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HOST RESPONSE AND INFLAMMATION

Genetic Background Affects the Mucosal Secretory IgA Levels, Parasite Burden, Lung Inflammation, and Mouse Susceptibility to *Ascaris suum* Infection

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ABSTRACT Ascariasis is a neglected tropical disease that is widespread in the world and has important socioeconomic impacts. The presence of various stages of worm development in the pulmonary and intestinal mucosae induces a humoral and cellular immune response. However, although there is much evidence of the protective role of mucosal immunity against various pathogens, including helminths, there is still a gap in the knowledge about the immune response and the mechanisms of action that are involved in protection against diseases, especially in the initial phase of ascariasis. Thus, the aim of this study was to evaluate the kinetic aspects of the immune parasitological parameters in intestinal and pulmonary mucosae in male mice with early ascariasis. Therefore, two mouse strains that showed different susceptibilities to ascariasis (BALB/c and C57BL/6J) when experimentally infected with 2,500 infective eggs of *Ascaris suum* from time point 0 were examined: the immune parasitological parameters were evaluated each 2 days after infection over a period of 12 days. The results were suggestive of a synergetic action of intestinal and pulmonary secretory IgA (S-IgA) contributing to protection against early ascariasis by reducing the amount of migrating larvae as well as the influx of leukocytes in the lung and the consequent impairment of pulmonary capacity.

KEYWORDS mucosal immunity, helminth, experimental ascariasis, pulmonary larval migration

During ascariasis, different phases of *Ascaris* spp. develop in the pulmonary and gastrointestinal mucosae, and their presence induces the mucosal immune response (1). However, despite this, there is a gap in knowledge about the immune response and the mechanisms of action that are involved in natural protection against infections, especially in the initial phase of ascariasis. It is known that protection against ascariasis is associated with soluble mediators and cells characteristic of the T helper 1 (Th1) cells, Th2, Th17, and regulator T cell (Treg) immune responses (2–4). In this context, different mouse strains show a diverse profile of immune responses and *Ascaris* resistance, in which mice of the most susceptible strain, C57BL/6J, are associated with a predominance of the Th1 immune response, while those of the most resistant strain, BALB/c, are associated with a Th2 response (5–7). It is interesting to highlight that

Editor De'Broski R. Herbert, University of Pennsylvania

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The authors declare no conflict of interest.

Received 7 November 2021
Accepted 11 November 2021
Accepted manuscript posted online 22 November 2021
Published

cytokines secreted in the Th2 immune profile, such as interleukin-5 (IL-5) and IL-4, have important role in regulating IgA expression, mainly the secretory IgA (S-IgA) on Peyer's patches (PP) and other mucosal sites (8–12). Furthermore, there is much evidence of the role of S-IgA in protecting the mucosal surface. For instance, swine IgA-positive cells have been associated with protection against ascariasis (13, 14), and the size of the Peyer's patch and the levels of total intestinal IgA have been related to *Trichinella* infection (15), reduction in fertility was observed when mucous IgA was added to adult worm cultures (16), and in the rat, the depletion of antigen-specific IgA reduced eosinophil antibody-dependent cellular cytotoxicity (ADCC) and was associated with impaired larval development (17). Likewise, reinforcing the protective role of IgA, there are many articles showing S-IgA associated with resistance against bacteria, protozoa, and helminths (16, 18–22). In addition, an S-IgA-mediated inflammatory response that included induction of neutrophil respiratory burst (23) and eosinophil degranulation (24) has been described as one of several protective mechanisms against pathogens. Therefore, the role of the mucosal immune response and its main immunoglobulin in protecting against ascariasis, as well as in helminthiasis in general, may be underestimated. Thus, the aim of this study was (i) to determine the kinetic aspects of immune parasitological parameters in the lung and intestine during experimental acute ascariasis in male mice and (ii) to assess whether total S-IgA may be associated with differences in male mouse strains' resistance to ascariasis. For this purpose, in this research, we compared the kinetic variation of the immune parasitological parameters in the intestinal lavage (INL) and bronchoalveolar lavage (BAL) fluids from C57BL/6J and BALB/c male mice during experimental infection by *Ascaris suum*. The result showed an association between higher concentrations of total S-IgA in INL fluid and lower parasite loads in the most resistant BALB/c mouse strain. On the other hand, in BAL fluid, the number of larvae was positively associated with increases in S-IgA production, regardless of the strain of mice. However, after the peak of larval migration in the lung, there was an abrupt reduction in total parasitism coinciding with the peak of S-IgA production. In summary, the data from the kinetic evaluation presented here are suggestive of a synergetic action of intestinal and pulmonary S-IgA contributing to protection against early ascariasis induced in an experimental murine model.

RESULTS

Kinetic patterns of S-IgA differed in intestinal and bronchoalveolar mucosal sites, and they were associated with resistance to larval ascariasis. To evaluate the kinetic variation of immune parasitological parameters in mucosal sites during larval migration in early experimental ascariasis, an experiment was performed for 12 days in which the variables were assessed every 2 days. The patterns of larval migration were similar in the two strains evaluated in this study, with a predominance of total larvae recovered from the liver, followed by the lung, and very few larvae recovered from the intestine (75 and 67%, 23 and 32%, and 2 and 1%, respectively, for BALB/c and C57BL/6J mice). In the liver, peak larval migration occurred at 2 to 4 days postinfection (dpi) in both mouse strains, with almost 100% of larvae recovered in this organ. On day 2, the result for BALB/c mice was 98% (confidence interval [CI], 97 to 99%), and the result for C57BL/6J mice was 97% (CI, 97 to 98%). On day 4, the result for BALB/c mice was 96% (CI, 96 to 97%), and the result for C57BL/6J mice was 94% (CI, 91 to 96%). A significant strain versus time interaction ($S \times T$) was observed ($S \times T$, $F_{6,80} = 2.764$, $P = 0.017$), as only at 2 dpi was it possible to note statistical difference between mouse strains, with the highest value observed for C57BL/6J mice (Fig. 1A). Peaks of larval migration in the lung were observed at 6 to 8 dpi in both mouse strains. Moreover, it was possible to observe higher means of retrieved larvae from the lung parenchyma of C57BL/6J mice ($S \times T$, $F_{6,81} = 5.742$, $P = 0.000$) (Fig. 1B). It is interesting to note that, in the bronchoalveolar lavage compartment, the numbers of larvae did not differ significantly between the two strains (main effect of time [T], $F_{6,81} = 25.911$, $P = 0.000$) (Fig. 1C). In the intestine, the highest means of recovered larvae were observed at times 6 to 10 dpi and 6 to 8 dpi in strains BALB/c and C57BL/6J, respectively. Differences between the parasite

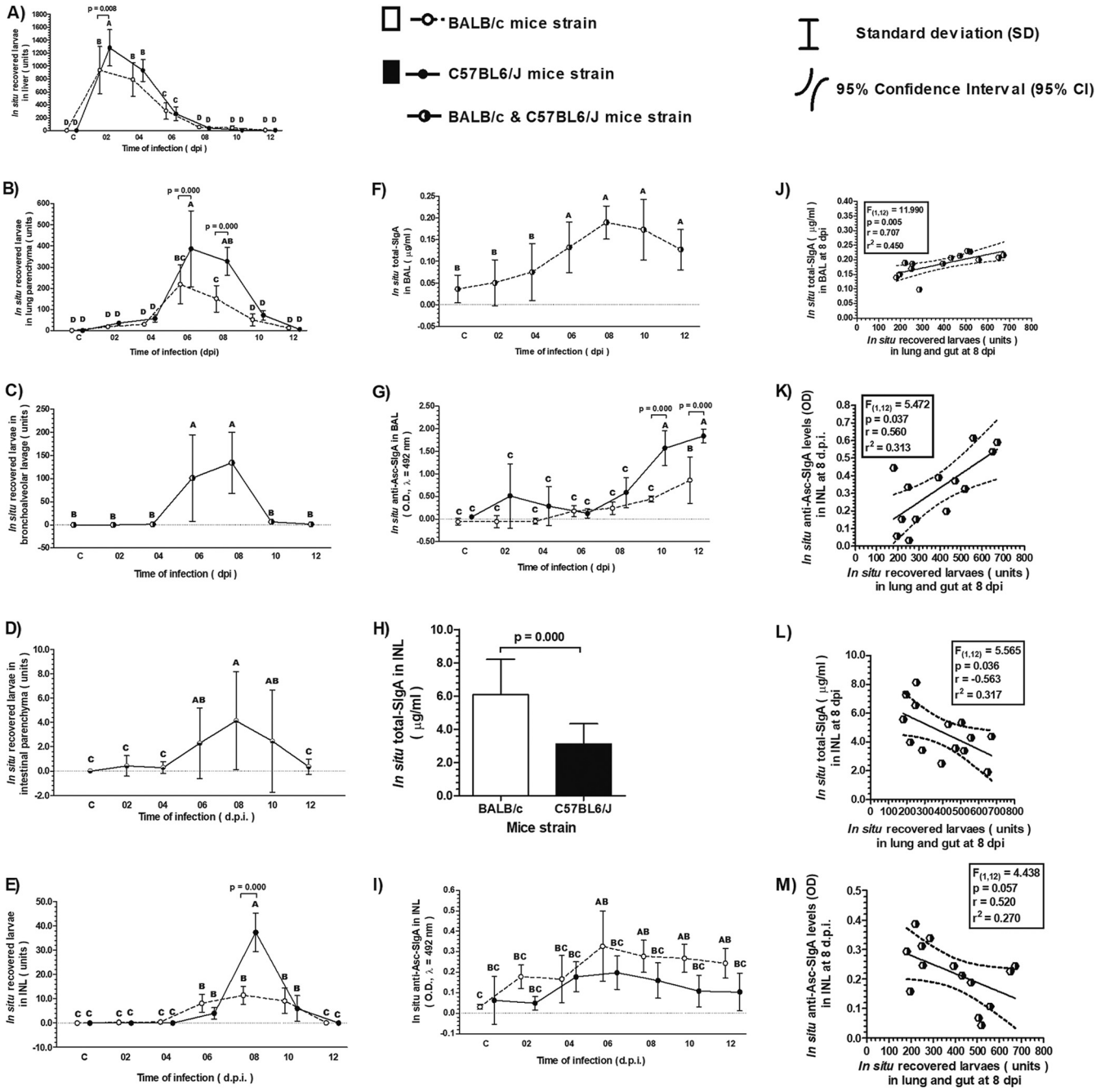

FIG 1 Kinetic evaluation of immune parasitological parameters during experimental ascariasis in mice. (A to E) Parasitic load in liver (A), lung parenchyma (B), BAL fluid (C), gut parenchyma (D), and INL fluid (E). (F to I) Levels of total S-IgA and specific S-IgA in BAL fluid (F and G) and INL fluid (H and I), respectively. (J to L) Scatterplot graphics showing the positive relationship between total S-IgA (J) and specific S-IgA (K) levels in BAL fluid, as well as the negative relationship between total total S-IgA (L) levels from INL versus parasitic load recovered from lung observed at 8 dpi. No significant differences were observed between specific S-IgA (M) levels from INL fluid versus parasitic load recovered from lung at 8 dpi. Two-way ANOVA followed by Tukey's multiple-comparison test was used to evaluate differences between the mean of the mouse strain versus infection time of groups in panels A to I, and Pearson's linear regression was used to evaluate the relationship between variables in panels J to M. Different letters on the means indicate statistically significant differences ($P < 0.05$), and the same letters denote nonsignificant differences in the kinetic comparison. Differences between strains are highlighted in the graphs with the respective *P* value. Asc, *Ascaris*; BAL, bronchoalveolar lavage fluid; C, control or noninfected group; HSD, honestly significant difference; INL, intestinal lavage fluid; *S*, mouse strain; *T*, time point of the kinetic evaluation.

loads of mice were detected only at the 8-dpi time point, with the highest parasite load observed in the intestinal parenchyma ($S \times T$, $F_{6,\ 80} = 5.566$, $P = 0.000$) of BALB/c mice and in the intestinal lavage fluid ($S \times T$, $F_{6,\ 80} = 33.363$, $P = 0.000$) of C57BL/6J mice (Fig. 1D and E).

Regarding the humoral response in mucosal sites, a different kinetic pattern was detected for total S-IgA measured in pulmonary (BAL fluid) and intestinal (INL) sites, since in lung, the statistically relevant differences were detected only in relation to time of infection, while in gut, significant difference was identified only in relation to mouse lineage (Fig. 1F to I). Thus, increases in total S-IgA levels in BAL fluid were identified from 6 dpi (peak of larval migration in the lung) in both mouse strains (T, $F_{6, 81}$ = 19.117, P = 0.000) (Fig. 1F). Relevant increases in specific anti-*Ascaris* S-IgA against crude extract of adult worms in the BAL fluid compartment were noted at the end of larval migration (12 dpi in BALB/c and10 to 12 dpi in C57BL/6J), with higher levels occurring in C57BL/6J mice ($S \times T$, $F_{6, 79}$ = 6.152, P = 0.000) (Fig. 1G). However, there was not any statistical difference in the levels of total S-IgA in INL fluid during kinetic evaluations in both strains of mice, with BALB/c mice showing higher levels of this immunoglobulin at all time points compared to C57BL/6J mice (S, $F_{6, 81}$ = 70.447, P = 0.000) (Fig. 1H). However, no differences were identified between the levels of specific anti-*Ascaris* S-IgA against the crude extract of adult worms from both strains of mice nor between the times of infection evaluated, except for the control of BALB/c mice that differed from the time points 6 to 12 dpi ($S \times T$, $F_{6, 81}$ = 2.384, P = 0.036) (Fig. 1I).

Reinforcing the observed patterns, at 8 dpi, a relationship between total S-IgA levels and parasite load was observed, with a positive trend for total and specific S-IgA in BAL fluid (Fig. 1J and K), as well as a negative trend for total S-IgA in INL fluid (Fig. 1L), although there was no relationship (P = 0.057) between specific S-IgA and parasitic load (Fig. 1M).

Intestinal S-IgA was associated with cellular immune response in BAL fluid as well as with pathological parameters in lung. Significant increases in the absolute number of total leukocytes and their subpopulation recovered from BAL fluid of both strains of mice were noted from 8 dpi, which corresponded to the peak of larval migration in the lung ($S \times T$, $F_{6, 81}$ = 6.278, P = 0.000) (Fig. 2A). Although higher absolute numbers of these immune cells were observed toward the end of the experimental infection period (10 to 12 dpi), there were some differences in the kinetic variation of these cell subpopulations, mainly when the relative contribution of each subpopulation was analyzed. In both strains, from the peak of larval migration in the lung, significant increases in the absolute number of macrophages in the BAL fluid were observed ($S \times T$, $F_{6, 81}$ = 7.662, P = 0.000) (Fig. 2B). Furthermore, only on the 12th dpi were significant differences between mouse strains detected, with higher values occurring in the C57BL/6J strain compared to BALB/c ($S \times T$, $F_{6, 81}$ = 16.753, P = 0.000) (Fig. 2F). However, analyses of their relative proportion showed that in controls and at the beginning of the experimental infection, macrophages represented more than 90% of the cells, being greater in C57BL/6J mice on days 6 and 10 dpi than in the BALB/c strain. Reductions in this proportion occurred from 6 dpi in C57BL/6J and 8 dpi in BALB/c mice (Fig. 2F).

Increases in the absolute number of lymphocytes also occurred from 6 dpi, with the BALB/c lineage showing a higher number than the C57BL/6J lineage at 12 dpi ($S \times T$, $F_{6, 81}$ = 8.137, P = 0.000) (Fig. 2C). Although, relative numbers of this subpopulation also showed an increase from 6 dpi, the proportion of lymphocytes were higher in the BALB/c lineage (T, $F_{6, 81}$ = 24.830, P = 0.000; S, $F_{6, 81}$ = 9.555, P = 0.027) (Fig. 2G and K). Eosinophils showed increases in absolute ($S \times T$, $F_{6, 81}$ = 4.785, P = 0.003) and relative values from the peak of migration, with higher values occurring in C57BL/6J on the 12th dpi ($S \times T$, $F_{6, 81}$ = 7.372, P = 0.000) (Fig. 2D, H, and L). Absolute values of macrophages and eosinophils in the BAL fluid at the end of the experimental infection (12 dpi) correlated positively with higher levels of total and specific S-IgA against the parasite, respectively (Fig. 2J and L). It was also verified that the kinetic variation of neutrophils contrasted with the general pattern observed for macrophages, lymphocytes, and eosinophils. In both mouse strains, the peak of neutrophils recovered, in absolute values (T, F6, 81 = 57.385, P = 0.000) and in relative proportion ($S \times T$, $F_{6, 81}$ = 19.220, P = 0.000) of this subpopulation, occurred at the 8th dpi, decreasing afterwards (Fig. 2E and I). Although significant difference between the strains was observed only in the

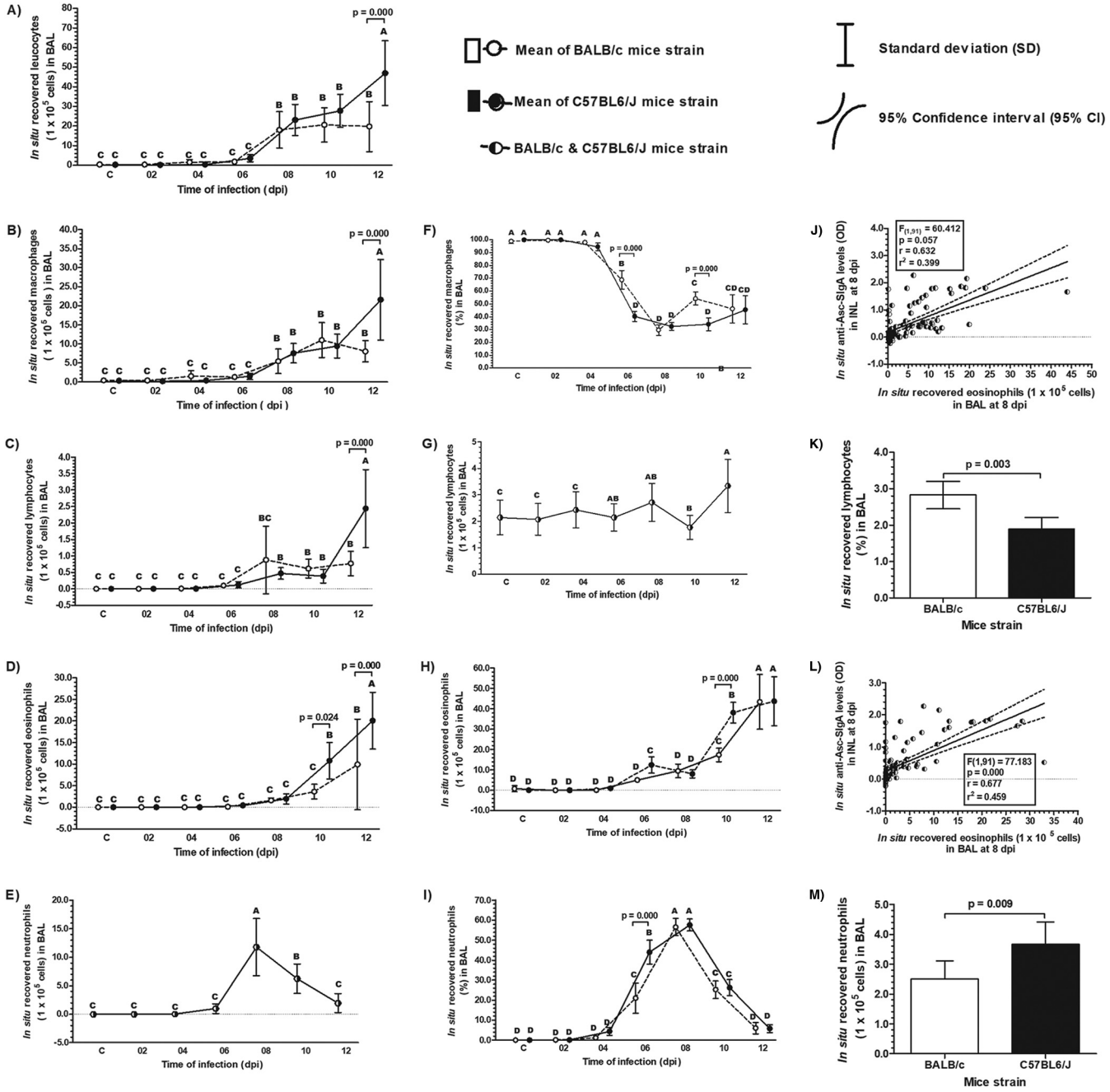

FIG 2 Kinetic evaluation of immune cells recovered from BAL fluid during experimental ascariasis in mice. Shown are the absolute number of total leucocytes (A), as well as absolute number and relative percentage of their subpopulations' macrophages (B and F), lymphocytes (C, G, and K), eosinophils (D and H), and neutrophils (E, I, and M), respectively. (J and L) Scatterplot graphics showing positive relationship between total S-IgA and specific S-IgA levels in BAL fluid versus macrophages (J) and eosinophils (L) recovered in BAL fluid, both measured at 12 dpi. Two-way ANOVA followed by Tukeý́s multiple-comparison test was used to evaluate differences between mean of mouse strain versus infection time of groups in panels A to I, K, and M. Pearson's linear regression was used to evaluate the relationship between variables in panels J and L. Different letters on the means indicate statistically significant differences ($P < 0.05$), and the same letters denote nonsignificant differences in the kinetic comparison. Differences between strains are highlighted in the graphs with the respective *P* value. Asc, *Ascaris*; BAL, bronchoalveolar lavage fluid; C, control or noninfected group; HSD, honestly significant difference; INL, intestinal lavage fluid; *S*, mouse strain; *T*, time point of the kinetic evaluation.

relative contribution of this subpopulation in the 6th dpi (Fig. 2I), it was possible to observe that the absolute number of neutrophils recovered from the BAL fluid of C57BL/6J was higher than that in strain BALB/c (S, $F_{6, 81} = 7.074$, $P = 0.000$) (Fig. 2M).

In the analyses of pathophysiological parameters, in both mouse strains, there was a tendency for the hemoglobin and total protein values to increase in the BAL fluid

coincidently with or shortly after the peak of larval migration. However, only in the BALB/c mice were there significant increases ($P < 0,05$) in hemoglobin values at 6 and 8 dpi (~2.4 times), as well as in total protein levels at 6 and 12 dpi, compared to initial levels. In addition, comparing the mouse strains, it was noted that hemoglobin ($S \times T$, $F_{6, 81} = 2.949$, $P = 0.012$) and total protein ($S \times T$, $F_{6, 81} = 4.708$, $P = 0.012$) values were higher in BALB/c than in C57BL/6J only at 6 to 10 dpi, respectively (Fig. 3A and B).

Finally, it was possible to note that mice in the infected group (SI) had a tendency in reduce the pulmonary functionality independent of mouse strain compared to mice in the not-infected (NI) group. This tendency was evidenced by the higher pulmonary resistance (T, $F_{1, 40} = 23.381$, $P = 0.000$), as well as lower compliance and functional capacity evaluated by the parameters chord compliance (Cchord) (T, $F_{1, 43} = 7.856$, $P = 0.008$; S, 18.955, $P = 0.000$), dynamic compliance (Cdyn) (T, $F_{1, 43} = 19.174$, $P = 0.008$; S, 18.955, $P = 0.000$), functional vital capacity (FVC) (T, $F_{1, 44} = 7.934$, $P = 0.007$), and forced expiratory volume at 100 ms (FEV100) (T, $F_{1, 40} = 8.302$, $P = 0.007$) (Fig. 3C to G). Moreover, the most susceptible mice, strain C57BL/6J, had lower compliance and functional capacity (Fig. 3C to G). In addition, the intestinal total S-IgA levels at the end of experimental infection (12 dpi) were positively associated with markers of preserved pulmonary function (Fig. 3H to K).

DISCUSSION

The murine experimental infection with *A. suum* performed in this study was consistent with those described in the literature with respect to infection time, organs affected by larval migration, and variation of infection intensity over time (4, 25–27). These results justify the use of this animal model to evaluate mucosal immunity. In addition, the comparison of the parasite burdens in the C57BL/6J and BALB/c strains also corroborated data already published in which the difference between the loads was not constant over time, being observed only in the peaks of hepatic-pulmonary migration (26). The results of the immune parasitological evaluation in intestinal mucosa were suggestive of the innate immune response impairing the infection progress and culminating in a lower associated pathology in the more resistant BALB/c mouse strain. A possible hypothesis is that the presence of innate S-IgA in the intestinal infection site would be a natural protection factor reducing the number of migrant larvae and, consequently, the hepatic-pulmonary pathologies associated with the development of early ascariasis.

The hypothesis of intestinal S-IgA as an innate protector factor is suggested by the higher concentrations of total S-IgA in the INL fluid regardless of the infection time and lower recovery of larvae in the lung of the BALB/c mice, allied to the existence of negative correlations between the concentration of this antibody and parasite burden and inflammation in lung, as well as positive associations with variables that measure pulmonary function. It is likely that this total S-IgA corresponds to polyreactive S-IgA (28–31), an important effector mechanism of gastrointestinal lymphoid tissue (GALT), which is constitutively active since fetal life (32). Recently, it was demonstrated that BALB/c mice show higher abundance and diversity of intestinal polyreactive S-IgA than C57BL/6J mice, and these characteristics in BALB/c mice were associated with bacterial protection (28). In this way, the intestinal S-IgA could contribute to reducing the number of larvae that reach the lung. Consequently, the small amount of cell infiltration and less inflammatory conditions would result in lower morbidity and better functional tissue capacity, as was found in our data.

In this context, the existence of a natural intestinal protection could explain epidemiologic studies showing a population pattern observed in areas of endemicity in which the majority of hosts have low parasite burden while a small group of individuals present the highest infection loads (33–39) and are more susceptible to becoming reinfected after chemotherapy (40).

Furthermore, the results of epidemiological studies also had reported multiple helminth infections as a common situation in areas of endemicity (41–46), which is

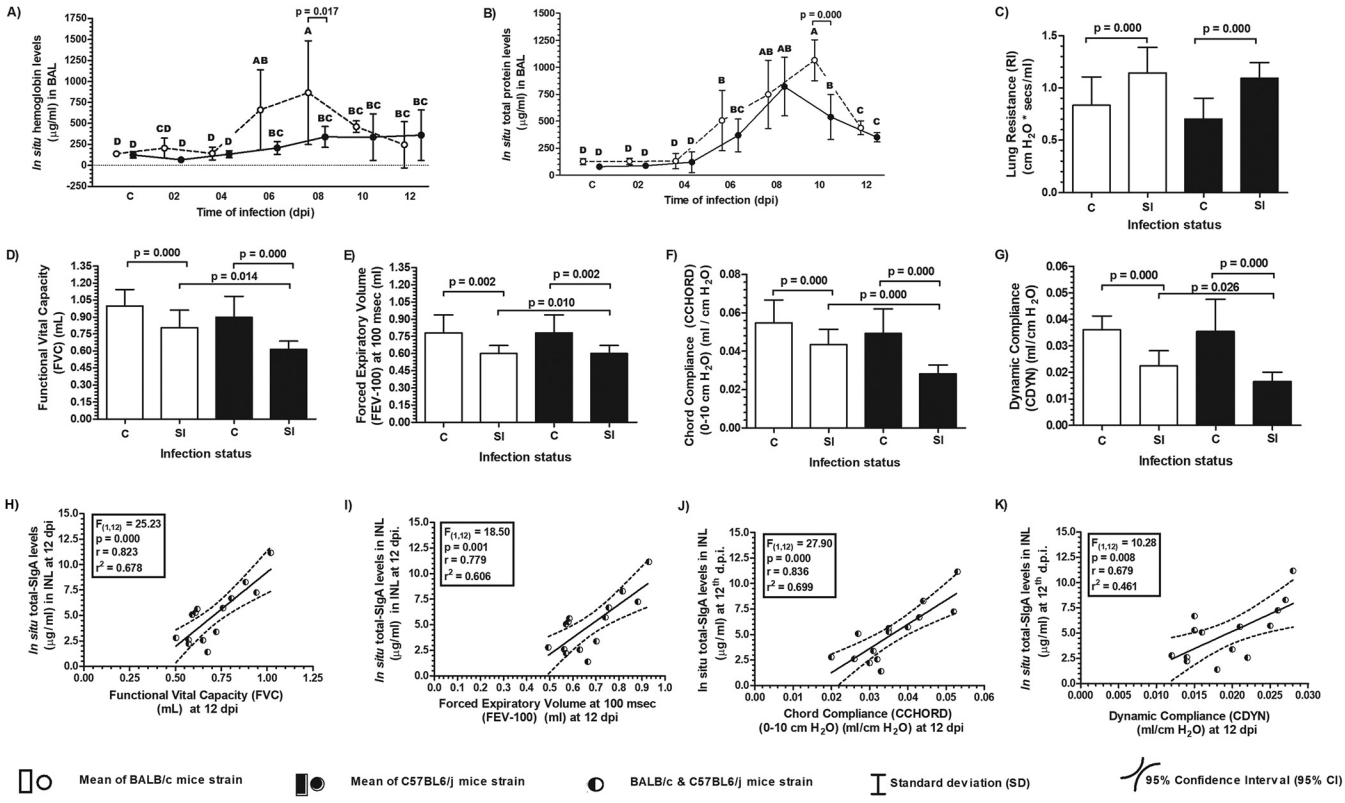

FIG 3 Impact of larval migration pathophysiological parameters in lung during ascariasis in mice. Shown is kinetic variation of hemoglobin (A) and total protein (B) levels in BAL fluid. Pulmonary functional capacity evaluation in the presence and absence of infection: pulmonary resistance (C), functional vital capacity (D), forced expiratory volume at 100 ms (E), chord compliance (F), and dynamic compliance (G). Two-way ANOVA followed by Tukey's multiple-comparison test was used to evaluate differences between the mean of mouse strain (MS) versus infection time (IT) or infection status (IS) of groups in panels A and B and C to G, respectively. Pearson's linear regression was used to evaluate the relationship between variables in panels H to K. Different letters on the means indicate statistically significant differences ($P < 0.05$), and the same letters denote nonsignificant differences in the kinetic comparison. Differences between strains are highlighted in the graphs with the respective *P* value. Asc, *Ascaris*; BAL, bronchoalveolar lavage fluid; C, control or noninfected group; HSD, honestly significant difference; INL, intestinal lavage fluid; *S*, mouse strain; *T*, time point of the kinetic evaluation.

suggestive of synergistic association, as can occur in coinfections with *Ascaris* sp. and hookworms, wherein greater parasite burdens are found than in monoinfections (47). This synergism may be explained by the existence of IgA proteases in extracted-secreted (ES) products from hookworms (48), which are considered virulence factors of various pathogens (20, 49–52). Studies need to be designed to check the hypothesis of intestinal S-IgA acting as a protector factor in areas of endemicity.

Although total S-IgA may be acting as a natural intestinal protector factor, the kinetic study demonstrated that some migrating larvae escape from the gastrointestinal tract (gut and liver) and reach the lung compartments at the same time in both mouse strains, but with C57BL/6J accumulating the highest parasite loading in this organ. Thus, the accumulation of these larvae in the lung parenchyma and in the BAL fluid induces the influx of leukocytes into the lung, which could contribute to an increase in total and local specific pulmonary S-IgA from the 8th dpi.

The effect of infection time on the increase in the levels of total and specific S-IgA, as well as on the counting of leukocyte subtypes in the BAL fluid compartment, is consistent with the biology of the bronchoalveolar lymphoid tissue (BALT), because in mice and humans, this mucosal immune site is not constitutive, but inducible (iBALT) only in the presence of infection (53–56). Moreover, the observation of a coincident reduction of approximately 75% of the total parasite burden after the peak of larval migration suggests that the pulmonary immune response represents another important step for resistance against larval ascariasis. In this context, a high number of IgA-positive cells in the bronchial and peribronchial regions and in the intestinal lamina propria (LP) of swine resistant to ascariasis had already been reported, reinforcing the

hypothesis of mucosal IgA involved in the protection against migrant larvae (13, 14). Furthermore, it was demonstrated that eosinophils mediate local S-IgA production triggered by Toll-like receptor 2 (TLR2) and TLR4, and these events were associated with the control of *Ascaris suum* infection in mice (71).

Acute inflammation with the release of several mediators and cytokines that result in cellular infiltrates (mainly neutrophils and eosinophils) and subepithelial fibrosis are immunopathological processes associated with larval migration in ascariasis (2, 4, 27). As described previously (26), in this kinetic evaluation, the most resistant mouse strain (BALB/c) showed a lower BAL fluid cellularity than susceptible strains, with the main differences observed in absolute and relative proportions of lymphocytes and neutrophils, In addition, a higher mean leukocyte count in BAL fluid was observed, as well as a lower mean of compliance measures (Cdyn and Cchord) in the most susceptible mouse strain (C57BL/6J), as can be seen in Fig. 2 and 3. Corroborating these findings, multiple exposures to ascariasis affect lung function due to lung lesions and the inflammatory immune response induced by larval migration (2). Variations in elastic resistance (caused, for example, by the fibrosis process) have repercussions on compliance measures (Cdyn and Cchord), as they compromise the pulmonary expansion and retraction process (57). In addition, excess secretions and obstructions of the airways (edemas and inflammatory processes) can also alter measures of resistance and compliance, as they lead to an increase in the peak of pressure (57).

Together, the data of kinetic evaluation reported here delineate a suggestive protective role for mucosal S-IgA. In this setting, as larvae migrate to the pulmonary parenchyma, iBALT induction occurs, inducing a consequent cellular and humoral immune response that contributes to the elimination of infection. Thus, in BALB/c mice, the synergism between higher intestinal total S-IgA and a fast local pulmonary immune response impairs the larval accumulation in lung, resolving the infection with a lower influx of leukocyte cells and lung pathologies associated with inflammation. Nevertheless, in C57BL/6J mice, the higher accumulation of larvae in lung during the peak of migration results in expressive influx of leukocyte cells and consequent lung pathologies associated with inflammation, which have a more intense impact on pulmonary capacity observed in this mouse strain. In relation to S-IgA as protector factor, it was reported by Fransen et al. (28) that the highest densities and diversity of intestinal polyreactive S-IgA in BALB/c mouse strain were associated with protection against microbial infection.

A range of protective mechanisms associated with antibodies have been described, and those may be related to the protective function of local S-IgA against helminths. Among them are potent eosinophil degranulation induced by S-IgA (17, 58), entrapment of helminth larvae in mucus (59, 60), opsonization mediated by alternative activation of the complement cascade (61), and neutralization of proteins excreted/secreted by the worm (62).

Another difference between the BALB/c and C57BL/6J mouse strains are the cytokine profiles. There are suggestive data that C57BL/6 mice produce a Th1 profile, while BALB/c mice produce a Th2 profile under physiological and pathological conditions (5, 6, 63–66). In a kinetic evaluation of early ascariasis in BALB/c mice, a polarized proinflammatory response was observed, with cytokines peaking immediately before (IL-5 at 4 dpi), during (IL-6 at 8 dpi), or at the end (tumor necrosis factor [TNF] at 12 dpi) of larval migration (4). We did not find data from kinetic aspects of cellular response induced by ascariasis for C57BL/6J mice in the literature. As evaluation of the cytokine profile was not the focus of our study, unfortunately, it was not possible to evaluate this aspect of immune response due to insufficient amounts of mucosal lavage fluids and immune cells. Another limitation on this study is that female mice were not included, and it is known that they show a more robust immune response than males (64–67). However, as the difference between strains in relation to Th1/Th2 profiles happens in both sexes (5, 6, 63–67), it is possible that the findings of this study can also be applied to female mice.

Therefore, further investigations of mucosal immunity in early ascariasis need to be performed to confirm the role of S-IgA in murine experimental ascariasis and the possible existence of a synergistic action between intestinal and pulmonary S-IgAs as the key to the resolution of the infection, as well as to determine the mechanisms involved in this process.

Conclusion. The data set from this kinetic study highlighted the importance of the time points from 6 to 10 dpi in early murine ascariasis, since at these time points occur the peaks of larval migration, as well as the main events of local immune response in lung. Also, although there was no difference in kinetic variation of intestinal S-IgA, only at the time point 8 dpi was it possible to identify an association between the levels of this local antibody with immune parasitological parameters in lung (the main organ infected during the loss cycle). Furthermore, at these time points there were evident immune parasitological differences between mouse strains. The data reported here are also indicative that the increased susceptibility of the C57BL/6J mouse strain may be caused by a greater accumulation of larvae in the lung during hepatic-pulmonary migration. In addition, this accumulation is likely associated with a lower production of natural and acquired intestinal S-IgA that results in a greater number of larvae evading the gastrointestinal tract, as well as a slow pulmonary immune response.

In summary, the data are consistent with a protective role of mucosal S-IgA against experimental murine ascariasis, probably through a synergistic action between intestinal and pulmonary S-IgAs that contributes to a quick and efficient resolution of early ascariasis with the lowest possible morbidity.

MATERIALS AND METHODS

Ethics statement. All procedures used for this study was approved by the Ethics Committee for Animal Experimentation (CETEA) of the Minas Gerais Federal University (UFMG), Brazil (protocol no. 54-2012 and 187/2014). All efforts were made to minimize animal suffering, and mice were maintained and used in strict accordance with the recommendations of the guidelines of the Brazilian College of Animal Experimentation (COBEA).

Experimental design. With the aim of minimizing interference of confounding factors, such as sex and age, in the immune parasitological and pathophysiological parameters, in this study only male mice (*Mus musculus*) were used. The mice were 6 to 8 weeks old and were obtained from the Central Animal Facility from the Universidade Federal de Minas Gerais (UFMG), Brazil, and housed in the Animal Facility at the Department of Parasitology, UFMG. The choice of male instead of female mice was made in order to permit us compare our results with existing literature about studies of early murine ascariasis that have been performed with male mice (4, 25–27).

This study was designed using a bifactorial arrangement in order to evaluate a kinetic analysis of immunoparasitological and pathophysiological parameters in two mouse strains presenting different susceptibilities for larval ascariasis (the most resistant strain, BALB/c, and the most susceptible strain, C57BL/6J). Thus, a total of 104 animals (52 animals from each strain) were randomly divided into two groups: the control group (20 noninfected mice [NI]) and the experimental group (84 mice with single infection [SI]). On day 0 of the kinetic experiment, all animals from the NI or SI groups received by gavage, respectively, 200 μL of phosphate-buffered saline (PBS) or suspension containing 2,500 fully embryonated *A. suum* eggs. Then, each 2 days for a period of 12 days after infection, 14 animals from the SI group (seven from each mouse strain) were randomly selected to be euthanized. In addition, 10 animals from the NI group (five from each mouse strain) were randomly selected to be submitted to be euthanized on the second day postinfection (2nd dpi) and on the 12th dpi, corresponding to the beginning and the end of the kinetic experiment, respectively.

Parasites and experimental infections. The *Ascaris suum* worms were maintained in Piau breed pigs at the Campus of the Faculty of Environment of Iguatama, Brazil. After chemical treatment of the animals with levamisole hydrochloride injection (Ripercol) and confirmation of negativity of fecal exams, infection was performed by gavage with approximated 1,000 embryonated eggs of *A. suum*. After the 15th day of infection, the presence of adult worms was verified daily by analysis of feces. Then, at the end of the prepatent period, animals were euthanized under general anesthesia followed by bleeding and necropsy. The intestine was opened with scissors, and the parasites were recovered and placed in properly labeled flasks containing PBS (pH 7.4). Eggs were isolated from uteri of female worms, purified by using of cell strainers (70-μm pore), and incubated with 0.2 M H_2SO_4 for embryonation, as described by Boes et al. (68). After the 100th day of culture (the peak of larval infectivity), embryonated eggs were obtained and used in new experimental infections (4).

Assessment of respiratory mechanics. At the beginning (2nd dpi) and end (12th dpi) of the kinetic experiment, pulmonary dysfunction was measured as we previously described (2). For invasive *in vivo* assessment, mice were anesthetized and tracheostomized, and then were placed in a whole-body plethysmograph connected to a computer-controlled ventilator (Forced Pulmonary Maneuver System, Buxco Research Systems, Wilmington, NC, USA) to maintain spontaneous breathing. Under mechanical

respiration, the dynamic compliance (Cdyn) and lung resistance (Rl) were determined by a resistance and compliance (RC) test. To measure the forced vital capacity (FVC), the pressure-volume maneuver was performed, which inflates the lungs to a standard pressure of +30 cm H_2O and then slowly lets the lungs exhale until a negative pressure of −30 cm H_2O is reached. To measure the forced expiratory volume (FEV) and flow-volume curve, fast-flow volume maneuvers were performed, and the flow-volume curve was recorded during these maneuvers.

BAL fluid. In order to assess the mucosal bronchoalveolar compartment and obtain the BAL fluid, a catheter was introduced into the trachea of the euthanized animals and a procedure involving two perfusions and aspirations of 1.0 mL of PBS was performed. Then, to recover the *A. suum* larvae present in the BAL fluid, it was filtered on 70-μm-pore cell strainers (BD, USA), and the recovered larvae were fixed with 2.0 mL of PBS containing 10% formaldehyde for posterior counting. To recover cells for determination of the total number of leukocytes and their subpopulations in BAL fluid, the filtered liquid was centrifuged at 3,000 $\times$ *g* for 10 min, and the pellet was used for cell analysis in BAL fluid. The supernatant was used to quantify the amount of total S-IgA, total protein, and hemoglobin content.

INL fluid. The intestinal lavage (INL) fluid was obtained by collection of the entire intestine, which was injected with 10 mL of PBS. The lavage fluid was recovered, and the mucus was totally removed by squeezing the intestine with tweezers. The mixture of lavage fluid and mucus was submitted to centrifugation (3,000 $\times$ *g* for 10 min), and the supernatant was used to quantify the content of total S-IgA and specific S-IgA. To recover the *A. suum* larvae present in the INL fluid, sediment was fixed with 2 mL of PBS containing 10% formaldehyde for counting of posterior larvae.

Recovery of larvae from parenchyma. The parasite burden was evaluated in lung and intestine parenchyma using a modified Baermann method (4). Briefly, after collection of mucosal lavage specimens (BAL fluid and INL fluid), the lung and intestine were collected, sliced with scissors, and placed in a modified Baermann apparatus for 4 h in the presence of PBS at 37°C. The living larvae recovered from parenchyma were then fixed with 2.0 mL of PBS containing 10% formaldehyde for counting of posterior larvae.

Parasite burden determination. All larvae recovered from mucosal lavage (BAL fluid and INL fluid) and parenchyma (lung and intestine) were counted under a microscope using a 10$\times$ objective. The result was expressed as number of larvae recovered.

Total protein and hemoglobin measurement. Possible protein leakage into the airways was evaluated in the supernatant of BAL fluid by the quantification of total protein using the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, USA) as previously described (69). The alveolar hemorrhage was carried out by measuring concentrations of hemoglobin (Hb) using the Drabkin method, as previously described (70). For both parameters, the measurements were interpolated into a respective standard curve, and the values were expressed as μg/mL of total protein or Hb.

Cell analysis in BAL fluid. The global leukocyte numbers were assessed in the BAL fluid sediment with a hemocytometer. The cell number was obtained, and the volume of cell suspension was adjusted to 500 cells/mL. Then, BAL fluid slides were prepared using a Cytospin model II (Shandon, Pittsburgh, PA) and then stained with Wright-Giemsa. Leukocyte differentials were measured on a count of 300 cells.

Immunoenzymatic assay for evaluation of total and *Ascaris*-specific S-IgA. To determine the levels of S-IgA, polystyrene microplates (Nunc, Roskilde, Denmark) were sensitized overnight at 4°C with 0.5 μg/mL goat anti-mouse Ig, human ads-UNLB (Southern Biotechnology, USA, catalog no 1010-01) for total S-IgA or with 10 μg/mL of adult *Ascaris suum* crude extract for specific S-IgA. Subsequently, the plates were washed with PBS containing 0.05% Tween 20 (Sigma Chemical Co., USA). The blocking was performed at room temperature for 2 h with PBS plus 3% bovine serum albumin (BSA) (Fitzgerald Industries, USA). The samples (BAL fluid or INL fluid) were added to the plates and incubated overnight at 4°C. Subsequently, the plates were washed and incubated at 37°C for 1 h with biotinylated anti-IgA (4 μg/mL; Southern Biotechnology, USA, catalog no. 1040-08). After washing, the plates were incubated for 1 h with horseradish peroxidase (HRP)-streptavidin-conjugated solution (R&D Systems, USA). Then, after a new washing, the plates were incubated for 20 min with the substrate *o*-phenylenediamine (OPD [plus 30% H_2O_2]) (Sigma Chemical Co.). Finally, the enzymatic reaction was stopped with 2 N H_2SO_4, and the intensity of the reaction was determined at a wavelength of 492 nm in an automated reader (VersaMax tunable microplate reader; Molecular Devices). Specific S-IgA levels were expressed as optical density (OD). For total S-IgA levels, serial dilutions of mouse IgA standard (Southern Biotechnology) were included in each plate in order to calculate the concentration per milliliter of total S-IgA by interpolating the sample's OD in a standard curve fitted by an equation of five logistic parameters (5-PL). For quality control, positive and negative controls were included on all plates.

Statistical analysis. Outliers in the samples were detect by Grubb's test and removed from the statistical analysis. The existence of differences between the means was verified by the application of the multifactor variance (two-way analysis of variance [ANOVA]) test after analysis of normality of the distribution and homogeneity of the variances (Shapiro-Wilk and Levene tests, respectively). To evaluate the degree of association between the immune parasitological parameters (parasite burden, leukocyte counts, and total-S-IgA in BAL fluid) and pathological parameters (FVC, Rl, Cdyn, FEV100, Cchord, and total protein), determine the existence of dependence between the variables, and explain the variability found, it a simple linear regression and simple linear correlation tests (Pearson's linear regression) were used. The initial (2-dpi) and final (12-dpi) time points of the controls (NI) were compared with each other, and as there was no statistically significant difference detected in the analyses, the data from the two time points were considered replicates, and the mean was used for the comparisons against the times

of the kinetic evaluation of the infected groups. All tests were considered significant when the *P* value was ≤ 0.05.

ACKNOWLEDGMENTS

This work was funded by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). This investigation received financial support from Fundação de Amparo a Pesquisa do Estado de Minas Gerais/FAPEMIG, Brazil (grant no. CBB APQ-03280-15), the Brazilian National Research Council (CNPq) (grant no. 303345/2018-7), and Pró-Reitoria de Pesquisa of Universidade Federal de Minas Gerais. A.M.C.F., L.L.B., R.C.R., and R.T.F. are Research Fellows (Bolsa de Produtividade em Pesquisa) from CNPq. D.S.N. and L.M.O. are supported by a postdoctoral fellowship from CAPES.

D.S.N., L.M.O., S.G., and R.T.F. conceived and designed the experiments. D.S.N., L.M.O., R.M.G., F.S.B., C.C.O.A., A.C.G.-G., N.M.R., N.P-R., L.R.K., M.S.M., N.M.R., and R.C.R. performed the experiments. D.S.N., L.M.O., R.M.G., R.C.R., and R.T.F. analyzed the data. R.T.F., R.C.R., S.G., L.L.B., and A.M.C.F. contributed reagents, materials, and analysis tools. L.M.O., D.S.N., R.C.R., and R.T.F. wrote and reviewed the paper.

We declare no conflict of interest.

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