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Inhibition of the Human Neutrophil NADPH Oxidase by *Coxiella burnetii*

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Abstract

Coxiella burnetii is an obligate intracellular Gram-negative pathogen. A notable feature of *C. burnetii* is its ability to replicate within acidic phagolysosomes; however, the mechanisms utilized in evading host defenses are not well defined. Here, we investigated human neutrophil phagocytosis of *C. burnetii* (Nine Mile, phase II; NMII) and the effect of phagocytosed organisms on neutrophil reactive oxygen species (ROS) production. We found that opsonization with immune serum substantially enhanced phagocytosis of NMII. Human neutrophils phagocytosing opsonized NMII generated very little ROS compared to cells phagocytosing opsonized *Staphylococcus aureus*, *Escherichia coli*, or zymosan. However, phagocytosis of NMII did not affect the subsequent ROS response to a soluble agonist, indicating inhibition was localized to the phagolysosome and was not a global effect. Indeed, analysis of NADPH oxidase assembly in neutrophils after phagocytosis showed that translocation of cytosolic NADPH oxidase proteins, p47^{phox} and p67^{phox}, to the membrane was absent in cells phagocytosing NMII, as compared to cells phagocytosing *S. aureus* or activated by phorbol myristate acetate. Thus, phagocytosed NMII is able to disrupt assembly of the human neutrophil NADPH oxidase, which represents a novel virulence mechanism for this organism and appears to be a common mechanism of virulence for many intracellular pathogens.

Keywords

NEUTROPHIL; NADPH OXIDASE; *COXIELLA*; PHAGOCYTOSIS; SUPEROXIDE ANION

Introduction

Coxiella burnetii is an obligate intracellular Gram-negative pathogen that causes a debilitating disease known as Q fever (reviewed in [1]). This zoonotic pathogen is highly infectious, and inhalation of aerosolized bacteria is the most common mode of transmission, although the ingestion of contaminated food has also been suggested as an infectious route [1]. One of the notable features of *C. burnetii* is its ability to replicate within acidic phagolysosomes of permissive host cells, including professional phagocytes (reviewed in [2]). Once internalized, these bacteria can persist in the phagolysosome in the form of small cell variants [3,4]. Approximately two days after infection, the bacteria-containing phagosomes fuse with each other and endocytic vesicles to form large replicative vacuoles (LRV), where the bacteria morph into the replicative large cell variant that multiplies [3,4].

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Currently, not much is known regarding the mechanisms utilized by *C. burnetii* to persist in the phagolysosome, and lipopolysaccharide (LPS) is the only known virulence factor for this organism [2]. Indeed, *C. burnetii* exists as two phase variants based on the LPS structure expressed on the cell surface [5]. Virulent phase I bacteria, which can be isolated from natural sources and infected individuals, express a full-length LPS (Nine Mile, phase I; NMI). In comparison, the much less virulent phase II bacteria express a truncated LPS (Nine Mile, phase II; NMII) [5]. While phagocytosis of NMI and NMII is different, with phase II generally being more susceptible, most studies indicate that once the bacteria are internalized, both phases replicate with similar kinetics within indistinguishable LRV in phagocytes from humans, non-human primates, and guinea pigs [2]. The one exception is macrophages isolated from some mouse strains, which apparently respond differently to NMI and NMII infections [6].

Activation of the innate immune system results in an inflammatory response, which is essential for rapidly controlling infections before they can spread. Phagocytes are especially critical to the acute inflammatory response, due to their capacity to efficiently engulf and destroy a variety of pathogens. Among the phagocytes, neutrophils are the most numerous and are usually the first cell to arrive at sites of inflammation. To defend the host, neutrophils utilize a wide range of microbicidal products, such as oxidants, microbicidal peptides, and lytic enzymes [7]. The generation of microbicidal reactive oxygen species (ROS) by neutrophils results from the activation of a multi-protein enzyme complex known as the NADPH oxidase (reviewed in [8]). Indeed, Brennan et al. [9] reported that the NADPH oxidase contributes to control of *C. burnetii* infections and that NADPH oxidase-deficient mice were attenuated in their ability to control *C. burnetii* infection.

Previous studies have shown that several intracellular pathogens are able to interfere with the neutrophil NADPH oxidase, which represents an important host evasion mechanism (reviewed in [10]). Likewise, it has been reported that NMI failed to stimulate human neutrophil superoxide production [11], suggesting the possibility that this organism may utilize a similar approach to establish itself during early stages of infection, although little is known regarding mechanisms involved in this process. Previously, Baca et al. [12] reported that *C. burnetii* possesses significant acid phosphatase activity and suggested this activity contributed toward inhibition of human neutrophil NADPH oxidase activity through an undefined mechanism. Aside from these studies, there have been no other reports addressing the effects of *C. burnetii* on neutrophil NADPH oxidase function.

In this report, we investigated the effect of NMII on human neutrophil NADPH oxidase activity and show that phagocytosis of NMII inhibits assembly of the oxidase in the phagosomal membrane, resulting in significantly reduced ROS production. The inhibition of NADPH oxidase assembly represents a potential virulence mechanism utilized by *C. burnetii* to evade killing in the phagolysosome.

Materials and Methods

Bacteria preparation and labeling

C. burnetii phase II (Nine Mile, phase II, clone 4, RSA493; NMII) was cultured in African green monkey kidney (Vero) fibroblasts and purified seven days post-infection using Renografin density gradients, as previously described [3]. For labeling, the bacteria were washed twice with 100 mM NaHCO₃ (pH 8.3), resuspended in the same buffer at 3×10^{10} /ml, and 182 µg/ml Alexa 488 was added. After incubation in the dark for 1 hr at room temperature, the bacteria were washed with DPBS and stored at 4°C until use.

Killed NMII were prepared by incubation of bacteria for 24 hr in 70% ethanol, followed by washing three times with RPMI. Killing was confirmed using a bacterial viability kit (LIVE/

DEAD® BacLight™, Invitrogen, Carlsbad, CA). Viability of ethanol-treated NMII was < 0.25%. Ethanol treatment was used instead of formalin because we found that treatment for 24 hr with 10% formalin did not kill all bacteria (~10% still viable), which is consistent with previous observations [13].

S. aureus (strain COL) was grown in tryptic soy broth containing 0.5% glucose. Bacterial cultures were inoculated from overnight cultures with a dilution of 1/100, and incubated at 37°C with shaking to mid-exponential phase (OD₆₀₀ of 0.75). For labeling, the bacteria were washed twice with 100 mM NaHCO₃ (pH 8.3), resuspended in the same buffer at 10⁹/ml, and 10 µg/ml Alexa 488 was added. After incubation in the dark for 1 hr at room temperature, the bacteria were washed with DPBS, resuspended in DPBS, and stored at 4°C until use.

E. coli (strain K91) was grown in LB medium overnight at 37°C with shaking. Bacterial cultures were inoculated from overnight cultures with a dilution of 1/100, and incubated at 37°C with shaking to mid-exponential phase (OD₆₀₀ of 0.75). The bacteria were labeled with Alexa 488 as described above for *S. aureus*.

Bacteria were opsonized by addition of an equal volume of human serum and incubation for 30 min at 37°C. The bacteria were then washed twice with DPBS and resuspended in RPMI containing 10 mM Hepes, pH 7.4 (RPMI/Hepes). Sera used for these studies included immune and non-immune serum. Convalescent immune serum was obtained from a donor with demonstrated antibodies to NMII antigens (John Hunter Hospital, NSW, Australia).

Neutrophil Isolation

Blood was drawn from healthy donors using a protocol approved by the Montana State University Institutional Review Board. Neutrophils were purified from human blood using dextran sedimentation followed by Histopaque 1077 gradient separation, as previously described [14]. Cell preparations were routinely >95% pure, as determined by light microscopy, and >98% viable, as determined by trypan blue exclusion.

Phagocytosis Assays

Flow cytometry—Purified human neutrophils, resuspended at 10⁷ cells/ml in RPMI/Hepes, were incubated with various concentrations of the indicated bacteria for the desired times at 37°C. The samples were then diluted with 2 volumes of ice-cold quench buffer (DPBS containing 5% heat inactivated fetal calf serum and 7.5 mM azide) and analyzed by flow cytometry using a FACSCalibur (BD Biosciences, San Jose, CA). A total of 10,000 events were collected for all samples.

Confocal microscopy—Purified human neutrophils plated in the wells of quartz-bottom microtiter plates were incubated for 30 min at 37°C with Alexa 488-labeled NMII or *S. aureus*. In some experiments, cells were labeled with the Vybrant Lipid Raft Labeling Kit (Molecular Probes) prior to phagocytosis to label the plasma membranes with Alexa Fluor 594-conjugated cholera toxin B. After phagocytosis, the samples were washed, fixed with 1% paraformaldehyde in DPBS⁺, and analyzed by confocal microscopy using a Zeiss LSM 510 Meta confocal laser-scanning microscope (Carl Zeiss, Inc., Thornwood, NY). Images were acquired using a 63× Plan-Apochromat oil immersion objective (1.4 NA) at 1024×1024 pixel resolution.

Measurement of ROS Production

Microscopic NBT Assay—Purified human neutrophils plated in the wells of quartz-bottom microtiter plates were incubated for 25 min at 37°C with opsonized Alexa 488-labeled NMII or *E. coli* and NBT solution, as described [15]. The reactions were stopped by addition of 1%

paraformaldehyde in DPBS⁺, and the cells were analyzed by confocal microscopy, as described above.

Quantitative NBT Assay—For enhanced sensitivity, quantitative NBT assays were performed to determine intracellular and extracellular O₂^{•−} production, as described by Choi et al. [16]. Briefly, NMII and zymosan were opsonized by incubation for 30 min at 37°C in RPMI containing 50% immune serum and 10 mM Hepes, pH 7.4. After washing with RPMI/10 mM Hepes, the opsonized bacteria were mixed with human neutrophils at the indicated ratios, NBT was added, and the samples were incubated for 30 min at 37°C. The cells were pelleted by centrifugation, and the supernatants were collected for analysis. The cell pellets were sonicated in 480 µl of 2 M KOH, and 560 µl of DMSO was added. After vortexing, these samples and samples from the intact cell supernatants were aliquotted into microtiter plate wells, and absorbance was measured at 620 nm.

Chemiluminescence Assay—Neutrophil ROS production was analyzed using the chemiluminescent probe, L-012, as described previously [17]. Neutrophils (10⁶ cells/tube) were incubated for 15 min with various concentrations of bacteria as indicated, pelleted by centrifugation, and resuspended in L-012 assay buffer containing HBSS, 25 µM L-012, and 5 µg/ml HRP with and without 50 U/ml SOD. Samples were aliquotted into the wells of black microtiter plates (10⁵ cells/well in 25 µl), and reactions were initiated by addition of 175 µl L-012 assay buffer (± SOD) containing fMLF (1 µM final concentration) or PMA (100 ng/ml final concentration). Chemiluminescence was monitored for 45 min with a Fluoroskan Ascent FL microtiter plate reader (ThermoElectron, Milford, MA) at 37°C. Chemiluminescence measured in the presence of SOD was subtracted from the total chemiluminescence, and the results are expressed as the integrated SOD-inhibitable response (arbitrary units).

NADPH Oxidase Translocation Assay

Translocation assays were performed, as described previously [18] with slight modifications. Briefly, neutrophils were incubated with NMII (500:1) or *S. aureus* (20:1) in RPMI/Hepes for 30 min or 100 ng/ml PMA for 10 min at 37°C and quenched by addition of 3 volumes of ice-cold quench buffer. The cells were pelleted, resuspended in quench buffer, treated with 0.5 µl/ml diisopropylfluorophosphate for 15 min on ice, washed, and resuspended at 10⁸ cells/ml in 10 mM Hepes, pH 7.4, 1 mM EGTA, 150 mM sucrose, Sigma protease inhibitor cocktail, and 1 mM PMSF. The cells were then lysed by N₂ cavitation at 4°C, and the cell lysates were fractionated on discontinuous sucrose density gradients. The membrane band was collected for each sample, and protein content was determined using the BCA method. Translocated NADPH oxidase proteins were analyzed by SDS-PAGE and immunoblotting of samples normalized to protein content, as described below.

Electrophoresis and Immunoblotting

SDS-PAGE using 7–18% polyacrylamide gradient gels and Western blotting were performed, as described previously [19]. Transfers were probed with previously characterized monoclonal antibodies against gp91^{phox} (54.1), p22^{phox} (44.1), and p67^{phox} (81.1), and a polyclonal antibody against p47^{phox} (R360) [19], followed by HRP-conjugated goat anti-mouse or anti-rabbit secondary antibodies (BioRad, Richmond, CA) and chemiluminescence development. Prestained molecular weight standards (Amersham Biosciences, Piscataway, NJ) were included on all gels for reference.

Results

Opsonization is required for phagocytosis of *C. burnetii* phase II by human neutrophils

Although a number of studies using different species and different types of phagocytes have evaluated *C. burnetii* phagocytosis, the results vary widely regarding the requirement for opsonization. Thus, we evaluated this issue in order to define a system for addressing the effects of NMII on NADPH oxidase assembly/activation following phagocytosis. As shown in Figure 1A, opsonization with *Coxiella*-immune serum significantly enhanced phagocytosis of NMII by human neutrophils (~4-fold increase). Phagocytosis of opsonized and unopsonized *S. aureus* was essentially the same (data not shown), so we opsonized in subsequent experiments to control for this variable. Although human neutrophils phagocytosed *S. aureus* and immune serum-opsonized NMII with similar kinetics (Figure 1B), confocal microscopy showed that a larger number of *S. aureus* was phagocytosed per neutrophil (~3-fold more than NMII at a similar ratio of bacteria to cells) (Figure 1C versus 1D), which explains the difference in fluorescence intensity observed in panel A. Nevertheless, it is clear that the human neutrophils effectively phagocytosed opsonized NMII, and this system was used to evaluate effects on NADPH oxidase function. Note that the enhancement of *Coxiella* phagocytosis by opsonization suggests that F_c-mediated uptake is important in phagocyte interactions with these bacteria. Furthermore, opsonization of all bacteria in our studies tends to equalize the systems, leading to comparable uptake of control bacteria and *Coxiella* involving F_c-mediated activation.

Phagocytosis of *C. burnetii* phase II attenuates human neutrophil ROS production

To determine if phagocytosis of *Coxiella* altered the ability of the cells to generate ROS, we performed a microscopic NBT assay. As shown in Figure 2, phagocytosis of positive control *E. coli* resulted in a large amount of formazan precipitation in many of the cells (Panel 2C, arrowheads) where the bacteria were being killed, as compared to control cells without bacteria (Panel 2A). In addition, some formazan precipitates were observed outside of the cells, which is possibly due to extracellular ROS or debris from lysed cells. Indeed, many of the *E. coli* were destroyed, resulting in fewer numbers of fluorescent bacteria seen in these samples. Surprisingly, very little NBT reduction occurred in neutrophils phagocytosing NMII, even though the cells had phagocytosed bacteria (Figure 2B, green fluorescence). We did observe an occasional cell with formazan precipitate (Figure 2C, arrowhead); however, there was a substantially lower generation of ROS compared to that induced by *E. coli*. Thus, these data suggested that NMII may indeed be inhibiting the NADPH oxidase, resulting in loss of oxidant production.

The microscopic NBT assay is a qualitative assay often used for clinical diagnosis [15]. To obtain a quantitative measurement of these results, we utilized a quantitative NBT assay, which allowed us to determine both intracellular and extracellular ROS production. Initially, we performed this assay using *S. aureus* as a positive control; however, we found that *S. aureus* alone gave a high background signal, possibly due to bacterial respiration (data not shown). Thus, we used opsonized zymosan for a positive control, as it had a low background in the absence of neutrophils and induced a high level of ROS. In confirmation of our microscopy results, phagocytosis of NMII induced very little neutrophil ROS production, even at a ratio of 500 bacteria per cell (Figure 3). In contrast, opsonized zymosan induced significant levels of ROS at all concentrations tested (Figure 3). Indeed, the level of ROS produced by neutrophils treated with one zymosan particle/cell was higher than that produced by cells exposed to the highest level of NMII. With both bacteria, the majority of ROS were produced intracellularly (>95%), as would be expected for cells phagocytosing particles. Furthermore, the ability of NMII to inhibit neutrophil ROS production appears to require, at least in part, metabolically-active bacteria, as phagocytosis of ethanol-killed NMII resulted in production of a significant

level of intracellular ROS, although still not as high as that induced by zymosan (Figure 4). Again, very little extracellular ROS production was observed in these cells (data not shown).

Recently, Carlyon et al. [20] reported that exposure of neutrophils to *Anaplasma phagocytophilum* mobilized neutrophil NADPH oxidase reserves so that subsequent activation by a soluble agonist was diminished. Using a similar assay, we found that pre-exposure of neutrophils to a range of NMII concentrations (up to 1000 bacteria per neutrophil) had no effect on subsequent activation of the cells by *N*-formyl peptide (fMLF), while pre-exposure of the cells to as low as 10 *S. aureus* per neutrophil resulted in a significant loss in ROS production induced by subsequent treatment with fMLF (Figure 5). Thus, phagocytosis of NMII attenuates phagocytosis-induced ROS production but does not seem to alter the subsequent response to a soluble agonist, suggesting inhibition is localized to the phagolysosome and is not a global inhibition of the neutrophil response.

Neutrophil NADPH oxidase assembly is inhibited in cells phagocytosing *C. burnetii* phase II

Our results clearly show that phagocytosis of NMII attenuates/inhibits the neutrophil respiratory burst, suggesting the possibility that NADPH oxidase assembly might be disrupted. Thus, we analyzed effects on NADPH oxidase assembly. Analysis of NADPH oxidase subunit translocation in neutrophils after phagocytosis showed cells phagocytosing NMII had substantially decreased translocation of cytosolic NADPH oxidase proteins (p47^{phox} and p67^{phox}) to the membrane, as compared to cells phagocytosing *S. aureus* or activated by phorbol myristate acetate (PMA) (Figure 6). Although PMA is a soluble agonist, the translocation of NADPH oxidase cytosolic proteins induced by PMA is qualitatively similar in to that observed in neutrophils phagocytosing opsonized zymosan, which has been reported previously by a number of groups (e.g., [21–23]). Note that the levels of flavocytochrome b subunits, p22^{phox} and gp91^{phox}, were similar in all treatments (Figure 6). Thus, it is clear that at least one of the mechanisms contributing to attenuated ROS production in neutrophils phagocytosing *C. burnetii* is the disruption of NADPH oxidase assembly.

Discussion

Obligate intracellular bacteria are adapted to persist and even proliferate in the extreme environment of the phagolysosome. Thus, these pathogens have developed a variety of evasion mechanisms to avoid phagocyte defense mechanisms, including disruption of phagosome maturation and perturbation of the respiratory burst (reviewed in [10]). Indeed, recent studies by several groups have shown that one of the key evasion mechanisms used by a variety of intracellular pathogens involves disruption of the NADPH oxidase. For example, Carlyon et al. [20] reported that *A. phagocytophilum* resides in a parasitophorous vacuole that excludes both flavocytochrome b subunits and also has the ability to directly detoxify ROS. Allen et al. [24] showed that NADPH oxidase targeting is disrupted by *Helicobacter pylori*, resulting in absence of targeting to the phagolysosome, and Lodge et al. [25] reported that *Leishmania donovani* promastigote lipophosphoglycan blocks NADPH oxidase assembly at the phagosome, possibly by disrupting phagosome lipid microdomains. *Francisella tularensis* has also been shown to inhibit NADPH oxidase assembly, and all NADPH oxidase components were excluded from phagosomes by an unknown mechanism, facilitating escape of this pathogen from the phagosome [26]. Finally, Lin and Rikihisa [27] recently reported that *Erichia chaffeensis* inhibits ROS production in human monocytes by actually degrading p22^{phox}. Thus, it is evident that many pathogens have targeted the NADPH oxidase as a key invasion strategy; however, the mechanisms involved are not well understood and seem to vary among different pathogens.

In the present studies, we show that an additional intracellular pathogen, *C. burnetii* phase II, also targets the phagocyte NADPH oxidase as a mechanism of evasion and intracellular

persistence. In neutrophils phagocytosing NMII, ROS production was substantially diminished, and this effect was due to disruption of NADPH oxidase assembly, even though normal amounts of flavocytochrome b were present in membranes of these cells. Thus, this evasion mechanism is similar to that used by *L. donovani*, where recruitment of the cytosolic NADPH oxidase proteins was inhibited [25]. Conversely, the presence of normal levels of gp91^{phox} and p22^{phox} in membranes of cells phagocytosing NMII distinguishes the oxidase-inhibition mechanism used by this pathogen from those used by *A. phagocytophilum*, *H. pylori*, *F. tularensis*, and *E. chaffeensis*.

The ability of NMII to disrupt human neutrophil NADPH oxidase assembly suggests a possible explanation why NMI failed to stimulate neutrophil ROS production in previous studies [11, 12] and why NMII also failed to stimulate this response in the present studies. Although the mechanisms involved in disruption of oxidase assembly are currently unknown, this process appears, at least in part, to be an active process that requires live bacteria, since phagocytosis of killed NMII did not inhibit, but actually activated neutrophil ROS production. One virulence factor that could possibly be involved in oxidase inhibition is an acid phosphatase. Indeed, Baca et al. [12] previously reported high levels of acid phosphatase activity in NMI and suggested this phosphatase may be involved in inhibition of NADPH oxidase activity by NMI lysates. In support of this idea, Remaley and coworkers [28] reported that acid phosphatases from *L. donovani* and *Legionella micdadei* also inhibited human phagocyte ROS production. Phosphatase inhibitors have been shown to enhance phagocyte NADPH oxidase activity [29], and it has been proposed that tyrosine phosphatases are negative regulators of the NADPH oxidase [30]. In addition, a protein tyrosine phosphatase (SHP-1) has been shown to inhibit gp91^{phox} and p47^{phox} expression by interfering with transcription in myeloid cell lines [31]. Interestingly, Forget and coworkers [32] showed that *L. donovani* could also induce macrophage protein tyrosine phosphatases and that modulation of phagocyte SHP-1 favors its survival and propagation within its mammalian host. Thus, *L. donovani* utilizes two approaches to increase phosphatase activity during infection, one through expression of its own phosphatase and one through activation of host phosphatases. Whether NMII phosphatases do indeed play an active role in inhibition of neutrophil NADPH oxidase assembly remains to be determined and is the focus of future studies. In addition to inhibiting NADPH oxidase assembly, *C. burnetii* also expresses antioxidant enzymes, such as SOD and catalase, and these have also been proposed to contribute to intracellular persistence [33,34]. Thus, like many other intracellular pathogens, *C. burnetii* appears to utilize multiple mechanisms to avoid the toxic ROS generated by phagocytic leukocytes, possibly at different stages of infection.

The requirement for opsonization of NMII for phagocytosis remains controversial and may depend on the cell type, bacterial variant, and species. Wisseman et al. [35] reported that killed NMII was phagocytosed more readily than killed NMI by human neutrophils and that immune serum enhanced phagocytosis of killed NMI but not killed NMII organisms. Akporiaye et al. [11] reported that human neutrophils incubated with either opsonized or unopsonized NMI failed to generate ROS; however, the level of phagocytosis was not evaluated. Here, we show that nonimmune serum-opsonized live NMII are not phagocytosed efficiently by human neutrophils, while opsonization with immune serum significantly enhanced phagocytosis. Thus, the rate of phagocytosis of live *C. burnetii* seems to be different from that of killed bacteria, which would explain some of the variance in our findings from previous studies. In addition, it is apparent that guinea pig macrophages more readily phagocytose unopsonized *C. burnetii*, as compared to human neutrophils [36]. Likewise, we recently found that human MonoMac 1 macrophage cells also readily phagocytosed unopsonized NMII (K. Lubick and M.A. Jutila, unpublished data), suggesting that at least part of the differences observed in phagocytosis rates between studies may be due to the type of phagocyte being evaluated. Clearly, future studies are necessary to evaluate this issue.

The ability of *C. burnetii* to evade initial phagocyte host defenses is essential in order for this intracellular pathogen to persist and replicate in the phagolysosome. Since phagocytes utilize a variety of approaches to eliminate pathogens, including the generation of toxic ROS, successful pathogens have developed a range of mechanisms to thwart phagocyte defenses. Here, we show that phagocytosed NMII is able to disrupt assembly of the human neutrophil NADPH oxidase, resulting in diminished ROS production by cells infected with this organism. The ability to disrupt ROS production by host phagocytes appears to be a common mechanism of virulence for many intracellular pathogens. In future studies, it will be important to define the molecular mechanisms and pathogen-derived products responsible for this important evasion strategy.

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Figure 1.

Phagocytosis of NMII by human neutrophils is enhanced after opsonization with immune serum. **Panel A:** Alexa 488-labeled NMII were opsonized with non-immune and *Coxiella*-immune human serum, as described. As a positive control, Alexa 488-labeled *S. aureus* were opsonized with the same serum (*S. aureus* phagocytosis was similar for all sera; shown are bacteria opsonized with the *Coxiella*-immune serum). The treated *S. aureus* (20:1) and NMII (100:1) were incubated for 30 min with human neutrophils, and the samples were analyzed by flow cytometry. Cells alone received no bacteria. **Panel B:** Opsonized Alexa 488-labeled *S. aureus* (20:1) or opsonized Alexa 488-labeled NMII (500:1) were incubated for 30 min with human neutrophils for the indicated times, and the samples were analyzed by flow cytometry.

Cells alone received no bacteria. In both panels, 10,000 events were collected for each sample, and the data are presented as the mean fluorescence intensity $\pm$ SEM and are representative of at least 3 independent experiments. Statistically significant differences compared to NMII opsonized with nonimmune serum are indicated (* P <0.05; ** P <0.001). **Panels C and D:** Human neutrophils were plated in quartz-bottom microtiter plate wells, stained with Alexa Fluor 594-conjugated cholera toxin B, and incubated for 30 min with opsonized Alexa 488-labeled *C. burnetii* NMII (100:1) or opsonized Alexa 488-labeled *S. aureus* (20:1). The cells were then washed, fixed, and analyzed by confocal microscopy. The cells shown are representative of three independent experiments.

Figure 2.

Analysis of human neutrophil ROS production in cells phagocytosing NMII or *E. coli* using a microscopic NBT assay. Human neutrophils were plated into quartz-bottom microtiter plate wells and incubated for 30 min with control buffer (Panel A), opsonized Alexa 488-labeled *E. coli* (20:1, Panel B), or opsonized Alexa 488-labeled NMII (100:1, Panel C) in the presence of NBT, as described. The cells were analyzed by confocal microscopy. All panels show differential interference contrast (DIC) images, and panels B and C show merged images of DIC and Alexa 488 fluorescence. The cells shown are representative of three independent experiments.

Figure 3.

Analysis of human neutrophil ROS production in cells phagocytosing NMII or zymosan using a quantitative NBT assay. Human neutrophils were incubated without bacteria (Control) or with the indicated concentrations of opsonized NMII or zymosan particles for 30 min at 37°C in the presence of NBT. The cells were then centrifuged, the supernatants (extracellular ROS) and pellets (intracellular ROS) were extracted, and absorbance at 620 nm was measured. The results are expressed as mean±SEM of triplicate samples and are representative of at least 3 independent experiments.

Figure 4.

Analysis of human neutrophil ROS production in cells phagocytosing live and killed NMII. Human neutrophils were incubated without bacteria (Control), opsonized zymosan (20:1), opsonized live NMII (100:1), or opsonized ethanol-killed NMII (100:1) for 30 min at 37°C in the presence of NBT. The cells were then centrifuged, the supernatants (extracellular ROS) and pellets (intracellular ROS) were extracted, and absorbance at 620 nm was measured. Similar to what is shown in Figure 3, little extracellular ROS was generated by cells phagocytosing zymosan or any of the NMII samples (not shown). The results are expressed as mean±SEM of triplicate samples and are representative of at least 3 independent experiments.

Statistically significant differences ($P < 0.001$) compared to control cells (a) and cells treated with live NMII (b) are indicated.

Figure 5.

Pre-exposure of human neutrophils to NMII does not diminish ROS production in response to subsequent stimuli. Human neutrophils were incubated without bacteria (Control) or with the indicated concentrations of *S. aureus* or NMII for 15 min at 37°C. The cells were washed, aliquotted into microtiter plate wells $\pm$ SOD, and activated by addition of 1 μ M fMLF (upper and middle panels) or 100 ng/ml PMA (lower panel). ROS production was monitored using a chemiluminescence-based assay, and the results are expressed as SOD-inhibitable chemiluminescence, as described. Representative of 5 independent experiments.

Figure 6.

Phagocytosis of NMII disrupts translocation of NADPH oxidase cytosolic proteins to the membrane. Human neutrophils were incubated with control buffer (lane 1), opsonized *S. aureus* (20:1, lane 2), or NMII (500:1, lane 3) for 30 min or with 100 ng/ml PMA (lane 4) for 10 min at 37°C, and membranes were prepared as described. Samples were normalized for protein content and analyzed by immunoblotting with antibodies against the indicated NADPH oxidase proteins, as described. The location of prestained molecular weight standards is shown for each blot. Representative of 3 independent experiments.