

# The Role of CD4+ and CD8+ T-Cells during *Angiostrongylus vasorum* Infection in Dogs

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## Abstract

A determinant factor in the pathology of canine angiostrongylosis seems to be related to the location of the parasite in the definitive host. Their presence inside the arteries and its branches, promoting mechanical and metabolic action on the wall of the vessels, may alter its homeostasis. Bronchoalveolar lavage (BAL) is a procedure that retrieves cells and other elements from the lungs for evaluation, and helps in the diagnosis of many pulmonary diseases. The aim of this study was to evaluate CD4+/CD8+ lymphocyte profile during the infection by *Angiostrongylus vasorum*, using cells retrieved using BAL. The identification of subpopulations of T lymphocytes by evaluating the co-expression of CD4 and CD8 receptor proteins has shown that despite the increase in both populations, there was a predominance of CD4+ T-cells, instead of CD8+ T-cells. These increases of CD4+ T-cells associated with the increase of the ratio between CD4+/CD8+ suggest polarization of a Th2 response. However, the immune cells, signaling factors, and cytokines that mediate such immunity and how and where they act within the body remain largely undefined during angiostrongylosis.

## Keywords

Citometria, Angiostrongyliasis, Bronchoalveolar Lavage

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## 1. Introduction

*Angiostrongylus vasorum* is a nematode that is part of the Metastrongyloidea superfamily that parasitizes domestic dogs (*Canis familiaris*) and wild carnivores. Disease caused by *Angiostrongylus vasorum* is increasingly diagnosed in dogs, as the geographic range of the parasite increases along with awareness among clinicians. A determinant factor in the pathology of canine angiostrongylosis seems to be related to the location of the parasite in the definitive host. The presence of the parasite inside the arteries and branches of the host promotes a mechanical and metabolic action on the vessels walls, which may alter its homeostasis [1] [2] resulting in pneumonia, loss of racing performance, coughing and anemia [3].

Flow cytometry is a technique widely used in human medicine that is also becoming a helpful tool in veterinary medicine, being applied to a greater variety of conditions for diagnosis and prognosis of diseases and particularly in small animal practice [4] [5].

The bronchoalveolar lavage (BAL) is an accurate technique for the diagnosis of canine angiostrongylosis, especially in the situations when the feces' parasitological is negative and the clinical symptomatology matches the infection [6] [7]. Considering that BAL is a technique that allows the retrieval of cells and other elements that line the lung surface (airway) for cytological evaluation, the goal of this experimental study was to perform flow cytometric analyses of cells retrieved using BAL to determine CD4+/CD8+ profile during the infection.

## 2. Material and Methods

### 2.1. Parasite Source

The *Angiostrongylus vasorum* strain used in the experiments was isolated from feces of a domestic dog [8]. The nematode was maintained as described by Barçante *et al.* (2012) [9].

### 2.2. Animals

Twelve one-year-old mongrel dogs (*Canis familiaris*) were used in this experiment. The dogs were born and bred in the breeding facilities of the Department of Parasitology (UFMG, Brazil) and were free from any *A. vasorum* infections. Following the manufacturer's directions, the dogs were treated as described by Barçante *et al.* (2012).

The experimentation protocols are in agreement with the Ethical Principles in Animal Experimentation, adopted by the Ethics Committee in Animal Experimentation (CETEA/UFMG), and were approved under number 060/03.

### 2.3. Parasitic Infection

The parasitic infection was procedure using third stage larvae of *A. vasorum* (L3) isolated from dog feces as described by Barçante *et al.* (2012).

The dogs used for infected group were orally inoculated with 100 L3 of *A. vasorum* per kilogram of body weight suspended in 5 mL of PBS. Five animals received an extract of non-infected snails mixed to 5 mL of PBS and were kept as uninfected control.

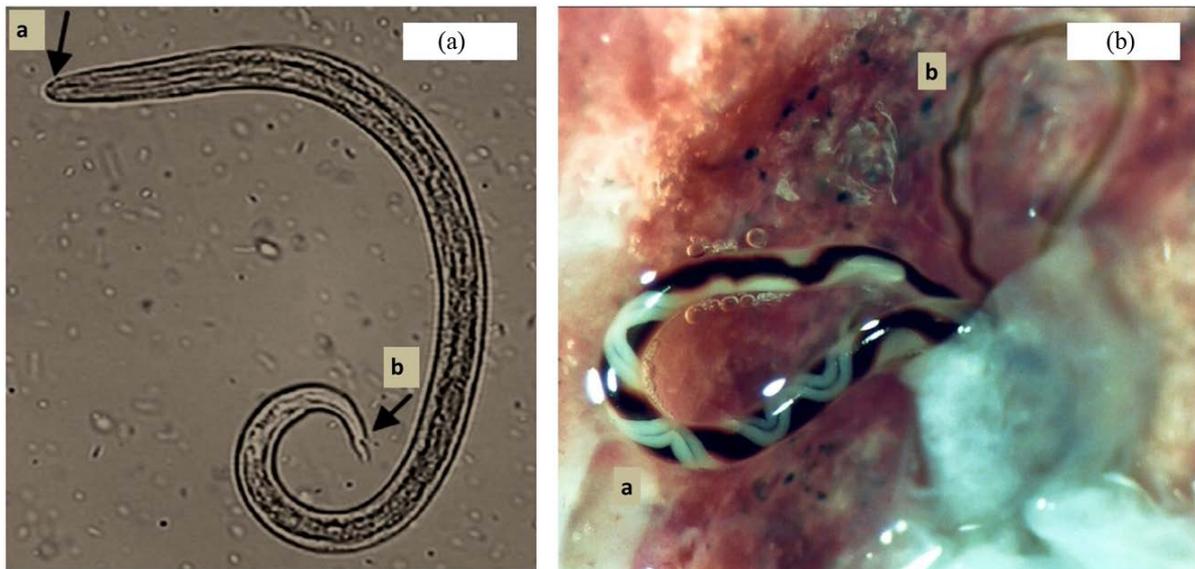
In order, to determine the pre-patent period (PPP), from 20 days post-infection (dpi) to 330 dpi, fecal samples were collected daily from the cage of each animal of the infected group and submitted to a modified Baermann apparatus to recover the first stage larvae (L1). The *A. vasorum* infections was confirmed by the presence of typically L1 in the dogs feces (Figure 1(a)) and by *post mortem* finding of adults worms (Figure 1(b)). Larva and adult worm were identified based on morphology (Lima *et al.*, 1985).

### 2.4. Bronchoalveolar Lavage Procedure

Bronchoalveolar lavage (BAL) was performed on days 0, 30, 60, 90, 120, 180, 240 and 330 after the infection with *A. vasorum* L3. The BAL procedure was performed as described by Barçante *et al.* (2008).

### 2.5. Immunophenotyping of Cells

The BALF of each animal was diluted 1:1 with PBS containing 1 mM of dithiotreitol (DTT) and kept in a water



**Figure 1.** Larvae and adults of *Angiostrongylus vasorum*. (a) Micrograph of a first-stage larva (L1) of *Angiostrongylus vasorum* recovered from feces using the Baermann technique: a—anterior end of L1; b—posterior end of L1 shows the larval tail. Note the kinked tail and the distinctive dorsal spine; (b) *Angiostrongylus vasorum* adults inside a branch of pulmonary artery of an infected dog: a—female and b—male.

bath for 30 min at 37°C to dissolve the mucus. Total cell counts were determined by using a Neubauer's chamber.

In short, the cell suspension was incubated with monoclonal antibodies (mAb) rat anti-canine CD4 and CD8 (Serotec Ltd., UK), fluorescein isothiocyanate conjugated (FITC). Stained cells were analysed on the FACS Calibur™ flow cytometer (Becton Dickinson, USA) using BD FACSTM tubes. A minimum of 10,000 events were acquired for each sample tube in list mode using the CellQuest™ software (BD). Lymphocytes were gated on established regions and percentages of CD4 and CD8 cells expressing were quantified.

For procedures with total cells counts for each animal did not reach the minimum number of cells required for immunophenotyping, it was decided to group the recovered cells in “pool” of normal animals and “pool” of animals infected with *A. vasorum*. In this situation, the markers were made with maximum obtained replicas.

## 2.6. Statistical Analysis

To assess the eventual significance of differences among the study parameters, the obtained results were compared with a standard T-test and the null hypothesis was rejected at  $p \leq 0.05$ .

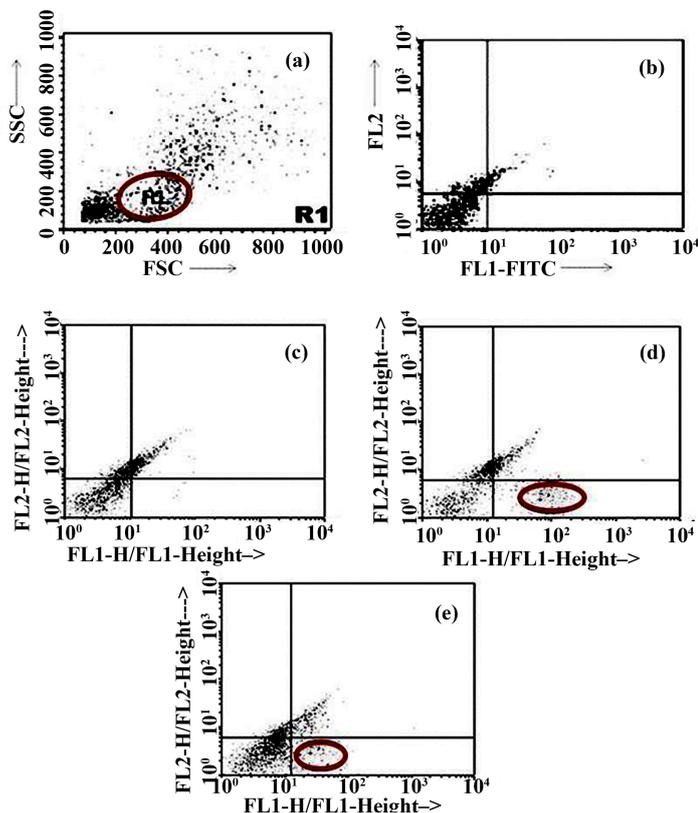
## 3. Results

The phenotypic analysis of recovered cells in the BAL was performed through the identification and demarcation of a R1 region, excluding debris (**Figure 2(a)**). **Figures 2(c)-(e)** show examples of marks of different cell populations recovered with the BAL of dogs and marked with primary ACMOS against cell surface receptor (CD4, CD8).

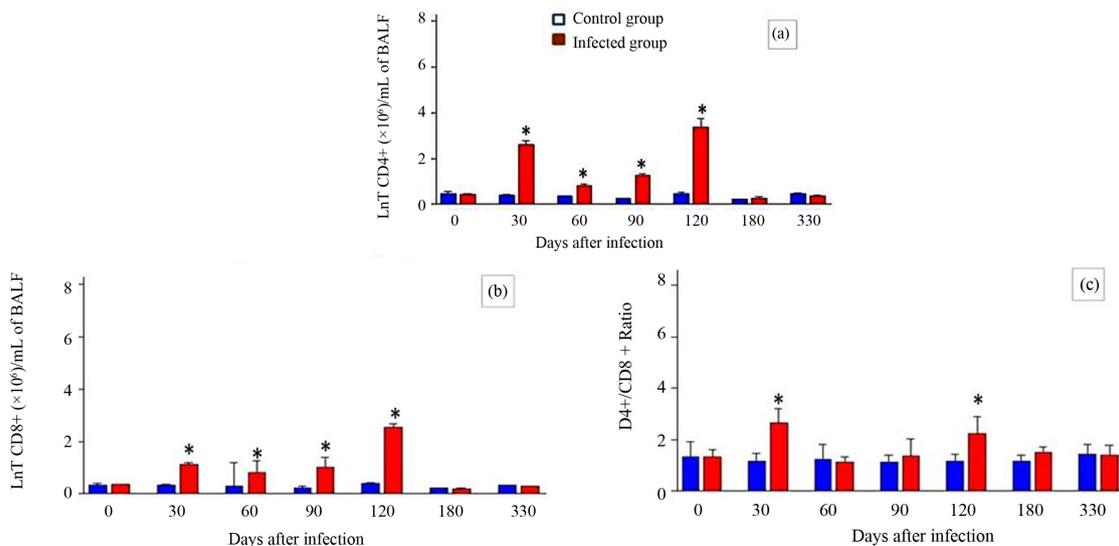
The absolute number and percentage of total, helper and cytotoxic T cells were estimated based on expression of CD4+ (helper T-cell) and CD8+ (cytotoxic T-cell) on the gated lymphocyte population. As shown outlined in **Figure 1(b)**, the cells population in region R1 showed no type 1 fluorescence, when not marked with specific cell surface markers MoAb.

The TCD4+ cell population was more representative in infected group from 30 to 120 DAI ( $p < 0.05$ ) (**Figure 3(a)**). The same profile was observed in relation to CD8+ T-cell population. Fluctuation on these T-cell markers was apparent as a significant difference in the absolute number of CD8+ T-cells detected between infected and non-infected animals ( $p < 0.05$ ) (**Figure 3(b)**).

There were significant differences in CD4+ ( $p < 0.05$ ) and CD4:CD8 ratio ( $p < 0.05$ ) infected group in rela-



**Figure 2.** Profile of cells recovered in the bronchoalveolar fluid of dogs identified by flow cytometric dot plot distributions based on: (a) their laser forward scatter (FSC) versus laser side scatter properties (SSC); (b) FL1 FITC versus FL2; (c) FL1 FITC versus FL2 control reaction; (d) FL1 CD4+ FITC versus FL2 gated CD4+ T lymphocyte population market with MoAb anti-CD4; (e) FL1 CD8+ FITC versus FL2 gated CD8+ T lymphocyte population market with MoAb anti-CD8. The red circle indicate the cell selected population for analysis.



**Figure 3.** Absolute number of cells in bronchoalveolar lavage and ratio between CD4+/CD8+, of dogs infected with *Angiostrongylus vasorum* and controls dogs. (a) cells expressing CD4; (b) cells expressing CD8; (c) ratio of CD4+/CD8+ cells. Group 2-Seven infected animals with 100 third-stage larvae of *A. vasorum* L3/kg live weight. Control five non-infected animals. Results are expressed as mean of each group of animals and the vertical bars represent the mean standard error. The asterisk indicates a significant statistical difference ( $p < 0.05$ ).

tion to non-infected one from 30 and 120 DAI (Figure 3(c)).

#### 4. Discussion

The gut and lung phases of helminth parasite invasion represent particular sites where the migrating larvae can be relatively easily eliminated from the host. Although this, tissue-migrating helminth parasites of many species have found that a relatively easy way to use mammalian hosts for their survival is to gain access to the blood circulation, often via the lung, where they mature, reproduce, and use the host excretory processes for dispersal and reinfection, as *Angiostrongylus vasorum* [10].

It has long been recognized that increasing host age and exposure to infections result in a state of Th2-mediated immunity which protects the host from reinfection [10]. Through the evaluation of lymphocytes surface markers by flow cytometry were possible to identify the phenotype of blood lymphocytes at different periods of infection. It was found that after 30 DAI the population of T lymphocytes (CD4+ and CD8+) was higher in infected animals than control animals.

The identification of subpopulations of T lymphocytes by evaluating the co-expression of CD4 and CD8 receptor proteins has shown that despite the increase in both populations, there was a predominance of CD4+ T-cells of CD8+ T-cells. These increases of CD4+ T-cells associated with the increase of the ratio between CD4+/CD8+ suggest polarization of a Th2 response.

Profound blood and tissue eosinophilia are among the hallmark features of parasitic helminth infection, observed in response to activation of CD4+ Th2 lymphocyte at specific stage of parasite life cycle [10]. Helminths infections generates a dominant type 2 response among both adaptive (Th2) and innate (macrophage, eosinophil, and innate lymphoid) immune cell types [12].

Although, at the present study, the profile of cytokines produced during infection by *A. vasorum* has not been evaluated, the cellular phenotype may suggest, indirectly, the action of certain cytokines in the activation of some specific cell types, such as eosinophils.

According to Barçante *et al.* (2012), absolute cell counts recovered from lungs of infected dogs revealed that eosinophils showed a significant increase in number from 30 reaching peaks at 30 and 60 DAI. The evaluation of lung cellularity in infected animals suggests that the phase of greatest antigenicity of *A. vasorum* happens in definitive host, when rapid growth and migration to the heart and lungs occur [13]. In this way, the increase of eosinophils could represent a consequent augment of a Th2 immune response, which agrees with the increase in the number of CD4 + cells and the CD4/CD8 ratio from infection, with significant changes to the corresponding period of 30 to 120 DAI. Still this focus, Harvie *et al.* (2010) who pointed the lung how an important site for priming immune protection. Furthermore, the lung-initiated, CD4 T-cell-dependent, and IL-4.

This study shows that the immunophenotyping of cells retrieved using BAL providing additional information about inflammatory diseases during infection by *A. vasorum*. However, the immune cells, signaling factors, and cytokines that mediate such immunity and how and where they act within the body remain largely undefined during angiostrongylosis

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