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Subversion of a Lysosomal Pathway Regulating Neutrophil Apoptosis by a Major Bacterial Toxin, Pyocyanin


Neutrophils undergo rapid constitutive apoptosis that is accelerated following bacterial ingestion as part of effective immunity, but is also accelerated by bacterial exotoxins as a mechanism of immune evasion. The paradigm of pathogen-driven neutrophil apoptosis is exemplified by the Pseudomonas aeruginosa toxic metabolite, pyocyanin. We previously showed pyocyanin dramatically accelerates neutrophil apoptosis both in vitro and in vivo, impairs host defenses, and favors bacterial persistence. In this study, we investigated the mechanisms of pyocyanin-induced neutrophil apoptosis. Pyocyanin induced early lysosomal dysfunction, shown by altered lysosomal pH, within 15 min of exposure. Lysosomal disruption was followed by mitochondrial membrane permeabilization, caspase activation, and destabilization of Mcl-1. Pharmacological inhibitors of a lysosomal protease, cathepsin D (CTSD), abrogated pyocyanin-induced apoptosis, and translocation of CTSD to the cytosol followed pyocyanin treatment and lysosomal disruption. A stable analog of cAMP (dibutyryl cAMP) impeded the translocation of CTSD and prevented the destabilization of Mcl-1 by pyocyanin. Thus, pyocyanin activated a coordinated series of events dependent upon lysosomal dysfunction and protease release, the first description of a bacterial toxin using a lysosomal cell death pathway. This may be a pathological pathway of cell death to which neutrophils are particularly susceptible, and could be therapeutically targeted to limit neutrophil death and preserve host responses. The Journal of Immunology, 2008, 180: 3502–3511.
generation and altered redox status (12). It is also unclear why neutrophils are exquisitely sensitive to pyocyanin. We therefore investigated the mechanisms of pyocyanin-induced apoptosis in neutrophils, and describe a novel pathway of pathogen-mediated neutrophil apoptosis, characterized by lysosomal acidification and activation of cathepsin D (CTSD).

Materials and Methods

Neutrophil isolation and culture
Human neutrophils were isolated by dextran sedimentation and plasma Percoll (Sigma-Aldrich) gradient centrifugation from whole blood of normal volunteers (15). The studies were approved by the South Sheffield Research Ethics Committee, and subjects gave written, informed consent. Purity of neutrophil populations (>95%) was assessed by counting >500 cells on duplicate cytopsins. Neutrophils were suspended at 2.5 × 10^6/ml in RPMI 1640 plus 1% penicillin/streptomycin and 10% FCS (all Invitrogen Life Technologies) and cultured in 96-well Flexiwell plates (BD Pharmingen).

Preparation and analysis of pyocyanin
Pyocyanin was prepared by photolysis of phenazine methosulfate (Sigma-Aldrich) and purified and characterized, as previously described (16).

Assessment of viability and apoptosis
Nuclear morphology was assessed on Diff-Quik-stained cytospins, with PE-labeled annexin V (BD Biosciences) and TOPRO-3 iodide (Molecular Probes) to identify apoptotic (annexin V+) and necrotic (TOPRO-3+) cells (17). Samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences). Twenty thousand events were recorded, and data were analyzed by CellQuest software (BD Biosciences).

Caspase activity assay
Caspase-3 activity was determined by measuring enzymatically cleaved fluorescent substrate 7-amino-4-methylcoumarin, N-acetyl-L-aspartyl-L-glutanyl-L-lysyl-L-aspartic acid amide (DEVD-AMC; Bachem), as previously described (18). Neutrophil lysates were prepared by resuspension of treated cells in lysis buffer (100 mM HEPES (pH 7.5), 10% v/v sucrose, 0.1% CHAPS, and 5 mM DTT) at a concentration of 1 × 10^9/ml. Lysates were frozen at −80°C until required. Using the FLUSYS software package for the PerkinElmer LS-50B fluorometer, lysate equivalents of 5 million neutrophils were coincubated with 20 μM M Ac-DEVD-AMC in DMSO and 50 μM pyocyanin (Sigma-Aldrich) and cultured in 96-well Flexiwell plates (BD Pharmingen). Kinetic data were collected for at least 20 min to ensure stability of activity. A known amount of free AMC was used to calibrate the system and allowed calculation of caspase-3 activity. In separate experiments, executioner caspase-3 and -7 activity was measured using a Caspase-Glo 3/7 Assay (Promega). Neutrophils were cultured at 5 × 10^6/ml and treated with medium (control), pyocyanin (50 μM), and pyocyanin with dibutyryl cAMP (dbcAMP; 100 μM) for 3 h. Cells were directly transferred to a white 96-well flat-bottom plate (Dynex Technologies) at a density of 62,500 cells/well in a 25-μl vol. An equivalent volume of Caspase-Glo 3/7 buffer mixed with substrate reagent was added to each well. The plate was read using a Lumistar Galaxy Luminometer (BMG Labtechnologies) at 25°C for 200 cycles.

ATP and glucose measurements
ATP was measured using a commercially available bioluminescent kit (Sigma-Aldrich) using a Lumistar Galaxy Luminometer. Glucose was assayed by detecting change in glucose concentration in lysates and culture supernatants using a commercial kit (Sigma-Aldrich), as previously described (19). Neutrophils were cultured in RPMI 1640 alone, with a glucose concentration of 2 mg/ml. Both assays were standardized using known concentrations of ATP and glucose, respectively (data not shown).

Modulation of pyocyanin-induced apoptosis
Neutrophils were incubated in the presence and absence of pyocyanin following preincubation with candidate modulators of pyocyanin-induced apoptosis. Except where indicated, a concentration of 50 μM pyocyanin was used because it significantly accelerates neutrophil apoptosis—5-fold at 5 h (12). The pan-caspase inhibitor, N-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fmk (Boc-D-fmk) (50 μM) was obtained from Calbiochem. McrOSic-Ala-Ala-Pro-Ala-chloromethylketone (Bachem) (10 μM) and elastatin (Sigma-Aldrich) (10 μM) were used as neutrophil elastase inhibitors, with optimal inhibitory concentrations determined by assessment of inhibition of fluorescent substrate digestion by purified neutrophil elastase (data not shown). BB94 (1 μM; gift from British Biotechnology, Oxford) was used as a pan matrix metalloproteinase inhibitor (20). Specific caspase inhibitors were CA-074Me (25 μM) for caspase B (Calbiochem), pepstatin A (10 μM; Sigma-Aldrich) and diazooacetyl-2-aminohecanoc acid-methyl ester (DAME; Bachem) for CTSD, and Ck-08 (1 μM; Enzyme Systems Products) for caspase G (21). Baflofinycin A1 (100 nM) (Sigma-Aldrich) inhibits the membrane vascular ATPase (22).

ROI production
ROI production was assessed by incubating 5 × 10^5 neutrophils in 200 μl of RPMI 1640 with 5 μM ditydrodorohamine (DHR; Sigma-Aldrich) for 30 min at 37°C, and measuring fluorescence in the FL-1 channel by flow cytometry.

Assessment of mitochondrial and lysosomal membrane permeability
To detect loss of Δψm, neutrophils were incubated with 10 μg/ml JC-1 (5,5′,6,6′-tetrachloro-1,1′,3′,3′-tetraethyl-benzimidazolyl-carbocyanine iodide; Molecular Probes) at 37°C. Loss of Δψm was assayed by observing a shift in fluorescence emission from red (590 nm) to green (530 nm) using flow cytometry (24). Neutrophils were treated with valinomycin (100 μM; Sigma-Aldrich) as a positive control. Lysosomal pH was measured by incubating neutrophils with 1 mg/ml FITC-dextran 70S (Sigma-Aldrich), a pH-sensitive fluorescent probe, for 30 min at 37°C. Increasing pH (i.e., loss of acidification) within the lysosomal compartment is associated with increased green fluorescence detected in the FL-1 channel (25). Loss of lysosomal acidification was determined by incubating neutrophils with 5 μM acidine orange (Sigma-Aldrich) for 30 min at 37°C, and loss of FL-3 fluorescence was measured by flow cytometry (21). Cytosins were also prepared and viewed by fluorescence microscopy. Neutrophils treated with 100 nM baflofinycin A1, a known inhibitor of vacuolar ATPase (a key regulator of lysosomal pH Ran, 2003 no. 965), were used as a positive control in these experiments.

CTSD translocation
Boron dipyrromethane difluoride (BODIPY FL)-pepstatin A (Molecular Probes) is a fluorescent pH-sensitive probe used to measure the subcellular distribution of CTSD (26). Neutrophils were treated with medium (control), baflofinycin A1 (100 nM), or pyocyanin (50 μM) for 30 min. The cells were washed and incubated with 1 μM BODIPY FL-pepstatin A (in medium) at 37°C for 30 min, after which they were washed, resuspended in medium, and incubated at 37°C for a further 60 min to allow endosomal trafficking. Cytosins were prepared and viewed under a fluorescence microscope (Leica AF6000, ×63 objective). CTSD-labeled BODIPY FL-pepstatin A was visible as punctate fluorescent inclusions.

SDS-PAGE and Western immunoblotting
Whole-cell extracts were used for Mc-I and actin immunoblots and prepared as described (27). Cytosolic and membrane fractions used in CTSD and cathepsin G (CTSG) immunoblots were prepared by sonication (three 10-s bursts in HBSS supplemented with protease inhibitor mixture III (Calbiochem), followed by 25,000 rpm microcentrifugation for 45 min). Proteins were separated by 15% v/v SDS-PAGE and blotted onto nitrocellulose membrane (Bio-Rad), and protein transfer was confirmed by Ponceau S (BDH) staining. Blots were incubated overnight at 4°C for Mc-I (S-19; Santa Cruz Biotechnology) and at room temperature for 2 h for actin (Sigma-Aldrich), CTSG (Abcam), and CTSD (Calbiochem); protein detection was
with HRP-conjugated IgG (DakoCytomation) and ECL (Amersham Biosciences).

Statistics

For multiple comparisons, means and SEM were analyzed by ANOVA with posttest, as indicated (GraphPad). For comparison of two sample means, Student’s t tests were used.

Results

Pyocyanin-induced neutrophil apoptosis is caspase dependent

Pyocyanin-induced cell death in neutrophils displays both morphological features (nuclear condensation, cell shrinkage) and cell surface changes (annexin V binding to exposed phosphatidylserine) of apoptosis (12). We therefore investigated whether pyocyanin-induced neutrophil death was also associated with caspase activation, using both morphology and annexin V binding to quantify neutrophil apoptosis. A pan-caspase inhibitor, zVAD.fmk, inhibited pyocyanin-induced apoptosis in a concentration-dependent manner, with significant reduction in neutrophil death at concentrations from 5 μM (Fig. 1A). A total of 50 μM zVAD.fmk delayed pyocyanin-induced death up to 10 h (Fig. 1B); thereafter, secondary necrosis in pyocyanin-treated cells made estimations of apoptosis unreliable. Apoptosis of control neutrophils at 5 h was 5.7 ± 1.2% and was not significantly inhibited by zVAD.fmk at any concentration used (data not shown). A second pan-caspase inhibitor, Boc-D.fmk, also inhibited pyocyanin-induced apoptosis at 5 h (pyocyanin-induced apoptosis in the absence (34.8 ± 3.8%) and presence (21.9 ± 5.5%) of 50 μM Boc-D.fmk). Because zVAD.fmk has some caspase-independent effects in neutrophils (28), we confirmed caspase activation by demonstrating cleavage of a caspase-3-specific fluorescent substrate, DEVD-AMC. Pyocyanin treatment (4 h) caused a significant increase in neutrophil intracellular caspase-3 activity compared with untreated controls (Fig. 1C). Thus, pyocyanin-induced neutrophil apoptosis is delayed by caspase inhibition and associated with caspase-3 activation.

Pyocyanin-induced oxidative stress mediates neutrophil apoptosis

The cytotoxic effects of pyocyanin on bacteria and eukaryotic cells are linked to its ability to undergo nonenzymatic redox cycling within cells, with resultant ROI formation, and further loss of reducing capacity by direct oxidation of NADH/NADPH and reduced glutathione (29). We found that pyocyanin induces prolonged generation of ROI in neutrophils as measured by oxidation of DHR (Fig. 2A), in keeping with our previous observations (12). To further corroborate the role of oxidative stress in pyocyanin-induced apoptosis, neutrophils were incubated in a hypoxic environment in the presence of pyocyanin for 5 h. We hypothesized that reduced availability of oxygen would hinder the ability of pyocyanin to induce apoptosis. Fig. 2B shows pyocyanin is unable to induce neutrophil apoptosis in hypoxia.

Metabolic activity is maintained in pyocyanin-treated neutrophils

Pyocyanin-induced ROI production is linked to depletion of intracellular ATP in epithelial cells (29, 30). To determine whether neutrophil death was associated with cellular ATP depletion in response to the profound ROI induction, we measured intracellular ATP in the neutrophil by a bioluminescence technique. We did not detect a reduction in ATP levels at time points up to 3 h following pyocyanin treatment (Fig. 3A). Metabolic pathways in pyocyanin-treated neutrophils remained viable, as shown by this maintenance of intracellular ATP, and by our subsequent experiments, which showed increased glucose uptake (Fig. 3B) and maintenance of intracellular glucose concentrations in these cells (Fig. 3C).

ROI are a feature of the stress pathway of apoptosis, and ROI production leads to loss of mitochondrial membrane potential (ΔΨm) (14). Because pyocyanin can interrupt mitochondrial respiration in epithelial cells as a result of ROI generation (29), we determined whether pyocyanin-mediated ROI production was inducing neutrophil apoptosis via loss of ΔΨm. We measured ΔΨm in neutrophils using the mitochondrial dye, JC-1. Fig. 4A shows flow cytometry dot plots illustrating the distribution of high-FL-1 fluorescent neutrophils (associated with a loss of ΔΨm (24, 31)) in control, pyocyanin-, and valinomycin-treated populations at 4 h.
Pyocyanin induced only modest changes in the proportions of cells showing loss of $\Delta \psi_{\text{m}}$ and these nonsignificant changes were only observed at later time points (Fig. 4B). These observations were confirmed using a second mitochondrial dye, 3,3-dihexyloxacarbocyanine iodide (data not shown). Thus, although loss of $\Delta \psi_{\text{m}}$ appears to occur in pyocyanin-induced neutrophil apoptosis, changes are not apparent until later time points, when there is already a significant increase in apoptosis. Loss of $\Delta \psi_{\text{m}}$ was therefore unlikely to be an initiating factor in the engagement of apoptosis.

Pyocyanin-induced apoptosis is preceded by changes in lysosomal pH, but these are not sufficient to induce death

A pathway of cell death is now recognized, activated primarily in pathological rather than homeostatic circumstances, in which lysosomal dysfunction may precede loss of $\Delta \psi_{\text{m}}$ (32). Lysosomal pH was measured using a pH-sensitive marker (FITC-conjugated dextran) that is taken up by acidic structures and increases in FL-1 channel fluorescence as pH rises due to loss of protonation (25, 33). Exposure of neutrophils to pyocyanin or bafilomycin A1 (an inhibitor of the vacuolar (H$^+$)-ATPase that maintains normal lysosomal pH gradients (34)) increased lysosomal pH compared with untreated controls (Fig. 5).

We confirmed loss of lysosomal acidification in neutrophils by staining with acridine orange, which is lysosomotropic and accumulates in acidic organelles (35). On fluorescence microscopy (Fig. 6A), a punctate staining pattern was seen in the cytosol of control neutrophils, consistent with lysosomal accumulation of the stain. In neutrophils treated with pyocyanin or bafilomycin A1, the punctate staining pattern was lost, in keeping with loss of lysosomal acidification (21, 32). Statistically significant losses of fluorescence were detected at 15 min following pyocyanin treatment (Fig. 6B). Once again, bafilomycin A1 was used as a positive control in these experiments and, at a concentration (100 nM) that inhibits V-ATPase function in neutrophils (36), caused loss of lysosomal acidification to an even greater degree than pyocyanin. However, this concentration of bafilomycin A1 was without effect on constitutive neutrophil apoptosis (Fig. 6C), in keeping with previous studies (37), although higher concentrations of bafilomycin are proapoptotic to neutrophils (data not shown). Lysosomal alkalization alone is not, therefore, sufficient to induce neutrophil apoptosis.

CTSD translocation and activation are associated with pyocyanin-induced apoptosis

Alterations in lysosomal pH alone do not induce apoptosis, and release of lysosomal proteases is critical for completion of the apoptotic program in a range of cell types (21, 35). No attenuation of pyocyanin-induced neutrophil apoptosis was seen by treatment with elastase inhibitors or a pan matrix metalloproteinase inhibitor (Fig. 7, A and B). In contrast, significant reductions in pyocyanin-induced apoptosis were observed with pepstatin A, an inhibitor of aspartyl proteases (21), both alone and in combination with inhibitors of the cathepsins B, G, and L (Fig. 7C). The latter inhibitors
were without effect, either alone or in combinations that excluded pepstatin A, on constitutive neutrophil apoptosis (data not shown) or on pyocyanin-induced apoptosis (Fig. 7C). Although pepstatin A is a specific inhibitor of CTSD, it has been reported to cause neutrophil activation similar to changes induced by FMLP treatment (38), and we found it also had an antiapoptotic effect upon neutrophils in the absence of pyocyanin (data not shown). We therefore investigated the potential of a second and unrelated CTSD inhibitor, DAME (39), to modulate pyocyanin-induced neutrophil cell death. DAME treatment significantly abrogated pyocyanin-induced neutrophil apoptosis (Fig. 7D) at concentrations that were without effect upon constitutive neutrophil death. CTSD is bound within the matrix of neutrophil azurophilic granules, but on activation is released to the cytosol (40).

BODIPY FL-pepstatin A is a pH-dependent fluorescent probe that binds to CTSD within the acidic lysosomal compartment (26). We demonstrated the expected pattern of CTSD localization with punctate cytosolic inclusions in control neutrophils (Fig. 8A). Neutrophils treated for 30 min with pyocyanin or bafilomycin A1 lost this typical granular staining pattern. To determine whether the decrease in lysosomal BODIPY FL-pepstatin A staining reflected alteration in lysosomal pH alone or lysosomal membrane permeability, we assessed translocation of CTSD by Western immunoblotting. Fig. 8B shows CTSD distribution in neutrophil lysates.
separated into membrane (upper panel) and cytosolic (lower panel) fractions. The predicted 44- and 31-kDa forms (41) are present within the membrane fraction of both control and bafilomycin A1-treated cells, whereas pyocyanin-treated cells show translocation of the 31-kDa form into the cytosol. We also studied translocation of another cathepsin, CTSG, and demonstrated that treatment with pyocyanin, but not bafilomycin, caused translocation to the cytosolic fraction (Fig. 8C), although as shown in Fig. 7C, CTSG inhibition did not abrogate pyocyanin-mediated apoptosis.

A stable cAMP analog retards pyocyanin-mediated neutrophil apoptosis by regulating CTSD translocation, caspase activity, and Mcl-1 expression

We previously reported that the synthetic cAMP analog dbcAMP is able to significantly abrogate pyocyanin-induced apoptosis of neutrophils (12). Having identified pathways of pyocyanin-induced apoptosis, we asked how dbcAMP might inhibit pyocyanin-induced death. dbcAMP was unable to inhibit pyocyanin-induced ROI generation or loss of lysosomal acidification (Fig. 9, A and B). However, translocation of CTSD to the cytosol in pyocyanin-treated cells was reduced in cells also treated with dbcAMP (Fig. 9C). Pyocyanin-induced caspase activity was also prevented by dbcAMP (Fig. 9D). Another potentially important effect of dbcAMP was also studied. The antiapoptotic bcl-2 family member, Mcl-1, plays a critical role in the regulation of neutrophil apoptosis (42), and cAMP analogues have been shown to stabilize Mcl-1 protein.
protein levels in neutrophils (43). We showed pyocyanin treatment of neutrophils reduced Mcl-1 protein levels, as did cycloheximide and sodium salicylate treatment, as previously described (44), but this effect of pyocyanin was reversed by coincubation with db-cAMP (Fig. 9E). These data suggest db-cAMP modulates pyocyanin-induced neutrophil apoptosis via multiple downstream mechanisms that include reduced CTSD translocation, caspase activation, and stabilization of Mcl-1.

Discussion

In these studies, we describe a novel mechanism of pathogen-induced subversion of neutrophil apoptosis that is critically dependent upon disruption of intracellular organelles and protease activity. This pathway is highly analogous to the recently described lysosomal death pathway, used in the regulation of cell survival in a range of pathological processes (reviewed by Guicciardi et al. (32)), providing additional insights into the pathways capable of regulating neutrophil survival.

Pyocyanin is a low m.w., bluish pigment secreted by *P. aeruginosa* that determines the characteristic color of infected pus and sputum. It is a major factor responsible for oxidant-dependent killing of *C. elegans* by *P. aeruginosa* through its ability to undergo redox cycling and to cause superoxide generation (45, 46), and production of pyocyanin is an important determinant of severity in murine models of sepsis (9). The important potential for pyocyanin to be an agent of immune subversion by prevention of neutrophil-mediated bacterial clearance has only recently been realized. To this end, we have shown pyocyanin both accelerates neutrophil apoptosis in vitro (12) and in vivo (13) and impairs clearance of *P. aeruginosa* from the lung (13).

In this work, we show that pyocyanin-induced neutrophil death is caspase dependent and associated with increased executioner caspase activity, which is likely to be attributable to caspase-3.

![FIGURE 9. A stable cAMP analog retards pyocyanin-mediated apoptosis. A, Neutrophils were preloaded with DHR and incubated with pyocyanin in the presence (●) or absence (□) of db-cAMP (100 μM) for 3 h. Increases in fluorescence were measured by flow cytometry (FL-1) and indicated enhanced ROI production. Chart shows mean ± SEM MFI from four independent experiments. Pyocyanin-induced ROI production was unaffected by db-cAMP (ANOVA, Bonferroni’s posttest). B, Neutrophils were treated with medium (control), bafilomycin A1 (100 nM), or pyocyanin (50 μM) alone or in combination with db-cAMP (100 μM) for 30 min. Loss of lysosomal acidification was assessed with acridine orange, and fluorescence was measured by flow cytometry. Decreases in FL-3 reflect loss of acridine orange due to lysosomal disruption. The chart shows mean ± SEM MFI from three independent experiments (**, p < 0.01; ***, p < 0.001; ANOVA, Bonferroni’s posttest). Pyocyanin-induced loss of lysosomal acidification was not prevented by db-cAMP. C, Membrane (upper panel) and cytosolic (lower panel) fractions were prepared from 4 × 10⁶ neutrophils treated for 30 min with medium (control), bafilomycin A1 (100 nM), or pyocyanin (50 μM) alone or in combination with db-cAMP (100 μM). SDS-PAGE immunoblots were probed for CTSD. D, Neutrophils were incubated for 3 h with medium (control) or pyocyanin (50 μM) alone or in combination with db-cAMP (100 μM). Executioner caspase (3 and 7) activity was measured using a Caspase-Glo 3/7 assay. Chart shows fold change from control. Caspase activity induced by pyocyanin is significantly greater than control and is inhibited by db-cAMP (***, p < 0.001; ANOVA, Bonferroni’s posttest). E, Whole cell protein lysates were prepared from neutrophils treated for 2 h with pyocyanin (50 μM), cycloheximide (CHX, 10 μg/ml) plus sodium salicylate (sal, 10 μM) or pyocyanin plus db-cAMP (100 μM) and subjected to SDS-PAGE immunoblotting. Blots were probed for Mcl-1 (upper panel) and actin (loading control, lower panel). db-cAMP prevented pyocyanin-induced degradation of Mcl-1.](http://www.jimmunol.org/)

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since it is the major executioner caspase in human neutrophils (47, 48). These data provided biochemical confirmation that the cell death induced was apoptotic and represented a subversion of important normal regulatory pathways controlling neutrophil lifespan. We then sought the apoptotic pathways upstream of caspase activation to determine the mechanism of pyocyanin-induced neutrophil killing. The dependence upon ROI for pyocyanin-induced death was implied by effects of antioxidants in our previous studies (12), and in this study we further show the proapoptotic effects of pyocyanin were prevented by culture in hypoxia. Recent studies in epithelial cells found pyocyanin, in addition to causing intracellular ROI generation, can directly oxidize both NADH and NAPDH (49). This loss of cellular reducing capacity is associated with impaired glycolysis (50) and reduced levels of cyclic nucleotides, particularly ATP (29, 51). Although detailed experiments studying the energetic status of the cell were not performed, intracellular levels of ATP and glucose were maintained following pyocyanin treatment, data that are in keeping with the requirement of neutrophils to maintain function in very demanding environments of low pH, low glucose, low oxygen tension, and high oxidative stress such as in purulent secretions (52). Indeed, preservation of ATP is necessary for coordinated execution of apoptotic programs, and ATP depletion favors necrotic cell death rather than classical apoptosis (53), providing further support for our data showing no significant loss of intracellular ATP. Neutrophils, again because of the environments in which they must be active, are unique in that the majority of ATP generation in these cells occurs via oxygen-independent glycolysis (54), and we found intracellular glucose levels were maintained in pyocyanin-treated neutrophils. Glucose uptake from the extracellular medium was measured and, for untreated neutrophils, was comparable to previous studies using the same methodology (19), with an increased uptake following pyocyanin treatment that was comparable to that of LPS- or PMA-activated neutrophils (55, 56).

We then sought evidence for mitochondrial inner transmembrane permeabilization, which is characteristic of the stress pathway of apoptosis characterized by ROI generation (14). Although mitochondria have a minimal role in ATP generation in neutrophils, they do have a critical role in apoptosis induction (24, 57). Mitochondria have a minimal role in ATP generation in neutrophils (55, 56). Although detailed experiments studying ATP depletion favors necrotic cell death rather than classical apoptosis (53), providing further support for our data showing no significant loss of intracellular ATP. Neutrophils, again because of the environments in which they must be active, are unique in that the majority of ATP generation in these cells occurs via oxygen-independent glycolysis (54), and we found intracellular glucose levels were maintained in pyocyanin-treated neutrophils. Glucose uptake from the extracellular medium was measured and, for untreated neutrophils, was comparable to previous studies using the same methodology (19), with an increased uptake following pyocyanin treatment that was comparable to that of LPS- or PMA-activated neutrophils (55, 56).

A number of studies have shown oxidative stress-induced cell death is associated with lysosomal destabilization (32) and that ROI can induce lysosomal permeabilization (35, 59). Within 15 min of pyocyanin treatment of neutrophils, there was evidence of alkalization of the lysosomal compartment that preceded any detectable changes in \( \Delta \phi_m \) or caspase-3 activity. A lysosomal pathway of apoptosis that precedes mitochondrial changes is recognized in other cell types, with critical proteases translocating from lysosomes and other secretory vesicles into the cytoplasm (21, 60). This pathway is activated primarily in pathological rather than homeostatic circumstances (32) and can be activated by death receptors or lipid mediators (61) and following accumulation of lysosomal agents (21). The azurophilic or primary granules are generally regarded as the lysosomal structures within neutrophils, because they are the major cellular reservoir of acid-dependent hydrolases, contain lysosomal membrane proteins, and are abnor-

Recent studies by Ran et al. (22) provided important insights into the actions of pyocyanin. Yeast mutants with reduced sensitivity to pyocyanin frequently had mutations in the V-ATPase, an enzyme complex involved in mitochondrial electron transport and ATP synthesis, but also a major regulator of lysosomal pH (65). Ran et al. (22) found pyocyanin both induced lysosomal membrane permeabilization and inhibited V-ATPase function in epithelial cells, with high concentrations (2 mM) of a V-ATPase inhibitor, bafilomycin A1, having similar effects to pyocyanin. In neutrophils, bafilomycin A1, at a concentration (100 nM) that inhibits neutrophil V-ATPase function (36), reduced lysosomal acidification to an even greater degree than pyocyanin. This concentration of bafilomycin A1 was, however, without effect on neutrophil apoptosis, in keeping with previous studies (37). Two other global regulators of intracellular pH, amiloride (an inhibitor of Na\(^+\)/H\(^+\) exchange) and zinc chloride (an inhibitor of NAPDH-oxidase-associated proton channels), were also without effect on pyocyanin-induced apoptosis (data not shown). Our findings support those of Ran et al. (22) in demonstrating pyocyanin-induced loss of lysosomal acidification in neutrophils that is most likely mediated via the lysosomal V-ATPase, but also show lysosomal alkalization alone is not sufficient to induce apoptosis. It is not clear whether the effect of pyocyanin upon V-ATPase function is indirect, perhaps resulting from ROI generation, or whether pyocyanin is lysosomotropic and binds and directly inhibits V-ATPase function, as has been described for other agents, e.g., quinolones (21) and concanamycin (66).

Lysosomes contain multiple potent proteases that contribute to bacterial killing (67), several of which have been associated with onset of apoptosis (32). Using a series of broad and narrow-spectrum protease inhibitors, we identified a role for CTSD in pyocyanin-induced apoptosis. We found that a specific CTSD inhibitor, pepstatin A, delayed pyocyanin-induced death, but also caused activation of neutrophils (38) (our data not shown). We therefore used a second specific CTSD inhibitor (DAME) that also delayed pyocyanin-induced neutrophil apoptosis. We identified CTSD staining in neutrophils, with the expected distribution in subcellular organelles, and we detected translocation of a 31-kDa fragment of CTSD from the membrane to the cytosolic fraction of neutrophils following pyocyanin treatment. These data are in keeping with data showing a role for CTSD in apoptosis of fibroblasts (59, 60) and endothelial cells (69) following oxidant stress. Importantly, neutrophil primary granules contain significant amounts of CTSD (40, 70), accounting for 38% of acidic protease activity of neutrophils (71). Although CTSG was also released into the cytosol by pyocyanin treatment, CTSG inhibition did not abrogate pyocyanin-induced apoptosis, suggesting a particular role for CTSD in this system. Our work showing that pyocyanin destabilizes neutrophil granules and releases CTSD into the cytosol may in part explain the exquisite susceptibility of neutrophils to pyocyanin-induced apoptosis (12), because cells with lower or absent numbers of CTSD-containing granules (such as epithelial cells) are not stimulated to die when exposed to pyocyanin. The mechanisms by which CTSD induces apoptosis are uncertain. CTSD release is upstream of caspase activation (72), although a recent overexpression study suggests that the catalytic activity of CTSD is not essential for its proapoptotic role (73). The recent studies of Blomgran et al. (74)
show a role for cathepsins, including CTSG, in mediating Esche-
richia coli-induced neutrophil apoptosis, with evidence of cathe-
psin-mediated Bid cleavage and down-regulation of Mcl-1. Our
studies also demonstrate reductions in Mcl-1 protein, and that res-
toration of Mcl-1 protein levels by dbcAMP is associated with
delay of pyocyanin-induced apoptosis.
A number of pathogens such as E. coli (75), Staphylococcus
aureus (76), and Streptococcus pyogenes (77) are associated with
neutrophil apoptosis following phagocytosis; this is likely to be a
host-mediated process to prevent intracellular persistence of bac-
teria (78). However, our studies and those of Blomgran et al. (74)
identify a relationship between lysosome function and apoptosis in
bacterial infection has not previously been recognized, despite the
prominent role of lysosomal proteases in antibacterial responses of
neutrophils. In these studies, pathogens effectively subvert these
processes, with release of granule proteases triggered by bacterial-
driven ROI generation.

In summary, we have demonstrated pyocyanin induces apopto-
sis by engagement of lysosomal pathways of cell death, and we
provide evidence it may be a pathological mechanism of cell death
to which neutrophils are particularly susceptible. Furthermore, this
is the first description of a bacterial toxin using this pathway of
mammalian cell apoptosis to subvert host defense. Our findings are
clinical relevance, because understanding mechanisms to inhibit
pyocyanin-induced neutrophil death in P. aeruginosa infections
may lead to development of therapies that favor an effective im-
une response to this major human pathogen.

Disclosures
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relating to a multi-centre asthma genetics study. There is no overlap with
this study in any way.

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