

Increased Cytokine and Nitric Oxide Levels in Serum of Dogs Experimentally Infected with *Rangelia vitalii*

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Abstract: This study aimed to measure the levels of interferon-gamma (IFN- γ), tumor necrosis factor-alpha (TNF- α), interleukin 1 (IL-1), interleukin 6 (IL-6), and nitrite/nitrate (NO_x) in serum of dogs experimentally infected with *Rangelia vitalii*. Twelve female mongrel dogs were divided into 2 groups; group A (uninfected controls) composed by healthy dogs (n=5) and group B consisting of dogs inoculated with *R. vitalii* (n=7). Animals were monitored by blood smear examinations, which showed intraerythrocytic forms of the parasite on day 5 post-infection (PI). Blood samples were collected through the jugular vein on days 0, 10, and 20 PI to determine the serum levels of IFN- γ , TNF- α , IL-1, IL-6, and NO_x. Cytokines were assessed by ELISA quantitative sandwich technique, and NO_x was measured by the modified Griess method. Cytokine levels (IFN- γ , TNF- α , IL-1, and IL-6) were increased ($P < 0.01$) in serum of infected animals. Serum levels of NO_x were also increased on days 10 PI ($P < 0.01$) and 20 PI ($P < 0.05$) in infected animals. Therefore, the infection with *R. vitalii* causes an increase in proinflammatory cytokines and nitric oxide content. These alterations may be associated with host immune protection against the parasite.

Key words: *Rangelia vitalii*, IFN- γ , TNF- α , IL-1, IL-6, nitric oxide, immunity, rangelirosis

Canine rangelirosis is a tick-borne protozoan disease caused by *Rangelia vitalii* that primarily affects rural and periurban young dogs in southern Brazil [1,2]. Infection with *R. vitalii* causes severe anemia, jaundice, fever, splenomegaly, lymphadenopathy, hemorrhage along the gastrointestinal tract, and persistent bleeding through the tips of the pinnae, external surface of the ears, nose, and oral cavity [3-6]. The influence of this disease on immune system parameters of infected animals is unknown.

Cytokines represent the major molecules involved in the communication among T-cells, macrophages, and other immune cells in the course of an immune response to infectious agents [7]. IFN- γ is the cytokine responsible for the function of CD4⁺ Th1 cells, which is the activation of macrophages to kill

intracellular microorganisms and synthesize other proinflammatory cytokines (TNF- α , IL-1, and IL-6) and nitric oxide (NO) [8].

NO is an important cytotoxic mediator on immune activated cells, capable to kill pathogenic agents [9]. This reactive nitrogen species is synthesized by the isoenzymes nitric oxide synthases (NOS), which include inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS) that catalyze the oxidation of L-arginine and molecular oxygen to citrulline and NO [10]. The iNOS is highly expressed in macrophages when activated by proinflammatory cytokines [11]. The half-life of NO is very short, and its indirect determination can be made by measurements of its oxidation products, nitrite (NO₂⁻) and nitrate (NO₃⁻), referred to as NO_x [12].

Numerous studies on the immune response of Th1 cells, characterized by the synthesis of proinflammatory cytokines and iNOS, have been performed in different hemoparasites [13-15]. However, the mechanism of host immune responses has not been explained in *R. vitalii* infection. Therefore, the aim of this study was to measure the levels of IFN- γ , TNF- α ,

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IL-1, IL-6, and NO_x in serum of dogs experimentally infected with *R. vitalii*.

Twelve female mongrel dogs (6- to 12-months old) were used in this study as previously described [16]. The animals, previously determined by molecular tests to be free from *Babesia* spp., *Hepatozoon* spp., and *Ehrlichia* spp. infection [16], were inoculated with *R. vitalii* (n=7) or served as uninfected controls (n=5). The *R. vitalii* strain used in this study was obtained from a naturally infected dog [17]. A fresh blood sample of this animal was inoculated (2 mL through the jugular vein) in another dog (Dog 13: male, 5-months old) for maintenance of the isolate in the laboratory. Each animal of Group B was infected through intravenous inoculation with 2 ml of fresh blood collected from Dog 13, containing an average of 6 parasites per slide, which were found inside of erythrocytes and leukocytes of blood smears [16].

The presence and degree of parasitemia were estimated for each animal every 2 days throughout the experiment. Peripheral blood smears were collected from the tip of the ear of each dog. The smears were Romanovsky stained and then examined under a microscope ($\times 1,000$ magnification), as previously described [16]. Blood collection of the jugular vein (3 ml) was performed on days 0, 10, and 20 post-infection (PI). The blood was placed in tubes without anticoagulant, centrifuged for 10 min to obtain serum and stored under freezing (-20°C). The serum was used for determination of proinflammatory cytokines (IFN- γ , TNF- α , IL-1, and IL-6) and NO_x.

The proinflammatory cytokines were quantified by ELISA, using the commercial Quantikine canine immunoassay kits (IFN- γ , TNF- α , IL-1, and IL-6), according to manufacturer's instructions (R&D Systems, Minneapolis, Minnesota, USA). Briefly, 96-well microplates were sensitized with the primary antibodies at room temperature (RT) for 30 min; the sample was added and incubated for 30 min at 37°C. After washing, the secondary antibody conjugated with peroxidase was added to each well, and a period of incubation followed. The presence and concentration of the cytokines were determined by the intensity of color measured by spectrometry by a micro-ELISA reader, Sunrine-Tecan (Tecan, Sunrise, Melbourne, Australia).

Nitric oxide levels in serum of dogs infected with *R. vitalii* were analyzed indirectly, by the quantification of nitrite/nitrate (NO_x) according to the technique described in detail by Tatsch et al. [12]. Therefore, NO_x was measured by the modified Griess method using the Cobas Mira automated analyzer (Roche Di-

agnostics, Basel, Switzerland). Results were expressed in $\mu\text{mol/L}$. The data were evaluated by the Student's t-test. Values with probability less than 5% were considered statistically different.

On day 5 PI, there were blood smears positive to *R. vitalii*. Parasitemia increased progressively until day 10 PI when a peak was observed. Then, the number of parasites was significantly reduced as described in detail by Da Silva et al. [16], and the parasite was found within erythrocytes, leukocytes, and the extracellular milieu. During the experimental period, clinical signs such as anorexia, apathy, weight loss, fever, anemia, and diarrhea were observed in dogs infected with *R. vitalii* [16]. The infected dogs showed no bleeding (a common finding in rangeliiosis) despite severe thrombocytopenia, reduction of platelet activity, and alteration in the concentrations of nucleotides and nucleosides present in platelets, participants in the process of homeostasis [18,19]. The infection by *R. vitalii* caused in these dogs reduction in serum levels of iron, zinc, and copper [20], as well as oxidative stress with increased lipid peroxidation, protein oxidation and, consequently, increase of antioxidant status to reduce cellular injury [21].

In this study, cytokine levels increased ($P < 0.01$) in infected animals compared to the control group (Fig. 1). A progressive increase in serum levels of IFN- γ (Fig. 1A), TNF- α (Fig. 1B), IL-1 (Fig. 1C), and IL-6 (Fig. 1D) was observed. An increase of nitrite/nitrate (NO_x) in serum of infected animals was observed on day 10 PI ($P < 0.01$) and 20 PI ($P < 0.05$) (Fig. 2).

This study is the first to demonstrate the increased serum levels of IFN- γ , TNF- α , IL-1, and IL-6 in dogs experimentally infected with *R. vitalii*. The increase of these proinflammatory cytokines can be attributed to activation of the immune response and parasitemia control, as related in other infections caused by hemoparasites, as babesiosis [22], ehrlichiosis [15], anaplasmosis [23], leishmaniasis [24], hepatozoonosis [14], and trypanosomosis [25].

On day 10 PI occurred the peak of parasitemia, when the levels of cytokines and NO increased significantly compared to the control group; thus this increase in the inflammatory mediators were able to reduce parasitemia of day 15 PI [16]. Serum levels of cytokines and NO increased during the course of infection, as detected on day 20 PI. Probably the high levels of inflammatory mediators on day 20 PI was the cause of low parasitemia observed during this period. According to the literature, these immunological parameters are responsible for controlling parasitemia in infections by other hemoparasites [14,15,22,24,25]. In trypanosomosis by *T. cruzi*, researchers

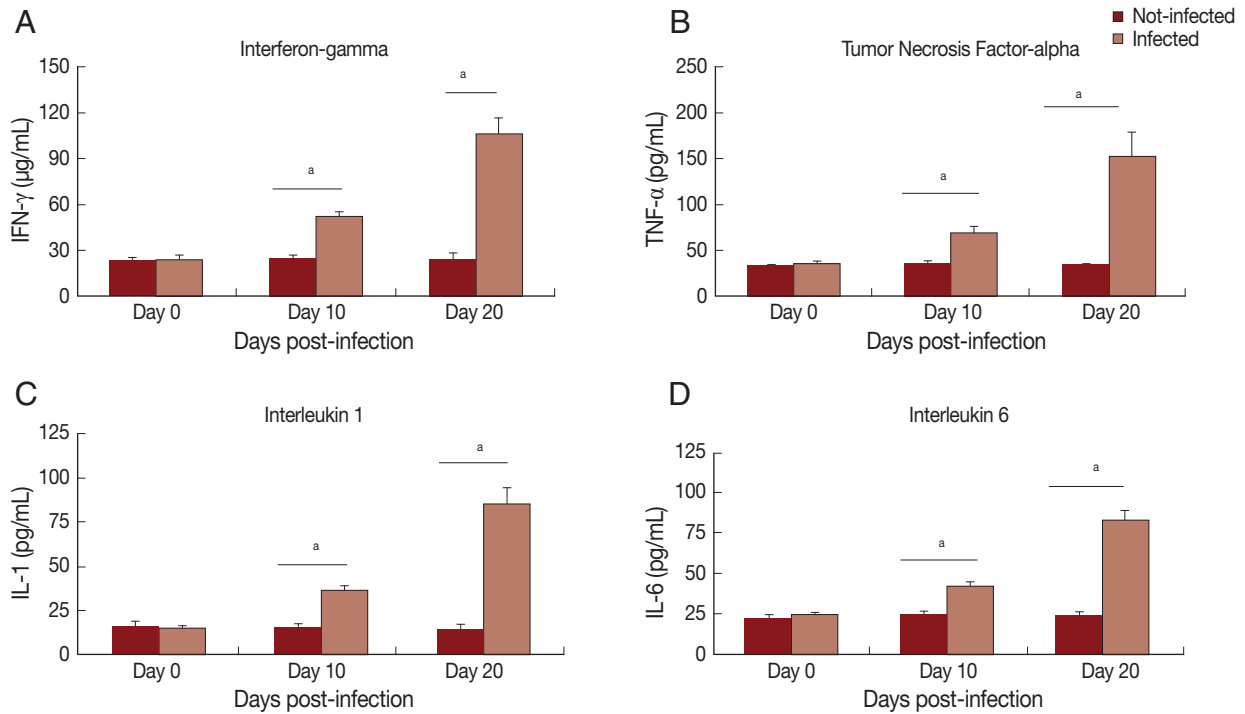


Fig. 1. Levels of proinflammatory cytokines in serum of *Rangelia vitalii*-infected dogs on days 0, 10, and 20 post-infection compared with uninfected controls. (A) IFN- γ , (B) TNF- α , (C) IL-1. (D) IL-6. ^aRepresents statistical difference between infected and control group ($P < 0.001$).

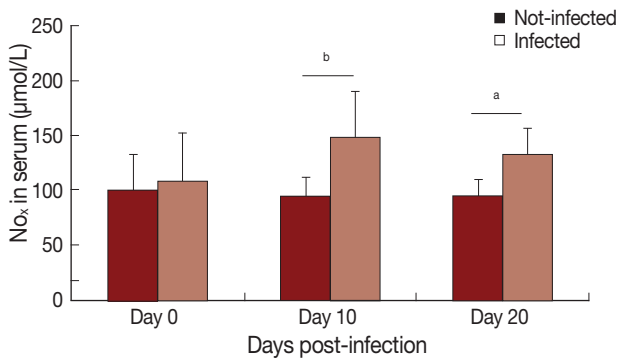


Fig. 2. Levels of nitrite/nitrate (NO_x) in serum of *Rangelia vitalii*-infected dogs on days 0, 10, and 20 post-infection compared with uninfected controls (^a $P < 0.05$, ^b $P < 0.01$).

concluded that NO may be directly related to parasitemia, severity of lesions, chronicity, and mortality of mice infected [26,27]. In vitro studies showed that NO has the ability to kill parasites like *Plasmodium* [28] and *T. cruzi* [26]. Therefore, this study suggests that both cytokines and NO are responsible for reduction and maintenance of low parasitemia in dogs infected with *R. vitalii*, as detailed below.

One early reaction of the host to infection with protozoan parasites is the secretion of an array of potent cytokines, including TNF, IL-1, and IL-6. These early responses contribute signif-

icantly to the outcome of infection by influencing its course directly and by regulating the specific immune responses against the parasite [29]. The overproduction of proinflammatory cytokines observed in this study contributes not only to control the infection, but also for the disease progress, similarly to which occurs in babesiosis [30]. The increased serum levels of proinflammatory cytokines (TNF- α , IL-1, and IL-6) could be associated with the onset of clinical signs in the acute phase of inflammatory responses (anorexia, apathy, weight loss, and fever) [8], as observed in this study.

This study found that the increase of proinflammatory cytokines coincides with the reduction of parasites in the bloodstream. A high expression of TNF- α was found in dogs experimentally infected with *Ehrlichia canis* [31]. Furthermore, TNF- α also played a role in controlling bacterial number because TNF- α receptor-knockout mice were highly susceptible in this infection [32]. Researchers reported an association between low parasite and increased IFN- γ and TNF- α expression in dogs naturally infected with *Leishmania chagasi*, indicating that these cytokines play a role in protection against infection [24]. Serum levels of TNF- α and IFN- γ increased in anaplasmosis, indicating a role of cellular immunity activation during the infection [33]. Enhanced levels of IL-1, IL-6, and TNF- α in bo-

vine babesiosis are important for stimulating immunity against protozoan pathogens [22].

High levels of NO are mediated by upregulated expression of the iNOS gene in response to the activating signals, in particular to the secretion of proinflammatory cytokines by Th1 cells [34]. Accumulating evidence indicates that parasitic diseases are commonly associated with elevated production of NO [35]. Production of proinflammatory cytokines predisposes to the increased synthesis of NO, which mediates host protection through either direct parasite killing or by limiting parasite growth [34]. The half-life of NO is very short. Because of this, the measurement of the circulatory stable end products of NO, i.e., nitrite/nitrate (NO_x), are most often used to evaluate the NO production [12].

In this study, it was observed that an increased level of NO_x which can be related to the induction of immune responses as demonstrated in *Babesia bovis* infection, in which increased NO production reflected a complex host-parasite interaction [36]. Furthermore, researchers have demonstrated that IFN- γ is responsible by the synthesis of NO by monocytes/macrophages, which show a high babesicidal activity [37]. In dogs infected by *Hepatozoon canis*, it was demonstrated that an increased production of NO enhances the host's defense against parasitic infections and helps to eliminate it [14]. In addition, protective responses were reported through high concentrations of nitrate in equids infected with *Theileria equi* and *Babesia caballi* [38]. Increased levels of NO and IL-6 were shown in cattle with anaplasmosis and may indicate the stimulation of the host immune system [23]. However, the determination of reactive nitrogen intermediates (RNI) is unlikely to be useful indicators of severity or outcome in dogs infected with *Babesia canis* [13].

We conclude that infection with *R. vitalii* causes an increase in proinflammatory cytokines and NO metabolites. This alteration may be associated with the host immune protection against the piroplasms, similar to what occurs in other hemoparasite infections.

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