

Chronic exposure to cigarette smoke transiently worsens the disease course in a mouse model of pulmonary paracoccidioidomycosis

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ABSTRACT

Paracoccidioidomycosis (PCM) may present as an acute/subacute clinical form, characterized by a progressive disease arising from the airborne initial infection, or, most often, as an asymptomatic or subclinical infection that may manifest later during an individual's life, the chronic form. Epidemiological studies show the existence of a strong association between smoking and the development of the chronic form. Current evidence demonstrates that cigarette smoke (CS) has immunosuppressive properties that could be implicated in the increasing susceptibility to the chronic form of PCM. To address this issue, we developed a murine model of a non-progressive pulmonary form of PCM that was exposed to CS at a magnitude that mimicked a moderate smoker. The chronic CS exposure started after 2 weeks and lasted up until 20 weeks post-infection, with the aim of mimicking human natural history, since it is estimated that individuals from endemic areas are infected early in life. The control group consisted of infected but not CS-exposed mice. We assessed the lung fungal burden (colony forming units [CFU]) and the area affected by the granulomatous inflammatory response, fungal dissemination to spleen and liver, and, by immunohistochemistry, the presence of CD4 and CD8 lymphocytes, CD68 and MAC-2 macrophages, and IFN- γ , IL-10 and TNF expressing cells within the granulomatous response. We detected a CS effect as early as 2 weeks after exposure (four weeks post-infection) when the lung CFU of exposed animals was significantly higher than in their non-exposed counterparts. At 12 weeks, the CS-exposed animals presented a more severe disease, as witnessed by the persistent higher lung fungal load (although it did not reach statistical significance [$p = 0.054$]), greater dissemination to other organs, greater affected area of the lung, decreased IFN- γ /IL-10 ratio, and higher TNF expression within the granulomas, compared with CS-non-exposed mice. The number of CD4 and CD8 lymphocytes infiltrating the granulomas was similar between both mice groups, but there was a decrease in the number of MAC-2+ macrophages. No difference was noted in the CD68+ macrophage number. However, the follow-up in week 20 showed that the immunological effects of exposure to CS ceased, with both CS and NCS mice showing the same infectious features, i.e., a trend for resolution of the infection. In conclusion, we show that chronic CS-exposure alters the course of the disease in an experimental model of subclinical pulmonary PCM, confirming the epidemiological link between CS-exposure and the chronic form of PCM. However, we also show that this effect is transitory, being detected between 4- and 12-weeks post-infection but not thereafter. The possible immune mechanisms that mediate this effect and the reasons for its transitory effect are discussed.

KEYWORDS: Paracoccidioidomycosis. Cigarette smoke. Subclinical infection. Immune response. Risk factors.

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INTRODUCTION

Paracoccidioidomycosis (PCM) may present as an acute/subacute clinical form, characterized by a progressive disease arising from the airborne initial infection, or, most often, as an asymptomatic or subclinical infection that may manifest later on in an individual's life, the chronic form. Epidemiological studies of PCM aiming to identify factors that lead the infected individual to manifest the disease have shown a strong association between smoking and the development of the chronic form of PCM¹⁻⁴. The epidemiological link between PCM and smoking is reinforced by a case-control study in which the authors demonstrated that smokers were 14 times more likely to develop the disease among patients from an endemic area in Brazil. Besides, the intensity of tobacco consumption also influences the disease, indicating that individuals who smoked twenty or more cigarettes/day became ill on average eight years earlier than the others⁵.

It is well established that cigarette smoke has a strong impact on immunity, affecting several aspects of both the innate and adaptive immune responses^{6,7}. Current evidence demonstrates that some cigarette components have immunosuppressive properties that could be implicated in increasing susceptibility to PCM^{6,7}, as it has been demonstrated for tuberculosis (TB)^{8,9}, a chronic granulomatous disease with some similarities to PCM. Strikingly, up to 10% of the CF PCM patients in Brazil concurrently present pulmonary TB¹⁰. Moreover, a previous study showed that monocytes isolated from CF PCM patients displayed an enhanced expression of components of the NLRP3-inflammasome, probably associated with the patients' exposure to smoke and hypoxemia¹¹.

In order to mitigate PCM within the smoke-exposed population, it is important to understand the mechanisms through which cigarette smoke (CS) exposure increases the risk of developing the CF of the disease. In the current study, we addressed this significant knowledge gap and investigated the effects of cigarette smoke exposure (CS) experimentally in a murine model of a non-progressive form of PCM.

MATERIALS AND METHODS

Animal use and ethics statement

Specific-pathogen-free 6- to 8-week-old male C57BL/6 mice were obtained from the Animal Facility at the School of Medicine of the University of Sao Paulo (USP) in pathogen-free conditions with *ad libitum* access to chow and water. The National Council for the Control of Animal

Experimentation (CONCEA) guidelines were strictly followed over the course of this project. All experimental protocols were approved by the Review Board for Human and Animal Studies at the School of Medicine of the University of Sao Paulo (USP), registry N° 066/17, and the Institute of Tropical Medicine, registry N° 000169A.

Fungus and inoculum preparation

The standard, virulent isolate *P. brasiliensis* 18 (Pb18) was used. It was obtained from reinoculated mice as a means to ensure its virulence as previously described¹², and was maintained on semisolid Fava Netto's culture medium for 7 days at 37 °C. To prepare the inoculum used in the experiments, Pb18 was harvested from the tubes and washed three times with phosphate buffered saline (PBS, pH 7.2). Thereafter, large and agglutinated yeast cells were separated by decanting and the small, isolated yeast cells were collected and counted by hemocytometer. The yeast cells used for intratracheal (IT) infection were >95% viable as determined by Trypan blue staining.

Inoculum standardization and intratracheal infection

Infection by the IT route was performed in C57BL/6 mice anesthetized intraperitoneally using a 200 µL solution of 80 mg/kg of Ketamine and 10 mg/kg of Xylazine. Under anesthesia, a small longitudinal incision was made in the neck, exposing the trachea, and 50 µL of PBS containing variable concentrations of the Pb18 were injected. The inoculum size (10³ yeast cells/mouse) used throughout the study was determined in pilot experiments (see Results). After 2 weeks of infection, the mice were randomly divided into two groups and transferred to the conventional area in the animal facility at the Institute of Tropical Medicine, USP. The experiments were performed twice independently, with each experimental group (NC and NCS) comprising 36 animals, with similar results. The data shown represent the combined results of the two experiments.

Cigarette smoke exposure

The C57BL/6 mice that had been IT infected two weeks earlier were split into two groups, one of which was exposed to CS in a whole animal exposure system using a modified cigarette smoke exposure device from Biselli *et al.*¹³. The cigarette smoke exposure was performed in a 28-L plastic box with two inlets (for air and smoke), one outlet and a fan (for the enhancing air and smoke mixture inside the box). In the first inlet, we applied synthetic air (10 L/min). The second inlet received synthetic air (1.5 L/min) that

passed through a Venturi valve connected to a lit cigarette that suctioned the cigarette smoke and pumped it inside the box. The mice were exposed twice daily for 120 min, 5 days a week to 12-16 cigarettes with filters removed for 20 weeks (100 days of actual CS exposure). The flow rate was set at 1.5 L/min, which generally resulted in blood levels of carboxyhemoglobin (COHb) of 10–15% after the first exposure, as determined in pilot experiments to mimic the levels found in moderate smokers¹⁴. Peripheral blood COHb was measured up to 20 min after blood collection using the Radiometer ABL800 Flex equipment (Radiometer, Bronshoi, Denmark). The other group, not exposed to CS, formed the infected-only control group (NCS). All mice were weighed before and at the end of the CS exposure period.

Fungal burden in the lungs of infected mice

Mice from each group were euthanized 4, 12 and 20 weeks after infection and one of the lungs (left and right side) was removed to analyze the fungal burden by colony forming units (CFU), and the other for histopathological analysis (below). A total of 20-24 animals from each group and time point was used. The lung was weighed, mashed and homogenized in PBS. Aliquots of 100 μ L were plated onto Brain Heart Infusion (BHI) agar supplemented with 5% spent supernatant from *P. brasiliensis* Pb192 cultures, 10 IU/ml Streptomycin-Penicillin (Cultilab, Brazil), 4% fetal bovine serum (Gibco), and 500 mg/ml Cycloheximide (Sigma, St. Louis, MO, United States)¹⁵. CFUs were determined every 5 days for 15 days of incubation at 37 °C. The number (\log_{10}) of viable *P. brasiliensis* colonies per gram of tissue was expressed as mean \pm standard error (SE). To assess fungal dissemination to liver and spleen, these organs were removed from 12 animals from each group and time point and processed as above.

Histological analysis

Histopathology studies were performed in weeks 4, 12 and 20 after infection in the other lung ($n = 20-24$ for each group and time point). The lung was removed, fixed in 10% formalin and embedded in paraffin. Five-micrometer sections were stained with hematoxylin-eosin (H&E) for histological analysis. Pathological changes were analyzed based on the size, morphology and cell composition of granulomatous lesions, presence of fungi and intensity of the inflammatory infiltrates. Morphometrical analysis was performed using the AxioVision release 4.8.2 SP3 software (Carl Zeiss Microscopy, White Plains, NY, USA) ($\times 10$ magnification). The area of granulomatous lesions (μm^2)

was measured in 20 microscopic high-power fields per slide. Results were expressed as the mean \pm standard error (SEM) of the total area of lesions for each animal. The area corresponding to 25% of the total lung area was determined by the AxioVision software.

Immunohistochemical analysis (IHC)

Deparaffinized lung sections (4 μm thick) from the lungs excised from 12 animals from each group and time point were incubated for 18h with the primary antibody at 4 °C, to detect the antigens CD4 (Santa Cruz, clone sc-13573, dilution 1:50); CD8 (Invitrogen, clone MA170041, dilution 1:50); TNF (Santa Cruz, clonesc-52746, dilution 1:90000); IFN- γ (Bioss, clone bs-0480R, dilution 1:40); IL-10 (Santa Cruz, clone sc-73309, dilution 1:50); MAC-2 (Cedarlane, clone CL8942AP, dilution 1:25000) and CD68 (Biorbyt, clone Orb-10343, dilution 1:200). Antigen retrieval was performed with TRIS-EDTA at pH 9.0, except for MAC2 and CD68, for which antigen retrieval was performed with citrate at pH 6.0. The amplification of the reaction was achieved by the polymer molecules conjugated to peroxidase (Reveal, Biotin-Free Polyvalent horseradish peroxidase diaminobenzidine; Spring Bioscience, Pleasanton, CA, USA, code SPD-125) and chromogen (Dako Liquid DAB (DAB) + substrate chromogen system; Dako, Carpinteria, CA, USA, code K3460). Finally, the slides were counterstained with Harris hematoxylin (Merck, Darmstadt, Germany). The immunostained cells in the granulomas, with brownish cytoplasm, were counted as positive for each primary antibody, also considering the lymphocyte typical morphology for CD4/CD8 staining, and the histiocyte typical morphology for CD68/MAC-2 staining. Consecutive high power fields (400x) were used to quantify positive immune cells, considering the entire circumference of each granuloma. IHC counts were performed in a blinded fashion regarding the CS exposure by two pathologists (ANDN and FLL).

Statistical analysis

GraphPad Prism 7 software (GraphPad, San Diego, CA, United States) was used to build the graphics. The results were expressed as the mean values \pm standard error of the mean (SEM). The variables analyzed were weight, CFU, affected granulomas area and IHC markers. To run the statistical analysis, Student's t-test was used for continuous or quantitative variables with normal distribution as determined by the Shapiro–Wilk's normality test. The Mann–Whitney's test was used for variables that do not have a normal distribution. Fisher's exact test was

used for non-continuous variables. The level of statistical significance adopted was 0.05.

RESULTS

Inoculum standardization

The established mouse models of PCM aimed to mimic the human disease are based on the IT challenge with high fungal inoculum, usually ranging from 10^5 to 10^6 yeast cells¹⁵. Although we know neither the initial steps of the infection in humans nor the fungal burden to which infected people from endemic areas have been exposed, it goes without saying that 10^5 to 10^6 yeast cells inoculum is greatly overestimated. For instance, the TB mouse models typically employ intratracheal inoculum of 10^2 *M. tuberculosis* bacilli^{16,17}. We thus sought to establish an inoculum size that could achieve a subclinical-like pulmonary infection. Inoculation experiments with 10^4 to 10^6 yeast cells all resulted in the involvement of $\geq 25\%$ of the lung area and lung CFU $\geq 5 \times 10^5$ /g tissue, by week 12 post-infection (Supplemental Figure S1). Surprisingly, the 10^3 -inoculum also yielded a CFU around 5×10^5 cells/g of tissue, reaching 25% of the lung area affected. Only a 10^2 -inoculum resulted in a low degree, “subclinical”-like,

infection, with $\leq 10^4$ CFU and $< 25\%$ area involved at all three times of the study: 4, 12 and 20 weeks post infection.

Effects of CS on fungal burden, area of lung involvement, and fungal dissemination

We obtained an average level of COHb of $14.45 \pm 1.50\%$ in CS mice during the experiments, which is within the range expected for moderate smokers¹⁴. The group that was not exposed showed an average COHb level of $1.22 \pm 0.24\%$. During the 20 weeks of experiments, we observed a marked weight increment in the NCS group while the CS group did not show significant weight gain, which resulted in a significantly lower weight at 12 and 20 weeks (Figure 1A). Nevertheless, we observed neither increased mortality nor altered behavior (e.g., water and food intake, nocturnal dynamic activity, etc.) during the study period in the CS mice compared to the NCS mice.

The NCS group showed small but detectable foci of granulomatous response as early as 4 weeks. These foci had markedly expanded by 12 weeks, slightly decreasing by week 20; at all three time-points, the involvement corresponded to less than 25% of the total lung area (Figure 1B). The CS group also presented small foci of granulomatous response at 4 weeks, which increased to

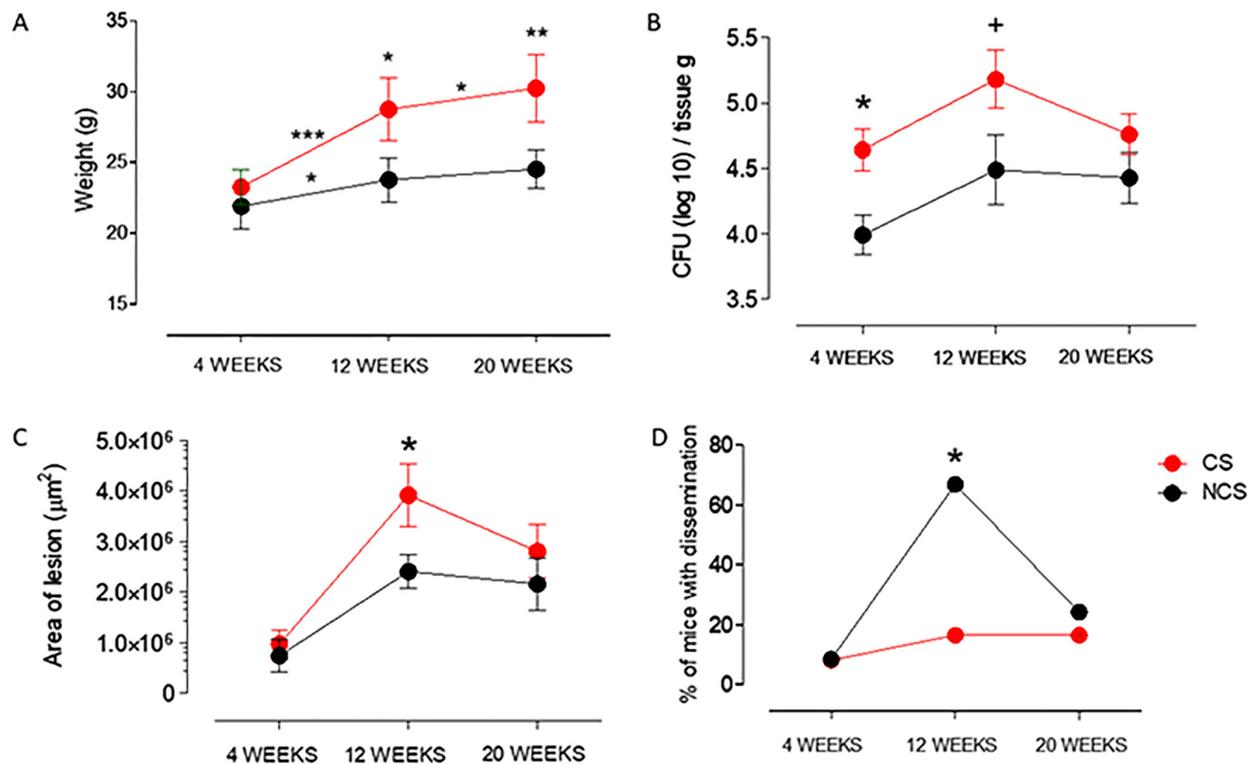


Figure 1 - Body weight (A), lung colony forming units (B), total lung area affected by the granulomatous inflammatory response (C), and rate of fungal dissemination (D) over time in the cigarette smoke exposed (●) and non-exposed (●) groups. A, B and C: data shown as mean and standard error; Mann-Whitney or Student's t-test was used. D) Fisher's exact test was used. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; + $p = 0.054$.

involve >25% of the total lung area at 12 weeks, returning to lower levels by week 20 (Figure 1B). CFU counts showed as early as 4 weeks, a significantly higher fungal burden in the CS than the NCS group (Figure 1C). The fungal burden consistently increased in both groups from week 4 to week 12, with the CS group persisting with higher values, although this difference did not reach statistical significance (5.18 ± 0.22 vs 4.49 ± 0.27 , $p = 0.054$). We observed a reduction in the CFU at 20 weeks, when both groups were comparable.

Dissemination to other organs (spleen and liver) was virtually absent in both groups at week 4 (Figure 1D). However, by week 12, dissemination to either one organ was observed in 66.7% of the mice in the CS group compared with only 16.7% in NCS mice ($p = 0.0078$). By week 20 the dissemination rate in the CS group decreased to 24.0%, close to that of the NCS group (16.7%).

Histology and IHC analyses

Within four weeks, we observed diffuse foci of histiocytes, epithelioid cells, lymphocytes, and some neutrophils aggregated around the yeast cells, identifiable as the granuloma's initial structures, usually close to an airway (Figure 2). With disease progression, from week 12 onwards, well-organized granulomas composed of histiocytes, epithelioid cells, and multinuclear giant cells aggregated around the yeasts, and surrounded by a border of lymphocytes were observed. It should be noted that no alterations outside the granulomatous response could be

found in the lung parenchyma, such as scarring fibrosis or other evidence of tissue repair, using H&E and Gomori's stainings. The overall 4-week lung cell counts tended to be low, in line with the smaller affected areas due to smaller and less mature granulomas (Figure 2). IHC analyses showed, in both groups and at the three-time points, a marked infiltration of cells of the myeloid lineage (either recently migrated monocytes, macrophages or dendritic cells), identified as MAC-2+ or CD68+ cells. Interestingly, after 4 weeks, MAC-2+ cells predominated over CD68+ cells, but from week 12 onwards, with granuloma maturation, CD68+ cells started to predominate (Figure 3) while MAC-2 counts tended to decrease. Furthermore, the CS group exhibited significantly lower density of MAC+ cells than the NCS group at the three-time points, while no differences between the two groups were seen with CD68 staining (Figure 3).

In contrast, there were small numbers of infiltrating CD4 and CD8 lymphocytes, ranging between 10-30 cells/mm³, without significant differences between CS and NCS groups at the three-time points for both subsets (data not shown). Some differences were noted regarding cytokine expression within the granulomas at week 12, but not with week 4 and week 20 lungs in the two groups (Figure 4). Higher expression of IL-10 was seen in CS than NCS mice, while there was no difference regarding IFN- γ expression. Interestingly, the IFN- γ vs. IL-10 balance, critical for the regulation of the immune response in both human and experimental PCM¹⁸⁻²⁰, distinguished the two groups: by week 4 the IFN- γ /IL-10 ratio was lower in NCS compared with the CS (3.0 vs 8.4, $p = 0.02$) (Figure 5). With disease

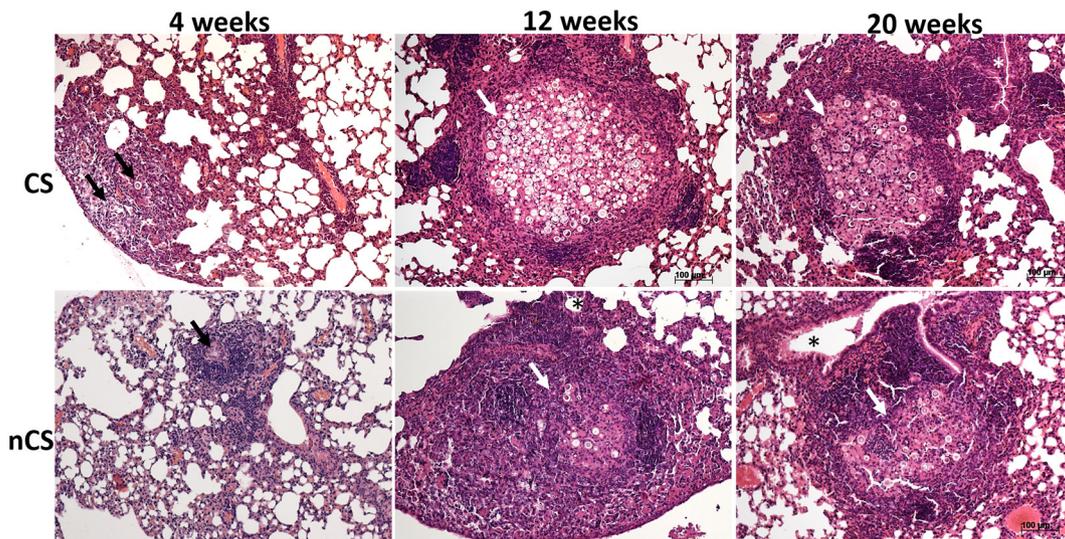


Figure 2 - HE staining of the lung granulomatous response over time in the cigarette smoke exposed (top row) and non-exposed (bottom row) groups. At week 4, most granulomas in both groups were small and/or incipient; *P. brasiliensis* yeast cells were already found within them, more numerous in exposed (arrows) than non-exposed mice; at 12 and 20 weeks, the granulomas were in general well-formed, with epithelioid histiocytes, multinucleated giant cells and an external rime of lymphocytes surrounding numerous typical *P. brasiliensis* yeast cells. Granulomas were in general associated with an airway (indicated by the symbol *), as the animals were infected through intratracheal inoculation. Magnification: 200x. Scale bar: 100 μ m.

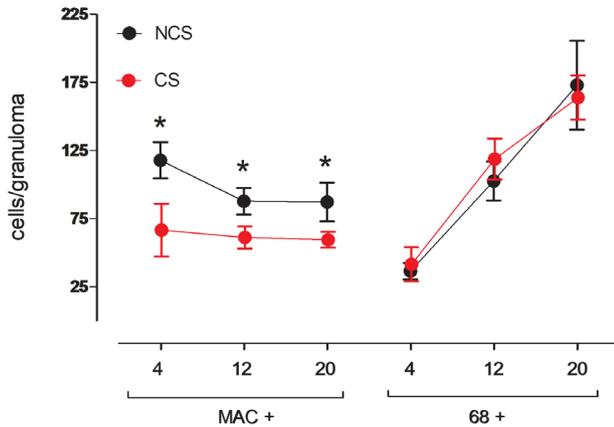


Figure 3 - Immunohistochemistry analyses of the lungs. Counts over time of CD68 and MAC-2 expressing cells with histiocyte morphology within the granulomas in the cigarette smoke exposed (●) and non-exposed (●) groups. Data shown as mean and standard error; Mann-Whitney's test was used; * $p < 0.05$.

progression (12 weeks) the ratio sharply increased to 9.4 in NCS mice but sharply decreased to 1.8 in CS mice ($p = 0.0047$), correlating with the less controlled course of the disease in the former. By week 20, both groups had similar IFN- γ /IL-10 ratios (~ 3.5). In addition, significantly greater TNF expression was found in week 12 lungs of CS mice, possibly reflecting the enhanced inflammatory status at this time point in CS-exposed animals (Figure 4).

Histological changes related to CS

In the fourth week of the experiment, the control group's lung tissue, not exposed to cigarette smoke, had alveolar septa and bronchiolar epithelium within the normal morphological aspect. In contrast, in the lung tissue of mice

exposed to cigarette smoke for two weeks, we could already observe high columnar epithelium and a slight dysplasia in it. At 12 and 20 weeks, we did not observe any lung tissue changes in the unexposed group's respiratory epithelium. In contrast, among the group exposed to cigarette smoke, we observed moderate dysplasia of the columnar epithelium and increased mucus production (Figure 6).

DISCUSSION

Although the epidemiological evidence strongly supports the hypothesis that smoking may lead to the development of the pulmonary CF of PCM¹⁻⁵, no study to date has demonstrated the possible mechanism for this association. In our study, we evaluated for the first time the influence of smoking in a murine experimental model of CF pulmonary PCM. We showed that cigarette smoke exposure alters, albeit transiently, the course of the mycosis in our mouse model.

A CS effect was discernible as early as 2 weeks after exposure, i.e., four weeks post-infection, when the lung CFU in exposed animals was significantly higher than in their non-exposed counterparts. However, the magnitude and characteristics of lung inflammation were still comparable. The granulomatous response was sparse (as expected for 4 weeks of infection²¹), affecting only $\sim 3\%$ of the total lung area. Furthermore, IHC analyses of this initial granulomatous response in CS and NCS mice showed that both were populated predominantly by myeloid lineage cells rather than by CD4 and CD8 lymphocytes. Monocytes predominated at week 4 (but were overcome onwards by macrophages, with granuloma maturation). 4-week CS and NCS lungs exhibited higher expression of IFN- γ than

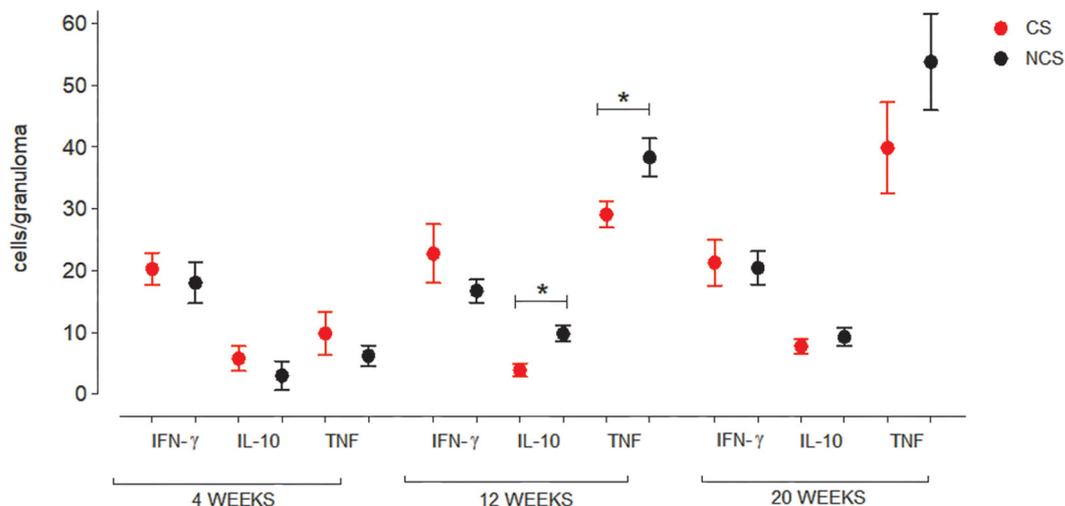


Figure 4 - Immunohistochemistry analyses of the lungs. Counts over time of IFN- γ , IL-10 and TNF expressing cells within the granulomas in the cigarette smoke exposed (●) and non-exposed (●) groups. Data shown as mean and standard error; Mann-Whitney's test was used; * $p < 0.05$; ** $p < 0.01$.

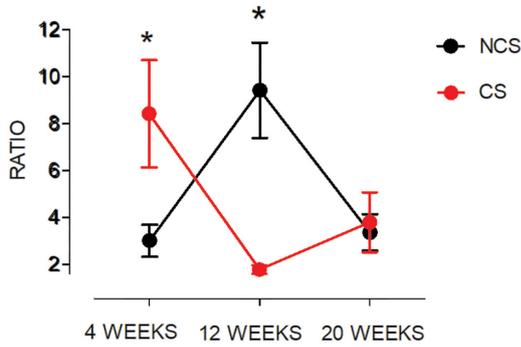


Figure 5 - Ratio of IFN- γ -expressing cells/IL-10-expressing cells within the granulomas over time in the cigarette smoke exposed (●) and non-exposed (●) groups; Data shown as mean and standard error; Mann-Whitney's test was used; *p < 0.05.

IL-10 and TNF; however, the IFN- γ /IL-10 ratio was much higher in the CS group. Strikingly, by 12 weeks, although the CD4 and CD8 lymphocytes infiltration did not change from 4 weeks, CS led to significantly wider affected lung areas, persistently higher CFUs, and a sharp reduction in the IFN- γ /IL10 ratio. As a correlate of the less controlled infectious process in the CS mice, TNF expression was also higher than in NCS mice.

However, the follow-up in week 20 showed that the CS exposure immunological effects ceased, with both CS

and NCS mice showing the same infectious features. The affected lung area and CFU decreased to levels comparable with those of NCS (which also decreased slightly), as did the IFN- γ /IL10 ratio. Furthermore, TNF expression by the granulomas persisted highly, but was also of similar magnitude between CS and NCS mice. Our hypothesis for the transitory CS effect is that it was overcome by the genetic background of the mouse strain, which characteristically leads to the control of the infection in the long term²².

Considering our experimental model, mice were exposed to cigarette smoke after two weeks of infection with *P. brasiliensis*, more truly mimicking what happens to patients: it is estimated that the patients are infected at young ages, before starting a smoking habit^{23,24}. We decided to wait 14 days, the time necessary for the infection to be established²¹. Furthermore, exposure to cigarette smoke was carried out according to the modified protocol by Biselli *et al.*¹³ and regardless of when the infection occurred, our mice maintained COHb levels within the desired range, which would correspond to an at least moderate smoker during the 20 weeks of experiments. This monthly control of COHb is crucial when discussing our results' validity since these mice were not exposed to toxic doses related to smoking. As observed in other experimental models²⁵, the

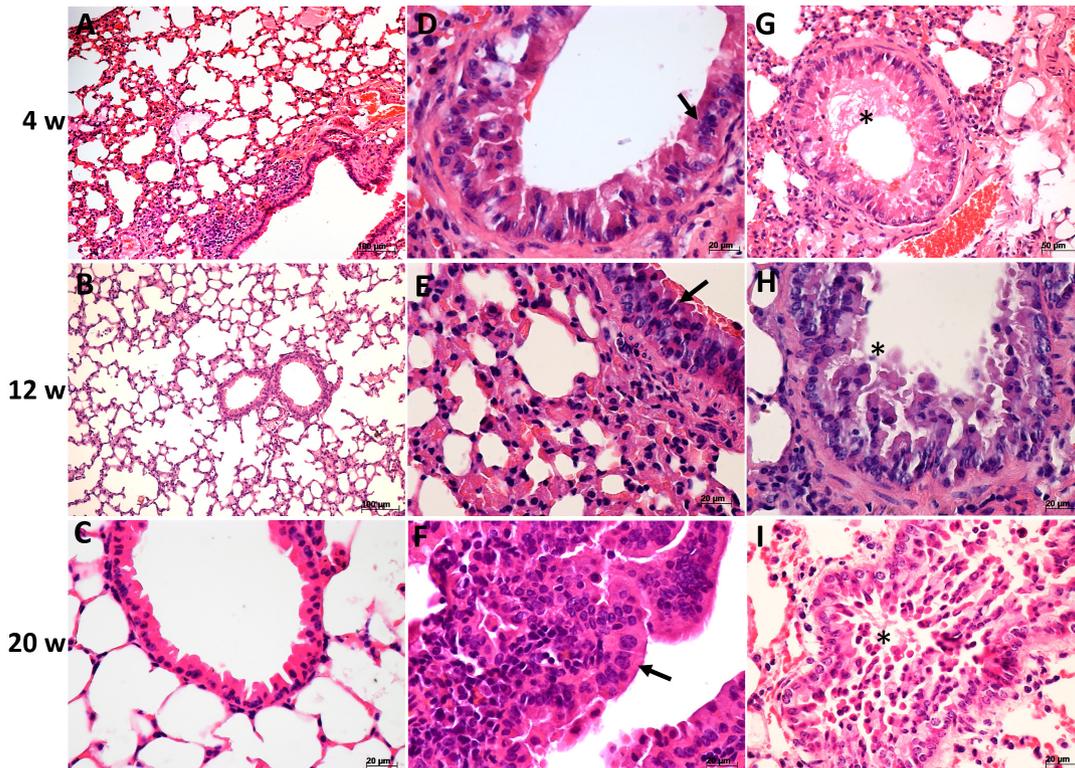


Figure 6 - HE staining of the airway epithelial alterations along the cigarette smoke exposure (4, 12 and 20 weeks). The left column (A-C) shows non-*P. brasiliensis* infected/non-exposed control mice. The middle column (D-F) shows the dysplasia of the ciliary epithelium (multilayered cells, with larger and hyperchromatic nuclei and coarse chromatin) in the exposed group (arrows), and the right column (G-I) shows increased mucus production in this group (indicated by the symbol *). Magnification: 100x (A, B); 200x (G); 400x (C, D, E, F, H, I). Scale bar: 20 mm (C, D, E, F, H, I); 50 mm (G); 100 mm (A, B).

exposed animals lost more weight than the unexposed group regardless of the inoculum administered, demonstrating that our experiment correctly simulated a whole-body exposure to cigarette smoke.

The possibility that the increase in the prevalence of diseases associated with cigarette smoke may, in part, be due to changes induced by tobacco smoke in the immune and inflammatory processes was recognized for the first time in the 1960s²⁶. However, recent reviews recognize that the influence of cigarette smoke on the immune system is complex due to its chemical heterogeneity and dual nature, pro-inflammatory and immunosuppressive^{6,7}. Understanding CS-induced effects is further complicated by the sometimes-contradictory results of human vs. experimental studies, the individual genetic susceptibility, and the variability in experimental designs such as time, frequency and mode of exposure⁶. CS effects in our PCM model concern particularly adaptive immune response, since the mice were first infected and only 2 weeks later submitted to CS. Regarding this response, the weight of evidence points to CSE dampening the Th1 type and promoting the Th2 type immune responses⁶, which markedly impact the course and outcome of some infectious diseases. This was best studied in TB^{8,9}, where it was shown that *M. tuberculosis*-mediated inhibition of DC function is exacerbated by smoking. CS-exposure led to decreased IL-12-production but enhanced IL-10-production by DCs, which was linked to reduced influx of IFN- γ -producing and TNF-producing CD4+ and CD8+ effector and memory T-cells¹⁷. In addition, CS has several direct detrimental impacts on T-cell function and proliferation and can induce T-cell apoptosis²⁷. The net result is enhanced permissiveness to *M. tuberculosis*, with increased mycobacterial loads^{8,9}.

CD68+ is a specific marker of blood monocytes and tissue macrophages as it spares DC, while MAC-2 is expressed by all three cell types^{28,29}. Interestingly, we observed in both the CS and NCS groups greater granuloma infiltration by MAC-2+ cells than by CD68+ cells early in the infection, and the reverse after its progression (20 weeks), when mature CD68+ macrophages predominate. A conceivable explanation for the higher MAC-2 counts is DC infiltration in the granulomas early in the infection. In addition, we also found sustained higher MAC-2+ cell counts in the NCS group than the CS group. This suggests an impaired infiltration of DC in the latter, which concurs with the described defective antigen presentation by DC caused by cigarette smoke exposure^{8,17}. This putative DC deficiency caused by CS may be further linked to the findings observed from week 4 to week 12 of the infection in the CS group: (i) higher fungal load, (ii) increase in the lung

area affected by the granulomatous response, (iii) increased dissemination to other organs, and (iv) sharp decrease in the IFN- γ /IL10 ratio as compared with non-exposed mice. Unfortunately, no staining with DC specific markers was included in the study to explore this mechanism. The net CS effects were, however, less persistent than those seen in TB models since by week 20 there were no marked differences between CS and NCS mice.

Some factors may help to explain the differences between PCM and TB. Firstly, *M. tuberculosis* is likely more pathogenic than *Paracoccidioides spp.*: 10% of the *M. tuberculosis*-infected persons will develop the disease, while it is roughly estimated that the PCM-disease will develop in less than 1% of the *Paracoccidioides*-infected population in endemic areas^{18,19}. Secondly, as opposed to our experimental protocol, the TB-CS association studies in mice were designed with CS taking place before infection, thus deeply affecting innate immune responses. Third, the mouse strain used, C57Bl/6 was shown to resist, at least partially, to a 10⁵ yeast cells intraperitoneal inoculum²¹: C57Bl/6 mice inoculated showed no difference in survival rate as compared with control animals, and our unpublished data show that the fungal load decreases further after 20 weeks in both CS and NCS animals (unpublished data). We thus may infer that our CS protocol was not able to overcome the innate resistant behavior of this strain, as witnessed by the progressive increase in the density of mature CD68+ macrophage cells within the granulomas in both CS and NCS mice, potentially contributing to the progressive control of the infection (along with other ill-defined features of the protective anti-*Paracoccidioides* adaptive immune response of this strain). This is in contrast with the TB mice model, where a chronic infection persists with up to 300 days of infection characterized by a high *M. tuberculosis* load¹⁷. In the human PCM infection, it was clearly shown that the balance between the IL-12-IFN- γ axis and IL-10 drives the clinical presentation of the disease^{19,20,30}, while in mice IL-10 has also been shown to be a major player³¹.

When comparing with the TB study by Shang *et al.*¹⁷, we observed similarities in the initial inflammatory response characterized by a mild perivascular infiltrate and predominant peribronchiolar infiltrates of lymphocytes. The exposed mice showed thickening of the alveolar septa wall during the experiment, a more diffuse inflammatory infiltrate of macrophages, lymphocytes, and neutrophils. However, unlike the experimental model of tuberculosis, granulomas remained well-formed and compact in our study despite being more prominent in size in the exposed group. It is also interesting to note that the histological changes related to smoking were shown earlier, with 30 days of

exposure, compared with the result of the area occupied by granulomas, in which the differences between the groups were more evident only after 10 weeks of exposure.

Our work has some limitations. Firstly, we observed individual variability in the results within the groups. This likely occurred due to the 10^2 -inoculum used: *Paracoccidioides* spp have a marked tendency to form clumps, eventually altering the final inoculum, despite our efforts and checks to avoid this issue. This variability may have precluded the observation of more significant differences between the two groups. In the same token, although we observed that the value of 100 yeasts apparently met the goal of generating a subclinical infection, perhaps the inoculum dose was too low to overcome the genetic background of the C57Bl/6 strain: the mice were able to control the disease at 20 weeks, despite the additional effect of smoking. Thirdly, it is worth noting that the mouse model of IT infection does not truly mimic the pathology of the human chronic pulmonary infection: the typical mouse's large granulomas with nests of numerous yeast cells are not usually found in the lungs of patients, while many features of the patients' lung histopathology are absent in mice, such as tissue necrosis, fibrosis, caseous necrosis and granulomatous inflammation, all intermixed and with a dispersed, lower number of fungal elements³².

CONCLUSION

In conclusion, we showed that CS exposure can alter the course of the disease in a subclinical pulmonary PCM mouse model, providing a putative mechanism underlying the close link between a smoking habit and the development of the chronic form of PCM. However, the CS exposure probably did not overcome the background for resolution of the infection in the long-term, characteristic of the mouse strain. While further studies are warranted to further clarify this issue, we believe that public health policies aimed at reducing the cigarette smoke burden in highly endemic areas will likely also reduce the PCM burden.

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