JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

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

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doi: 10.15414/jmbfs.2014.4.1.01-05

ARTICLE INFO

Received 27. 3. 2014
Revised 19. 5. 2014
Accepted 21. 5. 2014
Published 1. 8. 2014

Regular article

OPEN ACCESS

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 TE200 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 = 73.48 ± 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 comparison to acquired immunity **(Akira, Takeda 2004)**. 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 **(Miles and Bowman *et al*., 1998)**. The phagocytic and antimicrobial potential of such macrophages can be epitomized by the lack of infections within the body under normal conditions. It is suggested that a bacterial inoculum of 10^5 can be eliminated by macrophages without the assistance of other leukocytes, depending on the aggressiveness of the bacterial strain **(Lohnmann-Matthes and Stienmuller *et al*., 1994)**. Nevertheless, the process of recognition takes place prior to internalization and sterilization of infectious bacteria.

Initial bacterial-macrophage interactions may occur indirectly by opsonisation, or directly by pathogen associated molecules (PAMP) such as Gram negative bacterial-lipopolysaccharide (LPS) or Gram positive lipoteichoic acid (LTA), which are identified by pathogen pattern recognition receptors (PRR) such as toll-like receptors (TLR) found in macrophages **(Choi, Harkewicz *et al*., 2009)**. Fundamentally, ingestion and subsequent digestion of pathogens via lysosome dependent and independent mechanisms underpin innate immunity and subsequent adaptive immunity via antigen presentation **(Vincenti 2010)**.

Phagocytosis can be exemplified as the actin-dependent process of ingesting particles/pathogens larger than 0.5 µm by macrophages **(Aderem, Ulevitch 2000)**. Pathogen engulfment occurs by ensnaring the microorganism together with the accumulated extracellular fluid into a phagosome (vacuole) followed by maturation of the phagosome through a series of strictly choreographed events **(Flannagan *et al*., 2009)**. As a result of sequential fusions with various endosomes and lysosomes, phagosome-maturation reaches the concluding phase in which a phagolysosome is formed. Successively, phagolysosomes gain a range of antimicrobial properties such as microbicidal peptides, reactive oxygen and nitrogen species (ROS and RNS).

Nitric oxide (NO) as RNS in macrophages is believed to play a pivotal role in both immunity and inflammation. In macrophages, the cytoplasmic-enzyme inducible nitric oxide synthase (iNOS or NOSII) is responsible for NO production upon stimulation from cytokines and microbial compounds. Functionality of iNOS is dependent on its ability to form a dimer in which one subunit transfers electrons from NADPH to the iron group of the other subunit for the subsequent synthesis of NO free radicals (NO•) and citrulline with the use of oxygen and L-arginine **(Alderton *et al*., 2001)**.

NO is an uncharged gas which functions with antimicrobial and tumoricidal properties in both in vitro and in vivo. Despite the production of NO• from iNOS, it has been observed that other NO congeners such as nitrogen dioxide (NO_2), peroxynitrate ($OONO^-$), S-nitrosothiols (RSNO) and many other NO varieties are being produced as a result of rapid NO• conversions **(Bogdan 2001)**. Such conversions are a product of redox interactions between NO• and ROS derivatives including superoxide and O_2^- which generates RNS **(Wink et al., 2011)**. Essentially, NO• is a non-polar and highly diffusible molecule which is able to enter cells in a similar manner to oxygen on the basis of not being confined to interact with a defined receptor. Upon diffusing into a pathogen, NO• and its derivatives are able to implicate chemical insults via oxidative stress towards several essential constituents of a pathogen **(Liu *et al*., 1997)**. For instance, pathogenic DNA is modified by NO• through deamination, strand breaks and impairment of DNA repair mechanisms. Consecutively, pathogen proteins are denatured by oxidation and enzymatic inactivation while lipid components of the pathogen are modified by peroxidation **(Fang, 1997)**. Nevertheless, it has been suggested that the antimicrobial properties of NO• are not universal for all species. The above statement can be illustrated by pathogenic

Escherichia coli which is not susceptible to NO• 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 phagolysosome activity of murine macrophages revealed that Gram negative bacteria induce a significant increase of NO• production, mediated by TLR-4 while its counterparts induced a delayed NO• 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 crocus 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· production is conspicuous in comparison to human macrophages **(Tiscornia *et al*., 2009)**. Detection of NO· in this study is facilitated by the use of a novel fluorophore, 4-amino-5-methylamino- 2', 7'-difluorofluorescein diacetate (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.

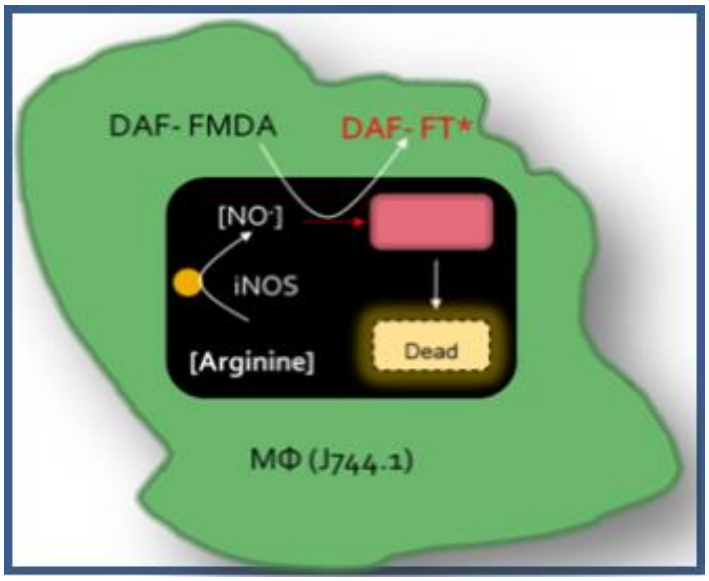

Figure 1 The interaction between produced nitric oxide free radicals (NO·) from inducible nitric oxide synthase enzyme (iNOS) and 4-amino-5-methylamino- 2', 7'-difluorofluorescein diacetate (DAF-FM DA), in which the fluorescent probe chemically converts to a triazole (DAF-FT) resulting in fluorescence.

MATERIAL AND METHODS

Bacterial cultures, enumeration

Pure cultures of *Staphylococcus epidermidis* (Oxoid, UK) and *Serratia marcescens* (Sigma-Alderich, UK) were cultured and maintained using nutrient Agar (Fisher scientific, UK). Cultures of each species were prepared separately by inoculating into Nutrient broth, which were incubated in an orbital incubator at 37°C in 150 rpm. Viable cell counts were determined through colony forming unit (CFU) counts using Miles & Misra agar plating method **(Miles *et al*., 1938)**. Bacterial growth curves were produced to determine the time to reach a mid-exponential phase cellular optical density of 0.5 for each species. Optical densities were substantiated using optical density readings via spectrophotometry at 600 nm standardised according to sterile broth. These cultures were then subjected to centrifugation at 13,500 *g* for 10 minutes at 4°C. Resulting pellets were individually suspended in 1 ml PBS (pH 7.0).

Cell-line culturing and enumeration

Murine alveolar macrophage J774 cell line was maintained in T25 flasks containing 5 ml Dulbecco's modified eagle media (DMEM; Fisher, UK) supplemented with 10% foetal bovine serum (FBS; Sigma-Alderich, 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_2. Concentration of confluent macrophages was justified by performing a trypan blue (0.4%; Sigma-Alderich, UK) exclusion assay using a 50 µl sample using an inverted microscope. A working concentration of 10^5 cells ml^{-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_2. Afterwards, experimental slides were suspended in 1 ml RPMI 1640 and 0.2% pluronic acid (Sigma-Alderich, UK) with 5 µM DAF- FM DA (Sigma-Alderich, 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^{-1} Lipopolysaccharides from *Escherichia coli* 055:B5 (Sigma-Alderich, 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 Nω-Methyl-L-arginine acetate salt [L-NMMA] (Sigma-Alderich, UK) at a working concentration of 10 mM followed by incubation for 16 hours at 37°C with 5% CO_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 Timelapse® 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 fluorescent unit (RFU) data. 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® 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 *t*-tests. Differences were regarded as significant when $p \leq 0.05$. Errors bars represent the mean standard error of trpilicates.

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· 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· production in macrophages challenged with Gram positive *Staphylococcus epidermidis*.

An instant production of NO· which peaked and plateaued overtime was observed in macrophages challenged with *S. epidermidis* (Figure 3-A). Synthesis of NO· 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

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. macrcescens* 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 challenged with *S. marcescens* produced significantly low amounts of free radicals over time as seen and figure 3-B.

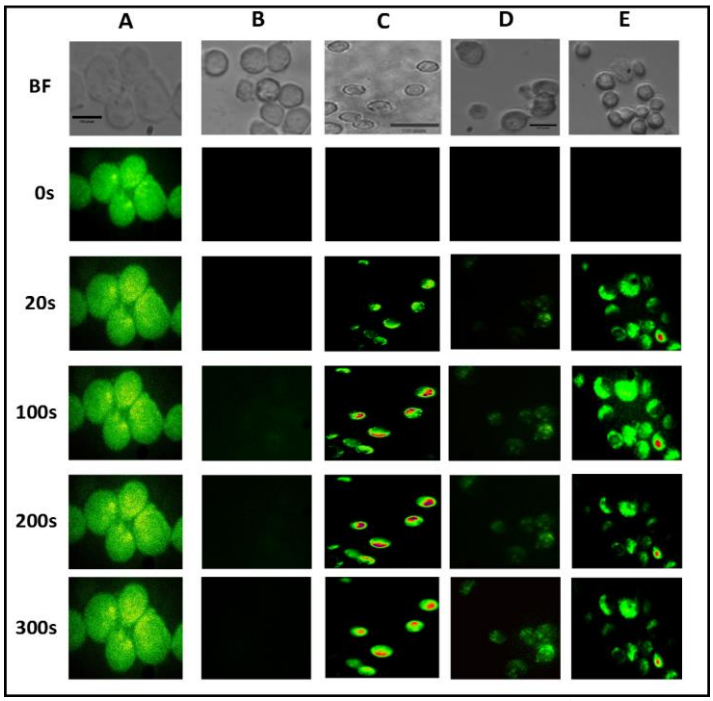

Figure 2 Real time pseudo-coloured mages taken at 400X magnification of nitric oxide free-radical production for a period of five minutes in J774 macrophages treated with 4-amino-5-methylamino- 2', 7'-difluorofluorescein diacetate (DAF-FM DA). **(A)** Primed with LPS derived from *Escherichia coli* 055:B5 (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 filed].

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 ($P <0.00001$).

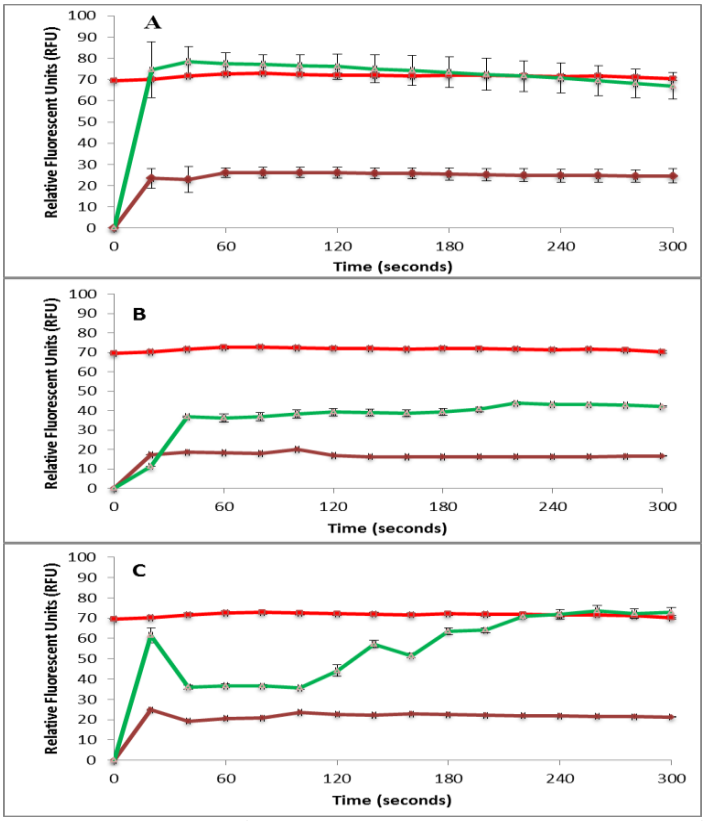

Figure 3 Average relative fluorescence unit variation (RFU) within a period of five minutes (300 seconds) for each experiment which were done in triplicates. **(A)** Macrophages challenged with *Staphylococcus epidermidis*, **(B)** Macrophages challenged with *Serratia marcescens*. **(C)** Macrophages challenged with the model bacterial assemblage. –– Macrophages primed with *E. coli* 055:B5 Lipopolysaccharide 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 (PepG) 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:B5 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 activity of iNOS within macrophages derived from chickens primed with *Serratia marcescens* have indicated 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 plausible explanation hypo-responsive iNOS activity **(Ishii *et al*., 2012)**.

In several different occasions, the polymicrobial assemblage mirrored either *S. marcescens* or *S. epidermidis*. In the beginning from 40 seconds to 120 seconds, the data suggested similar values to *S. marcescens* which then increased significantly. Beyond that point till the end of the experiment, the readings were similar to the readings demonstrated by *S. epidermidis*. Hence, the majority of the values were similar to the values resulted from *S. epidermidis*-macrophage interactions. It can be suggested that macrophages have preferentially phagocytosed *S. epidermidis* in that instance. Similarly, this could be considered as a possible indication of an immune evasion strategy implemented by *S. marcescens* in which phagocytosis is being avoided. On another note, it was observed in several occasions (t= 220 s and beyond) that the RFU values which mirrored or exceeded the values observed with macrophages treated with LPS possibly because of synergetic effect of LTA and LPS to induce high iNOS activity as described by **Paul-Clark *et al*., in 2006**. **Huang *et al*., (2011)** cited that it is generally accepted that macrophage response upon polymicrobial infections tend to diverge depending on the strains and composition of the bacterial assemblage.

NO• synthesis in phagocytosis is a result of recognition which takes place prior to internalization of bacteria. Customarily, this is achieved through interactions between a set of evolutionarily conserved TLR receptors found in macrophages and pathogen molecules found in Gram negative and positive bacteria **(Medzhitov 1997)**. In essence, the theoretical basis of NO• production in J774 macrophages subsequently challenged by either Gram negative *Serratia marcescens* and Gram positive *Staphylococcus epidermidis* can be elucidated using the interactions between the TLR and complementary ligands (**Takeuchi *et al*., 1999; Makimura *et al*., 2007; Hayashi *et al*., 2001; Schwandner *et al*., 1999)**.

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 phagolysosome activity have facilitated the identification weaknesses and discrepancies within the mechanism, employing different Gram positive and negative bacteria **(Nüsse 2011)**. Given that bacterial killing by phagocytosis is governed by an arsenal of antimicrobial 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 assemblage. On parallel, L-NMMA has been cited 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 series, negligible amounts of NO· were produced arguably due to the incomplete iNOS inhibitory ability of L-NMMA as described by McCartney-Francis et al in (2001).

The process of complete phagolysosomal killing is known to take from several minutes to ~2 hours depending on the bacterial strain **(Nüsse 2011)**. Yet, the resulting free radicals from cytoplasmic iNOS are short-lived, 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, studies involving NO• synthesis by cytosolic iNOS enzyme in macrophages have been quantified using techniques such as Griess assay or by monitoring mRNA expression profiles of the iNOS gene in macrophages. Within such studies, several variations such as heat-killed bacteria or extracted LPS/LTA contents have been used to obtain results. As oppose to such studies, fluorescence microscopy approach within study using live bacterial cultures have provided insights for macrophage interactions between Gram positive and negative bacteria, indicating initial immunological responses which cannot be obtained using Griess assay or any other method which focus on long-term immune responses.

From clinical point of view, studies carried out focussing phagolysosome activity have enabled to identify weaknesses within the mechanism under different Gram positive and negative bacterial infections **(Nüsse 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)**. Despite the 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 fluorescently 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 microbicidal properties, macrophages possess the ability to synthesize toxic n NO· through cytoplasmic iNOS. It has been evident that NO· production within macrophages as a short-term immune response is dependent on the bacterial species yet not necessarily on the Gram status. Studies which aimed examining the function of iNOS in macrophages suggested varied results. For instance, numerous studies have studied the long-term effects of iNOS utilizing Griess assay have concluded that typically macrophages are hyper-responsive to Gram negative species while it's the opposite for Gram positive bacteria. However, this statement can differ depending on the bacterial species and the immune evasion strategies they may possess. The latter study which examined the short term (five minutes) NO· in J774 cell line challenged with opportunistic pathogens such as Staphylococcus epidermdis (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 assemblage 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 mimicked the Gram positive *S. epidermidis* which could be an indication that the macrophages preferentially interacted with S .epidermidis. In contrast to studies which monitored the long-term function of iNOS as response to bacteria, this study which used a fluorescence microscopy approach with the highly responsive fluorophore DAF- 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.

Acknowledgments: This research project was conducted in the microbiology and tissue culture laboratories in Teesside University under the guidance of Dr. David Wright & Dr. Mosharraf Sarker whom I would like to thank for the support that I was given. I would also like thank Mrs. Yashaswini Nanjundaiah for her advice and the training she provided in order to complete this work successfully. Finally, I would like to thank all my friends and family for the support they have given me. The work was exclusively funded by Teesside University, Middlesbrough, United Kingdom.

REFERENCES

ADEREM, A., ULEVITCH, R.J., 2000. Toll-like receptors in the induction of the innate immune response. *Nature,* 406(6797), 782-787. http://dx.doi.org/10.1038/35021228

AKIRA, S., TAKEDA, K., 2004. Toll-like receptor signalling. *Nature Reviews Immunology,* 4(7), 499-511.

ALDERTON, W.K., COOPER, C.E., KNOWLES, R.G., 2001. Nitric oxide synthases: Structure, function and inhibition. *Biochemical Journal,* 357(3),593-615 http://dx.doi.org/10.1042/0264-6021:3570593

BAYDOUN, A., MANN, G., 1994. Selective targeting of nitric oxide synthase inhibitors to system y^+ in activated macrophages. *Biochemical and biophysical research communications,* 200, 726-736. http://dx.doi.org/10.1006/bbrc.1994.1511

BOGDAN, C., 2001. Nitric oxide and the immune response. *Nature immunology,* 2(10), 907-916. http://dx.doi.org/10.1038/ni1001-907

CHOI, S., HARKEWICZ, R., LEE, J.H. *et al*, 2009. Lipoprotein accumulation in macrophages via toll-like receptor-4-dependent fluid phase uptake. *Circulation research,* 104(12), 1355-1363. http://dx.doi.org/10.1161/CIRCRESAHA.108.192880

DIL, N., 2002. Molecular Regulation of Inducible Nitric Oxide Synthase Gene in Chicken Macrophages. North Caroline State university, http://www.lib.ncsu.edu/resolver/1840.16/3204 ,Accessed on 20/04/2013.

FANG, F.C., 1997. Mechanisms of nitric oxide-related antimicrobial activity. *Journal of Clinical Investigation,* 99(12), 2818-2825.

FLANNAGAN, R.S., COSÍO, G., GRINSTEIN, S., 2009. Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. *Nature Reviews Microbiology,* 7(5), 355-366. http://dx.doi.org/10.1038/nrmicro2128

FOLEY, E., O'FARRELL, P.H., 2003. Nitric oxide contributes to induction of innate immune responses to gram-negative bacteria in Drosophila. *Genes & development,* 17, 115-125. http://dx.doi.org/10.1101/gad.1018503

HAYASHI, F., SMITH, K.D., OZINSKY, A. *et al*., 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature,* 410(6832), 1099-1103. http://dx.doi.org/10.1038/35074106

HUANG, C., ALTIMOVA, Y., STRANGE, S. and EBERSOLE, J., 2011. Polybacterial challenge effects on cytokine/chemokine production by macrophages and dendritic cells. *Inflammation Research,* 60(2),119-125. http://dx.doi.org/10.1007/s00011-010-0242-0

ISHII, K., ADACHI, T., IMAMURA, K., *et al*., 2012. *Serratia marcescens* induces apoptotic cell death in host immune cells via a lipopolysaccharide-and flagella-dependent mechanism. *Journal of Biological Chemistry,* 287(43), 36582-36592. http://dx.doi.org/10.1074/jbc.M112.399667

JACOBS, F., CHAUSSABEL, D., TRUYENS, C., *et al*., 1998. IL-10 up-regulates nitric oxide (NO) synthesis by lipopolysaccharide (LPS)-activated macrophages: improved control of Trypanosoma cruzi infection. *Clinical & Experimental Immunology,* 113, 59-64. 10.1046/j.1365-2249(1). http://dx.doi.org/10.1046/j.1365-2249.1998.00637.x

JONES, K.J., PERRIS, A.D., VERNALLIS, A.B., WORTHINGTON, T., LAMBERT, P.A., ELLIOTT, T.S., 2005a. Induction of inflammatory cytokines and nitric oxide in J774. 2 cells and murine macrophages by lipoteichoic acid and related cell wall antigens from *Staphylococcus epidermidis*. *Journal of medical microbiology,* 54(4), 315-321. http://dx.doi.org/10.1099/jmm.0.45872-0

JONES, K.J., PERRIS, A.D., VERNALLIS, A.B., WORTHINGTON, T., LAMBERT, P.A., ELLIOTT, T.S.J., 2005b. Induction of inflammatory cytokines and nitric oxide in J774.2 cells and murine macrophages by lipoteichoic acid and related cell wall antigens from *Staphylococcus epidermidis*. *Journal of medical microbiology,* 54(4), 315-321. 315-321. http://dx.doi.org/10.1099/jmm.0.45872-0

KELLER, R., FISCHER, W., KEIST, R., BASSETTI, S., 1992. Macrophage response to bacteria: induction of marked secretory and cellular activities by lipoteichoic acids. *Infection and immunity,* 60(9), 3664-3672.

KENGATHARAN, K.M., DE KIMPE, S., ROBSON, C., FOSTER, S.J. and THIEMERMANN, C., 1998. Mechanism of gram-positive shock: identification of peptidoglycan and lipoteichoic acid moieties essential in the induction of nitric oxide synthase, shock, and multiple organ failure. *The Journal of experimental medicine,* 188(2), 305-315. http://dx.doi.org/10.1084/jem.188.2.305

KRZYMIŃSKA, S., RACZKOWSKA, M., KAZNOWSKI, A., 2010. Cytotoxic activity of *Serratia marcescens* clinical isolates. *Polish Journal of Microbiology,* 59(3), 201-205.

LIU, H., ANAND, A., BLOCH, K., CHRISTIANI, D., KRADIN, R., 1997. Expression of inducible nitric oxide synthase by macrophages in rat lung. *American Journal of Respiratory and Critical Care Medicine,* 156(1), 223-228.

LOHNMANN-MATTHES, M., STIENMULLER, C., FRANKE-ULLMANN, G., 1994. Pulmonary macrophages. *European Respiratory Journal,* 7(1), 1678-1689.

MAKIMURA, Y., ASAI, Y., SUGIYAMA, A., OGAWA, T., 2007. Chemical structure and immunobiological activity of lipid A from *Serratia marcescens* LPS. *Journal of medical microbiology,* 56(11), 1440-1446. http://dx.doi.org/10.1099/jmm.0.47327-0

MCCARTNEY-FRANCIS, N.L., SONG, X., MIZEL, D.E., WAHL, S.M., 2001. Selective inhibition of inducible nitric oxide synthase exacerbates erosive joint disease. *The Journal of Immunology,* 166(4), 2734-2740.

MEDZHITOV, R., JANEWAY Jr, C.A., 1997. Innate immunity: impact on the adaptive immune response. *Current opinion in immunology,* 9(1). 4-9. http://dx.doi.org/10.1016/S0952-7915(97)80152-5

MILES, A., MISRA, S. and IRWIN, J., 1938. The estimation of the bactericidal power of the blood. Journal of Hygiene, 38(6), 732-749.

MILES, P.R., BOWMAN, L., RENGASAMY, A., HUFFMAN, L., 1998. Constitutive nitric oxide production by rat alveolar macrophages. *American Journal of Physiology - Lung Cellular and Molecular Physiology,* 274(318-3), L360-L368.

NAGANO, T., 2009. Bioimaging probes for reactive oxygen species and reactive nitrogen species. *Journal of Clinical Biochemistry and Nutrition,* 45(2), 111-124. http://dx.doi.org/10.3164/jcbn.R09-66

NÜSSE, O., 2011. Biochemistry of the phagosome: the challenge to study a transient organelle. *The Scientific World Journal,* 11, 2364-2381. http://dx.doi.org/10.1100/2011/741046

PAUL-CLARK, M.J., MC MASTER, S.K., BELCHER, E., *et al*., 2006. Differential effects of Gram-positive versus Gram-negative bacteria on NOSII and TNFα in macrophages: Role of TLRs in synergy between the two. *British journal of pharmacology,* 148(8), 1067-1075. http://dx.doi.org/10.1038/sj.bjp.0706815

SCHWANDNER, R., DZIARSKI, R., WESCHE, H., ROTHE, M., KIRSCHNING, C.J., 1999. Peptidoglycan-and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. *Journal of Biological Chemistry,* 274(25), 17406-17409. http://dx.doi.org/10.1074/jbc.274.25.17406

SUN, J., ZHANG, X., BRODERICK, M., FEIN, H., 2003. Measurement of nitric oxide production in biological systems by using Griess reaction assay. *Sensors,* 3(8), 276-284.

TAKEUCHI, O., HOSHINO, K., KAWAI, T., et al., 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity,* 11(4), 443-451. http://dx.doi.org/10.1016/S1074-7613(00)80119-3

TARPEY, M.M., WINK, D.A. and GRISHAM, M.B., 2004. Methods for detection of reactive metabolites of oxygen and nitrogen: in vitro and in vivo considerations. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology,* 286(3), R431-R444.

TISCORNIA, A., CAIROLI, E., MARQUEZ, M., DENICOLA, A., PRITSCH, O., CAYOTA, A., 2009. Use of diaminofluoresceins to detect and measure nitric oxide in low level generating human immune cells. *Journal of immunological methods,* 342(1-2), 49-57. http://dx.doi.org/10.1016/j.jim.2008.11.014

VINCENTI, E.J., 2010. The influence of cell-free *Lactobacillus rhamnosus* GG supernatant on the phagocytic activity of macrophages. *Bioscience Horizons,* 3(2), 105-112. http://dx.doi.org/10.1093/biohorizons/hzq014

WINK, D.A., HINES, H.B., CHENG, R.Y., *et al*., 2011. Nitric oxide and redox mechanisms in the immune response. *Journal of leukocyte biology,* 89(6), 873-891. http://dx.doi.org/10.1189/jlb.1010550