A Functional NADPH Oxidase Prevents Caspase Involvement in the Clearance of Phagocytic Neutrophils

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ABSTRACT

Neutrophils play a prominent role in host defense. Phagocytosis of bacteria leads to the formation of an active NADPH oxidase complex that generates reactive oxygen species for bactericidal purposes. A critical step in the resolution of inflammation is the uptake of neutrophils by macrophages; however, there are conflicting reports on the mechanisms leading to the apoptosis of phagocytic neutrophils. The aim of this study was to clarify the role of effector caspases in these processes. Caspase activity was measured by DEVDase activity assays or immunofluorescence detection of active caspase-3. With normal human and wild-type murine neutrophils there was no caspase activation following phagocytosis of Staphylococcus aureus. However, caspase activity was observed in phagocytic neutrophils with a defective NADPH oxidase including neutrophils isolated from X-linked gp91phox knockout chronic granulomatous disease (CGD) mice. These results indicate that a functional NADPH oxidase and the generation of oxidants in the neutrophil phagosome prevent the activation of the cytoplasmic caspase cascade.
INTRODUCTION

Neutrophils play an integral role in the eradication of pathogens from the body. These cells contain a range of toxic compounds, and it is essential that they remain intact without releasing intracellular contents that might damage host tissue. Following maturation and release from the bone marrow, the circulating neutrophil has a lifespan of 1-2 days before undergoing apoptosis (26). This process has been well characterized, and includes caspase activation, phosphatidylserine (PS) exposure and the phagocytosis of the dying cell (8, 9, 27). The rate of this spontaneous apoptosis is susceptible to modulation. If neutrophils are exposed to pro-inflammatory cytokines such as interleukins, GM-CSF or bacterial lipopolysaccharide (LPS) the apoptotic process is delayed (3, 5), thereby prolonging neutrophil lifespan, and presumably, their ability to contribute to pathogen removal.

At an inflammatory site, neutrophils ingest pathogens into intracellular compartments called phagosomes, where killing occurs (11). Phagocytic neutrophils are themselves ingested by macrophages before they disintegrate. However, the mechanism of apoptosis in phagocytic neutrophils appears more complicated and controversial than the spontaneous apoptosis of unstimulated cells. Both acceleration (18, 21, 23, 34-36) and retardation (1, 23, 31) of neutrophil apoptosis in actively phagocytosing neutrophils has been reported.
Stimulated neutrophils generate vast amounts of oxidants upon activation of the NADPH oxidase membrane complex within the phagosome (11), and several studies have demonstrated that these oxidants promote the onset of neutrophil apoptosis (7, 14, 23, 24, 28, 34). However, the role of the caspases in this process is unclear. These enzymes play a key role in the cascade of proteolytic cleavage that occurs during apoptosis. Their activation is redox-sensitive (12, 13), and we have previously shown that triggering the oxidative burst with PMA blocks caspase activation (8). The situation during phagocytosis, however, will differ from that of the artificial PMA stimulus. In particular, oxidant production is generally considered to be restricted to the internal phagosome during phagocytosis, as compared with NADPH oxidase activation over the entire plasma membrane with PMA (32). The duration and extent of the oxidative burst will also vary between the two systems. There are two reports suggesting that in contrast to the PMA model, caspases are activated during the apoptosis of phagocytic neutrophils (24, 35).

In this study we investigated the effect of the NADPH oxidase on caspase activation in phagocytic neutrophils. The model involved phagocytosis of Staphylococcus aureus by neutrophils isolated from X-linked gp91phox knockout mice with a non-functional NADPH oxidase. We found that while neutrophil oxidant generation is required for PS exposure and uptake by macrophages, these oxidants clearly prevented caspase activation. Indeed inhibition of the oxidative burst led to enhanced caspase activation, but decreased clearance by macrophages. This indicates that the clearance of actively phagocytosing neutrophils is a caspase-independent process.
MATERIALS AND METHODS

Materials

*S. aureus* strain 502a (ATCC 27217) was obtained from the New Zealand Communicable Disease Centre (Porirua, New Zealand) and trypticase soy broth from Becton Dickinson (Cockeysville, MD). Cell culture media was supplied by Gibco-BRL (Grand Island, NY) and normal mouse serum by Biomeda (Foster City, CA). Caspase-3 substrate DEVD-AMC was from Peptide Institute Inc. (Osaka, Japan) and caspase inhibitor z-VAD-fmk from Enzyme Systems Products (Livermore, CA). Annexin V-fluorescein isothiocyanate (FITC) Apoptest kit was from Dakocytomation (Glostrup, Denmark for NeXins Research, Kattendijke, The Netherlands). Monoclonal rabbit anti-human-cleaved caspase-3 antibody was from Cell Signaling Technology Inc. (Beverly, MA), and Diff-Quik was from Dade Behring AG (Dudingen, Switzerland). Diphenyleneiodonium (DPI), paraformaldehyde (PFA), o-dianisidine and thioglycollate were all from Sigma Chemical Co. (St. Louis, MO).

Preparation of *S. aureus*

*S. aureus* were cultured overnight in trypticase soy broth, harvested by centrifugation, washed and resuspended in HBSS (10mM phosphate buffer pH 7.4 containing 140mM NaCl 0.5mM MgCl₂, 1mM CaCl₂, and 1mg/ml glucose). Bacterial cell density was measured spectrophotometrically at 550nm and the cell number calculated using a standard curve based on colony forming unit counts. Heat-killed propidium iodide (PI) labeled *S. aureus* were prepared by heating $10^9$/ml *S. aureus* at 90°C for 30 min before
adding 2μg/ml PI. Bacteria were then pelleted, the supernatant discarded to remove excess PI and the cells resuspended in HBSS. All bacteria were opsonized with 10% autologous human serum or commercially-obtained mouse serum and rotated end-over-end for 20 minutes at 37°C immediately before addition to neutrophils at defined ratios.

**Isolation of Human and Mouse Neutrophils**

Neutrophils were isolated from heparinized peripheral blood of healthy adult donors under sterile conditions by Ficoll/Hypaque centrifugation, dextran sedimentation, and hypotonic lysis. Mouse neutrophils were obtained from the peritoneal cavity of X-linked gp91phox knockout and C57BL/6 wild-type mice 18 h after injection with 1ml 4% thioglycollate, as approved by the University of Otago Animal Ethics Committee. Cells collected in HBSS supplemented with 0.1% bovine serumalbumin (BSA), were spun through 1.5ml of Ficoll/Hypaque at 1,000g for 20 min to concentrate neutrophil numbers.

**Neutrophil Stimulation**

Human or murine neutrophils (10⁷/ml) were incubated at 37°C in RPMI 1640 media with 10% autologous or mouse serum for 10 min in the presence or absence of 10μM DPI. DPI pretreated or untreated neutrophils were then incubated at 37°C in 5% CO₂ for up to 5 h with occasional mixing with opsonized *S. aureus* to give a ratio of 2, 10, 20, or 50 bacteria per neutrophil. At various intervals cells were harvested, and cytocentribs prepared by first removing any non-phagocytosed bacteria by differential centrifugation at 100g (15) followed by cytocentrifugation for 5 minutes at 250 rpm on to high-binding microscope glass slides. The spot was fixed with 4% (w/v) paraformaldehyde (pH 7.4)
and stained with Diff-Quik. The number of cells which had phagocytosed *S. aureus* was counted and morphology observed.

**Fluorometric Analysis of Effector Caspase Activity**

Neutrophils (10⁶ cells) untreated or pretreated with DPI were incubated with or without *S. aureus* at ratios 1:2, 1:10, 1:20, and 1:50 and centrifuged at selected times to remove media. The pellets were washed with PBS and caspase-3-like activity determined by the assessment of DEVD-AMC cleavage. Briefly, pellets were transferred to a microtitre plate and resuspended in a final volume of 100µl of caspase buffer solution (100mM HEPES, 10% sucrose, 0.1% NP-40, 0.1% CHAPS, 5mM DTT pH 7.2) with 50µM of the fluorogenic peptide substrate Ac-DEVD-AMC. The cleavage of the caspase substrate was monitored over a 30 minute period at 37°C in a Fluoroscan II plate reader using 390 nm excitation and 460 nm emission wavelengths.

**Immunofluorescence Analysis and Quantification of Caspase-3**

Neutrophils (10⁷ cells/ml) pretreated with or without DPI were incubated with or without PI labelled *S. aureus* (1:20) for 3 h at 37°C. Volumes of non-phagocytic and phagocytic neutrophils at 1:20 ratios of cells:bacteria were cytopun onto the same microscope slide to achieve a mixed population of cells. The active form of caspase-3 was detected by immunofluorescence using a previously described method (4), with a monoclonal rabbit anti-human-cleaved caspase-3 antibody and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G. A Leitz Aristoplan fluorescence microscope was used to capture images of cleaved caspase-3 immunofluorescence (green
fluorescence) and red fluorescent PI-labeled neutrophils. Color composite images were then processed using a high through-put image analysis program (Metamorph V.6.2.6, Molecular Devices) to determine the average fluorescence intensity of cleaved caspase-3 staining in phagocytic (red fluorescence) and non-phagocytic (no red fluorescence) neutrophils. A ‘Journal’ was written to automatically color separate the red and green channel, convert each image into a 16-bit image format for analysis and then insert each pair of 16-bit images into the Cell Scoring application on Metamorph. The Cell Scoring algorithm determined the average fluorescence intensity of cleaved caspase 3 staining in phagocytic (red fluorescence) and non-phagocytic (no red fluorescence) neutrophils and automatically logged this data into excel spreadsheets. The advantages of this method for quantifying imaging data are that it is fully automated, objective and standardized. Further information about this high through-put image analysis platform can be found at http://www.health.auckland.ac.nz/pharmacology/discovery-1/.

Exposure of Phosphatidylserine

The externalization of PS was assessed using annexin V-FITC according to the protocol outlined in the Apoptest-FITC kit. Untreated or DPI pretreated WT or CGD murine neutrophils were incubated with *S. aureus* at a 1:20 ratio in the presence or absence of the caspase inhibitor z-VAD-fmk (10µM) for 4 h at 37° C. Binding was also assessed using calcium-free Hepes buffer with EGTA, (10mM Hepes-NaOH, pH7.4, 150mM NaCl, 5mM KCl, 1mM MgCl₂, 1mM EGTA) to confirm that fluorescence increases were due to annexin V binding, which is calcium-dependent, and also assessed without any addition of annexin V-FITC. Flow cytometry was performed by a FACS Vantage from Becton
Dickinson (San Jose, CA) and the data analyzed using CellQuest software. Ten thousand events were analyzed and both the geometric mean fluorescence of viable cells and the percentage of cells displaying greater than 10 fluorescent units were recorded. PI-positive necrotic neutrophils (10-17%) were gated out of the final analysis.

**Uptake of Phagocytic Neutrophils by Macrophages**

Human monocyte-derived macrophages were prepared by adhering the peripheral blood monocyte (PBMC) layer after Ficoll/Hypaque centrifugation for 2 h on a 24-well tissue culture plate (5 x 10⁶/well) (14). Following vigorous washing with HBSS, adherent monocytes were cultured in Iscove’s modified Dulbecco’s medium (IMDM) with 10% mouse serum for 7 days. Mouse neutrophils (10⁶ cells) untreated or pretreated with DPI were incubated with or without *S. aureus* (1:20) for 4 h at 37°C, harvested and resuspended in 500 µl HBSS. IMDM media was removed from the 7 day macrophage wells and replaced with the neutrophils in HBSS and incubated for 1 h at 37°C. Wells were then fixed with 4% paraformaldehyde for 10 min and neutrophils visualized with a myeloperoxidase stain (1mM o-dianisidine, 5mM H₂O₂, 50mM sodium phosphate buffer, pH6). The number of neutrophils phagocytosed per 100 macrophages was counted.

**Statistics**

Statistical analysis was performed with the SigmaStat software package from Jandel Scientific (SPSS Science, Chicago, IL) using repeated measures analysis of variance (ANOVA) followed by the Bonferroni test for multiple comparisons.
RESULTS

Caspase activation in human phagocytic neutrophils

We have previously established that artificial stimulation of the oxidative burst in neutrophils by a phorbol ester can prevent activation of the redox-sensitive caspases (8). To determine whether this phenomenon is physiological we investigated the effect of NADPH oxidase-derived oxidants on caspase activity in neutrophils phagocytosing bacteria. In our system neutrophils were incubated with opsonized *S. aureus* at ratios from 2 to 50 bacteria per neutrophil and harvested at various intervals. We have previously demonstrated that with continual mixing *S. aureus* are rapidly internalized (t_{1/2}= 9 min (15)). In this system we used occasional mixing to prevent excessive neutrophil damage, however all neutrophils still had bacteria in their cytoplasm within 30 minutes. None of the inhibitors used in this study had any effect on the kinetics of uptake. Phagocytic neutrophils lost their characteristic multilobed nuclei at 3 h (Fig. 1C) and instead displayed nuclei that were swollen and irregular in shape. This was very different from the shrunken and condensed nuclei in neutrophils that had undergone spontaneous apoptosis at 48 h (Fig. 1B). At 5 h swollen nuclei were still apparent (Fig. 1D), and later time points were impossible to assess because the phagocytic neutrophils were too fragile to survive the cytocin process.

There was a 3-4 fold increase in the activity of effector caspase activity measured with the fluorescent substrate DEVD-AMC in unstimulated human neutrophils over 5 h (Fig. 2A). This reflects initiation of spontaneous apoptosis. In contrast, caspase activation was
delayed following phagocytosis of *S. aureus* (Fig. 2A). DPI treatment of phagocytic neutrophils resulted in enhanced caspase activation (Fig. 2A). DPI alone had no effect on the rate of spontaneous caspase activity. *S. aureus* themselves had no detectable caspase activity, and they were also not able to inhibit caspases as demonstrated by the significant levels of caspase activity seen in DPI-treated phagocytic neutrophils. Addition of the supernatant of cultured *S. aureus* had no influence on levels of caspase activation observed in non-phagocytic neutrophils (not shown), demonstrating that inhibition of caspase activity observed in neutrophils coincubated with *S. aureus* is indeed due to phagocytosis and not a soluble factor secreted by the bacteria.

We explored the effect of different neutrophil: *S. aureus* ratios on caspase activation. After 1 h incubation a ratio-dependent inhibition of caspase activity was observed (Fig. 3). However, over the 5 h time course there was little difference between ratios (Fig. 2B). Cytospins at 3 h showed that although the cultures began at a ratio of 1:2, bacterial growth during this period meant that the ratio was increased at later time points (not shown). Phagocytic neutrophils pretreated with DPI at all ratios showed caspase activity at levels either equivalent to or slightly greater than cells undergoing spontaneous apoptosis (Fig. 2A-D).

**Immunofluorescence analysis of active caspase-3**

To confirm the caspase activity assays we used an immunofluorescence technique that detects the active form of caspase-3 in individual cells. Neutrophils were incubated with or without propidium iodide-labelled *S. aureus* for 3 h and the two populations of cells
were mixed immediately prior to cytospin to enable a direct comparison between phagocytic and unstimulated neutrophils. Non-phagocytic neutrophils undergoing spontaneous apoptosis showed strong active caspase-3 staining within the cytoplasm (Fig. 3A). In contrast, neutrophils that had phagocytosed PI-labelled \textit{S. aureus} displayed reduced cytosolic active caspase-3 staining (Fig. 3A). Inhibition of the NADPH oxidase by DPI, however, resulted in these phagocytic neutrophils demonstrating caspase-3 fluorescence staining equal to or greater than non-phagocytic cells (Fig. 3B).

To quantify these observations we used a high through-put image analysis program (Metamorph V.6.2.6, Molecular Devices) to determine the average fluorescence intensity of cleaved caspase-3 staining in PI-labelled phagocytic (red fluorescence) and non-phagocytic (no red fluorescence) neutrophils (Fig. 3C). The results of this objective analysis confirmed our initial observations and showed a statistically significant reduction in cleaved caspase-3 in phagocytic neutrophils compared with non-phagocytic neutrophils, and a reversal of this suppression by DPI (Fig. 3C).

**Caspase activation in murine phagocytic neutrophils**

DPI is not a specific inhibitor of the NADPH oxidase and is known to have a variety effects on cells (20, 25). To validate our findings that caspase-3 activation is blocked by NADPH oxidase-derived oxidants upon phagocytosis we assessed caspase activation in phagocytic murine CGD neutrophils with a non-functional NADPH oxidase. Neutrophils from the mouse peritoneal cavity were incubated with opsonized \textit{S. aureus} at a 1:20 ratio and harvested at selected times. The extent of phagocytosis of \textit{S. aureus} was comparable
between wild-type and CGD murine neutrophils, and no apparent difference in nuclear morphology was observed (not shown).

There was a 3-fold increase in caspase-3 activation in unstimulated wild-type murine neutrophils, that was blocked in the phagocytic neutrophils (Fig. 5A). When the oxidative burst was inhibited by DPI there was increased caspase-3 activity induced in phagocytic cells (Fig. 5A). Unstimulated CGD mouse neutrophils showed no spontaneous caspase-3 activation during the 5 h incubation, but following phagocytosis of \textit{S. aureus} the levels of caspase activity were comparable to DPI-treated phagocytic wild-type neutrophils and increased more than 4-fold (Fig. 5B). In combination these results suggest that the process of phagocytosis can result in caspase activation, but that an active NADPH oxidase prevents this from occurring.

\textbf{Phosphatidylserine exposure and uptake of murine phagocytic neutrophils by macrophages}

We have previously shown by pharmacological inhibition that a functional NADPH oxidase is important for PS exposure in human phagocytic neutrophils (14). Therefore, we sought to investigate PS externalization and neutrophil uptake by macrophages in our CGD mouse model which we have now demonstrated to have elevated caspase activity. PS exposure was assessed by annexin V-FITC labeling in wild-type and CGD mouse neutrophils. Phagocytosis of \textit{S. aureus} triggered a 3-fold increase in annexin V-FITC positive wild-type murine neutrophils (Fig. 6B, D). This increase in fluorescence was not observed in the absence of annexin V-FITC, or when a calcium-free buffer was used to
prevent the binding of annexin V-FITC to PS (not shown). The increased PS exposure was blocked upon treatment with DPI (Fig. 6C, D). Similarly, neutrophils isolated from CGD mice did not express PS after phagocytosis of *S. aureus* (Fig. 6D) confirming the importance of a functional NADPH oxidase for PS externalization. The presence of the general caspase inhibitor zVAD.fmk had no significant effect on the NADPH oxidase-dependent externalization of PS observed in phagocytic neutrophils (Fig. 6B).

The key apoptotic process leading to clearance of cells from an inflammatory site is uptake by macrophages. To determine whether macrophages were able to recognize and engulf these phagocytic cells, neutrophils from wild-type and CGD mice were co-incubated with *S. aureus*, then layered onto human monocyte-derived macrophages. We found that macrophages actively engulfed wild-type neutrophils that had phagocytosed *S. aureus*, and that this was also blocked by DPI treatment (Fig. 6E). Furthermore, we observed impaired uptake of phagocytic CGD mouse neutrophils by macrophages (Fig. 6E).
DISCUSSION

Neutrophils undergo apoptosis following the phagocytosis and killing of pathogens, but it is not clear as to whether apoptosis proceeds via a conventional caspase-dependent pathway or an alternate pathway. Using enzymatic and immunofluorescence assays of effector caspases in phagocytic murine and human neutrophils, our results clearly demonstrate that caspase activation does not occur following phagocytosis. Caspases were only detected when the NADPH oxidase was inhibited or absent. However, it is known that the oxidative burst is critical for PS exposure and macrophage uptake. Therefore, caspase activation and the oxidative burst appear to be mutually exclusive events in the phagocytic neutrophil.

We have previously shown that sustained oxidant production can interfere with caspase activation (12, 13), and the same phenomenon was observed in neutrophils stimulated with the artificial stimulus PMA (8). It is possible that phagosomal production and consumption of oxidants could spare cytoplasmic caspases, but our data clearly shows that phagocytic cells have reduced caspase activity, as assessed by activity assays and immunohistochemistry. Our results differ from the reports of Zhang et al. (35) and Perskvist et al. (24), which demonstrated caspase activation in phagocytic neutrophils and suggested that this activity is a necessary requirement for apoptosis (24, 35). The basis of the apparent discrepancy between the studies is unclear, although it might relate to the nature of micro-organism used to activate the neutrophils. We explored the effect of different neutrophil: bacteria ratios and saw no difference, with significant inhibition
of caspase activation occurring even at low ratios. There was also no difference in the
effect of phagocytosis on caspase activation due to live or dead bacteria, as demonstrated
in our immunofluorescence study where neutrophils phagocytosed heat-killed *S. aureus.*
This rules out any contribution of killing defects in provoking the different responses.
Others have reported that inhibition of the NADPH oxidase had no effect on the
*Entamoeba*-induced cleavage of caspase-3 in human neutrophils (28), and that
phagocytosis results in a reduction of caspase activity (31) and caspase-3 gene expression
(2), thus supporting our results.

It is possible that phagocytic neutrophils secrete factors that dampen caspase activity in
surrounding neutrophils. However, the immunofluorescence assay clearly illustrates that
the NADPH oxidase-dependant block in caspase activation is occurring in the individual
phagocytic cells. DPI-treated or CGD neutrophils were the only phagocytic neutrophils
to show significant caspase activity. However, there was no PS exposure or uptake of
these cells. Furthermore, PS exposure in phagocytic neutrophils occurred in the presence
of a caspase inhibitor. This implies that caspases are not involved in the clearance of
phagocytic neutrophils, but rather an oxidant-dependent event is crucial. This is
consistent with the observations of Kagan and colleagues that oxidation of PS is
necessary for its externalization (16, 22, 30) and whom more recently showed in HL-60
cells exposed to nitrosative stress that PS exposure was dissociated from the common
apoptotic pathway (29). We have also recently reported that 24 h ascorbate-deficient
neutrophils fail to undergo PS exposure and uptake by macrophages despite activation of
caspases (33).
It has been proposed that the accumulation of neutrophils associated with the pathology of CGD is in part due to impaired clearance of these cells (8, 14, 19). Our observations now indicate that these neutrophils will also have active caspases. One possibility is that caspase activation in these neutrophils could enhance the structural dismantling of the cell, increasing the potential for release of neutrophil proteins and exacerbation of local tissue damage. Such a phenomenon is consistent with the increased gastric atrophy observed in CGD mice colonized with *Helicobacter pylori* (17).

Many studies have used morphological changes to the neutrophil nucleus as a key marker of apoptosis (2, 18, 34, 35). However, we found that nuclei of phagocytic neutrophils underwent morphological changes clearly different from that of neutrophils undergoing spontaneous apoptosis. Zhang and colleagues considered that morphological features of apoptosis such as nuclear condensation would become distorted by an overabundance of yeast and therefore used only 2 yeast particles per human neutrophil (35). Although the loss of the characteristic multi-lobed nuclei could result from space constraints within the neutrophil, the same morphology was observed in neutrophils incubated with *S. aureus* at lower ratios containing only one or two bacteria within the cytoplasm. Coxon *et al.* have also reported NAPDH oxidase-dependent changes in nuclear morphology in neutrophils phagocytosing serum opsonized target particles (6), and Fuchs and colleagues recently described morphology distinct from apoptosis and necrosis in stimulated neutrophils during neutrophil extracellular trap (NET) formation that also showed irregular swollen nuclei and required oxidants derived from the NADPH oxidase (10). Therefore, we do
not believe that nuclear morphological changes can be used to assess apoptosis in phagocytic neutrophils.

In summary, our studies indicate that roles for oxidants generated by the neutrophil NADPH oxidase upon phagocytosis extend beyond the former exclusive task of bacterial killing. We show that these neutrophil oxidants also serve as important signaling molecules in the phagocytic neutrophil. NADPH oxidase-derived oxidants trigger cell surface changes that result in macrophage recognition and engulfment of phagocytic cells. This phagocytic neutrophil clearance is a caspase-independent process; indeed the neutrophil-derived oxidants prevent caspase involvement.
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FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. Phagocytosis of *S. aureus* by human and murine neutrophils and subsequent changes in morphology. Photographs from a representative experiment are taken from cytospins of human (A-D) unstimulated neutrophils at 3 h (A), untreated neutrophils at 48 h incubation (B), with *S. aureus* at 3 h (C), and with *S. aureus* at 5 h (D). Murine (E-H) cytospins at 3 h are of WT unstimulated neutrophils (E), with *S. aureus* (F), and CGD unstimulated neutrophils (G), and with *S. aureus* coinubcation (H).

FIGURE 2. Caspase-3 activation is blocked by NADPH oxidase-derived oxidants in phagocytic human neutrophils. Caspase activity was assessed hourly in neutrophils incubated alone (CTRL), with *S. aureus* (SA) at ratios of 1:20 (A), 1:2 (B), 1:10 (C), and 1:50 (D) or treated with DPI prior to co-incubation with *S. aureus* (SA+DPI). Their active caspase-3 activity was measured by monitoring the arbitrary units of fluorescence liberated following cleavage of the fluorogenic peptide substrate DEVD-AMC. The means and SEM of four to nine experiments are plotted. * p<0.05 vs control and # p<0.05 vs phagocytic neutrophils with a functional NADPH oxidase.

FIGURE 3. Dose-dependant inhibition of Caspase Activity. Caspase activity in neutrophils incubated with *S. aureus* at ratios 1:2, 1:10, 1:20, and 1:50 after 1 h incubation expressed as a percentage of activity relative to that expressed by 1 h in unstimulated neutrophils.
FIGURE 4. Immunofluorescence staining for active caspase-3. Neutrophils were incubated either alone or with PI-labeled *S. aureus* (1:20) for 3 h, then both phagocytic and non-phagocytic neutrophils were mixed and cytospun onto the same microscope slide. Cells were treated with a cleaved caspase-3 antibody followed by a secondary antibody conjugated to fluorescein isothiocyanate (FITC) and visualized under a fluorescent microscope. Images of the green fluorescence (1) showing positive caspase-3 staining localized in the cytosol and red fluorescence (2) identifying phagocytic neutrophils were captured from the same field and the two images superimposed (3). Untreated phagocytic neutrophils (A2) surrounded by non-phagocytic neutrophils (A3) show negligible caspase-3 activity compared to the neighbouring non-phagocytic cells (A1). When phagocytic neutrophils were pretreated with DPI (B) to inhibit the NADPH oxidase, the phagocytic cells lying adjacent to non-phagocytic neutrophils (B3) displayed enhanced caspase activation (B1). The photographs are from representative experiments. Quantification of caspase-3 fluorescence within untreated and phagocytic neutrophils with or without DPI treatment analyzed using the Discovery-1 high throughput and high content screening machine (C). Fluorescence intensity was assessed in 300 cells from 27 photographs taken from three different experiments. * p<0.05 vs control and # p<0.05 vs phagocytic neutrophils with a functional NADPH oxidase.

FIGURE 5. Caspase-3 activation in phagocytic murine neutrophils. Neutrophils were incubated either alone (CTRL), with *S. aureus* (SA) (1:20), or treated with DPI prior to co-incubation with *S. aureus* (SA+DPI). Caspase-3 activity was assessed hourly by
monitoring the increase in fluorescence at 390nm excitation and 460nm emission following cleavage of the fluorogenic peptide substrate DEVD-AMC. The means and SEM of three to ten experiments are plotted. * p<0.05 vs control and # p<0.05 vs phagocytic neutrophils with a functional NADPH oxidase.

**FIGURE 6.** Phagocytosis triggers PS exposure and uptake by macrophages in murine neutrophils and requires a functional NADPH oxidase. After 4h incubation with *S. aureus* (1:20) cells were stained with annexin V-FITC and PI (A-C). Flow cytometry histograms are of a single representative experiment with mouse wild-type (WT) neutrophils. Unstimulated neutrophils (A), neutrophils with *S. aureus* (filled) (B), and DPI-treated neutrophils with *S. aureus* (filled) (C). Treatment of neutrophils with *S. aureus* in the presence of the caspase inhibitor Z-VAD-FMK is shown by the dotted line overlay (C). The percentage of murine WT and CGD (D) neutrophils exposing PS following incubation either alone (CTRL), with *S. aureus* (SA), or pretreated with DPI (SA+DPI) are shown. For macrophage uptake studies, murine (E) neutrophils were incubated with *S. aureus*, harvested and layered onto monocyte-derived macrophages media removed and the wells fixed and stained for myeloperoxidase with o-dianisidine enabling visualization of neutrophils. The number of neutrophils phagocytosed per 100 macrophages was counted. The means and SEM of three to eight experiments are plotted. * p<0.05 vs control and # p<0.05 vs phagocytic neutrophils with a functional NADPH oxidase.
FIGURE 1.
FIGURE 2

(A) Fold Induction Caspase Activity over time for CTRL, SA 1:20, and SA + DPI conditions.
(B) Fold Induction Caspase Activity over time for CTRL, SA 1:2, and SA + DPI conditions.
(C) Fold Induction Caspase Activity over time for CTRL, SA 1:10, and SA + DPI conditions.
(D) Fold Induction Caspase Activity over time for CTRL, SA 1:50, and SA + DPI conditions.
FIGURE 3.
FIGURE 4C.

Average Fluorescence Intensity of Cytoplasmic Caspase-3 Staining

- DPI
+ DPI

non-phagocytic neutrophils
phagocytic neutrophils

* #
FIGURE 5.
FIGURE 6.