THE USE OF NITRIC OXIDE FREE RADICAL SENSITIVE FLUOROPHORES TO DETECT MACROPHAGE PHAGOLYSOSOME ACTIVITY

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ABSTRACT

Macrophages are phagocytes which facilitate innate immunity via phagocytosis, averting antagonistic effects resulting from bacterial infections. This is a strictly choreographed event initiated by bacterial-macrophage interactions between pathogen associated molecular patterns and toll-like receptors in macrophages. Consequently, the pathogen is ingested by the macrophage through a vacuole which matures to obtain an arsenal of antimicrobial properties including nitric oxide free-radicals (NO•). Inducible nitric oxide synthase is an enzyme accountable for NO• production upon stimulation. This study utilized opportunistic pathogens Staphylococcus epidermidis, Serratia marcescens, an assemblage composed of the two species and a Lipopolysaccharide positive control to challenge the murine macrophage J774 Cell-line. Phagolysosome activity was assessed using NO• sensitive fluorophore, DAF-FMDA. Fluorescence activity was measured for 300 seconds using a Nikon Eclipse TE2000 fluorescence microscope and DXM1200F camera. In all treatments, maximal fluorophore activity was attained within 20 seconds; level of fluorophore activity was dependent on the treatment. S. epidermidis and the bacterial assemblage initiated relatively high activities (RFU = 73.86 ± 3.52 SD; RFU = 56.66 ± 4.74 respectively), comparable to the positive control (RFU = 71.66 ± 0.90). S. marcescens induction of fluorophore activity occurred, but to a lesser extent (RFU = 48.72 ± 3.36), over 20 seconds. The current study suggests the Gram positive S. epidermidis incites relatively high levels of NO• synthesis similar to the positive control which was primed with commercial Lipopolysaccharide in challenged macrophages while the NO• levels induced by Gram negative S. marcescens were inferior. The response to the bacterial assemblage largely mimicked the response to S. epidermidis alone suggesting macrophages preferentially phagocytosed this species. This study suggests that immune responses by macrophages depend on the bacterial species and therefore does not follow a consistent pattern, particularly in short term NO• synthesis.

Keywords: Macrophage, phagocytosis, phagolysosome, iNOS, Nitric Oxide, DAF-FM DA, J774

INTRODUCTION

Living organisms are constantly challenged by microorganisms present in the environment and therefore should manifest effective strategies to cope with infections which cause disease by such microorganisms. These strategies include immune responses, which can be categorised as innate and acquired immunity. Innate immunity represents the first line of defence which is relatively rapid in infections which cause disease by such microorganisms. These strategies include immune responses, which can be categorised as innate and acquired immunity. Innate immunity represents the first line of defence which is relatively rapid in infections which cause disease by such microorganisms. These strategies include immune responses, which can be categorised as innate and acquired immunity. Innate immunity represents the first line of defence which is relatively rapid in infections which cause disease by such microorganisms.

Macrophages are professional phagocytes which facilitate the primary line of immunological defence, preventing antagonistic effects resulting from inhalation of foreign particles, including bacteria. Macrophages represent an important fraction of the innate immunity, whereby pathogens are killed by the process of phagocytosis. Macrophages are professional phagocytes which facilitate the primary line of immunological defence, preventing antagonistic effects resulting from inhalation of foreign particles, including bacteria. Macrophages represent an important fraction of the innate immunity, whereby pathogens are killed by the process of phagocytosis.

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Escherichia coli which is not susceptible to NO\textsuperscript{•} damage despite being susceptible for its derivatives such as RNSO and ONOO (Fang, 1997). Differential effects of Gram negative and Gram positive bacteria upon phagocytosis are supported by a limited amount of literature. In particular, a study carried out by Paul-Clark, Mc Master et al., in 2006 using Staphylococcus aureus (Gram positive) and Escherichia coli (Gram negative) to assess the long-term phagolysoosome activity of murine macrophages revealed that Gram negative bacteria induce a significant increase of NO\textsuperscript{•} production, mediated by TLR-4 while its counterparts induced a delayed NO\textsuperscript{•} production, mediated by collaborative interactions between TLR-2, 1 and 6 (Aderem 2000). Responses as such are a result of interactions between bacterial constituents such as LPS and LTA. Therefore, this particular study sought to examine this hypothesis further, with the use of Staphylococcus epidermidis and Serratia marcescens in short term immunological responses. Additionally, the behaviour of a heterogeneous bacterial assemblage upon the phagolysosome activity of macrophages is described as a synergetic interaction between Gram positive and Gram negative bacteria (Paul-Clark et al., 2006). Thus, this association will be assessed using a model heterogeneous bacterial assemblage in comparison to a single bacterial colony using fluorescence microscopy. In light of this study, selected bacterial strains are either part of the human flora or commonly encountered nosocomial /community acquired opportunistic-pathogens. S. epidermidis is Gram positive coccus shaped nosocomial pathogen which causes endocarditis through medical transplant devices (Jones et al., 2005b). Similarly, S. marcescens is another Gram positive rod-shaped opportunistic-pathogen which can cause infections such as bacteraemia (Krzymińska et al., 2010). Phagocytosis assays are performed using the robust murine alveolar macrophage J774 cell-line in which NO\textsuperscript{•} production is conspicuous in comparison to human macrophages (Tiscornia et al., 2009). Detection of NO\textsuperscript{•} in this study is facilitated by the use of a novel fluorophore, 4-amino-5-methylamino- 2', 7'-difluoro-1,8-dihydro-acridine (DAF-FM DA), which is more sensitive and photostable in comparison to its other family members (Nagano 2009). The mechanism in which produced free radicals interact with the fluorescent probe to create fluorescence is depicted in Figure 1 below.

**Cell-line cultivating and enumeration**

Murine alveolar macrophage J774 cell line was maintained in T25 flasks containing 5 ml Dulbecco’s modified eagle medium (DMEM, Fisher, UK) supplemented with 10% foetal bovine serum (FBS; Sigma-Aldrich, UK) and 1% 50 mg/ml Streptomycin (Fisher, UK). At three day intervals, macrophages were passaged by scraping and inoculating into fresh T25 flasks containing fresh medium. Incubation conditions for the cell-line were 37°C with 5 % CO\textsubscript{2}. Concentration of confluent macrophages was justified by performing a trypan blue (0.4%; Sigma-Aldrich, UK) exclusion assay using a 50 μl sample using an inverted microscope. A working concentration of 10\textsuperscript{5} cells ml\textsuperscript{-1} was achieved by dilutions using fresh DMEM medium.

**Fluorescence microscopy**

This aspect of the study was conducted using a Nikon Eclipse TE2000 microscope equipped with B filters for DAF- FM DA encompassing a wavelength of 510 – 580 nm. Confluent macrophages were transferred to microscope slides with whole media (DMEM and supplementation) prior to incubating for an hour in 37°C with 5% CO\textsubscript{2}. Afterwards, experimental slides were suspended in 1 ml RPMI 1640 and 0.2% pluronic acid (Sigma-Aldrich, UK) with 5 μM DAF- FM DA (Sigma-Aldrich, UK) followed by incubation for 45 minutes. Subsequently, cover slips were washed with 1 ml PBS (pH 7.0) and incubated for 15 minutes for further esterification as mentioned in the manufacturer’s protocol. The first phase consisted of challenging macrophages with individual S. epidermidis and S. marcescens communities with 100:1 multiplicity of infection (MOI), for five minutes in triplicates for each experiment. Macrophages were then challenged with a model bacterial assemblage composed of S. epidermidis and S. marcescens (1:1) with a combined MOI of 100:1 for five minutes. Macrophages primed with pure LPS served as positive controls for this study. Fresh coverslips containing macrophages were incubated with 10 mg ml\textsuperscript{-1} Lipopolysaccharides from Escherichia coli 055:B5 (Sigma-Aldrich, UK) for 24 hours followed by an one hour incubation with media containing fluorescent probe before examining through fluorescence microscopy. In parallel, cover slips were incubated with potent iNOS inhibitor N\textsubscript{2}-Methyl-L-arginine acetate salt (L-NMMA) (Sigma-Aldrich, UK) at a working concentration of 10 mM followed by incubation for 16 hours at 37°C with 5% CO\textsubscript{2} prior to fluorescent staining with DAF- FM DA. These were then infected according to the above mentioned method which served as negative controls per experiment. All experiments were done in destructive triplicates. Multiple macrophages were analysed (typically n=7) in each cover slip for a total of three cover slips per experiment.

**Imaging protocol and software**

Images were taken using Time lapse\textsuperscript{(i)} imaging software. Imaging protocol included capturing a bright field picture at 20 seconds post-infection and capturing fluorescent images every 20 s for a period of five minutes. Such images were taken at a magnification of 400X. Resulting images were analysed using Image Hopper 2.0 (in house software) image analysis software to obtain relative fluorescence images from each fluorophore used. The process of pseudo-colouring fluorescent images with basic RGB (Red, Green and Blue) shades was carried out using Image Hopper 2.0 software.

**Statistical analysis**

Statistical analysis was performed using Microsoft\textsuperscript{(ii)} Excel 2010 office package. Data in this study are presented as means ± 1SD for each group, unless specified. Apparent differences in data were evaluated using ANOVA method which served as negative controls per experiment. All experiments were done in destructive triplicates. Multiple macrophages were analysed (typically n=7) in each cover slip for a total of three cover slips per experiment.

**RESULTS**

Macrophages primed with LPS (Positive control)

Macrophages primed with LPS derived from Escherichia coli 055:B5 virtually appeared to produced consistent amounts of NO\textsuperscript{•} over time for a period of five minutes as indicated in figure 2-A below. Readings obtained over time did not indicate any significant fluctuations as indicated in figure 3.

**NO\textsuperscript{•} production in macrophages challenged with Gram positive Staphylococcus epidermidis.**

An instant production of NO\textsuperscript{•} which peaked and plateaued overtime was observed in macrophages challenged with S. epidermidis (Figure 3-A). Synthesis of NO\textsuperscript{•} by macrophages was observed at 20 s post infection and was determined as significantly different in comparison to the negative control by a paired t-test (p<0.0001). As seen in Figure 2-C, free radicals were produced within the macrophage cytoplasm upon phagolysosome formation, which intensified in the presence of a heterogeneous bacterial assemblage compared to a single bacterial colony as shown in Figure 1. The first phase consis...
several instances before diminishing towards the end of the experiment. Colour changes from green to yellow or red indicate intense amounts of nitric oxide production due to the presence of ingested bacteria. The negative control in which L-NMMA treated macrophages were challenged with *S. epidermidis* produced significantly trivial amounts of NO (images not shown here). Fluorescent readings for this particular experiment including positive and negative controls are illustrated in figure 3-A.

**NO** production in macrophages challenged with Gram negative *Serratia marcescens*.

Macrophages challenged with Gram negative *S. marcescens* induced a relatively low production of NO which peaked and plateaued overtime (RFU = 48.72 ± 3.36). Nevertheless, a paired t-test suggested a statistically significant deference in comparison to the negative control (p=0.0001). Free radicals congregated within the cytoplasm and were formed by 20 s post infection. Relatively intense (yellow) NO formation was observed within the phagolysosome in which bacteria has been ingested (Figure 2-C). Significantly high concentrations of NO (as indicated by red or blue) which were observed in macrophages challenged with Gram positive *S. epidermidis* were not observed within macrophages challenged with Gram negative *S. marcescens* (p=0.00001). In comparison to LPS-primed macrophages, *S. marcescens* population was able to induce relatively low NO amounts in J774 macrophages (p=0.00001). Conversely, the negative control in which L-NMMA treated macrophages were challenged with *S. marcescens* produced significantly low amounts of free radicals over time as seen and figure 3-B.

![Figure 2](image28x282 to 566x638)

**Figure 2** Real time pseudo-coloured mages taken at 400X magnification of nitric oxide free-radical production for a period of five minutes in J774 macrophages treated with 4-amino-5-methylamino- 2’, 7’-difluorofluorescein diacetate (DAF-FM DA). (A) Primed with LPS derived from *Escherichia coli* 055:B5 (Positive control), (B) Potent iNOS inhibitor Nω-Methyl-L-arginine acetate salt treated macrophages challenged with the model bacterial assemblage composed of *Staphylococcus epidermidis* and *S. marcescens* (negative control ; other negative control images not shown here), (C) – challenged with *Staphylococcus epidermidis*, (D) challenged with *Serratia marcescens*, (E) challenged with the model bacterial assemblage composed of *Staphylococcus epidermidis* and *Serratia marcescens*. [Fluorescent images taken under B filters at a wavelength of 510 – 580 nm every 20 seconds; (BF) bright field].

**NO** production in macrophages challenged with the model polybacterial assemblage.

Macrophages instantly produced a high amount of NO which then diminished and gradually increased afterwards. The average value for the macrophages challenged with the bacterial assemblage between the start of the experiment till 120 seconds was 41.76 ± 10 RFU and the average value for macrophages challenged with *S. marcescens* was 38.14 ± 7.9 RFU. Hence, the start of this particular experiment provided readings which are numerically similar to the readings observed in macrophages challenged with *S. marcescens*. In addition, the reading proceeding from 180 seconds to 300 seconds was an average of 69.87 ± 4 RFU for this experiment. Similarly, the average reading from macrophage challenged with *S. epidermidis* was 73.48 ± 3.52 RFU, suggesting similarities between the above mentioned components within this study.

After 100 seconds, the readings gradually increased to a level which is similar to the readings observed in macrophages challenged with Gram positive *Staphylococcus epidermidis*. Some macrophages produced intense amounts of free radicals as indicated by red colour, whilst some macrophages produced relatively low amounts of NO. Therefore, it could be said that the macrophages behaved in an inconsistent manner. Changes which occurred within 5 minutes are illustrated in figure 3-C.

From 220 s post infection, macrophages challenged with the model bacterial assemblage produced similar readings to the readings observed in LPS-primed macrophages. However, trivial amounts of NO were produced in L-NMMA treated macrophages which were challenged with the heterogeneous model bacterial community. A paired t-test suggested that the NO production in macrophages which were not treated with L-NMMA were statistically different in comparison to the negative control (P <0.00001).

![Figure 3](image434x289 to 445x295)

**Figure 3** Average relative fluorescence unit variation (RFU) within a period of five minutes (300 seconds) for each experiment which were done in triplicates. (A) Macrophages challenged with *Staphylococcus epidermidis*, (B) Macrophages challenged with *Serratia marcescens*. (C) Macrophages challenged with the model bacterial assemblage. – Macrophages primed with *E. coli* 055:B5 Lipo polysaccharide as a positive control. – Macrophages challenged with bacteria; Methylarginine treated macrophages to inhibit iNOS function, which serves as negative controls.

**DISCUSSION**

In this study, Gram positive *Staphylococcus epidermidis* induced NO production in J774 cells within 20 seconds, which was in a numerically similar range to NO production in macrophages primed with LPS derived from *Escherichia coli* 055:B5. Many studies involving Griess assay which have used Gram positive bacterial species such as *Staphylococcus aureus* suggested a delayed response is the induction of iNOS (four hours onwards) and therefore the production of NO. Provided that Griess assay sensitivity (2-3 μM) and its detection (nitrite) is significantly deferent to fluorescence microscopy protocol used in this study, results can be anticipated to be dissimilar (**Tarpey et al., 2004**). Nevertheless, several researchers have concluded that different LTA species derived from different Gram-positive bacterial species have a varied capability of inducing NO production in macrophages and therefore is a possible explanation for observed results for macrophages challenged with *S. epidermidis* (**Keller et al., 1992, Jones et al., 2005a**). To elucidate further, a study by **Kengatharan et al., in 1998** suggested that the cooperative interactions between Peptidoglycan (PeptG) and LTA induces rapid iNOS function governed by poorly defined mechanisms, which also can be considered as theoretical explanations for the results obtained in this study.
On the other hand, studies carried out using other Gram negative bacterial species such as Escherichia coli have suggested relatively rapid induction of iNOS by using Griess assay, which again is not comparable to the technique used in the current study. Serratia marcescens in this study instantly produced NO within 20 seconds to a level which was significantly lower than the levels observed in macrophages primed with E. coli 055:BS LPS or S. epidermidis. Interestingly, a study conducted by Dil, N (2002) appreciated the differential capabilities of LPSs derived from several Gram-negative bacterial species including Serratia marcescens and Escherichia coli. Within that study it was observed that the activation of macrophages derived from S. marcescens have related relatively low induction of iNOS and thus production of NO. In addition, it has been suggested that the flagellum and LPS components within S. marcescens are believed to play an important role in immune evasion within several models including mouse peritoneal macrophages and therefore is a phenomenon known as non-activating inducible NO synthase (Nüssle 2011). Differential responses in terms of free radical production and cytokine production as a response to different bacterial-macrophage interactions have provided avenues for counteracting medical conditions such as septic shock resulting from both Gram negative and Gram positive bacteria (Kengatharan et al., 1998, Foley 2003). NO. It is evident that fluorescence microscopy facilitates the superficial visualisation of host-bacterial interactions, it is not able to provide precise structural details such as receptor-ligand interactions which may take place in bacterial-macrophage interactions. For future recommendations, it would be convenient to conduct similar work using fluorescein labelled bacteria (i.e. Fluorescein - isothiocyanate (FITC) & Rhodamine 6G) with the use of flow cytometry which will enable to monitor the TLR receptor activities and preferential ingestion of bacteria.

**CONCLUSION**

Macrophages are immune cells with ability to maintain the sterility within the host’s internal environment. Their aptitude for identification, ingestion and destruction of foreign matter such as microbes underpins the conservation of internal sterility. Within the acquired arsenal of microbial properties, macrophages possess the ability to synthesise toxic NO through cytoplasmonic iNOS. This in fact that fluorescence microscopy facilitates the superficial visualisation of host-bacterial interactions, it is not able to provide precise structural details such as receptor-ligand interactions which may take place in bacterial-macrophage interactions. For future recommendations, it would be convenient to conduct similar work using fluorescein labelled bacteria (i.e. Fluorescein - isothiocyanate (FITC) & Rhodamine 6G) with the use of flow cytometry which will enable to monitor the TLR receptor activities and preferential ingestion of bacteria.

Living organisms are constantly challenged by microorganisms present in the environment and thereby requires implicating effective strategies to avoid and cope with invasion and infection by such microorganisms. Innate immunity represents a strategy as such, whereby the first line of immunological defence is instated. Within this context, macrophages signify an important fraction of innate immunity, whereby pathogens such as bacteria are killed by the process of phagocytosis (Akira 2004). Studies which have focussed on the phagolysosomal activity have facilitated the identification weaknesses and discrepancies within the mechanism, employing different Gram positive and negative bacteria (Nüssle 2001). It has been demonstrated by phagocytosing a mix of antimonial properties comprised of free radicals (NO- and ROS) and other antimicrobial peptides, the existence of knowledge gaps for each element provides with new research avenues. Particularly, NO- production in commonly encountered Gram negative and positive opportunistic pathogens such as Serratia marcescens and Staphylococcus epidermidis has received little attention. Thus, this study facilitated to examine the NO- production in murine J774 cell-line as a response to the above mentioned bacteria. For experimental control purposes, LPS has been used in this study because of its standardised acceptance as a Gram negative bacterial endotoxin with a capability to stimulate iNOS to produce NO within J774 macrophages (Jacobs et al., 1998). This enabled comparative analysis between macrophages challenged with live bacterial communities or the model bacterial ensemble. On parallel, L-NMMA has been identified as a potent inhibitor of cytoplasmic iNOS which arrests NO- production in J774 cells (Baydoun 1994). Hence, L-NMMA treated macrophages were used as negative indicators to prove that NO- were produced by iNOS throughout the experiment (Kengatharan et al., 1998). However, during each experiment, negligible amounts of NO- were produced arguably due to the incomplete NO- inhibitory ability of L-NMMA as described by McCartney-Francis et al. (2001). The process of complete phagolysosomal killing is known to take from several minutes to ~2 hours depending on the bacterial strain (Nüssle 2011). Yet, the resulting intracellular-living bacteria are protected, given that the half-life of NO- is limited to a few seconds (Sun et al., 2003). These facts along with the ability of macrophages to modulate NO- synthesis over time was taken into consideration to assess the NO- synthesis within macrophages for period of five minutes using fluorescence microscopy (Wink et al., 2011). Typically, explanation on hyper-responsive iNOS activity (Ishii et al., 2012). The latter study which examined the short term (five minutes) NO- in J774 cell line challenged with opportunistic pathogens such as Staphylococcus epidermidis (Gram positive) and Serratia marcescens (Gram negative) provided observations which opposed observations from studies which have been carried out using typical model bacterial species such as Escherichia coli and Staphylococcus aureus. It was found that the Gram negative S. marcescens induced rapid NO- release in macrophages, yet to a significantly lower level than typical Gram negative species such as E. coli (RFU = 48.72 ± 3.36). On the other hand, Gram positive S. epidermidis induced an instant production of NO to a level which is higher than some Gram positive bacteria (RFU = 73.48 ± 3.52). Additionally, macrophages challenged with a polybacterial assemble which consisted of equal volumes of the above bacterial species suggested a mixed response, which at times mimicked each species. Yet, majority of the time macrophages preferentially interacted with S. epidermidis. In contrast to studies which monitored the short-term function of iNOS as response to bacteria, this study which used a fluorescence microscopy approach with the highly responsive fluorophore DAP- FM DA and provided insights which gives confidence to state that macrophage response in terms of NO- production is depends on the type of bacteria and could be significantly different to the patterns observed in long term studies.

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