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Nitric oxide production and nitric oxide synthase type 2 expression by cotton rat (*Sigmodon hispidus*) macrophages reflect the same pattern as human macrophages

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Abstract

Our knowledge of the antibacterial role of nitric oxide (NO) during infection is based on studies of murine macrophages, which secrete large amounts of NO. In contrast, human macrophages produce very little NO and its relevance as an antibacterial mediator during infection of humans is uncertain. We have defined bone marrow-derived macrophages from cotton rats (*Sigmodon hispidus*). These macrophages display phenotypical and functional characteristics similar to other rodent and human macrophages. The most interesting finding was the low level of NO production which is in contrast to findings for murine macrophages, but consistent with those of humans. In spite of these low levels, inhibition of NO production led to a decrease in killing of bacteria. Cotton rats are highly susceptible to a variety of human pathogens and therefore offer a rodent model of infectious diseases with similar characteristics to humans in terms of NO production.

Keywords

Susceptibility to infection; Macrophages; Superoxide; Nitric oxide; Immune system; Cotton rat

1. Introduction

Macrophages play a very important role in antimicrobial immunity by linking the innate and adaptive immune responses. As part of the innate immune response, macrophages are able to phagocytose and kill pathogens; as part of the adaptive immune response, they can stimulate antigen-specific immune responses through antigen presentation and secretion of cytokines. For this reason, a variety of pathogens have devised strategies to subvert macrophage functions (for review [1]). Through the study of mouse and rat macrophages it was determined that nitric oxide (NO) is a major antimicrobial molecule and produced in large quantities [2]. NO secretion is generated by nitric oxide synthase type 2 (NOS 2) which in turn is stimulated by a variety of pro-inflammatory signals like tumor necrosis factor, interferon gamma (IFN γ), and infection with a variety of pathogens. When investigators attempted to apply these findings from rodent macrophages to human mononuclear phagocytes they had difficulties demonstrating NOS2

expression and NO production (for review [3]). With improvements in techniques and reagents, recent studies have shown low levels of NOS2 expression and NO production in human macrophages ([4,5]). It is currently thought that NO expression in human macrophages is mainly involved in signal transduction rather than antimicrobial activity [3], although antimicrobial activity of NO produced by human macrophages has been established [5]. Whether this difference between human and rodent macrophages is important in the pathogenesis of infectious diseases and therefore constitutes an important aspect in which murine models differ from humans has not been established.

Cotton rats (*Sigmodon hispidus*) are a very useful animal model for infectious disease research due to their susceptibility to a variety of human pathogens (for review [6]). As a model for viral diseases, cotton rats have been used for either acute respiratory infection with measles virus [7,8], respiratory syncytial virus [9,10], metapneumovirus [11–13], parainfluenza virus [14] and influenza A virus [15,16] or chronic infection with human immunodeficiency virus [17,18], herpes simplex virus type 1 [19] and 2 [20] and adenovirus [21]. As a model for bacterial diseases cotton rats have been infected with *Helicobacter pylori* [22], *Staphylococcus aureus* [23], *Mycobacterium tuberculosis* [24], *Borrelia burgdorferi* [25,26] and *Francisella tularensis* [27]. The use of cotton rats as a model of infectious diseases has increased over the last decade, mainly due to commercially available inbred animals and the development of methods and reagents for the analysis of the immune system [6,28]. In this study, we have defined the phenotypical and functional characteristics of bone marrow-derived macrophages from cotton rats. This allows for the analysis of macrophage–pathogen interaction during infection of cotton rats with human pathogens. In contrast to other rodent and similar to human macrophages, cotton rat macrophages demonstrate low levels of NOS2 expression and NO production. The similarities between human and cotton rat macrophages might help to explain the high susceptibility of this rodent to human pathogens.

2. Materials and methods

2.1. Animals

Inbred cotton rats and C3H mice were obtained from Harlan, Indianapolis. Female animals from 6 to 10 weeks of age were used. The animals were bought specific pathogen free according to the breeder's specification and were maintained in a barrier system. Animals were kept under controlled environmental conditions of 22 ± 1 °C and a 12-h light cycle. All animals were euthanized by CO₂ inhalation.

2.2. Bone marrow-derived macrophages

Bone marrow was aspirated aseptically from the femoral diaphyseal marrow cavity of female cotton rats and cells were isolated after centrifugation. Bone marrow cells were then cultured in 10 cm perfluoroalkoxy polymer resin dishes for 7 days in the presence of 100 ng/ml recombinant mouse M-CSF (R&D Systems) in RPMI 1640 supplemented with 10% FCS, 1% non-essential amino acids, 1% sodium pyruvate, 2 mmol/L glutamine, 50 IU penicillin, 50 mg streptomycin/L and 5×10^{-5} M β -mercaptoethanol. After 7 days in culture plastic adherence of cells is characteristic of macrophage differentiation. Macrophages were primed in RPMI/10 (RPMI 1640 with 10% FCS, 1% non-essential amino acids, 1% sodium pyruvate, 2 mmol/L glutamine, 50 IU penicillin, 50 mg streptomycin/L, 5×10^{-5} M β -mercaptoethanol) supplemented with 20% tissue culture supernatant from Concanavalin A stimulated spleen cells (containing IFN γ). To produce this supernatant, $5\text{--}10 \times 10^7$ cotton rat splenocytes were cultured in RPMI/10 containing 2.5 μ g/mL Concanavalin A at 37 °C. After 36 h, 10 mg/mL methyl- α -mannopyranoside was added and supernatant obtained by centrifugation. The supernatant was filtered prior to use.

2.3. Non-specific esterase staining

Macrophages were stained for cytoplasmic non-specific esterase activity using α -naphthyl acetate (non-specific esterase) (Sigma) according to the manufacturer's instructions.

2.4. Cloning of cotton rat CD14 and production of antiserum

Isolation of the CD14 gene was performed by screening cDNA libraries constructed from LPS-stimulated cotton rat macrophages using standard recombinant DNA techniques. cDNA libraries were constructed using the SuperScriptTM Plasmid System for cDNA synthesis and plasmid cloning (Invitrogen) and transformed into DH5a maximum efficiency *Escherichia coli* (Invitrogen). To clone cotton rat CD14, sense and anti-sense degenerate primers based on the sequence homology of CD14 for various species (mouse, rat, rabbit and human) were synthesized. With these primers (see Table 1) a probe of 938 bp was amplified from cotton rat macrophage cDNA and used for screening of a cotton rat macrophage library. The full-length cotton rat CD14 gene was cloned into pcDNA-3. This plasmid was used to stably transfect L929 cells using lipofectin, and gentamycin was used as the selection agent. C3H mice were inoculated with 10^7 CD14-transfected L929 cells in weekly intervals for 3 weeks intraperitoneally. Three weeks later mice were euthanized and serum was obtained by cardiac puncture.

2.5. Flow cytometry

Spleen cells or macrophages were incubated with primary antibodies MHC I [29] (clone W6/32), MHC II [30] (clone 13/4) cotton rat-CD4 [31] or a mouse serum specific for cotton rat CD14. The secondary donkey anti-mouse serum labeled with FITC was pre-absorbed with cotton rat serum. Subsequently cells were analyzed by flow cytometry (Facsan, Becton Dickinson).

2.6. Reverse transcriptase polymerase chain reaction

Macrophages were primed with 20% supernatant from Concanavalin A stimulated spleen cells and stimulated further with 100 ng/mL LPS (*E. coli* 0127:B8; Sigma). RNA was isolated using the Qiagen RNeasy kit (Ambion). RNA concentration was calculated using 260/280 nm light absorbance (ND-1000 Spectrophotometer, Nano Drop Technologies). To ensure RNA sample integrity, each sample was probed for the housekeeping gene β actin. All reactions were also performed without reverse transcriptase to verify the absence of genomic DNA contamination. Standard RT-PCR was performed using M-MLV reverse transcriptase (Invitrogen). NOS2 was amplified by standard RT-PCR (Table 1 for primer sequences). Samples were analyzed by 2.0% agarose gel electrophoresis and stained with ethidium bromide.

Real time RT-PCR was performed using the LightCycler RNA Amplification Kit SYBR Green I (Roche) for IL12p35, IL18, TGF β , TNF α and β actin (Table 1 for primer sequences). Total SYBR Green fluorescence was measured at conditions that preserve specific gene product amplicons but not non-specific amplification products. Data were analyzed using LightCycler Software Version 3. Quantification was based upon fit points analysis with arithmetic baseline adjustment. Melting peak and melting curve analyses were performed using the polynomial calculation method. RT-PCR standards were generated by *in vitro* transcription. Template for these transcription reactions was an expression plasmid containing the complete mRNA sequence. Plasmid was linearized to introduce a consistent transcription stop site. The transcripts were treated with DNase (Ambion) to remove the template plasmid. NucAway columns (Ambion) were used to remove free nucleotides. RNA transcripts were quantified by optical density. Serial dilutions of the RNA transcripts were made for use as standards for the real time RT-PCR; samples were also analyzed by agarose gel electrophoresis (2.0%) and stained with ethidium bromide.

2.7. Bacterial killing

Bone marrow-derived macrophages (5×10^5 /well) were plated in 24-well plates, primed for 48 h with 0.5 μ g/ml recombinant cotton rat IFN γ and infected with *E. coli*. The cells were washed to remove extracellular bacteria. Cell lysates were harvested at times zero and 120 min post infection. Serial dilutions of the homogenates were plated in duplicate on LB agar plates to determine the number of viable intracellular bacteria. After incubation overnight at 37 °C, colonies were counted. In some experiments 1400 W (Sigma), an inhibitor of NOS2, was added to the macrophage media for 30 min prior to and during the 15 min incubation with *E. coli*.

2.8. Nitric oxide detection by Griess reaction

Cotton rat bone marrow-derived macrophages were plated in 96-well plates in triplicates and primed with RPMI containing 20% supernatant of Concanavalin A stimulated spleen cells (containing IFN γ). Macrophages were triggered with 100 ng/mL LPS (*E. coli* O127:B8, Sigma), infection with *Listeria monocytogenes* (EGD-P_{sod}-gfp; MOI of 10), PMA 100 ng/ml or TNF- α 10 pg/mL (R&D Systems). At 24, 36 and 48 h, 50 μ L of culture supernatant was collected and transferred to a 96-well plate. Serial dilutions of a 2mM stock of NaNO₂ solution were used to generate a standard (1.25 μ M through 100 μ M). 50 μ L of 0.1% (w/v) *N*-(1-naphthyl) ethylenediamine dihydrochloride (in 2.5% phosphoric acid) and 50 μ L of 1% (w/v) sulfanilamide (in 2.5% phosphoric acid) were added to each well. The absorbance was measured at 550 nm in a microwell plate reader.

2.9. Nitric oxide detection by electron paramagnetic resonance (EPR) spectroscopy

The nitric oxide generated by macrophages was measured using Fe-MGD complex (1mM Fe²⁺, 5 mM *N*-methyl-D-glucaminedithio-carbamate (MGD) in PBS, pH 7.4). Stimulated bone marrow-derived macrophages were incubated with Fe-MGD for 30 min, and then EPR spectra were recorded in a quartz flat cell at room temperature with a Bruker ER 300 spectrometer operating at X-band with 100 kHz modulation frequency and a TM110 cavity as described [33,34]. *S*-Nitroso-*N*-acetyl-penicillamine (SNAP, 10 μ M) was used as NO standard. NO scavenger, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO, Sigma) (50 μ M) was used to verify the specificity of the EPR signal.

2.10. Nitric oxide detection by DAF fluorescence

The intracellular NO production was determined using diaminofluoresceine-2 diacetate (DAF-2A) fluorescence. Macrophages grown on glass cover slips were preloaded with DAF-2A (10 μ M) for 30 min, washed to remove excess fluorophore and then stimulated with 100 ng/mL LPS for 6 h. SNAP (100 μ M) was used as a positive control which releases nitric oxide under physiological conditions. NO scavenger, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO, Sigma) (50 μ M) was used to verify the specificity of the DAF fluorescence. Macrophages were rinsed with PBS and examined using a Leica TCS SP2 AOBS Confocal Laser Scanning Microscope under a magnification of 40 $\times$ at 490 and 520 nm. The fluorescence intensity was quantitatively determined using Leica Confocal Software Lite.

2.11. Superoxide and hydrogen peroxide detection

Bone marrow-derived macrophages were plated in a black, clear-bottomed 96-well plate (10^6 cells/well) in replicates of six. Macrophages were primed with 10 U/mL recombinant cotton rat IFN γ (R&D Systems) for 48 h and then washed with Hank's balanced salt solution (HBSS). Subsequently, lucigenin (10 μ M) or luminol (100 mM) was added to the each well and macrophages were stimulated with PMA (100 ng/mL) or Zymosan A (100 μ g). Luminescence measurements were taken immediately (PMA) or after centrifugation (Zymosan

A) and then for every 5 min in the Microbeta Scintillation Counter (Perkin–Elmer). Cells were maintained at 37 °C between the readings.

3. Results

3.1. Culture conditions for the generation of cotton rat macrophages from bone marrow

In order to determine the antimicrobial activity of cotton rat macrophages, we established a method to culture a homogenous cell population from bone marrow and defined these cells as macrophages by phenotypical and functional criteria. These macrophages were used for phagocytosis assays and tested for their secretion of reactive oxygen and nitrogen intermediates. Based on protocols for the generation of mouse macrophages from bone marrow, we plated 5×10^6 bone marrow cells in a 10 cm Petri dish in RPMI medium supplemented with 10% FCS and 5×10^{-5} M β -mercaptoethanol. As a growth promoter L929 supernatant (which contains mouse M-CSF), recombinant mouse or human M-CSF was added. The best growth of cells from bone marrow was observed after the addition of recombinant mouse M-CSF that yielded 7.5×10^6 to 1×10^7 macrophages per plate (twice as much as after addition of human M-CSF). After the addition of L929 supernatant, cells grew poorly. The cells growing after 7 days in culture had a homogenous phenotype of large cells adherent to plastic and contained granules expressing non-specific esterase, an enzyme typically expressed by macrophages (data not shown).

3.2. Expression of cell surface markers on cotton rat macrophages

In order to define cotton rat macrophages, further, they were stained with antibodies against MHC class I (clone W6/32; [29]), MHC class II (clone 13/4; [30]), CD14 and CD4 (clone CR-CD4; [31]). W6/32 is an antibody specific for human MHC I and is cross-reactive with cotton rat MHC I by immunoprecipitation (data not shown). It demonstrates a typical pattern of MHC I expression by flow cytometry on spleen cells and macrophages obtained by peritoneal lavage (Fig. 1). 13/4 is an antibody specific for mouse MHC II; it did not immunoprecipitate cotton rat MHC II (data not shown). However, it inhibited the proliferation of measles virus specific CD4 T cells in tissue culture [31], and demonstrated a typical pattern of expression by flow cytometry on spleen cells and macrophages obtained by peritoneal lavage (Fig. 1). Staining with these two antibodies revealed that bone marrow-derived macrophages of cotton rats express MHC I, but little MHC II. In order to increase MHC II expression, macrophages were treated with recombinant IFN γ for 48 h, and this treatment increased MHC II expression (Fig. 1). For staining against CD14, L929 cells stably transfected with cotton rat CD14 were used to produce a mouse polyclonal antiserum specific for cotton rat CD14. All cells derived by culture from bone marrow expressed CD14 (Fig. 1). It has been shown that rat and human macrophages express CD4 but staining with CR-CD4 (a monoclonal antibody specific for cotton rat CD4 [31]) demonstrated no CD4 expression on cotton rat macrophages (data not shown).

3.3. Cytokine expression of bone marrow-derived macrophages

Macrophages typically express TNF α , TGF β , IL12 and IL18. The expression of these cytokines by bone marrow-derived cotton rat macrophages was determined by real time RT-PCR. TNF α , TGF β and IL18 were expressed in both stimulated and unstimulated bone marrow-derived macrophages (Fig. 2), and the expression of both TNF α and TGF β increased after stimulation. In contrast, IL12 p35 expression was found only after stimulation with IFN γ and LPS (Fig. 2).

3.4. Phagocytosis of *L. monocytogenes* by cotton rat macrophages

A hallmark of macrophage function is its antimicrobial activity through phagocytosis. Phagocytosis is defined as the binding, up-take and killing of bacteria in lysosomal compartments. In order to observe these different steps, we established both a confocal microscopy assay to visualize intracellular compartments of infected macrophages and a bacterial killing assay. For infection of macrophages a *L. monocytogenes* mutant was used in which the gene for superoxide dismutase (*sod A*) was replaced by green fluorescent protein (GFP) [32]. This mutant constitutively expresses GFP, is fully functionally in tissue culture and easily visible by UV-light microscopy (data not shown). In order to visualize different cellular compartments of macrophages and to follow the route of infection by the *Listeria* mutant, dyes staining DNA in the nucleus (Hoechst stain 33258), the F actin skeleton (Alexa Fluor 633 phalloidin) and the phagolysosome (LysoTracker Red) were used. Infected cells were analyzed at different time points by confocal microscopy. As previously published for mouse macrophages, infection with *L. monocytogenes* lead to binding and up-take of bacteria. *Listeria* infection stimulated the generation of phagolysosomes (containing bacteria) and disrupted the regularly structured cytoskeleton (actin F filaments, data not shown). In order to demonstrate bactericidal activity, macrophages were incubated with *L. monocytogenes* and lysates were prepared directly or after 2-h incubation. Cotton rat macrophages reduced the number of intracellular *L. monocytogenes* by $>0.2 \log 10$ (difference between T_0 and T_2 hours) which is considered to be bactericidal activity [35] although the difference did not reach statistical significance. As a facultative intracellular bacterium, *L. monocytogenes* is relatively resistant against lysis by macrophages. Relative to *L. monocytogenes*, activated cotton rat bone marrow-derived macrophages lysed *E. coli* (Fig. 5) more efficiently and *E. coli* was used for further phagocytosis assays.

3.5. ROI and NO generation in cotton rat macrophages

Killing of bacteria after phagocytosis by macrophages is typically achieved by a combination of methods including vesicle acidification, enzymatic digestion and reactive oxygen (ROI) and nitrogen intermediates. As a marker of antibacterial activity, the generation of superoxide, hydrogen peroxide and nitric oxide by cotton rat macrophages were measured. To stimulate the production of these molecules, cotton rat macrophages were primed with $\text{IFN}\gamma$ (2, 4, 6, 8 or 10 U/mL) or supernatant from Concanavalin A stimulated spleen cells (containing $\text{IFN}\gamma$), and triggered with 100 ng/mL LPS and *Listeria monocytogenes* (MOI = 10), PMA 100 ng/mL or $\text{TNF}\alpha$ 10 pg/mL. After the stimulation, there was an increase in the generation of superoxide and hydrogen peroxide by macrophages (Fig. 3A). However, no nitric oxide production by cotton rat macrophages was observed (Fig. 3B). In contrast, mouse bone marrow-derived macrophages secreted high amounts of nitric oxide (Fig. 3B). In order to exclude the possibility that the lack of NO secretion was specific for bone marrow-derived macrophages, peritoneal macrophages were obtained and tested with various stimuli. However, no secretion of NO was found (data not shown). The Griess reaction used to measure nitrite (a stable product of NO) has a limit of detection in the low micromolar range. Furthermore, we tested the production of NO in supernatants of cotton rat macrophages by electron paramagnetic resonance (EPR) spin trapping. Even with this sensitive technique, no nitric oxide production was observed (data not shown). To test for the presence of intracellular nitric oxide, cells were preloaded with the NO-specific intracellular fluorophore, diaminofluorescein-2 diacetate (DAF-2A), and results indicated the production of intracellular NO (Fig. 4). In addition, mRNA for inducible nitric oxide synthase 2 (NOS2) was detected by PCR from these macrophages (data not shown). These data indicate that cotton rat macrophages are very similar to human macrophages in that they express very little NO (in contrast to mouse macrophages). However, even low amounts of NO produced by human macrophages may have the antibacterial activity [5]. In order to determine whether this was also true for cotton rat macrophages, an inhibitor of NO production

was used. Macrophages treated with the NOS2 inhibitor 1400 W, demonstrated increased growth of bacteria as compared to bacterial growth in mock-treated macrophages (Fig. 5).

4. Discussion

In the present study, we have described the culture of bone marrow-derived macrophages from cotton rats and characterized them phenotypically and functionally. Culture conditions used for the generation of mouse bone marrow-derived macrophages proved to be satisfactory for cotton rat bone marrow-derived macrophages, too. Cotton rat macrophages display typical characteristics of macrophages including plastic adherence, the presence of granules containing non-specific esterase, expression of MHC I, MHC II, CD14, and production of TNF α , TGF β , IL12 and IL18 mRNA. One difference to other rodent macrophages was the expression of MHC II. In mouse and rat BMDM expression levels of MHC II molecules are high (60% [36] and 30% [37], respectively) whereas in cotton rat macrophages expression is low although it can be stimulated with IFN γ . Cotton rat macrophages also do not express CD4, similar to the mouse, but in contrast to rat and human macrophages [38]. In functional assays, cotton rat macrophages ingest bacteria into lysosomes and are able to kill them. In contrast to macrophages from a variety of rodents like mouse [39], gerbil, mastomys [40] and rats [2], cotton rat macrophages express low amounts of nitric oxide. Previous studies have shown that the induction of interferon regulatory factor 1 (IRF1) by IFN γ up-regulates the NOS2 promoter and this stimulation is counteracted by TGF β [41]. Constitutive TGF β mRNA expression is found in cotton rat macrophages. However, further studies have to elucidate whether this is the mechanical reason for the low level of NO expression.

The apparent difference between mouse and cotton rat is of particular interest for the study of infectious diseases. Mice have been used to study a variety of infectious diseases, as they are genetically and immunologically well-defined animal models. However, the increasing body of evidence demonstrate a large difference in the immune system of mouse and humans [42]. Of interest in the current study is to investigate the differences in NO generation. The production of NO by mouse macrophages in culture has been demonstrated and correlated with its antibacterial effects [39]. Further, the role of NO in the antibacterial actions has been confirmed with use of NO inhibitors and the deletion of the NOS2 gene [39]. In contrast, human macrophages secrete very little NO [43]. A large number of studies have demonstrated that the detection of NO production by human macrophages (peritoneal, alveolar or bone marrow-derived) is very difficult and often relies on the detection of NOS2 mRNA by PCR or Northern blotting or by immunohistochemistry in lung tissue [3]. These data have been interpreted as NO having more of a signal transduction function in human macrophages whereas reactive oxygen intermediates (like superoxide and hydrogen peroxide) are thought to be the effector molecules for antibacterial function. This seems to be true for the killing of a variety of bacteria, protozoa and helminthes, if chemokines, cytokines and bacterial products are used to activate macrophages [3]. However, activation of human macrophages with surfactant protein A resulted in NO production and killing of *Klebsiella* by human macrophages [5]. Interestingly enough, NO secretion by human macrophages is enhanced when alveolar macrophages are obtained from patients with lung injury, chronic lung inflammation [3] or patients with tuberculosis [44,45]. In the latter case, killing of mycobacteria *in vitro* by these macrophages was inhibited by NO inhibitors, thus indicating an antimicrobial role for NO, possibly also in humans during mycobacterial infection [46]. Currently, the mouse is an often-used animal model for the study of tuberculosis, and whether the differences in the antibacterial properties of human and mouse macrophages are relevant to study outcomes in the mouse has not been determined. Recently, it has been shown that cotton rats are an excellent model for infection studies with *Mycobacterium tuberculosis* [24]. As cotton rat macrophages display a NO secretion pattern similar to humans it would be of interest to study the role of NO in M. tuberculosis immunity in these animals.

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Fig. 1.

Expression of MHC I, MHC II and CD14 by cotton rat macrophages. Expression of MHC I, MHC II and CD14 on spleen cells, peritoneal macrophages and bone marrow-derived macrophages. MHC class II expression increased with stimulation of the bone marrow-derived macrophages for 48 h with recombinant cotton rat IFN γ prior to staining (gray no IFN γ treatment, black IFN γ treatment). Bone marrow-derived macrophages were stained with hybridoma tissue culture supernatant containing mouse anti-cotton rat monoclonal antibodies. These data are representative of three independent trials.

Fig. 2.

Levels of IL12p53, IL18, TNF α and TGF β mRNA in cotton rat macrophages. Expression levels of IL12p35, IL18, TNF α and TGF β mRNA were determined in untreated (white bars) and stimulated macrophages (gray bars) by SYBR green real time RT-PCR. The differences for mRNA expression in untreated versus stimulated macrophages were statistically significant for IL12p35 ($p < 0.001$), TNF α ($p < 0.001$) and TGF β ($p < 0.05$) whereas the difference in IL18 mRNA expression was not.

Fig. 3.

Secretion of superoxide, hydrogen peroxide and nitric oxide by cotton rat macrophages. Addition of zymosan to IFN γ primed cotton rat bone marrow-derived macrophages (10^6 per well) leads to secretion of extracellular superoxide (O_2^-) and hydrogen peroxide (H_2O_2) detected by lucigenin and luminol luminescence, respectively. Production of superoxide and hydrogen peroxide reaches a peak at 30 min post-trigger. Nitric oxide, measured as nitrite with the Griess reaction, was not detectable in the supernatant of IFN γ primed, LPS (*E. coli*) stimulated cotton rat bone marrow-derived macrophages. In contrast, nitrite was detectable in the supernatant of stimulated mouse bone marrow-derived macrophages.

Fig. 4.

Fluorescent microscopy of diaminofluorescein diacetate (DAF2A) stained cotton rat bone marrow-derived macrophages. Cotton rat macrophages were treated with the fluorophore DAF2A which can be detected by UV light (bottom) after reaction with nitric oxide as green fluorescence. The integrity of cotton rat macrophages is seen by light microscopy (top).

Priming with IFN γ does not stimulate secretion of nitric oxide (bottom left). Stimulation of IFN γ primed macrophages with LPS (*E. coli*) causes secretion of low levels of intracellular nitric oxide (bottom center, green fluorescence). Addition of 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), a nitric oxide scavenger, reduces nitric oxide specific fluorescence (bottom right). These data are representative of two independent trials.

Fig. 5. Inhibition of bacterial killing assay by inhibition of nitric oxide. Bone marrow-derived macrophages (5×10^5 /well) were plated in 24-well plates and primed for 48 h with 0.5 μ g/ml recombinant cotton rat IFN γ . Wells were incubated with 1400 W for 30 min, and then all wells were infected with *E. coli* in the presence or absence of 1400 W. Total CFUs were determined after 2 h. Killing ratio = initial viable intracellular bacterial at T0/viable intracellular bacteria at T2 h. *Significant difference between IFN and 1400 W treatment groups ($p < 0.001$). These data are representative of three independent trials.

Table 1

Sequences of primers used for the study of cotton rat macrophages.

Gene	Sense primer	Anti-sense primer	Size of amplicon in base pairs
CD14	C/A AGC G/T/C T/C G/A T/C C/T C/T C/T G/ TGCTTTGTGCTG	ACCT G/A T C/T CAG C/T C/T TGTTG T/C A A/ G CT	938
IL12p35	GGACCATAATCGGAAGCAGA	TGGAGGAGAATGCAGAGCTT	162
IL18	CACCAGAAGAAGGCTCTTGC	CTGGTCTGGGCTTCATTAGC	235
TGF β	GCCTTGTCCTACATTTGGA	CGCACGATCATGTTGGATAG	182
TNF α	TTGAGTGACAAGCCTGTAGC	CAAAGTAGACCTGCCCGGAT	433
β actin	CACCAGAAGAAGGCTCTTGC	CTGGTCTGGGCTTCATTAGC	246
NOS2	CCTCCAGGCACACAGTTGGTGG	GCCAAGAACGTGTTACCATGAG	229

The sequences for the primers used to amplify gene products from cotton rat macrophages by PCR and the expected size of the amplicon are shown.