Divergent Effects on Phagocytosis by Macrophage-Derived Oxygen Radicals

Kelly L. Brown  Karin Christenson  Anna Karlsson  Claes Dahlgren
Johan Bylund
Department of Rheumatology and Inflammation Research, University of Gothenburg, Gothenburg, Sweden

Abstract
Clearance of senescent cells from damaged tissue is a crucial step to successfully resolve an inflammatory response. Poor clearance has been attributed to defects in neutrophil apoptosis, a process that is regulated in part by reactive oxygen species (ROS) produced by activated neutrophils. Using ROS-deficient and wild-type macrophages treated with extracellular ROS scavengers we demonstrate that macrophage-derived ROS also influence phagocytosis and do so by direct and indirect mechanisms.

Key Words
Reactive oxygen species • Phagocytes • Chronic granulomatous disease • Inflammation

Introduction
The removal of apoptotic neutrophils from infected or damaged tissue prior to necrotic disintegration is required for the resolution of inflammation and restoration of tissue homeostasis. An inability to clear apoptotic neutrophils from tissues cannot only prolong inflammation and increase tissue damage but can contribute to the development of chronic inflammatory and autoimmune diseases. The aberrant removal of apoptotic neutrophils can manifest for a multitude of reasons that include impaired neutrophil function, for example, by a failure to enter apoptosis or to display appropriate apoptotic markers, and/or an inability of macrophages to recognize, bind or ingest (phagocytose) the apoptotic cells.

Reactive oxygen species (ROS) are produced by the NADPH oxidase complex and are well-known central components in the antimicrobial arsenal of activated phagocytes. Neutrophil oxygen radicals also regulate neutrophil lifespan, apoptosis and clearance [1, 2]. A condition known as X-linked chronic granulomatous disease (CGD) can arise in humans from a mutation in a gene, CYBB, encoding one subunit (gp91phox) of the NADPH oxidase complex. This mutation extinguishes the production of ROS and predisposes individuals to recurrent infections, granulomas and sterile inflammatory diseases [3]. Given that neutrophils are the classical ROS-producing leukocyte, investigations have tended to focus on the identification of ROS-dependent processes in neutrophils, such as apoptosis, that underlie the inflammatory diseases associated with CGD. The results have revealed, for example, that CGD neutrophils are delayed from entering apoptosis and fail to display markers of apoptosis, such as oxidized phosphatidylserine (PS), to phagocytic macrophages [1, 2]. It has also been suggested that the reduced exposure of PS (on neutrophils) hampers normal macrophage polarization and, in turn, phagocytic capacity for apoptotic neutrophils [4]. The general consensus is that ROS deficiency alters normal cellular functions that negatively impact neutrophil clearance from damaged or infected tissues. There is no evidence thus far that ROS are directly involved in the phagocytic process.
In our previous studies on inflammatory disease in individuals with X-linked CGD we discovered that, not unlike neutrophils, ROS deficiency impacted a broad range of basal cellular processes in monocytes. While resting monocytes produce miniscule levels of ROS compared to activated neutrophils, the low levels are clearly important since their absence was sufficient to induce a pro-inflammatory signature in CGD monocytes compared to ROS-competent control monocytes [5]. We hypothesized then that basic cellular functions in myeloid-derived macrophages would also be altered by a radical deficiency and questioned if macrophage-derived radicals influenced, indirectly or directly, the ability of macrophages to phagocytose apoptotic neutrophils.

Using a simple in vitro system, we compared the capacity of wild-type (WT) and gp91phox-deficient (CGD) murine macrophages to phagocytose WT apoptotic neutrophils. This system ensures that any observed changes could be attributed to ROS deficiency in the macrophages (rather than the neutrophils). While the ability to phagocytose microbes was intact in CGD macrophages, the ingestion of apoptotic neutrophils was appreciably compromised. A similar result was observed in human monocyte-derived macrophages (MDM) in the presence of inhibitors of extracellular ROS. Thus, ROS generated by macrophages are required specifically for phagocytosis of apoptotic neutrophils. Furthermore, the participation of these NADPH oxidase-derived extracellular radicals in augmenting phagocytosis can be attributed in part to the direct action(s) of radicals, presumably on the cell surface of the macrophage.

**Materials and Methods**

**Culturing of Cells**

Unless otherwise indicated, all cells were cultured at 37°C, 5% CO2 in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 1 mM sodium pyruvate and 50 U/ml PEST (PAA Laboratories GmbH, Pasching, Austria). Primary human and murine cells were obtained and used in accordance with the Ethical Committee of The University of Gothenburg and in cooperation with Sahlgrenska Hospital, Gothenburg, Sweden.

**Isolation of Human Peripheral Blood Monocytes and Differentiation to Macrophages (MDM)**

Monocytes were isolated from 1-day-olduffy coats and differentiated to macrophages as described [6]. On day 6, the MDM were detached using ice-cold Versene containing 4 mg/ml lidocaine (Sigma, St. Louis, Mo., USA), counted, seeded into 24-well plates at 3 × 105 cells/well and incubated overnight.

**Isolation of Murine Bone Marrow and Differentiation to Macrophages**

C57BL/6j and gp91phox-deficient (CGD) mice (B&K Universal AB, Sollentuna, Sweden) were maintained under pathogen-free conditions in the Department of Rheumatology and Inflammation Research’s animal facility. Mice were euthanized at 6–12 weeks of age. Bone marrow cells were flushed from the femur with a 25-gauge needle and seeded onto 100-mm Petri dishes (approx. 1 × 105 cells/plate) in DMEM (Sigma) containing 20% FCS, 2 mM l-glutamine, 1 mM sodium pyruvate, 50 U/ml PEST and 2.5–5% L929 cell-conditioned medium as a source of M-CSF. On day 5, bone marrow-derived macrophages (BMDM) were used immediately or cryopreserved in 90% FCS/10% DMSO for future use.

**Polarization of Murine Macrophages to an M1 or M2 Phenotype**

BMDM (2 × 105) were seeded into 24-well TC-treated plates or 8-well glass chamber slides (Lab Tek II; Nalgene Nunc, Roskilde, Denmark) in complete, phenol red-free RPMI-1640 medium supplemented with 2.5–5% L929 cell-conditioned medium. Adherent BMDM were polarized 18–20 h in complete medium containing, for M1-macrophages, 100 ng/ml ultra pure Escherichia coli 0111:B4 LPS (InvivoGen, Toulouse, France) and 50 ng/ml mouse recombinant IFN-γ (R&D Systems, Abingdon, UK) and, for M2-macrophages, 10 ng/ml of mouse recombinant IL-4 (R&D Systems).

**Isolation, CFDA Labeling and Induction of Apoptosis in Human Neutrophils**

Human neutrophils were isolated from 1-day-olduffy coats as described [7]. Isolated neutrophils were CFDA labeled according to the manufacturer’s instructions (Molecular Probes/Invitrogen, Carlsbad, Calif., USA) and incubated in the presence of FasL (anti-CD95; BD Biosciences, San Jose, Calif., USA) and incubated in the presence of FasL (anti-CD95; BD Biosciences, San Jose, Calif., USA) as described [6] to induce apoptosis. This method consistently induced apoptosis in more than 80% of neutrophils, as determined by flow cytometry, with apoptotic cells staining positive for annexin V (1/50, APC-conjugated; Molecular Probes) and negative for 7-amino-actinomycin D (7AAD, 1/100; BD Biosciences). ROS production could not be detected from neutrophils treated with FasL as described above.

**Phagocytosis of Apoptotic Neutrophils and Yeast**

Phagocytosis of apoptotic neutrophils was performed as described [6]. Briefly, macrophages and CFDA-labeled neutrophils or heat-killed, FITC-labeled bakers yeast were incubated at a 1:4 ratio (macrophage:neutrophil/yeast) at 37°C for a maximum of 2 h in RPMI with up to 2% FCS. FCS was heat inactivated, thus phagocytosis proceeds in the absence of antibody- or complement-opsonization of prey. Alternatively, human MDM were treated for 10 min with SOD and catalase (1/100 dilution, cleaned with Detoxi-Endotoxin removal gel from Pierce, Rockford, Ill., USA) before apoptotic neutrophils were added to the system for 2 h. The cells were then washed, detached from plates with Versene or Versene/lidocaine, fixed and stained for human CD14 or mouse CD14 (1/50, PE-Cy7 labeled; eBioscience, San Diego, Calif., USA) and, 50 ng/ml mouse recombinant IFN-γ (7AAD, 1/100; BD Biosciences). A total of 5,000–10,000 events were collected using a FACS-Calibur or FACSscan flow cytometer (Becton Dickinson, Mountain View, Calif., USA) and the percentage of F4/80+ mouse BMDM or CD14+ human MDM that were also CFDA+ (neutrophils) or L-glutamine, 1 mM sodium pyruvate, 50 U/ml PEST and 2.5–5%
FITC + (yeast) was determined using FlowJo 8.7.3 or WinMDI 2.8 software. Macrophages on the chamber slides were washed twice in cold PBS, fixed with 2% PFA, washed in PBS, mounted with glycerol and a cover slip and imaged on a Leica fluorescence microscope.

Results and Discussion

To determine if NADPH oxidase-derived ROS influence phagocytosis, C57BL/6 WT and CGD (gp91 phox deficient) macrophages were tested for the ability to phagocytose heat-killed, FITC-labeled yeast. Macrophages were polarized to an M1 pro-inflammatory phenotype and an M2 anti-inflammatory phenotype. It is expected that both types of macrophages would be present in inflamed tissues concurrent with apoptotic neutrophils [8]. The phenotypes were confirmed by monitoring the production of oxygen radicals, nitric oxide and arginase activity. Despite the obvious inability to produce ROS, the

Table 1. Phagocytosis by murine macrophages

<table>
<thead>
<tr>
<th>Macrophage</th>
<th>Target</th>
<th>% Phagocytosis ± SD</th>
<th>n²</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT M1</td>
<td>yeast</td>
<td>28 ± 2</td>
<td>2</td>
</tr>
<tr>
<td>WT M2</td>
<td></td>
<td>44 ± 3</td>
<td>2</td>
</tr>
<tr>
<td>CGD M1</td>
<td></td>
<td>36 ± 7</td>
<td>4</td>
</tr>
<tr>
<td>CGD M2</td>
<td></td>
<td>74 ± 8</td>
<td>4</td>
</tr>
<tr>
<td>WT M1</td>
<td>human apoptotic neutrophils</td>
<td>25 ± 8</td>
<td>4</td>
</tr>
<tr>
<td>WT M2</td>
<td></td>
<td>26 ± 5</td>
<td>3</td>
</tr>
<tr>
<td>CGD M1</td>
<td></td>
<td>10 ± 4</td>
<td>7</td>
</tr>
<tr>
<td>CGD M2</td>
<td></td>
<td>9 ± 5</td>
<td>6</td>
</tr>
</tbody>
</table>

¹ Average percent ± standard deviation of macrophages with bound and/or ingested prey.
² Number of experiments on macrophages derived from individual mice.
M1-polarized CGD macrophages retained other features of M1 cells such as the expression of nitric oxide and absence of arginase activity (data not shown).

Combined microscopic and flow cytometry analysis illustrated that both WT and CGD macrophages of an M1 and M2 phenotype are extremely effective phagocytes (fig. 1a–c; table 1). Phagocytosis was observed in a minimum of 30% of macrophages, often with multiple yeast particles engulfed by individual macrophages (fig. 1b). M2-polarized macrophages had a superior ability over M1-polarized macrophages to phagocytose yeast; phagocytosis was observed, on average, 1.6× and 2.0× more often in M2 than M1 macrophages from WT and CGD mice, respectively. This observation is in line with the proposed characteristics of M2 macrophages as anti-inflammatory cells conditioned to efficiently clear debris from tissue [8]. Both M1- and M2-polarized CGD macrophages demonstrated enhanced phagocytosis compared to their WT counterpart (fig. 1; table 1), illustrating that phagocytosis by oxygen radical-deficient macrophages is not only intact, it is enhanced.

These results are in accordance with previous reports regarding the impact of ROS on the phagocytic capacity of neutrophils. Fc receptor-mediated phagocytosis of opsonized bacteria, yeast and sheep erythrocytes was enhanced in neutrophils from individuals with CGD and myeloperoxidase deficiency as well as in neutrophils taken from healthy donors that were subsequently treated in vitro with radical scavengers [9–11]. These reports independently concluded that the nonoxidized state of phagocytic Fc receptors, that, for example, would be found on neutrophils from individuals with CGD or myeloperoxidase deficiency, enhance phagocytic activity of opsonized products. We extend this observation by demonstrating a role of macrophage-derived radicals in regulating the phagocytic capacity of macrophages for nonopsonized (yeast) particles.

We next wanted to determine if macrophage-derived ROS affected the phagocytosis of apoptotic cells. WT and
CGD macrophages were incubated with CFDA-labeled human apoptotic neutrophils. Approximately 25 ± 8% (n = 4) and 26 ± 5% (n = 3) of WT M1 and M2 macrophages ingested apoptotic neutrophils (fig. 2; table 1). In contrast, a mere 10 ± 4% (n = 7) and 9 ± 5% (n = 6) of CGD M1 and M2 macrophages phagocytosed apoptotic neutrophils, approximately half of that observed with WT macrophages. These data demonstrated that a deficiency in ROS production severely impaired the ability of macrophages to phagocytose apoptotic neutrophils. This result is consistent with a recent publication by Fernandez-Boyanapalli et al. [4] that demonstrated that ROS deficiency impaired macrophage phagocytosis of apoptotic thymocytes in vitro and in vivo. The results are considerably more remarkable in light of our initial observations (fig. 1) that ROS-deficient macrophages are capable, and in fact superior phagocytes with regards to the uptake of yeast.

To investigate whether NADPH oxidase-derived ROS are factors that directly promote the phagocytosis of apoptotic neutrophils, the phagocytosis assay was repeated using human MDM in the absence and presence of the extracellular radical scavengers superoxide dismutase (SOD) and catalase. Together, SOD and catalase scavenge the highly reactive radicals superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$). Neither SOD nor catalase can cross the plasma membrane, thus their action is confined to radicals produced in the extracellular environment by the NADPH oxidase. Figure 3 illustrates that the phagocytosis of human apoptotic neutrophils by MDM (40.7 ± 5.2%, n = 7) was reduced in the presence of SOD and catalase (36.1 ± 5.5%, n = 7). These data support the findings presented in figure 2 and establish a direct role for extracellular radicals derived from the NADPH oxidase in macrophage phagocytosis of apoptotic neutrophils.

It was reported previously that the oxidation state of particular molecules, PS for example, on the surface of apoptotic neutrophils will promote the uptake of neutrophils by macrophages [1]. Given that the neutrophils in our assay system are genotypically WT, apoptosis and the presentation of appropriate apoptotic markers, including oxidized PS, would have proceeded in a normal fashion. In addition, the oxidation of PS occurs prior to, not following, exposure to the extracellular environment and oxygen radicals [12]. Our results therefore suggest that neutrophils and their NADPH oxidase-derived radicals alone are not the sole determinants of clearance.

Extracellular oxygen radicals are highly reactive species that alter proteins and lipids through oxidation. The observed decrease in phagocytosis by human MDM treated with radical scavengers SOD and catalase (fig. 3) implies that cell surface molecules on macrophages are sensitive to oxidation by extracellular radicals and this...
modification has an impact, either negative or positive, depending on the prey, on phagocytosis. Cytochalasin B blocked phagocytosis in a similar percentage of WT and CGD macrophages (percent inhibition; 74.9 ± 3.6%, n = 5 for WT BMDM; 72.2 ± 8.5%, n = 4 for CGD BMDM), indicating that oxygen radicals must influence both binding and internalization of apoptotic neutrophils.

While we provide evidence that macrophage ROS participate directly in phagocytic processes, the milder inhibition of phagocytosis observed with the extracellular radical scavengers compared to that observed in CGD cells also implies that ROS can influence phagocytosis by indirect means. We cannot, however, exclude the possibility that intracellular macrophage ROS (that would not be neutralized by the scavengers used) may also contribute directly to phagocytosis. As for indirect effects of ROS, radical deficiency in macrophages may influence for example the expression of phagocytic receptors. Indeed, CD36, a component of the nonopsonic receptor CD36/oV that specifically recognizes oxidized PS on apoptotic neutrophils [13] and CD14, another receptor required for macrophage phagocytosis of apoptotic cells [14], are downregulated on resident, peritoneal CGD macrophages [4]. We have previously demonstrated altered gene expression in monocytes from persons with CGD [5, 15], thus expression and/or activity of the phagocytic machinery may also be regulated albeit indirectly by macrophage radicals.

We and others have provided evidence that NADPH oxidase-derived ROS alter basic neutrophil and macrophage functions that negatively influence the efficiency by which apoptotic neutrophils are phagocytosed by macrophages. ROS deficiency indirectly leads to, for example, enhanced neutrophil infiltration [16, 17], delayed neutrophil apoptosis [18], diminished oxidized PS on apoptotic neutrophils [16, 19], elevated production of inflammatory cytokines [5, 15], and a potential skewing towards pro-inflammatory M1 macrophages [4]. Herein we provide evidence that macrophage radicals also have immediate, direct effects on the phagocytosis of apoptotic neutrophils and this presumably occurs as the result of oxidation of macrophage cell surface molecules. These data emphasize the imperative and extensive role(s) that radicals play in the clearance of apoptotic neutrophils and, in a more general sense, as anti-inflammatory agents required for resolving inflammatory processes and preventing the development of sterile inflammatory or autoimmune diseases.

Acknowledgements

The research leading to these results has received funding from the European Community’s Seventh Framework Programme (FP7/2007–2013) under grant agreement number 221094 (J.B./K.L.B.), the Swedish Medical Research Council, King Gustaf V Memorial Foundation (Sweden), Gothenburg Rheumatism Association, Swedish Rheumatism Association and the Swedish state under the LUA-ALF agreement. The authors thank Dr. P. Johnson and D. Birkenhead (UBC, Canada) for technical advice and discussion on macrophage protocols as well as Mustafa Matlak (GU, Sweden) for valuable technical assistance.

References


