Specific Recognition of Apoptotic Cells Reveals a Ubiquitous and Unconventional Innate Immunity*

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The purpose of physiological cell death is the noninflammatory clearance of cells that have become inappropriate or non-functional. Consistent with this function, the recognition of apoptotic cells by professional phagocytes, including macrophages and dendritic cells, triggers a set of potent anti-inflammatory responses manifest on multiple levels. The immediate-early inhibition of proinflammatory cytokine gene transcription in the phagocyte is a proximate consequence of recognition of the apoptotic corpse, independent of subsequent engulfment and soluble factor involvement. Here, we show that recognition is linked to a characteristic signature of responses, including MAPK signaling events and the ablation of proinflammatory transcription and cytokine secretion. Specific recognition and response occurs without regard to the origin (species, tissue type, or suicidal stimulus) of the apoptotic cell and does not involve Toll-like receptor signaling. These features mark this as an innate immunity fundamentally distinct from the discrimination of “self” versus “other” considered to be the hallmark of conventional immunity. This profound unconventional innate immune discrimination of effete from live cells is as ubiquitous as apoptotic cell death itself, manifest by professional and non-professional phagocytes and nonphagocytic cell types alike.

Innate apoptotic immunity provides an intrinsic anti-inflammatory circuit that attenuates proinflammatory responses dynamically and may act systemically as a powerful physiological regulator of immunity.

The process of physiological cell death assures the elimination of functionally inappropriate cells in a manner that does not elicit inflammation (1, 2). Professional phagocytes, including resident macrophages and dendritic cells, participate in the efficient clearance of apoptotic corpses in vivo (3–6).

Studies of neutrophil death and resulting phagocytosis by macrophages provided the first experimental evidence that a variety of cytokines and chemokines associated with inflammation, including interleukin (IL)-6 and IL-8, are not secreted from phagocytes that engulf apoptotic targets (3, 7–10). More significantly, the lack of inflammatory cytokine release reflects an affirmative inhibitory response. For example, whereas stimulation of macrophages via the Toll-like receptor (TLR) 4 signaling complex (11) upon engagement with bacterial lipopolysaccharide (LPS) triggers significant cytokine secretion, the additional ingestion of apoptotic cells attenuates this response potently (9, 10, 12).

The ability of apoptotic cells to be cleared in a noninflammatory manner by professional phagocytes, such as macrophages, is a consequence of their specific expression of determinants for recognition and modulation of proinflammatory responses. The acquisition of these apoptotic determinants represents a gain of function and is common to all physiological cell deaths, without regard to suicidal stimulus (10, 13). Cells that die necrotically also are recognized by professional phagocytes; in contrast, however, necrotic corpses do not down-regulate inflammatory responses. Discrimination between apoptotic and necrotic corpses occurs on the level of binding and not engulfment and involves distinct and noncompeting mechanisms of recognition (10).

The modulatory activity of the apoptotic corpse is manifest as an immediate-early inhibition of macrophage proinflammatory cytokine gene transcription and is exerted directly upon binding to the macrophage, independent of subsequent engulfment and soluble factor involvement (13). Apoptotic cells target the proinflammatory transcriptional machinery of macrophages, with which they interact through a novel regulatory pathway. Inhibition appears to involve sequestration of critical transcriptional co-activator molecules without effect on proximal signaling events induced by inflammatory receptors, including innate immune receptors of the TLR family (13).

The ubiquity of apoptotic cells in all tissues throughout

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4 The abbreviations used are: IL, interleukin; EGF, epidermal growth factor; PI, propidium iodide; PS, phosphatidylserine; LPS, lipopolysaccharide; RLU, relative light units; CFDA, 5,6-carboxyfluorescein diacetate succinimidyl ester; PBS, phosphate-buffered saline; CMTMR, 5-(and 6)-((4-chloromethyl)benzoyl)aminotetramethyl rhodamine; TNF, tumor necrosis factor α; PMA, phorbol 12-myristate 13-acetate; NF-κB, nuclear factor κB; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase 1 and 2; TGRβ, transforming growth factor β; TLR, toll-like receptor.
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organismal life prompted us to ask whether this innate discrimination of apoptotic cells is limited to professional phagocytes. Certainly, normal homeostatic cell turnover in vivo, and especially in solid tissues and intact cellular strata, results in apoptotic cells that are in immediate contact with their neighbors independent of (and before the arrival of) mobile phagocytes (6, 14–17). We asked whether nonprofessional phagocytes discriminate and respond specifically to apoptotic cells in an anti-inflammatory manner. Here we describe studies that reveal that they do. Remarkably, this profound innate immune function is manifest fully and ubiquitously among professional and nonprofessional phagocytes and even nonphagocytic cell types.

EXPERIMENTAL PROCEDURES

Cells and Death Induction—RAW 264.7 murine macrophages, D011.10 murine T hybridoma cells, Jurkat human acute T leukemia cells, Ramos (RA-1) human Burkitt’s B lymphoma cells, and PLB-985 human myelomonoblastic leukemia cells (generously provided by Dr. Peter Henson, National Jewish Medical and Research Center) were cultured at 37 °C in a humidified, 5% (v/v) CO2 atmosphere in RPMI 1640 medium (Mediatech, Herndon, VA) supplemented with heat-inactivated fetal bovine serum (10% v/v), 2 mM L-glutamine, and 50 μM 2-mercaptoethanol. HeLa human cervical carcinoma cells and 293T human transformed kidney epithelial cells were grown in Dulbecco’s modified Eagle’s medium with 4.5 g/liter glucose (Mediatech) supplemented with fetal bovine serum (10% v/v); HyClone Laboratories, Logan, UT) and 2 mM L-glutamine. Chinese hamster ovary cells were grown in α-minimal essential medium (Invitrogen) supplemented only with fetal bovine serum (10%, v/v). Human umbilical vein endothelial cells were grown in supplemented endothelial growth medium (Cambrex Bio Science, East Rutherford, NJ) on gelatin-coated plates. Immortalized murine 3T3 fibroblast cell lines were derived from mouse embryo fibroblasts following the 3T3 protocol of Todaro and Green (18). Briefly, the embryo fibroblasts were cultured at 37 °C in a humidified, 5% (v/v) CO2 atmosphere in Dulbecco’s modified Eagle’s medium with 4.5 g/liter glucose (Mediatech) supplemented with fetal bovine serum (10%, v/v; HyClone Laboratories), 2 mM L-glutamine, and 50 μM 2-mercaptoethanol, replating at 3 x 10^5/60-mm diameter dish every 3 days. Immortalized cell lines were established from cells that grew from cultures that had become senescent.

Physiological cell death (apoptosis) was induced by treatment of target cells with the macromolecular synthesis inhibitors actinomycin D (200 ng/ml, 12 h) or cycloheximide (1 μg/ml, 12 h) (19). Cells were killed pathologically (necrotic death) by incubation in phosphate-buffered saline (PBS) at 55 °C for 20 min (until trypan blue uptake indicated compromise of membrane integrity) (10). In all cases, target cells (viable, apoptotic, and necrotic) were washed twice and resuspended in the medium of the responder cells to be tested. Plasma membrane vesicles were prepared from HeLa cells following the approach of Baumann et al. (20). Monolayers of cells, either untreated or induced to die with actinomycin D (and still adherent), were stimulated to vesiculate by incubation at 37 °C in Vesiculation Buffer (10 mM Hepes (pH 7.4), 150 mM NaCl, 2 mM CaCl2, 2 mM dithiothreitol, and 25 mM formaldehyde). Supernatants were collected after ~2.5 h (when abundant small membrane vesicles were apparent in the culture fluid). Nonadherent cells were removed (1,000 x g for 10 min), and vesicles were pelleted from the cleared supernatant by centrifugation (30,000 x g, 60 min, 4 °C). Cytofluorimetric analysis indicated that vesicles were ~0.8 μm in diameter and free of contaminating intact cells.

Phagocytosis Assay and Other Cytofluorimetric Analyses—Phagocytosis was assessed as previously described for macrophages (13). Target cells were labeled green with 5.6-carboxyfluorescein diacetate succinimidyl ester (CFDA; 0.2 μM; Molecular Probes, Inc., Eugene, OR) and were then induced to undergo apoptotic cell death, killed pathologically by heat treatment, or left untreated. Phagocytes (or cells being tested for phagocytic activity) were labeled red with 5-(and 6)-(bis-(4-chloromethyl)benzoxyl)tetramethyl rhodamine (CMTMR; 10 μM; Molecular Probes). In all cases, cells were labeled on the day preceding the experiment and cultured in serum-containing medium overnight to eliminate unbound label. Labeled phagocytes were co-cultured with the apoptotic, necrotic, or viable target cells for 30 min at 37 °C. Cells were harvested with PBS supplemented with 0.4 mM Na2EDTA and analyzed cytofluorimetrically on a FACSCaliber instrument (BD Biosciences). Cytofluorimetric data were processed with WinMDI software (Joe Trotter, Scripps Research Institute, La Jolla, CA). Cells that were both CMTMR-positive (Exλ = 488 nm, Emλ = 610 ± 15 nm) and CFDA-positive (Exλ = 488 nm; Emλ = 530 ± 15 nm) and that had scatter properties of the phagocyte population represented phagocytes that had engulfed targets. Engulfment is calculated as the fraction of double-positive phagocytes (all CMTMR-positive cells that also are CFDA-positive). Most targets that are bound but not engulfed are disrupted and do not remain adherent during the analysis, although they could be enumerated under static microscopic examination (10).

The accessibility of phosphatidylserine was revealed by the binding of fluorescein isothiocyanate-conjugated annexin V (Pharmins; San Diego, CA; Exλ = 488 nm, Emλ = 525 nm). Cells were harvested and washed twice with cold PBS. Cells were resuspended in 100 μl of binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2) and incubated with 5 μl of fluorescein isothiocyanate-conjugated annexin V for 15 min in the dark at 25 °C. After incubation, 400 μl of binding buffer was added per sample, and cells were analyzed cytofluorimetrically. Propidium iodide (PI) was employed to assess plasma membrane integrity. PI was added to cells at 1 μg/ml immediately before cytofluorimetric analysis (Exλ = 488 nm, Emλ = 610 nm).

Transfections and Luciferase Assays—Apoptotic modulation of specific transcription (e.g. dependent on nuclear factor κB (NF-κB) or the IL-8 promoter) was assessed in various cell types following transfection of relevant transcriptional reporter constructs, using a dual luciferase strategy, as described previously (13). The efficiencies of transfection were measured in parallel...
with farnesylated green fluorescent protein as a transfection marker (21). Cells were co-transfected with pNF-κB-Luc, a plasmid containing the firefly (Photinus pyralis) luciferase gene, the expression of which is driven by a basal transcriptional promoter linked to four copies of the κB motif (Clontech), together with pRL-SV40, a Renilla (sea pansy; R. reniformis) luciferase control vector, the constant expression of which is dependent on the SV40 early enhancer/promoter region (Promega, Madison, WI). RAW 264.7 macrophages (5.0 × 10⁶ cells/60-mm diameter dish) and HeLa, 293T, and Chinese hamster ovary cells, at ~75% confluence, were transfected using Effectene Transfection Reagent (Qiagen, Valencia, CA). 3T3 cells were transfected using the MEF1 Nucleofector Kit (AMAXA Biosystems; Gaithersburg, MD), with MEF Nucleofector Solution 1 and a machine setting of A-23. Jurkat, Ramos RA-1, and human umbilical vein endothelial cells were transfected using GenePORTER2 Transfection Reagent (Gene Therapy Systems, San Diego, CA).

The next day, the cells were replated in 24-well plates (1.0 × 10⁵ cells/well) and incubated without or with the indicated target cells (at a target cell/macrophage ratio of 10:1) and/or a proinflammatory stimulus in a final volume of 2 ml. The proinflammatory stimuli used included LPS (100 ng/ml; Escherichia coli O111:B4; Sigma), tumor necrosis factor-α (TNFα; 10 ng/ml; R&D Systems; Minneapolis, MN), IL-1β (5 ng/ml; R&D Systems), and phorbol 12-myristate 13-acetate (PMA; 1.3 ng/ml; EMD Biosciences, San Diego, CA) alone or with ionomycin (200 ng/ml; Molecular Probes).

Cell extracts were prepared after further incubation as indicated, and luciferase activities were measured by the Dual Luciferase Reporter Assay System (Promega) in an FB12 luminometer (Zylux; Oak Ridge, TN). Each condition was repeated in triplicate wells, and the luciferase activities in cells from each well were determined independently. Within any experiment, Renilla luciferase activities among samples varied less than 6%. The firefly luciferase activity in each sample was normalized with respect to the internal Renilla luciferase activity, and the relative level of normalized firefly luciferase activity compared with the activity in an untreated population was taken as a measure of specific (e.g. NF-κB-dependent) transcriptional activity.

Stably transfected reporter cells were generated by transfection of 293T cells with another NF-κB-Luc reporter construct, 4×NF-κB(HIV)tkluc (22), and an unlinked vector conferring hygromycin resistance. Cells resistant to hygromycin (50 μg/ml) were selected, cloned at limiting dilution, and tested for NF-κB-dependent responsiveness. Extracts were prepared and analyzed as above, except that the luciferase assay system (Promega) was used. Data with one clone, B2, are described here.

Quantification of Cytokine Release—Cytokine production was assessed following incubation of responder cells with target cells. Where indicated, proinflammatory stimuli were added simultaneously with the addition of targets. Culture supernatants were withdrawn from wells at the indicated times and frozen at −20 °C until analysis. Secreted cytokines were quantified by ELISA, using matched pair cytokine-specific capture and biotinylated reporter antibodies for murine IL-6 (eBio-sciences; San Diego, CA) or human IL-8 (BIOSOURCE, Camarillo, CA). The reporter reactions were developed with horseradish peroxidase-conjugated streptavidin (R&D Systems) and measured spectrophotometrically at 450 nm (corrected for turbidity at 550 nm; Microplate Autoreader model EL311; Bio-Tek Instruments, Winooski, VT).

Cellular Extract Preparation and Immunoblot Analysis—Activation of Akt and inhibition of ERK1/2 were assessed in 3T3 cells cultured overnight in serum-free medium and left unstimulated or stimulated for 15 min with a 5-fold excess of apoptotic DO11.10 cells (the apoptotic cells, which had been cultured under serum-free conditions, were centrifuged briefly onto the adherent 3T3 cells to initiate the interaction) and/or subsequent stimulation with epidermal growth factor (EGF; 10 nm; Calbiochem). After washing, cell extracts were prepared from the adherent 3T3 cells. Cells were lysed in lysis buffer (150 mM NaCl, 50 mM HEPES (pH 7.5), 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 200 μM orthovanadate). Lysates were centrifuged at 10,000 × g for 10 min at 4 °C, and the supernatants were stored at −70 °C.

Protein samples (20 μg each, determined by the bicinchoninic acid protein assay; Pierce) were boiled in 5× sample buffer, run on 12% SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride membranes (Millipore Corp., Billerica, MA). Blots were blocked with 5% dry milk in 150 mM PBS, 20 mM Tris HCl, pH 7.5, before probing with a phospho-Akt(Thr³⁰⁸)-specific rabbit anti-serum (Cell Signaling, Beverly, MA) or an affinity-purified phospho-ERK1/2 (Thr¹⁸³/Tyr¹⁸⁵)-specific polyclonal rabbit IgG (Promega, Madison, WI). Following incubation with an anti-rabbit secondary antibody conjugated to horseradish peroxidase, immunoreactive bands were visualized by the luminal reaction (ECLplus; Amersham Biosciences). Equivalent loading of protein samples was monitored by Ponceau S staining (0.25% (w/v) (Sigma) in 0.1% acetic acid; 5 min) of blotted proteins.

RESULTS

Specific Recognition and Response to Apoptotic Cells Is Not Limited to Macrophages—The anti-inflammatory response triggered in macrophages by their specific recognition of apoptotic cells is exerted on the level of cytokine gene transcription (13). Although key transcriptional activators of cytokine gene expression, such as NF-κB (23, 24), are not the molecular targets of apoptotic modulation, modulation is evident on the level of NF-κB-dependent transcription, and an NF-κB-dependent transcriptional reporter serves as a sensitive, reliable, and convenient readout for the modulatory effect exerted by apoptotic targets (13). The experiment in Fig. 1A exemplifies this analysis. We transiently transfected RAW 264.7 macrophages with pNF-κB-Luc, a plasmid containing the firefly luciferase gene, the expression of which is driven by a basal transcriptional promoter linked to four copies of the κB motif. Macrophages were co-transfected with a constitutive (NF-κB-independent) Renilla luciferase control vector, which served as an internal normalization control for transfection efficiency and cell viability. Following transfection, macrophages were incubated with different target cell populations and/or bacterial LPS, a potent proinflammatory agonist. Firefly and Renilla luciferase activi-
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FIGURE 1. Apoptotic modulation, revealed by a reporter of NF-\(\kappa\)B-dependent transcription, is evident in cells other than macrophages. A, murine RAW 264.7 macrophages were co-transfected with pNF-\(\kappa\)B-Luc and an NF-\(\kappa\)B-independent Renilla luciferase normalization control vector. 24 h later, macrophages were incubated without or with apoptotic, viable, or necrotic DO11.10 murine T cell or HeLa human epithelial cell targets (at a target/responder ratio of 10:1) and/or LPS (100 ng/ml) as indicated. Target cells were prepared as described under “Experimental Procedures”; apoptotic cell death was induced in DO11.10 cells by treatment for 12 h with cycloheximide and in HeLa cells by treatment with actinomycin D. Macrophage cell extracts were prepared after another 9 h, and luciferase activities were measured. Data are presented as normalized luciferase activities in treated macrophages relative to the uninduced control population (fold induction) and are the mean \pm S.E. of triplicate determinations. Results without target cells are represented as shaded bars, results with apoptotic targets are shown as black bars, results with necrotic targets are shown as stippled bars, and results with viable cells are shown as unshaded bars. In this experiment, the induced NF-\(\kappa\)B-dependent signal in RAW 264.7 cells was \(7.4 \times 10^{-1}\) relative light units (RLU) s\(^{-1}\) cell\(^{-1}\); whereas the uninduced background was \(6.0 \times 10^{-2}\) RLU s\(^{-1}\) cell\(^{-1}\); the transfection efficiency was \(-25\%\). The range of Renilla signals (mean \(4.0 \times 10^{-2}\) RLU s\(^{-1}\) cell\(^{-1}\)) varied less than 5.0% among all samples. B, NF-\(\kappa\)B-dependent luciferase activity was determined similarly in HeLa cells. Transfected HeLa cells were incubated without or with apoptotic, viable, or necrotic populations of target cells and/or TNF\(\alpha\) (10 ng/ml) as a stimulus of an NF-\(\kappa\)B-dependent transcriptional response. Presented data are representative of absolute values of NF-\(\kappa\)B-dependent luciferase activities. As indicated by these luciferase reporters, LPS-activated NF-\(\kappa\)B-dependent transcription (but not global transcription) in macrophages is inhibited specifically following their interaction with apoptotic cells; necrotic and viable cells do not exert this effect (13). This response is elicited by apoptotic cells generally, regardless of species, cell type, or suicidal stimulus. Here, murine DO11.10 T cells and human HeLa epithelial carcinoma cells, triggered to die with different suicidal stimuli (inhibitors of translation and transcription, respectively), were equally effective at triggering modulation in these murine macrophages.

As a first test of the ability of nonprofessional phagocytes to recognize and respond to apoptotic cells, we examined the responsiveness of HeLa cells, the same cells used as targets in Fig. 1A, utilizing the identical transcriptional reporter strategy. The transfected HeLa cells were incubated with apoptotic, necrotic, or viable populations of target cells and/or the inflammatory cytokine TNF\(\alpha\) as a stimulus of an NF-\(\kappa\)B-dependent transcriptional response. Significantly, HeLa cells do not die in response to TNF\(\alpha\) unless the NF-\(\kappa\)B-dependent response is attenuated (e.g. by an inhibitor of macromolecular synthesis) (25–27). Just as with LPS-stimulated macrophage responsiveness, robust TNF\(\alpha\)-activated NF-\(\kappa\)B-dependent transcription in HeLa cells was inhibited specifically and profoundly following the interaction of those cells with apoptotic, but not necrotic or viable, targets (Fig. 1B). It is notable that the ranges of absolute values of NF-\(\kappa\)B-dependent luciferase activities were quite different in HeLa cells and macrophages at comparably early times (as much as 300-fold; see Fig. 1), reflecting differences in the efficiencies of transfection and transgene expression. Still, expressed relative to basal luciferase levels, these data present a consistent pattern of modulation and reveal an identical response to apoptotic targets in different responder cell populations. Again, this selective response to apoptotic cells occurred without species restriction. Most dramatically, HeLa cells even were able to recognize and respond specifically to homotypic apoptotic cells.

The Characteristic Repertoire of Anti-inflammatory Responses Elicited Specifically upon Apoptotic Cell Recognition Is Evident in Murine Fibroblasts—To begin a more comprehensive exploration of the recognition and response to apoptotic targets by nonprofessional phagocytes, we examined nontransformed murine fibroblasts. Immortalized murine embryo fibroblast cells, derived by the 3T3 protocol of Todaro and Green (18), were highly phagocytic for dead cells (Fig. 2A; for consistency, we used DO11.10 cells as targets in this and the following experiments). Apoptotic and necrotic cell targets were engulfed rapidly and to equal extents by 3T3 fibroblasts, whereas viable cells were not ingested (Fig. 2A; we take the low level of engulfment of “viable” cells to reflect the small fraction of dead and dying apoptotic cells present in any cell culture).

These 3T3 fibroblasts secrete the inflammatory cytokine IL-6 in response to a variety of proinflammatory stimuli, including IL-1\(\beta\), TNF\(\alpha\), and, to a lesser degree, bacterial LPS (Fig.

with an uninduced background of \(1.1 \times 10^{2}\) RLU s\(^{-1}\) cell\(^{-1}\); the transfection efficiency was \(-50\%\). The range of Renilla signals (mean \(2.9 \times 10^{-2}\) RLU s\(^{-1}\) cell\(^{-1}\)) varied less than 3.4% among all samples.
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FIGURE 2. Profound apoptotic modulation is evident in murine fibroblasts. A, 3T3 fibroblasts were fluorescently labeled red with CMTMR, and apoptotic, necrotic, and viable DO11.10 murine T cell targets were prelabeled green with CFDA. 3T3 cells were incubated with apoptotic, necrotic, or viable DO11.10 targets at a target to 3T3 cell ratio of 10:1 for 30 min at 37 °C. Engulfing 3T3 cells, identified cytofluorimetrically as CMTMR- and CFDA-positive, were quantified. Results with apoptotic targets (induced to die by treatment with actinomycin D) are represented as black bars, with necrotic targets as stippled bars, and with viable cells as unshaded bars, and are expressed as the mean ± S.E. of triplicate determinations. B, 3T3 fibroblasts were incubated at 37 °C without or with IL-1β (5 ng/ml) and/or apoptotic, necrotic, or viable DO11.10 targets (at a target cell/fibroblast ratio of 10:1). Culture supernatants were collected after 9 h, and levels of secreted IL-6 were quantified. Under these conditions, target cells themselves produced no detectable levels (<15 pg/ml) of IL-6. C, inhibition of ERK1/2 activation and activation of Akt were assessed in 3T3 cells cultured overnight at 37 °C in serum-free medium. The cells then were incubated for 15 min without or with apoptotic DO11.10 cells (Apo; these cells also had been cultured without serum) at the indicated ratios. Adherent cells were washed and incubated for 15 min without (Un) or with EGF (10 ng/ml). After washing, cell extracts were prepared from the adherent 3T3 cells. The presence of each activated kinase was assessed by immunoblot analysis, probing the same filter with an affinity-purified phospho-ERK1/2 (Thr183/Tyr185),-specific rabbit antibody or a phospho-Akt(Thr308)-specific rabbit antiserum. Apoptotic cells alone (Apo only; note that this overrepresents the contribution of apoptotic cells in the apoptotic (Apo) samples) also were probed and found to express no activated ERK1/2 or Akt. Equivalent loading of protein samples also was confirmed by Ponceau S staining. D, NF-κB-dependent luciferase activity was assessed in 3T3 fibroblasts by the approach outlined in Fig. 1. 3T3 fibroblasts were transiently transfected with NF-κB-Luc and an NF-κB-independent Renilla luciferase normalization control vector. 48 h after transfection, cells were incubated as indicated without or with IL-1β (5 ng/ml) and/or apoptotic, viable, or necrotic DO11.10 targets (at a target/responder ratio of 10:1). Cell extracts were prepared after 12 h, and luciferase activities were measured. The induced NF-κB-dependent signal in the 3T3 cells was $3.4 \times 10^9$ RLU s$^{-1}$ cell$^{-1}$ with an uninduced background of $3.8 \times 10^8$ RLU s$^{-1}$ cell$^{-1}$; the transfection efficiency was ~40%. The range of Renilla signals (mean $8.8 \times 10^7$ RLU s$^{-1}$ cell$^{-1}$) varied less than 5.6% among all samples.

We characterized the release of IL-6 from fibroblasts following their interaction with target cells as one indication of inflammatory responsiveness; IL-6 secretion in macrophages reflects inflammatory responsiveness generally (10, 13).

IL-1β-stimulated IL-6 secretion was potently attenuated when fibroblasts interacted with apoptotic targets, but not with necrotic or viable targets (Fig. 2B). Apoptotic target cell contact was necessary for this response, since supernatants from apoptotic cell cultures could not substitute for the target cells themselves to modulate IL-6 secretion (data not shown). The ability of apoptotic cells to block IL-6 secretion by IL-1β-stimulated fibroblasts parallels their ability to abrogate the secretion of IL-6 and other inflammatory cytokines by LPS-stimulated macrophages (10). This is consistent with the view that, during the process of physiological cell death, apoptotic cells acquire a cell-associated, dominant acting anti-inflammatory signaling activity that suppresses inflammatory responsiveness independent of the particular proinflammatory stimulus (10, 13).

In contrast, necrotic cells did not affect IL-6 secretion in response to IL-1β (Fig. 2B) and did not trigger IL-6 secretion in the absence of IL-1β (data not shown). We have noted previously that intact native necrotic cells are not sufficient to stimulate an inflammatory response in macrophages and, at most, only modestly augment macrophage LPS responsiveness (10, 13). Others also have reported that intact necrotic cells are not proinflammatory (12, 29). Our new results with fibroblasts extend those observations. It is important to note that the necrotic targets we employ are intact cells (see Fig. 5C), as opposed to the freeze/thaw lysates that often are used as “necrotic” samples. The presumptive association of proinflammatory molecules, such as the high mobility group box
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FIGURE 3. Profound apoptotic modulation is evident in human epithelial 293T cells. A, NF-κB-dependent luciferase activity was assessed in 293T cells by the approach outlined in Fig. 1. Cells were transiently transfected with NF-κB-Luc and an NF-κB-independent Renilla luciferase normalization control vector. 48 h after transfection, cells were incubated as indicated without or with PMA (1.3 ng/ml) and/or apoptotic, viable, or necrotic DO11.10 targets (at a target/responder ratio of 10:1). Cell extracts were prepared after 12 h, and NF-κB-dependent luciferase activities were measured. The induced NF-κB-dependent signal in 293T cells was 3.0 × 10^4 RLU s⁻¹ cell⁻¹, with an uninduced background of 4.5 × 10^3 RLU s⁻¹ cell⁻¹; the transfection efficiency was greater than 80%. The range of Renilla signals (mean 9.8 × 10⁻² RLU s⁻¹ cell⁻¹) varied less than 2.7% among all samples. B, 293T cells were incubated at 37 °C without or with PMA and/or apoptotic, necrotic, or viable murine target cells (as above). Culture supernatants were collected after 12 h, and levels of secreted human IL-8 were quantified. Target cells produced no detectable levels (<20 pg/ml) of IL-8. C, the transcriptional activity of an IL-8 promoter construct in 293T cells also was assessed by a luciferase readout as in A. Cells were transiently transfected with pIL8-Luc and a constitutive Renilla luciferase normalization control vector. 48 h after transfection, cells were incubated as indicated without or with PMA (1.3 ng/ml) and/or apoptotic, viable, or necrotic DO11.10 targets (at a target/responder ratio of 10:1). Cell extracts were prepared after 12 h, and luciferase activities were measured. D, luciferase activity was determined similarly in 293T cells transfected with an IL-8 promoter construct (IL-8 ×B⁻) from which the cognate NF-κB binding site (34) had been deleted.

1 protein (30), with necrosis may instead relate to that method of preparation.

The discordant responses of 3T3 fibroblasts to apoptotic and necrotic cell targets with regard to phagocytosis and inflammatory cytokine secretion are striking and entirely reminiscent of the behavior we have characterized in professional phagocytes. We showed previously that, whereas the kinetics and magnitude of engulfment by macrophages of apoptotic and necrotic targets are comparable, the mechanisms employed for recognition of those classes of target cells are distinct (10) and, in the case of apoptotic recognition, linked directly to anti-inflammatory responsiveness (13). These new data again make clear that inflammatory responsiveness is dissociated from engulfment per se. It also is remarkable that the process of phagocytosis of dead cells by these amateur phagocytes is at least as extensive as it is with professional antigen-presenting phagocytes (10).

We have described recently novel signaling events within macrophages, associated with their recognition of apoptotic and necrotic cells (29, 31). In particular, their interaction with apoptotic cells inhibits activation of macrophage extracellular signal-regulated kinases 1 and 2 (ERK1/2), whereas exposure to necrotic cells triggers ERK1/2 activation. Apoptotic cells also strongly induce both Jun N-terminal kinase (JNK) and p38, whereas necrotic cells have no detectable effect on those mitogen-activated protein kinase (MAPK) modules (29, 31). Activation of Akt, a protein kinase associated with death-sparing function, results from interaction with both apoptotic and necrotic targets (29).

We explored these signaling events in 3T3 fibroblasts. The inhibition of ERK1/2 phosphorylation at its catalytic core (on Thr<sup>183</sup> and Tyr<sup>185</sup>) and the activation of Akt, indicated by specific phosphorylation on Thr<sup>308</sup> were assessed by immunoblot analysis with phosphospecific antibodies. As shown in Fig. 2C, ERK1/2 was present in an unactivated state in 3T3 cells cultured overnight in the absence of serum but was rapidly activated upon mitogenic stimulation of the cells with EGF. Apoptotic target cells did not trigger ERK1/2 activation in the responding 3T3 fibroblasts and, further, inhibited EGF-mediated ERK1/2 activation in a dose-dependent manner (Fig. 2C). Conversely, treatment of serum-deprived 3T3 cells, which contained little activated Akt, with apoptotic targets was as effective as EGF stimulation in promoting robust Akt activation. These data
expand our findings to demonstrate that specific signaling events triggered upon apoptotic recognition occur normally in nonprofessional phagocytes.

Finally, we examined apoptotic modulation in these nonprofessional phagocytes on the level of transcription, using the simple NF-κB-dependent luciferase transcriptional reporter assay. As shown in Fig. 2D, just as IL-1β treatment triggered IL-6 secretion, it activated NF-κB-dependent transcription. That transcriptional response was ablated profoundly by the interaction of the fibroblasts with apoptotic targets but not with necrotic or viable targets (Fig. 2D). Transcriptional modulation in 3T3 cells triggered by apoptotic targets anticipates completely their effect on IL-6 secretion (Fig. 2, compare B and D) and confirms the strong correlation between specific signaling involving ERK1/2 and other MAPKs and the modulation of inflammatory transcription triggered by apoptotic cells (32).

Again, the observation that apoptotic cells actively antagonize IL-1β-triggered proinflammatory responsiveness, just as they abrogate those responses triggered by other proinflammatory stimuli, corroborates our finding that apoptotic modulation is exerted independent of the particular proinflammatory stimulus (10, 13).

Together, these data illuminate a repertoire of apoptotic modulation in nonprofessional fibroblasts that is extensive and entirely consistent with that seen in macrophages. Importantly, apoptotic modulation, as indicated by blockade of specific transcription and cytokine release, is not a consequence of the death of the responding fibroblasts. By morphological criteria, the 3T3 cells retain viability following their incubation with apoptotic targets; the associated prosurvival signaling resulting from activation of Akt even may sustain their viability. Most definitively, their continued viability is confirmed by the ongoing macromolecular synthesis that we observe in the form of unimpaired Renilla luciferase activity from that co-transfected control reporter (Fig. 2). Additionally, these data reaffirm the reliability of the NF-κB-dependent reporter as a specific read-out of apoptotic modulation and, consequently, as an early measure of specific cellular responsiveness to apoptotic targets.

By all criteria, these nonprofessional phagocytes recognize and are highly and selectively responsive to apoptotic cell targets. That their responses correspond precisely to the signature of the responding fibroblasts. By morphological criteria, the 3T3 cells retain viability following their incubation with apoptotic targets; the associated prosurvival signaling resulting from activation of Akt even may sustain their viability. Most definitively, their continued viability is confirmed by the ongoing macromolecular synthesis that we observe in the form of unimpaired Renilla luciferase activity from that co-transfected control reporter (Fig. 2). Additionally, these data reaffirm the reliability of the NF-κB-dependent reporter as a specific read-out of apoptotic modulation and, consequently, as an early measure of specific cellular responsiveness to apoptotic targets.

The Signature Anti-inflammatory Responses to Apoptotic Cell Recognition Are Evident in Human Epithelial Cells—We expanded our analysis of specific apoptotic recognition to human tumor cells. In contrast to the 3T3 fibroblasts cells, which are difficult to transfect and which become stressed by the procedure (32) (see “Experimental Procedures”), 293T transformed human kidney epithelial cells transfect readily and efficiently.

The NF-κB-dependent responsiveness we observed from the transfected reporter in 293T cells was dramatic. With little transfection stress, basal NF-κB-dependent luciferase activity was low, and the magnitude of the induced response was enormous (greater than 60-fold; Fig. 3A). Apoptotic cells, but not necrotic or viable cells, modulated the induced response specifically. Although the absolute levels of induction and, consequently, the apparent extent of apoptotic modulation of the NF-κB-dependent signal are more dramatic than with macrophages and other cells, the qualitative pattern of apoptotic response is conserved among these cells. Clearly, 293T cells, which have been shown previously to engulf apoptotic targets (32, 33), also respond to them specifically.

We also assessed the production of endogenous IL-8 by these cells. The characteristic pattern of responsiveness to apoptotic cells was evident with respect to the vigorous secretion of that cytokine by 293T cells (Fig. 3B). That transcriptional modulation in B2 cells are expressed on a logarithmic scale to capture this dynamic range). Apoptotic modulation is directly responsive to target cell dose (Fig. 4C). The normal modulated expression and secretion of IL-8 in this clone (compare Figs. 5D and 3B) indicates that endogenous gene responsiveness is not altered. In addition, the convenience and sensitivity of these reporter clones make them particularly useful in the analysis of the determinants of apoptotic recognition (see below).
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Specific Apoptotic Recognition Is Evident in Other Nonprofessional Phagocytes and Nonphagocytic Lymphoid Cells—Employing the NF-κB-dependent reporter assay, we observed apoptotic modulation in additional types of cells, including human endothelial cells and Chinese hamster ovary cells (Table 1). We expanded our survey of the recognition and response to apoptotic cells by nonprofessional phagocytes to include lymphoid cells. As shown in Table 1, both T cell and B cell lines were fully and selectively responsive to apoptotic cells. Notably, whereas apoptotic cells triggered a robust modulation of transcriptional responsiveness in Jurkat T cells (Table 1), no engulfment of those apoptotic targets was detectable (data not shown). This provides independent confirmation that apoptotic recognition and inflammatory modulation can be dissociated from engulfment (13) and extends that finding to indicate that responsiveness to apoptotic cells is a ubiquitous property of phagocytic and nonphagocytic cells alike.

Specific Innate Apoptotic Recognition Is Dependent on Endogenous Membrane Determinants—From studies with macrophage responders, we have argued that phosphatidylserine (PS), which is taken to be the archetypal recognition molecule, is not a specific ligand for the recognition of apoptotic or necrotic cells (10). Externalization of PS occurs during both types of cell death and PS vesicles are equally effective at preventing the uptake of both classes of targets (10). Consistent with these findings, more recent data have demonstrated that PS and tethering molecules that may bind the phagocytosis (macropinocytosis) of already bound targets (35, 36).

Fadok et al. (37) described the unusual absence of PS externalization during apoptotic cell death of a human myelomonoblastic cell line, PLB-985 (38). We have exploited this cell line to test our previous conclusion. Apoptotic PLB-985 cells (triggered to die by treatment with actinomycin D) modulated NF-κB-dependent transcription in 293T epithelial cell line (Fig. 5, A and C) as well as in macrophages (data not shown), although no PS exposure, as assessed by calcium-dependent binding of fluorescently conjugated annexin V, was detectable (Fig. 5, A and B). In contrast, intact necrotic cells did not modulate NF-κB-dependent transcription, although they exposed PS (Fig. 5C).

The transcriptional effect exerted by PS-negative apoptotic PLB-985 cells also was paralleled by their modulation of the secretion of endogenous IL-8 from B2 cells was assessed as in Fig. 3B, E. NF-κB-dependent luciferase activity was assessed in B2 cells incubated (as in A) without or with PMA and/or apoptotic or viable HeLa targets that were either freshly prepared cells, cells fixed with formaldehyde, cells subjected to three rounds of freezing and thawing, or vesicles derived from apoptotic or viable HeLa cells.
tion of proinflammatory cytokines (IL-8 in 293T cells and TNFα in macrophages; data not shown). These findings demonstrate that the specific recognition of apoptotic cells, linked to the modulation of inflammatory responsiveness, is not dependent on phosphatidylserine exposure and affirm our conclusion that PS is not a ligand for specific apoptotic recognition (10).

It also is significant that the specific recognition and response to apoptotic cells occurs in the absence of serum. As shown in Fig. 4B, whereas NF-κB-dependent responsiveness is reduced by about 1 order of magnitude in B2 reporter cells cultured without serum, those cells retain fully their ability to recognize and respond to apoptotic targets specifically. The independence of specific apoptotic recognition from serum also is revealed by the characteristic MAPK signaling responses seen in macrophages (29, 31) and fibroblasts (see Fig. 2C; note that those assays are performed in cells cultured under serum-free conditions). These data suggest that serum-derived tethering molecules are dispensable for functional apoptotic recognition and that the requisite specific ligands for recognition and anti-inflammatory modulation are expressed endogenously by apoptotic cells.

In an effort to identify affirmatively the apoptotic cell determinants that are responsible for specific recognition and the triggering of anti-inflammatory modulation, we built on previous studies suggesting that those determinants were preserved following apoptotic cell fixation (12, 39). We found that the modulatory activity of apoptotic cells also was retained after several rounds of freezing and thawing and was recovered in the resulting washed cell membranes (Fig. 4E). These observations are consistent with our previous demonstration that apoptotic cell activity persists regardless of plasma membrane integrity (10, 31), and suggest that specific recognition and triggering determinants reside with those membranes. Indeed, isolated plasma membrane vesicles (20) derived from apoptotic cells mimic the intact cellular targets and exert specific anti-inflammatory modulation (Fig. 4E). Significantly, membrane vesicles derived from viable cells do not have such activity (Fig. 4E). Since membrane polarity is not maintained in vesicles (40), these findings reinforce our conclusion that apoptotic recognition determinants do not arise simply with the externalization of molecules, including phosphatidylserine, normally kept sequestered within the cell, and rather represent specifically acquired cryptic epitopes modified post-translationally from resident molecules (10, 13, 19).

### DISCUSSION

**Recognition and Response to Apoptotic Cells Represents a Profound and Unconventional Innate Immunity**—Our studies reveal that the specific recognition of apoptotic cells represents a potent and strikingly unconventional innate immunity. We have shown that apoptotic cell recognition triggers directly a robust anti-inflammatory effect in responding cells. The response is exerted on the level of transcription, involving an immediate-early modulation of proinflammatory cytokine gene transcription (13). It is engaged selectively upon recognition of apoptotic cells, regardless of the particular suicidal stimulus or cell type, and is not induced by necrotic or viable cells.

The discrimination of “self” from “other” is considered generally to be the defining hallmark of immune selectivity. By extension, innate immunity is held to function in the elimination of “nonself” invaders, typically in a phlogistic context. Our data challenge this concept and indicate that innate immunity directed to apoptotic cells represents a fundamentally distinct and unorthodox form of discrimination in which effete cells are recognized without regard to self. Indeed, we detect no qualitative or quantitative differences in responsiveness to self and nonself apoptotic targets nor to heterotypic and homotypic apoptotic targets (see Fig. 1). In essence, the innate immune recognition of apoptotic determinants represents a potent intrinsic anti-inflammatory circuit that opposes (or dampens) conventional innate inflammatory responsiveness triggered via Toll-like and other classical innate immune receptors.

**Innate Apoptotic Immunity Is Manifest Ubiquitously among Phagocytic and Nonphagocytic Cells**—It is remarkable that specific recognition and responsiveness to apoptotic cells is ubiquitous among professional and nonprofessional phagocytes and even nonphagocytic cells. We have found that a wide variety of cell types, including cells of epithelial, endothelial, neuronal, and lymphoid lineages, are able to recognize and respond to apoptotic corpses efficiently; notably, this immune activity exists in untransformed as well as transformed cells. Other studies have suggested that some nonprofessional phagocytes can engulf apoptotic targets. Neighboring homotypic cells in particular have been shown to engulf apoptotic cells (6, 14, 17), and mesenchymal cells have been demonstrated to replace macrophages in the clearance of dead cells during development in mice when the macrophage lineage is ablated (6). Metazoan species lacking professional phagocyte lineages also rely on neighboring cells to engulf apo-

### TABLE 1

| Responder cells | Stimulus   | Modulation of NF-κB-dependent response by target cells ± S.E. |
|-----------------|------------|-------------------------------------------------------------|
|                 |            | Apoptotic          | Viable            | Necrotic          |
| Jurkat          | PMA + ionomycin | 13.01 ± 1.30 | 99.89 ± 1.11  | 102.92 ± 8.47 |
| Ramos RA-1      | PMA + ionomycin | 15.53 ± 3.70 | 104.75 ± 8.95 | 100.95 ± 11.36 |
| HUVEC           | TNFα       | 16.29 ± 3.17 | 101.71 ± 6.17 | 103.36 ± 10.90 |
| CHO             | PMA        | 24.57 ± 6.10 | 106.06 ± 4.98 | 111.58 ± 7.19 |

Note: The table shows the modulation of NF-κB-dependent luciferase activity by apoptotic, viable, and necrotic targets in various cell types.
ptotic corpses (41). Our new data expand this view and demonstrate that the selective functional recognition of apoptotic cells, linked to specific cellular responsiveness, is ubiquitous among diverse cell types.

This finding is provocative. On the one hand, the existence of a specific and selective mechanism for the discrimination of apoptotic cells, distinct from engulfment, reinforces our previous observations that specific apoptotic recognition and response are dissociable from phagocytosis (10, 13). More broadly, we hypothesize that the ubiquity of apoptotic cell recognition implicates the presence of common molecular components that comprise an evolutionarily conserved apoptotic recognition complex.

An apoptotic target recognition structure that predates the development of committed phagocytic (or immune) cell lineages suggests that lineage-specific determinants do not play a central role. It remains that idiosyncratic differences in the handling of apoptotic corpses may apply in certain cases (36, 42, 43), and additional levels of apoptotic modulation may be exerted on the expression of particular cytokines (44). Fundamentally, however, apoptotic recognition represents a pervasive innate recognition process that is independent of signaling via TLR pathways and other lineage-specific molecules.

Further, whereas the clearance of functionally inappropriate cells is the obvious purpose of physiological cell death, these new data, which demonstrate apoptotic response by nonphagocytic cells, suggest that the exertion of immune control, as distinct from clearance, also may be critical. In fact, some data suggest that apoptotic cells, when administered in high numbers, can exert systemic anti-inflammatory effects (45, 46).

**A Characteristic Signature of Responses Is Associated with Innate Apoptotic Immunity**—We have described the repression of proinflammatory cytokine gene transcription as the direct consequence of apoptotic recognition (13). Here, we show that this effect is reflected reliably in all responder cell types as the inhibition of luciferase activity from a transcriptional reporter coupled to an apposite proinflammatory promoter, such as the human IL-8 gene promoter or, most simply, an NF-κB coupled to an apposite proinflammatory promoter, such as the human IL-8 gene promoter. Our new data confirm and extend these findings by demonstrating that transcriptional repression is exerted by the sequestration of limiting (51) transcriptional co-activators without effect on proinflammatory signaling via innate immune receptors (13). For example, although TLR signaling leading to activation of NF-κB ensues normally, transcriptional initiation is precluded (13). We speculate that apoptotic modulation of cytokine gene transcription is dependent on MAPK signaling. Jun family members, components of the AP1 transcription factor, are substrates for JNK, and their phosphorylation promotes co-activator association (49). Previous work has demonstrated that when active ERK1/2 is not present, the AP1 transcriptional complex can function as a transcriptional repressor (50). We propose that JNK activation and ERK1/2 inhibition following apoptotic target recognition leads similarly to the formation of inactive AP1 complexes that sequester co-activators and exert transcriptional trans-repression.

**FIGURE 6. Speculative model of apoptotic modulation mechanism.** Apoptotic target recognition triggers a signature repertoire of responses, most notably including the modulation of inflammatory cytokine gene transcription and engulfment of the apoptotic targets. These are associated with characteristic signaling events, involving a number of MAPK modules and the activation of several other protein kinase and G-coupled pathways. Transcriptional modulation and MAPK signaling, particularly the activation of p38 and JNK and the inhibition of ERK1/2, are linked specifically to apoptotic cell recognition (13, 29, 31, 32). Other consequential signaling responses, such as the activation of Ral1 and the phosphatidylinositol-3 kinase pathway (including Akt), do not reflect selective interactions with apoptotic targets and rather are associated with phagocytosis generally (29, 53). Transcriptional repression is exerted by sequestration of limiting (51) transcriptional co-activators without effect on proinflammatory signaling via innate immune receptors (13). For example, although TLR signaling leading to activation of NF-κB ensues normally, transcriptional initiation is precluded (13). We speculate that apoptotic modulation of cytokine gene transcription is dependent on MAPK signaling. Jun family members, components of the AP1 transcription factor, are substrates for JNK, and their phosphorylation promotes co-activator association (49). Previous work has demonstrated that when active ERK1/2 is not present, the AP1 transcriptional complex can function as a transcriptional repressor (50). We propose that JNK activation and ERK1/2 inhibition following apoptotic target recognition leads similarly to the formation of inactive AP1 complexes that sequester co-activators and exert transcriptional trans-repression. A Characteristic Signature of Responses Is Associated with Innate Apoptotic Immunity—We have described the repression of proinflammatory cytokine gene transcription as the direct consequence of apoptotic recognition (13). Here, we show that this effect is reflected reliably in all responder cell types as the inhibition of luciferase activity from a transcriptional reporter coupled to an apposite proinflammatory promoter, such as the human IL-8 gene promoter or, most simply, an NF-κB-dependent promoter.

It is important to emphasize that NF-κB is not the target of apoptotic modulation. Our previous data have demonstrated that apoptotic cells target the proinflammatory transcriptional machinery of macrophages with which they interact, without effect on proximal steps of TLR signaling leading to NF-κB activation (13). We have suggested that the sequestration of a common transcriptional co-activator may be the mechanism of transcriptional modulation. Our new data confirm and extend these findings by demonstrating that transcriptional modulation is exerted in different cell types and that this effect is not dependent on NF-κB (see Fig. 3). Furthermore, we observed the inhibitory effect regardless of the particular proinflammatory stimulus.

Whereas the modulation of inflammatory cytokine expression is the most obvious and global recognition-dependent effect, it is one component of a more extensive set of outcomes that follow recognition of apoptotic targets in all responding cells. Our data demonstrate that the specific signal transduction events characterized in macrophages as proximate consequences of apoptotic recognition (29, 31) ensue generally in response to the recognition of apoptotic cells. For example, the inhibition of ERK1/2 activation occurs selectively following interaction with apoptotic targets. Necrotic cells, which do not modulate inflammation, instead trigger the activation of ERK1/2 (28, 30). It is intriguing that the potent anti-inflammatory cytokine transforming growth factor β (TGFβ) also induces ERK1/2 activation (47), unlike the effect of apoptotic targets. TGFβ has been suggested to play a role in apoptotic modulation (9, 17, 47, 48). We have shown previously that modulation of inflammatory responsiveness in macrophages is initiated as a direct consequence of their specific recognition of apoptotic cells, independent of TGFβ (13). These new data broaden that conclusion to other cell types and reinforce the dissociation of responses initiated by apoptotic cells and TGFβ.

In contrast to ERK1/2 inhibition, Akt is activated in responder cells by their interaction with apoptotic targets. Akt activation does not mark apoptotic cell interactions uniquely, however; necrotic targets also trigger Akt activation in responder cells (29). In light of the death-sparing effects of Akt
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signaling, it is interesting to consider that dying cells may promote the survival of responder cells with which they interact. This is especially significant in the context of the blockade of NF-κB-dependent responses, including potential prosurvival effects (25, 26), exerted by apoptotic targets. Given the ability of homotypic cells to respond to dying neighbors, we conjecture that the clearance of effete neighbors may contribute to overall tissue vigor, reinforcing the view that cell death in metazoans reflects cellular altruism.

Toward the Molecular Characterization of Apoptotic Immunity—Specific modulation of cytokine gene transcription and MAPK signaling responses together constitute a characteristic and unambiguous signature reflective of selective apoptotic recognition and innate apoptotic immunity. We speculate that these signature responses are linked mechanistically and that transcriptional modulation is contingent on MAPK signaling. Previous studies have demonstrated that phosphorylation by JNK of Jun family members, components of the API transcription factor, promotes their association with co-activator molecules (49) and that when active ERK1/2 is not present, the API transcriptional complex functions as a transcriptional repressor (50). We propose that JNK activation and ERK1/2 inhibition resulting from apoptotic target recognition (29, 31, 32) similarly promote the formation of such API complexes, which sequester requisite and limiting (51) transcriptional co-activators and exert transcriptional trans-repression (Fig. 6).

Just as responsiveness to apoptotic cells is robust and widespread, recognition determinants are conserved and persistent on apoptotic targets. We have reported previously that cell-associated dominant acting anti-inflammatory signaling determinants are acquired post-translationally during the process of physiological cell death and that apoptotic cells retain their anti-inflammatory properties indefinitely (10). The studies reported here, in which isolated membrane preparations and “late” apoptotic cells that have lost membrane integrity continue to elicit innate immune responsiveness (see Fig. 5), confirm earlier findings. These results further refute the notion that distinctions between early and late apoptotic cells are consequential with regard to inflammatory outcome (31).

In addition to excluding TGFβ, our data indicate generally that soluble and serum factors are not essential for apoptotic modulation. Anti-inflammatory modulation appears to be dependent strictly on responder cell contact with apoptotic targets; modulation is not exerted by cell supernatants or trans-factors, nor upon physical (“transwell”) separation between cell populations (10, 13, 31, 52).

In summary, our studies reveal an unappreciated, unconventional, and ubiquitous arm of innate immunity that is evolutionarily ancient and appears to exert profound anti-inflammatory effects. The fuller molecular dissection of apoptotic immunity is fundamentally important for a more complete understanding of immune responsiveness and cell death in a physiological context. We anticipate as well that the profound modulation of inflammation exerted by apoptotic cells without species-specific restriction may reveal new targets for inflammatory control, with exciting potential for efforts to intervene in cases of pathological inflammatory response.

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