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### Original Article

# Optimization of the Timing of Induction for the Assessment of Nitric Oxide Production in *Leishmania major* Infected Macrophage Cells

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#### **Abstract**

**Background:** The present study was conducted to investigate the optimized timing for macrophages induction and nitric oxide (NO) production against invading *Leishmania* parasite.

**Methods:** The present study examined the murine macrophage cell line, B10R, in three different states. In the first state, the cells were first infected with *L. major* and then treated with IFN- $\gamma$  and LPS as stimulants. In the second state, the cells were infected after stimulation with IFN- $\gamma$  and LPS. In the third state, the cells were only exposed to stimulants as controls. In all the three states, cell culture supernatants were collected at three points in time (6, 24 and 48 h) and the amount of NO production was measured using Griess assay.

**Results:** The treatment of macrophages with inducers prior to infection with stationary phase parasite led to the secretion of significant amounts of NO, particularly at early time points quit contrary to the cells infected with parasites prior to induction. The amount of NO produced by cells induced after infection was detected significantly lower.

**Conclusion:** The induction of macrophages prior to infection with parasites leads to the production and secretion of greater amounts of NO, resulting in an increased ability to suppress and inhibit parasite proliferation even in the early stages of infection.

### Introduction

**L**eishmaniasis is a disease caused by a unicellular parasite called *Leishmania*. This parasite is transmitted to verte-

brate hosts including humans through sandfly bites. *Leishmania* is a flagellated parasite found in two forms during its life cycle: amastigotes,

which lack flagellum and reside in mammalian host macrophages, and promastigotes, which have anterior flagella found in the body of sandflies and culture media (1). Phagocytosis and anti-leishmanial activity of macrophage are the main factors in the elimination of *Leishmania* parasites. One way to estimate the infectivity of *Leishmania* and the anti-parasite immune response is to assess the germicidal activity of macrophages through the production of reactive oxygen and nitrogen intermediates, particularly nitric oxide (NO) (2, 3).

In *Leishmania* infections, the factors that induce Th1 or Th2 immune response are directly linked to resistance and control or sensitivity and exacerbation of the disease in BALB/c mice respectively. In addition to being an essential cytokine for distinguishing Th1 responses, IFN- $\gamma$  as main Th1 cytokine plays a major role in anti-leishmanial activity of macrophages (4). IFN- $\gamma$  promotes the stimulation and production of iNOS (Inducible Nitric Oxide synthase) by the ligands derived from pathogens such as LPS (lipopolysaccharide). These enzymes then cause the conversion of L-Arginine to L-Citrulline and the production of NO in the presence of NADPH (Nicotinamide Adenine Dinucleotide Phosphate-oxidase) cofactor. Researches have indicated a strong relationship between parasite persistence and the production of NO. Inhibiting the production of NO or disrupting iNOS genes make macrophages unable to control parasites. Administration of NO inhibitors to rats resistant to *Leishmania* leads to the loss of their infection inhibition abilities (5). However, *Leishmania* reduces the production of NO by activating of Protein-tyrosine phosphatase SHP-1 and by its subsequent reaction with the molecules involved in NO production such as JAK (6, 7). iNOS-mediated NO production plays a key role in fighting against bacterial infections (such as *Mycobacterium tuberculosis* (8) and *Salmonella typhimurium* (9)) and intracellular parasites (*Leishmania* (10) and *Trypanosoma* (11)) and prevents their proliferation.

The timing of the induction of macrophages for controlling the proliferation of parasites is a vital part of in vitro studies. The present study was therefore conducted to investigate the optimum timing for macrophages induction (stimulation prior to or after infection by parasite) and the in vitro production of NO.

## Materials and Methods

### Cell culture

The B10R cell line (an immortalized murine bone marrow-derived macrophage cell line, a gift from Prof. Martin Olivier, McGill University, Canada) were cultured in DMEM media (Sigma, Germany) supplemented with 10% hiFCS (heat-inactivated Fetal Calf Serum) and incubated at 37 °C in humidified atmosphere with 5% CO<sub>2</sub>. After addition of inducers or parasite, phenol red free media was exchanged with general cell media.

### Parasite culture

The amastigote form of *L. major* parasite (strain MRHO/IR/75/ER) was isolated from lymph node of infected mice and grown at 26 °C in M199 medium (Sigma, Germany) supplemented with 5% hiFCS (heat inactivated FCS, Gibco, UK), 40 mM HEPES, 0.1 mM adenosine, 0.5  $\mu$ g/ml hemin, 2 mM L-glutamine, and 50  $\mu$ g/ml Gentamycin (Sigma, Germany). Promastigotes in the stationary phase (at 5 days) were used to infect cells.

### Cell infection

The cells were seeded at a density of  $3 \times 10^6$  cell/ml in 96-well flat bottom culture plates (Orange, USA) for 24 h. Then, the cells were infected with *L. major* in stationary phase (1:5 cell/parasite) and incubated at 37 °C with 5% CO<sub>2</sub> overnight. After 4 h, non-internalized parasites were removed by washing with culture media or phosphate-buffered saline (PBS) for three times and replaced with phenol red free media. Non-treated cells were used as negative control. Supernatants were collected and kept in -20 °C.

### Addition of inducers

To optimize the best time point for induction, the cells were induced in two different conditions with inducers (IFN- $\gamma$  and/or LPS). In state 1, the B10R cells before infection were exposed to recombinant murine IFN- $\gamma$  (rmIFN- $\gamma$ , 10 ng/ml, Gibco) alone or in combination with LPS (derived from *Salmonella abortusequi*, 10 ng/ml, Gibco). Parasite-infected cells (state 2) were treated with 10 ng/ml of rmIFN- $\gamma$  alone or in combination with 10 ng/ml LPS. All cells were further incubated with phenol free media and incubated at 37°C for 6, 24 and 48 h. In addition, no infected / stimulated cells were used as control (state 3).

### Nitric oxide assay

To measure the produced nitric oxide, the Griess assay (12) was used that includes Griess reagent (1% sulphanilamide, 0.1% naphthylethylendiamine dihydrochloride, 3% H<sub>3</sub>PO<sub>4</sub> in H<sub>2</sub>O) (all reagent from Sigma, Germany) and sodium nitrite as standard. Equal volume of Griess reagent and supernatant were mixed and incubated for 5 min at room temperature and the absorbance was measured at 570 nm. The relative NO concentration was calculated using standard curve (a serial dilution from 1 to 100  $\mu$ M of sodium nitrite).

### Data analysis

Statistical data analysis was done using GraphPad Prism version 5 software through one way ANOVA and nonparametric (Mann-Whitney) tests. Statistical differences between samples were considered significant at  $P < 0.05$ . Each test was performed in duplicate-quadruplicate and repeated at least 3 times.

## Results

### Optimization of the timing of induction in the production of NO

To study the effect of the timing of induction on NO production and secretion against *Leishmania* infections, two distinct approaches were considered. In the first approach, B10R cells were first stimulated using IFN- $\gamma$  and LPS and then infected with parasites (24 h later). In the second approach, the cells were stimulated 24 hours after infection with parasites. In both cases, cells that were only exposed to stimulants were used as controls. The cell culture supernatants were then collected and the amount of produced NO was estimated through Griess assay at three different time points, 6, 24 and 48 hours after treatment of the cells with inducers or their infection with parasites (Table 1).

**Table 1:** NO measurement by Griess assay in different conditions

States	Inducers	6 h	24 h	48 h
First induced	IFN- $\gamma$ +LPS	20.75 $\pm$ 0.39	25.48 $\pm$ 1.3	25.48 $\pm$ 1.04
	LPS	7.51 $\pm$ 0.52	11.22 $\pm$ 0.26	11.77 $\pm$ 0.52
	Infected cell	6.12 $\pm$ 0.13	8.81 $\pm$ 0.26	7.05 $\pm$ 0.91
	B10R	1.59 $\pm$ 0.78	7.05 $\pm$ 0.39	7.5 $\pm$ 1.44
First infected	IFN- $\gamma$ +LPS	-	4.09 $\pm$ 3.53	8.9 $\pm$ 4.32
	LPS	-	3.81 $\pm$ 2.19	2.42 $\pm$ 0.39
	Infected cell	-	1.74 $\pm$ 2.6	1.4 $\pm$ 0.26
	B10R	-	-	-
MQ stimulated	IFN- $\gamma$ +LPS	2.33 $\pm$ 1.04	24.37 $\pm$ 2.6	30.75 $\pm$ 0.13
	LPS	0.85 $\pm$ 0.52	12.05 $\pm$ 0.65	14.74 $\pm$ 2.88
	Infected cell	-	-	-
	B10R	-	-	-

\* All units are in  $\mu$ M.

A significantly higher amount of NO at early detection hours in the cells that were stimulated prior to infection compared to other groups. NO production in IFN- $\gamma$ -stimulated cells was calculated as  $20.75 \pm 0.39 \mu\text{M}$  with LPS, while LPS alone was calculated as  $7.51 \pm 0.52 \mu\text{M}$ . The level of NO measured was less than  $1 \mu\text{M}$  in cell culture supernatants that had been stimulated after infection and  $2.3 \pm 1.04$  and  $0.85 \pm 0.52 \mu\text{M}$  in the cell culture supernatants that had been induced by LPS+IFN- $\gamma$  and by LPS alone respectively, which showed significant differences ( $P < 0.05$ ) with the NO amounts produced by the IFN- $\gamma$ -stimulated cells (Fig. 1A).

The NO secretion increased in the supernatant after 24 hours, calculated as  $25.48 \pm 1.3 \mu\text{M}$  in state 1 and  $24.37 \pm 2.61 \mu\text{M}$  in the control group (state 3) showing no statistically significant differences (Fig. 1B). However, the different in NO level compared to state 2 was statistically significant ( $4.09 \pm 3.53 \mu\text{M}$ ).

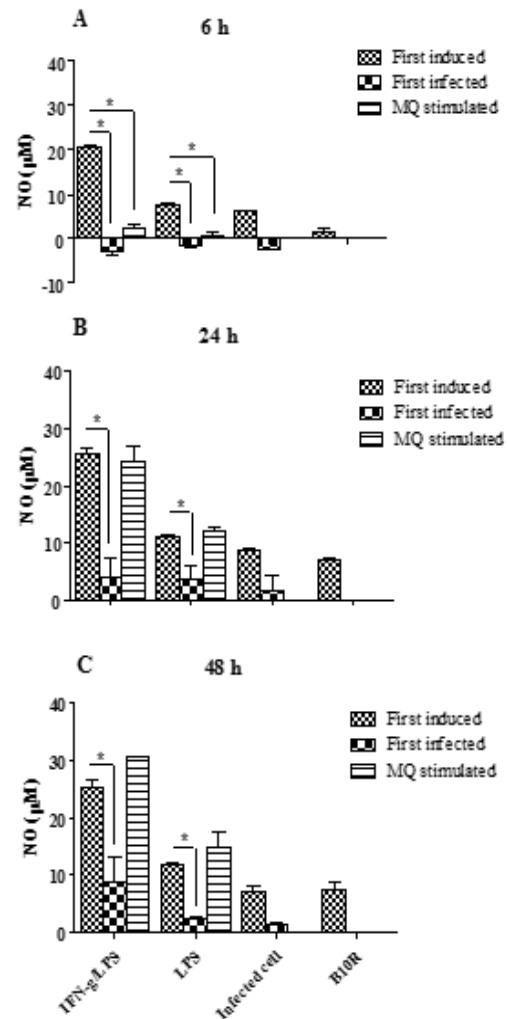
As shown in figure 1C, 48 hours later, no difference was observed between states 1 ( $25.48 \pm 1.04 \mu\text{M}$ ) and 3 ( $30.75 \pm 0.13 \mu\text{M}$ ) in amount of NO produced, but the difference with state 2 ( $8.90 \pm 4.32 \mu\text{M}$ ) still remained significant.

At different cell incubation times following stimulation with IFN- $\gamma$  and LPS, the production of NO increased with time and reached its peak 48 hours after cell stimulation.

Greater amounts of NO were produced in the cells stimulated before infection compared to the other states. The amount of NO production also increased with time in the cells that were merely exposed to stimulants. In contrast, the production of NO was inhibited in the cells that were first infected with parasites (Table 1).

## Discussion

Through the production of toxic nitrogen and oxygen products such as NO, macrophage is the main factor in the elimination of intracellular *Leishmania* (4).



**Fig. 1:** Quantification of NO production by infected/treated cells.

The NO production in B10R cells was measured in different states by Griess assay at different time points, 6, 24 and 48 h after infection or treatment: First induced: cells were first stimulated using IFN- $\gamma$ /LPS and then infected with *L. major*; First infected: cells were first infected with *L. major* then treated with *L. major*; MQ stimulated: the cells were induced/non infected. All results were shown as mean  $\pm$  SD and stars indicate significant difference between groups ( $P < 0.05$ )

IFN- $\gamma$  cytokine is the main factor in inducing the transcription of iNOS and the production of NO (4, 12). IFN- $\gamma$  causes tyrosine kinase phosphorylation, JAK1 and JAK2, and subsequently the phosphorylation and dimerization of transcription factor STAT. Activated STAT migrates to the nucleus and binds to

iNOS promoter sequences finally resulting in NO production (7). LPS is a microbial stimulant derived from the cell wall of gram-negative bacteria. In lower concentrations, LPS provides the second signal for the activation of macrophages inducing inflammatory conditions (13). Through the activation of the NF- $\gamma$  B pathway, LPS activates iNOS and leads to the production of NO. This process is augmented in the presence of IFN- $\gamma$  (14). Although NO is a lethal metabolite for various pathogens such as *Leishmania* parasites (15, 16). *Leishmania* is able to skip this lethal effect by different mechanisms including the inhibition of IFN- $\gamma$  receptor complex formation or activation of the host SHP-1 inhibitor, which inhibits phosphorylation of the JAK2 tyrosine kinase (6, 7). This finally ends in NO reduction. The inhibition of NO production is one of the principal mechanisms used by *Leishmania* to evade and suppress the host's immune system. However, cytokines such as IFN- $\gamma$  are able to inhibit parasites' evasion mechanism. Creating a balance between the activation and suppression of cytokines production throughout the anti-*Leishmania* immune responses and the elimination of parasites therefore play an essential role (17).

On the other side, the right timing for the use of cytokines for the stimulation and increasing of the anti-leishmanial activity of macrophages is therefore a vital part of *in vitro* studies (18). Pre-sensitization of macrophages by cytokines such as IFN- $\gamma$  before infection with parasites can affect intracellular functions such as parasitophorous vacuole development or the binding of vacuoles to lysosomes (18). In addition, IFN- $\gamma$  is able to regulate the expression of some *Leishmania* receptors on the macrophage surface, such as mannose (19) and complement receptor-3 (20). In animals vaccinated against *Leishmania*, IFN- $\gamma$ -activated macrophages kill intracellular parasites and thus increase the resistance to infection in tissues around the infected cell (18). The cytokine "IFN- $\gamma$ " was first used in the treatment of *Leishmania* infection in 1986 regarding due

its ability to increase the anti-leishmanial activity of macrophages and its synergy with pentavalent antimony drugs (21). The importance of the timing of interferon- $\gamma$  addition and its concentration are emphasized in articles investigating the use of this cytokine in the treatment of *Leishmania* infections. IFN- $\gamma$  administration is most effective in reducing parasitic infections before or in the early stages of infection (22). The activation of macrophages before infection with parasites thus induces anti-leishmanial mechanisms and produces cell resistance and immunity against the parasite, and the presence of NO as the main anti-leishmanial metabolite of macrophages can be helpful.

The results of the present study showed a greater amount of NO production in the B10R murine macrophage cells stimulated by IFN- $\gamma$  and LPS before infection with *Leishmania* parasite compared to the cells first infected with *Leishmania*. This difference was discernible even in the early stages of infection (6 h) and remained stable with time (24 and 48 h). The stimulation of macrophages prior to infection with parasites appears to help overcome their growth and proliferation. OTA and *et al.* reported a significantly reduced percentage of infectivity and live parasites in macrophages stimulated with IFN- $\gamma$ /IL-12 prior to infection with *Leishmania* compared to the cells infected with parasites before stimulation (18). In line with this study, Murray HW noted the crucial role of recombinant IFN- $\gamma$  and the inactive transmission of IFN- $\gamma$ -activated macrophages in inducing resistance against intracellular microorganisms including *Chlamydia trachomatis* (23).

The results of this study also showed a lower production of NO in the cells stimulated only by LPS alone compared to the cells stimulated by LPS+IFN- $\gamma$ , suggesting the heterogeneity of LPS-induced iNOS compared to the LPS+IFN- $\gamma$  induced enzyme and indicating the enzyme regulation mechanisms based on  $\beta$ -NADPH concentrations, which have been

proven in previous studies conducted on similar subjects (24).

The presence of soluble factors in the host cells such as cytokines plays an important role in the host's anti-leishmanial responses and protective Th1 responses form in mice resistant (C57Bl/6) to this parasite, which leads to the elimination of parasites and recovery from infection through macrophage activation. This type of response tends to produce large amounts of IFN- $\gamma$ . In contrast, in susceptible mice (BALB/c), a Th2 response tends to form that is characterized by IL-4 production (25, 26).

This hypothesis can also be justified by experimental studies, so that there is a significant reduction in the percentage of infected cells in the macrophage cells treated with cytokines before infection with *Leishmania* compared to controls (untreated cells). It can therefore be concluded that in vivo produced cytokines are effective in controlling infections in the host. Activated macrophages have shown less sensitivity to infection with intracellular microorganisms such as *Leishmania* (27), *Trypanosoma* (28), *Rickettsia* (29) and *Legionella* (30).

## Conclusion

Activation of macrophages and the subsequent NO production, considered as the main anti-leishmanial factor for macrophages, play an essential role in infection control, particularly during the early stages and before the parasite defense mechanisms are activated. In fact, this approach can be used in treatment procedures and the production of anti-*Leishmania* vaccines, thereby justifying further research into the anti-leishmanial mechanisms of cytokines and inducers such as IFN- $\gamma$  at play in *Leishmania* infections.

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