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Impairment of Apoptotic Cell Engulfment by Pyocyanin, A Toxic Metabolite of *Pseudomonas Aeruginosa*

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Abstract

Rationale—Cystic fibrosis lung disease is characterized by accumulation of apoptotic neutrophils, indicating impaired clearance of dying cells. *Pseudomonas aeruginosa*, the principal microbial pathogen in cystic fibrosis, manipulates apoptosis induction via production of toxic metabolites. Whether these metabolites, particularly pyocyanin, can also modulate apoptotic cell engulfment is unknown.

Objectives—To assess the effects of pyocyanin on apoptotic cell engulfment by macrophages *in vitro* and *in vivo* and to investigate potential mechanisms of the observed effects.

Methods—Human-monocyte-derived macrophages were treated with pyocyanin prior to challenge with apoptotic neutrophils, apoptotic Jurkat cells or latex beads and phagocytosis was assessed by light microscopy and flow cytometry. Effects of pyocyanin production on apoptotic cell clearance *in vivo* were assessed in a murine model, comparing infection by wild-type or pyocyanin-deficient *P. aeruginosa*. Oxidant production was investigated using fluorescent probes and pharmacological inhibition and Rho GTPase signaling by immunoblotting and inhibitor studies.

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Results—Pyocyanin treatment impaired macrophage engulfment of apoptotic cells *in vitro*, without inducing significant macrophage apoptosis, whilst latex bead uptake was preserved. Macrophage ingestion of apoptotic cells was reduced and late apoptotic/necrotic cells were increased in mice infected with pyocyanin-producing *P. aeruginosa* compared with the pyocyanin-deficient strain. Inhibition of apoptotic cell uptake involved intracellular generation of ROI and effects upon Rho GTPase signaling. Anti-oxidants or blockade of Rho signaling substantially restored apoptotic cell engulfment.

Conclusions—These studies demonstrate *P. aeruginosa* can manipulate the inflammatory micro-environment through inhibition of apoptotic cell engulfment, and suggest potential strategies to limit pulmonary inflammation in cystic fibrosis.

Keywords

Macrophages; phagocytosis; apoptosis; inflammation; cystic fibrosis

Introduction

Resolution of inflammation involves apoptosis of recruited inflammatory cells and their recognition and clearance by professional phagocytes (1). These mechanisms can clear substantial inflammatory infiltrates without significant inflammatory cell necrosis or bystander tissue injury (2), but dysregulation of these efficient clearance systems is now widely described in inflammatory disease (3). The pathology of cystic fibrosis lung disease is characterized by a massive chronic neutrophilic inflammation of the airways, with the infiltrate containing excessive numbers of both apoptotic and necrotic neutrophils on a scale not seen in other inflammatory lung diseases (4). These findings could reflect increased neutrophil apoptosis, or impairment of apoptotic cell clearance, or a combination of these processes. This accumulation of effete neutrophils has important functional consequences, particularly the liberation of granule proteases (4, 5), and there is evidence in cystic fibrosis that neutrophil elastase can impair apoptotic cell clearance (4).

Pseudomonas aeruginosa is an opportunistic pathogen that causes a range of infections in the immunocompromised host and is the principal cause of mortality in cystic fibrosis lung disease (6). *P. aeruginosa* produces a range of factors that modify host immune responses and contribute to its pathogenicity (7). We have begun to dissect the impact of *P. aeruginosa* on both induction of apoptosis and clearance of apoptotic cells. In host-pathogen interactions, pathogen-driven neutrophil apoptosis is a well-recognised mechanism of immune evasion employed by a number of bacteria (8) and we and others have shown *P. aeruginosa* can induce neutrophil apoptosis (9, 10). There are, however, no reports of a microbial factor modulating the engulfment of apoptotic cells by professional phagocytes such as macrophages. *P. aeruginosa* produces highly diffusible, pigmented toxic secondary metabolites, known as phenazines, that play a major role in killing infected organisms such as *C. elegans* and mice (11) and, in pneumonia models, cause extensive tissue damage (12). We have shown that pyocyanin, the principal phenazine generated by *P. aeruginosa*, induces rapid apoptosis of neutrophils (9, 13). Excess apoptotic neutrophils are detected in mice infected with a pyocyanin-producing, wild-type *P. aeruginosa* as compared to

pyocyanin-deficient strains (13), confirming acceleration of apoptosis but also raising the possibility that pyocyanin might alter clearance of apoptotic neutrophils.

We therefore examined whether pyocyanin modulated uptake of apoptotic neutrophils by macrophages. We show significant reductions in apoptotic cell engulfment *in vitro* that are confirmed in a murine pneumonia model *in vivo* and are not due to reduced macrophage viability. We further show that impairment of apoptotic cell engulfment is dependent upon ROS generation by macrophages and modulation of GTPase activity. To our knowledge this is the first description of a microbial factor modulating apoptotic cell engulfment and identifies a potentially important mechanism of host tissue damage in infection.

Methods

Materials

Pyocyanin was prepared by photolysis of phenazine methosulphate (Sigma, Poole, UK) and purified and characterized as described (14). Dihydroethidium (DHE), dihydrorhodamine (DHR), carboxyl-modified green-fluorescent latex beads were from Sigma. Hoechst 33342, MnTBAP and the Rho-kinase inhibitor (Y-27632) were from Calbiochem (CA, USA), C3 transferase (C3T) protein from Cytoskeleton (CO, USA) and Rho/Rac activity assay from Upstate (VA, USA). All media, antibiotics and sera were from Life Technologies (Glasgow, UK). The TUNEL ApopTag® Plus Peroxidase In Situ Apoptosis Detection Kit used for *in vivo* studies was from the Intergen Company (Oxford, UK), the TUNEL ApopTag® Fluorescein Direct *In Situ* apoptosis detection kit used for assessment of HMDM apoptosis from Chemicon (Hampshire, UK) the Caspase-Glo® 3/7 Assay from Promega (Southampton, UK), Annexin V from BD PharMingen (Oxford, UK) and To-Pro3 from Invitrogen (Paisley, UK).

Cell isolation and culture

Human peripheral blood cells, neutrophils and PBMCs, were isolated from whole blood of healthy volunteers by dextran sedimentation and plasma-Percoll gradient centrifguation (9). Ethical approval was obtained from the South Sheffield Research Ethics Committee (Sheffield, UK) and all subjects gave informed consent. Resulting neutrophil populations were >97% pure, with the majority of contaminating cells being eosinophils. PBMCs were plated and matured to monocyte-derived macrophages (HMDM) as previously described (1) and studied at day 6-8 in culture. Jurkat cells were obtained from ATCC and grown in RPMI with 10% FCS.

Induction and assessment of apoptosis

PMN (>95% purity) were cultured at 2.5×10^6/ml in RPMI with 1% penicillin/streptomycin and 10% FCS in 96-well "Flexiwell" plates (BD PharMingen, Oxford, UK) for 24 h. At the 24 h time point, cells were washed and shown to be typically 60-70% apoptotic by assessment of nuclear condensation on Giemsa-stained cytospins and necrosis was <2% by trypan blue exclusion. Jurkat cells were exposed to UV irradiation at 254 nm for 10 min, with similar levels of apoptosis. HMDM numbers and apoptosis were assessed by identification of characteristic nuclear morphology of apoptosis in Hoechst 33342 stained

cells on fluorescence microscopy (15). In further experiments, HMDM grown on coverslips were assessed for apoptosis by TUNEL, using an ApopTag® Fluorescein Direct *In Situ* apoptosis detection kit following the manufacturer's recommended protocol. Coverslips were mounted on slides using VectaShield mounting media (Vector Labs) containing DAPI (nuclear counter-stain). Caspase 3/7 activity of HMDMs was measured using a Caspase-Glo® 3/7 Assay, following the manufacturer's recommended protocol. Briefly, 24 hrs post treatment, MDM were incubated with Caspase-Glo reagent for 1 h in the dark, and the luminescence was measured with a Lumistar Galaxy luminometer (BMG Labtechnologies Ltd).

Phagocytosis assays

HMDM were co-cultured for 1 hour with "aged" neutrophils (range 60-70% apoptotic) suspended in 500 μl of Iscove's medium (without serum) in 24 well plates. Uningested cells were removed by washing with HBSS then plates were fixed and stained for myeloperoxidase (1). Phagocytosis was determined by visual counting of 500 HMDM in duplicate wells and was scored both as % of HMDM ingesting and as phagocytic index, generated by multiplying the percentage of macrophages that had phagocytosed cells by the average number of apoptotic cells ingested per macrophage (16). Uptake of fluorescent Cell Tracker-(Invitrogen, Paisley, UK) loaded apoptotic neutrophils or Jurkat cells was measured by flow cytometry (FACSCalibur) using CellQuest software (Becton Dickinson, CA, USA), as previously described (17). Uptake of mixed serum-opsonised carboxyl-modified green-fluorescent latex beads was scored by fluorescence microscopy and we confirmed there were no differences in ingested rather than adherent cells by confocal microscopy (data not shown). In experiments using pharmacological inhibitors, HMDM were treated with the inhibitor prior to the addition of pyocyanin for the following times; MnTBAP (1-100 μM): 30 mins, Y-27632 (10 μM): 30 mins C3T (1 μg/ml): 24 h. The inhibitors also remained present throughout the pyocyanin pre-treatment period.

Murine model of P. aeruginosa infection

This model of acute *P. aeruginosa* infection has been previously described (13). Briefly, C57BL6 mice (8-12 weeks) were instilled with 1×10^7 CFU of bacteria via the trachea, either a pyocyanin-producing wild-type strain, PA14, or a pyocyanin-deficient but otherwise genetically identical strain, *ΔphnAB* (11). At the time points indicated mice were killed by overdose and bronchoalveolar lavage (BAL) performed to obtain total and differential cell counts, including the proportion of neutrophils that were apoptotic (18). Macrophage ingestion of apoptotic cells was identified by morphologic criteria (16) and by TUNEL staining of BAL cytospins. Apoptotic and necrotic macrophages and neutrophils in BAL were identified by flow cytometry using Annexin V and ToPro-3 staining respectively (19).

Assessment of ROS Production

Production of ROS by HMDM was assessed using cell-permeable DHR staining (5μM for 30 min) and cells were analyzed by flow cytometry (20), with each sample run in triplicate. HMDM were also stained with the oxidant-sensitive fluorescent probe, DHE, and ROS production assessed by nuclear fluorescence via microscopy (21).

Rho/Rac activity assays

Rho/Rac activity assays were performed according to the manufacturer's instructions. Briefly, 2.5×10^7 HMDM and stimulated for timepoints as indicated. Active Rho and Rac were isolated by incubating lysates with sepharose beads bound to Rhotekin or PAK, respectively, which were run on an SDS-PAGE gel. Whole cell lysates were ran on separate gels and total Rho/Rac levels were evaluated using mouse anti-Rho (Cytoskeleton, CO) or mouse anti-Rac (Upstate). The densitometry was measured using Image J software (NIH) and expressed as a percentage of the untreated control.

Statistical analysis

Results are expressed as mean±SEM. Analysis was by ANOVA for multiple comparisons and post-tests were applied where appropriate. Statistically significant differences by ANOVA analysis are illustrated by * $p<0.05$, ** $p<0.01$, ***$p<0.001$ in all figures. Student's t tests were used for comparison of two sample means (illustrated by †, †† indicating significance values of $p \leq 0.05$, $p \leq 0.01$ respectively). Where important data comparisons did not reveal significant differences, this is indicated by NSD (no significant difference). All data were analyzed using GraphPad Prism (CA, USA).

Results

Pyocyanin impairs engulfment of apoptotic cells

To determine whether pyocyanin could inhibit apoptotic cell engulfment, we assayed phagocytosis of apoptotic PMN (APMN) by human monocyte-derived macrophages (HMDM) *in vitro*. Pyocyanin caused a concentration and time-dependent reduction in phagocytosis of APMN. Of note, pyocyanin reduced both the proportion of HMDM ingesting APMN and the average number of APMNs ingested (Figure 1). In 3 further experiments, we confirmed that the defect was of ingestion rather than adherence of apoptotic cells to HMDM using a flow-based assay to detect engulfment of Cell Tracker labelled APMN (see Table E1 in online supplement). Since pyocyanin causes acceleration of neutrophil apoptosis (9) and loss of macrophage viability would lead to loss of phagocytic capacity, we confirmed that pyocyanin was not causing macrophage death using three independent methods. First, pyocyanin-treated HMDM were stained with a fluorescent nuclear dye, Hoechst 33342, and no significant loss of cell numbers was detected in pyocyanin-treated HMDM compared to untreated controls (Figure 2A,B). Secondly, TUNEL staining showed there was minimal, if any, increase in nuclear changes of apoptosis (Figure 2C) and thirdly a caspase 3/7 activity assay detected no increase in caspase activation following pyocyanin treatment, in contrast to a positive control, staurosporine (Figure 2D).

We addressed whether the phagocytic defect was specific for apoptotic cell uptake or more generalised. Engulfment of fluorescently-labeled apoptotic Jurkat cells was assessed by flow cytometry. Ingestion of these cells by control macrophages was significantly greater than ingestion of apoptotic neutrophils, 80% compared to 35%, but was significantly reduced following pyocyanin pre-treatment (Figure 3A). In contrast, uptake of serum-opsonized carboxyl-modified green-fluorescent latex beads, an assay of Fc-receptor

mediated phagocytosis, did not differ between pyocyanin-treated and control HMDM (Figure 3B).

Mice infected with pyocyanin-producing *P. aeruginosa* have reduced clearance of apoptotic cells

To determine whether pyocyanin impaired clearance of apoptotic cells in an acute *P. aeruginosa* pneumonia model (13), mice infected with a wild-type, phenazine-producing strain were compared with mice infected with a phenazine-deficient strain that has only 10% of wild-type pyocyanin production (11). The model is associated with rapid neutrophil influx and, in mice infected with phenazine-producing bacteria, with accelerated neutrophil apoptosis by 18 h following bacterial instillation (13). To determine macrophage engulfment of apoptotic cells, we assessed apoptotic cell uptake by alveolar macrophages using TUNEL staining (Figure 4A) at 18 and 30 h following instillation of bacteria. At 18 h total neutrophil counts were similar in mice infected with both strains (PA14 $1.92\pm0.23 \times 10^6$, Δphn $1.80\pm0.12 \times 10^6$) but were increased in the pyocyanin-deficient infection at 30 h (PA14 $2.02\pm0.14 \times 10^6$, Δphn $3.90\pm0.54 \times 10^6$, $p<0.05$). There were increased numbers of apoptotic PMN in BAL from mice infected with the wild-type strain at 18 h (PA14 $8.20\pm0.91 \times 10^5$, Δphn $1.96\pm0.08 \times 10^5$, $p<0.01$) and at 30 h (PA14 $3.96\pm0.80 \times 10^5$, Δphn $1.57\pm0.37 \times 10^5$, $p<0.05$), in keeping with our previous findings (13). Despite the excess of apoptotic cells in the PA14 infected mice, the proportion of macrophages containing apoptotic cells was lower in these mice (Figure 4B, C) and ingesting macrophages also contained reduced numbers of apoptotic bodies (Figure 4D, E) in wild-type infection compared with mice infected with the phenazine-deficient strain. Importantly, increased numbers of late apoptotic/necrotic (To-Pro3 positive) cells were detected at 30h in mice infected with the pyocyanin-producing strain (Figure 4F, G) suggesting the wave of early apoptotic cells detected at 18 h had not been efficiently cleared. Moreover, this data supports the view that the reduced numbers of apoptotic cells detected within macrophages in wild-type *P. aeruginosa* infection reflect impaired engulfment rather than increased efficiency of apoptotic body degradation by macrophages. In keeping with our previous data (13), there was no difference in BAL macrophage numbers in the two groups of mice and we detected no differences in macrophage death, assessed by To-Pro3 staining, between mice infected with the different strains (please see Figure E1 in the online supplement). There was a trend towards increased bacterial numbers in mice infected with wild-type *P. aeruginosa* (log10 cfu was PA14 5.82±0.47, Δphn 4.09±0.55 at 18 h and PA14 4.98±0.16, Δphn 4.02±0.54 at 30 h). These colony numbers were not significantly different, perhaps reflecting the sample size, although significant differences were found only at 48 hours and beyond in our previous study (13).

ROS production mediates reduced apoptotic cell uptake by pyocyanin-treated macrophages

The cytotoxic effects of pyocyanin on bacteria and eukaryotic cells are linked to its ability to undergo non-enzymatic redox cycling within cells, with resulting ROS generation (22). *P. aeruginosa*-induced killing of *C. elegans* and in a murine model of sepsis is dependent upon both pyocyanin production and ROS generation (11) and pyocyanin-mediated neutrophil apoptosis is associated with massive and sustained generation of ROS (9). We measured

ROS production in HMDM using a fluorescent probe, dihydrorhodamine (DHR). We observed a significant increase in ROS 30 min after pyocyanin treatment (Figure 5A,B) that remained elevated to 24 h. The magnitude of ROS generation by macrophages appeared less than that of neutrophils (data not shown) possibly reflecting their greater anti-oxidant levels (23), which may also explain their resistance to pyocyanin-induced apoptosis. An anti-oxidant and superoxide dismutase-mimetic, MnTBAP, significantly reduced ROS levels at 6 h (Figure 5C) at concentrations of 1μM and above. We confirmed these findings using a second fluorescent probe, dihydroethidium (DHE), demonstrating nuclear fluorescence following pyocyanin treatment that was inhibited by MnTBAP (Figure 5C). A previous study showed that some fluorescent probes, including DHR, can be directly oxidised by redox-active compounds, including pyocyanin, but the increased fluorescence signal that results is not prevented by anti-oxidants (24). Our data shows the pyocyanin-induced increase in macrophage fluorescence is significantly inhibited by anti-oxidant treatment (Figure 5C). Crucially, MnTBAP, at a concentration that inhibited ROS production, also reversed pyocyanin-mediated impairment of apoptotic cell uptake, demonstrating dependence upon ROS generation (Figure 5D).

Pyocyanin inhibits apoptotic cell uptake by effects on small GTPases

Pathways originally defined in *C. elegans* involve the Rho family of low molecular weight GTPases in signaling pathways mediating apoptotic cell engulfment (25). The Rho GTPase, Rac, facilitates engulfment of apoptotic cells, whereas Rho inhibits uptake (25). Rho-kinase is a downstream mediator of RhoA inhibition of uptake of apoptotic cells and blockade of Rho-kinase enhances apoptotic cell uptake (26). We therefore investigated whether pyocyanin-induced ROS might mediate its actions by alterations in Rho signalling. Rho activity was measured using Rhotekin to pull down active Rho and analyzing both total and active Rho by Western blotting. We found Rho activity was significantly increased following pyocyanin treatment (Figure 6A, B), whereas Rac-1 activity, measured using PAK to pull down active Rac, was significantly reduced by pyocyanin treatment (Figure 6C, D). Thus pyocyanin treatment of HMDM inhibited apoptotic cell uptake, inhibited Rac and activated Rho.

We next examined the ability of C3 transferase (from *Clostridium botulinum*) which inactivates RhoA, as well as Y-26732, a specific inhibitor of Rho-kinase, to reverse pyocyanin-impaired macrophage engulfment of apoptotic neutrophils. We found both compounds, at concentrations previously shown to inhibit their respective targets (26), substantially restored engulfment of APMN by pyocyanin-treated macrophages (Figure 6E). These data place regulation of GTPase activity downstream of induction of ROS in macrophages and are consistent with other recent data from one of our labs that identified a role for ROS production and inactivation of small GTPases in impairment of apoptotic cell uptake following TNF-α treatment of macrophages (21).

Discussion

In this study we show that pyocyanin, a major secondary metabolite of *P. aeruginosa*, impairs macrophage engulfment of apoptotic cells as a result of intracellular ROS generation

and modulation of small GTPase signaling. This finding identifies a novel and potentially important mechanism by which pathogens could disrupt efficient clearance of inflammatory cells, increasing host tissue injury.

Chronic infection with *P. aeruginosa* is a major cause of pulmonary damage and mortality in patients with cystic fibrosis (27) and a number of different *P. aeruginosa* products have been shown to modify host immune responses (7). A central feature of cystic fibrosis lung disease is abnormal neutrophil recruitment and persistence in the airway, often beginning early in childhood (28). There is also evidence for aberrant neutrophil death in the cystic fibrosis lung, leading to DNA release and resulting sputum hyperviscosity (29), and leakage of major proteases such as neutrophil elastase that exacerbate lung injury (30). The proportion of neutrophils in CF sputum that are apoptotic is in the region of 30-40% (4), vastly in excess of the levels of <1% found in patients with community-acquired pneumonia (31). This in turn suggests delayed clearance of apoptotic cells in cystic fibrosis airways, which could reflect either macrophage capacity for phagocytosis being overwhelmed by vast numbers of effete neutrophils or a more specific defect of macrophage engulfment. Against the former is the observation of effective clearance of apoptotic cells and subsequent resolution of acute lobar pneumonia where the burden of neutrophils is also very substantial (32). Vandivier *et al* showed that the presence of neutrophil elastase in CF sputum specifically impairs engulfment of apoptotic neutrophils, demonstrating a “vicious circle” in which the presence of large numbers of effete neutrophils will further impair apoptotic cell clearance (4).

We investigated the possibility that *P. aeruginosa* might directly impair apoptotic cell engulfment. There is some evidence that dysregulation of clearance mechanisms may be pathogen-dependent. Watt *et al.* analyzed sputum samples from CF patients and identified a large excess of late apoptotic or secondarily necrotic neutrophils in patients infected with *P. aeruginosa* or *Burkholderia cenocepacia* compared with those infected with other gram-negative pathogens. The findings suggest these pathogens are causing accelerated neutrophil apoptosis, as previously described for both *P. aeruginosa* (9) and *B. cenocepacia* (33), as well as other bacterial pathogens (8), but could also imply that phagocytosis of effete neutrophils by macrophages was impaired in these infections (34). The cystic fibrosis patients studied by Vandivier *et al.*, who showed evidence both of profoundly impaired apoptotic cell clearance and of neutrophil necrosis, were also all infected with *P. aeruginosa* (4). We studied the major toxic metabolite of *P. aeruginosa*, pyocyanin, since it has been shown to be a key agent of *P. aeruginosa* pathogenicity in multiple experimental models and to cause massive oxidant stress by intracellular redox generation (11, 35). We found pyocyanin impaired macrophage engulfment of apoptotic cells *in vitro*, at concentrations that have been reported in sputum from patients chronically colonized with *P. aeruginosa* (36). Although there are reports of *P. aeruginosa* inducing apoptosis of a macrophage-like cell line *in vitro* (37), pyocyanin treatment did not cause significant macrophage apoptosis. Using a murine model of acute resolving *P. aeruginosa*, we showed that infection with a pyocyanin-producing strain of *P. aeruginosa* was associated with reduced numbers of apoptotic cells within airway macrophages and with increased numbers of necrotic neutrophils that had not been engulfed. There were no differences in macrophage numbers nor evidence of increased macrophage apoptosis in mice infected

with the pyocyanin-producing strain of *P. aeruginosa*, compared with a phenazine-deficient strain in which pyocyanin production was shown to be 10% of wild-type (11). There is a previous report of *P. aeruginosa* inducing apoptosis of a macrophage-like cell line via type III secretion-dependent mechanisms (37). We cannot exclude effects of other *P.aeruginosa* virulence factors on macrophage numbers, but there were no differences in macrophage numbers between the two strains, so the impact of any such factors was similar between wild-type and phenazine-deficient bacteria. The reduction in apoptotic cell engulfment in mice infected with the wild-type strain was observed at both time-points studied, but was more marked at 30 hours. Since we were unable to measure pyocyanin production *in vivo* in the murine model, we cannot determine whether this reflects a delayed pyocyanin effect or that an increase in "available" apoptotic cells "unmasks" the defect at 30 hours. However, it is notable that pyocyanin produces long-lasting effects *in vitro:* oxidant production persisted 6 hours after pyocyanin treatment (the latest time-point at which it was measured), down-regulation of Rac activity was significant only at 24 hours after pyocyanin treatment and maximal inhibition of apoptotic cell engulfment was also detected after 24 hours. These data identify a pathological process that could exacerbate host tissue injury both directly by release of proteases and other toxic products from secondarily necrotic neutrophils (4) and indirectly by failure of apoptotic cell uptake to generate macrophage release of anti-inflammatory cytokines (38). A more chronic model of *P. aeruginosa* infection, as opposed to the acute, resolving model used in these experiments, could further address effects upon host tissue injury.

As predicted from other cell types, including neutrophils (9, 35) and pulmonary epithelial cells (22), pyocyanin treatment caused generation of ROS in macrophages, using pyocyanin concentrations detected in clinical samples. Pyocyanin-induced ROS generation induces apoptosis in neutrophils (9) and in pulmonary epithelial cells although over a longer time course (9, 22, 39). We did not detect significant macrophage apoptosis following pyocyanin treatment and it is possible the much greater sensitivity of neutrophils to pyocyanin-induced apoptosis reflects their low basal activity of anti-oxidant enzymes, in contrast to macrophages which have much higher levels, particularly of GSH and glutathione peroxidase (23). The macrophage thus exhibits a more subtle phenotype of impaired apoptotic cell engulfment, without loss of viability. Engulfment was partially restored by treatment with an anti-oxidant, MnTBAP, confirming the effects of pyocyanin were ROS dependent. A previous study had shown that monocyte-derived macrophage engulfment of apoptotic cells was impaired by hydrogen peroxide treatment, supporting a specific role for ROS in impairing apoptotic cell engulfment (40). The phagocytic defect appears specific to apoptotic cell uptake, as evidenced by the signalling pathway implicated, and also by preserved Fc-mediated uptake of fluorescent beads. The latter is in keeping with studies by Muller et al (41), who showed macrophage uptake of bacteria was preserved in the presence of pyocyanin.

A large number of receptors for surface changes on apoptotic cells have been implicated in the tethering, engulfment and signalling uptake of apoptotic cells in mammalian systems, with evidence for considerable redundancy (42). Studies of deficiencies of engulfment in *C. elegans*, in contrast, have identified a number of genes in signalling pathways for cell clearance, including evidence the Rho-family GTPases are involved in engulfment of

apoptotic cells (43) and in regulation of subsequent maturation of the phagosome (44). We found pyocyanin treatment of HMDM increased Rho activity and reduced Rac-1 activity, a pattern previously associated with impaired apoptotic cell engulfment (25, 26). There is evidence the Rho/Rac balance within cells might determine the rate of phagosome maturation and that, in monocyte-derived macrophages, inhibition of Rho signaling pathways delays intracellular disposal of ingested apoptotic cells (44). Theoretically, therefore, activation of these pathways by pyocyanin might accelerate degradation of ingested apoptotic cells and thus contribute to the finding of smaller numbers of apoptotic cells detected within macrophages following pyocyanin treatment or in the *Pseudomonas aeruginosa* infection model. However, the increased numbers of uningested late apoptotic/necrotic neutrophils observed in the mice infected with pyocyanin producing *P. aeruginosa* support the view there is impaired engulfment of apoptotic cells. Moreover, inhibition of Rho signaling pathways does not alter the numbers of ingested apoptotic neutrophils detected within monocyte-derived macrophages *in vitro*, as shown in Figure 6E, showing accelerated degradation of apoptotic cells is not an explanation for our findings.

We showed the effects of pyocyanin upon apoptotic cell engulfment could be reversed by treatment with an anti-oxidant, MnTBAP, or by Rho-kinase inhibition and thus are amenable to therapeutic targeting. The specific defect of apoptotic cell engulfment could be abrogated by specific Rho-kinase inhibitors, novel compounds which have been used as vasodilators in clinical trials in patients with stable angina (45) and ischemic stroke (46). Recent evidence also suggests statins, which inhibit prenylation of Rho GTPases, can also enhance apoptotic cell engulfment by both monocyte-derived macrophages and, importantly, human alveolar macrophages (47). More generally, there is evidence of increased oxidative stress in the airways in cystic fibrosis, which is a major factor in lung damage and is thought to be caused by excessive neutrophil activation and oxidant release (48). Oxidative stress is associated with airway inflammation (49) and is increased in infection with *P. aeruginosa* or *B. cenocepacia* (50). Treating with anti-oxidants may be of clinical benefit (48) and has specifically been shown to reduce neutrophilic inflammation and neutrophil elastase activity in the airways (48). Our data suggest that one important effect of anti-oxidant treatment might be restoration of apoptotic cell engulfment that has been impaired by *P. aeruginosa* infection, in addition to other beneficial effects of these compounds.

In conclusion, we show that pyocyanin, a major toxin produced by *P. aeruginosa*, impairs macrophage engulfment of apoptotic cells both *in vivo* and *in vitro* and that these effects are substantially reversed by anti-oxidants or by blockade of Rho signaling pathways. Since more than 95% of those with significant cystic fibrosis lung disease are infected with *P. aeruginosa* and pyocyanin also accelerates neutrophil apoptosis, therapies directed at the toxic effects of pyocyanin could abrogate both acceleration of neutrophil apoptosis and impairment of apoptotic cell clearance, with the potential to reduce chronic tissue injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Impact of this research

Engulfment of apoptotic cells by macrophages plays a crucial role in the resolution of inflammation. This study is the first to report the modulation of apoptotic cell engulfment by a bacterial toxin, pyocyanin, the major toxic metabolite of *Pseudomonas aeruginosa*. The studies identify a novel mechanism by which pathogens subvert the immune response that could be targeted to limit inflammation during *P. aeruginosa* infection.

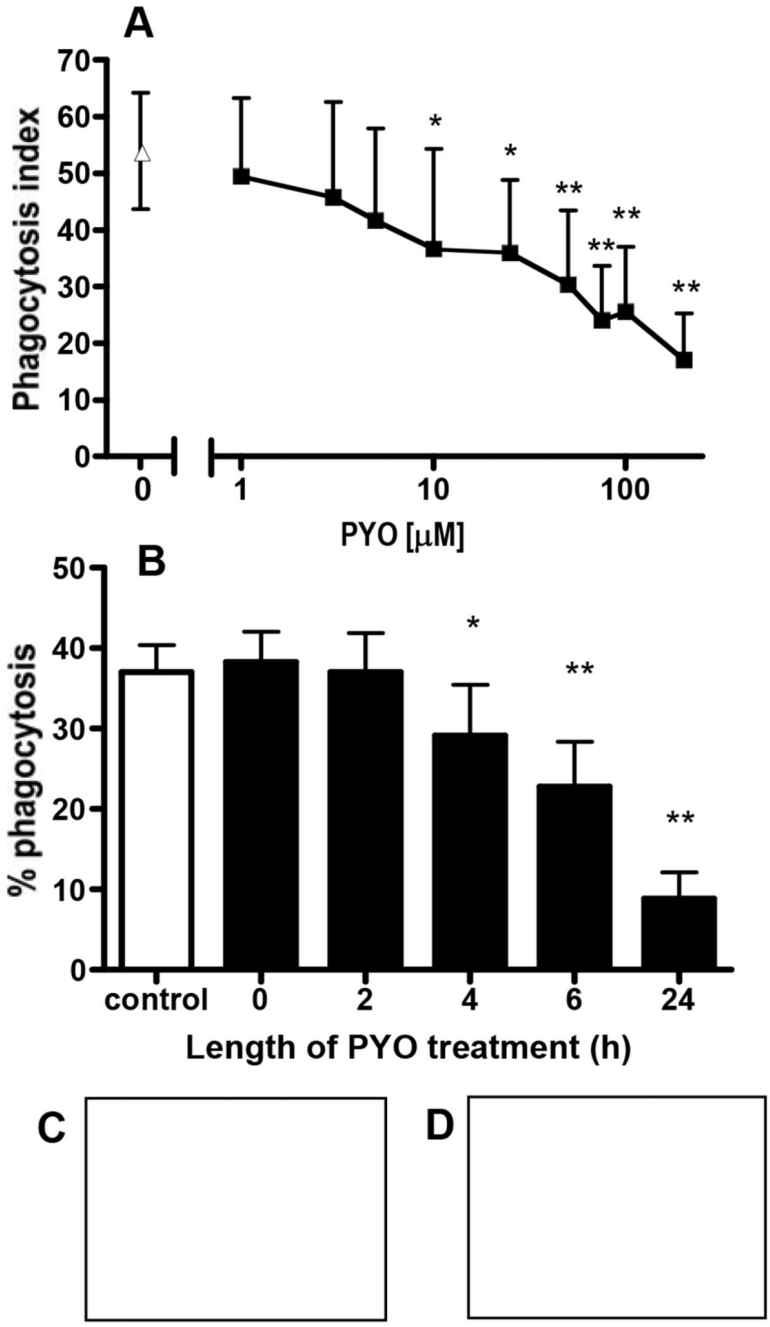

Figure 1. Pyocyanin impairs macrophage uptake of apoptotic neutrophils.

HMDM were exposed to pyocyanin for up to 24 h prior to incubation with APMN for 1 h and phagocytosis of apoptotic PMN (APMN) was quantified by staining for myeloperoxidase. (**A**) Pyocyanin inhibition of apoptotic neutrophil engulfment is concentration dependent. Phagocytosis is expressed as mean±SEM phagocytic index (% HMDM phagocytosing × mean number APMN per phagocytosing HMDM) after exposure to varying concentrations of pyocyanin for 24 h. Data shown is from three independent experiments and significant differences from untreated cells are indicated for

each concentration of pyocyanin (significance calculated by ANOVA with Dunnet's post test). (**B**) The percent of HMDM ingesting APMN after varying lengths of pre-incubation with pyocyanin (50 μM) in five independent experiments and significant differences from cells harvested at time 0 are indicated (significance calculated by ANOVA with Dunnet's post test). (**C, D**) Ingestion of apoptotic cells is quantified by counting myeloperoxidase-stained inclusions in HMDM by light microscopy. Arrowheads highlight the presence of MPO-stained apoptotic neutrophils within untreated HMDM (control, **C**) whereas apoptotic neutrophils are largely extracellular in HMDM pre-treated with pyocyanin for 24 h (**D**).

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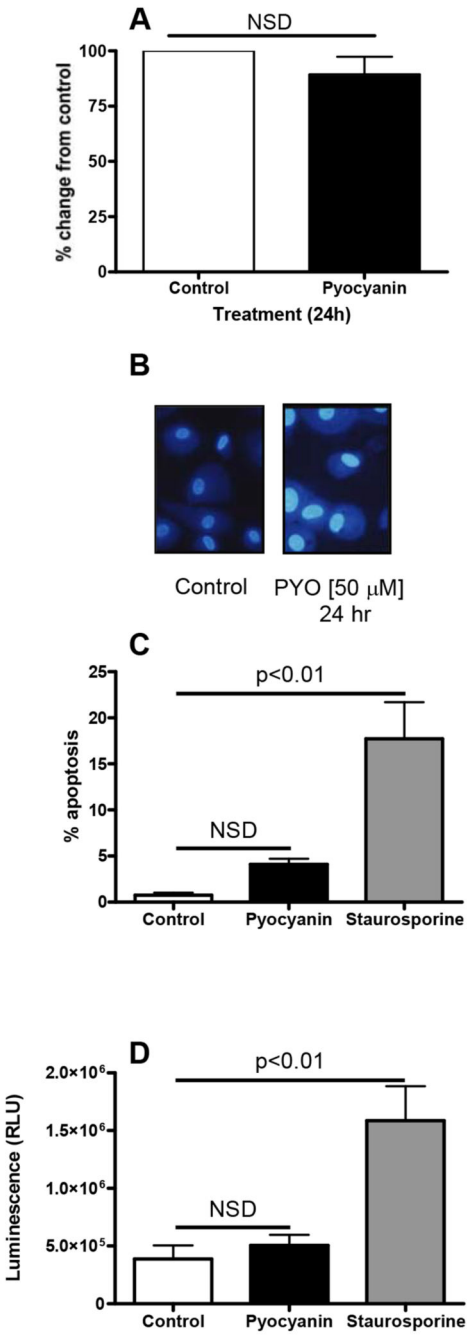

Figure 2. Pyocyanin treated monocyte-derived macrophages remain viable.

HMDM viability was assessed using Hoechst 33342 staining and fluorescence microscopy. Cell counts were performed for cell number and cells were also assessed for morphologic changes of apoptosis. (**A**) There was no loss of cell number in HMDM pre-treated with pyocyanin (50 μM) for 24 h (solid bars) relative to controls (open bars) in three independent experiments. NSD signifies no significant difference. Chart shows data normalised to 100% in controls. (**B**) Photomicrographs illustrating typical morphology of viable cells in both control and pyocyanin-treated populations. (**C**) HMDM apoptosis was assessed by TUNEL

staining in cells pre-treated with pyocyanin (50 μM, solid bars) or staurosporine (10 μg/ml, shaded bars) for 24 h relative to controls (open bars) in four independent experiments. The percentage of apoptotic cells was not significantly increased in pyocyanin-treated cells. (**D**) Caspase 3/7 activity was measured in HMDM pre-treated with pyocyanin (50 μM, solid bars) or staurosporine (10 μg/ml, shaded bars) for 24 h compared with controls (open bars) (n=4).

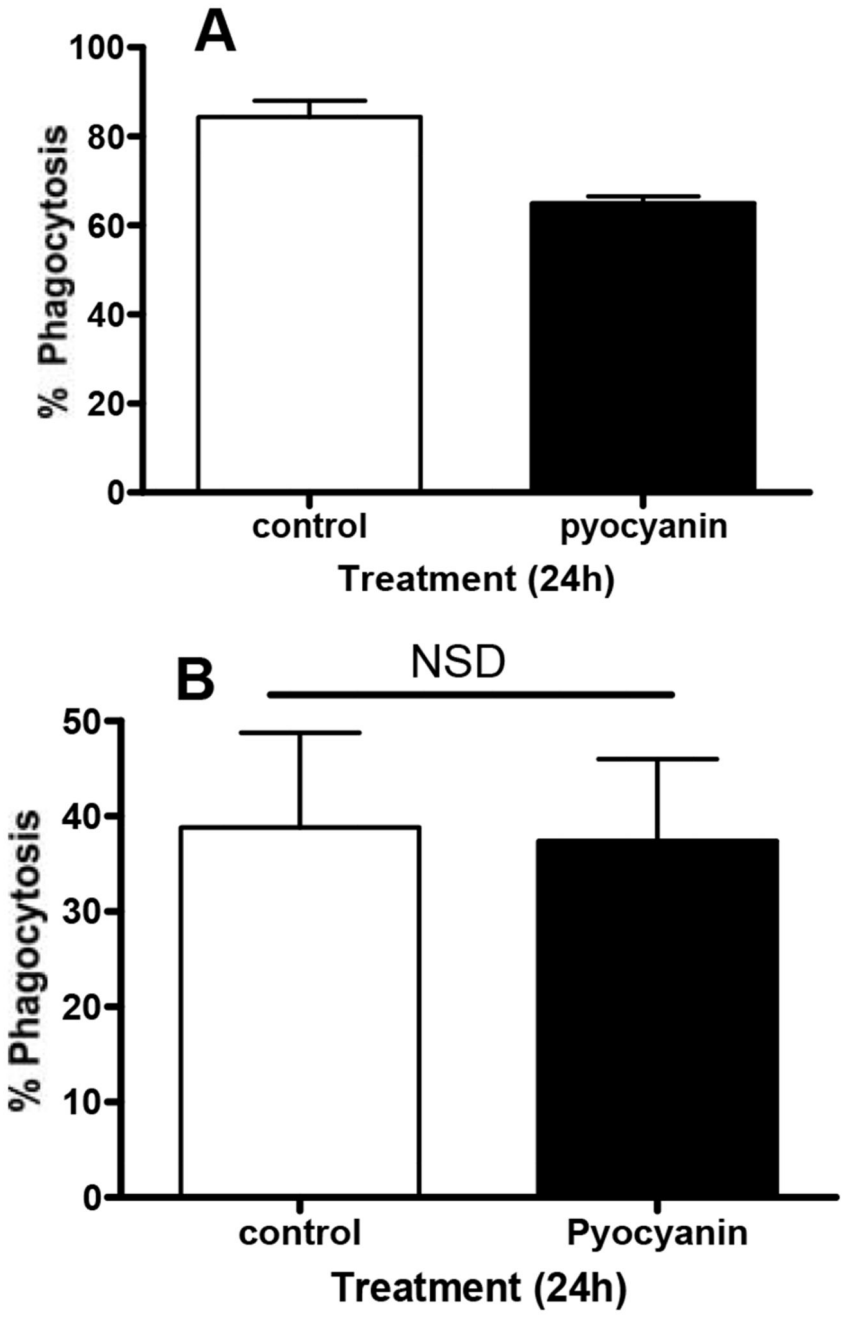

Figure 3. The pyocyanin-mediated phagocytic defect is specific for apoptotic cells.

(**A**) Apoptotic Jurkat cells were labelled with Cell-Tracker and co-incubated with HMDM for 1 h following pre-treatment of HMDM with 50 μM pyocyanin (solid bars) or media control (open bars) for 24 h. Engulfment of apoptotic cells was measured by flow cytometry in three independent experiments and was significantly reduced by pyocyanin treatment. (**B**) HMDM were pretreated with media (open bars) or 50 μM pyocyanin (solid bars) for 24 h before being co-incubated with carboxyl-modified green fluorescent latex beads at a ratio of 1:5 (HMDM:bead) for 1 h. Phagocytosis was scored by fluorescence microscopy

based on the percentage of macrophages containing at least one latex bead and did not differ significantly between the groups.

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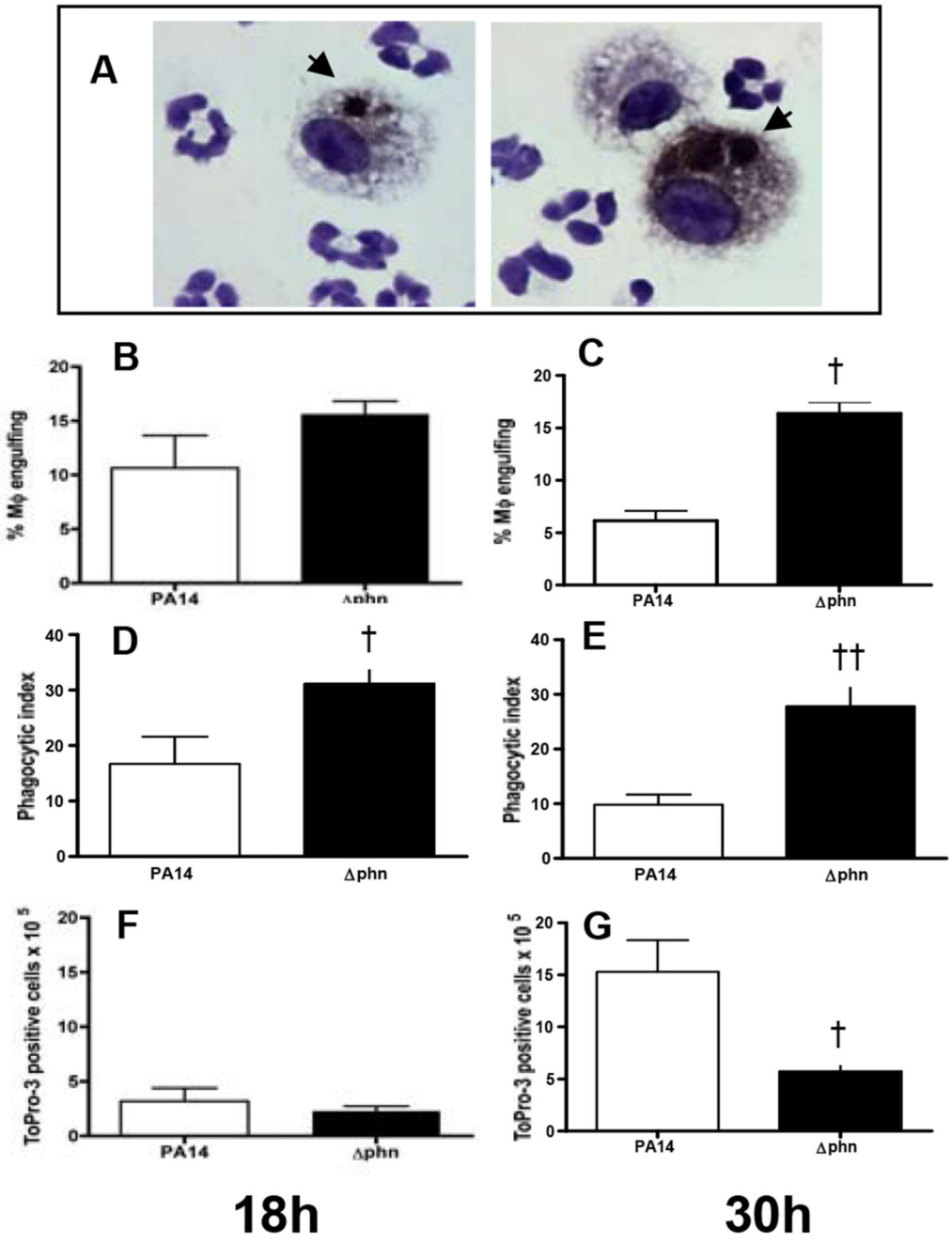

Figure 4. Pyocyanin production is associated with impaired apoptotic cell clearance *in vivo*.

C57BL6 mice were instilled with 10^7 CFU live WT (PA14, open bars) or phenazine-deficient (ΔphnAB, solid bars) of *P. aeruginosa* and BAL performed after 18 (B,D,F) or 30 (C,E,G) h. Total and differential cell counts were obtained by hemocytometer and cytospin counts. Data shown were obtained from 3 independent experiments. (**A**) Cytopsins were TUNEL stained and macrophage phagocytosis of apoptotic bodies (TUNEL positive inclusions, indicated by arrowheads) visualised by microscopy. (**B,C**) Phagocytosis of APMN by macrophages was assessed and represented as % macrophages engulfing APMN

(**D,E**) Phagocytic index was similarly calculated from TUNEL-stained cytospins. (**F,G**) The presence of necrotic cells in BAL was assessed by ToPro-3 uptake on flow cytometry.

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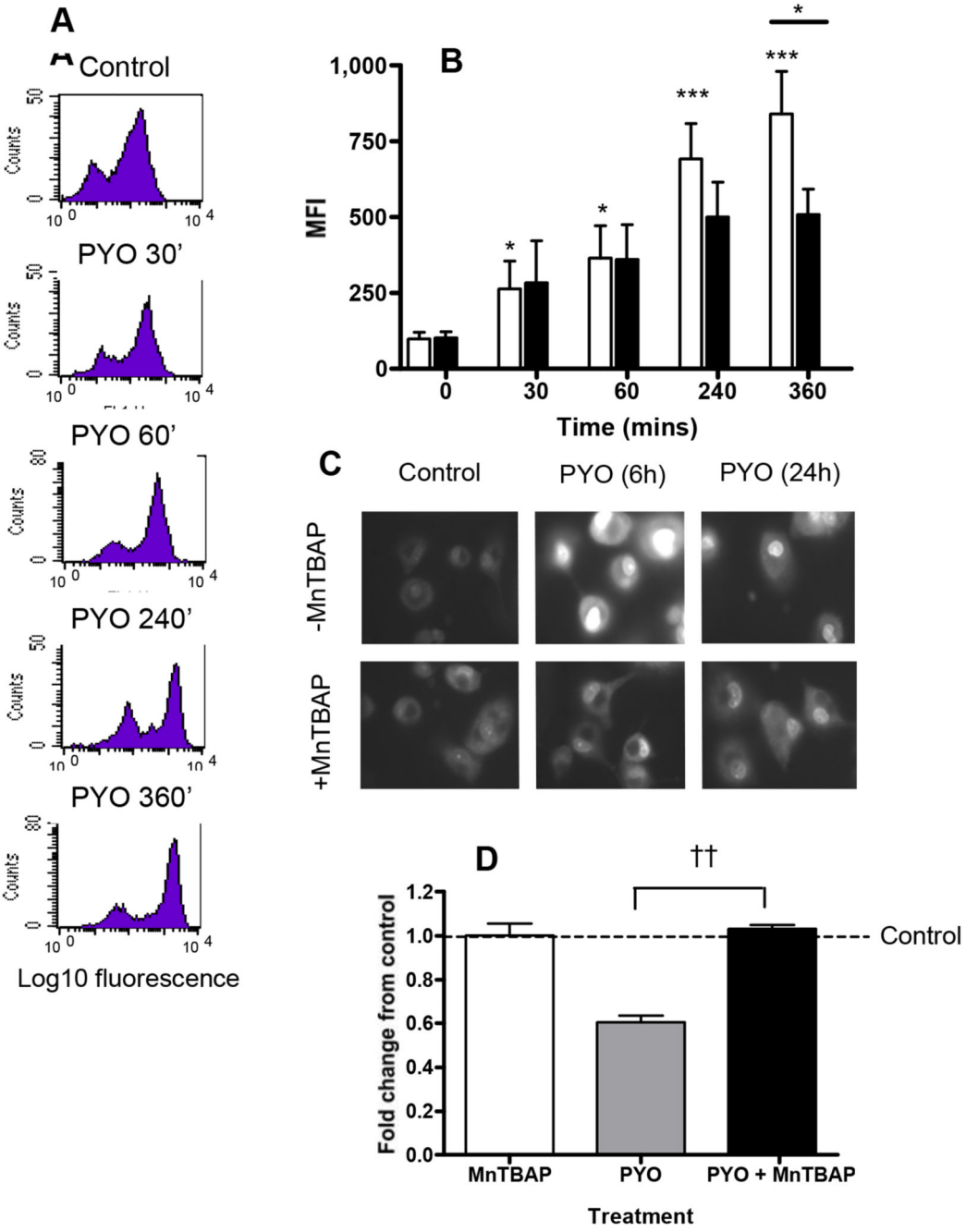

Figure 5. Pyocyanin impairment of macrophage apoptotic cell uptake is mediated via ROS generation.

(**A**) ROS production by HMDM was measured by flow cytometry. DHR-loaded HMDM were treated with 50μM pyocyanin over a range of time points (30, 60, 240 and 360 min). Representative histograms show increases in fluorescence (oxidant production) as right shifts on the × axis (FL-1). (**B**) Mean data for ROS production, expressed as geometric mean fluorescence intensity (MFI) over time in HMDM treated with 50μM pyocyanin in the presence (solid bars) or absence (open bars) of an antioxidant MnTBAP (1μM). Data were

from three independent experiments and significant differences are indicated for pyocyanin-treated cells (open bars) for each time point compared to cells at time 0. Results for cells incubated with MnTBAP and pyocyanin compared with pyocyanin alone were significantly different only at 360 min as indicated by the bar (significance calculated by ANOVA with Bonferroni post test). (**C**) HMDM were treated for 6 h or 24 h with media (control), pyocyanin (50μM), MnTBAP (100μM) or pyocyanin and MnTBAP in combination. Nuclear fluorescence was visualised by DHE staining and fluorescence microscopy. Representative images are shown from a single experiment of a set of 3. (**D**) HMDM were treated for 24 h with media (control) or pyocyanin (50μM), in the presence (solid bar) or absence (shaded bar) of MnTBAP (1μM). MnTBAP alone is represented by the open bar. HMDM were subsequently incubated with APMN for 1 h and ingestion scored by myeloperoxidase staining and microscopy. Data from 3 independent experiments are shown as fold change from control (illustrated by dashed line).

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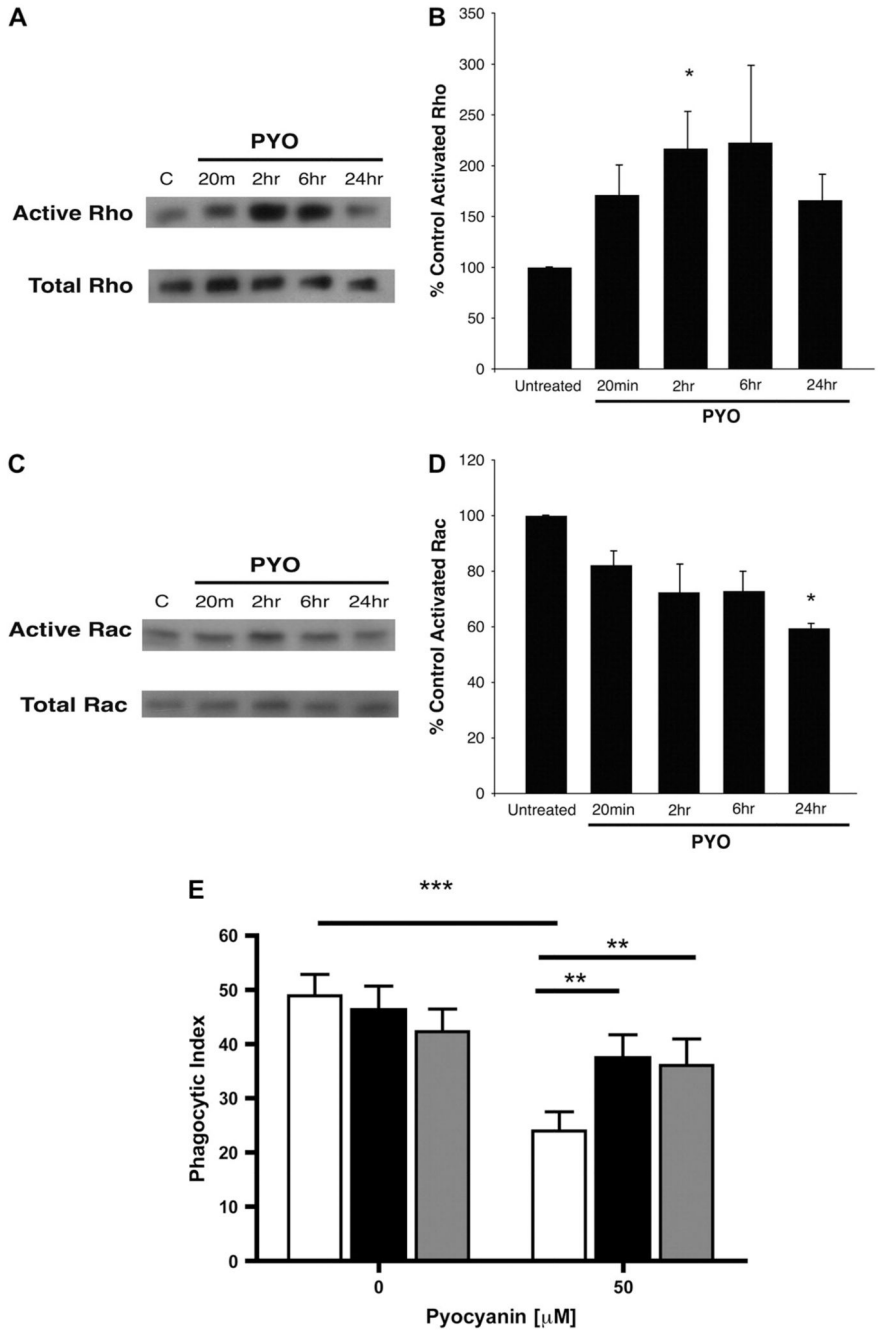

Figure 6. Pyocyanin inhibits apoptotic cell uptake via effects on Rho/Rac GTPases.

HMDM were treated with PYO for the indicated times and analyzed for active and total Rho (**A,B**) and Rac (**C,D**). Representative gels are shown from a single experiment of a set of 4 and the mean±SEM amounts of active Rho/Rac were quantified using Image J densitometry software (n=4). Where amounts of Rho/Rac were significantly different from levels in untreated cells this is indicated (*). (**E**) HMDM were treated with media (control, open bars) or pyocyanin (50μM) (open bars) for 24 h in the presence or absence of Y-27632 (10μM) (solid bars) or C3 transferase (1μg/ml) (shaded bars). HMDM were subsequently

incubated with APMN and ingestion scored by microscopy (times indicated above represent incubation periods prior to addition of APMN) and expressed as phagocytic index. Data are obtained from seven independent experiments (significance calculated by ANOVA with Bonferroni post test).

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