

# The effects of nitric oxide on the immune response during giardiasis

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## ABSTRACT

Nitric oxide (NO) is a free radical synthesized from L-arginine by different isoforms NO-synthases. NO possesses multiple and complex biological functions. NO is an important mediator of homeostasis, and changes in its generation or actions can contribute or not to pathological states. The knowledge of effects of NO has been not only important to our understanding of immune response, but also to new tools for research and treatment of various diseases. Knowing the importance of NO as inflammatory mediator in diverse infectious diseases, we decided to develop a revision that shows the participation/effect of this mediator in immune response induced against *Giardia* spp. Several studies already demonstrated the participation of NO with microbicidal and microbiostatic activity in giardiasis. On the other hand, some works report that *Giardia* spp. inhibit NO production by consuming the intermediate metabolite arginine. In fact, studies *in vitro* showed that *G. lamblia* infection of human intestinal epithelial cells had reduced NO production. This occurs due to limited offer of the crucial substrate arginine (essential aminoacid for NO production), consequently reducing NO production. Therefore, the balance between giardial arginine consumption and epithelial NO production could contribute to the variability of the duration and severity of infections by this ubiquitous parasite.

**Keywords:** nitric oxide; immune system; giardiasis.

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## INTRODUCTION

Nitrogen monoxide, also called nitric oxide (NO) is a radical with a small molecular weight (30 kDa) that performs multiple biological activities.<sup>1,2</sup> Since its discovery, an increasing number of studies have attempted to address the biochemistry of this freely diffusible second messenger and the connections with its biological properties. This molecule became the focus of intensive biological and clinical research.<sup>3,4</sup> As a consequence, pharmaceutical industries have shown increasing interest in NO manipulation in the last years, because this free radical could be used as a potential therapeutic target in a vast variety of conditions, ranging from inflammation to degenerative diseases.

It is known that NO can directly and indirectly modulate the immune response through diverse mechanisms such as mediating microbicidal effects of NO-derived free radicals, mostly within macrophages and other phagocytes. Modulation of leukocyte effector mecha-

nisms during the infection has been also observed. However, due to its regulatory effects, NO plays a dual role in the elicited response, dictated by the concentration in the inflammatory microenvironment. Of note, a deregulated NO production is observed in infected individuals, which is associated with collateral toxicity for host cells, leading to immunopathology, autoimmune responses or persistence of the parasite due to immune evasion.

## Nitric oxide production

From an evolutionary point of view, NO formation may have originated as a first-line defense for metazoan cells against intracellular pathogens. This is confirmed by the wide occurrence of the enzyme responsible for NO production: NO-synthase (NOS) responses in several species, ranging from invertebrates such as *Limulus polyphemus*,<sup>5</sup> insects,<sup>6,7</sup> to mammals and non-mammal vertebrates.<sup>8-10</sup> The NO signaling mechanisms would evolve only later for signaling. In mammals, the NO response is

raised in response to infection by a wide variety of intracellular pathogen unicellular organisms such as bacteria, yeast and parasites (e.g., *Leishmania* spp. and *T. cruzi*).<sup>11</sup> It appears that evolutionary diversity has been acquired at the level of the transcription factors involved in NO synthesis in response to different kinds of stress stimuli.

Three forms have been described: neuronal (nNOS, NOS1), endothelial (eNOS, NOS3) and inducible (iNOS, NOS2).<sup>12,13</sup> The first two are constitutively expressed (NOS1 and NOS3), but can also be upregulated to release substantial amounts of NO mainly in the central and peripheral nervous system and vascular endothelial cells, respectively. The iNOS is induced mainly in macrophages, but can be induced in a vast variety of cells when stimulated by cytokines and polysaccharides. NO is biosynthesized by one of the NO synthases (NOS) in an oxidative reaction that mediates the incorporation of molecular oxygen (O<sub>2</sub>) into the unstable intermediate NO-hydroxy-L-arginine (NOHA), and subsequently into L-citrulline.

This reaction consumes reducing equivalents in the form of NADPH. The final products of this reaction are NADP<sup>+</sup>, L-citrulline and NO.<sup>14</sup>

L-arginine is the main substrate for NO synthesis. The participation of L-arginine in the inflammatory response may occur through two basic mechanisms: (I) as precursor for NO production through the activation of iNOS, mainly in macrophages; and (II) as precursor for the synthesis of metabolites required for T lymphocyte proliferation and effector function.<sup>15,16</sup>

The regulation of NO synthesis within cells can be exerted at various checkpoints such as: compartmental distribution of NOS, changes in NOS gene expression, enzymatic competition by Arginase. Arginase activity is a major regulatory mechanism for NO biosynthesis. It leads to the production of ornithine and urea. Moreover, it can modulate inflammatory response at several levels including (I) the production of ornithine, a precursor of proline, favoring cellular regeneration, wound healing and repair;<sup>17</sup> (II) the production of polyamines, also from ornithine, which can regulate macrophage function;<sup>18</sup> (III) regulating arginine availability and, therefore, decreasing NO production and other arginine mediated processes such as normal T lymphocyte proliferation.<sup>19</sup> The decisive mechanism by which L-arginine affects the balance of the cellular immune response is ultimately determined by the predominant cytokine pattern expressed in a given pathogenic process. While NO synthases are induced or up-regulated mainly during T-helper 1 inflammatory conditions, the arginase system is mainly induced in macrophages, when the predominance of T-helper 2 cytokines.<sup>20</sup> Moreover, uncoupling NOS from L-arginine results in the generation of NO scavenger, superoxide, and peroxynitrite, repression of the translation and stability of inducible NOS protein, inhibition of inducible NOS activity

via the generation of urea, and by sensitization of NOS to its endogenous inhibitor, asymmetric dimethyl-L-arginine.<sup>21</sup>

Moreover, they are also differentially modulated according to the stimulus and the type of cytokines present in the inflammatory microenvironment.<sup>20</sup> The products and intermediary molecules from both pathways act as modulators of the immune response.

The expression and activity of enzymes involved in NO synthesis are differentially modulated depending on different biological milieu conditions.<sup>13,22</sup> The diverse actions of NO could be explained by its capacity to chemically attack several molecules containing metals. Moreover, soluble mediators of immune responses can differentially modulate the inducible isoform of NOS. It can be quickly enhanced in cells of the innate<sup>23</sup> and adaptive immune response after some cytokines are produced, mainly IFN $\gamma$ , TNF $\alpha$ , IL-1b. These cytokines are produced in response to pathogen associated molecular patterns (PAMPs), which can be sensed mainly by the action of pattern recognition receptors (PRR), such as Toll-like receptors (TLRs).<sup>24,25</sup> Signaling through these innate immune recognition receptors leads to activation of transcription factors NF- $\kappa$ B and AP-1, which regulate the expression of iNOS.<sup>26</sup>

### Effects of nitric oxide on the immune system

One of the most prominent functions of NO in the immune system is its participation in protective immunity against various intracellular pathogens including viruses, bacteria and protozoa. Furthermore, the killing activity of NO has also been showed effective in host defense against tumor cells<sup>27</sup> and alloantigens.<sup>28</sup> Considering its direct microbial toxicity, NO can exert a microbistatic or microbicidal effect, or even act as a microbial metabolic product.<sup>29</sup> Although these direct cellular effects of NO over pathogen biology are not yet fully understood, the strong oxidative capacity of NO and its sub-products appears to act in synergism with other lethal reactive oxidant species (H<sub>2</sub>O<sub>2</sub>, etc), accounting for most of the host's microbial toxicity. Peroxynitrite (ONOO) induces modifications of selected molecular targets in a parasite and is a major mechanism for direct effects of NO, and such targets are starting to be revealed.<sup>30-33</sup> Peroxynitrite can induce nitration of tyrosine residues as well as the reversible binding of NO to metal centers in microbial targets that are involved in vital processes, including nutrition and respiration. In various infections, cysteine-containing proteins, metalloproteins, calcium transport systems,<sup>34</sup> as well as essential enzymes of energy metabolism<sup>35</sup> have been identified as important intracellular targets for the toxic actions of peroxynitrite. Among them, cysteine proteinases are critical factors for virulence or replication of many pathogens (including viruses, bacteria, fungi, and parasites).<sup>36-38</sup>

Depending on the individual characteristics of molecules, it can lead to either the activation or inactivation of the respective protein function. Exacerbated production of NO and the consequent exaggerated levels of “reactive nitrogen species” (RNS) can unbalance the homeostatic mechanisms of the host, mediating collateral host toxicity and this could be the basis for certain pathologies related to nitrosative and oxidative stress.<sup>39-41</sup> Indeed, the presence of nitrosylated tyrosine residues (Nitro tyrosine) has been recognized as an indicator of cell damage and inflammation, as well as of the production of ONOO. In addition to the direct toxicity, other effects of NO over leukocyte biology were described, which can influence several physiological processes ranging from DNA transcription<sup>42</sup> and replication<sup>43</sup> to protein synthesis<sup>44</sup> and secretion. The signaling processes through which NO acts to regulate immune cells are extremely complex and are only just beginning to be revealed, but are largely indirect through generation of reactive nitrogen oxide species that chemically modify enzymes, signaling proteins and transcription factors. The role of NO might depend on the stage of a disease (i.e., early or late disease stages). And for a given cell, the response to NO will depend on its reactivity state and on the microenvironment as well.

### Nitric oxide in giardiasis

*Giardia intestinalis* is the causative agent of giardiasis, a widespread intestinal infectious disease in humans.<sup>45</sup> It is known that human infection with *Giardia lamblia* often results in severe abdominal cramps and malabsorptive diarrhea. While most infections are controlled by an effective immune response, some individuals develop chronic diseases that occur occasionally in the absence of apparent immunodeficiency.<sup>46,47</sup> Have been shown that NOS1, but not NOS2, is necessary for clearance of *Giardia* infection. The authors also suggested that increased gastrointestinal motility contributes to the parasite elimination and this is the first example of NOS1 being involved in the elimination of an infection.<sup>48</sup> In fact, the intestinal hypermotility is an important host mechanism of defense against *Giardia*. This defense appears to depend on the development of a normal adaptive immune response against the parasite, as it did not occur in mice lacking T and B-cells, although it is possible, in principle, that T or B-cells contribute to hypermotility independent of their role in adaptive anti-giardial immunity.<sup>49</sup> It is likely that similar changes in intestinal transit in humans contribute to the symptoms associated with this infection. However, it is not yet clear what immune mechanisms are responsible for effective control of infections.<sup>50,51</sup> Therefore, further investigation into the pathophysiology of giardiasis is needed as no pathogenic mechanisms have yet been identified. Based on this consideration, the following sections focus on the importance of specific host effector mechanisms against *Giardia*. Elucidation of these mechanisms is not only important for understanding mucosal immune defense

against this parasite, but also provides a crucial basis in the rational development of therapeutic strategies for activating the most effective host defenses against *Giardia*. It is believed that antimicrobial products synthesized by epithelial cells in the small intestine are good candidates. One of these is NO, which has an antimicrobial effect (microbiostatic and microbicidal) on a wide range of microorganism<sup>52-66</sup> and multiple functions, including increased cytotoxicity of activated macrophages.<sup>67</sup> In polarized intestinal epithelial cells, the stable NO end products, nitrite and nitrate, are preferentially detected on the apical side,<sup>68</sup> suggesting that *Giardia* could be a relevant target for epithelial cell-derived NO and its metabolites. The underlying mechanisms for apical NO release are not known, but might be related to the preferential localization of iNOS at the apical side of polarized epithelial cells underneath the cell membrane.<sup>69,70</sup> Consistent with a role of epithelial cell-produced NO as a potential anti-giardial effector molecule, NO was found to inhibit proliferation of *G. lamblia* trophozoites *in vitro*, but not to kill them.<sup>68</sup> Thus, NO was cytostatic rather than cytotoxic for trophozoites in these studies. Another report suggested that NO can kill trophozoites *in vitro*,<sup>71</sup> although that study used minimal media (i.e., buffered salt solution) for culture, which may have exaggerated the results, as such media do not support prolonged trophozoite viability and growth even in the absence of NO. In addition, it was observed that NO also inhibited *G. lamblia* excystation and encystation of *in vitro*.<sup>68</sup> Growth inhibition may be important for the infected host, because local trophozoite growth is probably crucial for the ability of *G. lamblia* to establish and maintain infection of the proximal small intestine. In contrast, inhibition of encystation by NO could reduce the formation and passing of infectious cysts and, thereby, transmission to other potential hosts.

Fernandes and Assreuy<sup>71</sup> studied the role of nitric oxide and superoxide in *G. lamblia* killing and verified that from the vast array of cytotoxic molecules potentially produced by defense cells, NO seems to account for the majority, if not all, of macrophage giardicidal effects. The genome from *Giardia* spp. has been completely sequenced,<sup>72</sup> leading to the conclusion that *Giardia* is an early diverging protozoon with very simplified metabolic pathways. Although *Giardia* spp. have a relatively poor tolerance to O<sub>2</sub>, they preferentially colonize the fairly aerobic upper part of the small intestine (duodenum and jejunum). They lack the conventional respiratory oxidases, as well as the systems (catalase, superoxide dismutase, glutathione reductase) responsible for the scavenging of radical oxygen species (ROS).<sup>73</sup>

Data from Li *et al.*<sup>48</sup> involving human infection with *G. lamblia*, indicate that signals mediated by enzymes nitric oxide synthases (NOS1 but not NOS2) play a key role in eliminating this infection.

The hypothesis of contact of *G. lamblia* with intestinal epithelial cells might lead to release of specific proteins.<sup>74</sup> Results have shown host-cell stimulated secretion of giardial proteins, which have the potential to be involved in the immunological events during initiation of infection and subsequent survival of the parasite in the host. *In vitro* studies with recombinant arginine deiminase showed that the secreted *Giardia* proteins could disable host innate immune factors such as nitric oxide production.

Eckmann *et al.*<sup>68</sup> detected that *G. lamblia* and intestinal epithelial cells compete for arginine and have several implications for understanding the pathogen-host interaction [between pathogen and host]. According to Eckmann, the reduction of arginine availability could be considered a virulence mechanism of the pathogen, because it inhibited epithelial NO production, thereby subverting a potential host defense against *G. lamblia*. Both intestinal epithelial cells and *G. lamblia* trophozoites have highly efficient arginine transporter systems with comparable substrate affinities suggesting that *Giardia* may have an advantage over the host in taking up arginine. Thus, in models of human intestinal epithelium, *G. lamblia* inhibited epithelial NO production by consuming arginine, the crucial substrate used by epithelial NO synthase to form NO. These results indicate that contact of *Giardia* spp. with epithelial cells triggers metabolic enzyme release, which might facilitate effective colonization of the human small intestine.

It is known that the flavodiiron proteins are widespread among strict or facultative anaerobic prokaryotes, where they are involved in the response to nitrosative and/or oxidative stress. These flavodiiron proteins were fairly recently identified in a restricted group of microaerobic protozoa, including *G. intestinalis*. Ringqvist *et al.*<sup>74</sup> proposed that in *G. intestinalis* the primary function of flavodiiron proteins is to efficiently scavenge O<sub>2</sub>, allowing this microaerobic parasite to survive in the human small intestine, thus promoting its pathogenicity.

### **B and T-cells, antibodies, cytokines and nitric oxide in human giardiasis**

Despite the clinical symptoms, diarrhea, abdominal pain, malabsorption and weight loss, infection is not accompanied by significant mucosal inflammation.<sup>75</sup> The infections are normally self-limiting, as immunocompetent hosts can control and typically eradicate *G. lamblia*, a process that involves T and B-cells.<sup>50,51,76,77</sup>

Several studies suggest an important role for B-cells in clearing *Giardia* infection. For example, infections of humans with *G. lamblia* result in the production of anti-giardial antibodies of the IgA, IgM and IgG isotypes in mucosal secretions and serum. The specific antibody production correlates with giardial clearance.<sup>50,78</sup>

Moreover, secretory antibodies of the IgA and IgM isotypes are attractive candidates for immune defense against *Giardia* spp., because they are secreted in large quantities into the intestinal lumen and their actions are antigen-specific. In this regard, parasite-specific IgA antibodies in infected children has been evaluated by several investigators.<sup>79,80</sup> In previous studies, a 31-kD protein and 57-kD heat-shock protein (HSP), strong candidates for peptide vaccine, were recognized by specific IgA antibody to *Giardia* spp.<sup>79</sup> Additionally, it was shown by immunoblotting that children with chronic infections with *G. intestinalis* did not show an IgA response to the 57-kD *G. intestinalis* HSP, which suggested an impairment of the switch from an IgM response to an IgG or IgA response.<sup>80</sup>

These results together showed that the clearance of human *G. intestinalis* infection is dependent on antibodies directed against parasite proteins and T-cell activity. However, it remains to be determined if and how cellular immune responses might contribute to control *G. lamblia* infections in humans.

Of the T-cells, CD4+ but not CD8+ cells are responsible for cytokine production operating in the recruitment of cells and the immunopathogenesis of the disease.<sup>81</sup> One of the main cytokines that appears to be associated with allergy cases in human giardiasis is the IL-6.

In fact, Mahmoud *et al.*<sup>82</sup> demonstrated that there is a direct relationship between IL-6 levels increases and improved expression of adhesion molecules: the intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) as mediators in development of skin allergy caused by giardiasis. Byraktar *et al.*<sup>83</sup> found that pro-inflammatory mediators, such as IL-6, and IL-1 $\beta$ , and a chemokine, IL-8 that attracts neutrophils to the site of infection, were not elevated in giardiasis children without allergy, suggesting that these cytokines may have no influence on immunity to giardiasis. However, in allergy-associated giardiasis patients, strong correlations between IL-6 and nitric oxide were seen.

The role of transforming growth factor-beta1 (TGF- $\beta$ 1) in intestinal parasitic infections has attracted significant attention, as *in vitro* tests showed that TGF- $\beta$ 1 stimulates the isotype switch to IgA, as well as IgA secretion by LPS-stimulated B cells from Peyer's patches and spleen.<sup>84,85</sup> Knowing this peculiar property, Taherkhan *et al.*<sup>86</sup> confirm that there was a relationship between TGF- $\beta$ 1 cytokines polymorphism and susceptibility to giardiasis. They found that the prevalence of allele C and CC genotypes of TGF- $\beta$ 1 T+869C polymorphism was significantly higher in the patients with symptomatic giardiasis and these patients had significantly lower levels of S-IgA compared to the asymptomatic and control groups.

Based in previous report, secretory IgA antibodies have a central role in anti-giardial defense. B-cell-

independent mechanisms also exist and can contribute to eradication of the parasite, although their identity and physiological importance are poorly understood currently, mainly in human models. So, the elucidation of key anti-giardial effector mechanisms will be important to understand mucosal immune defense against this parasite and suggest new pharmacological targets for drug therapy.

## CONCLUSIONS

A delicate, not completely understood interplay do exist between the components of immune response and the levels of nitric oxide. Vast scientific evidence show that NO can exert its effects on the immune response either directly or through the activity of its derivatives (mainly oxygen and nitrogen reactive species), which are able to induce structural modifications, thus altering their biological activities. In the same way, NO is able to affect the biology of *Giardia*, either by direct toxicity, or through affecting essential metabolites, or even by enhancing the immune response against the parasite. Despite the potent anti-giardial activity of NO, *G. lamblia* is not simply a passive target for host-produced NO, but has strategies to evade this potential host defense. Studies define NO and arginine as central components in a novel cross talk between a pathogen and immune response in intestinal epithelium, but the balance between giardial arginine consumption and epithelial NO production could contribute to the variability in the duration and severity of infections by this ubiquitous parasite. However, additional studies are required to further understand the multiple roles played by NO in giardiasis.

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