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ORIGINAL ARTICLE

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Evaluation of the serum cytokines profile and nitric oxide in experimental murine leprosy

Avaliação do perfil de citocinas séricas e óxido nítrico na hanseníase experimental murina

Evaluación del perfil sérico de citoquinas y óxido nítrico en la lepra experimental murina

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ABSTRACT

Introduction: leprosy is a chronic infectious disease caused by *Mycobacterium leprae* (*M. leprae*), an obligate intracellular parasite. Thus, host resistance to this pathogen depends on cellular immunity. Experimental models have made it possible to study leprosy from an immunological, microbiological, and therapeutic point of view. However, the differences in the progression of the infection between the most used models (immunocompetent mice, BALB/c, and congenitally athymic mice, nude) have yet to be studied. **Objective:** to compare the evolution of *M. leprae* infection in BALB/c and

nude mice regarding bacillary multiplication and evaluate the systemic inflammatory profile by quantifying serum cytokines and nitric oxide (NO). **Methods:** the mice were inoculated with *M. leprae* in the footpads and assessed 3, 5, and 8 months after infection. **Results:** nude mice showed progressive bacillary multiplication in the footpads. In BALB/c mice, the number of bacilli was higher at five months. Regarding cytokine quantification, BALB/c mice showed an increase in IL-2 and IL-17A and a decrease in IL-6 and NO at eight months of inoculation. In the nude mice, there was an increase in TNF at eight months of inoculation and maintenance of NO levels. **Conclusion:** the results suggest that BALB/c mice activate an immune response capable of controlling the multiplication of *M. leprae*, whereas in nude mice, the infection is progressive despite high levels of TNF.

Keywords: *Leprosy. Models Animal. Cytokines. Nitric Oxide. Immunity.*

RESUMO

Introdução: a hanseníase é uma doença infecciosa crônica causada pelo *Mycobacterium leprae* (*M. leprae*), um parasita intracelular obrigatório. Assim, a resistência do hospedeiro a esse patógeno depende da imunidade celular. O uso de modelos experimentais tem permitido o estudo da hanseníase do ponto de vista imunológico, microbiológico e terapêutico, entretanto, as diferenças na progressão da infecção entre os modelos mais empregados (camundongos imunocompetentes, BALB/c, e camundongos congenitamente atímicos, *nude*) são pouco estudadas. **Objetivo:** comparar a evolução da infecção pelo *M. leprae* em camundongos BALB/c e *nude* quanto à multiplicação bacilar e avaliação do perfil inflamatório sistêmico pela quantificação sérica de citocinas e óxido nítrico (NO). **Métodos:** os camundongos foram inoculados com *M. leprae* nos coxins plantares e avaliados aos 3, 5 e 8 meses após a infecção. **Resultados:** camundongos *nude* apresentaram multiplicação bacilar progressiva nos coxins plantares. Em camundongos BALB/c, o número de bacilos foi maior aos 5 meses. Em relação à quantificação de citocinas, nos camundongos BALB/c houve aumento de IL-2 e IL-17A e diminuição de IL-6 e NO aos 8 meses de inoculação. Nos camundongos *nude*, verificou-se o aumento do TNF aos 8 meses de inoculação e manutenção dos níveis de NO. **Conclusão:** os resultados encontrados sugerem que em camundongos BALB/c ocorre a ativação de uma resposta imune capaz de controlar a multiplicação do *M. leprae*, em contrapartida em camundongos *nude* a infecção é progressiva a despeito de altos níveis de TNF.

Palavras-chave: *Hanseníase. Modelos Animais. Citocinas. Óxido Nítrico. Imunidade.*

RESUMEN

Introducción: la lepra es una enfermedad infecciosa crónica causada por *Mycobacterium leprae* (*M. leprae*), un parásito intracelular obligado. Así pues, la resistencia del huésped a este patógeno depende de la inmunidad celular. La utilización de modelos experimentales ha permitido estudiar la lepra desde un punto de vista inmunológico, microbiológico y terapéutico. Sin embargo, las diferencias en la evolución de la infección entre los modelos más utilizados (ratones inmunocompetentes, BALB/c, y ratones congénitamente atímicos, nude) han sido poco estudiadas. **Objetivo:** comparar la evolución de la infección por *M. leprae* en ratones BALB/c y nude en términos de multiplicación bacilar y evaluación del perfil inflamatorio sistémico mediante la cuantificación de citoquinas séricas y óxido nítrico (NO). **Métodos:** los ratones fueron inoculados con *M. leprae* en las almohadillas plantares y evaluados a los 3, 5 y 8 meses tras la infección. **Resultados:** los ratones nudes mostraron una multiplicación bacilar progresiva en las almohadillas plantares. En los ratones BALB/c, el número de bacilos fue mayor a los 5 meses. En cuanto a la cuantificación de citoquinas, los ratones BALB/c mostraron un aumento de IL-2 e IL-17A y una disminución de IL-6 y NO a los 8 meses de la inoculación. En los ratones nude, se produjo un aumento del TNF a los 8 meses de la inoculación y un mantenimiento de los niveles de NO. **Conclusión:** los resultados sugieren que los ratones BALB/c activan una respuesta inmunitaria capaz de controlar la multiplicación de *M. leprae*, mientras que en los ratones nude la infección es progresiva a pesar de los altos niveles de TNF.

Palabras clave: *Lepra. Modelos animales. Citoquinas. Óxido nítrico. Inmunidad.*

INTRODUCTION

Leprosy is an infectious disease with a broad clinical spectrum, prevalent in developing countries. It is caused by *Mycobacterium leprae* (*M. leprae*), an acid-fast bacillus (AFB)[1].

Cultivating the bacillus in vitro remains a challenge that has not been overcome. Still, the development of experimental models such as the nine-banded armadillo (*Dasypus novemcinctus*) and BALB/c congenitally athymic (nude) mice has enabled the study of leprosy from microbiological, immunological, and therapeutic perspectives[2,3].

In 1960, Shepard demonstrated that injection of 10^4 bacilli into the footpad of BALB/c mice reached a plateau of multiplication at about eight months

and declined one year after inoculation. Bacillus growth in this mice strain is limited, and bacilli do not spread to other tissues and organs because these are immunocompetent animals[4].

Another milestone in experimental leprosy was the inoculation of *M. leprae* into nude mice deficient in T-lymphocyte-mediated immune response, resulting in intense bacillary proliferation and dissemination to the liver and spleen 18 months after the initial inoculation in the footpad[5,6].

M. leprae is an obligate intracellular parasite with a predilection for Schwann cells and macrophages. It can cause sensory and motor damage through injury to the affected nerves, resulting in neuropathy, impairment, and deformity of the hands and feet[7].

The pathophysiology of leprosy is influenced by several factors, including genetic, immunological, and environmental aspects, which determine individual susceptibility to the bacillus. Both innate and adaptive immune responses play a role in controlling the bacillus. However, studies have indicated a correlation between the clinical forms of the disease and the cytokine profile. Patients with tuberculoid leprosy (TT), a limited manifestation of the disease, exhibit a predominance of the Th1 profile characterized by the production of IL-2 and IFN-y, while patients with the disseminated form of leprosy, named lepromatous leprosy (LL), exhibit a predominance of Th2 cytokines (IL-4, IL-5, and IL-10)[8].

Between the TT and LL forms, there is the borderline group with variable clinical, bacteriological, and histopathological characteristics according to the degree of immune response to *M. leprae*[9]. These patients have different cytokine profiles in their skin lesions, predominating the Th1 profile in borderline-tuberculoid patients (BT) and the Th2 profile in borderline-lepromatous patients (BL)[10].

More recently, studies have shown that in addition to the classic Th1/Th2 paradigm, regulatory T cells (Treg), Th9, Th17, and Th25 are involved in the host response, with the Th1/Th9/Th17 profile predominating in the tuberculoid pole (TT/BT) and Th2/Treg/Th25 in the lepromatous pole (LL/BL)[11-13].

It is believed that the Th17 response may play a pivotal role in modulating macrophage activity, as IL-17 can induce the production of TNF and iNOS, leading to the generation of reactive oxygen and nitrogen species that facilitate the destruction of the bacillus[12].

Concerning the immune response in experimental models, BALB/c and athymic nude mice exhibit distinct behaviors when inoculated with *M. leprae* in the footpad. In BALB/c mice, the bacilli remain exclusively in the footpad, with

granulomatous lesions similar to those observed in TT patients[14], while in nude mice, there is intense bacillary multiplication with the dissemination of the bacilli to other areas besides the footpad, showing predominantly macrophagic lesions with numerous bacilli inside, similar to those of LL patients[14].

Given the absence of studies comparing the evolution of *M. leprae* infection in immunocompetent (BALB/c) and immunocompromised (nude) mice, this study evaluated the systemic response to *M. leprae* based on the dosage of cytokines and nitric oxide (NO) at different stages of infection.

MATERIAL E METHODS

Animals: this study utilized 80 mice, 40 BALB/c mice, and 40 nude mice (NU-*Foxn1*^{nu}). The mice were of both sexes and aged 6 to 8 weeks. They were obtained from the animal house of the Lauro de Souza Lima Institute in Bauru, São Paulo, Brazil, and maintained in mini-isolators in ventilated racks in a pathogen-free environment, with access to water and food *ad libitum*.

Ethical aspects: all procedures followed the ethical standards established by CONCEA and were approved by the Ethics Committee for the Use of Animals (CEUA) of the Lauro de Souza Lima Institute, Bauru, São Paulo, Brazil (number 001/23).

Inoculum: the suspension of *M. leprae* was obtained from nude mice footpad, previously inoculated with the Thai-53 strain to maintain the bacillus. After macerating the footpad in Hank's saline solution (Sigma, St. Louis, MO, USA), the suspension was filtered through a sterile 40 µm diameter nylon membrane (BD Falcon, Bedford, MA, USA) to eliminate tissue debris[15]. The total bacilli was then calculated according to the protocol described in the Manual of Laboratory Techniques for Leprosy[16].

Inoculation: the mice were inoculated intradermally on both footpads with 0.03 ml of *M. leprae* suspension, containing $1x10^4$ bacilli for BALB/c mice and $3x10^6$ bacilli for nude mice.

Euthanasia: the animals were euthanized at 3, 5, and 8 months post-inoculation (10 per group). Ten healthy BALB/c and nude mice were used as a control group. Following euthanasia, whole blood was collected to obtain serum, which was aliquoted and kept at -80 °C until it was used. Footpads were removed to determine the number of bacilli.

Determination of the number of bacilli in the footpad: following the footpad's soaking in Hank's saline solution (Sigma, St. Louis, MO, USA), the suspension was filtered through a sterile 40 µm diameter nylon membrane (BD Falcon, Bedford, MA, USA) to eliminate tissue debris[15]. The bacillary suspension

was fixed on slides and stained using the Ziehl-Neelsen technique. The total bacilli was calculated according to the protocol described in the Laboratory Techniques Manual for Leprosy[16].

Serum cytokine quantification: the quantification of the cytokines IL-2, IFN-γ, TNF, IL-4, IL-6, IL-10, and IL-17A was conducted using the CBA method (Cytometric Bead Array, catalog number 560485, Becton Dickinson Industries, BD, United States). The analyses were performed according to the manufacturer's instructions on the BD FACSAria™ Fusion analyzer (Becton Dickinson Industries, BD, United States). The results were generated using the FCAP Array software (BD), based on a standard curve, and expressed in pg/ml. Serum cytokine levels below the detection limit were expressed as 0 pg/ml.

Nitric oxide (NO) production: NO is decomposed into nitrite (NO_2^-) and nitrate (NO_3^-), and thus the output of this element was estimated by detecting NO_2^- using the Griess colorimetric method[17]. In a 96-well plate were pipetted 100 µl of serum and 100 µl of Griess reagent containing n-(1-naphthyl)-ethylenediamine (NEED) diluted to 0.1% in distilled water and sulfanilamide diluted to 1% in 5% H_3PO_4 in equal volumes. The plate was maintained at room temperature for 10 minutes and then read by an automated ELISA micro-reader at a wavelength of 540 nm, compared to a blank consisting of a Griess reagent. The results were expressed in micromolar (µM) units by comparing the optical density (OD) to a standard curve established for each test.

Statistical analysis: comparisons of NO and serum cytokine data were done using the Kruskal-Wallis test, followed by Dunn's post-test, with a significance level of 5% ($p < 0.05$). All analyses were carried out using the GraphPad Prism 7.0 statistical program.

RESULTS

In BALB/c mice, infection progressed up to five months; after this period, there was a decline in the number of bacilli. This parameter did not show a significant difference between three and eight months after infection, as seen in Figure 1. BALB/c mice did not show macroscopic lesions on the footpads during the study period, as seen in Figure 2. Infection was progressive in nude mice, with bacillary proliferation in the footpads (Figure 1) and the appearance of macroscopic lesions by five months, which increased in size and often involved the entire footpads by eight months post-inoculation (Figure 2).

Figure 1 – Quantification of bacilli in the footpads of BALB/c and nude mice inoculated with *M. leprae*. The bars represent the median and interquartile ranges. *$p < 0.05$; ****$p < 0.0001$.

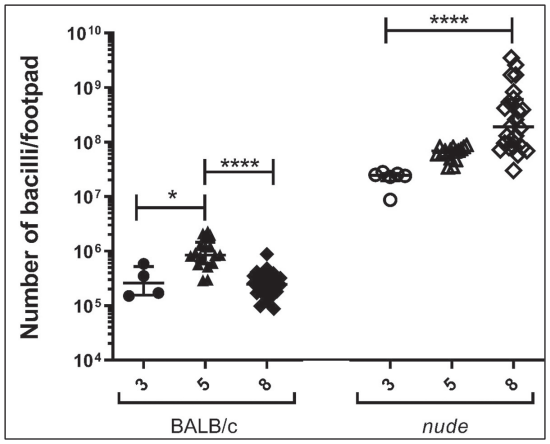

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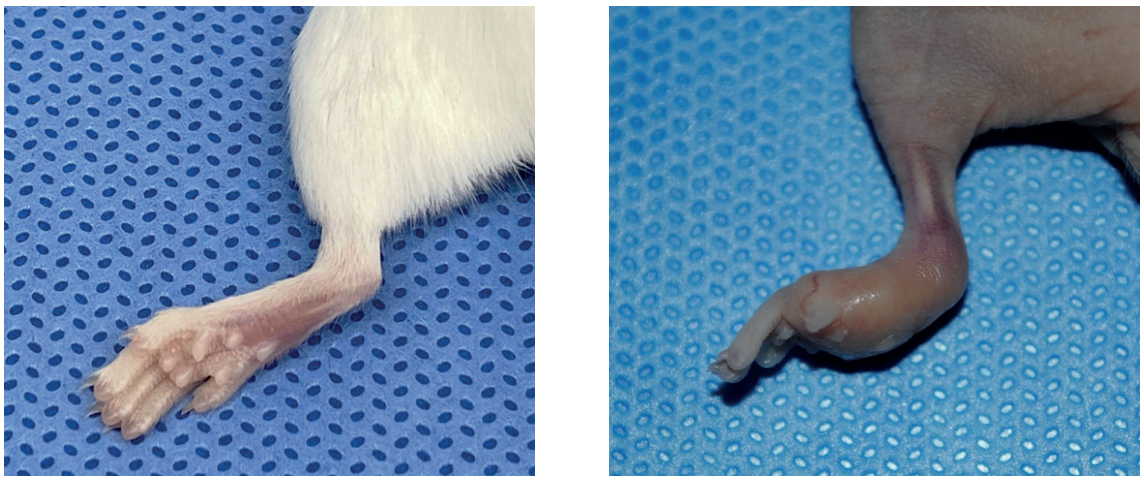
Figure 2 – Mice inoculated with *M. leprae* in the footpad. A) Footpad with no apparent macroscopic lesion eight months after inoculation in a BALB/c mouse. B) Macroscopic lesion eight months after inoculation in a nude mouse.



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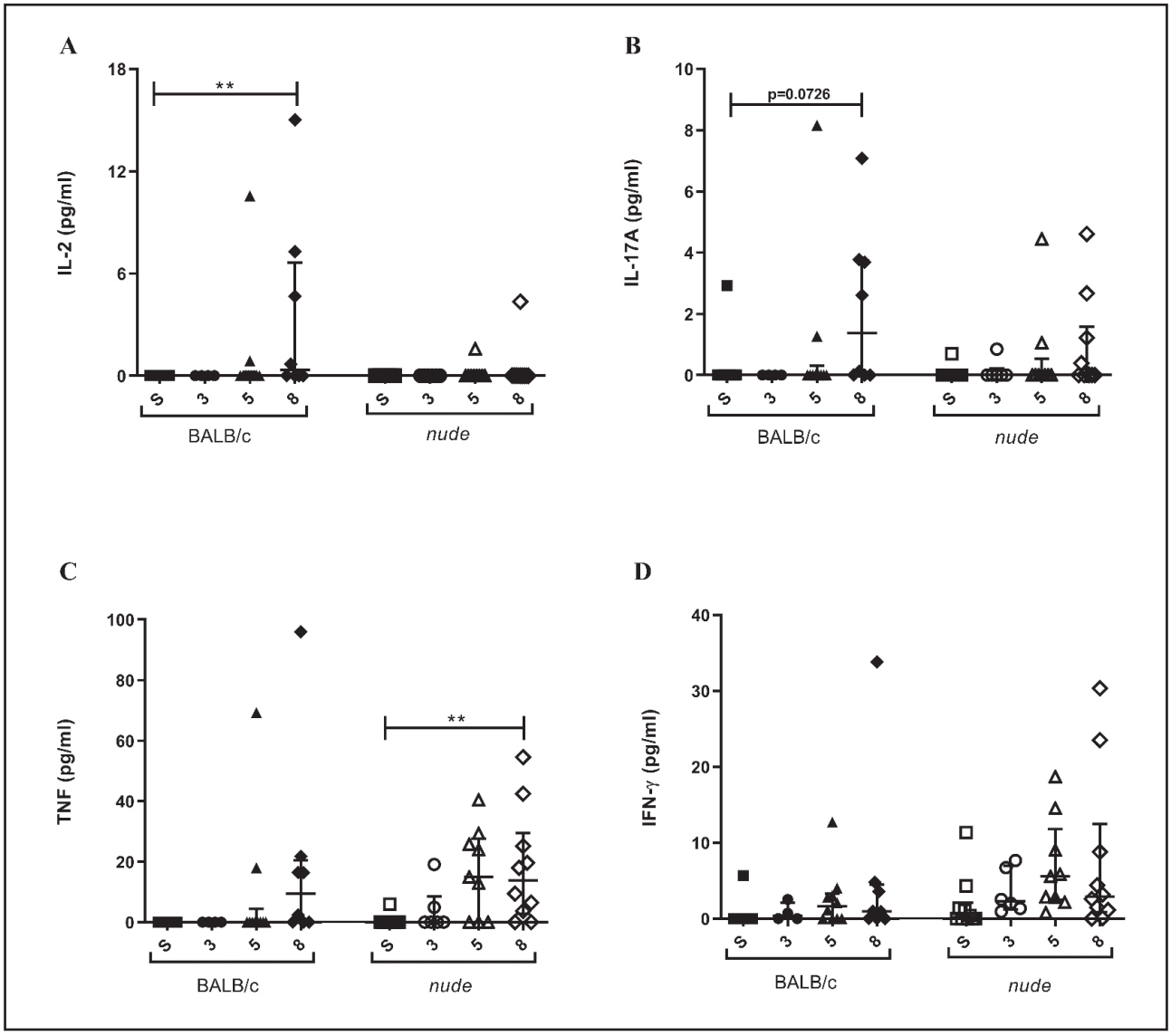
The quantification of serum cytokines in the BALB/c strain revealed higher levels of IL-2 and IL-17 at eight months compared to the control group (Figures 3A and 3B). In contrast, IL-6 showed higher levels in the control group compared to 8 months post-inoculation (Figure 4A), while TNF, IFN-γ, IL-10, and IL-4 showed no significant changes (Figures 3 and 4).

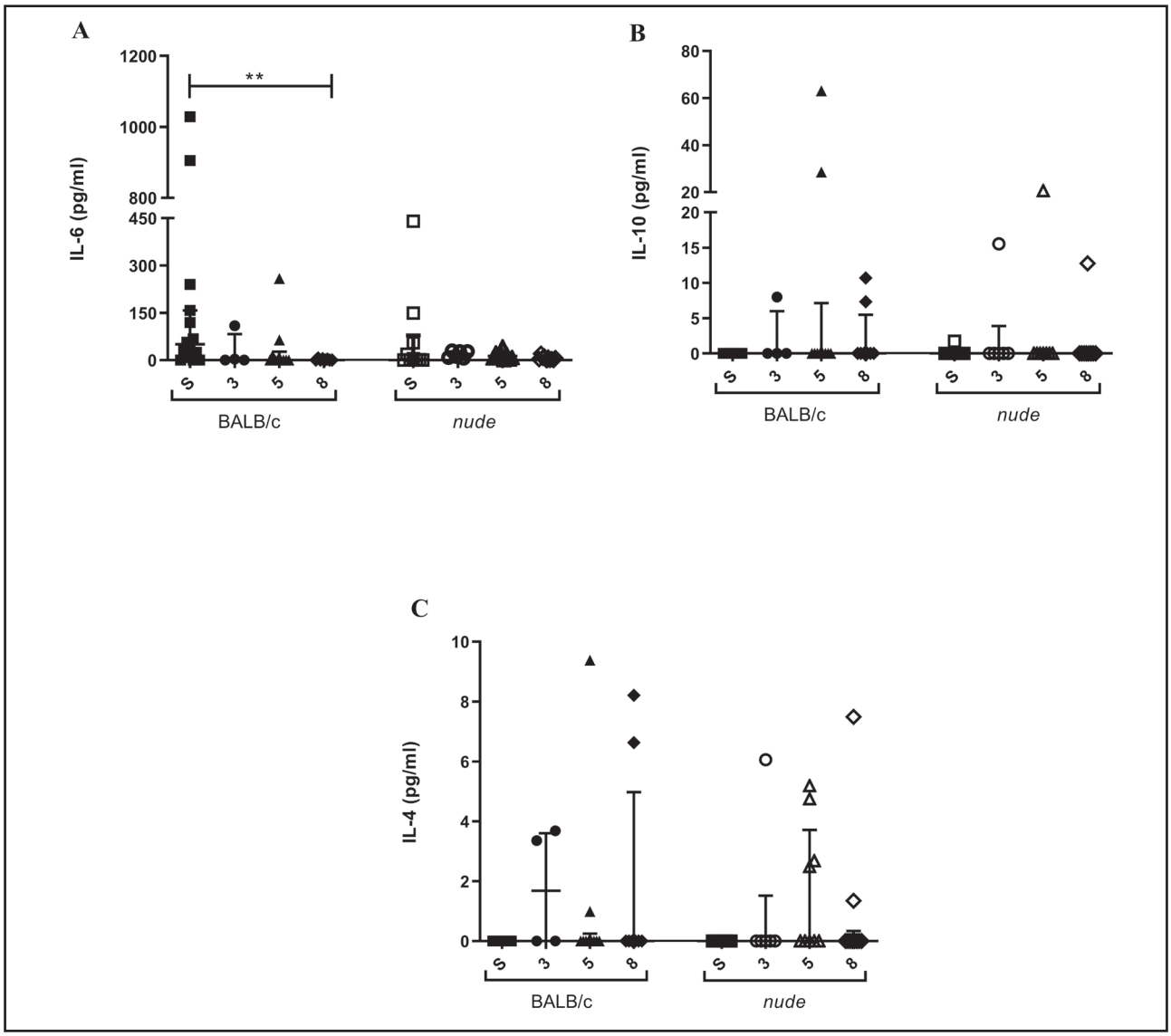
The quantification of cytokines in nude mice revealed higher levels of TNF at eight months when compared to the control group (Figure 3C). There was no significant difference in the levels of the cytokines IL-2, IL-17, IFN-γ, IL-4, IL-6, and IL-10 (Figures 3 and 4).

Figure 3 – Quantification of serum cytokines (IL-2, IL-17A, TNF, and IFN-γ) in BALB/c and nude mice inoculated with *M. leprae* in the footpad and euthanized at 3, 5 and 8 months post-inoculation. H = healthy mice. $^{**}p < 0,01$.



Source: Created by the authors.

Figure 4 – Quantifying serum cytokines (IL-4, IL-6, and IL-10) in BALB/c and nude mice inoculated with *M. leprae* in the footpad and euthanized at 3, 5, and 8 months post-inoculation. H = healthy mice. $^{**}p < 0{,}01$.



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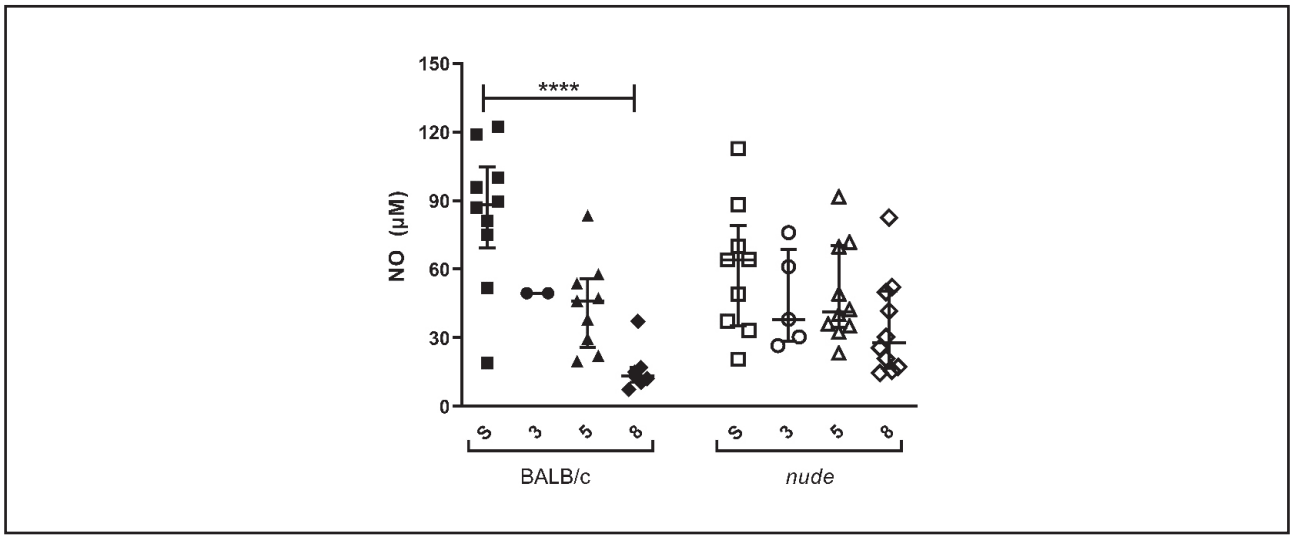
NO levels in BALB/c mice were higher in the control group compared to 8 months post-inoculation (Figure 5). There were no significant differences in nude mice during the course of infection (Figure 5).

Figure 5 – Serum nitric oxide (NO) levels in BALB/c and nude mice inoculated with *M. leprae* in the footpad and euthanized at 3, 5, and 8 months post-inoculation. H = healthy mice. ****$p < 0.0001$.



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DISCUSSION

The infectious process in the nude mice was progressive and intense, with the multiplication of the bacilli in the footpads. In contrast, the infection reached a plateau of bacillary multiplication at five months in the BALB/c mice, followed by regression at eight months. The earlier evolution of the infectious process in the BALB/c animals in comparison with Shepard's findings may be attributed to the viability of the bacilli present in the inoculum, as they originated from nude mice (used to maintain the Thai-53 strain) and not from lesions of untreated multibacillary patients, as described by Shepard[4].

Because *M. leprae* is an obligate intracellular parasite of Schwann cells and Schwann cells and macrophages, host resistance to this pathogen depends on the cellular immune response, with the Th1/Th2 paradigm being classic in leprosy[18].

Serum cytokine levels in BALB/c mice revealed higher levels of IL-2 and IL-17 at eight months compared to the control group. In particular, concerning IL-17, high levels of this cytokine were observed in the tuberculoid pole, which contributes to the recruitment of inflammatory cells that activate endothelial cells, thereby promoting the maintenance of the chronic inflammatory process. It has been demonstrated that the Th17 response plays a pivotal role in regulating macrophage activity, as this cytokine can stimulate the production of TNF and iNOS, ultimately leading to the destruction of the bacilli[19,20].

In BALB/c mice, TNF and IFN-γ serum levels were higher at eight months, although no statistically significant difference was identified. This suggests

a possible Th1 profile consistent with TT patient observations. Furthermore, increased levels of these cytokines were also observed in peritoneal cell culture supernatant from BALB/c mice inoculated with *M. leprae* after eight months, which corroborates our findings[14].

Concerning IL-6, healthy BALB/c mice (control group) exhibited higher levels of this cytokine compared to 8 months of infection. This cytokine is known to have a pro-inflammatory function and is detected mainly in LL patients[22]. It is possible that this decrease in IL-6 production in mice is due to the greater expression of Th1 cytokines during the infection process.

In the nude mice, the results showed higher levels of TNF at eight months compared to the control group. In light of these findings, we can suggest that the production of this cytokine is related to the high bacillary load at eight months of infection since, in nude mice, there is the dissemination of *M. leprae* to sites other than the footpad[12,22]. Consequently, the lipopolysaccharides present in the bacillus cell wall may directly stimulate TNF production despite the absence of a cellular immune response, given that these animals are athymic[21]. Regarding the other cytokines, there were no significant differences over the course of the infection, although higher levels of IL-17 and IFN-γ were observed at 5 and 8 months compared to the control group.

It is important to point out that the cytokine levels found in the serum may not reflect the changes found in the footpad, and additional in situ studies are needed to better assess the immune response of these experimental models.

It is well established that cytokines produced during the immune response can activate or inhibit macrophages' microbicidal action. When the macrophage is activated, many processes are initiated, including NO production, which is essential in controlling infections due to its microbicidal action[12].

Macrophages have microbicidal activity, attributed to the production of reactive oxygen and nitrogen species by the NADPH-oxidase complex and iNOS, respectively. A higher expression of iNOS has been described in skin lesions of TT/BT patients compared to LL/BL lesions, which can be attributed to the Th1-type immune response[23].

Serum NO levels were higher in BALB/c mice in the control group than at eight months post-inoculation. This observation was noteworthy given that higher NO production would be expected during the infectious process since these animals exhibit a cellular immune response against *M. leprae*. A decrease in NO can also be observed in other infectious disease models. For instance, Anstead et al.[24] observed lower NO production in mice inoculated with *Leishmania*

donovani than in healthy mice. Similarly, Barbosa et al.[25] observed decreased NO production in mice inoculated with the fungus *Lacazia loboi*.

In contrast to BALB/c mice, nude mice exhibited no statistically significant difference in NO levels throughout the infection. This could indicate the high TNF production that accompanied the growth in the number of bacilli in an attempt to contain the infection, even in the absence of a thymus-dependent response.

In conclusion, BALB/c mice exhibited elevated serum levels of IL-2 and IL-17 at eight months post-inoculation. In contrast, nude mice increased serum TNF levels at this period. However, due to their athymic nature, these animals cannot eliminate *M. leprae* and have a high bacillary load. The results indicate that BALB/c mice exhibited an immune response capable of controlling the multiplication of *M. leprae*, in contrast to the progressive infection observed in nude mice, despite a systemic pro-inflammatory environment mediated by high TNF production.

ETHICAL APPROVAL AND INFORMED CONSENT: *the Ethics Committee for the Use of Animals of the Lauro de Souza Lima Institute approved the study, registered under CEUA number ILSL 001/23. The study was conducted following the Ethical Principles in Animal Experimentation, drawn up by the Brazilian Society for Science on Laboratory Animals (BSSLA).*

CONFLICTS OF INTEREST: *the authors have no conflicts of interest to declare.*

AUTHORS' CONTRIBUTIONS: Lima TS *and* ***Sartori BGC*** *contributed to the study's conception and design.* ***Diorio SM****,* ***Nogueira MRS****, and* ***Silva SMUR*** *contributed to the analysis and interpretation of the results.* ***Rosa PS****,* ***Pedrini SCB****, and* ***Brito-de-Souza VN*** *contributed to the drafting and critical revision of the manuscript.* ***Barbosa ASAA*** *and* ***Vilani-Moreno FR*** *contributed to the study's conception, design, analysis, interpretation of the results, drafting, and critical revision of the manuscript. All authors critically reviewed the manuscript.*

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