Phagocytosis of complement-opsonized targets is a primary function of neutrophils at sites of inflammation, and the clearance of neutrophils that have phagocytosed microbes is important for the resolution of inflammation. Our previous work suggests that phagocytosis leads to rapid neutrophil apoptosis that is inhibited by antibody to the β2 integrin, Mac-1, and requires NADPH oxidase-derived reactive oxygen species (ROS) generated during phagocytosis. Here we report that phagocytosis-induced cell death (PICD) does not occur in Mac-1-deficient murine neutrophils, suggesting that PICD proceeds through a *bona fide* Mac-1-dependent pathway. A sustained, intracellular oxidative burst is associated with PICD. Furthermore, PICD does not require traditional death receptors, Fas, or tumor necrosis factor (TNF) receptor. TNF but not Fas synergizes with phagocytosis to enhance significantly PICD by increasing the oxidative burst, and this is Mac-1-dependent. Phagocytosis-induced ROS promote cleavage/activation of caspases 8 and 3, key players in most apoptotic pathways. Phagocytosis also triggers a competing MAPK/ERK-dependent survival pathway that provides resistance to PICD likely by down-regulating caspase 8 activation. The anti-apoptotic factor granulocyte-macrophage colony-stimulating factor (GM-CSF) significantly enhances ROS generation associated with phagocytosis. Despite this, it completely suppresses PICD by sustaining ERK activation and inhibiting caspase 8 activation in phagocytosing neutrophils. Together, these studies suggest that Mac-1-mediated phagocytosis promotes apoptosis through a caspase 8/3-dependent pathway that is modulated by NADPH oxidase-generated ROS and MAPK/ERK. Moreover, TNF and GM-CSF, likely encountered by phagocytosing neutrophils at inflammatory sites, exploit pro-(ROS) and anti-apoptotic (ERK) signals triggered by phagocytosis to promote or suppress PICD, respectively, and thus modulate the fate of phagocytosing neutrophils.

Circulating neutrophils are recruited to sites of inflammation as a first line of defense against microbes. The resolution of inflammation is associated with neutrophil clearance, which occurs upon apoptosis of neutrophils *in situ* and their subsequent engulfment by tissue macrophages. Human peripheral blood neutrophils (PBNs) have a short life span in vivo (t½, 7 h) and in vitro and undergo spontaneous apoptosis within 24 h of culture (3). During inflammation, the life span of neutrophils is extended by cytokines, growth factors, and the activated endothelium (4–8) and thus likely contributes to the accumulation of inflammatory cells in tissues. On the other hand, neutrophil apoptosis is accelerated by at least three different mechanisms. First, engagement of well described death-inducing receptors, TNFR or Fas, induces apoptosis in peripheral blood neutrophils (9). Second, stress stimuli such as UV irradiation and rapid temperature shift lead to neutrophil apoptosis (10–12). Finally, phagocytosis of complement or IgG opsonized targets triggers rapid apoptosis (13–17), which recent studies suggest may be in part due to the transcriptional regulation of “apoptotic” genes in the phagocytosing neutrophils (18, 19). Thus, phagocytosis may have a dual role in clearing microbial infections and promoting resolution of inflammation by triggering cell death of neutrophils that have reached the end of their useful life span.

Phagocytosis of complement-opsonized targets requires the β2 integrin, Mac-1 (CD11b/CD18, CR3), which is a complement-binding receptor important in cell activation, recruitment, and phagocytosis (20). Treatment of PBNs with a functional blocking antibody to Mac-1 inhibits phagocytosis and the subsequent apoptosis suggesting a role for this integrin in promoting cell death (13). Mac-1-mediated phagocytosis triggers a robust oxidative burst by the NADPH oxidase, a reactive oxygen species (ROS)-generating system in phagocytic cells that is required for host defense (21). Although much is known about the interaction of ROS with microbes, little is known of their effect on the phagocyte itself. Previous studies (13, 14) showed that the

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**Elucidation of Molecular Events Leading to Neutrophil Apoptosis following Phagocytosis**

**CROSS-TALK BETWEEN CASPASE 8, REACTIVE OXYGEN SPECIES, AND MAPK/ERK ACTIVATION**

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The abbreviations used are: PBN, human peripheral blood neutrophil; CGD, chronic granulomatous disease; cGMP, cyclic guanosine monophosphate; CCR, chemokine (C-C motif) receptor; GM-CSF, granulocyte-macrophage colony-stimulating factor; LPS, lipopolysaccharide; MAPK/ERK, mitogen-activated protein kinase/extracellular signal-regulated kinase; PICD, phagocytosis-induced cell death; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; m.o.i., multiplicity of infection; BSA, bovine serum albumin; PBS, phosphate-buffered saline; HBSS, Hank’s buffered saline solution; FITC, fluorescein isothiocyanate; FCS, fetal calf serum; AMC, aminomethylcoumarin; FMK, fluorescent activated cell sorter; CHX, cycloheximide WT, wild type.
NADPH oxidase-derived ROS were essential for triggering apoptosis following phagocytosis. In particular, PBNs treated with an inhibitor of the NADPH oxidase, diphenyleneiodonium (DPI) (22), as well as neutrophils from chronic granulomatous disease (CGD) patients that lack a functional NADPH oxidase-phagocytosed complement oposened targets but failed to undergo PICD (13). Neutrophil apoptosis has also been reported to be enhanced in neutrophils treated with Mac-1 antibody in combination with TNF or Fas ligation (23, 24). A recent study suggests (25) that NADPH oxidase-stimulated activation of the SH2-containing inositol-5-phosphatase SHIP leads to Akt down-regulation and that this is responsible for the observed apoptosis in this system.

Most apoptotic signaling pathways originating from death receptor engagement or stress stimuli converge on caspases that are cysteine proteinases activated by diverse apoptotic stimuli and are key executors of apoptosis. Apoptosis triggered by ligation of death receptors such as Fas and TNF receptors is referred to as an extrinsic pathway of apoptosis and utilizes caspases 8, 10, and 3. Ligand engagement of preassembled receptor complexes leads intracellularly to the formation of the death-inducing signaling complex in which recruited initiator pro-caspases 8 or 10 are activated through a series of proteolytic cleavage steps. The intrinsic pathway for apoptosis is triggered by stress signals and involves a reduction in mitochondrial membrane potential, the release of cytochrome c from the mitochondria into the cytosol, and activation of caspase 9. Activation of initiator complexes by either of these routes leads to activation of the effector caspases, caspase 3 and 7, which are the proteolytic executioners of cell death. The extrinsic and intrinsic pathways are modulated by regulatory proteins such as FLICE inhibitory protein (FLIP), which blocks the signaling pathway at a point before caspase 8 activation and release, or Bcl-2, which can inhibit or favor apoptosis via its effects on the mitochondria (26). Several studies suggest that ROS affect the intrinsic apoptotic pathway, with mitochondria (through aerobic metabolism) being the major source and the primary target of these ROS. ROS may oxidize mitochondrial pores that lead to cytochrome c release and caspase 9 activation due to disruption of the mitochondrial membrane potential. Indeed, protection from apoptosis by the Bcl-2 and Bcl-xL molecules may be related to their ability to serve as "antioxidants" in this system (27–29). ROS generation may also change the redox status of cells with subsequent effects on specific kinases, phosphatases, and transcription factors that alter the sensitivity of the cell to apoptotic stimuli (4, 30, 31). Paradoxically, a number of competing survival signals are also activated by death-inducing signals. These include phosphatidylinositol 3'-kinase and the serine/threonine kinase Akt, the mitogen-activated protein kinase MAPK/ERK cascade, the transcription factor NFκB, and the Bcl-2 family of proteins. In many cell types, the Akt and Bcl-2-mediated survival pathways inhibit components of the intrinsic mitochondrial pathway of apoptosis, and recent studies (26, 32) suggest that the ERK cascade functions as a survival pathway by inhibiting caspase 8 activation, an important component of the extrinsic death receptor pathway of apoptosis. Thus competing pro- and anti-apoptotic signaling mechanisms triggered by death-inducing stimuli likely modulate the fate of cells.

Here we have explored the molecular mechanisms that contribute to PICD. We show that Mac-1 is required for PICD and that the other well described death-inducing receptors Fas and TNF on neutrophils do not play a role. We show that an intracellular, sustained ROS generation by the NADPH oxidase that reaches a threshold is associated with apoptosis. We demonstrate that phagocytosis leads to activation of caspases 8 and 3, and we reveal a central role for these caspases in induction of PICD. Importantly, NADPH oxidase-generated ROS triggered by phagocytosis are responsible for caspase 8 and 3 processing, suggesting a previously unappreciated role for ROS in regulating caspases of the extrinsic apoptotic pathway. Indeed, agonists such as TNF that increase ROS production in phagocytosing neutrophils increase caspase 8 activation and cell death. Finally, we demonstrate that MAPK/ERK activation in phagocytosing neutrophils provides resistance to phagocytosis-induced apoptosis by down-regulating caspase 8 activation. GM-CSF, a known survival factor for neutrophils (33, 34), exploits this signaling pathway to avert apoptosis in phagocytosing neutrophils.

EXPERIMENTAL PROCEDURES

Materials and Preparation of FITC-labeled Yeast—Human TNF, TNF neutralizing antibody, and murine TNF were purchased from R&D Systems; Fas agonist antibody (clone CH11) and Fas antagonist antibody (clone ZB4) were purchased from Upstate Biotechnology, Inc.; superoxide dismutase (SOD) and catalase were purchased from Roche Applied Science; Baker's yeast, diphenyleneiodonium chloride (DPI), cytochalasin D, cycloheximide, luminol, isoluminol, LPS, and IMLP were from Sigma; FITC-labeled Escherichia coli was from Molecular Probes and MEK inhibitor, PD-098059 and U0126, from Sigma. Cell-permeable caspase 3 inhibitor (Z-DEVD-FMK), caspase 8 inhibitor (Z-IETD-FMK), and caspase 9 inhibitor (Z-LEHD-FMK) were from Calbiochem. Fluorometric caspase 3 substrate (Ac-DEVD-AMC), caspase 3 inhibitor (DEVD-CHO), fluorometric caspase 8 substrate (Ac-IETD-AMC), and caspase 8 inhibitor (IETD-CHO), fluorometric caspase 9 substrate (Ac-LEHD-AMC), and caspase 9 inhibitor (LEHD-CHO) were from BIOSOURCE International. GM-CSF was purchased from Immunix. FITC-labeled yeast particles were prepared as follows. Yeast particles (Baker's yeast) were autoclaved and resuspended at 1 × 10^9 cells/ml. Heat-killed yeast particles were then washed in PBS, resuspended in a carbonate buffer, pH 9.5, containing FITC (0.25 mg/ml), and incubated at room temperature for 30 min. Samples were washed in PBS and stored in aliquots at 4 °C.

Mice—Mac-1-deficient mice and their wild-type counterparts (13) backcrossed 9 generations to C57BI/6 were bred and maintained in the Viral Antigen Free facility at the Longwood Medical Research Center animal housing facility at Harvard Medical School. The lpr mice (B6) and TNFβRII-deficient mice (B6/129Sv) and their respective wild-type counterparts, which were matched for genetic strain, sex, and age, were purchased from The Jackson Laboratory (Bar Harbor, ME).

Isolation of Human and Mouse Neutrophils—Human anti-oxidated peripheral blood was collected, and neutrophils were isolated by lymphocyte separation medium (ICN Biomedicals Inc., Aurora, Ohio) and depicted in a manner essentially as described previously (36). Briefly, neutrophils were isolated from the cell suspension by density gradient centrifugation on Percoll (Amersham Biosciences) using stepwise gradients of 55, 65, and 75% Percoll. After centrifugation at 1600 rpm for 10 min at 4 °C without the brake, the band between 65 and 75% of Percoll was collected. Cells (>98% neutrophils), referred to as PBNs, were resuspended in HBSS (minus Ca^2+ and Mg^2+) supplemented with 0.5% BSA and 25 μM Hepes, pH 7.4, at a concentration of 1 × 10^6 cells/ml. Human breast milk neutrophils were collected as follows. Breast milk was spun at 1200 rpm for 5 min, and the supernatant was removed. The cell pellet was washed twice with PBS and resuspended in DPBS (minus Ca^2+ and Mg^2+) at a concentration of 1 × 10^6 cells/ml. IRB approval was granted for the collection of human blood and breast milk from healthy volunteers.

Murine bone marrow was collected from two femurs by washing out the bone marrow with 1 ml of ice-cold RPMI with 5% FCS followed by NH4Cl lysis. Bone marrow neutrophil isolation was accomplished essentially as described previously (36). Briefly, neutrophils were isolated from the cell suspension by density gradient centrifugation on Percoll (Amersham Biosciences) using stepwise gradients of 55, 65, and 75% Percoll. After centrifugation at 1600 rpm for 30 min at 4 °C without the brake, the band between 65 and 75% of Percoll was collected. Cells (>98% neutrophils) were then washed with HBSS and suspended in HBSS supplemented with 0.5% BSA and 25 μM Hepes, pH 7.4, at a concentration of 1 × 10^6 cells/ml.

Phagocytosis Assay—FITC-labeled heat-killed E. coli or yeast particles were incubated with fresh human or mouse serum mixed at equal volumes and incubated at 37 °C for 30 min to allow opsonization with complement (C3). Isolated human neutrophils, breast milk neutrophils, or bone marrow-derived mouse neutrophils (1 × 10^7 cells per sample) were resuspended in DPBS (plus Ca^2+ and Mg^2+) immediately prior to the following assays. Cells were incubated for 10 min in the presence or absence of 10 μM cytochalasin D. Then cytochalasin D-pretreated or untreated neutrophils were incubated with FITC-labeled bioparticles at...
defined ratios for 30 min at 37 °C. For quantitative analysis of neutrophil phagocytosis, 1 yeast/human or murine neutrophil or 25 E. coli human or murine neutrophils were used. The mixture was spun to remove the supernatant, and ice-cold 0.2% trypan blue in PBS (100 μl) was added to the mixture for 5 min to quench extracellular fluorescence. The samples were then transferred to 96 culture wells (CoStar), and associated fluorescence was measured by the fluorescence multwell plate reader (CytoFluor II, PerSeptive Biosystems, Framingham, MA) with the excitation wavelength at 485 nm and emission wavelength at 530 nm. Phagocytosis was calculated by subtracting the fluorescence reading of cytochalasin D-pretreated neutrophils from untreated neutrophils.

In cases where apoptosis following yeast phagocytosis was analyzed, the yeast pellets were cooled to room temperature to inhibit apoptosis before the next sample was added. Human neutrophil and 3 particles/murine neutrophils. This was done so that the morphological features of apoptosis such as nuclear condensation and fragmentation could be readily identified without distortion of these characteristics by an overabundance of phagocytosed yeast. In measuring oxygen radical production, the rate of yeast to neutrophils was consistently used at 5 particles/human or murine neutrophils.

Assessment of Reactive Oxygen Species—Respiratory burst products were measured using the luminol chemiluminescence assay as described previously (37). Human neutrophils (1 × 10^6 cells) were mixed with E. coli or yeast particles and Luminol (50 μM), and the production of light over time (relative fluorescence units) was recorded by a luminometer (Analytical Luminescence Laboratory, San Diego). To determine intracellular generation of ROS products, superoxide dismutase (SOD) (50 units/ml) and catalase (2000 units/ml) were included in addition to luminol. To determine extracellular release of respiratory burst products, 0.1 ml of isoluminol (50 μM) and horseradish peroxidase (4 units/ml) were added instead of luminol. In some experiments, human neutrophils were pretreated with cytochalasin D (10 μM) or DPI (20 μM) for 10 min before addition of target particles for phagocytosis.

Treatment of Neutrophils and Assessment of Apoptosis—Neutrophils (1 × 10^6 cells) were incubated in DPBS (plus Ca^2+ and Mg^2+) in the presence or absence of a variety of pro-apoptotic stimuli at 37 °C for 30 min. These included human TNF (25 ng/ml) or murine TNF (30 ng/ml), Fas agonist antibody (10 μg/ml), Fas antagonist antibody (500 ng/ml), as well as caspase 3 substrate (Ac-DEVD-AMC), the caspase 8 sub-see above). In indicated samples, the anti-apoptotic stimuli, TNF-neutralizing antibody (10 μg/ml), Fas antagonist antibody (500 ng/ml), as well as cell-permeable caspase 3 inhibitor (50 μM), caspase 8 inhibitor (30 μM), and caspase 9 inhibitor (30 μM) were incubated with neutrophils for 30 min at 37 °C prior to the addition of the pro-apoptotic stimuli. Similarly, in indicated samples, neutrophils were pretreated for 30 min with the MEK inhibitors, PD98059 (30 μM) or U0126 (20 μM), for 10 min with LPS (100 μg/ml), GM-CSF (4 nM), cytokinin D (10 μM), diphosphenyliondium (DPI) (20 μM), or cycloheximide (5 μM) prior to the addition of the pro-apoptotic stimuli. At the end of all the indicated incubation periods, neutrophils were spun to remove the media, and the cell pellet was resuspended in 1 ml of IMDM with 10% FCS and incubated in 1.5-ml polypropylene Eppendorf tubes at 37 °C on an end-to-end shaker to prevent adherence. All the aforementioned reagents were included throughout the culture period, except for DPI and unbound phagocytic targets. Aliquots of the cell suspension were removed at 4 and 6 h after culture and spun down. Samples were resuspended in 0.05% trypsin/EDTA (0.53 mM) for 4 min at room temperature to obtain a single cell suspension because culturing neutrophils leads to variable aggregation over time particularly in samples with phagocytic targets. FCS (final 10%) was added to the samples to neutralize trypsin, and samples were then cytospun onto a slide, fixed in methanol, stained with Wright-Giemsa, and examined by light microscopy using a 1000x magnification (1-2). The percentage of apoptotic neutrophils was determined by counting the number of cells showing features associated with apoptosis (chromatin condensation and fragmented nuclei) as described previously (3). For all samples analyzed, 200 cells per slide were counted by the researcher without prior knowledge of the sample. We and others (13, 38) have demonstrated that morphological assessment of apoptosis closely correlates with results obtained using other methods to assay apoptosis such as propidium iodide staining, annexin V binding, and decreased surface CD16 expression.

Western Blot Analysis of Caspase 8 Cleavage—1 × 10^7 PBNs were treated with yeast, TNF, cycloheximide, DPI, or combinations of these agents as indicated above. Samples were placed on ice and then harvested after 2 and 4 h. Cells were lysed in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA and EGTA, protease inhibitors including diisopropyl fluorophosphate), and samples were electrophoresed on a 4–20% gradient gel. Western blot analysis was undertaken using 1 μg/ml mouse anti-caspase 8 monoclonal antibody (Clone 1C12, Cell Signaling Technology) and anti-mouse horseradish peroxidase secondary antibody (1:10,000) (Bio-Rad).

Immunocytochemistry of Active Caspase 3—PBNs were cytospun onto glass slides and fixed in 1% paraformaldehyde for 20 min at 4 °C followed by 3 washes in TBS containing 0.1% Triton X-100 (TBS/T). Cells were incubated with rabbit anti-cleaved caspase-3 polyclonal antibody 1:100 (Cell Signaling Technology, Beverly, MA) in TBS/T containing 5% BSA at 12 h at 4 °C, followed by incubation with biotinylated secondary goat anti-rabbit antibody 1:500 (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. After 3 washes with TBS/T, cells were treated with 0.6% hydrogen peroxide for 30 min at room temperature to inhibit endogenous peroxidase activity. Next, specimens were treated with an avidin-biotin complex (Vector Laboratories) for 1 h, and the reaction was developed with DAB reagent (Vector Laboratories). The cells were counterstained with Gill’s hematoxylin solution (Polysciences, Warrington, PA).

Fluorometric Analysis of Caspase 3, 8, and 9 Activity—PBNs (5 × 10^6 cells) untreated or pretreated with cycloheximide were mixed with fresh serum-opsonized inactive and unlabeled yeast particles in an Eppendorf tube at ratio of 2 yeast to 1 neutrophil. After 30 min at 37 °C, the tubes were spun to remove the media, and each sample was resuspended in 1 ml of IMDM with 10% FCS. The mixture was further cultured at 37 °C for 30 min and 2 h. The media were removed, and neutrophil pellets were washed with PBS and lysed for 10 min in ice-cold lysis buffer (50 mM Heps, 1 mM EDTA, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 mM dithiothreitol, pH 7.5). Cell lysates were collected after spinning the extract at 14,000 rpm for 15 min at 4 °C to remove detergent-insoluble material. The protein concentration of the supernatant was determined by colorimetric measurement using the Bio-Rad protein assay reagent. An aliquot of each sample (20–30 μg) was diluted to a final volume of 200 μl of assay buffer (50 mM Heps, 50% sucrose, 0.1% Triton X-100, 10 mM dithiothreitol) supplemented with 62.5 μM of the fluorogenic caspase 3 substrate (Ac-DEVD-AMC), the caspase 8 substrate (Ac-IETD-AMC), or the caspase 9 substrate (Ac-LEHD-AMC) with or without 6.25 μM of the corresponding inhibitors. DEVD-CHO for caspase 3, IETD-CHO for caspase 8, and LEHD-CHO for caspase 9. Samples were incubated for 1 h at 37 °C and measured at an excitation wavelength of 360 nm and an emission wavelength of 460 nm using a Fluorescence multimode plate reader (CytoFluor II, PerSeptive Biosystems, Framingham, MA).

Analysis of ERK Activation—To examine the phosphorylation of ERK, PBNs (5 × 10^6 cells) were incubated with DPI, and/or GM-CSF for 15 min in the indicated samples. Samples plus and minus DPI and/or GM-CSF were then incubated with media alone or serum-opsonized yeast. Aliquots of samples were removed and incubated at 37 °C for 4 h after which the percent neutrophil apoptosis was assessed. Further- to, samples were treated, at 2, 10, and 30 min after addition of yeast, ice-cold cell lysis buffer as added to aliquots of the samples for analysis of ERK activation. Cell lysates were clarified by centrifuging for 10 min at 14,000 rpm at 4 °C. Samples were then heated at 95 °C for 5 min in Laemmli sample buffer. The proteins were resolved on 8.75% SDS-PAGE, and the separated proteins were transferred to PVDF membrane (Bio-Rad) and probed with the phospho-specific ERK p42/44 antibody (BioSource). To confirm equivalent amount of loaded proteins, the membranes were stripped with 0.2 N NaOH for 5 min at room temperature and probed with ERK p42/44 antibody (Santa Cruz Biotechnology).

RESULTS

Effect of Target Pathogen to Neutrophil Ratio, and Comple- ment Opsonization, on PICD—Our previous work (13) demon- strated that PBNs fed complement-opsonized lipid particles underwent rapid apoptosis. Here we used complement-opson- ized E. coli and yeast as target pathogens to evaluate the effect of pathogen density on PICD. PMNs cocultured with complement-opsonized heat-killed E. coli at multiplicity of infection (m.o.i.) ratios of 1:10 and 1:25 underwent a dose-de- pendent apoptosis (Fig. 1A) which is consistent with data pub- lished previously (14). To evaluate whether phagocytosis is required for apoptosis, we assessed the effect of cytochalasin D, an inhibitor of actin polymerization, on apoptosis of PBNs incubated with complement-opsonized E. coli. Cytochalasin D inhibited phagocytosis as assessed by neutrophil uptake of

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Mac-1-deficient neutrophils did not (Fig. 2A). Phagocytosis led to significant apoptosis in wild-type neutrophils compared with untreated (control) samples, whereas Mac-1-deficient murine neutrophils fed yeast demonstrated levels of apoptosis similar to that in control samples (Fig. 2B). These studies suggest an essential role for Mac-1 in PICD.

The TNF and Fas Receptor Are Not Required for Phagocytosis-induced Cell Death—The phagocytosis of complement opsonized particles by PBNs leads to the release of TNF (39) and in monocytes increases FasL in the supernatant (40), suggesting the possibility that autocrine/paracrine pathways of apoptosis involving the TNF and Fas receptors may be operative in PICD. Thus PICD was examined in PBNs treated with functional blocking antibodies against the Fas receptor or TNF. First, the ability of blocking antibodies to the Fas receptor or TNF to reproducibly inhibit Fas and TNF-mediated cell death, respectively, in PBNs was evaluated. Indeed, Fas agonistic antibody (CH11)-induced neutrophil apoptosis was significantly attenuated (>80%) by the antagonistic anti-Fas antibody ZB4. TNF alone has variable effects on neutrophil apoptosis but in the presence of cycloheximide (CHX) reproducibly induced apoptosis in PBNs (24, 41) which could be partially blocked by anti-TNF antibody (Fig. 3A). On the other hand, neither the antagonistic anti-Fas nor the anti-TNF antibody blocked apoptosis following phagocytosis of yeast (Fig. 3A). Furthermore, anti-Fas and anti-TNF antibody together had no effect on PICD (data not shown). It is possible that regions of the Fas and TNF receptor not blocked by functional blocking antibodies may contribute to PICD. Thus PICD was evaluated in bone marrow-derived neutrophils isolated from mice deficient in TNF receptors 1 and 2 (TNFR1/II) or Fas (lpr mice) and their wild-type counterparts. Neutrophils from all four sets of mice had comparable phagocytosis of yeast (WT, 2.9 ± 1.9; lpr, 3.9 ± 0.2; WT, 6.6 ± 1.9; TNFR1/II, 3.1 ± 0.4 × 104 yeast/106 neutrophils, p = 0.6). The conclusion that there is no significant difference in phagocytosis between the knock-outs and strain-matched wild types was supported by the finding that the phagocytosis-induced oxidative burst was similar in all sets of mice (data not shown). Importantly, PICD was comparable in neutrophils from lpr, TNFR1/II deficient mice, and their wild-type counterparts (Fig. 3B). Together these data demonstrate that PICD proceeds

FITC-labeled E. coli (data not shown) and inhibited the oxidative burst (see Fig. 5). Cytochalasin D treatment had no effect on spontaneous apoptosis but inhibited the induction of apoptosis in PBNs fed opsonized E. coli (Fig. 1A). Complement opsonization of phagocytic targets was required for significant apoptosis. E. coli, or E. coli opsonized with heat-inactivated serum was not as efficiently phagocytosed as complement-opsonized counterparts and induced significantly less neutrophil apoptosis compared with neutrophils fed complement-opsonized E. coli (control, 3.58 ± 1.00%; + E. coli, 11.1 ± 2.8%; + heat killed serum opsonized E. coli, 18.3 ± 8.2%; + serum-opsonized E. coli, 28.3 ± 4.3%). Thus, complement likely promotes PICD by increasing neutrophil phagocytosis of the target pathogen.

PBN phagocytosis of another pathogen, complement-opsonized, heat-killed Saccharomyces cerevisiae (yeast), also led to apoptosis. A PBN:yeast ratio of 1:2 (m.o.i. = 2) led to rapid apoptosis similar to that seen with E. coli m.o.i. = 25 (Fig. 1, B and C). A lower PBN:yeast ratio (m.o.i. = 1) was ineffective in inducing PICD (data not shown). Pretreatment of PBNs with cytochalasin D inhibited phagocytosis (data not shown), and blocked PICD (Fig. 1B).

Mac-1 Is Required for PICD; Analysis of Neutrophils from Knock-out Mice—Our previous studies (13) using functional blocking Mac-1 antibodies in human peripheral blood neutrophils demonstrated that Mac-1 was required for PICD. Here we used neutrophils from Mac-1-deficient mice to assess directly the importance of Mac-1 to PICD. First, the ability of bone marrow-derived wild-type and Mac-1-deficient murine neutrophils to phagocytose yeast and generate an oxidative burst were assessed. Wild-type murine neutrophils phagocytosed yeast and generated a sustained oxidative burst, whereas
through a pathway that does not require Fas and TNF receptors. 

**TNF Synergizes with Phagocytosis to Induce Neutrophil Apoptosis That Is Mac-1-dependent**—TNF is a potent stress stimulus present during inflammation that has been variably reported to induce, delay, or have no effect on neutrophil apoptosis. Indeed, TNF has been shown to promote consistently apoptosis only in the presence of CHX. TNF is also known to increase quantitatively Mac-1 expression and activation (42). Thus, we evaluated what effects TNF may have on PICD. We analyzed neutrophil apoptosis following incubation of PBNs with *E. coli* at different m.o.i. and a range of TNF doses. A low m.o.i. of *E. coli* (m.o.i. = 1) and a dose of TNF (0.1 ng/ml) which alone do not trigger significant apoptosis were sufficient to trigger neutrophil apoptosis as early as 2 h following their incubation with neutrophils (Fig. 4A). Similarly, PBNs fed cytochrome in combination with TNF exhibited significantly enhanced apoptosis compared with neutrophils treated with either pro-apoptotic stimulus alone (Fig. 4B). The synergistic increase in neutrophil apoptosis was associated with an increase in the oxidative burst in these samples compared with those treated with either stimulus alone (see Fig. 5 for oxidative burst). The apoptosis was blocked with DPI suggesting that the synergistic effect was ROS-dependent (data not shown). On the other hand, Fas receptor engagement did not synergize with phagocytosis to enhance the oxidative burst and did not promote apoptosis, whether PBNs were treated with the agonistic Fas antibody prior to or at the same time as presentation of cytochrome (Fig. 4B). The synergism of phagocytosis and TNF in PICD was Mac-1-dependent as the combined stimuli failed to induce apoptosis in Mac-1-deficient murine neutrophils (Fig. 4C).

The susceptibility of extravasated neutrophils to PICD, TNF, or Fas is unclear (43–45). Thus, we examined apoptosis in extravasated neutrophils in human breast milk which comprise ~60% of the leukocytes and have high surface levels of Mac-1 (data not shown) (46). In parallel, the apoptosis of PBNs was examined. PBNs underwent apoptosis in response to TNF, agonistic Fas antibody CH11, or cytochrome plus and minus TNF, as expected. In contrast, breast milk neutrophils exhibited minimal induction of apoptosis by TNF and a small induction with the Fas agonistic antibody. On the other hand, breast milk neutrophils did undergo PICD. Furthermore, TNF treatment significantly enhanced PICD in these cells suggesting that the TNFR is functional (Fig. 4D). Thus the combination of phagocytosis and TNF provides a potent pro-apoptotic stimulus in extravasated neutrophils which otherwise appear to be largely resistant to death-inducing signals triggered by Fas and TNF engagement alone.

**A Robust, Sustained Oxidative Burst Is Required for Neutrophil Apoptosis**—NADPH oxidase-generated ROS are critical triggers of PICD (13). Thus, to understand how ROS produced during phagocytosis triggers apoptosis, we first determined whether the kinetics of ROS production, the quantities produced over time, and/or the intracellular or extracellular release of oxygen radicals were predictive of apoptosis. We used the chemiluminescence system amplified by luminol which is membrane-permeable and thus a sensitive method for detecting intracellular and extracellular respiratory burst products generated by the NADPH oxidase in phagocytes (37). The intracellular oxygen radicals were specifically evaluated by incubating PBN samples with superoxide dismutase (SOD) and catalase, which scavenge extracellular oxygen radicals. The extracellular release of oxygen radicals was detected by isoluminol which is membrane-impermeable. We observed that phagocytosis of cytochrome generated a rapid, robust ROS production that was sustained over time in PBNs (Fig. 5). The oxidative burst detected in neutrophils following the phagocytosis of opsonized yeast with luminol was not readily detected with isoluminol which is membrane-impermeable. We observed that phagocytosis of cytochrome generated a rapid, robust ROS production that was sustained over time in PBNs (Fig. 5). The oxidative burst detected in neutrophils following the phagocytosis of opsonized yeast with luminol was not readily detected with isoluminol or blocked by SOD/catalase (data not shown) suggesting that the majority of oxygen radicals are released intracellularly, a finding that is consistent with previous reports (37). Indeed, blocking oxygen radicals released to the extracellular compartment with SOD or catalase (which are largely membrane-impermeable) only marginally affected PICD (control, 3.3 ± 1.9%; + phagocytosis, 16.3 ± 1.9%; control + SOD/catalase, 5.0 ± 0.6; + phagocytosis + SOD/catalase, 14.3 ± 0.9% apoptosis, n = 3) suggesting a predominant role for intracellularly released oxygen radicals in triggering PICD (Fig. 5). In contrast, PBNs stimulated with fMLP generated an oxidative burst that was almost entirely extracellular (data not shown) (37), and apoptosis in these cells was slightly reduced compared with that observed in untreated cells (data not shown) (33, 48). Both phagocytosis of complement-opsonized targets and fMLP stimulate an NADPH oxidase-generated burst (49). Together these data led us to propose that a threshold of NADPH oxidase derived oxygen radicals released intracellularly is a prerequisite for triggering PICD.

Next, the ROS profile in PBNs was examined following treatment with cytochalasin D which averts and TNF which enhances PICD. PBNs treated with cytochalasin D treatment prior to being incubated with cytochrome showed an inhibition of
ROS production. Phagocytosis of cyeast plus TNF led to an increase and more sustained oxygen radical production compared with cells fed cyeast alone (Fig. 5). This is likely due to enhanced membrane expression of NADPH oxidase flavocytochrome b_558 through exocytosis of intracellular granules and/or Mac-1 ligation (50, 51). On the other hand, PBNs incubated with cytochalasin D, TNF, and cyeast exhibited an oxidative burst that reached a threshold (that was actually greater than that seen with neutrophils fed cyeast alone) but was not sustained. This correlated with an inability to undergo apoptosis over and above that present in neutrophils treated with TNF alone (Fig. 5). Thus, in addition to reaching a threshold of ROS production, a sustenance of the oxidative burst appears to be necessary for triggering PICD.

Caspase 8 and 3 Activation Occurs in Phagocytosing Neutrophils and Requires NADPH Oxidase-derived Reactive Oxygen Species.—To begin delineating the intracellular mechanism of PICD, we examined whether phagocytosis leads to activation of caspases and if NADPH oxidase-derived ROS, key intracellular triggers of PICD, played a role in this process. We first focused on caspase 8 which plays a key role in the extrinsic death receptor pathway of apoptosis. Activation of caspase 8 occurs in two steps. The initial cleavage of the precursor gives rise to the p43/p41 and p12 subunit, whereas the next two cleavage steps within the p43/p41 subunit generate the active p18 and p10 subunits (52). Examination of pro-caspase 8 processing in

Fig. 4. TNF but not Fas synergizes with phagocytosis to induce apoptosis, the synergism requires Mac-1. A, PBNs were treated with low doses of TNF (0.1–5 ng/ml) in combination with serum-opsonized E. coli (m.o.i. = 1–12.5) as indicated. B, neutrophils were treated with a combination of TNF (25 ng/ml) and serum-opsonized yeast particles or Fas agonist antibody (Fas) and serum-opsonized yeast particles (Y) (m.o.i. = 2). C, bone marrow neutrophils from wild-type or Mac-1-deficient mice were incubated with TNF (30 ng/ml), serum-opsonized yeast, or both TNF and yeast. Apoptosis was quantitated after 4 h in culture (n = 3). D, PBNs or neutrophils harvested from breast milk (BMN) were left untreated or treated with Fas agonist antibody (Fas, 100 ng/ml), TNF (25 ng/ml), or serum-opsonized yeast particles (Y, m.o.i. = 2). Apoptosis was assessed 4 h after culture. Data are shown as the fold induction of apoptosis in treated compared with untreated samples because the percent apoptosis in untreated BMN samples (spontaneous apoptosis) varied between independent experiments (n = 3).

Fig. 5. Oxygen radical generation and correlation with apoptosis. PBNs were incubated with yeast alone (Y), TNF (25 ng/ml) alone, a combination of both, or they were pretreated with cytochalasin D (CD, 10 μM) prior to the addition of yeast or the combination of TNF and yeast. Total oxygen radical release in the presence of luminol (RLUs) was examined. An aliquot of each sample was put in culture for 4 h, and the percent apoptosis in each was determined (shown in parentheses).
PIPs using immunoblot techniques revealed that pro-caspase 8 was cleaved to the p43/41 subunit 4 h following phagocytosis (Fig. 6). Although further p43/41 processing was not observed at the 4-h time point (Fig. 6), the p18 subunit was detectable at later time points (6 h after phagocytosis) (data not shown). Notably, phagocytosing PBNs additionally treated with CHX or TNF exhibited enhanced apoptosis that correlated with an increase in pro-caspase 8 cleavage compared with samples fed yeast alone. TNF treatment alone did not lead to caspase 8 cleavage, and samples treated with both TNF and CHX exhibited significant caspase 8 processing. To examine the role of ROS in caspase 8 activation following phagocytosis, PBNs were subjected to treatment with the NADPH oxidase inhibitor DPI and analyzed. These neutrophils exhibited a block in pro-caspase 8 processing. Similar results were observed in DPI-treated PBNs fed yeast and additionally treated with TNF, although in this case caspase 8 cleavage was not completely prevented (Fig. 6). Together, these data suggest that PICD is associated with caspase 8 cleavage and that ROS play a critical role in this processing step.

In previous work, treatment of PBNs with Mac-1 antibody and TNF was reported to enhance neutrophil apoptosis compared with neutrophils treated with either stimulus alone (5, 23, 24). Because ROS generation under these conditions is minimal (1:10) compared with that seen in samples fed yeast (25, 53, 54), we were interested in determining whether caspase 8 activation was observed under these experimental conditions. Indeed, caspase 8 processing to the p43/41 subunit was detected in PBN samples treated with Mac-1 (VIM12) or CD18 antibody and TNF but not in controls (control antibody plus TNF), and this correlated with enhanced apoptosis (data not shown). This suggests that Mac-1 engagement in the presence of a potent cell-activating agonist TNF triggers caspase 8 activation, despite the small oxidative burst. The implications of this are discussed under “Discussion.”

Next, the activation of caspase 3, an executioner caspase downstream of caspase 8, was examined by determining the number of PBNs that immunostain positively for the cleaved product of activated caspase 3. Caspase 3-positive staining was observed in a significant number of neutrophils fed yeast compared with unfed neutrophils. Furthermore, pretreatment of PBNs with DPI prior to them being fed yeast blocked phagocytosis-induced active caspase 3 (Fig. 7).

To examine whether the processed caspases detected by immunoblot and immunostain techniques were catalytically active, we evaluated the enzymatic activity of caspase 8 and 3 toward synthetic peptide targets in PBN extracts. The positive control for caspase 8 activity was PBNs treated with TNF plus CHX. Following phagocytosis, significant caspase 8 activity was observed. This activity was consistently observed only in samples assayed in the presence of CHX. The need to include CHX to observe reliable caspase 8 activity has been reported previously (32) for TNF-induced caspase 8 activation in other cell systems and may be a direct result of CHX-induced down-regulation of an inhibitor of caspase 8, cFLIP (55, 56). In CHX-treated neutrophils fed yeast, caspase 8 activity was increased 2–3-fold, which was comparable with the 4-fold induction in caspase 8 activity observed in neutrophils stimulated with TNF and CHX (Table I). Pretreatment of phagocytosing PBNs with DPI significantly attenuated caspase 8 activity thus supporting the results obtained by immunoblot studies (Fig. 6). Concurrently, the activities of caspase 3 (a downstream target of caspase 8) and caspase 9, a component of the intrinsic apoptotic pathway, were examined. The positive control for these assays was neutrophils treated with Fas agonistic antibody which exhibited robust caspase 9 and 3 activation. We observed a 2-fold induction in activation of caspase 3 in cells fed yeast compared with PBNs incubated in media alone, and this was inhibited by DPI (Table I). This is consistent with the results of Fig. 7. On the other hand, there was no detectable increase in caspase 9 activity following phagocytosis (Table I). In conclusion, caspases 8 and 3, and not caspase 9, are activated by phagocytosing neutrophils, and this activation appears to be dependent on NADPH oxidase-derived ROS.

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2 B. Zhang, J. Hirahashi, X. Cullere, and T. N. Mayadas, unpublished data.

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**FIG. 6. Immunoblot analysis of caspase 8 cleavage.** PBNs were treated with cyeast (Y), TNF (T, 25 ng/ml), and a combination of these stimuli (YT), and samples were additionally pretreated with DPI (D, 20 μM) or cycloheximide (X, 5 μM) as indicated. After 4 h, neutrophil extracts were prepared, and caspase 8 was detected by immunoblot analysis. Neutrophils fed cyeast exhibited caspase 8 processing to the p43/41 product which was enhanced in samples also containing TNF (YT). Neutrophil samples treated with TNF alone (T) had minimal caspase 8 cleavage, whereas TNF plus CHX (TX) induced neutrophil apoptosis (data not shown) that correlated with caspase 8 cleavage. CHX enhanced caspase 8 cleavage in neutrophils phagocytosing cyeast (XY) that was associated with increased neutrophil apoptosis compared with samples incubated with cyeast alone (data not shown). DPI treatment reduced caspase 8 cleavage in both phagocytosing neutrophils (YT) and phagocytosing neutrophils treated with TNF (YT).

**FIG. 7. Immunostaining of neutrophils for active caspase 3.** PBNs were placed in culture for the indicated time points following treatment with media (Con), complement-opsonized E. coli, or complement-opsonized E. coli plus DPI (E. coli + DPI, 20 μM). Increased caspase 3 activation was observed 45 min after neutrophils fed E. coli were placed in culture, although the intensity of staining was weak in all samples (including controls) at this time point. At the 2-h time point more robust staining for active caspase 3 was observed in all samples. Samples of neutrophils fed E. coli had ~2.5-fold more active caspase 3-positive cells compared with controls. On the other hand, DPI pretreatment of phagocytosing neutrophils led to a significant decrease in the number of active caspase 3-positive cells, whereas it had no effect on control samples (data not shown). Representative images of active caspase 3-stained neutrophil cytosplasm from the 2-h time point are shown. An arrow points to an active caspase 3-positive neutrophil. Note that neutrophils fed E. coli have multiple E. coli present in their cytoplasm (see arrowheads). n = 2.
Mechanism of Phagocytosis-induced Cell Death in Neutrophils

**TABLE I**

| Activity ± S.E. | Con | CHX | Y | CHX + DPI | Y + CHX + DPI | TNF + CHX |
|----------------|-----|-----|---|-----------|--------------|-----------|
| Caspase 8       | 54.8 ± 29.8 | 9.0 ± 0.6 | 122.7 ± 59.1 | 32.7 ± 9.1 | 75.5 ± 12.4 | 13.7 ± 5.2 |
| Caspase 9       | 36.7 ± 21.7 | 3.7 ± 0.9 | 32.6 ± 4.2 | 2.3 ± 1.9 | 45.4 ± 2.4 | 16.3 ± 3.3 |
| Caspase 3       | 251.1 ± 78.7 | 3.7 ± 0.9 | 224.9 ± 77.9 | 2.3 ± 1.9 | 831.1 ± 270.7 | 16.3 ± 3.3 |
| Fas/CH11        | 269.8 ± 139.2 | 53.3 ± 8.3 | 2533.1 ± 1124.6 | 53.3 ± 8.3 |

Con, control.

**Caspase 8 and 3 Are Required for PICD—**To assess directly the functional significance of caspase activation in PICD, we assayed the ability of cell-permeable specific peptide substrate inhibitors of caspase 8 (Z-DEVD-FMK), 3 (Z-LEHD-FMK), and 9 (Z-LEHD-FMK directly) to block PICD in PBNs. As compared, the effect of these inhibitors on spontaneous and agonist Fas antibody-induced apoptosis was analyzed. Agonistic Fas antibody led to rapid cell death which was blocked by inhibitors of caspases 8 and 3. These inhibitors also attenuated spontaneous cell death likely through the inhibition of the Fas/FasL-induced pathway of cell death (Fig. 8) (57, 58). Phagocytosis alone led to significant apoptosis that was inhibited by both caspase 8 and 3 inhibitors. This was despite an increase in the oxidative burst in these samples compared with untreated neutrophils fed yeast (data not shown). The caspase 9 inhibitor inhibited Fas-induced cell death; caspase 9 has been shown to be essential for Fas-induced apoptosis in certain cell types (59). The caspase 9 inhibitor had no significant effect on PICD (Fig. 8) suggesting a non-essential role for the mitochondrial/caspase 9 pathway of cell death in PICD. To investigate further the possible role for the mitochondrial dependent cell death pathway, we examined cytochrome c release following phagocytosis by fluorescence-activated cell sorter analysis, and we assessed PICD in the presence of the mitochondrial membrane-stabilizing agent, bongkrekic acid, that has been shown previously to inhibit Fas-induced apoptosis in neutrophils (60). Cytochrome c release was not evident at 30 min, 2 h, and 4 h following the incubation of neutrophils with opsonized yeast particles (data not shown). Furthermore, PBNs treated with bongkrekic acid and fed yeast exhibited similar levels of apoptosis compared with untreated cells (data not shown). Together, these data suggest that caspases of the death receptor pathway are required for PICD, whereas the caspase 9/cytochrome c/mitochondrial dependent pathway is not essential.

The MAPK/ERK Attenuates PICD by Inhibiting Caspase 8 Activation; GM-CSF Treatment Enhances ROS Generation in Phagocytosing Neutrophils but Delays Apoptosis by Sustaining ERK—GM-CSF and LPS are inflammatory mediators that prime neutrophil functions such as the oxidative burst but also delay spontaneous PBN apoptosis (33, 34). The effects of these compounds on PICD in PBNs were examined. Treatment of PBNs with LPS or GM-CSF prior to cytochrome was delayed an increase in the oxidative burst compared with samples fed cytochrome alone (Fig. 9A). The oxidative burst was sustained and intracellular, which fulfilled our criteria for the induction of cell death. Despite this, PICD was averted (Fig. 9B). Thus GM-CSF and LPS can delay PICD in the presence of a significant respiratory burst suggesting the stimulation of a potent survival pathway upon treatment with these stimuli. We sought to elucidate the mechanism by which one of these stimuli, GM-CSF, mediated survival following phagocytosis. GM-CSF is known to activate ERK signaling pathways that can lead to cell survival signals (61). A potential role for ERK in GM-CSF-mediated survival of PBNs was assessed by two methods. ERK activation was evaluated biochemically using antibody that recognizes the activated form of ERK. Next, the effect of an inhibitor of ERK activation, the MEK inhibitor PD98059, on PICD was examined. A time course of ERK activation revealed that it is activated as early as 2 min following yeast phagocytosis but declines by 10 min. However, in the presence of GM-CSF, ERK activation triggered by phagocytosis was significantly sustained (Fig. 10A). Furthermore, inhibition of the NADPH oxidase in GM-CSF-treated phagocytosing PBNs markedly increased and further sustained ERK activation. Together, the biochemical data suggest that phagocytosis triggers rapid ERK activation which is enhanced and sustained by GM-CSF but that ERK activation is tempered by ROS. To test directly the importance of ERK activation in PICD, the effect of PD98059 on PICD and GM-CSF-mediated delay in PICD was assessed. PD98059, shown in biochemical assays to attenuate significantly ERK activation (by 80% in yeast + GM-CSF samples; data not shown), accentuated apoptosis in phagocytosing neutrophils (Fig. 10B). PD98059 had no effect on neutrophil phagocytosis or generation of an oxidative burst (data not shown) suggesting that these processes are not ERK-dependent and is consistent with data published previously (62). Importantly, PD98059 reversed the GM-CSF-mediated delay of PICD to that seen in neutrophils phagocytosing cytochrome alone (Fig. 10B). PD98059 also increased spontaneous cell death which may be due to the increased susceptibility of PD98059-treated neutrophils to the Fas/FasL pathway of spontaneous neutrophil apoptosis (32). Thus ERK plays a central role in the survival of neutrophils during PICD with the anti-apoptotic factor GM-CSF exploiting this pathway to avert PICD.

Although we attempted to strengthen the data on the anti-apoptotic role of ERK by using another described inhibitor of MEK, U0126 (20 μM), we found that although this inhibitor had no effect on phagocytosis, it significantly inhibited the oxidative burst in phagocytosing PBNs and thus PICD (data not shown). This precluded using U0126 for assaying the role of ERK in apoptotic signaling events downstream of ROS production. A possible explanation for the discrepancy between U0126 and PD98059 in their effects on the NADPH oxidase is that in neutrophils, U0126 may inhibit other signaling molecules in addition to ERK1/2. For example, U0126 has been shown to block significantly the p70S6K pathway, whereas PD98059 did not (63). Schonhoff et al. (64) also demonstrated that U0126 completely blocked nitric-oxide synthase activity in PC12 cells, whereas PD98059 had no effect. They argued that the discrepancy may arise from the increased effectiveness of U0126 over PD98059 in blocking the Ras-ERK pathway (65, 66) with the residual MEK activity in PD98059-treated cells being sufficient for nitric-oxide
synthase activity. By analogy, it is possible that the residual ERK1/2 activity in PD98059-treated cells in our study (i.e., 20% remaining activity) may be sufficient for NADPH oxidase activity.

Activated ERK Inhibits Caspase 8 Activation—The rapid effect of the MEK inhibitor in enhancing PICD suggested direct signaling effects of ERK and not ERK-mediated transcriptional activation in modulating PICD. A recent study (32) suggested that the ERK pathway prevents cell death induced by a number of death receptors (Fas, TNFR, and TRAIL) through the inhibition of caspase 8 activation. Therefore, we assessed caspase 8 activation in untreated PBNs or PBNs fed yeast in the presence or absence of PD98059 (Table II). We observed that PD98059 treatment increased caspase 8 activity in untreated neutrophils or yeast-fed neutrophils. Neutrophils pre-treated with GM-CSF and fed yeast had reduced levels of caspase 8 activity, whereas those samples additionally treated with PD98059 exhibited increased caspase 8 activity. Together, the data suggest that activated ERK likely makes neutrophils refractory to PICD by tempering activation of caspase 8.

DISCUSSION

Previous work has demonstrated that neutrophil phagocytosis leads to rapid apoptosis and that apoptosis is critically
dependent on ROS generated during phagocytosis (13, 14, 16). Our present findings provide several insights into the mechanisms and signal transduction pathways that regulate PICD. Our data support a model wherein Mac-1 promotes PICD through a caspase 8-dependent pathway. NADPH oxidase-derived ROS generated by phagocytosing neutrophils promotes apoptosis by amplifying caspase 8 and 3 activation. Phagocytosis also triggers competing survival signals through MAPK/ERK. Activated ERK provides resistance to PICD by down-regulating caspase 8 activation. Thus cross-talk between pro- and anti-apoptotic signals generated by the phagocytosing neutrophil places checks and balances on the apoptotic pathway triggered by phagocytosis. Phagocytosing neutrophils at sites of inflammation are likely to encounter several cytokines and other inflammatory mediators. We present evidence that exogenous pro- and anti-apoptotic cytokines exploit intracellular pathways triggered by phagocytosis to enhance or suppress apoptosis, respectively. TNF augments Mac-1-mediated oxidative burst and thereby enhances caspase 8 activation and PICD. GM-CSF also augments the oxidative burst in phagocytosing neutrophils but in addition sustains ERK activation triggered by phagocytosis thus down-regulating caspase 8 activation. This shifts the balance in phagocytosing neutrophils toward survival. Our data suggest that ROS triggered by phagocytosis are pro-apoptotic as a result of their effects on two targets. 1) The activation of caspase 8 in phagocytosing neutrophils, and 2) the down-regulation of GM-CSF-mediated ERK activation that has the effect of tempering the GM-CSF-mediated survival pathway. ERK likely inhibits caspase 8 activation upstream of ROS-mediated caspase activation because significant ERK activity generated by GM-CSF protects the cell against ROS pro-apoptotic effects and shifts the balance in the phagocytosing cell toward survival (Fig. 11). Indeed, a recent study suggests that the MAPK/ERK may inhibit Fas and TRAIL-induced apoptosis downstream of the assembly of death-inducing signaling complex but upstream of caspase 8 cleavage (67).

The primary receptor required for PICD is Mac-1. This has been demonstrated definitively in our studies because an absence of Mac-1 abrogates PICD. PICD appears not to require other death-inducing receptors on neutrophils, Fas, and TNFRs suggesting that Mac-1 may directly regulate neutrophil apoptosis. Importantly, Mac-1-mediated phagocytosis led to caspase 8 cleavage/activation; caspase 8 processing was not complete at early time points after phagocytosis but did correlate with increased caspase activity. This is consistent with recent studies (68, 69) demonstrating that caspase 8 processing limited to the first step (p43/41 cleavage) is sufficient to expose the caspase 8 active site. Activation of caspase 8 and its downstream effector caspase, caspase 3, were required for Mac-1-dependent PICD. This supports the notion that Mac-1 may be a death-inducing receptor because caspase 8 activation is a signature of such receptors. Although Mac-1 does not have a recognized death effector domain this does not rule it out as a possible death receptor candidate because the cytoplasmic tail of CD27, a pro-apoptotic member of the TNFR family, also lacks the death domain but triggers apoptosis by binding an adaptor protein Siva with a death-domain homology region (70). On the other hand, the role of Mac-1 in apoptosis may be limited to promoting phagocytosis-induced ROS production which in turn triggers cell death.

The increase in oxygen consumption following Mac-1-mediated phagocytosis occurs through the activity of the NADPH oxidase that generates superoxide anion and hydrogen peroxide. The NADPH oxidase is a complex of proteins that assemble upon stimulation with agonists to form the active oxidase. Our previous study used PBNs from CGD patients and DPI to document a role for the NADPH oxidase in apoptosis (13). Our current study has relied on pharmacological inhibition of the NADPH oxidase with DPI which reacts with the heme and FAD prosthetic redox groups of the membrane-bound flavocytochrome b of the NADPH oxidase (71, 72). DPI also inhibits other flavoprotein-using enzymes which in neutrophils would include the nitric-oxide synthase (73). However, nitric oxide is not essential for PICD because CGD patients, which have a functional nitric synthase, phagocytosed complement-opsonized targets as efficiently as normal neutrophils yet failed to undergo apoptosis (13). It is also noteworthy that DPI treatment of normal neutrophils has been shown to very closely mimic the phenotype of CGD neutrophils (74) which fail to generate ROS in response to diverse stimuli including complement and antibody-opsonized phagocytic targets, FMLP and phorbol 12-myristate 13-acetate (13, 49, 75–77). GM-CSF, LPS, and TNF have been shown to prime the NADPH oxidase for an oxidative burst in response to the aforementioned stimuli.

### Table II

**ERK activation leads to the inhibition of phagocytosis-induced caspase 8 activity**

| Conditions          | Caspase 8 activity (% apoptosis) |
|---------------------|----------------------------------|
| C                   | 38.0 (7%)                        |
| C + PD              | 77.0 (11%)                       |
| Y                   | 84.1 (19%)                       |
| Y + PD              | 123.7 (25%)                      |
| Y + GM              | 42.3 (5%)                        |
| Y + GM + PD         | 60.0 (17%)                       |

*Human peripheral blood neutrophils were untreated or pretreated with the MEK inhibitor PD98059 (+PD, 30 μM) for 30 min and/or GM-CSF (GM, 4 ng/ml) for 15 min prior to incubation with or without serum-opsonized yeast particles (Y) for 30 min. Cells were further incubated for 4 h. Apoptosis was quantitated morphologically in aliquots of samples, the results of which are shown in parentheses. The remaining cells were lysed, and *in vitro* caspase 8 activity was quantitated. One of two representative experiments is shown.

**FIG. 11. Model of phagocytosis-induced cell death.** Mac-1-mediated phagocytosis generates a robust NADPH oxidase-mediated burst. This promotes caspase 8 activation leading to subsequent activation of the executioner caspase 3 and apoptosis. Phagocytosis also triggers a parallel ERK-mediated survival pathway which attenuates PICD through down-regulation of caspase 8. Extracellular stimuli such as TNF and GM-CSF influence PICD by potentiating the oxidative burst or sustaining ERK activation, respectively. Oxygen radicals suppress GM-CSF-mediated ERK activation thus tempering this survival pathway. The dotted lines depict anti-apoptotic pathways.
through enhanced assembly or expression of the cytosolic oxidase factors (51, 78–80). The oxidase, localized to the phagosome (95% of total) and the plasma membrane (about 5%), generates ROS that are intracellular and/or extracellular depending on the stimulus (37). Phagocytosis leads to a robust and sustained intracellular oxidative burst which is well known to be required for microbial killing but may also be essential for triggering apoptosis. Reagents that blunted ROS production, such as cytochalasin D, blocked apoptosis, and agonists that enhanced the oxidative burst, such as TNF, increased the number of apoptotic cells. Indeed it is possible that ROS are pro-apoptotic only at concentrations that overwhelm the endogenous antioxidant defense mechanisms.

It is known that the caspase 9/mitochondrial pathway of apoptosis is activated by stress-induced apoptotic signals such as ROS (28). Despite this, the mitochondrial pathway of apoptosis was not essential for PICD, i.e. phagocytosis did not lead to increased caspase 9 activity, and cytochrome c release and inhibitors of caspase 9 and a mitochondrial stabilizing agent had no effect on PICD. Although this is unexpected, the link of oxidative stress to mitochondrial dependent apoptosis was previously made primarily in studies in which hydrogen peroxide was added extracellularly or ROS were generated by the mitochondria (27, 81–84). We propose that ROS generation by the NADPH oxidase (a non-mitochondrial source of ROS), the intracellular localization of oxygen radicals, the type of radicals generated, and the kinetics and threshold of production may significantly dictate the intracellular targets of ROS. This in turn may determine whether ROS induced apoptosis and if so the pathway of apoptosis that is triggered. TNF appears to enhance apoptosis in phagocytosing neutrophils by increasing ROS production through the NADPH oxidase; the effect of TNF may be to enhance Mac-1 expression at the cell surface, Mac-1 activation (42, 85), and/or translocation of NADPH oxidase components (51).

The ability of non-lethal doses of TNF to trigger apoptosis in neutrophils engulfing as little as one E. coli suggests that the density of the offending pathogen and the concentrations of TNF present at inflammatory sites may directly correlate with the extent of cell death observed. On the other hand, Fas had no effect on ROS generation in phagocytosing neutrophils or the subsequent PICD. Previous data suggest that activated neutrophils are resistant to Fas-induced cell death due to alterations in the redox potential of activated cells (87) and that superoxide anions are natural inhibitors of Fas-mediated apoptosis (88). These data support our findings. We also found that neutrophils present in an extravascular compartment are largely resistant to Fas or TNF-induced cell death but were sensitive to PICD. PICD in extravasated neutrophils is enhanced by TNF suggesting that the TNFR is functional but does not deliver a pro-apoptotic signal when engaged alone. Thus, PICD represents a potent pro-apoptotic pathway in extravasated neutrophils. The finding that CHX accelerates PICD in PBNs as it does TNF suggests that neutrophil survival may be regulated by the expression of transiently expressed “survival” proteins. It is possible that inflammatory neutrophils have an increase in these CHX-sensitive survival pathways (e.g. NFκB-dependent transcription) that are overcome most efficiently by the combination of phagocytosis and TNF.

A principal mechanism by which the NADPH oxidase-derived ROS trigger apoptosis is through activation of caspase 8 that results in activation of the executioner caspase, caspase 3. The effect of ROS on caspase 8 processing suggests that ROS may be a receptor proximal focal point for regulation of cell death in phagocytosing neutrophils. The possible role of ROS in caspase 8 activation is largely unexplored as some studies suggest that caspase activation does not have apparent redox-sensitive components (27). Because pro-caspase 8 processing itself is blocked by DPI, we propose that the effect of ROS is on modulating regulatory proteins of the intrinsic cell death pathway that directly affect caspase 8 activation rather than on negative regulators of active caspase 8. This possibility is being currently investigated. We found that a combination of Mac-1 ligation by antibody and TNF (but not Mac-1 ligation alone) promotes caspase 8 activation and apoptosis despite a minimal oxidative burst. Although ROS appear to be required for apoptosis (25), the target of ROS in this system is unclear. That is, TNF mediated Mac-1 activation (42), which is required for triggering neutrophil apoptosis (5) may be ROS-dependent (85). In contrast, the ROS target in PICD is downstream of Mac-1 activation required for phagocytosis because phagocytosis proceeds normally both in normal neutrophils pretreated with DPI and in neutrophils from CGD patients (13). We speculate that clustering of Mac-1 with antibody and TNF may facilitate caspase 8 autoactivation and bypass the need for oxygen radical generation to activate caspase 8 because of the strength of the apoptotic signal generated by saturating levels of Mac-1 antibody and TNF. On the other hand, a pathogen that may comparatively engage a much smaller fraction of the total Mac-1 receptor population (89) may lead to inefficient caspase activation requiring amplification signals from ROS for full caspase 8 activation.

Aside from generating pro-apoptotic signals, phagocytosis also triggers potent survival pathways. Mac-1-mediated phagocytosis of cyest alone leads to ERK activation which is consistent with published data (47, 62). We have identified a role for this ERK activation in PICD. Inhibition of ERK by the MEK inhibitor PD98059 increased PICD suggesting that phagocytosis-mediated ERK activation induces resistance to PICD. We present evidence that the protective effect of ERK during phagocytosis is through direct suppression of caspase 8 activity. The generation of a MAPK-dependent competing survival pathway following Mac-1-mediated phagocytosis has similarities with the TNF-mediated cell death pathway. TNFR or Fas ligation triggers activation of ERK which can override the pro-apoptotic signaling pathway of these death receptors (32). Thus, ERK may have a generally protective effect on death receptor-induced apoptosis, which could be used under conditions when death receptor responses have to be rapidly switched off. A case in point is the ability of GM-CSF to suppress PICD despite a robust oxidative burst by sustaining activation of ERK initiated by phagocytosis. Activated ERK overcomes the pro-apoptotic effects of the phagocytosis-induced oxidative burst by inhibiting caspase 8 activation, a proximal intracellular trigger of PICD. On the other hand, the robust oxidative burst in GM-CSF-treated phagocytosing neutrophils tempers the GM-CSF mediated survival by diminishing ERK activation. A previous study has suggested that ROS can inhibit insulin-mediated ERK activation (86). Thus extracellular stimuli shown previously to inhibit spontaneous neutrophil apoptosis in vitro (9) and in vivo (8) may exploit ERK-dependent anti-apoptotic pathways to delay PICD at sites of inflammation.

In summary, our studies indicate that Mac-1-mediated phagocytosis is a formidable pathway of cell death in neutrophils and that the robust, intracellular and sustained ROS production is essential for triggering apoptosis. Our studies suggest ROS produced during phagocytosis triggers an “extrinsic” apoptotic pathway that is distinct and may have similarities and differences with the previously well described TNF and Fas receptor-mediated pathways of apoptosis. Phagocytosis leads to competing death and survival pathways with cross-talk between components of these pathways eventually favoring cell...
Mechanism of Phagocytosis-induced Cell Death in Neutrophils

dearth. The dynamic nature of these signals in phagocytosing neutrophils is likely what gives the neutrophils the ability to integrate rapidly exogenous pro- and anti-apoptotic factors converging on neutrophils during inflammation such as TNF and GM-CSF, respectively. This would ensure that extravasated neutrophils have an extended life span to complete their phagocytic functions but would then be eliminated once they have reached the end of their useful life span. This would limit tissue injury by containing the release of cytotoxic contents of phagocytosing neutrophils.

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Elucidation of Molecular Events Leading to Neutrophil Apoptosis following Phagocytosis: CROSS-TALK BETWEEN CASPASE 8, REACTIVE OXYGEN SPECIES, AND MAPK/ERK ACTIVATION

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