UNIVERSIDADE FEDERAL DE ALFENAS

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AGING-DEPENDENT RESPONSES TO HUMAN SYSTEMIC PROTOZOOSIS: PROOF OF PRINCIPLE IN AN EXPERIMENTAL MODEL OF *Trypanosoma cruzi* INFECTION

Alfenas/MG

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Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre em Biociências Aplicadas à Saúde pela Universidade Federal de Alfenas.
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Orientador: Prof. Dr. Rômulo Dias Novaes
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AMANDA APARECIDA FELIZARDO

"AGING-DEPENDENT RESPONSES TO HUMAN SYSTEMIC PROTOZOOSIS: PROOF OF PRINCIPLE IN AN EXPERIMENTAL MODEL OF TRYPANOSOMA CRUZI INFECTION..".

A Banca Examinadora, abaixo assinada, aprova a Dissertação apresentada como parte dos requisitos para a obtenção do título de Mestre em Biociências Aplicadas à Saúde pela Universidade Federal de Alfenas . Área de concentração: Fisiopatologia

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ABSTRACT

Immunosenescence, a process that develops throughout aging, is characterized by decline in the phenotype and function of organs and cells of the immune system. In general, the increased susceptibility of elderly organisms to infectious diseases has been related to immunosenescence. Although extreme age groups are more susceptible to pathogenic microorganisms, especially bacteria and virus, the impact of parasitic infections in elderly organisms is poorly understood. Therefore, we developed an integrated model based on systematic review and preclinical studies to compare the evolution of systemic protozooses in young and elderly organisms. In the first approach based on systematic review, we investigated preclinical models of human systemic protozooses (Chagas disease, Leishmaniasis, Malaria, Sleeping sickness, Toxoplasmosis). In this study, we identified the evidence that throughout aging, parasitemia and mortality were reduced in Chagas disease and malaria, but similar or increased in Leishmaniasis and highly variable in Toxoplasmosis. While a humoral response in older animals was protective in Chagas disease, a cellular Th1 response was protective against Plasmodium infections. In Leishmaniasis, the most severe infections were related to the imbalance between Th1 and Th2 phenotypes. To reinforce the evidence found in the systematic review, we used a preclinical model (*in vitro* and *in vivo*) to compare the evolution of *T. cruzi* infection in young (8 weeks-old) and elderly (72 weeks-old) mice. Our findings indicate that young and elderly mice infected by *T. cruzi* express divergent parasitic control and myocarditis severity, which is potentially related to differences in cytokine expression and activation of Arg-1 and iNOS pathways. The severe myocarditis identified in elderly animals was consistent with higher parasitemia and parasitic load, indicating that the upregulated IgG2b and IL-17 production was unable to counteract heart parasitism and damage. Thus, the higher susceptibility of elderly mice to *T. cruzi* infection was related to differential activation of Arg-1 and iNOS pathways. Therefore, our findings indicated that the aging of immune cells is associated with an attenuated response to antigenic stimulation, in which iNOS downregulation and increased activation of the arginase pathway creates favourable conditions for heart parasitism and myocarditis development.

Key words: Aging. Chagas disease. Immunosenescence. Parasitic diseases. Systematic review.

RESUMO

A imunossenescência é caracterizada por um declínio no fenótipo e função do sistema imunológico que se desenvolve ao longo do envelhecimento. Em geral, o aumento da susceptibilidade em organismos idosos às doenças infecciosas tem sido relacionado à imunossenescência. Embora grupos etários extremos sejam mais susceptíveis a microrganismos patogênicos, o impacto das infecções parasitárias em organismos idosos é pouco compreendido. Assim, desenvolvemos um modelo integrado baseado em revisão sistemática e estudos pré-clínicos para comparar a evolução de protozooses sistêmicas em organismos jovens e idosos. A partir da revisão sistemática, investigamos modelos pré-clínicos de protozooses sistêmicas humanas (Doença de Chagas, Leishmaniose, Malária, Doença do Sono, Toxoplasmose). Neste estudo, identificamos evidências de que ao longo do envelhecimento, a parasitemia e a mortalidade foram reduzidas na Doença de Chagas e na Malária, mas semelhantes ou aumentadas na Leishmaniose e altamente variáveis na Toxoplasmose. Enquanto uma resposta humoral em animais mais velhos foi protetora na doença de Chagas, uma resposta celular Th1 foi protetora contra infecções por Plasmodium. Na Leishmaniose, as infecções mais graves foram relacionadas ao desequilíbrio entre os fenótipos Th1 e Th2. Para reforçar as evidências encontradas na revisão sistemática, utilizou-se um modelo pré-clínico (*in vitro* e *in vivo*) para comparar a evolução da infecção pelo *T. cruzi* em camundongos jovens (8 semanas de idade) e idosos (72 semanas). Nossos achados indicaram que camundongos jovens e idosos infectados por *T. cruzi* expressam controle parasitário e gravidade da miocardite divergentes, o que está potencialmente relacionado a diferenças na expressão de citocinas e ativação das vias Arginase-1 (Arg-1) e óxido nítrico sintase induzível (iNOS). A miocardite grave identificada em animais idosos foi consistente com maior parasitemia e carga parasitária, indicando que a produção aumentada de IgG2b e IL-17 foi incapaz de neutralizar o parasitismo e os danos no coração. Assim, a maior susceptibilidade dos camundongos idosos à infecção pelo *T. cruzi* foi relacionada à ativação diferencial das vias Arg-1 e iNOS. Portanto, nossos achados indicaram que o envelhecimento das células imunes está associado a uma resposta atenuada à estimulação antigênica, na qual a regulação negativa da iNOS e o aumento da ativação da via da arginase cria condições favoráveis para o parasitismo cardíaco e o desenvolvimento da miocardite.

Palavras-chave: Envelhecimento. Doença de Chagas. Imunossenescência. Doenças parasitárias. Revisão sistemática.

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SUMMARY

1 GENERAL INTRODUCTION

The human systemic protozooses (Malaria, Leishmaniasis, Toxoplasmosis, Sleeping sickness and Chagas disease) are neglected diseases directly related with adequate environmental conditions to parasite development, low socioeconomic population status, and inadequate hygiene habits (LOZANO et al., 2012; POLLITT et al., 2011). These diseases are endemic in subtropical and tropical countries, and infections such as Chagas disease and especially leishmaniasis are raising in non-endemic areas, with higher impact in North America, Europe and Middle East (ANTINORI et al., 2017; KETTLER; MARJANOVIC, 2004; RASSI; RASSI; MARIN-NETO, 2010). These systemic protozooses are responsible for important socioeconomic impact and high rates of morbidity and mortality worldwide, especially in endemic countries (MACKEY et al., 2014)

Although the etiological agents, infection pathways and pathogenesis are widely variable in human systemic protozooses, the immunological system invariably constitutes the pivotal host defense line in all infectious diseases (PODACK; MUNSON, 2016). Thus, in Malaria, Leishmaniasis, Toxoplasmosis, Sleeping Sickness, and Chagas Disease; innate and acquired immunological processes, humoral and cellular responses modulated by different immunological phenotypes (Th1 / Th2 / Th17) are activated from host-pathogen interaction (BASSO, 2013; DAMSKER; HANSEN; CASPI, 2010). Invariably, the balance between the different immunological response profiles seems to be the central element that establishes the interface of the parasite-host relationship and the interactions that direct the infection to the ecological balance that maintains the disease stable or to the dynamic imbalance in favor of the host, which leads to cure, or in favor of the parasite, which more frequently determines disease progression (CHAPLIN, 2010). Thus, investigating the variants of the immune response activated in parasitic diseases may represent a rational and valuable strategy to understand more deeply the host-pathogen relationship and the mechanisms that regulate resistance and susceptibility to infection.

In the general population, extreme age groups (children and elderly) are most susceptible to pathogenic microorganisms, developing severe forms of infection and suffering disproportionately high mortality rates compared to intermediate age groups (FELIZARDO et al., 2018; SIMON; HOLLANDER; MCMICHAEL, 2015). In these vulnerable groups, infection susceptibility has been attributed to an attenuated immunological ability to contain infectious challenges, especially due to incomplete immunological maturation in children and

immunological dysfunction in aged organisms (SIMON; HOLLANDER; MCMICHAEL, 2015). Understanding the biological cycle of each etiological agent and the physiopathological mechanism linked to the infections is essential to control transmission and treat human parasitic diseases (MOLYNEUX, 2006; FELIZARDO et al., 2018). Furthermore, the rational design of more effective public health programs also depends on the identification of vulnerable population groups, as well as on clear delimitation of factors associated with the greater resistance or susceptibility to infectious diseases (GIEFING-KRÖLL et al., 2015), including those determined by age and aging (FERNÁNDEZ-MAYORALAS et al., 2015).

Over the last decades, age pyramid modification has been clearly identified, with a significant increase in the proportion of elderly individuals in the general population (WHO, 2015a). Demographic studies estimate that by the year 2050 the population will increase from 8% to 16% of the current world population (WHO, 2015b). Throughout aging, in addition to physical modifications, functional decline in multiple systems are also described, especially modifications in the phenotype and function of the immune system (LINTON; DORSHKIND, 2004; LÓPEZ-OTÍN et al., 2013). The process of progressive decline in the functions of the immune system due to aging is called immunosenescence. There is evidence that this process increases the organism susceptibility to infectious diseases (BOE; BOULE; KOVACS, 2017). Among the most striking changes that occur in the immune system, there is a significant reduction of peripheral naïve T lymphocytes, impairing the antigenic recognition and the combat against new pathogens (CARUSO et al., 2009). Even more striking and worrisome is the loss of receptor diversity in T lymphocytes (TCR) and B cells, a condition that indicates a reduction in the diversity of circulating cell subpopulations beyond those present in the bone marrow and thymus. In addition to reduced phenotypic variability, the accumulation of dysfunctional cells with limited ability to recognize and respond efficiently to the broad spectrum of pathogen-associated antigens has also been attributed to immunosenescence (FULOP et al., 2014). From this immunological dependence, it seems consistent that increased immunological fragility in population groups with extremes ages is associated with disproportionately high susceptibility to infectious diseases (GIEFING-KRÖLL et al., 2015). As the immune response is an essential element in the fight against infections, if it is in decline due to the aging process, it becomes more difficult to combat pathogenic microorganisms and to resist the onset of infectious diseases. Despite the biotechnological advances observed in recent decades, the impact of immunosenescence on the evolution of infectious diseases, especially in parasitic diseases, remains poorly understood (FELIZARDO et al., 2018). Thus, in this dissertation we developed an integrated model based on systematic

review and pre-clinical studies *in vitro* and *in vivo* to evaluate and compare the evolution of human systemic protozooses in young and old organisms. In the systematic review, preclinical models of the most prevalent systemic protozooses in humans (Malaria, Leishmaniasis, Toxoplasmosis, Sleeping Sickness, Chagas disease) were investigated. In the original *in vitro* and *in vivo* studies, a Chagas' disease model was developed to compare parasitological, immunological, molecular and morphological characteristics of the infection in young and elderly mice.

2 JUSTIFICATION

The human protozooses Malaria, Leishmaniasis, Toxoplasmosis, Sleeping Sickness and Chagas disease represent a public health problem that have a serious medical and social impact associated with high rates of morbidity and mortality, mainly in subtropical and tropical endemic areas. As biological aging is associated with the physical and functional decline of multiple organs and systems, disturbances in the immunological system, called immunosenescence, can determine divergences in the evolution of human protozooses in young and elderly organisms. Considering that studies investigating the impact of age and aging on the development of parasitic diseases are scarce, and due to the strong immunological basis that regulates resistance and susceptibility to parasitic infections; it is relevant to compare the impact of age and aging on the evolution of Chagas Disease. Elucidating potential differences in response to *T. cruzi* infection may help develop more rational and efficient strategies for controlling Chagas Disease in different age groups, which may take into account the morphofunctional biological status of the host's immune system, especially immunosenescence.

3 OBJECTIVES

3.1 GENERAL OBJECTIVE

To compare the impact of age and aging on the evolution of systemic protozooses in animals models.

3.2 SPECIFIC OBJECTIVES

a) To synthesize preclinical *in vivo* scientific evidence on the impact of age and aging on the evolution of Chagas Disease, Leishmaniasis, Malaria, Sleeping Sickness and Toxoplasmosis;
b) To analyze blood and heart parasitism in young and elderly mice infected by *T. cruzi*;
c) To evaluate cytokines and antibodies production in young and elderly mice infected by *T. cruzi*;
d) To analyze the cardiac microstructural organization in young and elderly mice infected by *T. cruzi*;

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4 CHAPTER 1: EFFECT OF AGE ON THE HOST RESPONSE TO SYSTEMIC PROTOZOAN DISEASES: A SYSTEMATIC REVIEW OF ANIMAL STUDIES

ABSTRACT

The impact of aging in the evolution of systemic protozooses is poorly understood. Thus, we conducted a systematic review from preclinical models of Chagas disease, leishmaniasis, malaria, sleeping sickness and toxoplasmosis. From a structured and comprehensive search in electronic databases, 29 studies were recovered and included in the review. Beyond the characteristics of the experimental models, parasitological and immunological outcomes, we also discussed the quality of current evidence. Our findings indicated that throughout aging, parasitemia and mortality were consistently reduced in Chagas disease and malaria, but were similar or increased in leishmaniasis and highly variable in toxoplasmosis. While a marked humoral response in older animals was related to the anti-*T. cruzi* protective phenotype, cellular responses mediated by a polarized Th1 phenotype were additionally associated with a more effective defense against Plasmodium infection. Conversely, in leishmaniasis, severe infections and high mortality rates were potentially related to attenuation of humoral response and an imbalance between Th1 and Th2 phenotypes. Due to the heterogeneous parasitological outcomes and limited immunological data, the role of aging on toxoplasmosis evolution remains unclear. From a detailed description of the methodological bias, more controlled research could avoid the systematic reproduction of inconsistent and poorly reproducible experimental designs.

Key words: Aging. Human protozooses. Parasitic diseases. Methodological quality. Preclinical research.

RESUMO

O impacto do envelhecimento na evolução das protozooses sistêmicas é pouco compreendido. Assim, realizamos uma revisão sistemática de modelos pré-clínicos da doença de Chagas, leishmaniose, malária, doença do sono e toxoplasmose. A partir de uma pesquisa estruturada e abrangente em bases de dados eletrônicas, 29 estudos foram recuperados e incluídos na revisão. Além das características dos modelos experimentais, resultados parasitológicos e imunológicos, também discutimos a qualidade da evidência atual. Nossos achados indicaram que, ao longo do envelhecimento, a parasitemia e a mortalidade foram consistentemente reduzidas na doença de Chagas e na malária, mas foram semelhantes ou aumentadas na leishmaniose e altamente variáveis na toxoplasmose. Enquanto uma resposta humoral bem definida em animais mais velhos foi relacionada a um fenótipo protetor contra o *T. cruzi*, as respostas celulares mediadas por um fenótipo Th1 polarizado foram adicionalmente associadas a uma defesa mais eficaz contra a infecção por Plasmodium. Por outro lado, na leishmaniose, infecções graves e altas taxas de mortalidade foram potencialmente relacionadas à atenuação da resposta humoral e ao desequilíbrio entre os fenótipos Th1 e Th2. Devido aos resultados parasitológicos heterogêneos e dados imunológicos limitados, o papel do envelhecimento na evolução da toxoplasmose permanece obscuro. A partir de uma descrição detalhada do viés metodológico, pesquisas mais controladas poderiam evitar a reprodução sistemática de desenhos experimentais inconsistentes e pouco reprodutíveis.

Palavras-chave: Envelhecimento. Protozooses humanas. Doenças parasitárias. Qualidade metodológica. Pesquisa pré-clínica

5 INTRODUCTION

Malaria, Leishmaniasis, Toxoplasmosis, Sleeping sickness and Chagas disease are systemic protozooses responsible for dramatic economic and medico-social impact caused by human parasitic diseases worldwide (POLLITT et al., 2011; LOZANO et al., 2012). Taken together, they are the main neglected diseases responsible for the highest morbidity and mortality rates reported for parasitic diseases in tropical and subtropical regions (MACKEY et al., 2014). The etiological agents of each disease are hyper endemic in developing countries, especially in Africa, the Middle East, Central and South Americas; areas with favorable environmental conditions for parasite development, poor socioeconomic status and limited access to formal health services (KETTLER; MARJANOVIC, 2004; RASSI; RASSI; MARIN-NETO, 2010; ANTINORI et al., 2017). Children and the elderly are the most susceptible to parasitic diseases, developing severe forms of infection and suffering disproportionately high mortality rates compared to intermediate age groups (SIMON et al., 2015). In these vulnerable groups, infection susceptibility has been attributed to an immunological inability to contain the infection, especially due to incomplete immunological maturation in children and immunosenescence in aged people (SIMON; HOLLANDER; MCMICHAEL, 2015). Understanding the biological cycle of each etiological agent and the physiopathological mechanism linked to the infections is essential to control transmission and treat human protozooses (MOLYNEUX, 2006). Furthermore, the rational design of more effective public health programs also depends on the identification of vulnerable population groups, as well as on clear delimitation of factors associated with the greater susceptibility to infections (GIEFING-KRÖLL et al., 2015), including those determined by age and aging (FERNÁNDEZ-MAYORALAS et al., 2015).

Malaria is the most frequent systemic protozoozis worldwide. This disease is caused by parasites of the genus Plasmodium, which are transmitted through the bites of female *Anopheles* mosquitoes (COX-SINGH; DAVIS, 2008; OLIVEIRA-FERREIRA et al., 2010). According to recent estimates, more than 212 million new cases of malaria were registered worldwide in 2015, especially in Africa (90%), South-East Asia (7%) and Eastern Mediterranean areas (2%). For the same period, about 438,000 deaths were reported, 92% of which occurred in African countries (WHO, 2017a). Without treatment, the infection progresses with fever, headache and vomiting to marked anemia, respiratory distress and cerebral malaria, which is the severe condition leading most often to death (WHO, 2017a).

Leishmaniasis is an anthropopathy and zoonotic disease caused by protozoan parasites of the genus Leishmania, which are transmitted by the bite of female phlebotomine sand flies. This disease is endemic in Africa, the Middle East, Central Asia, South and Central America (WHO, 2017b). Leishmaniasis is closely correlated to poverty, poor domestic sanitary conditions, malnutrition, immunological debility, and precarious socio-environmental support. Recent estimates indicated an annual occurrence of 700,000 to 1 million new cases, and 20,000 to 30,000 deaths worldwide (WHO, 2017b). Leishmaniasis encompasses three different clinical forms: cutaneous, mucosal, and visceral. Visceral leishmaniasis the most severe form, and affects multiple organs such as spleen, liver, and bone marrow, and causes vomiting, diarrhea, malnutrition, weight loss, hepatosplenomegaly, pancytopenia, jaundice and eventually death (PAHO, 2014).

The protozoan parasite *Toxoplasma gondii* is the etiological agent of toxoplasmosis (CDC, 2015). Due to its wide distribution (<10 to 95% in some populations) this disease represents an important health problem worldwide (TORGERSON; MASTROIACOVO, 2013), especially in areas with precarious hygiene and eating habits (BABAIE et al., 2013). In the United States toxoplasmosis is a leading cause of death due to food-borne illness (OZ, 2014). In this country, at least 11% of the population of 6 years and older is infected by *T. gondii* (CDC, 2015), in contrast with countries such as Korea and Brazil, in which seroprevalence is around 6.7% and 68.6%, respectively (SHIN et al., 2009; SROKA et al., 2010). Food borne, animal-to-human (zoonotic), and mother-to-child (congenital) are the main forms of *T. gondii* infection. Although limited tissue damage and symptoms are reported in immunocompetent individuals, pregnant women and immunocompromised people can develop severe toxoplasmosis, which causes pulmonary necrosis, myocarditis, hepatitis, chorioretinitis, encephalitis and death (BHOPALE, 2003).

Sleeping sickness is a vector-borne disease caused by protozoan parasites belonging to the genus Trypanosoma, which are transmitted by tsetse flies (*Glossina* genus). While *Trypanosoma brucei gambiense* is endemic in 24 countries in west and central Africa and accounts for 97–98% of infection cases, *Trypanosoma brucei rhodesiense* is endemic in 13 countries in eastern and southern Africa, representing about 2–3% of all infection cases (WHO, 2017c). In 2015, 2804 cases of sleeping sickness were recorded, and currently, about 20,000 cases are estimated; being that 65 million people still live in areas at risk of infection. The initial stages of infection proceeds with fever, headaches, joint pains and itching, progressing to central nervous system infection, sensorial and motor disturbances, meningoencephalitis and death (PEREIRA et al., 2017; WHO, 2017c).

Chagas disease is caused by the protozoan *Trypanosoma cruzi*, which is mainly transmitted by contact with feces or urine of triatomine bugs. This disease is endemic in 21 Central and South American countries, and about 70 million people are at risk of infection. Approximately 6 to 7 million people are infected and 12,000 deaths are recorded per year worldwide (WHO, 2017d). Increasing incidence and prevalence rates of *T. cruzi* infection have been described in non-endemic areas, especially the United States, Europe and Australia; aspects directly related to the migratory flow of infected individuals from endemic countries (ANTINORI et al., 2017). Chronic Chagas cardiomyopathy is the most severe and incapacitating manifestation of *T. cruzi* infection (CUNHA-NETO; CHEVILLARD, 2014; PEREIRA et al., 2017), which is responsible for varying mortality rates according to the severity of heart damage (MEDEIROS; GOMES; CORREA-OLIVEIRA, 2017).

Although the etiological agents, infection pathways and pathogenesis are widely variable in human systemic protozooses, the immunological system invariably constitutes the pivotal host defense line in all infectious diseases (PODACK; MUNSON, 2016). From this immunological dependence, it seems consistent that increased immunological fragility at the extremes of population age groups is associated with disproportionately high susceptibility to infectious diseases (GIEFING-KRÖLL et al., 2015). Given that the world undergoes a gradual process of demographic transition, the elderly age groups have assumed increasing importance as vulnerable population segments (WHO, 2015). Estimates indicate that between 2015 and 2050, the world's population of 60 years and older will nearly double, from 12 to 22%, so that at least 80% of these older people will be living in low- and middle-income countries. Furthermore, by 2020 the number of people over 60 years will outnumber children younger than 5 years (WHO, 2015).

Although aging is associated with the progressive morphofunctional decline in all biological systems and general homeostatic mechanisms (GUPTA et al., 2013; GORONZY et al., 2014; PAWELEC; GOLDECK; DERHORRANESSIAN, 2014), immunosenescence is of special relevance in host-pathogen interaction and infectious disease development (KRONE et al., 2014). Immunosenescence is a complex and multifactorial process, characterized by general and progressive dysfunction of innate and acquired immunological mechanisms, which increases the susceptibility of aged organisms to autoimmune, neoplastic and infectious diseases (PASSTOORS et al., 2015). In parasitic diseases caused by intracellular protozoa, immunosenescence is potentially dangerous, due to poor modulation of immunological phenotypes (Th1 and Th2) that establish the interface of the host-pathogen relationship and the interactions that direct the infection to the ecological balance, either in favor of the host,

maintaining the disease stable or leading to cure, or in favor of the parasite, which determines disease progression and eventually death (TORRÃO et al., 2014).

Taken into account that the impact of the age of the host on the evolution of parasitic diseases is poorly understood, investigating how these diseases develop in response to variants of the aging process, especially immunosenescence, may represent a rational and valuable strategy to elucidate the parasite-host relationship. Furthermore, this strategy can be useful in identifying specific mechanisms that regulate the relationship between resistance and susceptibility to infection, with a direct impact on the understanding of pivotal variables that make older organisms more vulnerable. Considering that the literature provides only fragmented data, and no objective overview on the impact of age and aging on the evolution of parasitic diseases, we conducted a systematic review of preclinical models of human systemic protozooses. Our focus was to define the accumulated evidence on how malaria, leishmaniasis, toxoplasmosis, African and American trypanosomiasis develop in different-age hosts, determining in detail the characteristics and relevance of the preclinical models used; the potential convergences and divergences in physiopathological mechanisms; and the pathological manifestations of important neglected tropical diseases worldwide. From a detailed bias analysis, the methodological quality of current evidence was also evaluated, pointing out the main sources of bias that constitute important research barriers in the area and hinder advances in the understanding of the relationship between host age and parasitic diseases.

6 METHODOLOGY

6.1 SEARCH STRATEGY

All studies included in the systematic review were selected according the standardized guideline PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analyses) (MOHER et al., 2009). A two-level search was designed to identify relevant studies on the influence of aging on the host response against protozoan parasites associated with human systemic protozoozis. The primary (direct) search was based on three comprehensive electronic databases in biomedical literature: PubMed/MEDLINE, SCOPUS and Web of Sciences. The secondary (indirect) search was carried out by the screening of the reference lists of all relevant studies identified in the database search.

For electronic databases, retrieval of indexed records was based on structured search filters (Table S1). Initially, a reference filter was constructed by considering the search algorithm used in the PubMed interface to recover studies indexed by the MEDLINE database. Standardized descriptors registered in MeSH databases - Medical Subject Headings (https://www.ncbi.nlm.nih.gov/mesh) were used to construct the search filters, which were developed in three categories: (i) animal models, (ii) biological condition (aging), and (iii) disease (systemic protozoozis). To detect all *in vivo* preclinical studies in PubMed, a standardized and optimized animal filter was obtained (HOOIJMANS et al., 2010). In each filter, the search algorithms [MeSH Terms] and [TIAB] were also applied, to identify indexed records and those recently published in indexing process, respectively. The same search filters used for disease and biological condition (aging) were adapted for Scopus and Web of Science. The Scopus animal filter (Keyword – animals [limit to]) was used in this database, and another animal filter was created for Web of Science by considering the animal models identified in PubMed/MEDLINE and SCOPUS. No chronological or language limits were applied in the articles search. Thus, all studies identified and published up to April 2017 were included in the systematic review. The complete search strategy is detailed in the supplementary archives (Table S1).

6.2 SELECTION STRATEGY

The indexed studies recovered in the primary search were analyzed for duplicate removal by comparing the journal of publication, title, year, and authors. After the initial screening, all potentially relevant studies were recovered and evaluated in full-text for eligibility according well-defined inclusion and exclusion criteria. Only original preclinical studies investigating the impact of aging on the host response to protozoan parasites associated with human systemic protozoozis were included. The exclusion criteria were based on: (i) epidemiological and observational studies, (ii) secondary studies (i.e. literature reviews, letters to the editor, commentaries, and editorials), (iii) no full-text available, and (iv) studies in which the influence of age on measured outcomes could not be isolated due to the presence of confounder factors (i.e. multiple stimulations in the same experimental panel).The researchers analyzed eligibility independently, and disagreements were resolved by consensus. To increase the comprehensiveness of the research strategy, the reference list of each relevant study identified from all research repositories was screened for additional papers.

6.3 METHODOLOGICAL BIAS

Analysis of the reporting bias was determined according (PEREIRA et al., 2010), considering basic requirements reported in animal studies of parasitic diseases. The strategy adopted provided a methodological support for a complete screening of all manuscript sessions (abstract to acknowledgement and funding) to evaluate the completeness of the scientific reports of animal research. Criteria of bias were based on short descriptions of essential study characteristics, such as experimental design, sample size, randomization, animal allocation, experimental concealment, statistical methods, baseline data, ethnical statement and external validity (generalizability). Considering the specificity of the research subject and the aims of the systematic review, a table summarizing all applicable and relevant aspects described in the bias analysis was constructed. The overall mean adherence and individual quality criteria were expressed as absolute and relative values (PEREIRA et al., 2017).

6.4 DATA EXTRACTION AND SYNTHESIS

Considering a detailed characterization all studies included in the review, data extraction was based on basic methodological requirements for preclinical studies (PEREIRA et al., 2017). Thus, from the research reports were extracted essential data grouped into four descriptive levels as follows: (i) publication characteristics: authors, publication year, and countries; (ii) characteristics of the animal models: species, lineage, sex, age, and weight; (iii) disease model: parasite species and strain, inoculum size, route of parasite administration, duration of infection; and (iv) main measure outcomes (i.e. parasitemia, parasitic load, immunological markers, histopathological findings, and mortality). Considering the consolidation of the evidence from a mechanistic point of view, all phenotypic characteristics of the hosts related to resistance and susceptibility to the different types of infection were grouped into synthesis tables.

7 RESULTS

7.1 PRISMA-GUIDED STUDIES SELECTION

From our search strategy, 2498 research records were identified, of which 29 original and relevant studies were recovered and included in the systematic review. The search strategy and the results obtained in each step of evaluation are summarized in Fig. 1. In all relevant studies, diseases distribution was heterogeneous (Malaria = 11, Leishmaniasis = 6, Toxoplasmosis = 7, and American Trypanosomiasis = 5). No study investigating African Trypanosomiasis was identified in the primary or secondary search.

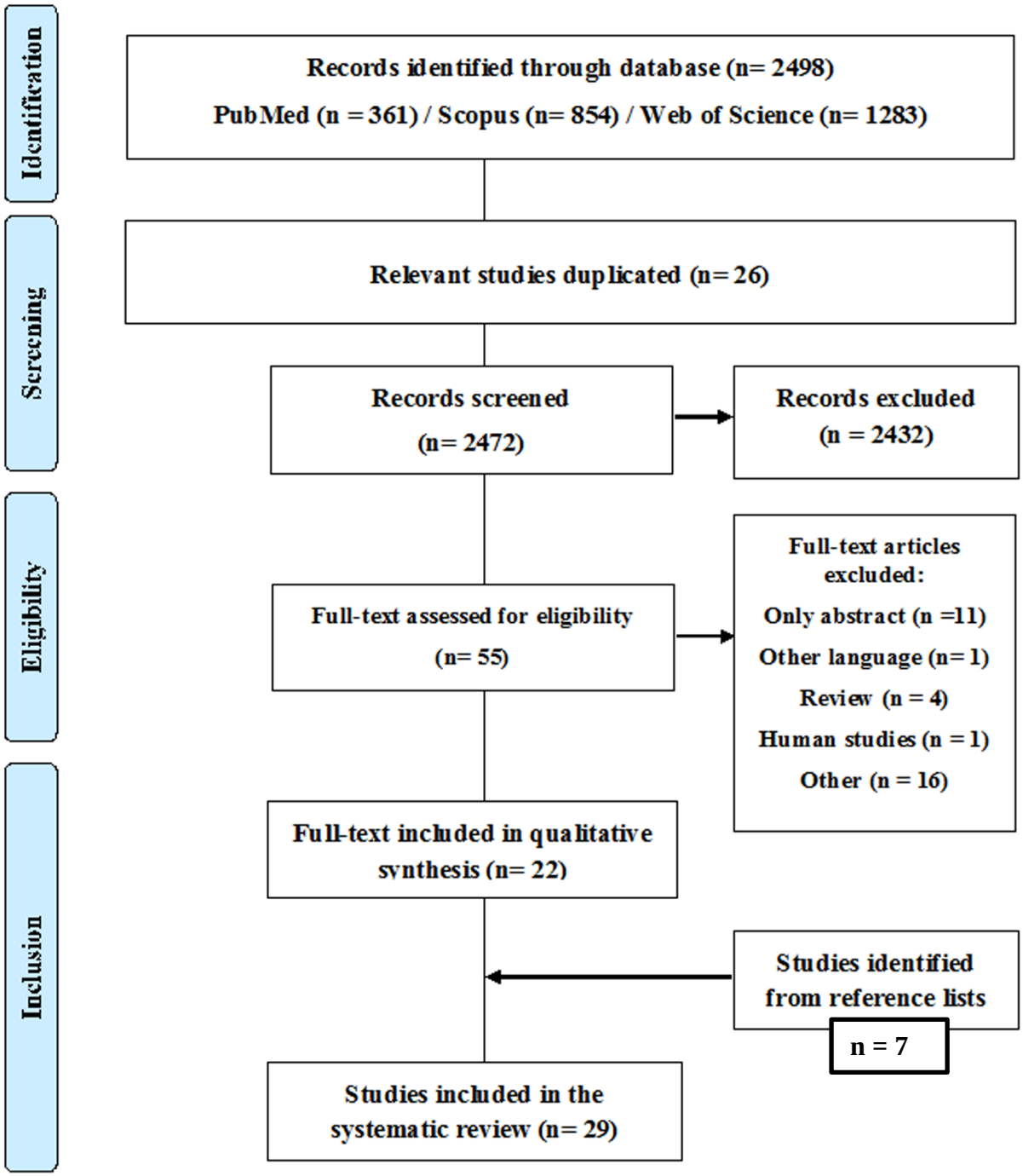

Figure 1 - Flow diagram the systematic review literature search results. Based on PRISMA statement "Preferred Reporting Items for Systematic Reviews and Meta-Analyses". www.prisma-statement.org

Source: From the author

7.2 REPORTING BIAS

The general reporting bias of each study is shown in Fig. 2 and all bias measurements, stratified by domain, are presented in Table S2. The level of reporting bias was similar in all studies, regardless of the disease investigated. No study fulfilled 100% of the 69 bias criteria analyzed. In general, a mean of 46.97 ± 6.84% (min. 35.56%, max. 64.4%) criteria were completed (about 21 items [min. 16, max. 29]). Information such as rationale for inoculum size, route and order of inoculation, data excluded of the analysis, adaptation in experimental protocol, comments on experimental limitations, and funding sources, were not reported in all studies. Experimental blindness, animal weight, baseline procedures, housing of experimental animals, welfare-related assessments, animal allocation (randomization), health status before inoculation, and relevance to human biology, were reported in less than 30% of all studies included. Conversely, clear and structured research background, research objectives, experimental approach, animal and parasite species and strain, inoculum size and route, time of infection, clear animal age, measured outcomes, and clear interpretation of the results were consistently reported (90–100%).

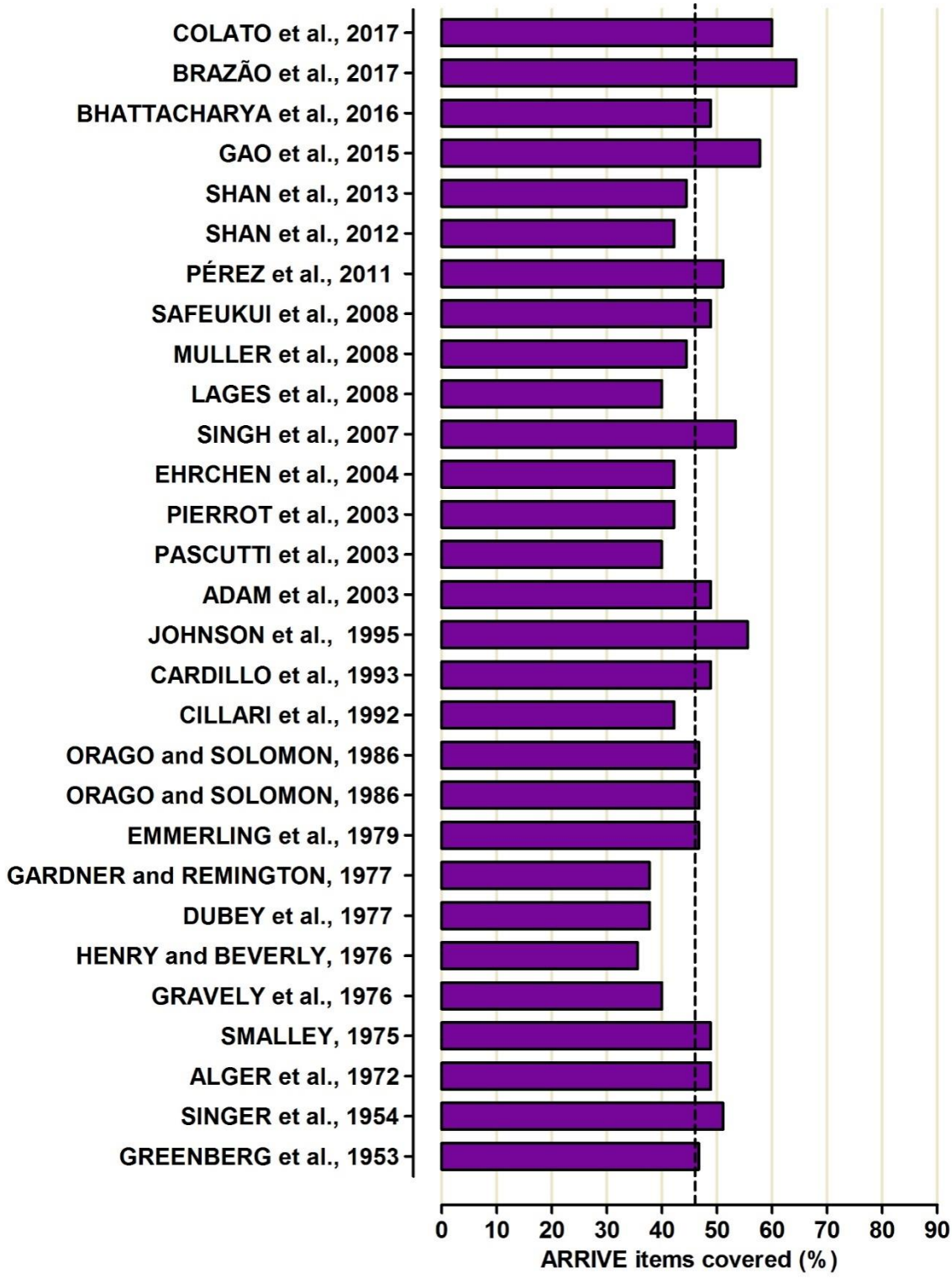

Figure 2 - Methodological quality (reporting bias) for each study included in the systematic review.
Source: From the author
Subtitle: The dotted line indicates the mean methodological score (%). The complete bias analysis stratified by domains is shown in supplementary files (Table S2).

7.3 MODELS OF MALARIA

Studies on malaria originated mainly from France, United Kingdom and USA (27.27%, n = 3each), followed by United Kingdom and China (18.18%, n = 2), and London (9.09%, n = 1). Rats (63.63%, n = 7), mice (27.27% n = 3) or both (9.09%, n = 1) were used as animal models. C57BL/6, (27.27%, n = 3), BALB/c, A/J, and Carworth CF (9.09%, n = 1 each) were the main mice lineages used; while Sprague Dawley and Fischer, (27.27%, n = 3 each), and Wistar and Lewis (9.09%, n= 1 each) were the most frequent rat lineages. Female animals were predominant (72.72%, n= 8) and the age ranged from 21 to 300 days. Malaria was induced by *Plasmodium berghei* in all studies (100%, n = 11). ANKA (36.36%, n= 4), followed by NK65 (27.27%, n = 3), KASAPA and 17XLN (9.09%, n= 1 each) were the most common parasite strains used. While the infection was consistently induced by intraperitoneal route, inoculum size was heterogeneous, ranging from 1×10^{6} to 2×10^{7}parasitized erythrocytes. In general, the infection period ranged from 20 to 30 days, except in one study (86 days) (Table S3).

7.4 MODELS OF LEISHMANIASIS

Studies on leishmaniasis had a heterogeneous origin (Germany, India, Tunisia, Australia, USA, Italy and United Kingdom). BALB/c (66.66%, n= 4) followed by C57BL/6 (33.33%, n= 2) mice were used preferentially as animal models. Hamsters (*Mesocricetus auratus*) were reported in 1 study (16.66%). Female animals were predominant (66.66%, n= 4) and animal weight and age ranged from 45 to 110g and 21 to 540 days, respectively. *Leishmania major* (66.66%, n= 4) and *Leishmania donavani* (33.33%, n= 2) were used as etiological agents. Parasite strain was highly heterogeneous, involving LV39 (33.33%, n= 2), MHOM/IL/81/FE/BNI, LdWT, MHOM/IL/80/Friedlin and MHOM/IN/80/Dd8 (16.06%, n= 1each). Subcutaneous (83.33%, n= 5) or intracardiac (16.66%, n= 1) routes were used for parasite inoculation. Inoculum size ranged from 1×10^{3} to 5×10^{6} promastigotes. In general, the infection period was 30 to 80 days, except in one (16.66%) study (160 days). This data was not reported in two studies (33.33%) (Table S3).

7.5 MODELS OF TOXOPLASMOSIS

Studies on toxoplasmosis originated from USA (42.86%, n = 3), Germany, China and England or both France and England (14.28%, n = 1 each). Mice (57.14%, n = 4), rats (28.57%, n = 2) and cats (14.28%, n = 1) were used as animal models. Female animals or mixed groups (male and female) were mainly used (42.85%, n = 3 each). This parameter was neglected in one study (14.28%). Only one study (14.28%) reported animal weight (150–200g). Mouse and rats ages ranged from 5 to 720 days, while the cats ranged from 7 to 1170 days. Each study used a different *Toxoplasma gondii* strain (ME49, BK, DX, C37, Prugniaud, RH, M-7741, CR-6 and H17), alone or in combination, to induce the infection. Intraperitoneal (42.85%, n = 3); intravenous, subcutaneous or oral (14.28%, n = 1 each); and both intraperitoneal and oral (14.28%, n = 1) routes were used for parasite inoculation. The infection period ranged from 21 to 42 days, except in one (14.28%) study (98 days) (Table S3).

7.6 MODELS OF AMERICAN TRYPANOSOMIASIS

Brazil (60%, n = 3) and Argentina (40%, n = 2) produced all identified studies of American trypanosomiasis. Wistar rats (40%, n = 2) and BALB/c, C57BL/6 and DBA/2 mice (20%, n = 1) were used as animal models. This parameter was neglected in two studies (40%). Male animals were predominant (80%, n = 4). Rats were used but the lineage not reported in two studies (40%, n= 2). Rat weight (100–600g) was reported in only two (40%) studies. Mouse and rat ages ranged from 150 to 360 days and from 21 to 540 days, respectively. *T. cruzi* Y strain (60%, n= 3), followed by Tulahuén strain (40%, n= 2) were used to induce Chagas disease. Intraperitoneal route was used in all cases of inoculation (20%, n= 5) and in one case, subcutaneous route was additionally used. The infection period ranged from 9 to 28 days (Table S3).

7.7 AGE-DEPENDENT BIOLOGICAL RESPONSES IN SYSTEMIC PROTOZOOSES

Parasitemia, parasitic load, mortality, immunological mediators (cytokines, antibodies, innate and acquired immune cells) and histopathological manifestations were the main measured outcomes used to determine the impact of host age on infection development (Table S4). The general age-dependent manifestations of all parasitic disease are summarized in Fig. 3. All measured outcomes exhibited some degree of variability between different age groups. In general, throughout aging, parasitemia and mortality were reduced in Chagas disease and malaria, were similar or increased in leishmaniasis and highly variable in toxoplasmosis. With increasing age, anti-parasitic antibodies levels were increased in Chagas disease and malaria, and reduced in leishmaniasis. Pro-and anti-inflammatory molecules (i.e. NO and TNF) and immune cells (i.e. TCD4+, B lymphocytes) and MHC II expression were downregulated in Chagas disease and upregulated in malaria, which presented variable IL-10 levels, macrophages and NK cells distribution. TCD8+ lymphocytes were increased in all diseases, except in Chagas disease, which exhibited a variable profile with increasing age. Cytokines such as IL-2, IL-4, IL-6, IL-12, IL-10, IFN-γ, and CD4+ T cells presented variable profiles in leishmaniasis. Immunological outcomes were poorly investigated in all studies on toxoplasmosis.

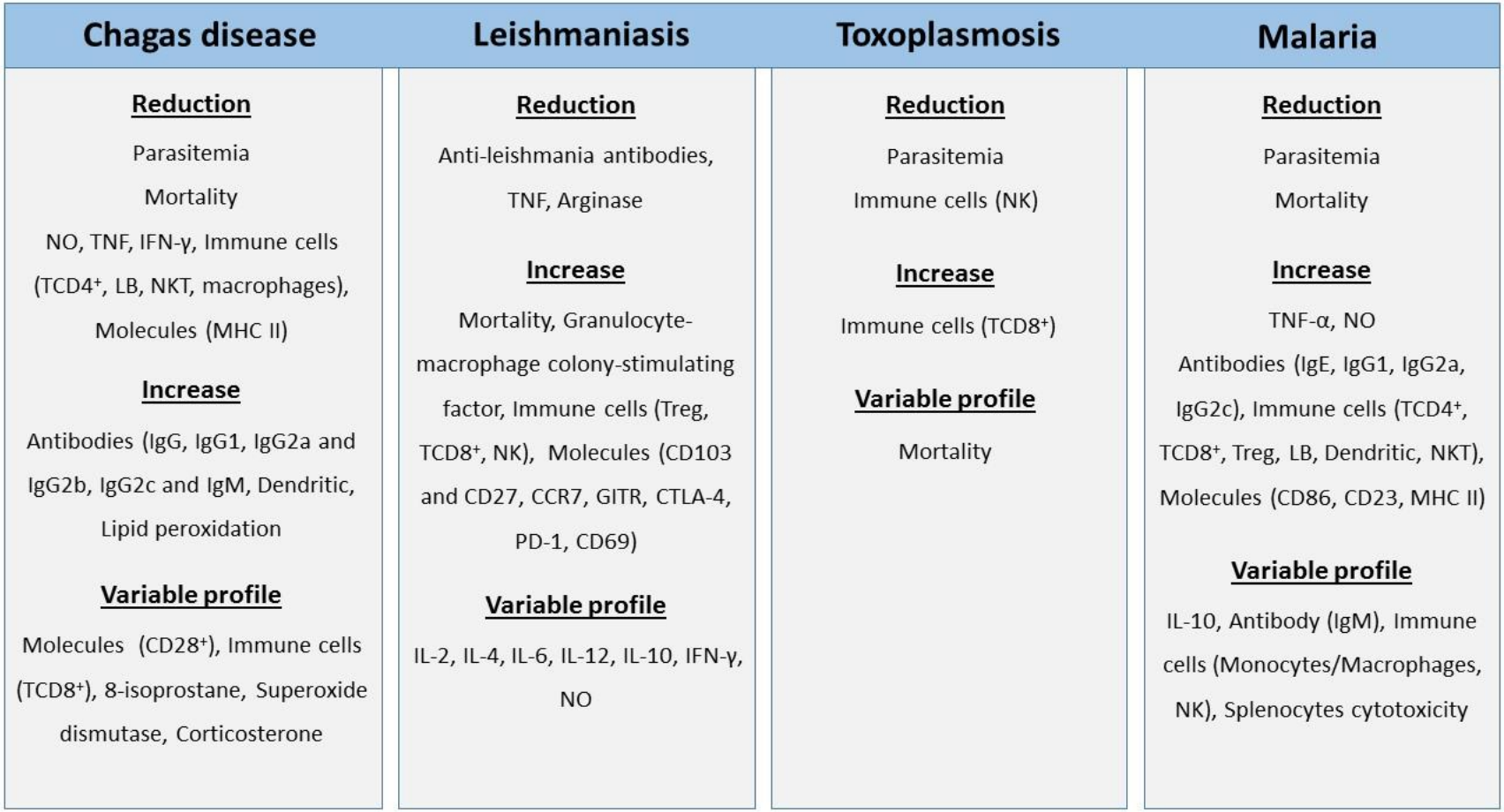

Figure 3 - Impact of aging on mortality, parasitological and immunological parameters *in-vivo* preclinical models of Chagas disease, Leishmaniasis, Toxoplasmosis and Malaria.

Source - From the author

Subtitle: Since animals with different ages were compared, all data presented in this figure are relative to older animals used in each experimental model. Detailed results obtained from specific age groups in each study identified are described in supplementary files (Table S2).

Since parasitemia/parasitic load and mortality are key outcomes in all protozooses investigated, the set of mediators related to host resistance or susceptibility to infection are shown in Fig. 4. In malaria, only upregulation of resistance factors such as IgE and IgG antibodies; TCD4+/Treg, TCD8+, LB, dendritic and NKT cells; TNF, NO, CD86, CD23 and MHC II expression, were identified with increasing age. In Chagas disease, dendritic cells, IgG and IgM antibodies were the resistance factors increased, while TCD4+,LB, NKT, APC cells; NO, TNF, IFN-γ, CD28 and MHC II expression were reduced. In leishmaniasis, the resistance factors Treg, TCD8+ and NK cells; CD103, CD27, CCR7, GITR, CTLA-4, PD-1, CD69 expression were increased; while anti-*Leishmania* antibodies and TNF expression were reduced. Increased TCD8+ and reduced NK cells were the only parameters significantly changed with increasing age, and were related to resistance or susceptibility to toxoplasmosis, respectively (Fig. 4).

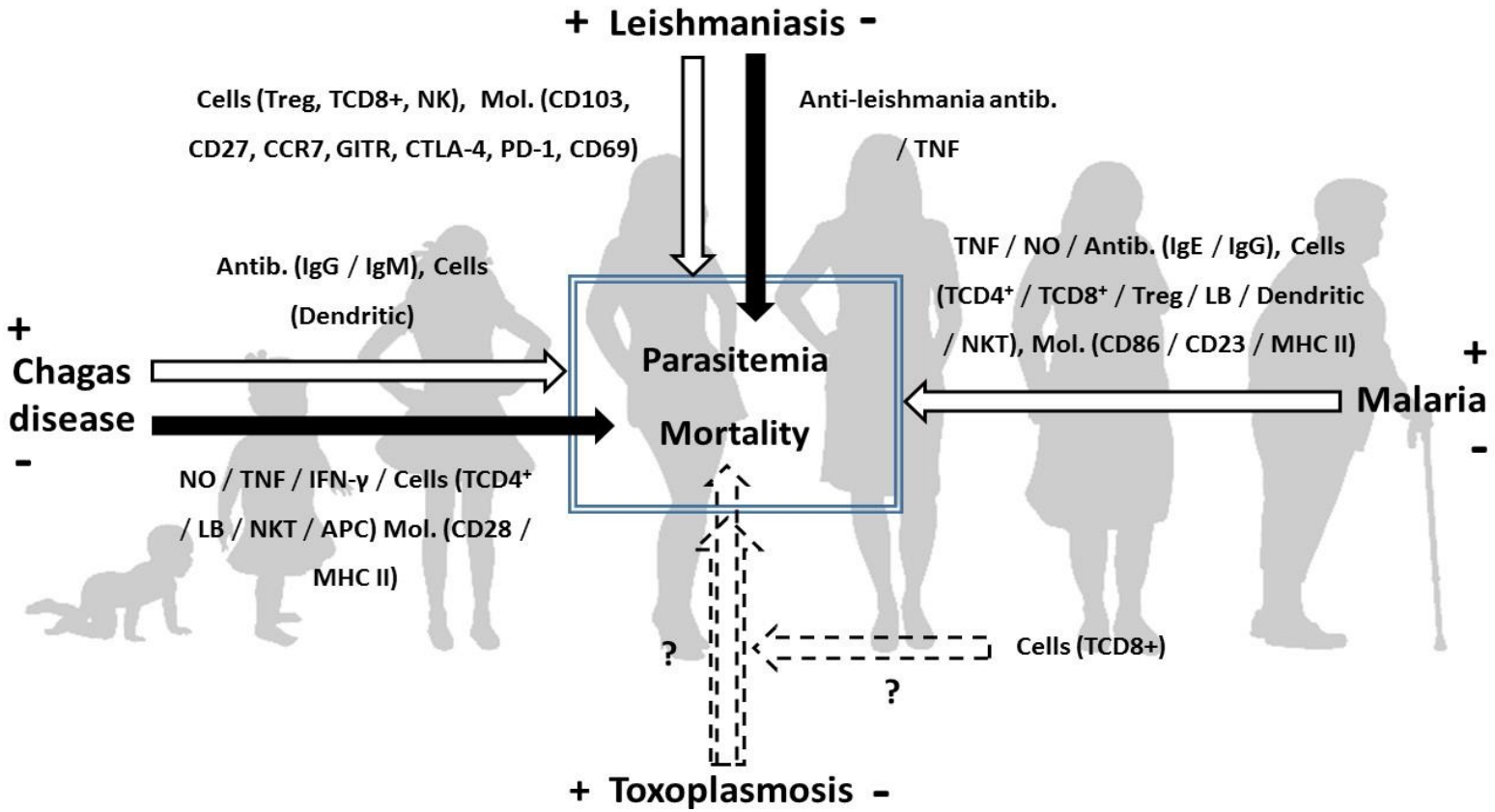

Figure 4 - *In-vivo* preclinical evidence of the impact of aging on the balance of immunological effectors involved in the control of resistance and susceptibility to systemic protozoozis.

Source - From the author.

Subtitle: Since animals with different ages were compared, all data presented in this figure are relative to older animals used in each experimental model. Black arrows: Promotes increased parasitemia and mortality. White arrows: Mitigates parasitemia and mortality. Dotted arrow: Uncertain impact on parasitemia and mortality. Parameters upregulated (+) and downregulated (-) throughout aging. APC: antigen-presenting cells; Antib: antibodies; LB: lymphocytes B; Mol: molecules. Detailed results obtained in each study identified are described in supplementary files (Table S2).

8 DISCUSSION

The studies investigating the impact of age on systemic protozooses presented heterogeneous origin. Except for American trypanosomiasis, which was reported only by South American research groups, the other parasitic diseases were analyzed in studies mainly from developing countries. Research initiatives originated from low- and middle-income countries were coherent with the epidemiological profile of each infectious disease analyzed (GBD, 2015; LI; ZHOU, 2013; WHO, 2015). Although Chagas disease is a serious health problem in Latin America, epidemiological compression has been registered in the last decades, especially due to the successful implementation of vector control strategies (BERN, 2015; DIAS et al., 2016) and improvements in parasite screening in tissue and organ banks (DIAS et al., 2016). Conversely, as malaria, leishmaniasis and toxoplasmosis are parasitic diseases with difficult control and broad geographic distribution, more investment in research coming from developed countries was not surprising and is an important contribution to coping with these neglected diseases (MONTOYA; LIESENFELD, 2004; ANDREWS; FISHER; SKINNER-ADAMS, 2014).

Despite geographic origin, age-dependent evolution of parasitic diseases was the primary concern of the most studies identified. As the research motivation was not always explicit, population aging may not have be directly related to research efforts in the area. Even considering population aging to be an objective reality, especially in developing countries, the number of research initiatives was not aligned with the demographic transition over the last decades (GAVAZZI; HERRMANN; KRAUSE, 2004; HOLMES; JOSEPH, 2011). Thus, the focus of the preclinical studies was directed potentially towards the investigation of the age-dependent susceptibility and/or resistance profiles to systemic protozooses, in order better to understand the host-pathogen relationship. Considering that the natural history of parasitic diseases is complex and highly variable in different geographic areas and populations (GAVAZZI; HERRMANN; KRAUSE, 2004), the current evidence on the mechanistic basis that regulates host-pathogen relationship in old organisms is almost exclusively based on animal studies. While clinical studies exhibit critical limitations in internal and external validity related to the biological variability of the populations (i.e. age, sex, comorbidities, nutritional and immunological status) (KHORSAN; CRAWFORD, 2014; HENDERSON et al., 2013) and diseases (i.e. form, time and severity of infection, occurrence of reinfections and coinfections)

(KILKENNY et al., 2010); these sources of bias can be strictly controlled in preclinical studies (PANNUCCI; WILKINS, 2011; HENDERSON et al., 2013).

Even in preclinical research, methodological consistency should be considered in order properly to interpret the evidence available (SENA et al., 2014; RAMIREZ et al., 2017). Thus, although individual scores of bias have been variable among the studies analyzed, they have not presented a clear temporal influence (year of publication). This finding indicated that reporting bias has been systematically reproduced through the research process, despite advances in the analytical methods and regulatory strategies to stimulate accurate reports of preclinical studies (KILKENNY et al., 2010; GULIN; ROCCO; GARCÍA-BOURNISSEN, 2015). However, simple constructs such as animal weight and housing, randomization, experimental blindness, route and order of inoculation, welfare-related assessments, and relevance to human biology, were underreported and were the main source of bias in all studies analyzed. As these methodological constructs are easily adjustable (BATH; MACLEOD; GREEN, 2009; VESTERINEN et al., 2011; PEREIRA et al., 2017), the design of more rigorous protocols aligned with acceptable internal validity is a feasible task in further research initiatives.

Although the studies analyzed have shown wide methodological variability, some points of convergence were observed for each disease investigated. In general, C57BL/6 and BALB/c mouse models were often used to study leishmaniasis and toxoplasmosis, while mainly Wistar, Sprague-Dawley and Fischer 344 rats were used in Chagas disease and malaria models. Considering the biological cycle of Leishmania and *T. gondii*, dogs and cats are highly relevant models to investigate leishmaniasis and toxoplasmosis, since they are the main reservoirs of parasites in the domestic environment (TENTER; HECKEROTH; WEISS, 2000; QUINNELL; COURTENAY, 2009). However, no study analyzed used dogs and only one investigated cats. Independent of the animal model of infection, the use of larger animals (i.e. cats, dogs and non-human primates) was limited. Although these models are useful, and are closely correlated with the natural history of specific parasitic diseases, it is possible that their restricted applicability is related more to operational limitations (i.e. availability, cost and ethical considerations) (PEREIRA et al., 2017) than methodological intentionality. A proper selection of animal species and genetic background are crucial in preclinical studies of infectious diseases, since these characteristics are closely correlated to the host susceptibility and resistance profiles to different parasite species (ANDRADE; CHIARI, 2002; LEÓN et al., 2017). From our findings, the broad applicability of isogenic mice was coherent with the susceptibility of the lineages selected to the etiologic agents used to induce the infections

(CARDILLO et al., 1993; JOHNSON; GIBSON; SAYLES, 1995; EHRCHEN et al., 2004; SHAN et al., 2012). Although heterogenic mice can be also applicable, they exhibit high genotypic and phenotypic variability, which compromises the experimental control and internal validity of the research. Thus, outbreed strains introduce a critical source of intrinsic bias (i.e. biological variability) often associated with heterogeneous and poorly reproducible pathological manifestations (POSTAN; DVORAK; MCDANIEL, 1983; MOROCOIMA et al., 2012).

Although most studies used rats, this species has opposite relevance in preclinical models of Chagas disease and malaria. Mice and rats are classically used in experimental malaria to induce multi-organ damage similar to human disease, including lung, liver and brain damage, thrombocytopenia, microvascular obstruction and anemia (CRAIG et al., 2012; KEITA ALASSANE et al., 2017). When used together with appropriate parasite strains with specific characteristics of virulence and pathogenicity, these models offer sufficient applicability to evaluate infection severity and the appropriate pathological outcomes within the broad spectrum of changes induced by malaria (KEITA ALASSANE et al., 2017). However, the use of rats in studies on Chagas disease indicated an important methodological limitation with a negative implication to the internal validity (i.e. cause-effect relationships). As rats are naturally resistant to *T. cruzi* infection, they are a limited model, and require high parasite inoculum to develop the low parasitemia, and minor or absent classical organ injuries (i.e. cardiomyopathy and digestive disturbances [mega syndromes]) that characterize Chagas disease (RIVERA-VANDERPAS et al., 1983; MELO; MACHADO, 2001). As parasitic control and complete parasitemia suppression is achieved early after *T. cruzi* inoculation in rats, the age-dependent changes can be obscured by the natural host resistance to infection, which is determined by an upregulated and effective Th1 immunological response (CARDOSO; REIS CUNHA; BARTHOLOMEU, 2015; CHATELAIN; KONAR, 2015).

Beyond genetic background, sex-dependent variability in immunological response has also been related to the host resistance or susceptibility to parasitic infections (MANGANO; MODIANO, 2014; ZUK; STOEHR, 2010). Considering the heterogeneous use of male and female animals in the studies analyzed, it is essential to consider that host sex exhibits a relative relevance according the model of infection. As the relationship between sex and host susceptibility is poorly understood, and due to the high rates of infectivity and similar histopathological manifestations, both sexes are potentially useful in Chagas disease (SCHUSTER; SCHAUB, 2001) and malaria models (SUCKOW; DANNEMAN; BRAYTON, 2000; LOPES et al., 2016). However, mechanistic characterizations pointed

more consistently that, due to opposite profiles of immunological polarization, leishmaniasis progresses differently in male and female animals (ALEXANDER, 1988; MOCK; NACY, 1988; SATOSKAR; CENTRE, 1995; ROBERTS; SATOSKAR; ALEXANDER, 1996). As female mice show stronger Th2 responses to the infection, they are highly susceptible to Leishmania (BATCHELOR; CHAPMAN, 1965; EIDINGER; GARRETT, 1972; PAAVONEN; ANDERSSON; ADLERCREUTZ, 1981). Conversely, male animals are more resilient to the infection due to marked Th1-polarized phenotype, which attenuates parasite replication and survival (SANTOLI et al., 1976; ANDRADE; CHIARI, 2002; ALEXANDER, 1988; LIEW; O'DONNELL, 1993). As most studies on leishmaniasis used female animals, a coherent experimental construct was established, since more susceptible models make it easier to isolate the effects of aging from the outcomes induced by an exacerbated immune response.

The high variability in parasite species and strains identified in the studies should also be considered when evaluating the adequacy of the experimental models used. There is no doubt that parasite biology is highly relevant to infection evolution, especially considering the heterogeneous profiles of infectivity, virulence and pathogenicity (SAEIJ; BOYLE; BOOTHROYD, 2005; LEÓN et al., 2017). As these elements exhibit a strong genetic basis, parasite selection should be strictly aligned with the measured outcomes and phase of infection in analysis (LEÓN et al., 2017; PEREIRA et al., 2017). Thus, highly virulent and pathogenic parasites are only relevant in acute models, since they induce early mortality before the animals develop chronic infections. Conversely, models using pathogenic strains with low virulence are suitable when chronic manifestations are an additional target of analysis (CHATELAIN; KONAR, 2015; PEREIRA et al., 2017).

Considering that studies on Chagas disease investigated short periods of infection by highly virulent Y or Tulahuén strains (MARTÍNEZ-DÍAZ et al., 2001; PEREIRA et al., 2017), the authors consistently aligned experimental design with critical acute outcomes such as parasitemia, parasite load and mortality. For toxoplasmosis, short periods of infection by strains of all main genotypes (type I: RH, BK; type II: ME49, Prugniaud; and type III: c56, M-7741) were also coherently applied. Similar to human disease, these strains are highly infective but rarely induce fatal infections in imunocompetents organisms (ARAUJO; SLIFER, 2003; KHAN et al., 2005). As each clonal population exhibits divergent pathogenicity, type I strains were consistently used in acute models, since they induced higher parasitemia and parasite loads than type II or type III strains (HOWE; SIBLEY, 1995; SAEIJ; BOYLE; BOOTHROYD, 2005). In addition, type II strains were mainly used, considering

that they are associated with high risks of toxoplasmic encephalitis (HOWE; SIBLEY, 1995), which is not observed in infections by type III strains (SAEIJ; BOYLE; BOOTHROYD, 2005). In studies on malaria, *P. berghei* and *P. yoelii* were the only species used. As these parasites and their strains exhibit high genomic similarity (OTTO et al., 2014) and are highly adapted to rodent biology, they were suitable to induce acute infections in all studies investigated (GONÇALVES; LIMA; FERREIRA, 2014). In addition, *P. berghei* exhibits high molecular homology (i.e. cell invasion receptors) and pathogenic mechanisms (i.e. red blood cell sequestration and neurovascular syndrome) compared to *P. falciparum*, the most deadly species to cause malaria in humans (DE NIZ et al., 2016). Thus, although specific parasite clones might not represent the genetic diversity of Plasmodium infections in humans (CRAIG et al., 2012), *P. berghei* rodent model has been considered an invaluable tool for understanding malaria physiopathology and for testing new anti-parasitic strategies (DE NIZ et al., 2016). Finally, *L. major* or *L. donovani* were coherently applied to induce cutaneous and visceral leishmaniasis in rodent models. In both inbred mice (i.e. BALB/c and C57BL/6) and humans, strains from both species exhibit divergent profiles of virulence and pathogenicity (LOEUILLET; BAÑULS; HIDE, 2016). However, clonal strains of the same species are almost invariably associated with a single form of the disease (cutaneous or visceral) (LOEUILLET; BAÑULS; HIDE, 2016). Thus, as in models with a homogeneous genetic background (inbred mice) the same strain induces reproducible experimental outcomes (KÉBAIER et al., 2001), an important experimental construct of methodological quality was respected in the studies on leishmaniasis.

In response to the parasitic infections analyzed, parasitological, immunological and mortality outcomes exhibited heterogeneous profiles in different age groups. Throughout aging, while parasitemia and mortality were consistently reduced in Chagas disease and malaria, these parameters were similar or increased in leishmaniasis and highly variable in toxoplasmosis. Except for toxoplasmosis, our findings indicated that host age exerted a marked influence on critical indicators of systemic protozoozis progression (SHAN et al., 2012; IZHAR; BEN-AMI, 2015). As immunological response is the most prominent mechanism to control parasitemia, parasitism, tissue damage and mortality (RODRIGUES; OLIVEIRA; BELLIO, 2012; CARDOSO; REIS CUNHA; BARTHOLOMEU, 2015), age-dependent oscillations of the immunological phenotype were potentially related to the parasitological outcomes. In fact, older age groups exhibited marked divergences in cellular and molecular immunological mediators that control host resistance and susceptibility to infection. In Chagas disease, the most prominent positive adaptation with aging was increased

anti-*T. cruzi* antibody levels (i.e. IgM and IgG), while negative events were reductions in the production of Th1 molecules (i.e. IFN-γ, TNF, NO) and in leukocyte distribution (i.e. $TCD4^+$, NKT and APC). In leishmaniasis, while the protective phenotype was associated with an increase in mediators of cell immunity (i.e. $TCD8^+$, NKT, Treg, and expression of cellular activation molecules), humoral response exhibited an age-dependent attenuation. Conversely, all these parameters were consistently increased in malaria, indicating the acquisition of a complex and more comprehensive age-dependent protective phenotype. Besides age-dependent differences in disease evolution, studies on toxoplasmosis were restricted to parasitological (parasitemia and mortality) and pathological (organ damage) outcomes, and the only reference to reduction in $TCD8^+$ lymphocytes did not allow establishment of a relationship between age, immune response and disease severity.

In general, our findings indicated that beyond the divergences attributed to animal models, the evolution of Chagas disease, malaria and leishmaniasis can be also influenced by variable immunological responses in different age groups. The immunoregulation of host-pathogen interactions is complex, and involves cellular and humoral effectors (SHISHIDO et al., 2012). In this process, an appropriate balance between the antagonistic T helper 1 (Th1) and T helper 2 (Th2) immunological profiles is critical in parasitic diseases (BASSO, 2013; MILLS; MCGUIRK, 2004). In infections by intracellular parasites, Th1 molecules such as IL-12, IFN-γ, TNF-α and NO are essential to induce host resistance to both parasites analyzed. Conversely, Th2 molecules such as IL-4, IL-5, IL-6 and IL-13 are essential against extracellular parasites, but increase the susceptibility to infection caused by intracellular parasites (ANTHONY; RUTITZKY, 2007; SIBLEY, 2013; TRINCHIERI, 1997). In addition, B cells and a broad spectrum of parasite-specific neutralizing antibodies reinforce host defenses in systemic protozooses (CARDOSO; REIS CUNHA; BARTHOLOMEU, 2015; SULLIVAN et al., 2015). During extracellular periods, opsonization by IgG, IgM and IgE antibodies subclasses enhance parasite recognition by phagocytic cells (i.e. macrophages and dendritic cells) and complement fixation, which act together to attenuate host cell invasion and stimulate parasite death (MOSSER; ZHANG, 2011; UMEKITA; MOTA, 2000).

In studies on Chagas disease and malaria, reduced parasitemia and mortality rates were compatible to different age-dependent immunological phenotypes. Although reduction in Th1 effectors has been observed in older animals infected by *T. cruzi*, a concurrent increased in humoral response was also identified, with intense production of IgG and IgM. There is strong evidence showing the affectivity of humoral response in parasitic control in Chagas disease (SPINELLA; LIEGEARD; HONTEBEYRIE-JOSKOWICZ, 1992;

PITCOVSKY et al., 2001; BERMEJO et al., 2010). In preclinical models, depletion of B and T cells, or inactivation of specific anti- *T. cruzi* antibodies in animals infected by *T. cruzi* were associated to increased parasitemia, parasitic load and mortality (TARLETON, 1990; CARDOSO; REIS CUNHA; BARTHOLOMEU, 2015; SULLIVAN et al., 2015). Conversely, passive immunization with anti-*T. cruzi* antibodies obtained from previously infected animals was effective in reducing parasitemia and mortality in experimental Chagas disease (MIKHAIL; ROWLAND, 1990). Although poorly understood with regard to parasitic diseases, younger hosts seems develop a delayed B-cell activation and a nonspecific humoral response (FOLCH; WAKSMAN, 1974; WEIGLE, 1973); an aspect potentially related to the excess of suppressor T cells in the spleen of younger hosts (FOLCH; WAKSMAN, 1974). Conversely, the complete immunological maturation in older hosts (i.e. differentiation of competent cells) determines fast antigen recognition and effective production of specific anti-*T. cruzi* neutralizing antibodies (JACOBS; RADZIOCH; STEVENSON, 1995; PIERROT et al., 2003; SHAN et al., 2012).

As identified in the studies on malaria, similar humoral mechanisms were also related to reduced parasitemia and mortality in older animals. However, consistent increases in Th1 molecules, and in innate and acquired immune cells, indicated a more complex and effective defense line against Plasmodium infection. In fact, previous studies indicated that beneficial age-dependent immunological adaptations observed in malaria are related to enhancement of the ability of leukocytes to recognize a broader repertoire of antigenic variants of parasite-derived proteins (MAITLAND; MARSH, 2004). In this process, the increased number and effectivity of immunocompetent cells, such as $TCD4^+$ (WEISS et al., 1993; PHILLIPS; MATHERS; TAYLOR-ROBINSON, 1994), $TCD8^+$ (DOOLAN; HOFFMAN, 1999), and B cells (MEDING; LANGHORNE, 1991; VON DER WEID; HONARVAR; LANGHORNE, 1996) identified in older hosts are associated with protection against malaria (ADAM et al., 2003). In addition, the expansion of NKT cells in older animals is also pointed out as a resistance factor, since it plays an important role in the onset of infection, by stimulating B-cell activation and antibody production (SU; STEVENSON, 2002). From the increases in leukocyte number and the expression of antigen-recognizing molecules, increased IgG1, IgG2a, IgG2c, and IgE production was not surprising, as these are relevant serum effectors against Plasmodium (JACOBS; RADZIOCH; STEVENSON, et al., 1995; PIERROT et al., 2003; DUARTE et al., 2007; SHAN et al., 2012). Beyond the classical opsonizing and neutralizing role of IgG subclasses on protozoan parasites, IgE protects against malaria by activating monocytes and macrophages by their CD23 (low affinity) receptors, stimulating the

production of pro-inflammatory cytokines TNF-α (PERLMANN et al., 1999) and NO (DUGAS et al., 1995). Together, these molecules contribute to inhibiting parasite growth through direct anti-parasitic mechanisms (BALMER et al., 2000) or by stimulating phagocytosis by macrophages (SAFEUKUI et al., 2008).

A contrasting behavior was identified for leishmaniosis, in which groups of animals of older age presented more severe infections, high mortality rates and quite heterogeneous immunological responses. In leishmaniosis, although older animals showed a marked expansion of Treg, $TCD8^+$ and NK cells, as well as increased expression of receptors of antigen recognition and cell activation, these adaptations did not improve host resistance against Leishmania. Conversely, attenuation of humoral response and imbalance between Th1 and Th2 phenotypes were potentially associated with poor host response to infection. There is no doubt that resistance in older hosts is related to an increased Th1 cell response and efficient activation of dendritic cells and macrophages (BOGDAN; ROLLINGHOFF; DIEFENBACK, 2000). However, delayed and attenuated anti-Leishmania response can be observed in older hosts due to limited or unsustained IL-12 production, which is essential to Th1 polarization (EHRCHEN et al., 2004). Although the variable expression of Th1 and Th2 molecules is poorly understood, preclinical data indicated that the poor parasitological outcomes are linked to the limited ability of older hosts to develop an adjusted immunological phenotype. Thus, the excessive activation of both phenotypes can be equally damaging, since unbalanced Th1 responses determine intense inflammatory processes and tissue damage, while upregulation of Th2 phenotype inhibits central anti-parasitic defenses and makes the host highly susceptible to Leishmania replication and dissemination (BASSO, 2013; GIMBLET et al., 2015).

Although the immunological principles that modulate host-pathogen interaction in infections by intracellular protozoans are also applied to toxoplasmosis (DUPONT; CHRISTIAN; HUNTER, 2013; WALKER et al., 2013), the impact of age and aging on cellular and humoral mechanisms associated with susceptibility or resistance to *T. gondii* is still unclear. Besides the toxoplasmosis studies being the oldest identified, they were almost exclusively limited to parasitological and histopathological parameters. Although the elderly animals showed consistent reduction in parasitemia, the impact on mortality rates was variable. As an increased number of $TCD8^+$ cells and reduced activity of NK lymphocytes were reported in only one study, it was not possible to analyze the impact of the immune response on age-dependent parasitological outcomes. Thus, more controlled and comprehensive studies relating aging and toxoplasmosis are needed. As age-dependent

manifestations presented marked differences in each parasitic disease analyzed, is not prudent to infer a similar behavior in infections by *T. gondii*. Due to the central role of the immune response in infection control, only from more comprehensive phenotypic characterizations will be possible to understand how aged organisms respond to the challenge induced by this parasite.

9 CONCLUSIONS

Taken together, our findings indicated that host age exerts profound and varying influences on the evolution of systemic protozoozis. Throughout aging, parasitemia and mortality were consistently reduced in Chagas disease and malaria, but similar or increased in leishmaniasis and highly variable in toxoplasmosis. While a marked humoral response in older animals was related to the anti-*T. cruzi* protective phenotype in Chagas disease, a cellular response mediated by a polarized Th1 phenotype was additionally associated with a more effective defense against Plasmodium infections. Conversely, in leishmaniasis, the most severe infections and highest mortality rates were potentially related to the attenuation of humoral response and an imbalance between Th1 and Th2 phenotypes. Due to the heterogeneous parasitological outcomes and limited immunological data, the role of aging in toxoplasmosis evolution remains poorly understood. Besides the heterogeneity in experimental protocols, animal models (animal species and lineage), parasites (virulence and pathogenicity) and measured outcomes (time-dependent manifestations) were in general consistently aligned, representing an important element of internal validity. Although the limited methodological quality of the studies identified points to the need for careful analysis of current evidence, the main sources of bias were related to incomplete reporting of simple constructs. From a detailed description of these elements of bias, more comprehensive and controlled research may benefit by avoiding the systematic reproduction of inconsistent and poorly reproducible experimental designs.

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APPENDIX

Table S1 - Complete search strategy with search filters and number of studies recovered in databases PubMed-Medline, Scopus and Web of Sciences. (continua)

PubMed-MEDLINE – Search filters
#1 Diseases (human systemic protozoozis): (“Chagas disease”[TIAB] OR “American trypanosomiasis”[TIAB] OR “Trypanosomacruzi”[TIAB] OR “African sleeping sickness”[TIAB] OR “African sleeping sicknesses”[TIAB] OR “Sleeping sicknesses”[TIAB] OR “African trypanosomiasis”[TIAB] OR “Trypanosomabrucei”[TIAB] OR “Trypanosomagambiense”[TIAB] OR “Trypanosomarhodesiense”[TIAB] OR “African trypanosomiases”[TIAB] OR Leishmaniasis[TIAB] OR “Leishmaniasis, Visceral”[MeSH Terms] OR Malaria[TIAB] OR Plasmodium[TIAB] OR “Plasmodium falciparum”[TIAB] OR “Plasmodium vivax”[TIAB] OR “Plasmodium ovale” [TIAB] OR “Plasmodium cynomolgi”[TIAB] OR “Plasmodium malariae”[TIAB] OR “Entamoebahistolytica”[TIAB] OR Entamoebiasis[TIAB] OR Toxoplasmosis[TIAB] OR Toxoplasma[TIAB] OR “Toxoplasma gondii”[TIAB])
#2 Biological condition (aging): (“Aging”[MeSH Terms] OR “Aging”[TIAB] OR “Senescence”[TIAB] OR “Senility”[TIAB])
#3 First animal filter:("animal experimentation"[MeSH Terms] OR "models, animal"[MeSH Terms] OR "invertebrates"[MeSH Terms] OR "Animals"[Mesh:noexp] OR "animal population groups"[MeSH Terms] OR "chordata"[MeSHTerms:noexp] OR "chordata, nonvertebrate"[MeSH Terms] OR "vertebrates"[MeSHTerms:noexp] OR "amphibians"[MeSH Terms] OR "birds"[MeSH Terms] OR "fishes"[MeSH Terms] OR "reptiles"[MeSH Terms] OR "mammals"[MeSHTerms:noexp] OR "primates"[MeSHTerms:noexp] OR "artiodactyla"[MeSH Terms] OR "carnivora"[MeSH Terms] OR "cetacea"[MeSH Terms] OR "chiroptera"[MeSH Terms] OR "elephants"[MeSH Terms] OR "hyraxes"[MeSH Terms] OR "insectivora"[MeSH Terms] OR "lagomorpha"[MeSH Terms] OR "marsupialia"[MeSH Terms] OR "monotremata"[MeSH Terms] OR "perissodactyla"[MeSH Terms] OR "rodentia"[MeSH Terms] OR "scandentia"[MeSH Terms] OR "sirenia"[MeSH Terms] OR "xenarthra"[MeSH Terms] OR "haplorhini"[MeSHTerms:noexp] OR "strepsirhini"[MeSH Terms] OR "platyrrhini"[MeSH Terms] OR "tarsii"[MeSH Terms] OR

"catarrhini"[MeSHTerms:noexp] OR "cercopithecidae"[MeSH Terms] OR "hylobatidae"[MeSH Terms] OR "hominidae"[MeSHTerms:noexp] OR "gorilla gorilla"[MeSH Terms] OR "pan paniscus"[MeSH Terms] OR "pan troglodytes"[MeSH Terms] OR "pongopygmaeus"[MeSH Terms])
#4 Second animal filter: ((animals[tiab] OR animal[tiab] OR mice[Tiab] OR mus[Tiab] OR mouse[Tiab] OR murine[Tiab] OR woodmouse[tiab] OR rats[Tiab] OR rat[Tiab] OR murinae[Tiab] OR muridae[Tiab] OR cottonrat[tiab] OR cottonrats[tiab] OR hamster[tiab] OR hamsters[tiab] OR cricetinae[tiab] OR rodentia[Tiab] OR rodent[Tiab] OR rodents[Tiab] OR pigs[Tiab] OR pig[Tiab] OR swine[tiab] OR swines[tiab] OR piglets[tiab] OR piglet[tiab] OR boar[tiab] OR boars[tiab] OR "susscrofa"[tiab] OR ferrets[tiab] OR ferret[tiab] OR polecat[tiab] OR polecats[tiab] OR "mustelaputorius"[tiab] OR "guinea pigs"[Tiab] OR "guinea pig"[Tiab] OR cavia[Tiab] OR callithrix[Tiab] OR marmoset[Tiab] OR marmosets[Tiab] OR cebuella[Tiab] OR hapale[Tiab] OR octodon[Tiab] OR chinchilla[Tiab] OR chinchillas[Tiab] OR gerbillinae[Tiab] OR gerbil[Tiab] OR gerbils[Tiab] OR jird[Tiab] OR jirds[Tiab] OR merione[Tiab] OR meriones[Tiab] OR rabbits[Tiab] OR rabbit[Tiab] OR hares[Tiab] OR hare[Tiab] OR diptera[Tiab] OR flies[Tiab] OR fly[Tiab] OR dipteral[Tiab] OR drosphila[Tiab] OR drosophilidae[Tiab] OR cats[Tiab] OR cat[Tiab] OR carus[Tiab] OR felis[Tiab] OR nematoda[Tiab] OR nematode[Tiab] OR nematoda[Tiab] OR nematode[Tiab] OR nematodes[Tiab] OR sipunculida[Tiab] OR dogs[Tiab] OR dog[Tiab] OR canine[Tiab] OR canines[Tiab] OR canis[Tiab] OR sheep[Tiab] OR sheeps[Tiab] OR mouflon[Tiab] OR mouflons[Tiab] OR ovis[Tiab] OR goats[Tiab] OR goat[Tiab] OR capra[Tiab] OR capras[Tiab] OR rupicapra[Tiab] OR chamois[Tiab] OR haplorhini[Tiab] OR monkey[Tiab] OR monkeys[Tiab] OR anthropoidea[Tiab] OR anthropoids[Tiab] OR saguinus[Tiab] OR tamarin[Tiab] OR tamarins[Tiab] OR leontopithecus[Tiab] OR hominidae[Tiab] OR ape[Tiab] OR apes[Tiab] OR pan[Tiab] OR paniscus[Tiab] OR "pan paniscus"[Tiab] OR bonobo[Tiab] OR bonobos[Tiab] OR troglodytes[Tiab] OR "pan troglodytes"[Tiab] OR gibbon[Tiab] OR gibbons[Tiab] OR siamang[Tiab] OR siamangs[Tiab] OR nomascus[Tiab] OR symphalangus[Tiab] OR chimpanzee[Tiab] OR chimpanzees[Tiab] OR prosimians[Tiab] OR "bush baby"[Tiab] OR prosimian[Tiab] OR bush babies[Tiab] OR galagos[Tiab] OR galago[Tiab] OR pongidae[Tiab] OR gorilla[Tiab] OR gorillas[Tiab] OR pongo[Tiab] OR pygmaeus[Tiab] OR "pongopygmaeus"[Tiab] OR orangutans[Tiab] OR pygmaeus[Tiab] OR lemur[Tiab] OR lemurs[Tiab] OR

lemuridae[Tiab] OR horse[Tiab] OR horses[Tiab] OR pongo[Tiab] OR equus[Tiab] OR cow[Tiab] OR calf[Tiab] OR bull[Tiab] OR chicken[Tiab] OR chickens[Tiab] OR gallus[Tiab] OR quail[Tiab] OR bird[Tiab] OR birds[Tiab] OR quails[Tiab] OR poultry[Tiab] OR poultries[Tiab] OR fowl[Tiab] OR fowls[Tiab] OR reptile[Tiab] OR reptilia[Tiab] OR reptiles[Tiab] OR snakes[Tiab] OR snake[Tiab] OR lizard[Tiab] OR lizards[Tiab] OR alligator[Tiab] OR alligators[Tiab] OR crocodile[Tiab] OR crocodiles[Tiab] OR turtle[Tiab] OR turtles[Tiab] OR amphibian[Tiab] OR amphibians[Tiab] OR amphibia[Tiab] OR frog[Tiab] OR frogs[Tiab] OR bombina[Tiab] OR salientia[Tiab] OR toad[Tiab] OR toads[Tiab] OR "epidaleacalamita"[Tiab] OR salamander[Tiab] OR salamanders[Tiab] OR eel[Tiab] OR eels[Tiab] OR fish[Tiab] OR fishes[Tiab] OR pisces[Tiab] OR catfish[Tiab] OR catfishes[Tiab] OR siluriformes[Tiab] OR arius[Tiab] OR heteropneustes[Tiab] OR sheatfish[Tiab] OR perch[Tiab] OR perches[Tiab] OR percidae[Tiab] OR perca[Tiab] OR trout[Tiab] OR trouts[Tiab] OR char[Tiab] OR chars[Tiab] OR salvelinus[Tiab] OR "fathead minnow"[Tiab] OR minnow[Tiab] OR cyprinidae[Tiab] OR carps[Tiab] OR carp[Tiab] OR zebrafish[Tiab] OR zebrafishes[Tiab] OR goldfish[Tiab] OR goldfishes[Tiab] OR guppy[Tiab] OR guppies[Tiab] OR chub[Tiab] OR chubs[Tiab] OR tinca[Tiab] OR barbels[Tiab] OR barbus[Tiab] OR pimephales[Tiab] OR promelas[Tiab] OR "poeciliareticulata"[Tiab] OR mullet[Tiab] OR mullets[Tiab] OR seahorse[Tiab] OR seahorses[Tiab] OR mugilcurema[Tiab] OR atlantic cod[Tiab] OR shark[Tiab] OR sharks[Tiab] OR catshark[Tiab] OR anguilla[Tiab] OR salmonid[Tiab] OR salmonids[Tiab] OR whitefish[Tiab] OR whitefishes[Tiab] OR salmon[Tiab] OR salmons[Tiab] OR sole[Tiab] OR solea[Tiab] OR "sea lamprey"[Tiab] OR lamprey[Tiab] OR lampreys[Tiab] OR pumpkinseed[Tiab] OR sunfish[Tiab] OR sunfishes[Tiab] OR tilapia[Tiab] OR tilapias[Tiab] OR turbot[Tiab] OR turbots[Tiab] OR flatfish[Tiab] OR flatfishes[Tiab] OR sciuridae[Tiab] OR squirrel[Tiab] OR squirrels[Tiab] OR chipmunk[Tiab] OR chipmunks[Tiab] OR suslik[Tiab] OR susliks[Tiab] OR vole[Tiab] OR voles[Tiab] OR lemming[Tiab] OR lemmings[Tiab] OR muskrat[Tiab] OR muskrats[Tiab] OR lemmus[Tiab] OR otter[Tiab] OR otters[Tiab] OR marten[Tiab] OR martens[Tiab] OR martes[Tiab] OR weasel[Tiab] OR badger[Tiab] OR badgers[Tiab] OR ermine[Tiab] OR mink[Tiab] OR minks[Tiab] OR sable[Tiab] OR sables[Tiab] OR gulo[Tiab] OR gulos[Tiab] OR wolverine[Tiab] OR wolverines[Tiab] OR minks[Tiab] OR mustela[Tiab] OR llama[Tiab] OR llamas[Tiab] OR alpaca[Tiab] OR alpacas[Tiab] OR camelid[Tiab] OR

camelids[Tiab] OR guanaco[Tiab] OR guanacos[Tiab] OR chiroptera[Tiab] OR chiropteras[Tiab] OR bat[Tiab] OR bats[Tiab] OR fox[Tiab] OR foxes[Tiab] OR iguana[Tiab] OR iguanas[Tiab] OR xenopuslaevis[Tiab] OR parakeet[Tiab] OR parakeets[Tiab] OR parrot[Tiab] OR parrots[Tiab] OR donkey[Tiab] OR donkeys[Tiab] OR mule[Tiab] OR mules[Tiab] OR zebra[Tiab] OR zebras[Tiab] OR shrew[Tiab] OR shrews[Tiab] OR bison[Tiab] OR bisons[Tiab] OR buffalo[Tiab] OR buffaloes[Tiab] OR deer[Tiab] OR deers[Tiab] OR bear[Tiab] OR bears[Tiab] OR panda[Tiab] OR pandas[Tiab] OR "wild hog"[Tiab] OR "wild boar"[Tiab] OR fitchew[Tiab] OR fitch[Tiab] OR beaver[Tiab] OR beavers[Tiab] OR jerboa[Tiab] OR jerboas[Tiab] OR capybara[Tiab] OR capybaras[Tiab]) NOT medline[subset])

#5 Combined search: (#1 AND #2) AND (#3 OR #4)

SCOPUS – *Search filters*

#1 Disease (human systemic protozoozis): (TITLE-ABS-KEY("Chagas disease") OR TITLE-ABS-KEY("American trypanosomiasis") OR TITLE-ABS-KEY("Trypanosomacruzi") OR TITLE-ABS-KEY("African sleeping sickness") OR TITLE-ABS-KEY("African sleeping sicknesses") OR TITLE-ABS-KEY("Sleeping sicknesses") OR TITLE-ABS-KEY("African Trypanosomiasis") OR TITLE-ABS-KEY("Trypanosomabrucei") OR TITLE-ABS-KEY("Trypanosomagambiense") OR TITLE-ABS-KEY("Trypanosomarhodesiense") OR TITLE-ABS-KEY("African trypanosomiases") OR TITLE-ABS-KEY("Leishmaniasis") OR TITLE-ABS-KEY("Leishmaniasis, Visceral") OR TITLE-ABS-KEY("Malaria") OR TITLE-ABS-KEY("Plasmodium") OR TITLE-ABS-KEY("Plasmodium falciparum") OR TITLE-ABS-KEY("Plasmodium vivax") OR TITLE-ABS-KEY("Plasmodium ovale") OR TITLE-ABS-KEY("Plasmodium cynomolgi") OR TITLE-ABS-KEY("Plasmodium malariae") OR TITLE-ABS-KEY("Entamoebahistolytica") OR TITLE-ABS-KEY("Entamoebiasis") OR TITLE-ABS-KEY("Toxoplasmosis") OR TITLE-ABS-KEY("Toxoplasma") OR TITLE-ABS-KEY("Toxoplasma gondii"))

#2 Biological condition (aging): (TITLE-ABS-KEY("Aging") OR TITLE-ABS-KEY("Senescence") OR TITLE-ABS-KEY("Senility"))

#3 Combined search: #1 AND #2

#4 Search limits (Keyword): Animals

WEB OF SCIENCE – *Search filters*
#1 Disease (human systemic protozoozis): TS=Chagas disease OR TS=American trypanosomiasis OR TS=Trypanosomacruzi OR TS=African sleeping sickness OR TS=African sleeping sicknesses OR TS=Sleeping sicknesses OR TS=African trypanosomiasis OR TS=Trypanosomabrucei OR TS=Trypanosomagambiense OR TS=Trypanosomarhodesiense OR TS=African trypanosomiases OR TS=Leishmaniasis OR TS= Leishmaniasis, Visceral OR TS=Malaria OR TS=Plasmodium OR TS=Plasmodium falciparum OR TS=Plasmodium vivax OR TS=Plasmodium ovale OR TS=Plasmodium cynomolgi OR TS=Plasmodium malariae OR TS=Entamoebahistolytica OR TS=Entamoebiasis OR TS=Toxoplasmosis OR TS=Toxoplasma OR TS=Toxoplasma gondii
#2 Biological condition (aging): TS=Aging OR TS=Aging OR TS=Senescence OR TS=Senility
#3 Animal filter:TS=animal OR TS=mice OR TS=mouse OR TS=rat OR TS=rats OR TS=dog OR TS=dogs OR TS=rabbits OR TS=murine model OR TS=guinea pig OR TS=hamster OR TS=pig OR TS=animal model
#4 Combined search: #1 AND #2 AND #3

Source: From the author

Table S2 - Bias analysis in all studies included in the systematic review.

Studies / Quality criteria	*Malaria*										
	SHAN et al. 2013	SHAN et al., 2012	SAFEUKUI et al. 2008	ADAM et al. 2003	SINGER et al. 1954	ALGER et al. 1972	GRAVELY et al. 1976	SOLOMON, 1986	ORAGO and SOLOMON, 1986	SMALLEY, 1975	PIERROT et al. 2003
Title											
Accurate and concise description of the content of the article	√	√	√	√	√	√	√	No	√	√	√
Abstract											
Summary of the background, objectives, methods, main findings and conclusions	√	√	No	√	No	√	√	√	√	√	√
Introduction											
Sufficient scientific background	√	√	√	√	√	No	√	√	√	√	√
Rational explanation of the experimental approach	√	√	√	√	√	No	√	√	√	√	√
Objectives											
Clear primary and secondary objectives	√	√	√	√	√	√	√	√	√	√	√
Materials and Methods											
Ethical statement											
Ethical review permissions, licenses and official guidelines for use of animals	No	No	√	No	No	No	No	√	No	No	√
Study design											
Number of animals per group	No	No	No	No	√	√	No	√	√	√	No
Information on whether the experiment was performed as a blind controlled study	No	No	No	No	No	√	No	No	No	No	No
Experimental procedures											
Parasite species	√	√	√	√	√	√	√	√	√	√	√
Parasite strain	√	√	√	√	√	√	No	√	√	No	√
Parasite inoculum	√	√	√	√	√	√	√	√	√	√	√
Inoculum route	√	√	√	√	√	√	√	√	√	√	No
Time of infection	√	√	√	√	√	√	√	√	√	√	√
Rationale for choice of parasite inoculum	No	No	No	No	No	No	No	No	No	No	No
Rationale for choice of route of administration	No	No	No	No	No	No	No	No	No	No	No

Note: √, Criteria completed. No, criteria not completed.

Table S2 - *(continuation)*. Bias analysis in all studies included in the systematic review.

Studies / Quality criteria	*Malaria*										
	SHAN et al. 2013	SHAN et al., 2012	SAFEUKUI et al. 2008	ADAM et al. 2003	SINGER et al. 1954	ALGER et al. 1972	GRAVELY et al. 1976	SOLOMON, 1986	ORAGO and SOLOMON, 1986	SMALLEY, 1975	PIERROT et al. 2003
Experimental animals											
Information regarding animal species	√	√	√	√	√	√	√	√	√	√	√
Strain of the animals	√	√	√	√	√	√	√	√	√	√	√
Sex of the animals	√	√	√	√	√	√	√	√	√	√	√
Weight range of the animals	*No*	No	No	No	√	No	No	No	√	√	No
Age of the animals	√	√	√	√	√	√	√	√	√	√	√
Information related to previous procedures performed on the animals	No	No	No	No	No	No	No	No	No	No	No
Housing and husbandry											
Housing of experimental animals (facility, animals/cage, material)	No	No	No	No	√	√	No	No	No	No	No
Breeding program, light/dark cycle, temperature, quality of water	No	No	No	No	√	√	√	No	No	No	No
Welfare-related assessments before, during, or after the experiment	No	No	No	No	No	√	No	No	No	No	No
Sample size											
Number of animals used in each experiment and group	No	No	No	No	√	√	No	√	√	√	No
Explanation regarding number of animals and sample size calculation	No	No	No	No	No	No	No	No	No	No	No
Allocating animals to groups											
Full details of how animals were allocated to groups (randomization)	No	No	No	No	No	No	No	No	No	No	No
Order of animals inoculated and evaluation	No	No	No	No	No	No	No	No	No	No	No
Experimental outcomes											
Clear experimental outcomes assessed	√	√	√	√	√	√	√	√	√	√	√
Statistical methods											
Statistical methods used for each analysis	√	√	√	√	No	No	No	No	No	No	No
Specification of the unit of analysis for each dataset	No	No	√	√	No	No	No	No	No	No	No
Methods used to assess adequacy of the statistical approach	No	No	√	√	No	No	No	No	No	No	No

Note: √, Criteria completed. No, criteria not completed.

Table S2 - *(continuation)*. Bias analysis in all studies included in the systematic review.

Quality criteria \ Studies	*Malaria*										
	SHAN et al. 2013	SHAN et al., 2012	SAFEUKUI et al. 2008	ADAM et al. 2003	SINGER et al. 1954	ALGER et al. 1972	GRAVELY et al. 1976	SOLOMON, 1986	ORAGO and SOLOMON, 1986	SMALLEY, 1975	PIERROT et al. 2003
Results											
Baseline data											
Description of health status of animals before inoculation	No	No	No	No	No	No	No	No	No	No	No
Numbers analyzed											
Number of animals in each group included in each analysis	No	No	No	No	√	√	No	√	√	√	No
Data not included in the analysis (explanation of exclusion)	No	No	No	No	No	No	No	No	No	No	No
Outcomes and estimation											
Information regarding parasitemia (Mean ± SD)	√		√	√	√	No	No	√	√	√	√
Information regarding inflammation (Mean ± SD)	√	√	√	√	No	No	√	No	No	√	√
Adverse events											
Information regarding mortality	√	√	√	√	√	√	No	√	√	√	√
Modifications to the protocols to reduce adverse events	No	No	No	No	No	No	No	No	No	No	No
Discussion											
Interpretation / scientific implications											
Interpretation of the results, consider objectives, hypotheses, current theory	√	√	√	√	√	√	√	√	√	√	√
Comments on limitations (bias, limitations of the model, imprecision of results)	No	No	No	No	No	No	No	No	No	No	No
Generalizability/translation											
Comments on how the findings are likely to translate to other species and relevance to humans	No	No	No	No	No	No	√	No	No	No	No
Funding											
List of funding sources and the role of the funder(s) in the study	No	No	No	No	No	No	No	No	No	No	No
Criteria completed (n)	20	19	22	22	23	22	18	21	22	22	19
Criteria completed (%)	44.4	42.2	48.9	48.9	51.1	48.9	40.0	46.7	48.9	48.9	42.2

Note: √, Criteria completed. No, criteria not completed.

Table S2 - *(continuation)*. Bias analysis in all studies included in the systematic review.

Studies / Quality criteria	Leishmaniasis						*Chagas disease*				
	EHRCHEN et al. 2004	LAGES et al. 2008	BHATTACHARYA et al. 2016	MULLER et al. 2008	CILLARI et al. 1992	SINGH et al. 2007	COLATO et al. 2017	PÉREZ et al. 2011	PASCUTTI et al. 2003	CARDILLO et al. 1993	BRAZÃO et al. 2017
Title											
Accurate and concise description of the content of the article	√	√	√	√	√	√	√	√	√	√	√
Abstract											
Summary of the background, objectives, methods, main findings and conclusions	√	√	√	√	√	√	√	√	√	√	√
Introduction											
Sufficient scientific background	√	√	√	√	√	√	√	√	√	√	√
Rational explanation of the experimental approach	√	√	√	√	√	√	√	√	√	√	√
Objectives											
Clear primary and secondary objectives	√	√	√	√	√	√	√	√	√	√	√
Materials and Methods											
Ethical statement											
Ethical review permissions, licenses and official guidelines for use of animals	√	√	√	√	√	√	√	√	√	√	√
Study design											
Number of animals per group	No	No	No	No	No	No	No	No	No	No	√
Information on whether the experiment was performed as a blind controlled study	No	No	No	No	No	No	√	No	No	No	√
Experimental procedures											
Parasite species	√	√	√	√	√	√	√	√	√	√	√
Parasite strain	√	√	√	√	√	√	√	√	√	√	√
Parasite inoculum	√	√	√	√	√	√	√	√	√	√	√
Inoculum route	√	√	√	√	√	√	√	√	√	√	√
Time of infection	√	No	√	√	√	√	√	√	√	No	√
Rationale for choice of parasite inoculums	No	No	No	No	No	No	No	No	No	No	No
Rationale for choice of route of administration	No	No	No	No	No	No	No	No	No	No	No

Note: √, Criteria completed. No, criteria not completed.

Table S2 - *(continuation)*. Bias analysis in all studies included in the systematic review.

Studies / Quality criteria	Leishmaniasis						*Chagas disease*				
	EHRCHEN et al. 2004	LAGES et al. 2008	BHATTACHARYA et al. 2016	MULLER et al. 2008	CILLARI et al. 1992	SINGH et al. 2007	COLATO et al. 2017	PÉREZ et al. 2011	PASCUTTI et al. 2003	CARDILLO et al. 1993	BRAZÃO et al. 2017
Experimental animals											
Information regarding animal species	√	√	√	√	√	√	√	No	No	No	√
Strain of the animals	√	√	√	√	√	√	√	√	No	√	√
Sex of the animals	√	√	√	√	√	√	√	√	√	No	√
Weight range of the animals	No	No	No	No	No	√	√	No	No	No	√
Age of the animals	√	√	√	√	√	√	√	√	√	√	√
Information related to previous procedures performed on the animals	No	No	No	No	No	No	√	No	No	No	No
Housing and husbandry											
Housing of experimental animals (facility, animals/cage, material)	No	No	No	No	No	√	No	√	No	√	√
Breeding program, light/dark cycle, temperature, quality of water	No	No	No	No	No	√	√	√	No	√	√
Welfare-related assessments before, during, or after the experiment	No	No	No	No	No	No	√	No	No	√	√
Sample size											
Number of animals used in each experiment and group	No	No	No	No	No	No	No	No	No	No	√
Explanation regarding number of animals and sample size calculation	No	No	No	No	No	No	No	No	No	No	No
Allocating animals to groups											
Full details of how animals were allocated to groups (randomization)	No	No	No	No	No	No	No	No	No	No	√
Order of animals inoculated and evaluation	No	No	No	No	No	No	No	No	No	No	No
Experimental outcomes											
Clear experimental outcomes assessed	√	√	√	√	√	√	√	√	√	√	√
Statistical methods											
Statistical methods used for each analysis	√	No	√	No	No	√	√	√	No	√	√
Specification of the unit of analysis for each dataset	No	No	√	No	√	√	√	√	No	√	√
Methods used to assess adequacy of the statistical approach	No	No	√	No	No	√	√	√	No	√	√

Note: √, Criteria completed. No, criteria not completed.

Table S2 - *(continuation)*. Bias analysis in all studies included in the systematic review.

Studies / Quality criteria	Leishmaniasis						*Chagas disease*				
	EHRCHEN et al. 2004	LAGES et al. 2008	BHATTACHARYA et al. 2016	MULLER et al. 2008	CILLARI et al. 1992	SINGH et al. 2007	COLATO et al. 2017	PÉREZ et al. 2011	PASCUTTI et al. 2003	CARDILLO et al. 1993	BRAZÃO et al. 2017
Results											
Baseline data											
Description of health status of animals before inoculation	No	No	No	No	No	No	√	No	√	No	No
Numbers analyzed											
Number of animals in each group included in each analysis	No	No	No	No	No	No	No	No	No	No	√
Data not included in the analysis (explanation of exclusion)	No	No	No	No	No	No	No	No	No	No	No
Outcomes and estimation											
Information regarding parasitemia (Mean ± SD)	No	No	√	√	No	√	No	√	√	√	No
Information regarding inflammation (Mean ± SD)	√	√	√	√	No	No	√	√	√	No	No
Adverse events											
Information regarding mortality	No	No	No	No	√	No	No	No	No	√	No
Modifications to the protocols to reduce adverse events	No	No	No	No	No	No	No	No	No	No	No
Discussion											
Interpretation / scientific implications											
Interpretation of the results, consider objectives, hypotheses, current theory	√	√	√	√	√	√	√	√	√	√	√
Comments on limitations (bias, limitations of the model, imprecision of results)	No	No	No	No	No	No	No	No	No	No	No
Generalizability/translation											
Comments on how the findings are likely to translate to other species and relevance to humans	No	√	No	√	No	No	No	No	No	No	No
Funding											
List of funding sources and the role of the funder(s) in the study	No	No	No	No	No	No	No	No	No	No	No
Criteria completed (n)	19	18	22	20	19	24	27	23	18	22	29
Criteria completed (%)	42.2	40.0	48.9	44.4	42.2	53.3	60.0	51.1	40.0	48.9	64.4

Note: √, Criteria completed. No, criteria not completed.

Table S2 - *(continuation)*. Bias analysis in all studies included in the systematic review.

Studies / Quality criteria	Toxoplasmosis							Total bias	
	HENRY and BEVERLY, 1976	DE CHAMPS et al. 1998	GAO et al. 2015	EMMERLING et al. 1979	GARDNER and REMINGTON, 1977	JOHNSON et al. 1995	DUBEY et al., 1977	**Bias score (n)**	**Bias score (%)**
Title									
Accurate and concise description of the content of the article	√	√	√	No	√	√	√	27	93.1
Abstract									
Summary of the background, objectives, methods, main findings and conclusions	√	√	√	√	√	√	√	27	93.1
Introduction									
Sufficient scientific background	√	√	√	√	√	√	√	28	96.6
Rational explanation of the experimental approach	√	√	√	√	√	√	√	28	96.6
Objectives									
Clear primary and secondary objectives	√	√	√	√	√	√	√	29	100.0
Materials and Methods									
Ethical statement									
Ethical review permissions, licenses and official guidelines for use of animals	No	No	√	No	No	√	No	16	52.2
Study design									
Number of animals per group	√	√	√	√	No	No	√	11	37.9
Information on whether the experiment was performed as a blind controlled study	No	No	No	No	No	No	No	3	10.3
Experimental procedures									
Parasite species	√	√	√	√	√	√	√	29	100.0
Parasite strain	No	√	√	√	√	√	√	26	89.7
Parasite inoculum	√	√	√	√	√	√	√	29	100.0
Inoculum route	√	√	√	√	√	√	√	28	96.6
Time of infection	√	√	No	√	√	√	√	26	89.7
Rationale for choice of parasite inoculum	No	No	No	No	No	No	No	0	0.0
Rationale for choice of route of administration	No	No	No	No	No	No	No	0	0.0

Note: √, Criteria completed. No, criteria not completed.

Table S2 - *(continuation)*. Bias analysis in all studies included in the systematic review.

Studies / Quality criteria	Toxoplasmosis							Total bias	
	HENRY and BEVERLY, 1976	DE CHAMPS et al. 1998	GAO et al. 2015	EMMERLING et al. 1979	GARDNER and REMINGTON, 1977	JOHNSON et al. 1995	DUBEY et al., 1977	**Bias score (n)**	**Bias score (%)**
Experimental animals									
Information regarding animal species	No	√	√	√	√	√	No	24	82.8
Strain of the animals	No	√	√	√	√	√	No	26	89.7
Sex of the animals	√	√	√	√	√	√	No	27	93.1
Weight range of the animals	No	No	√	No	No	No	No	7	24.1
Age of the animals	√	√	√	√	√	√	√	29	100.0
Information related to previous procedures performed on the animals	No	No	No	No	No	No	No	1	3.4
Housing and husbandry									
Housing of experimental animals (facility, animals/cage, material)	No	No	√	No	No	No	√	8	27.6
Breeding program, light/dark cycle, temp., quality of water	No	No	√	No	No	√	√	11	37.9
Welfare-related assessments before, during, or after experiment	No	No	√	No	No	√	No	6	20.7
Sample size									
Number of animals used in each experiment and group	√	√	√	√	No	No	√	11	37.9
Explanation regarding number of animals and sample size calculation	No	No	No	No	No	No	No	0	0.0
Allocating animals to groups									
Full details of how animals were allocated to groups (randomization)	No	No	No	No	No	No	No	1	3.4
Order of animals inoculation and evaluation	No	No	No	No	No	No	No	0	0.0
Experimental outcomes									
Clear outcomes assessed	√	√	√	√	√	√	√	29	100.0
Statistical methods									
Statistical methods used for each analysis	No	No	√	√	No	√	No	14	48.3
Specification of the unit of analysis for each dataset	No	No	√	No	No	√	No	11	37.9
Methods used to assess adequacy of the statistical approach	No	No	√	No	No	√	No	10	34.5

Note: √, *Criteria completed. No, criteria not completed.*

Table S2 - *(conclusion)*. Bias analysis in all studies included in the systematic review.

Quality criteria \ Studies	Toxoplasmosis							Total bias	
	HENRY and BEVERLY, 1976	DE CHAMPS et al. 1998	GAO et al. 2015	EMMERLING et al. 1979	GARDNER and REMINGTON, 1977	JOHNSON et al. 1995	DUBEY et al., 1977	**Bias score (n)**	**Bias score (%)**
Results									
Baseline data									
Description of health status of animals before inoculation	No	No	No	√	No	√	No	4	13.8
Numbers analyzed									
Number of animals in each group included in each analysis	√	√	√	√	No	No	No	10	34.5
Data not included in the analysis (explanation of exclusion)	No	No	No	No	No	No	No	0	0.0
Outcomes and estimation									
Information regarding parasitemia (Mean ± SD)	No	No	No	No	No	No	No	14	48.3
Information regarding inflammation (Mean ± SD)	No	No	No	No	No	√	No	15	51.7
Adverse events									
Information regarding mortality	No	√	No	√	√	√	No	16	55.2
Modifications to the protocols to reduce adverse events	No	No	No	No	No	No	No	0	0.0
Discussion									
Interpretation / scientific implications									
Interpretation of the results, consider objectives, hypotheses, current theory	√	√	√	√	√	√	√	29	100.0
Comments on limitations (bias, limitations of the model, imprecision of results)	No	No	No	No	No	No	No	0	0.0
Generalizability/translation									
Comments on how the findings are likely to translate to other species and relevance to humans	No	No	No	No	No	No	No	3	10.3
Funding									
List of funding sources and the role of the funder(s) in the study	No	No	No	No	No	No	No	0	0.0
Criteria completed (n)	16	20	26	21	17	25	17		
Criteria completed (%)	35.6	44.4	57.8	46.7	37.8	55.6	37.8		

√, Criteria completed. No, criteria not completed.

Source: From the author

Table S3 - General characteristics of all studies included in the systematic review.

Study	Country	Species	Lineage	Sex	Weight (mean or range)	Age (mean or range)
Malaria						
SHAN et al., 2013	China	Mice	C57BL/6 mice	Female	(-)	**Young mice:** 21 days and **Old mice:** 240 days
SHAN et al. 2012	China	Mice	C57BL/6 mice	Female	(-)	**Young mice:** 21 days **Old mice:** 240 days
SAFEUKUI et al. 2008*	France	Rats	Lewis	Female	(-)	**Rats:** 28, 42 and 56 days
ADAM et al. 2003	France	Rats	Fischer F344	Male	**Young:** 60g **Adult:** 190 g	**Young rats:** 28 days **Adult rats:** 56 days
SINGER et al. 1954	USA	Rats	Sprague-Dawley	Female	47.9g to 217.6g	**Rats:** 28, 30, 33, 39,41, 42, 49, 51, 57, 76, 81, 93 days
ORAGO and SOLOMON, 1986	UK	Rats	Sprague Dawley	Female	50g	**Rats:** 30, 50 days
PIERROT et al. 2003	France	Mice / Rats	BALB/c, C57B1/6J, Fischer F344	Male	(-)	**Rats and mices:** 28, 42, 56, 70, 84, 112 days
SMALLEY, 1975	UK	Rats	Wistar	Male	50 g	**Rats:** 30, 50, 90, 100, 300 days
SOLOMON, 1986*	UK	Rats	Sprague Dawley	Female	(-)	**Rats:** 30, 50 days
GRAVELY et al. 1976	USA	Rats	Fischer F344	Female	(-)	**Rats:** 28, 126 days

*Note: *In vitro and in vivo. (-) Data not reported. (?) Incomplete information.*

Table S3 - *(continuation)*. General characteristics of all studies included in the systematic review.

Study	Country	Species	Lineage	Sex	Weight (mean or range)	Age (mean or range)
ALGER et al. 1972	USA	Mice	A/J, Carworth CF	Female	(-)	**Young mice:** 4-120 days **Old mice:** 180-240 days
Leishmaniosis						
EHRCHEN et al. 2004*	Germany	Mice	BALB/c, C57BL/6	Female	(-)	**Young mice:** 56, 70 days **Old mice:** 540 days
CILLARI et al., 1992	UK	Mice	BALB/c	Female	(-)	**Mice:** 70 to 840 days
SINGH et al. 2007	India	Hamsters	Mesocricetus auratus	Male	**Young:** 45-50g **Adult:** 100-110g	**Young hamsters:** 21-28 days **Adult hamsters:** 105 -112 days
MULLER et al. 2008	Tunísia	Mice	BALB/c	Female	(-)	**Young mice:** 21 and 28 days **Old mice:** 42 and 56 days
BHATTACHARYA et al. 2016	Australia	Mice	BALB/c	Female	(-)	**Young mice:** 77 days **Old mice:** 504 days
LAGES et al. 2008*	USA	Mice	C57BL/6	(-)	(-)	**Young mice:** 42-56 days **Old mice:** 140 days
Toxoplasmosis						
JOHNSON et al. 1995	USA	Mice	C57BL/6J, DBA/2J, A/J, C57BL/6J	Female	(-)	**Young adult mice:** 60-90 days **Old mice:** 660-720 days
EMMERLING et al. 1979	Germany	Mice	NMRI	Female	(-)	**Young mice:** 42-84 days **Adult mice:** 330 days **Old mice:** 600-720 days
GARDNER and REMINGTON, 1977	USA	Mice	BALB/c and C57BL/6	Female	(-)	**Young mice:** 60 to 150 days **Old mice:** 270- 540 days

*Note: *In vitro and in vivo. (-) Data not reported. (?) Incomplete information.*

Table S3 - *(continuation)*. General characteristics of all studies included in the systematic review.

Study	Country	Species	Lineage	Sex	Weight (mean or range)	Age (mean or range)
GAO et al. 2015	China and England	Rats	Brown Norway, Fischer 344, Lewis	Female / Male	150-200g	**Newborn rats:** 5, 10, 15, 20 days **Adult rats:** 56 -70 days
DE CHAMPS et al. 1998	France	Rats	Fischer F344, Wistar, Sprague-Dawley	Female / Male	(-)	**Rats:** 7, 11, 21, 24 and 46 days
HENRY and BEVERLEY, 1976	England	Mice	(-)	Female / male	N	**Mice:** 28, 49, 70, 105, 140, 210 and 280 days
DUBEY et al. 1977	USA	Cats	(-)	(-)	(-)	**E1:** 7- to 1170 days **E2:** 330-1530 days
Chagas disease						
CARDILLO et al. 1993*	Brazil	Mice	BALB/c, C57BL/6, DBA/2	(-)	(-)	**Young mice:** 150 - 180 days **Old mice:** 240-360 days
PÉREZ et al. 2011	Argentina	Rats	(-)	Male	(-)	**Young rats: 21-28 days** **Adult rats:** 80 days
PASCUTTI et al. 2003*	Argentina	Rats	(-)	Male	(-)	**Young rats:** 21–28 days **Adult rats:** 70–120 days
COLATO et al. 2017	Brazil	Rats	Wistar	Male	**Young rats:** 180-250g **Old rats:** 500-600g	**Young rats:** 35 days **Old rats:** 540 days
BRAZÃO et al. 2017*	Brazil	Rats	Wistar	Male	**Young rats:** 100-150g **Old rats:** 500-600g	**Young rats:** 35 days **Old rats:** 540 days

*Note: *In vitro and in vivo. (-) Data not reported. (?) Incomplete information.*

Table S3 - *(continuation)*. General characteristics of all studies included in the systematic review.

Study	Parasite species	Parasite strain	Parasite inoculum / rout	Infection period (days)
Malaria				
SHAN et al., 2013	*Plasmodium berghei*	ANKA	1×10^6 parasitized erythrocytes / Ip.	20 days
SHAN et al. 2012	*Plasmodium yoelii*	17XNL	1×10^6 parasitized erythrocytes / Ip.	24 days
SAFEUKUI et al. 2008*	*Plasmodium berghei*	ANKA	1×10^7 parasitized erythrocytes / Ip.	20 days
ADAM et al. 2003	*Plasmodium berghei*	ANKA	1×10^7 parasitized erythrocytes / Ip.	25 days
SINGER et al. 1954	*Plasmodium berghei*	KASAPA	5,000 parasites / Ip.	25 to 28 days
ORAGO and SOLOMON, 1986	*Plasmodium berghei*	NK 65	2×10^6 parasitized erythrocytes / Ip.	(-)
PIERROT et al. 2003	*Plasmodium berghei*	ANKA	1×10^6 and 10^7 parasitized erythrocytes / (?)	30 days
SMALLEY, 1975	*Plasmodium berghei*	(-)	2×10^6 parasitized erythrocytes 50 g bw. / Ip.	18 days
SOLOMON, 1986	*Plasmodium berghei*	NK65	1×10^8, 20-25% parasitized erythrocytes / Ip.	(-)
GRAVELY et al. 1976	*Plasmodium berghei*	(-)	2×10^7 parasitized erythrocytes / Ip.	25 days
ALGER et al. 1972	*Plasmodium berghei*	**Exp. 1:** NK65C35 **Exp. 2:** NK65RR20 **Exp. 3:** NK65C40 and NK65C18 **Exp. 4:** NK65C17 **Exp. 5:** NK65RR7	**Exp. 1 and 2:** 2.5×10^1 **Exp. 3:** 1.2×10^4 **Exp. 4:** 1.2×10^4 **Exp. 5**: 1.2×10^4 / Ip.	86 days

*Note: *In vitro and in vivo. (-) Data not reported. (?) Incomplete information. Ip. Intraperitoneal; Iv. Intravenous; Ic. Intracardiac.*

Table S3 - *(continuation)*. General characteristics of all studies included in the systematic review.

Study	Parasite species	Parasite strain	Parasite inoculum / rout	Infection period (days)
Leishmaniosis				
EHRCHEN et al. 2004*	*Leishmania major*	MHOM/IL/81/FE/BNI	5×10^6 promastigotes / Sc.	80 days
CILLARI et al., 1992	*Leishmania major*	LV39	2×10^4 promastigotes / Sc.	160 days
SINGH et al. 2007	*Leishmania donovani*	(MHOM/IN/80/Dd8)	1×10^7 / Ic.	60 days
MULLER et al. 2008[#]	*Leishmania major*	LV39	2×10^6promasmtigotes / Sc.	30 days
BHATTACHARYA et al. 2016	*Leishmania donovani*	LdWT and Ld1S2D centrin1 gene-deleted	3×10^6 promastigotes / Sc.	(-)
LAGES et al. 2008*	*Leishmania major*	Clone V1 (MHOM/IL/80/Friedlin)	1×10^3 promastigotes / Sc.	(-)
Toxoplasmosis				
JOHNSON et al. 1995	*Toxoplasma gondii*	ME49	**Analysis of cytokines:** 500 cysts **Other experiments:**ME49 cysts in 200-µl brain suspensions / Ip.	40 days
EMMERLING et al. 1979	*Toxoplasma gondii*	BK and DX	2,000 and 2×10^4 cysts / Ip.	42 days
GARDNER and REMINGTON, 1977	*Toxoplasma gondii*	C56 and C37	4×10^5, 8×10^4, 4×10^4, 2×10^5 cysts / Iv.	24 days
GAO et al. 2015	*Toxoplasma gondii*	Prugniaud	**Newborn rat:** 50 cysts / Ip. **Adult rat:** 200 cysts / Oral	(-)

*Note: *In vitro and in vivo. (-) Data not reported. (?) Incomplete information. Ip. Intraperitoneal; Iv. Intravenous; Ic. Intracardiac.*

Table S3 - *(conclusion)*. General characteristics of all studies included in the systematic review.

Study	Parasite species	Parasite strain	Parasite inoculum / rout	Infection period (days)
DE CHAMPS et al. 1998	*Toxoplasma gondii*	RH	1×10^2, 1×10^4, 1×10^8, 5×10^7 tachyzoites / Ip.	28 -42 days
HENRY and BEVERLEY, 1976	*Toxoplasma gondii*	(-)	20 cysts / Sc.	21-42 days
DUBEY et al. 1977	*Toxoplasma gondii*	M-7741, CR- 6 and H17	**E1:** 10^4brain cysts / Oral **E2:** 10^4 brain + muscle cysts / Oral	**E1:** 7 and 98 days
Chagas disease				
CARDILLO et al. 1993*	*Trypanosoma cruzi*	Y	$1 \times l0^3$trypomastigotes / Ip.	(-)
PÉREZ et al. 2011	*Trypanosoma cruzi*	Tulahuen	1×10^6 or 7×10^6trypomastigotes (?) bw/ (?)	28 days
PASCUTTI et al. 2003*	*Trypanosoma cruzi*	Tulahuen	1×10^6 or 7×10^6 trypomastigotes / Sc. Ip.	21-28 days
COLATO et al. 2017	*Trypanosoma cruzi*	Y	1×10^5trypomastigotes / Ip.	16 days
BRAZÃO et al. 2017*	*Trypanosoma cruzi*	Y	1×10^5trypomastigotes / Ip.	16 days

*Note: *In vitro and in vivo. (-) Data not reported. (?) Incomplete information. Ip. Intraperitoneal; Iv. Intravenous; Ic. Intracardiac.*
Source: From the author

Table S4 - General measure outcome extracted from all studies included in the systematic review.

Author	Parasitemia / Parasitic load	Mortality	Cytokines
Malaria			
SHAN et al. 2013	**21 days C57BL/6 mice:** Early and reduced parasitemia (7%, 8 d.p.i.) **240 days mice:** Delayed and high parasitemia (40%, 16-18 d.p.i)	**21 days C57BL/6 mice:** Early mortality, 100% (6 to 10 d.p.i.) **240 days C57BL/6 mice:** Delayed mortality, 100% (13 to 20 d.p.i.)	**(21 x 240 days C57BL/6 mice)** **240 days C57BL/6 mice:** **Increased**-TNF-α and IL-10 levels. **Similar**- IFN-γ levels
SHAN et al. 2012	**21 days C57BL/6 mice:** Highest parasitemia (42%, 16-20 d.p.i.), **240 days mice:** Reduced parasitemia (20%, 12-16 d.p.i.)	**21 days C57BL/6 mice:** About 100% mortality, (20 d.p.i.) **240 days mice:** 0% mortality	**(21 x 240 days C57BL/6 mice)** **240 days mice: Increased**- IL-4 and NO levels. **Similar**- IFN-γ, IL-10
SAFEUKUI et al. 2008	**28 days Lewis rats:** mean parasitemia 32.3% **42 days rats:** mean parasitemia 12.6% **56 day rats:** mean parasitemia about 5.10%	**28 days Lewis rats:** 47%, (13 d.p.i.) **56 and 42 days rats:** 0% mortality	**(28 and 56 days Lewis rats)** **56 days rats: Increased:** Nitrite level
ADAM et al. 2003	**(28 x 56 days Fischer rats)** **28 days rats:** Higher parasitemia (42%, 12-16 d.p.i.) **56 days rats:** Reduced parasitemia (11%, 12 d.p.i.)	**(28 x 56 days Fischer rats)** **28 days rats:** 100% mortality (16 d.p.i.) **56 days rats:** 0% mortality	**(28 x 56 days Fischer F344 rats)** **56 days rats: Reduced-** IL-10 level
SINGER et al., 1954	**(Sprague-Dawley rats) parasites / 10,000 rbc:** **33 days rats:** 8040 **39 days rats:** 6096 **42 days rats:** 4964 **49 days rats:** 2025 **57 days rats:** 993 **76 days rats:** 899 **93 days rats:** 736	**<28 days Sprague-Dawley rats:** 92% Mortality **33 days rats:** 82% Mortality **39 days rats:** 57% Mortality **42 days rats:** 33% Mortality **49, 57, 76, and 93 days rats:** 0% Mortality	(-)

Note: (-) Data underreported or not analyzed. d.p.i., days post-infection; i.p., intraperitoneal; rbc, red blood cells.

Table S4 - (*continuation*). General measure outcome extracted from all studies included in the systematic review.

Author	Parasitemia / Parasitic load	Mortality	Cytokines
Malaria			
ORAGO and SOLOMON, 1986	**(30 x 50 days Sprague Dawley rats)** **30 days rats:** Highest parasitemia (26.3%) **50 days rats:** Reduced parasitemia (3.5%)	**(30 x 50 days Sprague Dawley rats)** **30 days rats:**100% mortality, 13 d.p.i. **50 days rats:**0% mortality	(-)
PIERROT et al. 2003	**28 days C57BL/6 mice:** mean parasitemia 19.35% **70 days C57BL/6 mice:** mean parasitemia 21.3% **112 days C57BL/6 mice:** mean parasitemia 19.34% **28 days BALB/c mice:** mean parasitemia 40.8% **70 days BALB/c mice:** mean parasitemia 44.3% **112 days BALB/c mice:** mean parasitemia 43.5% **28 days Fischer rats:** mean parasitemia 35.54% **42 days Fischer rats:** mean parasitemia 23.33% **56 days Fischer rats:** mean parasitemia 4.7% **84 days Fischer rats**: mean parasitemia 6.82%	**28, 70 and 112 days BALB/c and C57BL/6 mice:** 100% mortality in all age groups **28 days Fischer rats:** 100% mortality (14-19 d.p.i.) **42 days Fischer rats:** 10% mortality (14-25 d.p.i.) **56 and 84 days Fischer rats:** 0% mortality	(-)
SMALLEY et al. 1975	**(30, 50 and 100 days Wistar rats)** **30 days rats:** peak of parasitemia of 46.6%, (13 d.p.i.) **50 days rats:** peak of parasitemia of 3.2%, (5 d.p.i.) **100 days rats:** peak of parasitemia of 1.0 %, (4 d.p.i.)	**(30, 50 and 100 days Wistar rats)** **30 days rats:** 67% mortality **50 days rats:** 4.5% mortality **100 days rats:** 0% mortality	(-)
GRAVELY et al. 1976	**(28 x 126 days Fischer F344 rats)** **28 days rats:** mean parasitemia about 43.75% **126 days rats:** parasitemia of 0%	(-)	(-)

Note: (-) Data underreported or not analyzed. d.p.i., days post-infection; i.p., intraperitoneal; rbc, red blood cells.

Table S4 - (*continuation*). General measure outcome extracted from all studies included in the systematic review.

Author	Parasitemia / Parasitic load	Mortality	Cytokines
Malaria			
ALGER et al. 1972	(-)	**Exp. 1 to 3 (A/J mice):** 95-100% mortality in 28 days mice, and 7-70% mortality in 180-240 days mice (25 d.p.i.) **Exp. 4 and 5 (CF1 mice):** 100% mortality in 28 days mice (36 d.p.i.), 100% mortality in 180 days mice (28 d.p.i.**)**	(-)
Leishmaniasis			
EHRCHEN et al. 2004	**(56 , 70 and 540 days BALB/c and C57BL/6 mice:)** **56 days and 70 days mice (in vitro):** 72%phagocytes infection, and 37% parasite elimination **540 days mice (in vitro):** 67% phagocytes infection and 33% parasite elimination	(-)	**(56 days, 70 days and 540 days BALB/c mice)** **540 days mice (in vitro):** **Increased-**IL-12andIFN- γ levels. **Reduced**- IL-4 levels. **Similar**- NO production
CILARI et al. 1992	(-)	**(455 x 840 days BALB/c mice)** **455 days mice:** 20% mortality (25 d.p.i.) **840 days mice:** 100% mortality (20 d.p.i.)	**(350, 700, 840 days BALB/c mice)** **Increased**- IL-4 as age increase **Reduced**- IFN-γ and IL-2 as age increase

Note: (-) Data underreported or not analyzed. d.p.i., days post-infection; i.p., intraperitoneal; rbc, red blood cells.

Table S4 - (*continuation*). General measure outcome extracted from all studies included in the systematic review.

Author	Parasitemia / Parasitic load	Mortality	Cytokines
Leishmaniasis			
BHATTACHARYA et al. 2016	**(77 x 504 days BALB/c mice)** ***In vivo:*** **Similar**- parasitic load	(-)	**(77 x 504 days BALB/c mice)** **504 days mice (in vitro) : Reduced**- NO and TNF **Similar:** IL-12, IFN- γ, IL- 6, IL-10 level and IFN- γ and IL-10 levels **504 days mice (in vivo): Reduced:**IFN- γ **Increased:**TNF, IL-10 and IL-6. **Similar-** IL-12 levels
MULLER et al. 2008	(-)	(-)	**(21-28, 42-56, 70 and 360 days BALB/c mice)** **Similar**- IFN-γ, IL-4, IL-10 levels **42-56 and 360 days mice (in vitro): Similar** - NO and NO_2S production by macrophages **21-28, 70 and 280 days mice (in vitro): Similar:** NO production by macrophages
SINGH et al. 2007	(-)	(-)	**(21-28 and 105-112 days hamsters)** **105-112 days hamsters: Increased:** nitrite levels
LAGES et al. 2008	(-)	(-)	**(150 and 630 daysC57BL/6 mice)** **630 days mice (in vitro): Increased**- Granulocyte-macrophage colony-stimulating factor, IL-2 and IL-10

Note: (-) Data underreported or not analyzed. d.p.i., days post-infection; i.p., intraperitoneal.

Table S4 - (*continuation*). General measure outcome extracted from all studies included in the systematic review.

Author	Parasitemia / Parasitic load	Mortality	Cytokines
Toxoplasmosis			
JOHNSON et al. 1995	(-)	**60 and 90 days B6D2F1 mice (o.)-** 20% mortality **60 and 90 days B6D2F1 mice (i.p.)-** 0% mortality **660 and 720 days B6D2F1 mice (o.)**- 100% mortality **660 and 720 days B6D2F1 mice (i.p.)-** 80% mortality	**(60, 90, 660 and 720 days B6D2FI mice)** **Similar**- IFN-γ levels
GARDNER and REMINGTON, 1977	(-)	**120 days BALB/c mice:** 100% mortality (7-12 d.p.i.) **150 days BALB/c mice:** 0-40% mortality (20-22 d.p.i.) **270 days BALB/c mice**: 100% mortality, (8-11 d.p.i.) **450 days BALB/c mice:** 40-100% mortality (6-20 d.p.i.) **150 days C57BL/6 mice:** 0% mortality **450 days C57BL/6 mice:** 55% mortality (16 d.p.i.)	(-)
GAO et al. 2015	**(5, 10, 15 and 20 days F344, BN, SD, WST, LEW rats x 57 and 70 days F344, BN, SD, WST, LEW rats)** **57 and 70 days rats:Reduced**- parasitic load **Reduced-** Infection rate in LEW and WST mice with increasing age	(-)	(-)

Note: (-) Data underreported or not analyzed. d.p.i., days post-infection; i.p., intraperitoneal; o., oral.

Table S4 - (*continuation*). General measure outcome extracted from all studies included in the systematic review.

Author	Parasitemia / Parasitic load	Mortality	Cytokines
Toxoplasmosis			
EMMERLING et al. 1979	(-)	**(60 and 720 days NMRI mice)** **60 days mice (2,000 cysts)-**0% mortality (21 d.p.i.) **60 days mice (2 x 10^4 cysts)-** 60% mortality (15-21 d.p.i.) **720 days mice (2,000 cysts)-** 96% mortality (14-21 d.p.i.) **720 days mice (2 x 10^4 cysts)-**87% mortality (12-21 d.p.i.)	(-)
DE CHAMPS et al. 1998	(-)	**(7, 11, 21, 24 and 46 days rats)** **Similar**- after tachyzoites inoculation. **Reduced**- after brain cysts inoculation as the age increased	(-)
Chagas disease			
PÉREZ et al. 2011	**(21,28 and 180 days rats)** **21-28 days rats:** mean parasitemia 384 parasites/ mL **180 days rats:** mean parasitemia 174 parasites/ mL	(-)	**(21-28 x180 days rats)** **180 days rats: Reduced-** TNF-α levels **Similar -** IL10 levels
PASCUTTI et al. 2003	**(21-28 , 70- 120 days male rats)** **21 and 28 days rats:** 12.8 parasites/50 fields **70-120 days rats:** 0 parasites	(-)	**(21-28, 70-120 days male rats)** **70-120 days rats (in vitro):Reduced -** NO and TNF levels **70-120 days rats: Reduced -** NO and IFN-γ levels
CARDILLO et al. 1993	**(35, 42, 240, 360 days BALB/c mice)** **35, 42 days mice:** parasitemia 1.7 / mm^3 **240, 360 days mice:** parasitemia 0.6 mm^3	**(35, 42, 240 and 360 days BALB/c mice)** **35 and 42 days mice:** 70% mortality (20 d.p.i.) **240 and 360 days mice:** 0% mortality (20 d.p.i.)	(-)

Note: (-) Data underreported or not analyzed. d.p.i., days post-infection; i.p., intraperitoneal; o., oral.

Table S4 - (*continuation*). General measure outcome extracted from all studies included in the systematic review.

Author	Antibodies	Cells of the immune system	Other findings
Malaria			
SHAN et al. 2013	(-)	**(21 x 240 days C57BL/6mice)** **240 days mice: Increased:** Treg cells	**(21 x 240 days C57BL/6 days mice)** **21 days:** rapid respiration and immobility **240 days mice:** No clinical manifestations
SHAN et al. 2012	**(21 x 240 days C57BL/6 days mice)** **240 days mice: Increased** - IgG1 and IgG2a antibodies levels	**(21 x 240 days C57BL/6 days mice)** **240 days mice: Increased** - dendritic and Treg cells, MHC II and CD86 expression	(-)
SAFEUKUI et al. 2008b	**(28 and 56 days Lewis rats)** **56 days rats: Increased**- IgE antibody levels	**(28 and 56 days Lewis rats)** **56 days rats: Increased**- CD23 expression	**(28, 42 and 56 days Lewis rats)** **28 days rats:** reduced hematocrit, body weight and temperature, than 42 and 56 days mice
ADAM et al. 2003	**(28 x 56 days Fischer F344 rats)** **56 days rats: Increased**- IgG2c levels **Similar**- IgM levels	**(28 x 56 days Fischer F344 rats)** **56 days rats (in vitro): Increased**- CD8 + and CD4+ T cells, B lymphocytes, macrophages, NK and NKT cells. **Reduced**- CD4+ CD25+ T cells	**(28 and 56 days Fischer F344 rats)** **Similar**- splenomegaly and absence of signs of cerebral malaria
SINGER et al., 1954	(-)	(-)	**30 and 41 days Sprague-Dawley rats:** **Increased**- body mean weight and anemia started earlier (4-6 d.p.i.) **51 and 81 days rat: Increased**- body mean weight. Anemia varied according infection severity

Note: (-) Data underreported or not analyzed. d.p.i., days post-infection; i.p., intraperitoneal.

Table S4 - (*continuation*). General measure outcome extracted from all studies included in the systematic review.

Author	Antibodies	Cells of the immune system	Other findings
Malaria			
ORAGO and SOLOMON, 1986	**(30 x 50 days Sprague Dawley rats)** **50 days mice: <u>Increased</u>-** antibody levels	**(30 x 50 days Sprague Dawley rats)** **50 days rats: <u>Reduced</u>-** antibody-dependent and natural cytotoxicity by spleen cells	**(30 x 50 days Sprague Dawley rats)** **30 days rats:** severe anemia **50 days rats: <u>Reduced</u>-** splenomegaly, natural cytotoxicity and antibody-dependent cytotoxicity by spleen cells
PIERROT et al. 2003	**(28 x 42 days Fischer rats)** **42 days rats: <u>Reduced-</u>** IgM levels **<u>Increased-</u>** IgG2c levels	(-)	**(28, 42, 56, 70, 84 and 112 days BALB/c, C57BL/6 mice and Fischer rats)** **BALB/c, C57BL/6 mice and Fischer rats:** Increased body weight and hemoglobin according to the increase in age **BALB/c and C57BL/6 mice:** Reduced hemoglobin according to increasing age
SMALLEY et al. 1975	**(30, 50, 100 days Wistar rats)** Increased antibody levels according to increasing age	(-)	(-)
GRAVELY et al. 1976	(-)	**(30 x 50 days Sprague Dawley rats) *In vitro:*** **50 days rats: <u>Increased</u>-** Natural cytotoxicity and antibody-dependent cytotoxicity by spleen cells	(-)

Note: (-) Data underreported or not analyzed. d.p.i., days post-infection; i.p., intraperitoneal.

Table S4 - (*continuation*). General measure outcome extracted from all studies included in the systematic review.

Author	Antibodies	Cells of the immune system	Other findings
Leishmaniasis			
EHRCHEN et al. 2004	(-)	**(56 days, 70 days and 540 days BALB/c and C57BL/6 mice)** ***In vitro:*** **Similar**- percentages macrophages and proportions of CD4+ and CD8+ T cells	**(56 days, 70 days and 540 days BALB/c and C57BL/6 mice:)** **540 days BALB/c mice: Reduced**- foot pads ulceration, swelling and spleen parasitism
CILARI et al. 1992	(-)	(-)	**(70, 224, 455 and 840 days BALB/c mice)** **70 days mice:** Reduced wound progression **224, 840 and 455 days mice:** Rapid wound appearing and progression
BHATTACHARYA et al. 2016	(-)	**(77 and 504 days BALB/c mice).** **In vitro: Similar**- distribution and proliferation of CD4+ T cells	(-)
MULLER et al. 2008	(-)	**(42-56 and 360 days BALB/c mice)** ***In vitro*: Similar** CD86, CD40, CD80 and CD206 expression by macrophages	**(21- 28, 42-56, 70, 280 and 360 days BALB/c mice)** **360 days mice: Reduced-** lesions size, arginase-1 levels and activity compared to 42-56 days mice. **280 days mice: Reduced-** lesion size, arginase activity compared to 21-28 and 70 days mice
SINGH et al. 2007	**(21-28 and 105-112 days hamsters)** **105-112 days hamsters: Reduced**- Anti-leishmania antibodies levels	**(21-28 and 105-112 days hamsters)** **105-112 days hamsters: Increased**- lymphocyte response to antigens	**(21-28 and 105-112 days hamsters)** **Similar:** weight loss and parasitic burden **105-112 days hamsters: Reduced**- spleen mass

Note: (-) Data underreported or not analyzed. d.p.i., days post-infection; i.p., intraperitoneal.

Table S4 - (*continuation*). General measure outcome extracted from all studies included in the systematic review.

Author	Antibodies	Cells of the immune system	Other findings
Leishmaniasis			
LAGES et al. 2008	(-)	**(60, 90, 120, 150, 450, 550, 600, 630 and 840 days C57BL/6 mice)** **600 and 840 days mice (in vitro): Increased-** Tregs number and function markers (CD4, CD103, GITR, CTLA-4, PD-1, CD69) and NK cells. **90 days mice (in vitro)**: **Increased-** Tregs, CD27 and CCR7 expression (activation marker) in T lymphocytes and NK cells compared to 60 days mice	**(60, 90, 120, 150, 450, 550, 600, 630 and 840 days C57BL/6 mice)** **Increased**- reactivation of the lesion in the ear with increasing age
Toxoplasmosis			
JOHNSON et al. 1995	(-)	**(60, 90, 660 and 720 days B6D2FI mice)** **660 and 720 days B6D2FI mice: Increased-** CD8+ T cells, and Thy-1+ CD4- CD8- cells. **Reduced**- NK cells activity. **Similar**- CD4+ T cells	**(60, 90, 660 and 720 days B6D2FI mice - 500 ME49 cysts)** **660 days and 720 days B6D2FI mice:** Reduced lung and liver inflammation and degeneration
HENRY and BEVERLEY, 1976	(-)	(-)	**(28, 49, 70, 105, 140, 210 and 280 days mice)** Greater morphological lymph node damage with increasing age
DUBEY et al. 1977	(-)	(-)	**(7, 60, 120, 180, 210, 420, 540, 1080 and 1170 days cats)** Variable oocysts shedding in different ages Reduced- tissue infection in increasing age

Note: (-) Data underreported or not analyzed. d.p.i., days post-infection; i.p., intraperitoneal.

Table S4 - (*conclusion*). General measure outcome extracted from all studies included in the systematic review.

Author	Antibodies	Cells of the immune system	Other findings
Chagas disease			
LAGES et al. 2008	**(21-28 x 180 days rats)** **180 days rats:** **Increased-** IgG1, IgG2a, IgG2b and IgG2c antibodies levels	(-)	**(21-28 x 180 days rats)** **Similar-** testosterone and estradiol levels 1**80 days rats: Reduced-** corticosterone levels, heart parasitism and myocarditis, **Similar-** Heart and testes weights
JOHNSON et al. 1995	**(21-28 and 70-120 days rats)** **70-120 days rats:** **Increased:** IgM and IgG levels	**(21-28 and 70- 120 days male rats)** **Similar-** number of phagocytic cells	(-)
HENRY and BEVERLEY, 1976	(-)	**(35 days x 540 days Wistar rats)** **Similar-** CD28-/CD4+ cells, CD86 on antigen-presenting cells **Increased-** TCD8+ and dendritic cells **Reduced-** Macrophages, MCH-II and CD80 expression macrophages, CD28+ on TCD4+, TCD8+ and B lymphocytes	**(35 days x 540 days Wistar rats)** **Increased-** 8-isoprostane levels **Similar-** Activity of SOD and glutathione
DUBEY et al. 1977	(-)	**(35 x 540 days Wistar rats)** **540 days rats: Reduced -** CD28+, TCD4+, TCD8 +, B and NKT cells. **Similar:** NK cells	**(35 x 540 days Wistar rats)** **540 days rats: Reduced-** Activity of superoxide dismutase, 8-isoprostane levels. **Increased:** TBARS, NO, corticosterone levels. **Similar:** GSH activity
	(-)	**(35, 42, 240 and 360 days BALB/c mice)** **240 and 360 days mice (in vitro): Reduced-** splenocytes suppressor activity and MHC expression	**(35, 42, 240 and 360 days BALB/c mice)** **240 and 360 days mice: Increased-** Tissue inflammation

Note: (-) Data underreported or not analyzed. d.p.i., days post-infection; i.p., intraperitoneal. Source: From the author.

10 CHAPTER 2: IMPACT OF AGING ON THE HOST RESPONSE TO *TRYPANOSOMA CRUZI* INFECTION

ABSTRACT

Elderly organisms are more susceptible to infectious diseases. However, the impact of aging on antiparasitic mechanisms, especially the nitric oxide pathway, is poorly understood. Using an integrated *in vivo* and *in vitro* model, we compared the severity of *Trypanosoma cruzi* infection in young and elderly (8 or 72 weeks old) mice. Forty C57BL/6 mice were randomized into four groups: Y-inf, young infected; Yn-Inf, young uninfected; A-inf, aged infected; An-Inf, aged uninfected. Parasitemia was measured daily, and animals were euthanized after 15 days of infection. *Trypanosoma cruzi*-induced inflammatory processes were analyzed in blood and heart samples, as well as in bone marrow-derived macrophages (BMDMs) co-cultured with splenocytes isolated from young or elderly mice. Our results indicated upregulated IgG2b and IL-17 production in elderly animals, which was not sufficient to reduce parasitemia, parasitic load and myocarditis to levels observed in young animals. The higher susceptibility of elderly mice to *T. cruzi* infection was accompanied by reduced cardiac inducible nitric oxide synthase (iNOS) gene expression, nitric oxide (NO) and IFN-γ levels, as well as an antagonistic upregulation of arginase-1 expression and arginase activity. The same responses were observed when BMDMs co-cultured with splenocytes from elderly mice were stimulated with *T. cruzi* antigens. Our findings indicate that elderly mice are more susceptible to *T. cruzi* infection, which is potentially related to an attenuated response to antigenic stimulation, inhibition of iNOS gene expression and NO production, and antagonistic upregulation of arginase gene expression and activity, which created favorable conditions for heart parasitism and myocarditis development.

Key words: Chagas disease, Cardiovascular pathology, Experimental parasitology, Nitric oxide.

RESUMO

Organismos idosos são mais susceptíveis à doenças infecciosas. No entanto, o impacto do envelhecimento nos mecanismos antiparasitários, especialmente na via do óxido nítrico, é pouco compreendido. Utilizando um modelo *in vivo* e *in vitro* integrado, nós comparamos a gravidade da infecção pelo *Trypanosoma cruzi* em camundongos jovens e idosos (8 ou 72 semanas de idade). Quarenta camundongos C57BL/6 foram randomizados em quatro grupos: Y-inf, jovens infectados; Yn-Inf, jovem não infectado; A-inf, idoso infectado; An-Inf, idoso não infectado. A parasitemia foi avaliada diariamente e os animais foram eutanasiados após 15 dias de infecção. Os processos inflamatórios induzidos pelo *Trypanosoma cruzi* foram analisados em amostras de sangue e coração, bem como em macrófagos derivados da medula óssea (BMDMs) co-cultivados com esplenócitos isolados de camundongos jovens ou idosos. Nossos resultados indicaram aumento da produção de IgG2b e IL-17 em animais idosos, o que não foi suficiente para reduzir a parasitemia, carga parasitária e miocardite aos níveis observados nos animais jovens. A elevada susceptibilidade de camundongos idosos à infecção pelo *T. cruzi* foi acompanhada pela redução da expressão gênica de óxido nítrico sintase indutível (iNOS), níveis de óxido nítrico (NO) e IFN-γ, bem como aumento da expressão da arginase-1 e atividade da arginase. As mesmas respostas foram observadas quando BMDMs co-cultivados com esplenócitos de camundongos idosos foram estimulados com antígenos de *T. cruzi*. Nossos achados indicam que camundongos idosos são mais susceptíveis à infecção por *T. cruzi*, potencialmente relacionada a uma resposta atenuada à estimulação antigênica, inibição da expressão gênica da iNOS e da produção de NO, e regulação antagônica da expressão gênica e atividade da arginase, o que gerou condições favoráveis para o parasitismo cardíaco e o desenvolvimento da miocardite.

Palavras-chave: Doença de Chagas. Patologia cardiovascular. Parasitologia experimental. Óxido nítrico.

11 INTRODUCTION

Chagas disease is a neglected parasitic disease caused by the protozoan *Trypanosoma cruzi*, which is closely associated with poverty (DURRANCE et al., 2017). Estimates indicate that approximately 7 million people are infected by *T. cruzi* worldwide, and at least 41,000 new cases are reported each year (WHO, 2015, 2017). The disease is endemic in Latin American countries, in which around 13% of the population is at risk of infection, and more than 10,000 deaths attributed to *T. cruzi* are registered per year (FERREIRA; OLIVEIRA; ANDRICOPULO, 2016; WHO, 2015). Due to the immigration of infected people, vertical transmission and the donation of infected tissues and organs, the disease has spread to non-endemic areas such as Europe, Japan, North America and Australia (FARAZ et al., 2017; LIU; ZHOU, 2015).

Chronic cardiomyopathy is the most serious manifestation of Chagas disease, occurring in 30–40% of infected individuals (GROOM; PROTOPAPAS; ZOCHIOS, 2017; NOVAES et al., 2016a). This condition is related to a higher risk of death due to heart failure, which is invariably associated with severe reactive myocardial fibrosis, cardiomegaly, microvascular and electromechanical dysfunction (COURA, 2007; HIGUCHI et al., 2003). Although cardiomyocytes parasitism plays an important role in the direct heart damage associated with acute infections (DIAS et al., 2017), the organ deterioration observed in chronic infections is mainly due to immunomediated lesions associated with *T. cruzi*-induced upregulation of the T helper 1 (Th1) phenotype (RIBEIRO et al., 2012; SANTOS et al., 2015).

The immunological response is directly related to parasitic control, and plays a central role in the host's susceptibility and/or resistance to *T. cruzi* infection (AYO et al., 2013). Aligned with the response typically observed in infections caused by intracellular pathogens, the main line of defense against *T. cruzi* occurs via a Th1 response (TEIXEIRA et al., 2011). In this immunological response, cytokines such as IL-12, TNF-α and IFN-γ, as well as high nitric oxide (NO) production, are important markers of Th1 polarization and act as antitrypanosomal effectors (GOMES; ROCHA; GAZZINELLI, 2003). In contrast, the T helper 2 (Th2) phenotype, typically established in infections caused by extracellular pathogens, is associated with increased host susceptibility to *T. cruzi* infection, the effects of which are partially mediated by the cytokines IL-4 and IL-10 (MORENO et al., 2004; SANTOS et al., 2015). The greater susceptibility to *T. cruzi* infection related to the Th2

response is also associated with reduced NO synthesis, an event that occurs secondary to the metabolic shift of L-arginine for the production of arginase 1-dependent polyamines, a phenotype that favors the parasitism of target organs (ABRAHAMSOHN; COFFMAN, 1996; HUNTER et al., 1997; STEMPIN et al., 2002, 2004).

In addition to Th1 and Th2 profiles, elevated levels of IL-21, IL-23 and IL-1β can induce a third immunological phenotype (Th17) characterized by high IL-17 production (OUYANG; KOLLS; ZHENG, 2008). Studies have shown that an absence of Th1 and Th17 interleukins increase the host's susceptibility to infection (MACHADO et al., 2013; RODRIGUES et al., 2012), and that the major cytokines modulating Th1 (IFN-γ) and Th2 (IL-4) profiles are able to inhibit Th17 polarization (ERDMANN et al., 2013; MICHAILOWSKY et al., 2001). Thus, the balance between the Th1, Th2 and Th17 profiles modulates host susceptibility or resistance to infection, thereby determining the pathological outcome of Chagas disease and having a direct impact on the severity of the tissue lesions and on host mortality (BASSO, 2013; TEIXEIRA et al., 2011).

Aging is related to morphological and functional changes of the host immune system, which may significantly impact on the Th1/Th2/Th17 balance (DE ALBA-ALVARADO et al., 2018). However, the impact of aging on the host's ability to resist *T. cruzi* infection remains poorly understood. The progressive immunological decline with aging, termed immunosenescence, is known to increase the organism's susceptibility to autoimmune diseases, cancer and infections (BOE; BOULE; KOVACS, 2017; TU; RAO, 2016). Changes such as a reduction in the number and phenotypic diversity of peripheral T and B lymphocytes, combined with the accumulation of dysfunctional cells, are among the most striking changes that impair the ability to efficiently recognize and respond to the broad spectrum of pathogen-associated antigens in elderly organisms (CARUSO et al., 2009; FULOP et al., 2014; MONTECINO-RODRIGUEZ; BERENT-MAOZ; DORSHKIND, 2013). As the immune response is the central line of defense against pathogenic microorganisms (CHAPLIN, 2010), deterioration in immunological mechanisms due to aging, especially activation of the NO pathway by leucocytes, could impair the host's ability in resist to parasitic infections. In this study, we used an integrated *in vivo* and *in vitro* model to compare how young and elderly organisms respond to *T. cruzi* infection, especially considering the response of immune cells to parasite antigens, as well as the relationship between cardiac damage, cytokine production and activation of the antagonistic NO and arginase pathways.

12 MATERIAL AND METHODS

12.1 ANIMALS AND INFECTION

C57BL/6 mice were randomized into four groups with 10 animals in each group: Yinf, young infected; Yn-Inf, young uninfected; Ainf, aged infected; An-Inf, aged uninfected. Young animals were 8 weeks old (ROGGERO et al., 2002) and old mice were 72 weeks old (CARDILLO; NOMIZO; MENGEL, 1998; FELIZARDO et al., 2018). The animals were maintained under controlled temperature (22 ± 2ºC), humidity (60–70%) and photoperiod (12-h/12-h light/dark cycle) conditions. Commercial rodent food and water were provided *ad libitum*. Animals in the infected groups were intraperitoneally inoculated with 5000 blood trypomastigote forms of *T. cruzi* (Y strain) (SANTOS et al., 2015). The infection was allowed to develop for 16 days, and after 24h the animals were euthanized under anesthesia. Blood and heart samples were collected for use in morphological, immunological and molecular analyses. The study was approved by the Animal Ethics Committee of the Federal University of Alfenas (protocol 48/2017).

12.2 BLOOD PARASITISM

Parasitemia was evaluated daily during the 15-day infection period. The number of circulating trypomastigotes was determined according to a previously described technique (BRENER, 1962). Briefly, 5 µL of peripheral blood collected from the tail was distributed on glass slides (22 × 22 mm) and observed under a bright-field microscope. The volumetric distribution of parasites for each animal was determined by counting the number of parasites in 50 non-coincident microscopic fields using a 40× objective lens. The mean parasitemia was calculated daily, and the parasitemia curve of all infected groups was plotted. Blood of uninfected animals was also evaluated to simulate experimental stress and confirm the absence of infection.

12.3 PARASITE LOAD

The parasite load was estimated by the quantification of *T. cruzi* DNA in heart samples. Total genomic DNA was extracted from control mice and those infected with *T. cruzi* using a commercial kit (Assistente® Genomic DNA Purification Kit; Promega) (NOVAES et al., 2016a) and adjusted to 25 ng/µl (GeneQuant; Pharmacia Biotech, Piscataway, NJ, USA). Polymerase chain reaction (PCR) was performed, with the reaction mixture consisting of 10 µl (50 ng) of genomic DNA, 5 µl of SYBR Green PCR Mastermix (Applied Biosystems, Carlsbad, CA, USA) and 0.35 µM 195-bp repeat *T. cruzi* DNA or 0.50 µM murine TNF-α primers. The TNF-α primers (TNF-5241, 5'-TCCCTCTCATCAGTTCTATGGCCCA-3', and TNF-5411, 5'-CAGC AAGCATCTATGCACTTAGACCCC-3') amplify a 170-bp product. The *T. cruzi* DNA primers (TCZ-F, 5'-GCTCTTGCCCACAMGGGTGC-3', and TCZ-R, 5'-CCAAGCAGCGGATAGTTCAGG-3') amplify a 182-bp fragment (CUMMINGS; TARLETON, 2004). The PCR conditions, including the time, temperature and cycles, are detailed in a previous study [13]. The 96-well reaction plates contained standards to create a standard curve, two negative controls with *T. cruzi* or TNF-α primers but without DNA, as well as tissue DNA from uninfected mice. The *T. cruzi* DNA levels were normalized to data obtained with TNF-α primers (normalized value = [mean *T. cruzi* DNA/mean TNF-α DNA] × 1000), where "1000" represents the expected value for TNF-α in 30 mg of tissue.

12.4 IMMUNOGLOBULIN ASSAY

Blood samples (500 µl) treated with heparin (5 µl) were centrifuged at 20,000 *g* for 10 min, and the obtained plasma was used in immunological assays. Specific anti-*T. cruzi* antibodies were detected by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well polystyrene microplates were sensitized with *T. cruzi* antigens and incubated with plasma from each animal. Total anti-mouse immunoglobulin G (IgG), as well as IgG1, IgG2a and IgG2b isotypes, all conjugated with peroxidase, were applied to the pre-sensitized wells (Bethyl Laboratories, Montgomery, TX, USA). After treatment with the substrate (O-

fenilenodiamino-OPD), the optical density was read at 490 nm in a microplate spectrophotometer (Anthos Zenyth 200; Biochrom, Cambridge, UK).

12.5 CYTOKINE IMMUNOASSAYS

Plasma cytokine levels were measured by the sandwich ELISA method (NOVAES et al., 2017). The IL-4, IL-10, IL-17, TNF-α and IFN-γ cytokines were measured using commercial kits according to the manufacturer's instructions (PeproTech, Rocky Hill, NJ, USA). The reaction was developed with a streptavidin-peroxidase-conjugated antibody, followed by incubation with the chromogen 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (Sigma-Aldrich). The optical densities of all samples were detected at 450 nm (Anthos Zenyth 200; Biochrom, Cambridge, UK). Plasma cytokine levels were calculated by extrapolating the optical densities obtained from a standard curve for each recombinant cytokine (NOVAES et al., 2017).

12.6 HISTOPATHOLOGY AND STEREOLOGY

Heart sections of all animals were fixed in 4% paraformaldehyde (1 M, pH 7.2). The samples were then embedded in glycol methacrylate resin and cut into 3-μm thick sections using glass knives (Leica Biosystems, Wetzlar, Germany). The sections were evaluated in semi-series, using one out of every 20 sections, which were stained with toluidine blue and basic fuchsine. For each histological section, 10 microscopic images were captured using a 40× objective lens (Axioscope A1; Carl Zeiss, Germany). Histopathological analysis was performed by evaluating the distribution and organization of the organ parenchyma and connective stroma, tissue necrosis and inflammatory infiltrate, myocyte hypertrophy, morphology and distribution of blood vessels and interstitial cells, areas of tissue fibrosis and distribution of *T. cruzi* amastigote nests (NOVAES et al., 2016a).

The intensity of myocardial inflammation was assessed by the stereological method (NOVAES et al., 2013). For this, tissue cellularity per histological area was determined by quantifying the number of interstitial cell nuclei (Σinf) in the test area (At = 25×10^3 μm^2) from the notation QAinf = Σinf/At. Tissue cellularity was quantified from 10 randomly chosen histological fields of heart sections from each animal using a 40× objective lens (400× magnification; Axioscope A1, Carl Zeiss, Germany). A total tissue area of 12.9×10^5 μm^2 was analyzed for each group. Stereological analysis was performed using the image analysis

software Image-Pro Plus® (version 4.5; Media Cybernetics Inc., Silver Spring, Maryland, USA).

12.7 BONE MARROW-DERIVED MACROPHAGES

Cellular response to *T. cruzi* antigens was evaluated using macrophage and splenocyte co-culture. Bone marrow-derived macrophages (BMDMs) were obtained from four 8-week-old C57BL/6 mice (ROGGERO et al., 2002). Briefly, after euthanasia, the tibia and femur were collected and bone marrow cells were extracted by intramedullary injection of 5 mL sterile 0.9% NaCl solution. The obtained cell suspension was centrifuged at 20,000 *g* for 10 min, then the precipitate was resuspended in 1 mL Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 50 µl penicillin/streptomycin and 500 µl amphotericin B. Samples were filtered through nylon separators with 70-µm mesh to remove debris and rupture bonds between cells.

The cells were plated in petri dishes and incubated at 37°C in a 5% CO_2 atmosphere to obtain undifferentiated cells. For this, non-adherent cells were collected, centrifuged at 1200 rpm for 10 min, then resuspended in 1 mL of supplemented DMEM. Viable cells were stained with trypan blue and counted using a Neubauer's chamber. To induce differentiation of BMDMs, 5×10^5 viable cells were distributed in 24-well plates with supplemented DMEM medium and 10% L929 cell conditioned medium (LCCM) as a source of monocyte colony-stimulating factor (M-CSF). The plates were incubated at 37°C in a 5% CO_2 atmosphere. After 3 days, 100 µL of M-CSF was added to each well, and at day 7, the culture medium was replaced with new medium also containing M-CSF. After 10 days of culture, the differentiated BMDMs were used in the co-culture assay.

12.8 MACROPHAGE AND SPLENOCYTES CO-CULTURE

Splenocytes were obtained from uninfected C57BL/6 control mice and those that had been intraperitoneally infected with 5000 blood trypomastigotes of *T. cruzi* (Y strain). After 16 days of infection, the animals were euthanized under deep anesthesia (concentrated

isoflurane) followed by cardiac puncture. The spleen was collected with the aid of sterile tweezers and scissors, macerated with the aid of a steel sieve, then placed in falcon tubes containing 9 mL of saline and centrifuged for 10 min at 1200 rpm. The supernatant was discarded and the cells were resuspended in 1 mL of lysis solution and incubated at room temperature for 5 min to induce osmotic lysis of the red cells. Subsequently, 10 mL of saline was added to each falcon tube and centrifuged again. After discarding the supernatant, the cells were resuspended in RPMI medium supplemented with 10% fetal bovine serum. The concentration of cells was determined with a Neubauer chamber, using 0.02% trypan blue as a dye for the visualization of viable cells. Splenocytes were cultured at a concentration of 1×10^6 cells/well in 96-well U-bottom plates. Twenty-four hours later, splenocytes (1×10^6 cells/well) were added to *T. cruzi* antigen-stimulated BMDMs (5×10^5), and this co-culture was incubated at 37°C in a 5% CO_2 atmosphere. DMEM medium (negative control) or 1 μg/mL LPS (positive control) were added to wells containing splenocytes and BMDMs. After 24 h of incubation, the cells were collected for RNA extraction and gene expression analysis (WANG et al., 2013).

12.9 CYTOKINE, INDUCIBLE NITRIC OXIDE SYNTHASE AND ARGINASE GENE EXPRESSION ASSAYS

Expression of the genes encoding arginase-1 and inducible nitric oxide synthase (iNOS) in heart samples and the BMDM/splenocyte co-culture were determined by quantitative polymerase chain reaction (qPCR) as previously described (PEREIRA et al., 2017). Complementary DNA was constructed from mRNA using a commercial reverse-transcription kit following the manufacturer's instructions (ThermoFisher Scientific Waltham, MA, USA). Validated primers for arginase-1 and iNOS (DAVIS et al., 2013), IFN-γ (YANG et al., 2007), IL-4 and IL-17 (CHUNG et al., 2006) were used (Thermo Fisher Scientific, Waltham, MA, USA) (Table 1). Quantitative PCR reactions used SYBR Green PCR Mastermix (Applied Biosystems, Carlsbad, CA, USA), and were performed according to the manufacturer's instructions. Expression values for all genes analysed were normalized to GAPDH expression.

Table 1. Primers used in quantitative polymerase chain reaction*.

iNOS[a]	Forward	5'-TTTGCTTCCATGCTAATGCGAAAG-3'
	Reverse	5'-GCTCTGTTGAGGTCTAAAGGCTCCG-3'
Arginase-1[a]	Forward	5'-GGAAGCATCTCTGGCCACGCC-3'
	Reverse	5'-TCCCAGAGCTGGTTGTCAGGGG-3'
IFN-γ[b]	Forward	5'-ATGCATTCATGAGTATTGCCAAGT-3'
	Reverse	5'-GTGGACCACTCGGATGAGCTC-3'
IL-10[c]	Forward	5'-CCCTTTGCTATGGTGTCCTT-3'
	Reverse	5'-TGGTTTCTCTTCCCAAGACC-3'
IL-17[d]	Forward	5'-CTGGAGGATAACACTGTGAGAGT-3'
	Reverse	5'-TGCTGAATGGCGACGGAGTTC-3'
GAPDH[1]	Forward	5'-ACTCCACTCACGGCAAATTC-3'
	Reverse	5'-TCTCCATGGTGGTGAAGA CA-3'

Note: *All primers were validated previous studies: [a]MBio 2013; 4:e00264-13; [b]J. Biol. Chem. 2007; 282:9358-63; [c]J. Immunol. 2017; 198:986-992; [d]Cell. Res. 2006; 16:902-7.

12.10 ARGINASE ACTIVITY ASSAY

Heart and cell samples were macerated and homogenized in 0.5 mL Tris-HCl buffer (50 mM, pH 7.5). After the addition of 0.5 mL Triton X-100 (0.1%), the mixture was vortexed for 10 min then centrifuged at 3000 *g*. Arginine activity was measured according to Hesse et al. (HESSE et al., 2001). Briefly, the enzyme was activated by heating the homogenate with 1 mL $MnCl_2$ (10 mM) at 56°C for 10 min. Arginine cleavage was obtained from incubation with 10 mL of L-arginine (0.05 M) at 37°C (pH 9.7) for 15–120 min. The reaction was stopped with H_2O/H_3PO_4 (85%)/H_2SO_4 (96%) (7/3/1, v/v/v). The enzyme activity was estimated based on the formation of 1 mM of urea per minute. This was determined at 540 nm after the addition of 4 mL α-isonitrosopropiophenone, which was heated at 95°C for 30 min (RODRIGUES et al., 2017).

12.11 NITRIC OXIDE ASSAY

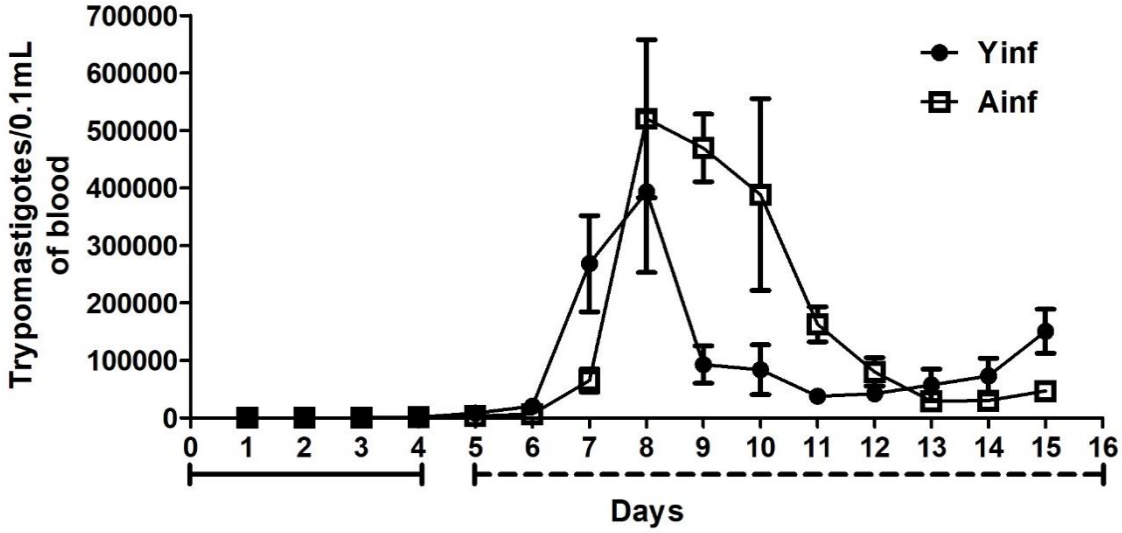
The NO concentration was estimated in heart samples and co-culture supernatant from the nitrite/nitrate levels determined using the Griess reaction (TSIKAS, 2007). Heart homogenate or cell supernatant (50 μL) samples were incubated with Griess reagent (1% sulfanilamide, 0.1% naphthalene diamine dihydrochloride and 2.5 % phosphoric acid; 1:1 v/v) at room temperature for 10 min. The optical density was read at 550 nm using a microplate spectrophotometer (Anthos Zenyth 200, Biochrom, Cambridge, UK).

12.12 STATISTICAL ANALYSIS

The results are presented as either the percentage, median and interquartile interval or as the mean and standard deviation (SD). Normality in data distribution was verified by the D'Agostino-Pearson test. Parametric data were compared between groups by analysis of variance (ANOVA), followed by the Student Newman-Keuls test for multiple comparisons. Nonparametric variables were compared using the Kruskal-Wallis test. Correlation between nitric oxide levels and parasite load was analyzed by linear regression. The confidence level of all tests was set at 95% ($P \leq 0.05$).

13 RESULTS

Both young and elderly animals infected with *T. cruzi* presented a similar prepatent period (4 days) and time until peak parasitemia (day 8). The clearance of parasites started later in elderly animals, reaching values similar to the young animals after 12 days of infection (Fig. 1).



Figur
e 1 - Parasitemia curve in young and elderly mice infected by *Trypanosoma cruzi*.
Source: From the author
Subtitle: Y: young, A: aged, inf: infected. Continuous line: pre-patent period. Dotted line: patent-period.

Although mean parasitemia was higher ($P < 0.05$), final parasitemia was lower in elderly mice when compared with young mice ($P < 0.05$). The parasitemia peak was similar in young and elderly mice ($P > 0.05$; Table 1).

Table 2 - Parasitemia in young and elderly mice infected with *T. cruzi*.

Group	Mean parasitemia (Tryp. / 0.1 mL blood)	Peak parasitemia (Tryp. / 0.1 mL blood)	Final parasitemia (Tryp. / 0.1 mL blood)
Y-inf	99383.1 ± 5185.45	393125.0 ± 396898.1	93571.4 ± 68166.7
A-inf	150276.6 ± 193648.3*	520500.0 ± 435318.9	34629.6 ± 12354.1*

Source: From the author
Subtitle: Parasitemia is expressed as mean ± standard deviation. * Statistical difference among the groups (P <0.05).

Plasma levels of IFN-γ, TNF-α, IL-10 and IL-17 cytokines were higher in both infected groups compared to uninfected groups (P < 0.05). Only IL-17 was increased in the elderly group compared to the young group of animals infected with *T. cruzi* (P < 0.05; Fig. 2).

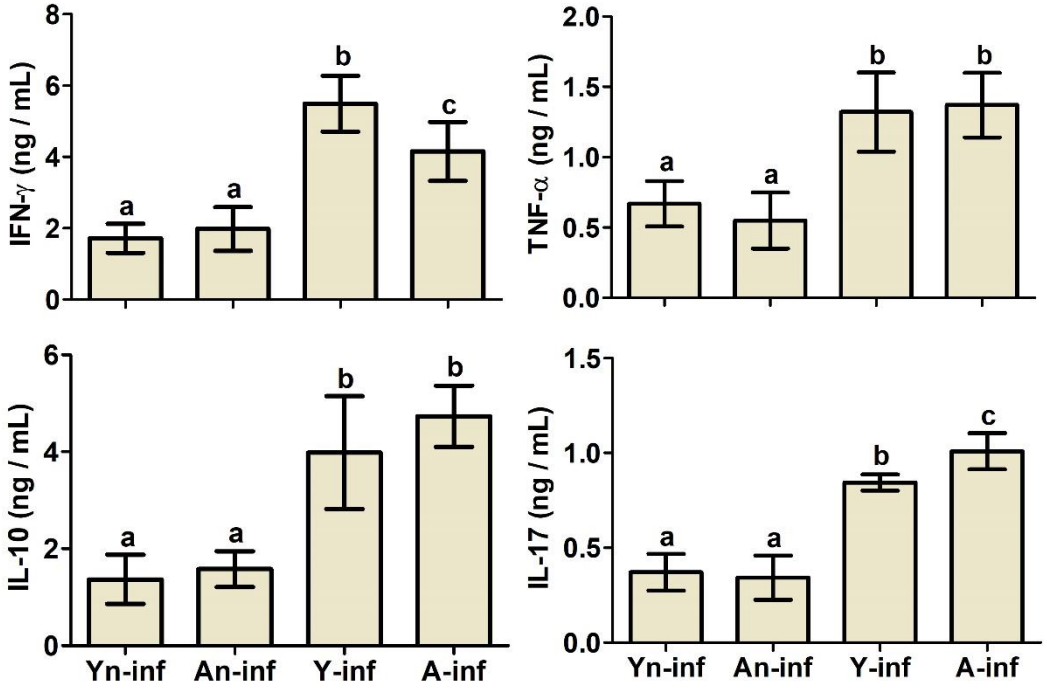


Figure 2 - Cytokines plasma levels in control and *Trypanosoma cruzi*-infected mice.
Source: From the author
Subtitle: Y: young, A: aged, inf: infected, n-inf: uninfected. a,b,c Different letters in the columns denotes statistical difference among the groups (p≤0.05), and groups with similar letters do not differ statistically (p>0.05).

Plasma levels of total IgG and IgG subclasses were higher in both infected groups compared to the uninfected groups ($P < 0.05$). Only IgG2b was increased in aged compared to young infected animals ($P < 0.05$; Fig. 3).

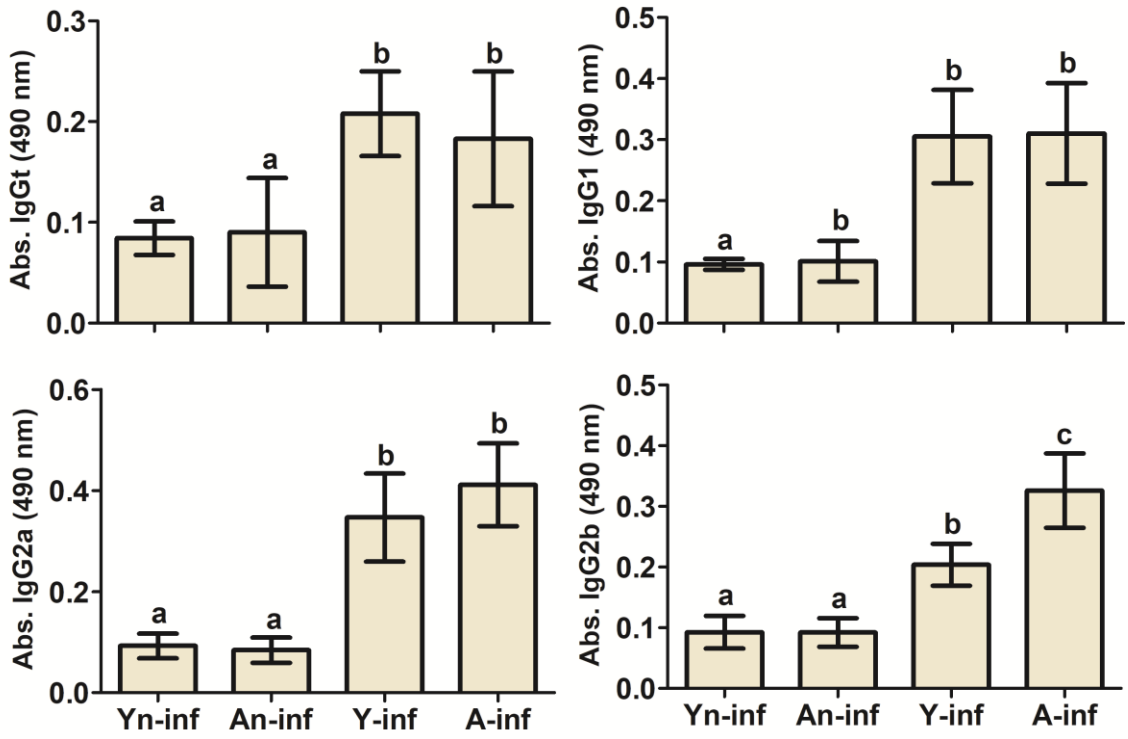


Figure 3 - Plasma levels of anti-*Trypanosoma cruzi* immunoglobulin G (IgG) subclasses in control and *Trypanosoma cruzi*-infected mice.
Source: From the author
Subtitle: Abs: absorbance, Y: young, A: aged, inf: infected, n-inf: uninfected. a,b,c Different letters in the columns denotes statistical difference among the groups ($p \leq 0.05$), and groups with similar letters do not differ statistically ($p > 0.05$).

Uninfected animals, both young and elderly, exhibited a well-organized cardiac microstructure with scarce connective tissue and reduced tissue cellularity, which was mainly composed of mononuclear cells. Young infected animals presented higher and more diffuse myocardial cellularity, as well as evident *T. cruzi* amastigote nests in cardiomyocytes. Elderly infected animals exhibited marked and diffuse inflammatory infiltrate, focal accumulation of mononuclear and polymorphonuclear leucocytes, mild connective tissue expansion and large *T. cruzi* amastigote nests in cardiomyocytes (Fig. 4 and 5B).

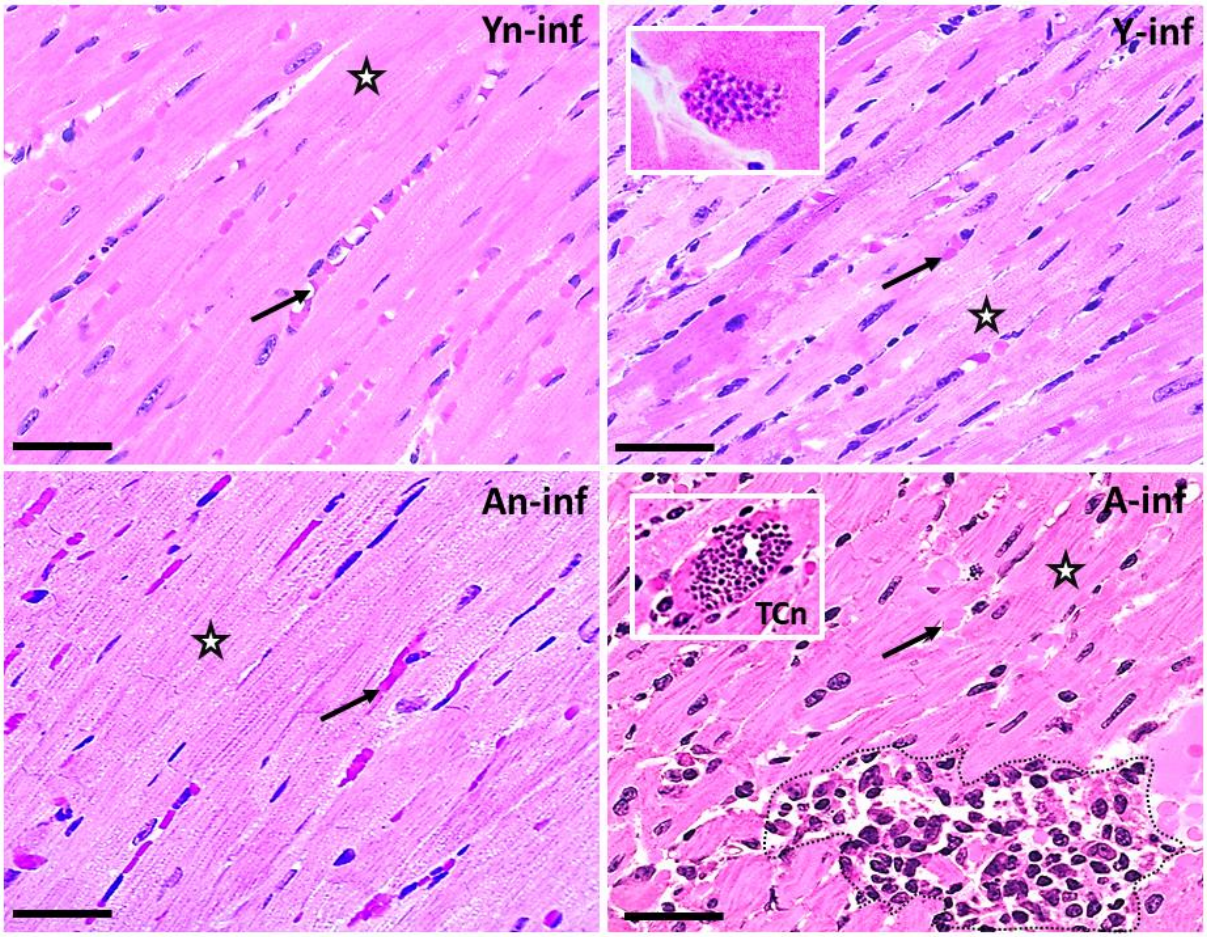


Figure 4 - Representative photomicrography's of the cardiac tissue from control and *Trypanosoma cruzi*-infected mice (Toluidine blue and basic fuchsine staining, bar= 50 µm).
Source: From the author
Subtitle: Arrow: blood vessels, Star: cardiomyocytes, Y: young, A: aged, inf: infected, n-inf: uninfected. In A-inf, the dotted line delimits a focal inflammatory infiltrate.

Using PCR analysis, parasite DNA was not identified in heart samples of either group of uninfected animals. For infected mice, elderly animals exhibited higher levels of *T. cruzi* DNA compared to young mice ($P < 0.05$; Fig. 5A). Uninfected animals also exhibited low interstitial cellularity and no evidence of myocarditis, while young and elderly mice infected with *T. cruzi* exhibited marked leucocyte infiltration and evident myocarditis, which was exacerbated in the elderly group (Figs. 4 and 5B). The parasite load was inversely and significantly correlated with heart NO levels in elderly mice ($r^2 = 0.82$, $P < 0.05$), but not in young infected mice ($r^2 = 0.19$, $P= 0.21$) (Fig. 5C and 5D).

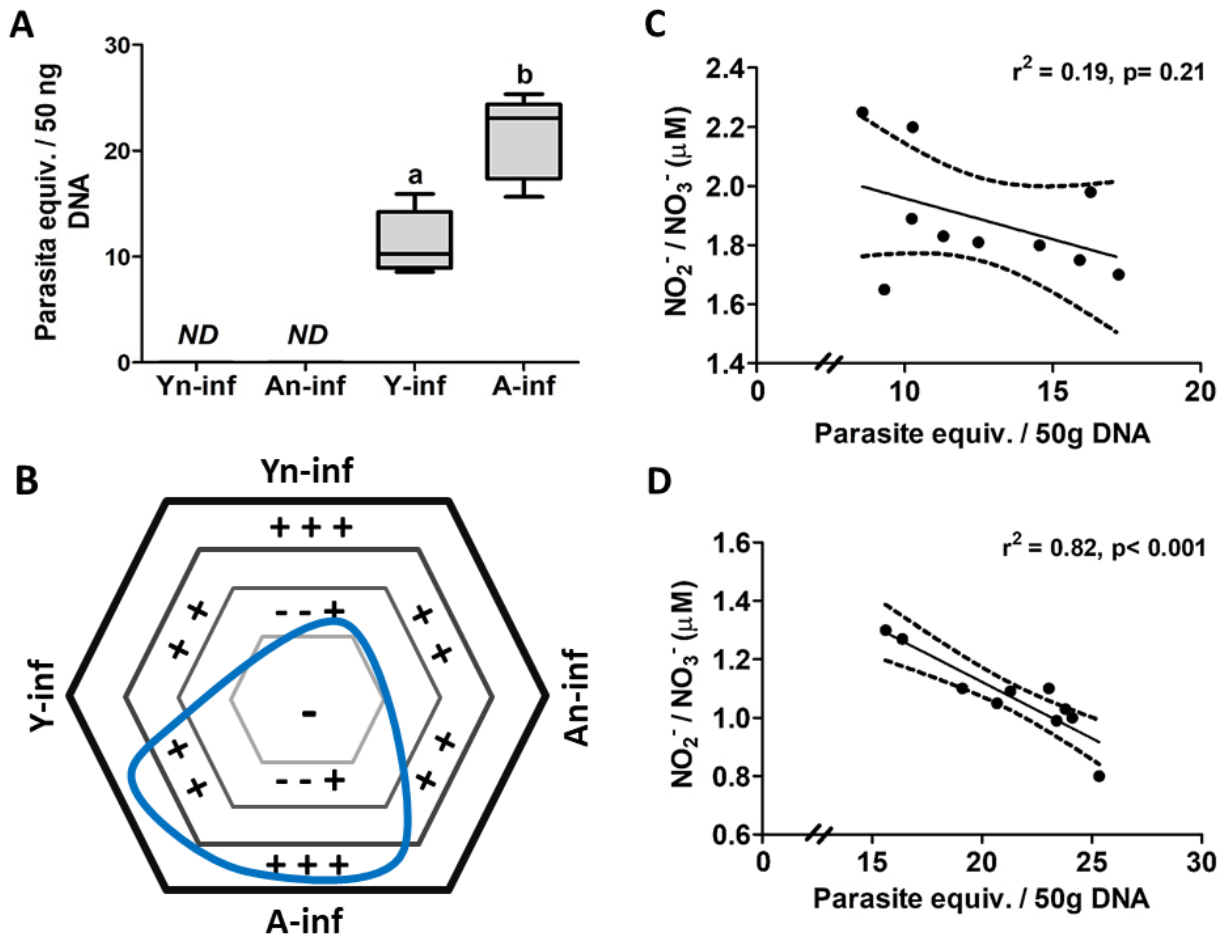


Fig. 5. Relationship between parasite load, inflammatory myocardial damage, and nitric oxide (NO_2^- /NO_3^-) in control and *Trypanosoma cruzi*-infected mice.

Source: From the author

Subtitle: Y: young, A: aged, inf: infected, n-inf: uninfected. In the graphic of parasite load (A), different letters (a,b,c) in the columns denotes statistical difference among the groups ($p \leq 0.05$), and groups with similar letters do not differ statistically ($p > 0.05$). In the diagram (B), the symbols indicate (-) normal/mild, (+ -) moderate or (+ +) intense inflammatory infiltrate. The area delimited by the blue line indicates the status of leucocytes infiltration determined by all groups, and the line direction indicates the influence of each group in this status. (C and D) Regression linear analyses correlating parasitic load with NO_2^-/NO_3^- heart levels in young (C) and elderly (D) mice.

Cardiac gene expression of arginase-1 and iNOS, as well as NO (NO_2^-/NO_3^-), were higher in young and elderly animals infected with *T. cruzi* compared to the uninfected groups ($P < 0.05$). Infected animals showed higher arginase-1 expression and arginase activity, while iNOS expression and NO tissue levels were lower in elderly animals compared to young animals ($P < 0.05$; Fig. 6).

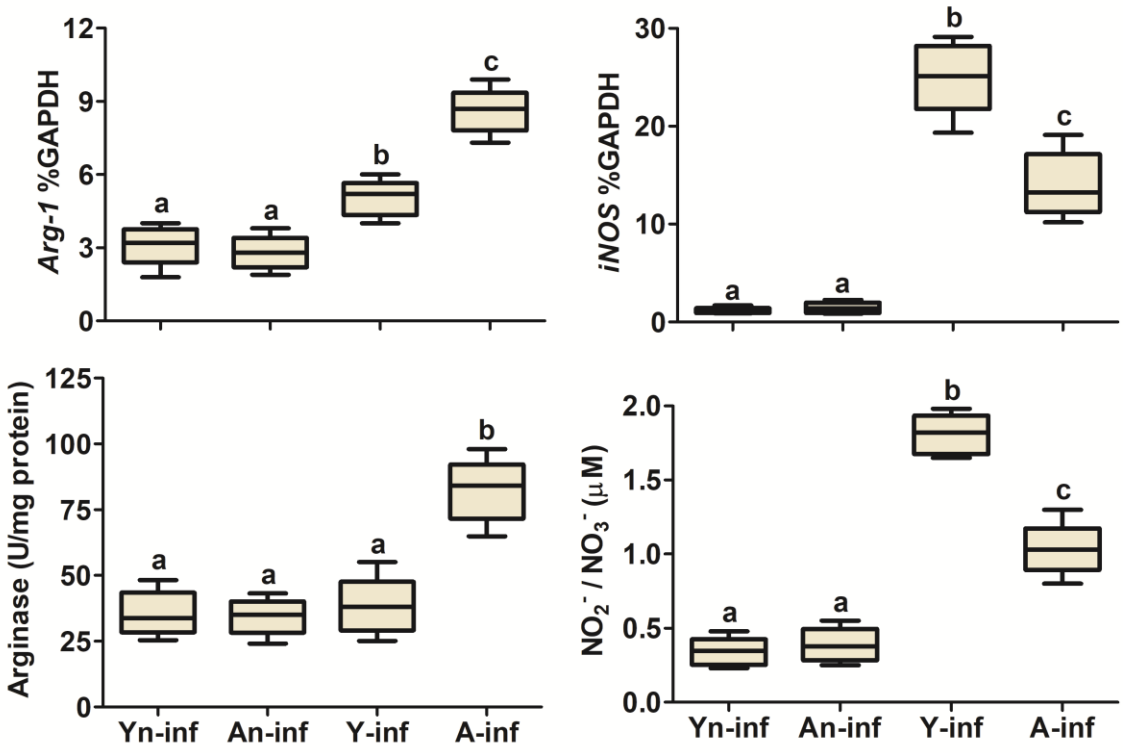


Fig. 6. Gene expression of arginase-1 (Arg-1) and inducible nitric oxide (iNOS), arginase activity and levels of nitrite/nitrate (NO_2^-/NO_3^-) in cardiac tissue from control and *Trypanosoma cruzi*-infected mice.

Source: From the author

Subtitle: Y: young, A: aged, inf: infected, n-inf: uninfected. a,b,c Different letters in the columns denotes statistical difference among the groups ($p \leq 0.05$), and groups with similar letters do not differ statistically ($p > 0.05$).

Gene expression levels of IL-10, IFN-γ, IL-17, Arg-1 and iNOS were increased in BMDMs stimulated with *T. cruzi* antigens, and levels were further elevated when BMDMs were co-cultivated with splenocytes when compared to control cells (BMDM and splenocytes alone) ($P < 0.05$). Cardiac IL-10 expression was similar in BMBDs that were co-cultivated with splenocytes derived from elderly and young mice ($P > 0.05$). While Arg-1 and IL-17 expression levels were increased, expression of IFN-γ and iNOS were reduced in BMBDs co-cultured with splenocytes from elderly animals compared to those from young animals ($P < 0.05$). The Arg-1/iNOS ratio was higher in elderly than young mice infected with *T. cruzi* ($P < 0.05$; Fig. 7).

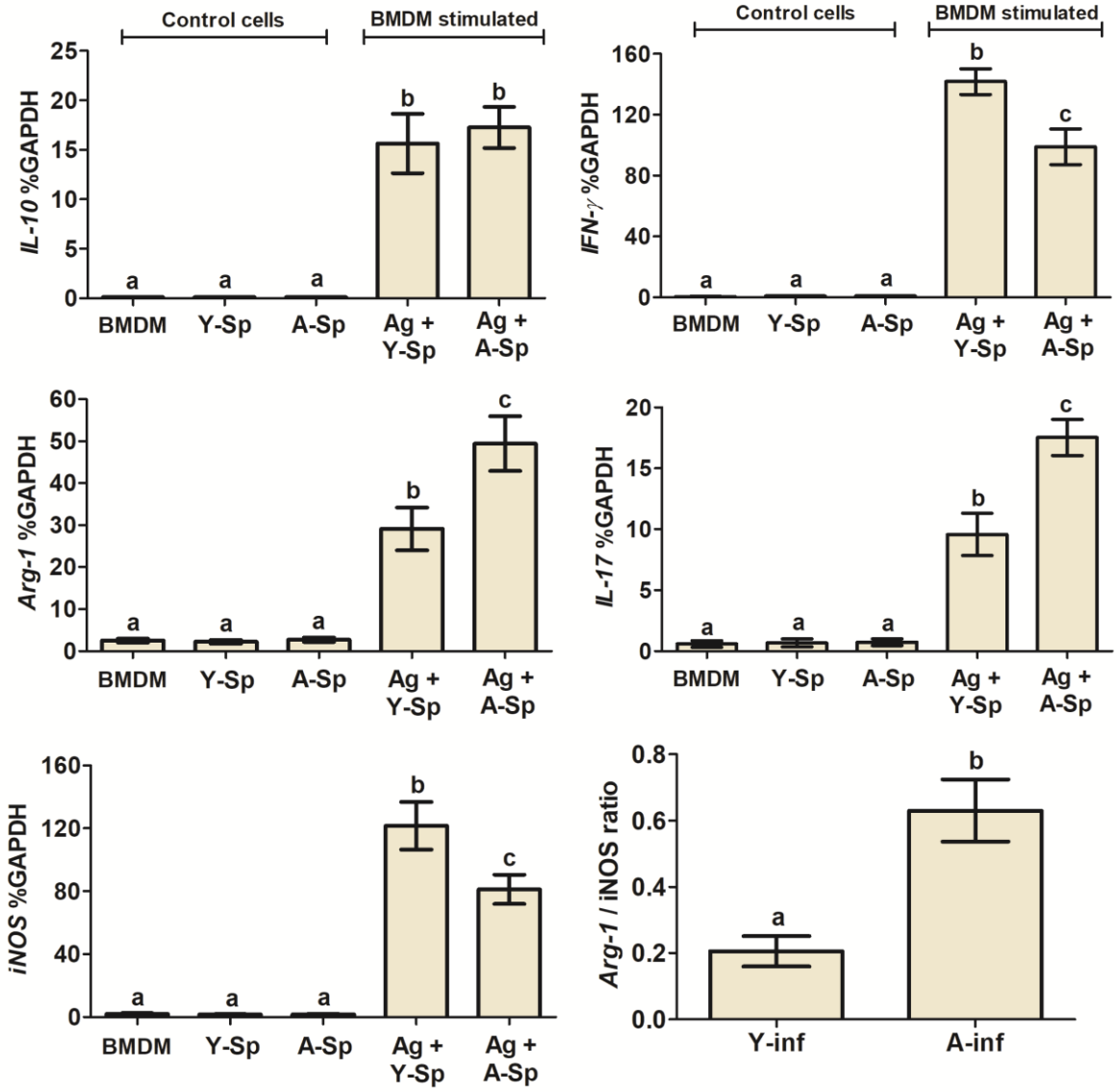


Figure 7 - Gene expression in macrophages and splenocytes co-culture stimulated with *Trypanosoma cruzi* antigens (Ag).

Source: From the author

Subtitle: BMDM: bone marrow-derived macrophages, Y: young, A: aged, Sp: splenocytes, Arg-1: arginase-1, iNOS: inducible nitric oxide, IL: interleukin, GAPDH: glyceraldehyde 3-phosphate dehydrogenase. a,b,c Different letters in the columns denotes statistical difference among the groups ($p \leq 0.05$), and groups with similar letters do not differ statistically ($p > 0.05$).

Arginase activity and NO levels were increased in BMDMs stimulated with *T. cruzi* antigens, especially when co-cultured with splenocytes compared to control cells (BMDM and splenocytes alone) ($P < 0.05$). In the infected group, arginase activity was increased and

the NO level was reduced in BMBDs co-cultured with elderly splenocytes than young splenocytes ($P < 0.05$; Fig. 8).

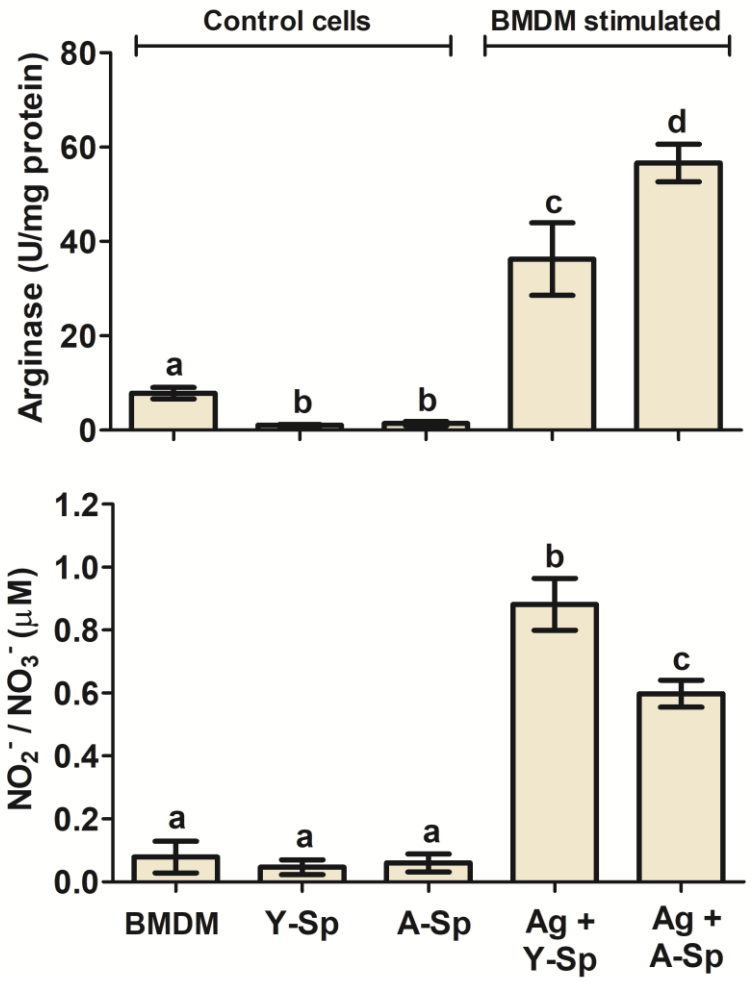


Figure 8 - Arginase activity and nitric oxide levels in bone marrow-derived macrophages and splenocytes co-culture stimulated with *Trypanosoma cruzi* antigens (Ag).

Source: From the author

Subtitle: Y: young, A: aged, Sp: splenocytes. a,b,c Different letters in the columns denotes statistical difference among the groups ($p \leq 0.05$), and groups with similar letters do not differ statistically ($p > 0.05$).

14 DISCUSSION

In this study, we compared the resistance of young and elderly organisms to *T. cruzi* infection using both *in vitro* and *in vivo* assays. Despite having a similar prepatent period and parasitemia peak, elderly animals showed delayed parasite clearance and elevated mean parasitemia levels. However, elderly animals exhibited stable parasitemia at the end of the experimental period, while young animals exhibited a progressive increase in parasitemia. As studies are scarce and present conflicting results, there is still no consensus on the impact of aging on Chagas disease evolution. While CARDILLO et al., (1993), and. REVELLI et al., (1987) reported enhanced parasitic control in elderly *T. cruzi*-infected mice or rats, COLATO et al., (2017) indicated a similar response between young and elderly rats. In a recent systematic review published by our research group, it was clear that these divergent results are directly related to the wide variability in methodologies among studies on aging and Chagas disease, especially in relation to the age of the host (FELIZARDO et al., 2018). There is no doubt that resistance against pathogens is directly dependent on the host's immunological heath (COBEY, 2014; RAUW, 2012). Due to the age-dependent curvilinear characteristic of the immune response (MONTECINO-RODRIGUEZ; BERENT-MAOZ; DORSHKIND, 2013), antimicrobial resistance varies throughout the host's life cycle (FELIZARDO et al., 2018; SAHUQUILLO-ARCE et al., 2011). While young hosts are more susceptible to systemic protozooses due to incomplete immunological maturity (FELIZARDO et al., 2018; SIMON; HOLLANDER; MCMICHAEL, 2015), the increased susceptibility of elderly animals is related to a decline in immune function characteristic of immunosenescence (MA; FANG, 2013; PERA et al., 2015). Therefore, different resistance and susceptibility profiles are natural and expected in different age brackets and between extremes of age (FANG; ROSCOE; SIGAL, 2010; SHAN et al., 2012).

Consistent with our findings for parasitemia, TOAPANTA; ROSS, (2009) indicated that changes in the innate immune system during aging leads to late activation of the adaptive immune response, thereby prolonging the disease. The slow rate of migration to infected tissues, in addition to delayed activation, clonal expansion and production of cytokines by T cells, can also contribute to slower parasitic clearance in elderly animals (FULTON; VARGA, 2009; PO et al., 2002). There is no doubt that cytokine production was triggered in both young and elderly animals infected by *T. cruzi*. However, elderly mice exhibited reduced IFN-γ and higher IL-17 levels than young animals. Both IFN-γ and TNF-α are Th1 cytokines

with potent anti-*T. cruzi* effects (PISSETTI et al., 2011; SILVA et al., 2015). Reduced IFN-γ production has been reported in elderly animals due to an imbalance in Th1/Th2 responses (OUYANG; KOLLS; ZHENG, 2008; RINK; CAKMAN; KIRCHNER, 1998). Although Th2 phenotypes are associated with increased susceptibility to *T. cruzi* infections (NOVAES et al., 2016b), our findings for IFN-γ and TNF-α cytokines do not provide sufficient evidence to support the increased Th2 activation previously suggested in elderly animals (RINK; CAKMAN; KIRCHNER, 1998). Thus, this requires further investigation.

The anti-inflammatory cytokine IL-10 was produced at similar levels in young and elderly animals infected with *T. cruzi*. As severe heart damage often occurs from exacerbated Th1 responses, IL-10 production is required to attenuate the inflammatory process in order to reduce the risk of early mortality due to acute *T. cruzi* infection (NOVAES et al., 2016b; ROFFÊ et al., 2012). We identified increased IL-17 levels in elderly infected mice; however, this was unable to prevent the development of myocarditis in these animals. While it is not clear how the aging process affects Th17 cells, there is no doubt that IL-17 plays a role in the host defenses against *T. cruzi* (LIM et al., 2014; MIYAZAKI et al., 2010). This cytokine is mainly released by $CD4^+$ T lymphocytes, and its protective role against heart damage has been suggested in both animals (GUEDES et al., 2010) and humans (RODRIGUEZ et al., 2015; SOUSA et al., 2017). GUEDES et al., (2010) showed that neutralization of IL-17 increases leucocyte recruitment, myocarditis and mortality rates in *T. cruzi*-infected mice, despite reduced organ parasitism. Moreover, MIYAZAKI et al., (2010) reported reduced IFN-γ production and higher mortality and parasitemia in *T. cruzi*-infected IL-17-deficient C57BL/6 mice, indicating that this cytokine could play a protective role in acute infections when the production of Th1 molecules is reduced. In humans, studies by MAGALHÃES et al., (2013) and SOUSA et al., (2017) both showed that reductions in IL-17 expression and Th17 cells are associated with greater deterioration of cardiac function, indicating a protective role of this cytokine against Chagas cardiomyopathy.

Together with cellular immunity, humoral effectors have an important inhibitory effect on *T. cruzi* infection (ALVAREZ et al., 2016; CARDOSO; REIS-CUNHA; BARTHOLOMEU, 2016). As expected, we found increased anti-*T. cruzi* IgG serum levels in all infected animals. Of the IgG isotypes, only IgG2b was found to be higher in elderly animals than young animals. Passive immunization of naïve mice indicated a central neutralizing role of anti-*T. cruzi* IgG against circulating trypomastigotes (BRYAN; GUYACH; NORRIS, 2010). The current evidence indicates that IgG1 and IgG2 antibodies are essential for controlling parasite spread, contributing to parasite clearance and reduced

parasitemia in acute infections (BRYAN; GUYACH; NORRIS, 2010; PYRRHO et al., 1998). Although IgG2a antibodies exert a more pronounced neutralizing effect (BRYAN; GUYACH; NORRIS, 2010; PYRRHO et al., 1998), the protective role of IgG2b against *T. cruzi* has been confirmed in previous studies (BRYAN; GUYACH; NORRIS, 2010; TAKEHARA et al., 1981), shown to be mediated by its ability to activate complement fixation (TAKEHARA et al., 1981). The impact of aging on the host's anti-*T. cruzi* humoral response is poorly understood. In a recent systematic review without chronological restriction (FELIZARDO et al., 2018), humoral mediators were reported in only two studies comparing young (21–28 days old) and adult (70–120 days old) rats (PASCUTTI et al., 2003; PÉREZ et al., 2011). In both studies, adult animals exhibited increased anti-*T. cruzi* IgG and IgM production compared to young animals, which were associated with higher parasitic resistance. Although it is tempting to suggest a more effective age-dependent humoral response, our data is not sufficient to affirm that the improved response observed in adult rodents is maintained in very old animals. Thus, further studies are needed to clarify the impact of immunosenescence on the humoral mechanisms activated during *T. cruzi* infection. Although the pathophysiology of Chagas cardiomyopathy is not completely understood, the production of non-specific autoantibodies in response to *T. cruzi* infection (immunological cross-reactivity) has been suggested to be a major cause of myocarditis in Chagas disease (CALDAS et al., 2017; GEORG et al., 2017). Interestingly, together with increased IgG2b production, elderly animals exhibited marked heart inflammation in the present study. Although we cannot determine a causal relation between IgG2b and inflammation, the most severe myocarditis was aligned with a higher parasitic load in elderly mice. PCR-based quantification of *T. cruzi* DNA is frequently used to estimate the heart parasite load (NOVAES et al., 2016a; SANTOS et al., 2016). In addition to its diagnostic value, parasite DNA load is a good predictor of leukocyte infiltration and damage to the cardiac microstructure (CALDAS et al., 2012; NOVAES et al., 2017).

Similar to that observed in previous studies RODRIGUES et al., (2017); SANTOS et al., (2016), our findings indicated that inflammation and parasite load were consistent with the increased Arg-1 expression and arginase activity observed in elderly infected animals, as well as the reduced iNOS expression and NO production. From the correlation between parasite load and heart NO levels, our findings indicated that the heart antitrypanosomal defense of elderly mice is potentially more dependent on iNOS pathway than that of young mice. COLATO et al., (2017) indicated similar NO production in young and elderly animals infected with *T. cruzi*. Although the age groups are compatible with our model, these authors

used Wistar rats to induce Chagas disease, a model whose resistance to *T. cruzi* infection is clearly associated to high NO production (FELIZARDO et al., 2018; PEREIRA et al., 2017). As no histopathological evidence was reported by COLATO et al., (2017), it was not possible to determine the relationship between NO production and Chagas-related myocarditis. Nitric oxide is an important antitrypanosomal effector, known to be induced in the Th1 polarized phenotype and inhibited by Th2 responses (RODRIGUES et al., 2017). By metabolizing L-arginine through the iNOS or arginase pathways, macrophages are the main source of NO (STEMPIN et al., 2004). Triggered by Th1 cytokines, the iNOS pathway is activated in M1 macrophages, and this pathway plays an important role in controlling *T. cruzi* infection due to its nitrosative and cytotoxic effects (CARVALHO et al., 2012; PEREIRA et al., 2014). As NO is also associated with host cell damage and myocarditis severity, the balance between Th1 and Th2 responses is essential for controlling the intensity of inflammatory processes in *T. cruzi*-infected hosts (BASSO, 2013; TEIXEIRA et al., 2011). In this sense, arginase pathway can alternatively be induced by Th2 cytokines, which block NO production by converting L-arginine into urea and L-ornithine (RATH et al., 2014). As Th2-polarized cells are ineffective against *T. cruzi* (RODRIGUES et al., 2017; TEIXEIRA et al., 2011), our results for parasitemia and parasitic load are potentially related to attenuation of NO-dependent parasitic death in elderly mice, a proposition which is reinforced by the iNOS/arginase imbalance observed in our *in vitro* model.

From a mechanistic approach, we developed a co-culture model to measure cytokine expression and the activation of arginase-1 and iNOS pathways in mononuclear leucocytes of young and elderly mice, recognized as the main cells involved in Chagasic myocarditis (CECÍLIO et al., 2011; WEISSER et al., 2013). Surprisingly, our *in vitro* results corroborated our *in vivo* findings. When BMDMs exposed to *T. cruzi* antigens were co-cultured with splenocytes from young and elderly mice, we found that IL-10 expression was similar, IFN-γ was reduced and IL-17 was increased in the culture containing cells from elderly animals. These findings support our hypothesis that age-dependent immunological variability *in T. cruzi*-infected mice is partially associated with divergent recognition and/or response to parasite antigens. This divergent response was clearly observed from the increased antagonistic Arg-1 and attenuated iNOS gene expression response in BMDMs co-cultured with elderly splenocytes. Accordingly, these cells also exhibited higher arginase activity and reduced NO production, indicating that splenocytes obtained from elderly mice are less efficient in activating the iNOS pathway. Although the age-dependent immunological regulatory mechanisms are poorly understood, reduced IFN-γ levels may be involved in this

attenuated response as this cytokine is a key inducer of NO production in macrophages (CUMMINGS; TARLETON, 2004).

Taken together, our findings indicate that young and elderly mice infected by a virulent strain of *T. cruzi* express divergent parasitic control and myocarditis severity, which is potentially related to differences in cytokine expression and activation of Arg-1 and iNOS pathways. In general, the severe myocarditis identified in elderly animals was consistent with higher parasitemia and parasitic load, indicating that the upregulated IgG2b and IL-17 production was not enough to counteract heart parasitism and damage. Thus, the higher susceptibility of elderly mice to *T. cruzi* infection was potentially related to differential activation of the antagonistic Arg-1 and iNOS pathways, which modulate the pathogen-host interactions. At the same time that these pathways contribute to keep the disease stable, they can also lead to a dynamic imbalance in favor of the parasite. Therefore, our findings provide initial evidence that the aging of immune cells is associated with an attenuated response to antigenic stimulation, in which iNOS downregulation and increased activation of the arginase pathway creates favorable conditions for heart parasitism and myocarditis development.

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AMANDA APARECIDA FELIZARDO

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15 GENERAL CONCLUSION

15.1 SYSTEMATIC REVIEW: IMPACT OF AGE AND AGING ON THE EVOLUTION OF SYSTEMIC PROTOZOOSES IN ANIMALS MODELS

From a comprehensive literature review, we identified that host age exerts profound influences on the evolution of systemic protozoozis. Throughout aging, parasitemia and mortality were consistently reduced in Chagas disease and malaria, but similar or increased in leishmaniasis and highly variable in toxoplasmosis. While a humoral response in older animals was related to the anti-*T. cruzi* protective phenotype in Chagas disease, a cellular response mediated by a polarized Th1 phenotype was associated with a more effective defense against Plasmodium infections. Conversely, in leishmaniasis, the severe infections and highest mortality rates were potentially related to the attenuation of humoral response and an imbalance between Th1 and Th2 phenotypes. Due to the heterogeneous parasitological outcomes and limited immunological data, the role of aging in toxoplasmosis evolution remains poorly understood. Besides the heterogeneity in experimental protocols, animal models (animal species and lineage), parasites (virulence and pathogenicity) and measured outcomes (time-dependent manifestations) were in general consistently aligned, representing an important element of internal validity. Although the limited methodological quality of the studies identified points to the need for careful analysis of current evidence, the main sources of bias were related to incomplete reporting of simple constructs. From a detailed description of these elements of bias, more comprehensive and controlled research may benefit by avoiding the systematic reproduction of inconsistent and poorly reproducible experimental designs.

15.2 ORIGINAL STUDY: IMPACT OF AGING ON THE EVOLUTION OF CHAGAS DISEASE

According our *in vitro* and *in vivo* findings, young and elderly mice infected by a virulent strain of *T. cruzi* express divergent parasitic control and myocarditis severity, which

was potentially related to differences in cytokine expression and activation of Arg-1 and iNOS pathways. In general, the severe myocarditis identified in elderly animals was consistent with higher parasitemia and parasitic load, indicating that the upregulated IgG2b and IL-17 production was not enough to counteract heart parasitism and damage. Thus, the higher susceptibility of elderly mice to *T. cruzi* infection was potentially related to differential activation of the antagonistic Arg-1 and iNOS pathways, which modulate the pathogen-host interactions. At the same time that these pathways contribute to keep the disease stable, they can also lead to a dynamic imbalance in favor of the parasite. Therefore, our findings provide initial evidence that the aging of immune cells is associated with an attenuated response to antigenic stimulation, in which iNOS downregulation and increased activation of the arginase pathway creates favorable conditions for heart parasitism and myocarditis development.

16 ARTICLES

16.1 ARTICLE 1: COULD AGE AND AGING CHANGE THE HOST RESPONSE TO SYSTEMIC PARASITIC INFECTIONS? A SYSTEMATIC REVIEW OF PRECLINICAL EVIDENCE **Impact factor: 3,340**

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Review

Could age and aging change the host response to systemic parasitic infections? A systematic review of preclinical evidence

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ABSTRACT

The impact of age and aging in the evolution of systemic parasitic infections remains poorly understood. We conducted a systematic review from preclinical models of Chagas disease, leishmaniasis, malaria, sleeping sickness and toxoplasmosis. From a structured and comprehensive search in electronic databases, 29 studies were recovered and included in the review. Beyond the characteristics of the experimental models, parasitological and immunological outcomes, we also discussed the quality of current evidence. Our findings indicated that throughout aging, parasitemia and mortality were consistently reduced in Chagas disease and malaria, but were similar or increased in leishmaniasis and highly variable in toxoplasmosis. While a marked humoral response in older animals was related to the anti-*T. cruzi* protective phenotype, cellular responses mediated by a polarized Th1 phenotype were associated with a more effective defense against Plasmodium infection. Conversely, in leishmaniasis, severe infections and high mortality rates were potentially related to attenuation of humoral response and an imbalance between Th1 and Th2 phenotypes. Due to the heterogeneous parasitological outcomes and limited immunological data, the role of aging on toxoplasmosis evolution remains unclear. From a detailed description of the methodological bias, more controlled researches could avoid the systematic reproduction of inconsistent and poorly reproducible experimental designs.

1. Introduction

Malaria, leishmaniasis, toxoplasmosis, African (sleeping sickness) and American (Chagas disease) trypanosomiasis are systemic protozooses responsible for dramatic economic and medico-social impact worldwide (Pollitt et al., 2011; Lozano et al., 2012). Taken together, they are the main neglected diseases responsible for the highest morbidity and mortality rates reported for parasitic diseases in tropical and subtropical regions (Mackey et al., 2014). The etiological agents of each disease are hyperendemic in developing countries, especially in Africa, the Middle East, Central and South Americas; areas with favorable environmental conditions for parasite development, poor socioeconomic status and limited access to formal health services (Kettler and Marjanovic, 2004; Rassi et al., 2010; Antinori et al., 2017). Children and the elderly are the most susceptible to parasitic diseases, developing severe forms of infection and suffering disproportionately high mortality rates compared to intermediate age groups (Simon et al., 2015). In these vulnerable groups, infection susceptibility has been attributed to an immunological inability to contain the infection, especially due to incomplete immunological maturation in children and immunosenescence in aged people (Simon et al., 2015). Understanding the biological cycle of each etiological agent and the physiopathological mechanism linked to the infections is essential to control transmission and treat human protozooses (Molyneux, 2006). Furthermore, the rational design of more effective public health programs also depends on the identification of vulnerable population groups, as well as on clear delimitation of factors associated with the greater susceptibility to infections (Giefing-Kröll et al., 2015), including those determined by age and aging (Fernández-Mayoralas et al., 2015).

Malaria is the most frequent systemic protozoozis worldwide. This disease is caused by parasites of the genus *Plasmodium*, which are transmitted through the bites of female *Anopheles* mosquitoes (Cox-Singh et al., 2008; Oliveira-Ferreira et al., 2010). According to recent estimates, > 212 million new cases of malaria were registered

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16.2 ARTICLE 2: IMBALANCE BETWEEN INDUCIBLE NITRIC OXIDE SYNTHASE AND ARGINASE EXPRESSION AND ACTIVITY IS INVOLVED IN AGE-DEPENDENT RESPONSE TO *TRYPANOSOMA CRUZI* INFECTION **Impact factor: 5,606**

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Title	Imbalance between inducible nitric oxide synthase and arginase expression and activity is involved in age-dependent response to Trypanosoma cruzi infection
Article type	Original article

Abstract

Abstract Elderly organisms are more susceptible to infectious diseases. However, the impact of aging on antiparasitic mechanisms, especially the nitric oxide pathway, is poorly understood. Using an integrated in vivo and in vitro model, we compared the severity of Trypanosoma cruzi infection in young and elderly (8 or 72 weeks old) mice. Forty C57BL/6 mice were randomized into four groups: Y-inf, young infected; Yn-Inf, young uninfected; A-inf, aged infected; An-Inf, aged uninfected. Parasitemia was measured daily, and animals were euthanized after 15 days of infection. Trypanosoma cruzi-induced inflammatory processes were analyzed in blood and heart samples, as well as in bone marrow-derived macrophages (BMDMs) co-cultured with splenocytes isolated from young or elderly mice. Our results indicated upregulated IgG2b and IL-17 production in elderly animals, which was not sufficient to reduce parasitemia, parasitic load and myocarditis to levels observed in young animals. The higher susceptibility of elderly mice to T. cruzi infection was accompanied by reduced cardiac inducible nitric oxide synthase (iNOS) gene expression, nitric oxide (NO) and IFN-γ levels, as well as an antagonistic upregulation of arginase-1 expression and arginase activity. The same responses were observed when BMDMs co-cultured with splenocytes from elderly mice were stimulated with T. cruzi antigens. Our findings indicate that elderly mice are more susceptible to T. cruzi infection, which is potentially related to an attenuated response to antigenic stimulation, inhibition of iNOS gene expression and NO production, and antagonistic upregulation of arginase gene expression and activity, which created favorable conditions for heart parasitism and myocarditis development.

Keywords	Chagas disease, cardiovascular pathology, experimental parasitology, nitric oxide.
Taxonomy	Nitric Oxide Synthase, Arginine
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