilide; Calbiochem) in elastase reaction buffer was added, and the samples were incubated at 37°C for 1 h. Elastase levels were determined by measuring absorbance at 410 nm, using serial dilutions of porcine elastase (Calbiochem) as standards. Specificity was determined by incubating the samples and standards in the presence or absence of Elastase Inhibitor III (methoxy succinyl-ala-ala-pro-val-chloromethylketone (Calbiochem).

**Statistical analysis**

For Figs. 1–3 and 6–7, data were evaluated by ANOVA and the Tukey Kramer method. For Figs. 4 and 5, ANOVA and Dunnett’s method were used, designating macrophages treated with lyzed neutrophils as the control for Fig. 4, and macrophages without target cells added as the control for Fig. 5. Significance was set at p < 0.05.

**Results**

We first studied the effects of the binding and/or uptake of apoptotic vs lysed human neutrophils on HMDM. The percent macrophages positive for uptake of apoptotic neutrophils was 49 ± 8.5% and for uptake of lyzed neutrophils was 57.5 ± 10.5%. There appeared to be no measurable cytokine production by the apoptotic or lyzed cells when incubated without macrophages (data not shown); thus the cytokine levels shown here appear to reflect macrophage production. As shown in Fig. 1, exposure to apoptotic human neutrophils in the absence of serum had the expected effects: increased production of TGF-β1, with no stimulation of IL-10, TNF-α, or a chemokine (IL-8), and suppression of IL-8, TNF-α, and IL-10 when macrophages were costimulated with zymosan. In contrast, exposure to lyzed neutrophils induced significantly lower levels of TGF-β1 than apoptotic cells, and stimulated production of IL-10, TNF-α, and IL-8 (p < 0.05). Although lyzed neutrophils stimulated proinflammatory cytokine production, the levels were lower than those induced by zymosan. When the macrophages were stimulated with zymosan to produce inflammatory...
cytokines, both apoptotic and lysed neutrophils were able to significantly suppress TNF-\(\alpha\) and IL-10 production, although apoptotic cells were significantly more effective \((p < 0.05)\). Similar effects were seen when mouse BMDM were used (Fig. 2). Percent uptake of apoptotic cells by BMDM was 45 ± 7.8% and of lysed cells 52.9 ± 11.2%. If the serine protease inhibitor PMSF was added, the proinflammatory response of the unstimulated macrophage to lysed neutrophils was significantly inhibited (Fig. 2A), as was the production of IL-10 \((p < 0.05)\). When PMSF was added to the lysed neutrophils coincubated with zymosan-stimulated macrophages, the lysed neutrophils behaved like apoptotic cells (Fig. 2B). Treatment with PMSF did not alter the effects of apoptotic cells or zymosan-stimulated macrophages, the lysed neutrophils behaved like apoptotic cells (Fig. 2B). Treatment with PMSF did not alter the effects of apoptotic cells or zymosan on macrophage cytokine production. Lysed Jurkat T cells had the same effects on macrophage cytokine production as on human macrophages, but lysed Jurkat T cells behave like apoptotic cells. Addition of PMSF abrogates the effects of lysed neutrophils. A. Apoptotic or lysed human neutrophils or Jurkat T cells were fed to mouse BMDM for 1.5 h then washed out. Medium was added, and supernatants were collected for evaluation of cytokine production by ELISA. B, Apoptotic or lysed human neutrophils or Jurkat T cells were added to BMDM as described in A; however, after washing, fresh medium containing 75 \(\mu\)g/ml zymosan was added, and supernatants were collected 24 h later for cytokine measurement by ELISA. * Statistically different from all other samples \((p < 0.05)\); **, statistically significant difference from macrophages incubated with apoptotic cells \((p < 0.05)\). In all cases, results from macrophages stimulated with apoptotic cells are significantly different from macrophages without neutrophils added.

![FIGURE 1](image1.png) Lysed neutrophils stimulate chemokine, TNF-\(\alpha\), and IL-10 production by human macrophages. Apoptotic or lysed human neutrophils were coincubated with HMDM in the presence or absence of zymosan (25 \(\mu\)g/ml) for 24 h. Supernatants were collected and cytokines evaluated by ELISA. n = 15 (± SEM). * Statistically significant difference from macrophages without addition of cells or macrophages incubated with apoptotic neutrophils; **, statistically significant difference from macrophages incubated with apoptotic cells \((p < 0.05)\). In all cases, results from macrophages stimulated with apoptotic cells are significantly different from macrophages without neutrophils added.

![FIGURE 2](image2.png) Lysed neutrophils have similar effects on mouse macrophages as on human macrophages, but lysed Jurkat T cells behave like apoptotic cells. Addition of PMSF abrogates the effects of lysed neutrophils. A. Apoptotic or lysed human neutrophils or Jurkat T cells were fed to mouse BMDM for 1.5 h then washed out. Medium was added, and supernatants were collected for evaluation of cytokine production by ELISA. * Statistically different from all other samples \((p < 0.05)\); **, significantly different from macrophages incubated with apoptotic cells and from macrophages incubated with lysed cells in the presence of PMSF. Macrophages incubated with either apoptotic or lysed Jurkat T cells showed statistically significant increased TGF-\(\beta\) production. B. Apoptotic or lysed human neutrophils or Jurkat T cells were added to BMDM as described in A; however, after washing, fresh medium containing 75 \(\mu\)g/ml zymosan was added, and supernatants were collected 24 h later for cytokine measurement by ELISA. n = 18 (± SEM). * Statistically different from zymosan-treated macrophages and from zymosan-treated macrophages preincubated with apoptotic cells. Macrophages incubated with either apoptotic or lysed Jurkat T cells showed statistically significant increased TGF-\(\beta\) production.
tain a crude membrane fraction and a cytosolic fraction; each of these were added to mouse macrophages in the presence or absence of zymosan to determine their effects on cytokine production. We also used membranes and cytosol from lysed Jurkat cells. As shown in Fig. 3, the cytosolic fraction from neutrophils contained the activity that stimulated MIP2, TNF-α, and IL-10. The membranes were anti-inflammatory as they induced the secretion of TGF-β, and inhibited zymosan-induced MIP2, TNF-α, and IL-10. We also used membrane and cytosolic fractions from viable and apoptotic neutrophils; as for neutrophils lysed by freeze-thaw cycles, the cytosolic fraction stimulated MIP2, TNF-α, and IL-10, whereas the membranes stimulated TGF-β (data not shown).

In the next set of experiments, we used a variety of protease inhibitors, as well as Abs against human neutrophil elastase and human cathepsin G, to determine their effects on lysed neutrophil-induced cytokine production. In Fig. 4, we show that the serine protease inhibitors PMSF and AEBSF were strong inhibitors of the proinflammatory response. The serine protease inhibitors aprotinin and leupeptin were weak inhibitors. Inhibitors of cysteine proteases (E-64), metalloproteases (bestatin), aspartic proteases (pepstatin), and calpain had no effect. Anti-human elastase Ab (50 μg/ml) was a strong inhibitor, but anti-human cathepsin G (50 μg/ml) was only a weak inhibitor; increasing the concentration of the latter did not improve the inhibition, and the isotype control (sheep IgG) had no effect. Neither of these Abs at the concentration used stimulated cytokine production from control HMDM (data not shown). These results support the interpretation that neutrophil elastase plays a major role in the stimulation of macrophage cytokine production.

Next, the production of cytokines from mouse BMDM exposed to early apoptotic neutrophils (cultured for 3 h after UV irradiation, trypan blue positivity ≤4%), late apoptotic neutrophils (cultured for 24 h after UV irradiation, trypan blue positivity 45%), and lysed neutrophils was assessed. As shown in Fig. 5, late apoptotic cells are similar to early apoptotic cells in that they induce TGF-β secretion and do not significantly induce MIP2, TNF-α, and IL-10.

It was important to determine whether neutrophil elastase could induce the same pattern of cytokine production as lysed neutrophils. Therefore, HMDM and mouse BMDM were exposed to purified human neutrophil elastase and human cathepsin G. As shown in Fig. 6, neutrophil elastase at 0.01 U/ml was a potent stimulator of mouse macrophage TNF-α, MIP2, and IL-10 production; cathepsin G virtually no activity, requiring 5 U/ml to see a small amount of IL-10 production only. Although not shown, the effects on HMDMs were identical.

Last, elastase concentrations in the pellet and medium from early and late apoptotic neutrophils were compared with those from freshly isolated and lysed neutrophils. Fig. 7 shows that early and late apoptotic neutrophils released virtually no elastase into their medium. Lysed neutrophils, as expected, released a large amount of elastase, and the proportion remaining in the cell pellet was significantly lower than that in the pellets from the apoptotic cells (p < 0.05). Lysed Jurkat T cells, as expected, were negative for elastase (data not shown).

Discussion:

The purpose of the experiments presented here was to determine whether lysed cells, used as a model for necrotic cells, could directly stimulate macrophages to release proinflammatory mediators in the absence of serum. We found that only lysed neutrophils could do so, and that the activity could be attributed to released neutrophil elastase. The binding and/or uptake of apoptotic neutrophils and lymphocytes by either mouse or human primary macrophages suppressed zymosan-induced production of proinflammatory cytokines as expected, exemplified by decreased levels of TNF-α, IL-10, and either MIP-2 or IL-8, as we have shown previously (3, 4). This down-regulation results in large part from secretion of TGF-β1, at least part of which is bioactive (3, 4), and the...
effects of apoptotic cells can be mimicked by treatment of macrophages with PtdSer-containing liposomes or a mAb against a Ptd-Ser receptor (5). Thus, uptake of apoptotic cells before their lysis not only prevents the release of potentially toxic or immunogenic intracellular contents but also induces an anti-inflammatory phenotype in the macrophage. We also determined that late apoptotic neutrophils, even though becoming permeable to propidium iodide and trypan blue, behave more like early apoptotic cells (Fig. 5) because they induced production of TGF-β but did not significantly stimulate TNF-α, IL-10, or the chemokine MIP2. Furthermore, late apoptotic cells released very little elastase into their culture medium (Fig. 7). These results are in keeping with the earlier observations of Ren and Savill, who observed that apoptotic neutrophils beginning to become permeable to trypan blue were not proinflammatory (14). The response to lysed cells in the unstimulated macrophages was expected (2) in that they induced TNF-α and chemokine production; however, the levels were low relative to those seen with a strong proinflammatory stimulus such as zymosan or LPS (the latter not shown). Lysed cells also consistently induced IL-10 production, in contrast to apoptotic cells.

When macrophages were stimulated with zymosan, they produced robust levels of MIP-2 or IL-8, TNF-α, and IL-10. Interestingly, lysed neutrophils were found not to have additive effects in the absence of serum; rather, they partially (but significantly, p < 0.05) inhibited zymosan-stimulated production of TNF-α and IL-10, although having no effect on the chemokines MIP-2 or IL-8, both of which are CXC chemokines chemotactic for neutrophils. Lysed neutrophils also induced significantly less TGF-β1 than did

**FIGURE 5.** Late apoptotic neutrophils and early apoptotic neutrophils stimulate TGF-β secretion, and fail to stimulate MIP2, TNF-α, or IL-10. Early and late apoptotic neutrophils, and lysed neutrophils, were prepared as described in Materials and Methods, and added to mouse BMDM. Supernatants were collected and evaluated for cytokine production by ELISA. n = 5 (±SEM); *, significantly different (p ≤ 0.05) from macrophages incubated in the absence of added cells.

**FIGURE 4.** Serine protease inhibitors and anti-human elastase Ab inhibit the ability of lysed neutrophils to stimulate MIP2, TNF-α, and IL-10 from mouse and human macrophages. Statistically significant differences (p < 0.05) are illustrated by solid black bars. n = 10 (±SEM). Not shown are the isotype controls for the anti-protease Abs, which had no inhibitory effect. None of the inhibitors shown inhibited zymosan stimulation of MIP2, TNF-α, or IL-10, suggesting that the protease inhibitors and Abs were not toxic.
The activity represented in this figure was completely inhibited by elastase or apoptotic neutrophils (SEM). Elastase and zymosan (positive control) were added to macrophages in X-Vivo medium without serum, and supernatants were collected for cytokine measurements 24 h later. n = 5 (±SEM).

As expected, lysed neutrophils appeared to be more potent inducers of macrophage cytokine production in the absence of serum than were lysed Jurkat T cells, which behaved, for the most part, like apoptotic cells. Given that granulocytes produce high levels of several different types of proteases, it seemed reasonable to suggest that the differing effects of lysed neutrophils compared with lysed lymphocytes were related to protease release. The serine protease inhibitors PMSEF and AEBSF significantly decreased the lysed neutrophils were homogenized and separated into membrane and soluble fractions, the effects of the membranes were indistinguishable from apoptotic cells, which express phosphatidylserine and are identifiable from apoptotic cells, which express phosphatidylserine. Given that granulocytes produce high levels of several different types of proteases, it seemed reasonable to suggest that the differing effects of lysed neutrophils compared with lysed lymphocytes were related to protease release. The serine protease inhibitors PMSEF and AEBSF significantly decreased the proinflammatory effects of lysed neutrophils on macrophage cytokine production, while leading to an up-regulation of TGF-β, permitting them to have anti-inflammatory effects similar to those induced by apoptotic cells. The strong inhibitory effect of the anti-serine proteases, particularly neutrophil elastase (27–29). Although IL-10 is not believed to affect the constitutive rate of neutrophil apoptosis, it enhances apoptosis of neutrophils at inflammatory sites, promotes the survival of macrophages, and enhances macrophage removal of apoptotic neutrophils (22–26). Thus, the protease-mediated release of IL-10 in inflammatory sites may represent a protective mechanism designed to promote resolution of inflammation by enhancing neutrophil apoptosis. Certainly, in its absence, inflammation is more severe in inflammatory cytokine production is not yet known. It may bind directly to protease-activated receptors. For example, Ishihara and coworkers recently demonstrated specific binding of neutrophil elastase to macrophages; binding was accompanied by enhanced production of chemokines, which was inhibited by PMSEF (15). In vivo, the proinflammatory actions of the proteases likely result not only from their actions on macrophages but on other cell types and on extracellular matrix. Alternatively, or in addition, the proteases may cleave a critical membrane signal or signals that mediate the down-regulation of proinflammatory cytokines associated with the uptake of apoptotic cells. In support of this notion is our observation that the PDMA receptor can be cleaved off the cell surface by trypsin (5); in addition, we have preliminary data to suggest that neutrophil elastase may cleave it as well (W. Vandivier, V.A.F., and P.M.H., unpublished data). In fact, the predicted extracellular domain of the PDMA receptor has several potential cleavage sites for elastase, given that the preference for P1 is A>V>T>I (16). This receptor, when stimulated by apoptotic cells, phosphatidylserine-containing membranes, or a stimulatory mAB, induces the release of TGF-β and the down-regulation of proinflammatory cytokines (5). Our studies suggest that the cellular carcass of a cell progressing to the late stages of apoptosis can retain anti-inflammatory activity. With regard to the observations of Ren and Savill that neutrophils that have progressed from early to late apoptosis (i.e., becoming permeable to trypan blue) fail to stimulate macrophage cytokine production (14), we suggest that the anti-inflammatory signal of the exposed phosphatidylserine on the late apoptotic cell predominates, as the cells have not released proteases to cleave the PDMA receptor. In any event, we suggest that activated, necrotic, or lysed cells can, by releasing proteases, overwhelm the anti-inflammatory effects of macrophage PDMA receptor interactions with PDMA-expressing apoptotic cells or membranous debris, and that prolonged inflammation would be the predicted end stage of cell death in the absence of clearance by phagocytes. In vivo, endogenous anti-proteases also contribute to the complexity of the final response.

One of the interesting observations from this study was that lysed cells induced release of IL-10, which could be attributed to the effect of serum proteases, particularly neutrophil elastase. Others have observed that IL-10 is up-regulated early and concomitantly with TNF-α and chemokines in a variety of inflammatory conditions (17–21). Although IL-10 is not believed to affect the constitutive rate of neutrophil apoptosis, it enhances apoptosis of neutrophils at inflammatory sites, promotes the survival of macrophages, and enhances macrophage removal of apoptotic neutrophils (22–26). Thus, the protease-mediated release of IL-10 in inflammatory sites may represent a protective mechanism designed to promote resolution of inflammation by enhancing neutrophil apoptosis. Certainly, in its absence, inflammation is more severe in a number of models (27–29).

Both TGF-β (resulting from stimulation with early or late apoptotic cells) and IL-10 (resulting from stimulation with lysed cells) have anti-inflammatory activities, including the down-regulation of inflammatory mediator production by macrophages (30–35). However, in our experiments, down-regulation of chemokines and TNF-α was associated with TGF-β production; the pattern of IL-10 secretion in the absence of serum was similar to that for proinflammatory cytokines. Both TGF-β and IL-10 can be immunosuppressive by exerting effects on both lymphocytes and APCs. The presence of elasstase was completely inhibited by elastase directly stimulated IL-10, TNF-α, and chemokine production, and only lysed neutrophils released elastase into the medium.

How neutrophil elastase directly promotes macrophage proinflammatory cytokine production is not yet known. It may bind directly to protease-activated receptors. For example, Ishihara and coworkers recently demonstrated specific binding of neutrophil elastase to macrophages; binding was accompanied by enhanced production of chemokines, which was inhibited by PMSEF (15). In vivo, the proinflammatory actions of the proteases likely result not only from their actions on macrophages but on other cell types and on extracellular matrix. Alternatively, or in addition, the proteases may cleave a critical membrane signal or signals that mediate the down-regulation of proinflammatory cytokines associated with the uptake of apoptotic cells. In support of this notion is our observation that the PDMA receptor can be cleaved off the cell surface by trypsin (5); in addition, we have preliminary data to suggest that neutrophil elastase may cleave it as well (W. Vandivier, V.A.F., and P.M.H., unpublished data). In fact, the predicted extracellular domain of the PDMA receptor has several potential cleavage sites for elastase, given that the preference for P1 is A>V>T>I (16). This receptor, when stimulated by apoptotic cells, phosphatidylserine-containing membranes, or a stimulatory mAB, induces the release of TGF-β and the down-regulation of proinflammatory cytokines (5). Our studies suggest that the cellular carcass of a cell progressing to the late stages of apoptosis can retain anti-inflammatory activity. With regard to the observations of Ren and Savill that neutrophils that have progressed from early to late apoptosis (i.e., becoming permeable to trypan blue) fail to stimulate macrophage cytokine production (14), we suggest that the anti-inflammatory signal of the exposed phosphatidylserine on the late apoptotic cell predominates, as the cells have not released proteases to cleave the PDMA receptor. In any event, we suggest that activated, necrotic, or lysed cells can, by releasing proteases, overwhelm the anti-inflammatory effects of macrophage PDMA receptor interactions with PDMA-expressing apoptotic cells or membranous debris, and that prolonged inflammation would be the predicted end stage of cell death in the absence of clearance by phagocytes. In vivo, endogenous anti-proteases also contribute to the complexity of the final response.

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(36–40). However, TGF-β has been shown to inhibit full maturation of dendritic cells, even in the presence of inflammatory stimuli such as TNF-α, effectively preventing the development of potentially harmful immune responses from a resolving inflammatory site (41). TGF-β also mediates bystander suppression associated with physiological self-tolerance in vivo, thereby preventing the activation of autoreactive lymphocytes (42). These activities may be critical when apoptotic cells are removed during physiological cell death or with apoptotic cell uptake associated with resolution of inflammation.

Exposure to necrotic vs apoptotic cells also has differential effects on maturation of dendritic cells for Ag presentation to lymphocytes. Sauter et al. recently showed that exposure of dendritic cells to necrotic tumor cells or to supernatants derived from these cells enabled them to mature into fully functional APCs (43). They also found differences in cell type in that only necrotic tumor cells, but not necrotic primary cells, could induce this effect. They could not define the active factor; however, released intracellular proteases may be worth evaluating in this system as well.

In summary, we have learned that exposure to apoptotic cells, whether neutrophils or lymphocytes, inhibited macrophage proinflammatory cytokine by a mechanism involving TGF-β. Whether neutrophils or lymphocytes, inhibited macrophage proinflammatory cytokines (including chemokines), and increased release of TGF-β. These data suggest that the release of serine proteases, particularly those from neutrophils, can be proinflammatory by directly stimulating macrophages to produce cytokines. Therefore, lack of removal of apoptotic cells before lysis will prolong inflammation, increasing the potential for tissue damage, and thus providing signals to promote an immune response.

It is important to note that the experiments presented here have been conducted in the absence of serum to avoid the confounding effects of complement activation. Giclas and colleagues showed many years ago that mitochondria can activate the complement cascade, thereby contributing to the proinflammatory effects of lysed cells (10, 11). Future work will focus on the effects of serum in our in vitro system and how it influences the interaction between apoptotic cells and macrophages; however, preliminary data suggest that exposing macrophages to either apoptotic neutrophils or Jurkat T cells in the presence of serum still causes down-regulation of TNF-α and chemokine production, as is seen in the absence of serum; TGF-β production is also up-regulated (V.A.F., unpublished data). These observations suggest that the anti-inflammatory effects of apoptotic cells are dominant to any proinflammatory effects of complement, as apoptotic cells have been shown to activate both the classical and alternative complement pathways (44). Interestingly however, in the presence of serum, we found that IL-10 production by macrophages was enhanced by apoptotic cells of either type, whereas in the absence of serum, its production was inhibited. Whether IL-10 contributes to the anti-inflammatory effects of apoptotic cells in our system remains to be determined. Furthermore, when serum was present, necrotic Jurkat T cells demonstrated proinflammatory activity in that unstimulated macrophages secreted TNF-α and chemokines. These preliminary results are in keeping with Giclas’ observations that intracellular organelles can fix complement (10, 11), particularly because heating serum to 56°C abolished these effects (V.A.F., unpublished data).

In conclusion, it seems reasonable to hypothesize that apoptotic neutrophils are anti-inflammatory for two reasons: first, they fail to release their serine proteases even when they begin to become leaky (as assessed by uptake of propidium iodide and trypan blue), and because the phosphatidyserine exposed on their surfaces is significantly anti-inflammatory. One can predict that the outcome of inflammation will be determined by the balance between proinflammatory signals generated by the release of granulocyte proteases and anti-inflammatory signals generated by exposure to phosphatidyserine-containing membranes associated with apoptotic cells or membranous cellular debris. The balance between proteases and anti-proteases will also contribute significantly to whether an inflammatory lesion resolves or not. Importantly, the anti-inflammatory potential of apoptotic neutrophils appears to be maintained even through the late stages, providing multiple opportunities for resolution of inflammation.

Note added in proof. Cocco and Ucker very recently showed that the inhibitory effects of apoptotic cells on macrophage cytokine production were dominant to the proinflammatory effects of necrotic cells (45). Jaffray and coworkers recently showed that pancreatic elastase induces macrophages to produce TNF-α in a NF-κB-dependent manner, supporting the hypothesis of surface receptors for elastase on macrophages (46).

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References


