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Original Article

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Itraconazole in combination with neutrophil depletion reduces the expression of genes related to pulmonary fibrosis in an experimental model of paracoccidioidomycosis

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Abstract

Itraconazole (ITC) is the drug of choice for treating paracoccidioidomycosis (PCM); nonetheless, patients with the chronic form of this mycosis develop fibrosis, a residual pulmonary abnormality, even after treatment. Recently, we observed that the depletion of neutrophils with a specific monoclonal antibody (mAb-anti-Ly6G) during the chronic stages of PCM was associated with a decrease in the fungal burden, the inflammatory response and a reduction of fibrosis. Herein, we aimed to evaluate the effect of ITC in combination with the mAb-anti-Ly6G in an experimental model of pulmonary PCM. BALB/c male mice were challenged with *Paracoccidioides brasiliensis* yeasts and treated with the mAb-anti-Ly6G and/or ITC at 4th week post-infection (p.i.) and then sacrificed at 12th week p.i. to assess neutrophil subpopulations, fungal load, collagen, expression of fibrosis- and pro-inflammatory-related genes and histopathology. We observed that combination of ITC/mAb-anti-Ly6G favored the control of infection and diminished the inflammatory response. Of note, such therapeutic strategy reduced the expression of IL-1β, IL-6, IL-17, IL-10, TNF-α, TGF-β1, TGF-β3, GATA-3, RORc, Ahr, MMP-1α, MMP-8 MMP-15, TIMP-1, and TIMP-2 genes in an additive manner compared to those mice treated with the mAb or ITC alone. Interestingly, ITC induced an increase of type-II neutrophils even in those mice treated with the mAb-anti-Ly6G. These results indicate that combination ITC/mAb-anti-Ly6G reduced the infection and pulmonary fibrosis through down-regulation of inflammatory and pro-fibrotic genes. Additionally, we confirmed the

immunomodulatory properties of this antifungal *in vivo*. This work emphasizes the importance of exploring new potential combination treatments to treat fungal infections.

Key words: Itraconazole, *Paracoccidioides brasiliensis*, paracoccidioidomycosis, neutrophils, fibrosis.

Introduction

Currently, triazole drugs such as itraconazole (ITC) and voriconazole (VRC) are considered as the treatment of choice for endemic and systemic fungal infections including histoplasmosis, coccidioidomycosis and paracoccidioidomycosis.[1,2] Of note, more recently it has been described that in addition to exerting an antifungal effect, ITC exhibits immunomodulatory properties.[3]

Paracoccidioidomycosis (PCM) is caused by the thermodimorphic fungal pathogen from the genus *Paracoccidioides*; this mycosis is considered one of the most important systemic mycosis with a high number of patients reported in Brazil (about 80% of the cases), followed by Colombia, Venezuela, and Argentina.[4] The clinical presentation of PCM covers the acute or subacute form (also known as juvenile type) and the chronic or adult type form, the latter representing about 90% of cases.[5] The chronic form of the disease may begin as asymptomatic with the primary lesion in the lung that may progress to a granulomatous inflammatory response with tissue damage;[6,7] this progressive chronic inflammation leads to the appearance of structural and functional pulmonary alterations and the development of a fibrotic sequelae that can be observed in approximately 60% of patients with this clinical presentation even after treatment with ITC for long periods of time.[5,8–11] Despite substantial morbidity and mortality associated with this condition, there is no effective therapy for pulmonary fibrosis (FP) in patients with PCM. In animal models, combined therapies have been tested using immunosuppressive drugs such as pentoxifylline and ITC with promising results demonstrating a reduction in both the inflammatory response and fibrosis in lungs of mice infected with *P. brasiliensis*.[11] Nonetheless, the evidence to support the therapies using immunosuppressive drugs in infectious diseases is limited, and there is a higher potential risk to develop latent infections.[12,13]

More recently, using an animal model of pulmonary PCM treated with a specific monoclonal antibody (mAb anti-Ly6G, clone 1A8) to neutrophils during the chronic stages of infection, a better control of infection was observed accompanied by an attenuation of pulmonary fibrosis through down-regulation of important pro-fibrotic molecules including transforming growth factor (TGF)-β1, tumor necrosis factor (TNF)-α, interleukin (IL)-17, matrix metalloproteinase (MMP)-8 and tissue inhibitor metalloproteinase (TIMP)-2.[14] Therefore, in the present work, we focused on evaluating the efficacy of a combined therapy using ITC with the specific mAb-anti-neutrophils for the treatment of infection and fibrosis development in an experimental model of pulmonary PCM.

Methods

Fungal strain

Paracoccidioides brasiliensis (Pb18), a highly virulent isolate was used in this study. This fungus was maintained and handling as described previously.[15] Briefly, Pb18 yeast cells were grown on Saboraud Dextrose Broth (Difco Laboratories, Detroit, MI, USA), supplemented with 0.14% L-asparagine, 0.01% thiamine hydrochloride (Sigma-Aldrich, Saint Louis, MO, USA) and antibiotics solution [100 U/ml Penicillin, 100 µg/ml Streptomycin (Gibco Invitrogen Corporation, Carlsbad, CA, USA)] incubated at 36°C, 150 rpm for 4 days. The yeast cells were washed twice in phosphate-buffered saline (PBS; pH 7.2, Gibco Invitrogen Corporation, Carlsbad, CA, USA). Viability was determined with Janus Green B vital dye (Acros Organics, Geel, Belgium) and cell suspensions were adjusted 2.5×10^7 cells/ml based on hemocytometer counts.

Mice and model infection

BALB/c male mice, 7–8 weeks old and 18–20 g in weight, were obtained from the breeding colony maintained at Corporación para Investigaciones Biológicas (CIB) (Medellín, Colombia). The experimental infection model was performed as previously described.[14] Briefly, animals were anesthetized by intramuscular injection of a solution of ketamine (80 mg/kg, Laboratorios Biosano, Santiago, Chile) mg) and Xylazine (8 mg/kg, Bayer S.A., Bogota, Colombia). Then, cell suspensions (1.5×10^6 yeasts cells in 60 µl PBS) were instilled intranasally in two equal portions. Non-infected (control) mice were inoculated with 60 µl PBS.

Ethics statement

All animal experiments were approved by our Institutional Ethics Committee (#PRE00501023095, Acta no. 92) and following Colombian (Law 84/1994, Resolution no.

8430/1993), European Union and Canadian Council on Animal Care Regulations.

Monoclonal antibody-mediated neutrophils depletion and antifungal treatment

Infected and non-infected subgroups of mice were injected intraperitoneally with a solution (200 μl) of mAb anti-Ly6G, clone 1A8 (1 μg/μl) or isotype control IgG2a, clone 2A3 (1 μg/μl) (Bio X Cell, West Lebanon, NH, USA). Treatment was administered from 4-week post-inoculation (PBS or *P. brasiliensis*) at doses every 48 h for 2 weeks.[14] Additionally, ITC (100 μl) oral solution (Sporanox®, Janssen-Cilag S.A, Mexico) was given at a dose of 1 mg/day/mouse, necessary to achieve serum levels of 1 μg/ml.[16] Antifungal treatment was administrated daily for 8 weeks by gavage started at 4-week post-infection (p.i.)

Fungal load

Mice were sacrificed as described before at 12 weeks p.i. The lungs, livers, and spleens were removed, weighed, and homogenized in 2 ml sterile solution of PBS added with Penicillin (100 U/ml) and Streptomycin (100 μg/ml) (GIBCO Invitrogen Corporation, Carlsbad, CA, USA) using a gentleMACS Dissociator (Miltenyi Biotec, Teterow, Germany). Homogeneous suspensions were diluted (1:100, 1:1000, and 1:10 000) and 0.5 ml of each dilution was plated on petri dishes with Brain Heart Infusion (BHI) agar medium (BD BBL, Franklin Lakes, NJ, USA) supplemented with 0.5% D-(+)-Glucose (Sigma-Aldrich, Saint Louis, MO, USA), 4% horse serum, previously heating inactivated at 56°C for 30 min (GIBCO Invitrogen Corporation, Carlsbad, CA, USA) and 300 μM EDTA (Sigma-Aldrich, Saint Louis, MO, USA), followed by incubation at 36°C, 5% CO_2. Colony-forming units (CFU) counts were assessed 11 days after cultivation. The number of viable *P. brasiliensis* yeast per gram of tissue are expressed in $\log_{10}$ CFU/g tissue.

Collagen assay

Lung homogenates obtained as describe above was used for the isolation and measurement of total collagen. The assay was made according to the manufacturer´s instructions of the Sircol soluble Collagen kit using for this study (Biocolor, Northern Ireland, UK).

qPCR

Total RNA was obtained from lung homogenates from groups of mice sacrificed at 12 weeks p.i. using TRIzol® (Invitrogen, Carlsbad, CA, USA). RNA isolated was treated with DNase I (Thermo Fisher Scientific Inc, Waltham, MA, USA). Complementary DNA (cDNA) was synthesized using 500 ng of treated RNA with Maxima First Strand cDNA synthesis kit for reverse-transcription quantitative polymerase chain reaction (RT-qPCR) according to the manufacturer´s instructions (Thermo Fisher Scientific Inc, Waltham, MA, USA). Real-time PCR was done using Maxima SYBR Green/Fluorescein qPCR Master Mix (2X) according to the manufacturer´s instructions (Thermo Fisher Scientific Inc, Waltham, MA, USA). The CFX96 Real-Time PCR Detection System (Bio-Rad, Headquarters Hercules, California, USA) was employed to measure gene expression levels. Melting curve analysis was performed after the amplification phase of real time PCR assays to eliminate the possibility of nonspecific amplification or primer-dimer formation. Measuring of messenger RNA (mRNA) expression was obtained by relative quantification comparing the level of target gene expression relative to Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; housekeeping gene). Each expression level measured was performed in triplicate. Specific primers for each different pulmonary fibrosis- and pro-inflammatory-related gene have been reported elsewhere,[14] and its sequences are listed in the supplemental material (Table S1).

Identification of neutrophils subsets

Experimental subgroups animals were sacrificed at 12 weeks p.i. Lungs of mice were removed and homogenized using 40 and 70 μm sterile cell strainers (Thermo Fisher Scientific Inc, Waltham, MA, USA) in RPMI supplemented with 1% fetal bovine serum (FBS) (Sigma-Aldrich, Saint Louis, MO, USA). Cell suspension was centrifuged at 1500 rpm, 10°C for 10 min, and red blood cells were lysed using ACK Lysing Buffer (GIBCO Invitrogen Corporation, Carlsbad, CA, USA). Then, the cells were resuspended in RPMI, 10% FBS and counted in hemocytometer. Fc receptors were blocked using a purified rat anti-mouse CD16/CD32 (BD Pharmingen, San Diego, CA, USA). Flow cytometric analysis was used to determine the percentage of neutrophils subsets within the lung suspension. The following fluorochrome-conjugated antibodies were used; Fluorescein isothiocyanate (FITC)-Rat $igG_{2b}\kappa$ (A95-1), FITC-anti-CD45 (30-F11), Phycoerythrin (PE)-Rat $IgG_{2a}\kappa$ (R35-95), PE-anti-CD11b (M1/70), Allophycocyanin (APC)-Rat $IgG_1\kappa$ (R3-34), APC-anti-Ly6G (1A8), APC-Cy7-Rat $IgG_1\kappa$ (R3-34), and APC-Cy7-anti-Ly6G/Ly6C (Gr1) (RB6-8C5) (BD Pharmingen, San Diego, CA, USA). The absolute number of total leukocytes was quantified by multiplying the total number of cells observed

by hemocytometer counting by the percentage of $CD45^+$ cells. The absolute number of each neutrophils subsets Type I ($CD45^+$, $Ly6G^+$, $Gr1^+$, $CD11b^-$) and Type II ($CD45^+$, $Ly6G^+$, $Gr1^+$, $CD11b^+$) was determined by multiplying the percentage of each gated population by the total number of $CD45^+$ cells.

Histopathology

Mice groups were sacrificed at 12 weeks p.i. The lungs were perfused and removed using 1X PBS and formalin [4% formaldehyde solution (EM Science, Gibbstown, NJ), 0.15 M sodium dihydrogen phosphate (Merck, Darmstadt, Germany) and 0.11 M sodium hydroxide (Sigma-Aldrich, Saint Louis, MO, USA)] to wash out red bloods cells and fix the tissue, respectively. Fixed tissues were embedded in paraffin, cut, and stained with Masson´s trichrome to determine the lung inflammatory response and to differentiate collagen fibers and methenamine silver to identify *P. brasiliensis* yeast cells. Sliced and stained tissues were analyzed using a Nikon Eclipse Ci-L microscope/Nikon DS-Fi2 digital camera and examined with NIS Elements 4.30.02 Laboratory Image software (Nikon Instruments Inc., Melville, NY, USA).

Statistical analysis

Data analysis was performed using Graph Pad Prism software v5 (San Diego, CA USA). Medians and interquartile range (IQR) were used to analyze fungal load and flow cytometry. For all developed methodologies, Kruskal Wallis test was used for comparisons between three or more groups and U-Mann–Whitney test was used for comparisons between two groups. Values of $P < .05$ were considered significant.

Results

Itraconazol therapy altered the number of neutrophils subsets in lungs of *P. brasiliensis* infected mice

In previous studies, we demonstrated the capacity of the mAb anti-Ly6G (clone 1A8) to deplete efficiently and specifically neutrophils at early or chronic stages of experimental pulmonary PCM.[14,15] Likewise, in the present study we observed that neutrophils remained significantly lower in the lungs of mice treated with the mAb anti-Ly6G. Interestingly, all ITC-treated groups showed a decrease in the number of type I neutrophils ($CD45^+Gr1^+Ly6G^+CD11b^-$) (Fig. 1A), while an increase in the number of type II neutrophils ($CD45^+Gr1^+Ly6G^+CD11b^+$) were observed even in those mice treated with the mAb anti-Ly6G in comparison with those infected untreated-mice (control group) (Fig. 1B).

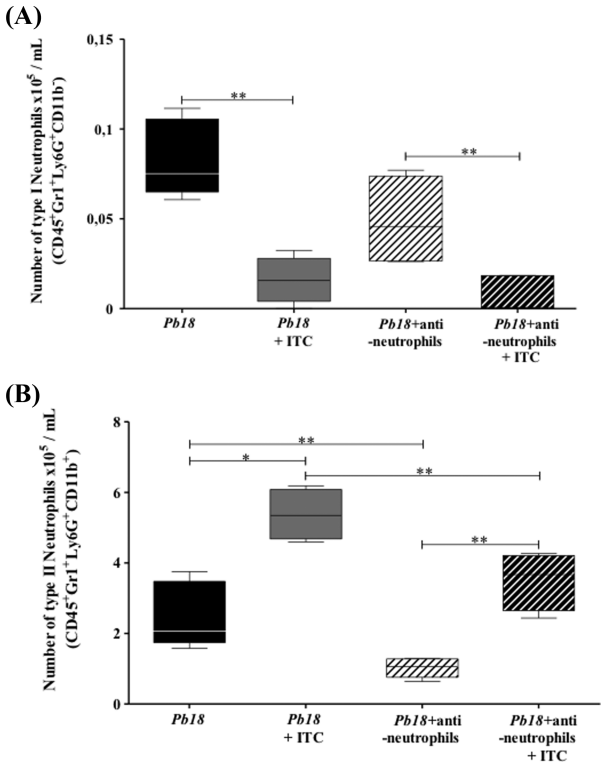

Figure 1. Effect of ITC in combination with the mAb anti-Ly6G on the number of neutrophils subsets in lungs of *P. brasiliensis* infected mice. BALB/c mice were inoculated with 1.5×10^6 *P. brasiliensis* (Pb18) yeast cells and treated with 1 mg of ITC contained in 100 μl of Sporanox® oral solution daily for 8 weeks and/or 200 μg of a mAb anti-neutrophils (Ly6G) contained in 200 μl intraperitoneally (i.p.) at doses every 48 h for 2 weeks; all treatments started at 4th week post-infection (p.i.) and analysis done at 12th week p.i. (**A**) Type I neutrophils ($CD45^+Gr1^+Ly6G^+CD11b^-$) and (**B**) type II neutrophils ($CD45^+Gr1^+Ly6G^+CD11b^+$) were assessed by flow cytometry as described in the Materials and Methods section. Data shown represent median and IQR ($n = 4$–5 mice/group; representative of two independent experiments). $^*P < .05$ and $^{**}P < .01$ comparing infected-untreated mice versus ITC-treated, mAb-treated or ITC/mAb-treated and infected mice. *Pb18*, infected-untreated mice; *Pb18* + ITC, infected mice treated with itraconazole; *Pb18* + anti-neutrophils, infected mice treated with specific mAb anti-Ly6G (monoclonal antibodies anti-neutrophils).

Effect of combined therapy ITC-mAb anti-Ly6G on fungal burden

Our next step was to determine the effect of the combined therapy ITC-mAb anti-Ly6G on the fungal burden with respect to the antifungal effect exert by the ITC or the mAb alone. The fungal burden was evaluated at 12th week p.i. in lungs, liver, and spleen of infected and untreated, mAb anti-Ly6G and/or ITC treated mice. We observed a significant

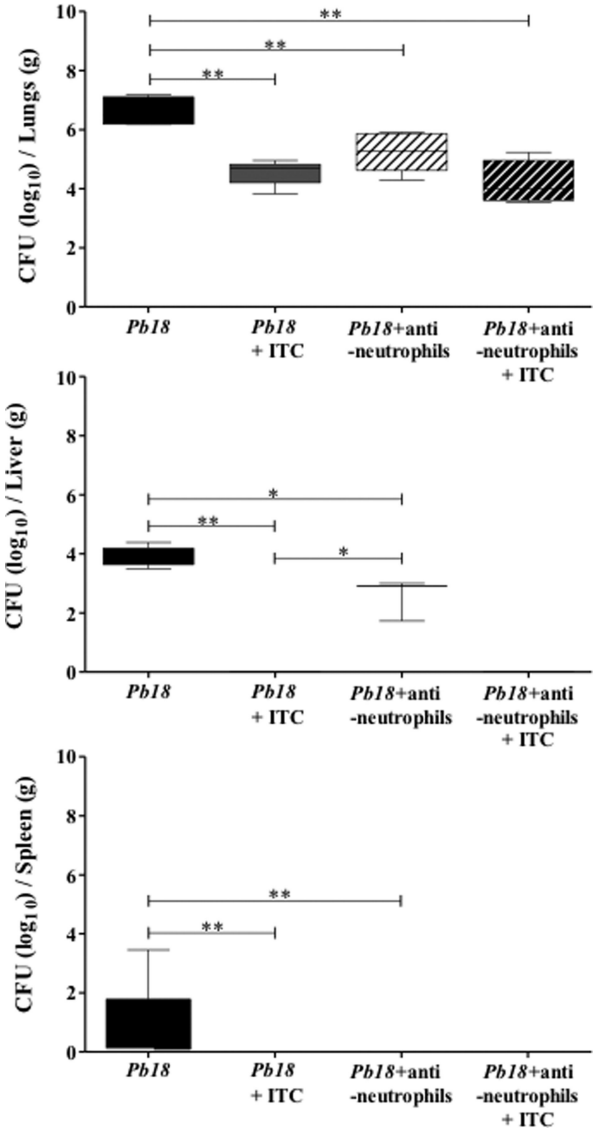

Figure 2. ITC and mAb specific to neutrophils treatment reduced the fungal burden in lungs, liver and spleen from mice infected with *P. brasiliensis* at the chronic stages of infection. Fungal burden was measured in lungs, liver and spleen in mice infected with 1.5×10^6 *P. brasiliensis* (Pb18) yeast cells and treated with 1 mg of ITC contained in 100 μl of Sporanox® oral solution daily for 8 weeks and/or 200 μg of a mAb anti-neutrophils (Ly6G) contained in 200 μl intraperitoneally (i.p.) at doses every 48 h for 2 weeks; all treatments started at 4th week p.i. and analysis done at 12th week p.i. Data shown represent median and IQR ($n = 4$–5 mice/group; representative of two independent experiments). $^{*}P < .05$ and $^{**}P < .01$ comparing infected-untreated mice versus ITC-treated, mAb-treated or ITC/mAb-treated and infected mice. *Pb18,* infected-untreated mice; *Pb18* + ITC, infected mice treated with itraconazole; *Pb18* + anti-neutrophils, infected mice treated with specific mAb anti-Ly6G (monoclonal antibodies anti-neutrophils).

reduction on fungal burden in lungs on those mice treated with ITC, mAb anti-Ly6G or combination of both therapies, but there was no difference between them (Fig. 2). Undetected fungal burden was noticed in liver and spleen of those mice treated with ITC (Fig. 2).

Effect of ITC and combined therapy ITC-mAb anti-Ly6G on collagen levels

We observed that soluble total collagen was significantly diminished only in those mice treated with the mAb anti-Ly6G alone or in combination with ITC, while ITC alone did not have any effect on collagen production in comparison with those infected untreated-mice (Fig. 3A). Of note, a significant increase of Col-1α2 and a significant reduction of Col-3α1 genes expression was observed on those mice treated with ITC, mAb anti-Ly6G or combination of both therapies in comparison with those infected untreated-mice (control group) (Fig. 3B, C). No difference was observed between treated groups.

ITC administration and combined therapy ITC-mAb anti-Ly6G alter the expression of mRNA levels of pulmonary fibrosis- and pro-inflammatory-related genes

We evaluated the expression of genes coding for TGF-β, matrix metalloproteinases (MMP), and tissue inhibitor metalloproteinases (TIMP), which are associated with pulmonary fibrosis development. We confirmed that the specific mAb to neutrophils reduced significantly the expression of TGF-β1, MMP-8, and TIMP-2, while increased the expression TGF-β3, MMP-12, and MMP-14 in comparison with those untreated infected-mice (Fig. 4). To highlight, for the first time we observed that ITC administration reduced significantly the expression of TGF-β1, MMP-1α, MMP-8 and MMP-13, while increased the expression of TGF-β3, MMP-12 and MMP-14 in comparison with those untreated infected-mice (Fig. 4). Interestingly, the combined therapy ITC/mAb anti-Ly6G decreased the expression of TGF-β1, TGF-β3, MMP-1α, MMP-8, TIMP-1 and TIMP-2 in an additive manner compared to those infected-ITC-treated mice or infected-neutrophil-depleted mice (Fig. 4).

Expression of genes coding for pro-inflammatory and anti-inflammatory molecules was also evaluated. Thus, we observed that the specific mAb to neutrophils increased significantly the expression of IL-6 and TNF-α (Fig. 5), while ITC treatment reduced significantly the expression of interferon (IFN)-γ, IL-6, IL-17, and IL-10 in comparison with those untreated infected-mice (Fig. 5). The combined treatment using the antifungal with the mAb specific to neutrophils reduced the expression of IL-1β, IL-6, IL-17, IL-10, and TNF-α in an additive manner compared to those infected-ITC-treated mice or infected-neutrophil-depleted mice (Fig. 5).

In addition, expression of genes encoding transcription factors related to immune patterns as follow: inducible nitric oxide synthase (iNOS) and T-bet (both Th1), arginase

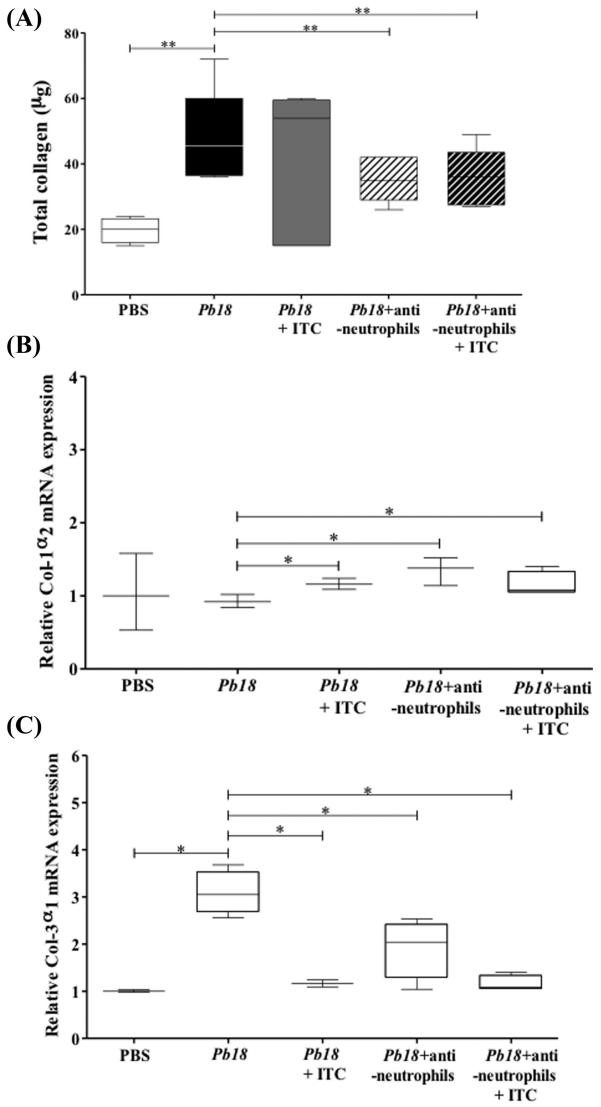

Figure 3. Effect of ITC in combination with mAb anti-Ly6G on collagen levels in lungs of mice infected with *P. brasiliensis*. BALB/c mice were infected with 1.5×10^6 *P. brasiliensis* (Pb18) yeast cells and treated with 1 mg of ITC contained in 100 µl of Sporanox® oral solution daily for 8 weeks and/or 200 µg of a mAb anti-neutrophils (Ly6G) contained in 200 µl intraperitoneally (i.p.) at doses every 48 h for 2 weeks; all treatments started at 4th week post-infection (p.i.) and analysis done at 12th week p.i. Total soluble collagen (A) and relative quantification of mRNA expression for Collagen-1 (*Col-1α 2*) (B) and Collagen-3 (*Col-3α 1*) (C) were assessed in lungs of mice ($n = 4$–5 mice/group; representative of two independent experiments). Results are expressed as median and IQR. $^*P < .05$ and $^{**}P < .01$ comparing infected-untreated mice versus ITC-treated, mAb-treated or ITC/mAb-treated and infected mice. *Pb18,* infected-untreated mice; *Pb18* + ITC, infected mice treated with itraconazole; *Pb18* + anti-neutrophils, infected mice treated with specific mAb anti-Ly6G (monoclonal antibodies anti-neutrophils).

(Arg)-1 and GATA-3 (both Th2), Spi-1 (Th9), RORc (Th17), Ahr (Th22), and FoxP3 (Treg) were determined. We confirmed that the depletion of neutrophils increased significantly the expression of iNOS and T-bet genes, while reduced the expression of GATA-3, RORc, Ahr, and FoxP3 genes in comparison with those untreated infected-mice (Fig. 6). ITC administration reduced significantly the expression of genes coding for iNOS, T-bet, Arg-1, GATA-3, Spi-1, RORc, Ahr, and FoxP3 in comparison with those untreated infected-mice (Fig. 6). The administration of ITC in combination with the mAb anti-Ly6G reduced the expression of GATA-3, RORc, and Ahr genes in an additive manner compared to those infected-ITC-treated mice or infected-neutrophil-depleted mice (Fig. 6).

Effect of ITC in combination with the mAb anti-Ly6G on the granulomatous inflammatory response in lungs of mice infected with *P. brasiliensis*

In order to evaluate the effect of the therapeutic strategy using the combination of ITC and a mAb specific to neutrophils in the pulmonary parenchyma (*in situ*), lungs were processed for histopathological analysis as described in the Materials and Methods section. We observed that the lungs of untreated infected-animals showed an extend granulomatous cellular infiltrated with abundant *P. brasiliensis* yeast cells surrounded by collagen fibers (Fig. 7B–C); in contrast, the lungs of those mice treated with the ITC significantly reduced fungal burden, inflammatory area and collagen fibers (Fig. 7E–F); therefore, the administration of the mAb anti-Ly6G reduced the inflammatory response, fungal structures and the fibrotic sequela more markedly (Fig. 7H–I). No differences in the histopathological analysis were observed between those mice treated with the combined therapeutic strategy (ITC-anti-Ly6G) and those animals treated only with the mAb anti-Ly6G (Fig. 7K–L).

Discussion

The chronic form of paracoccidioidomycosis (PCM) is characterized by an inflammatory and granulomatous response, which can promote the development of fibrosis and other pulmonary alterations in a substantial number of patients.[17] Despite the clinical manifestations and auscultatory findings related to this entity are minor, the radiologic abnormalities are extensive, this fact is associated with a silent course of the disease, which results in a late consultation, and in the majority of cases, with an irreversible damage of the lungs in those patients.[8] Itraconazole (ITC) is considered the therapeutic agent of choice for treating PCM due that it requires shorter courses of administration, is associated with fewer secondary effects, is effective in 95% of the patients, and relapses occur in < 5%[5,18–20]; however, the development of the fibrotic sequelae does not appear to be modified by this antifungal treatment.[7,8]

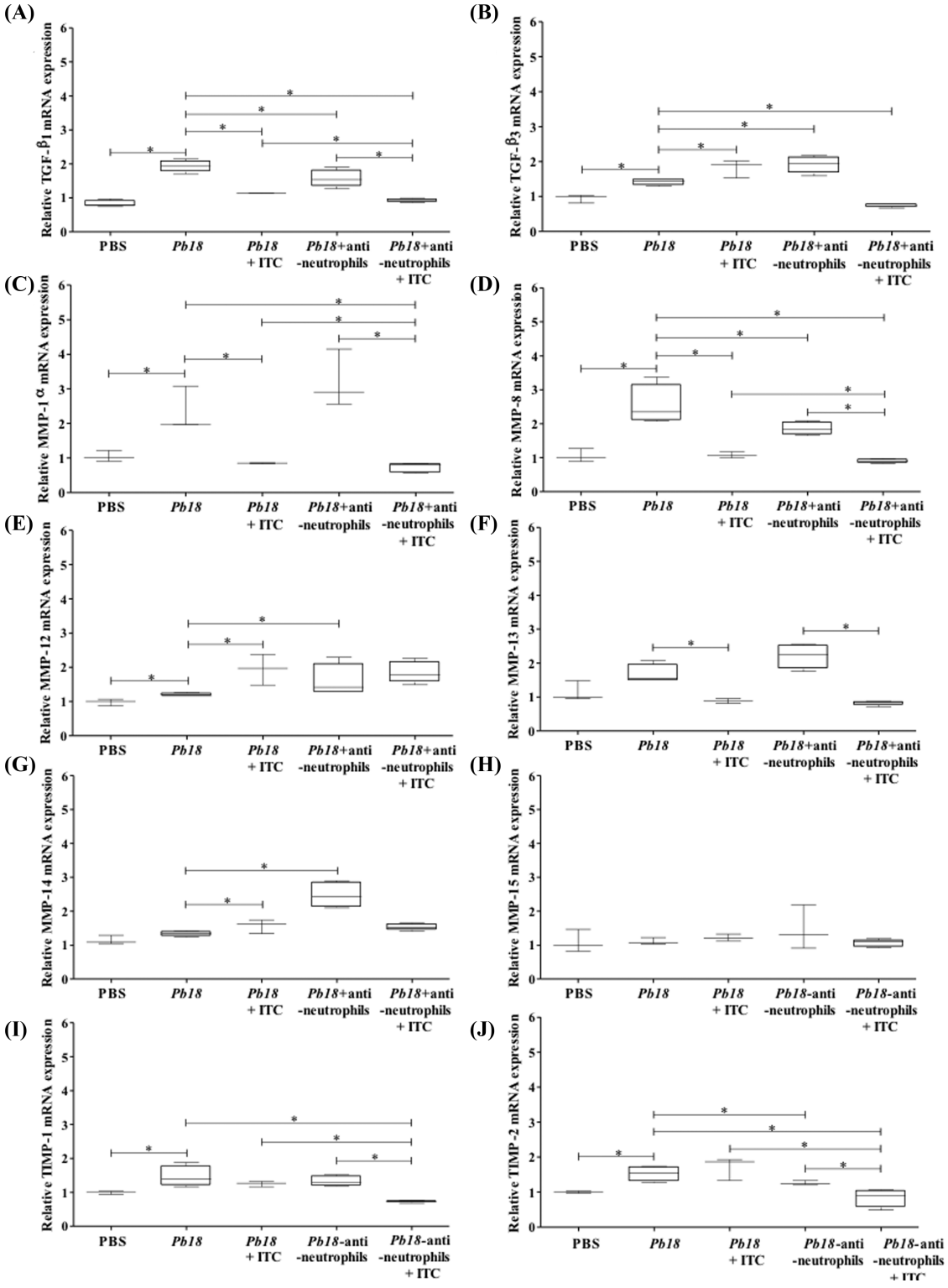

Figure 4. Effect of ITC and mAb anti-Ly6G combined therapy on mRNA expression of pulmonary fibrosis-related genes. BALB/c mice were infected with 1.5×10^6 *P. brasiliensis* (Pb18) yeast cells and treated with 1 mg of ITC contained in 100 μl of Sporanox® oral solution daily for 8 weeks and/or 200 μg of a mAb anti-neutrophils (Ly6G) contained in 200 μl intraperitoneally (i.p.) at doses every 48 h for 2 weeks; all treatments started at 4th week post-infection (p.i.) and analysis done at 12th week p.i. Relative quantification of mRNA expression for TGF-β1 (A), TGF-β3 (B), matrix metalloproteinase (MMP)-1$^{\alpha}$ (C), MMP-8 (D), MMP-12 (E), MMP-13 (F), MMP-14 (G), MMP-15 (H), tissue inhibitor metalloproteinase (TIMP)-1 (I), and TIMP-2 (J) were performed. Data are expressed as median and IQR (n = 4–5 mice/group; representative of two independent experiments). $^*P < .05$ comparing infected-untreated mice versus ITC-treated, mAb-treated or ITC/mAb-treated and infected mice. *Pb18*, infected-untreated mice; *Pb18* + ITC, infected mice treated with itraconazole; *Pb18* + anti-neutrophils, infected mice treated with specific mAb anti-Ly6G (monoclonal antibodies anti-neutrophils).

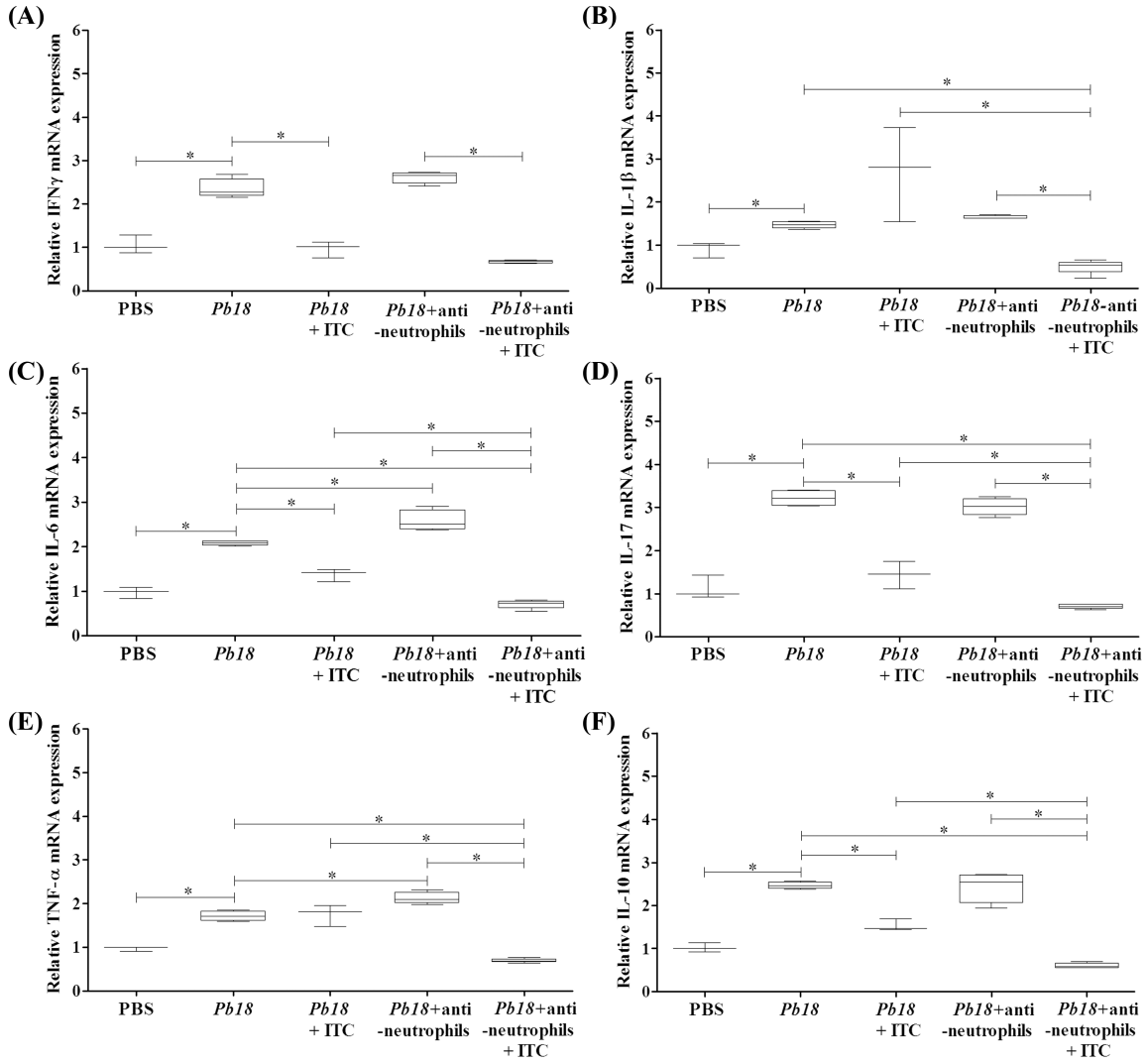

Figure 5. Effect of ITC and mAb anti-Ly6G combined therapy on mRNA expression of pulmonary pro-inflammatory cytokines-related genes. BALB/c mice were infected with 1.5 × 10^6 *P. brasiliensis* (Pb18) yeast cells and treated with 1 mg of ITC contained in 100 μl of Sporanox® oral solution daily for 8 weeks and/or 200 μg of a mAb anti-neutrophils (Ly6G) contained in 200 μl intraperitoneally (i.p.) at doses every 48 h for 2 weeks; all treatments started at 4th week post-infection (p.i.) and analysis done at 12th week p.i. Relative quantification of mRNA expression for IFNγ (A), IL-1$^{\beta}$ (B), IL-6 (C), IL-17 (D), TNF-$^{\alpha}$ (E), and IL-10 (F) were evaluated. Data are expressed as median and IQR (n = 4–5 mice/group; representative of two independent experiments). *$P < .05$ comparing infected-untreated mice versus ITC-treated, mAb-treated or ITC/mAb-treated and infected mice. *Pb18,* infected-untreated mice; *Pb18* + ITC, infected mice treated with itraconazole; *Pb18* + anti-neutrophils, infected mice treated with specific mAb anti-Ly6G (monoclonal antibodies anti-neutrophils).

ITC acts inhibiting the fungal lanosterol 14α-demethylase (Erg11), which in turn blocks the synthesis of ergosterol (an essential component of fungal cell membrane) and finally leads to fungal death.[21,22] Interestingly, more recently, it has been reported that in addition to the antifungal effect, ITC exhibits immunomodulatory properties; thus, ITC alone or in combination with LPS induced the expression and production of pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α by human monocyte THP-1 cells line.[3] In this direction, Naranjo et al.[23] using a murine model of chronic pulmonary PCM demonstrated that ITC not only reduces the fungal burden but also influences the immune response decreasing the production of some pro-inflammatory cytokines and changing the cellular infiltrate composition. Herein, we described that ITC alone or in combination with the mAb anti-neutrophils (mAb-Ly6G) exerts immunomodulatory effects *in vivo* in mice infected with *P. brasiliensis*; thus, this combined therapy had a beneficial effect on chronic stages of pulmonary PCM allowing not only the control of infection but also the reduction of the inflammatory response and the fibrotic sequelae through the down-regulation of gene expression associated with both inflammation and fibrosis process. We observed that ITC decreased significantly the expression of IL-6,

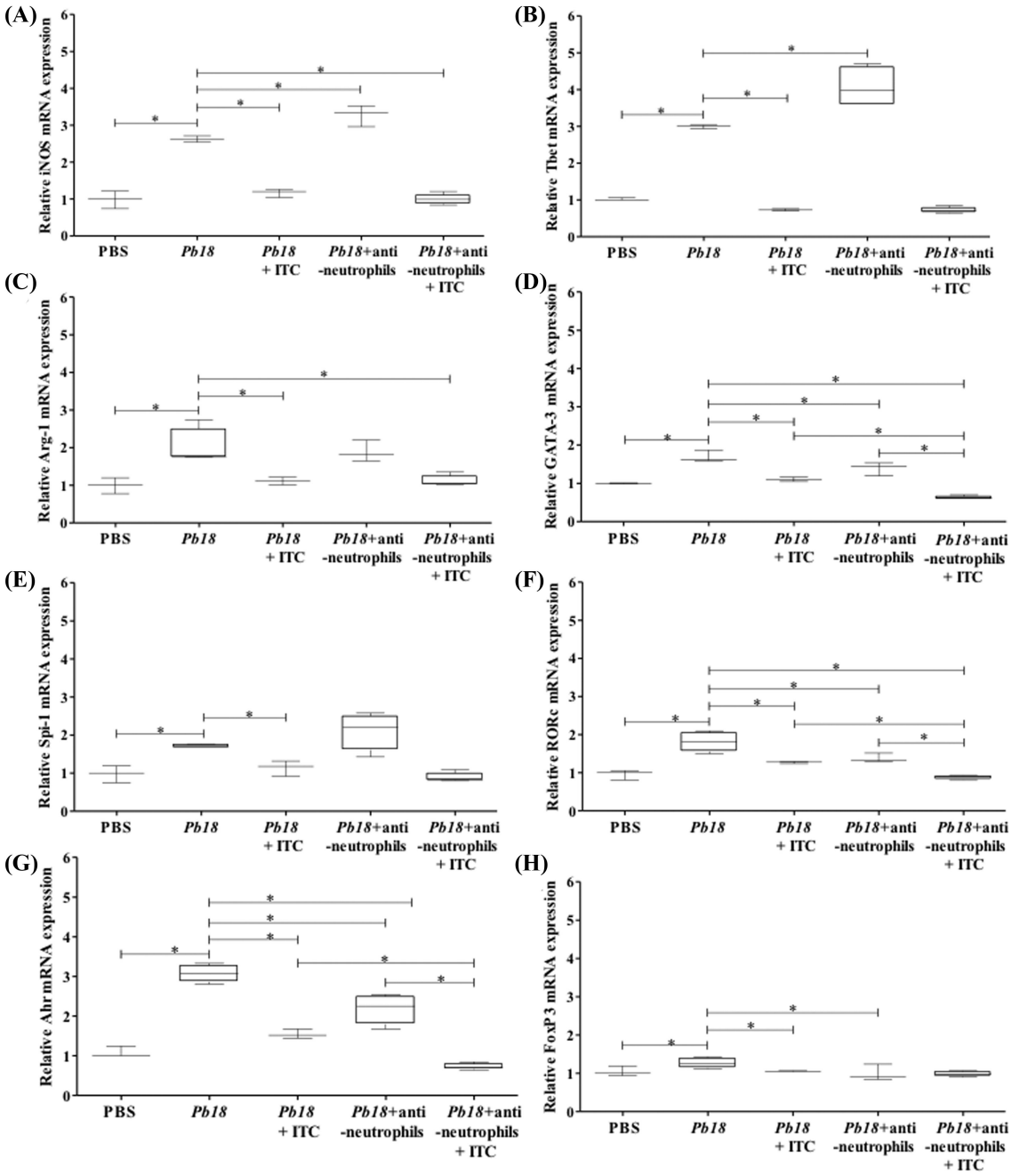

Figure 6. Effect of ITC and mAb anti-Ly6G combined therapy on mRNA expression of transcription factors genes-related to immune patterns. BALB/c mice were infected with 1.5×10^6 *P. brasiliensis* (Pb18) yeast cells and treated with 1 mg of ITC contained in 100 μl of Sporanox® oral solution daily for 8 weeks and/or 200 μg of a mAb anti-neutrophils (Ly6G) contained in 200 μl intraperitoneally (i.p.) at doses every 48 h for 2 weeks; all treatments started at 4th week post-infection (p.i.) and analysis done at 12th week p.i. Relative quantification of mRNA expression for inducible nitric oxide synthase (iNOS) (A), T-bet (B), arginase (Arg)-1 (C), GATA-3 (D), Spi-1 (E), RORc (F), Ahr (G), and FoxP3 (H) were analyzed. Data are expressed as median and IQR (n = 4–5 mice/group; representative of two independent experiments). $^*P < .05$ comparing infected-untreated mice versus ITC-treated, mAb-treated, or ITC/mAb-treated and infected mice. *Pb18*, infected-untreated mice; *Pb18* + ITC, infected mice treated with itraconazole; *Pb18* + anti-neutrophils, infected mice treated with specific mAb anti-Ly6G (monoclonal antibodies anti-neutrophils).

IL-10, IL-17, IFNγ, iNOS, Arg-1, T-bet, GATA-3, RORc, FoxP3, Spi-1, Ahr, TGF-β1, Col-3α1, MMP-1α, MMP-8, and MMP-13 genes; moreover, the combined therapy ITC plus a mAb anti-neutrophils (mAb anti-Ly6G) decreased the expression of IL-1β, IL-6, IL-10, IL-17, TNF-α, GATA-3, RORc, Ahr, TGF-β1, TGF-β3, MMP-1α, MMP-8, TIMP-1, and TIMP-2 genes in an additive manner when compared to those mice treated only with either ITC or mAb anti-neutrophils. On the same token, we recently reported that depletion of neutrophils using the mAb anti-Ly6G was

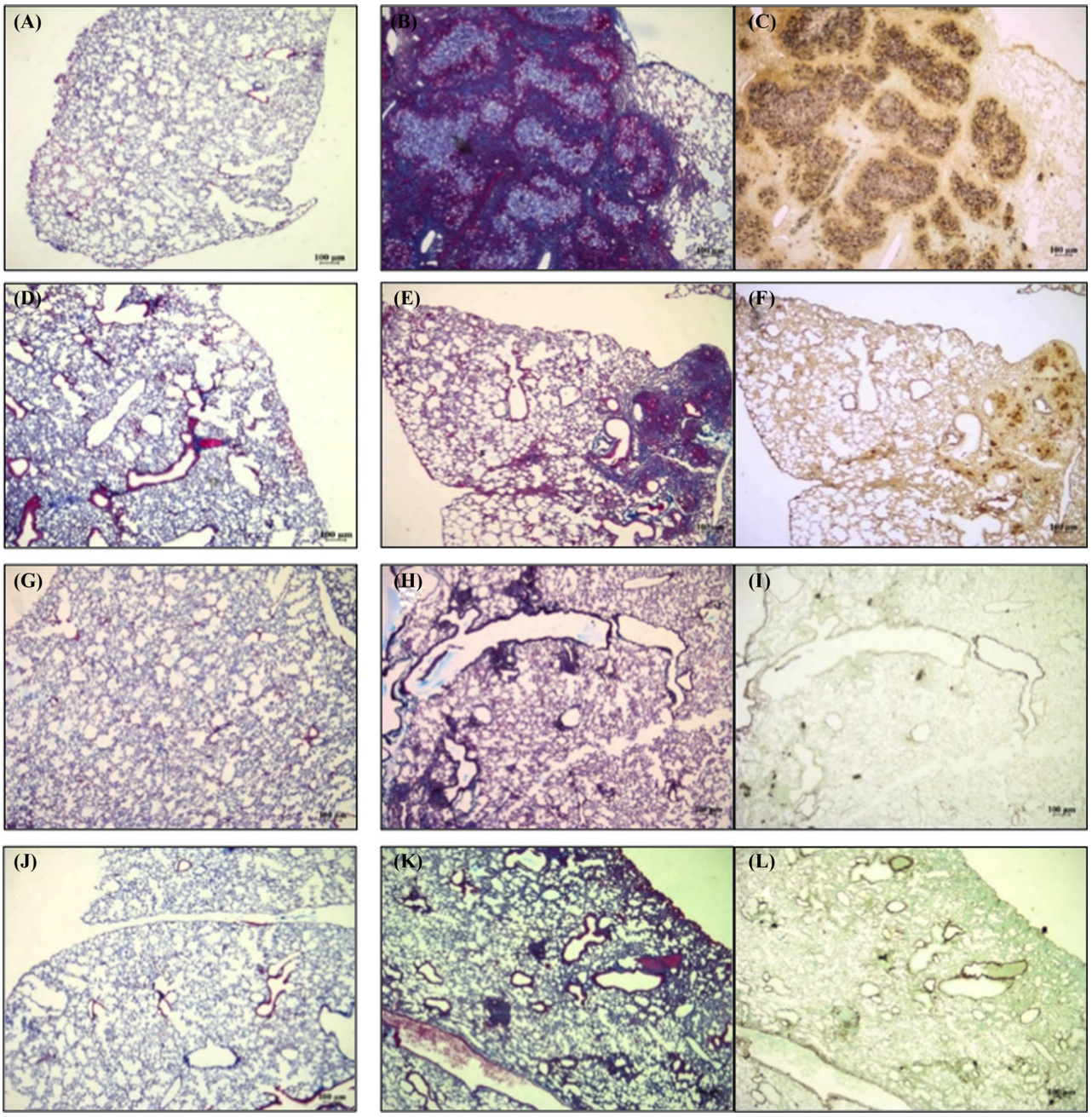

Figure 7. ITC and mAb anti-neutrophils reduce the granulomatous inflammatory response and fibrosis in lungs of mice infected with *P. brasiliensis.* BALB/c mice were infected with 1.5×10^6 *P. brasiliensis* (Pb18) yeast cells and treated with 1 mg of ITC contained in 100 μl of Sporanox® oral solution daily for eight weeks and/or 200 μg of a mAb anti-neutrophils (Ly6G) contained in 200 μl intraperitoneally (i.p.) at doses every 48 h for 2 weeks; all treatments started at 4th week post-infection (p.i.) and analysis done at 12th week p.i. Microphotographs are representative of lungs tissue from uninfected-untreated (A), uninfected-ITC treated (D), uninfected-mAb treated (G), uninfected-ITC/mAb-Ly6G treated (J), infected-untreated (B, C), infected-ITC treated (E, F), infected-mAb-Ly6G treated (H, I) and infected ITC-mAb-Ly6G treated (K, L) mice ($n = 4$–5 mice/group). Lungs were fixed, embedded in paraffin, cut, and stained using Masson's trichrome to determine lung inflammatory response and fibers collagen (A, B, D, E, G, H, J, K) and methenamine silver staining (C, F, I, L) to identify *P. brasiliensis* yeast cells as described in the Methods section. Magnification 10 ×.

associated with a significant decrease in the levels of several pro-inflammatory cytokines, including IL-17, TNF-α, and TGF-β1, levels of collagen, expression of TIMP-2 and MMP-8 genes as well as a high expression of TGF-β3, MMP-12, and MMP-14, accompanied of control of infection and reduced fibrosis.[14] In addition, it has been described that prevalence of Th17 immunity is associated with tissue damage in lungs of mice infected with *P. brasiliensis*;[24] in this study, we observed a reduced expression of IL-17 and RORc (its transcription factor) gene expression, confirming the important role of this immunity pattern in the development of tissue pathology. Of note, we previously

reported that IL-10 significantly increases after neutrophil depletion during the early stages of *P. brasiliensis* infection, fact that was associated with a worsening of the disease; thus, anti-inflammatory cytokine appears to modulate in a negative way the innate immune response, possibly dampening the antifungal effect exerted by activated macrophages and subsequently worsening the disease outcome.[15] In the present study, we observed a reduced expression of IL-10 associated with control of infection and diminished inflammatory response; this result clearly indicates that neutrophils type II are no the main source of IL-10.

On the other hand, it has been demonstrated that neutrophil plays a dual role in experimental PCM, being beneficial during the early stages and detrimental during the chronic course of *P. brasiliensis* infection.[14,15] Additionally, some studies have demonstrated the presence of different subsets of neutrophils during the development of the immune response, each associated with different immunological functions and biological properties.[25–27] Tsuda et al. postulated that there are at least two different subsets of murine neutrophils. The first subset (type I neutrophils) is related to a pro-inflammatory response with IL-12 and iNOS production; in contrast, type II neutrophils are associated to an anti-inflammatory response with IL-10 and Arg-2 production.[28] In this study, it was observed a greater number of type II neutrophils, which could be related to the incapacity of the host to control *P. brasiliensis* infection without the appropriated treatment. Of note, some authors have described the existence of other populations cells $Gr1^{+}CD11b^{+}$ (myeloid-derived suppressor cells [MDSc]) associated with a Th2-profile and immunosuppressive activities,[29] which has also been found increased during the *P. brasiliensis* infections,[14] and since both cell populations could be confused, it may be possible that a part of the active type II neutrophils being a populations of granulocyte-lineage immature myeloid cells. Surprisingly, ITC increased the number of type II and decreased the number of the type I neutrophils even in those neutrophil-depleted and *P. brasiliensis*-infected mice. Despite little is known about the interaction between ITC and neutrophils, *in vitro* studies have suggested that azoles could exert an effect on fungicidal activity and other biological functions of these phagocytic cells.[30–34] Likewise, other studies have associated the Th2-like response with the development of pulmonary fibrosis;[35,36] thus, we could hypothesize that the presence of type-II neutrophils, increased by ITC therapy, could encourage the appearance and perpetuation of fibrosis, as seen in patients with PCM even after treatment with the antifungal.[8] Nonetheless, ITC did not increase o altered the production of collagen in lungs of mice infected with *P. brasiliensis*, by the contrary ITC alone or in combination with the mAb anti-neutrophils decreased the expression of important genes related to the development of fibrosis.

As described above, we previously demonstrated that usage of mAb-Ly6G during the chronic stages of PCM promotes a better control of infection and decrease the granulomatous inflammatory response and fibrosis through down-regulation of genes associated with tissue remodeling process and fibrosis including MMPs and TIMPs.[14] Then, we hypothesize that combination ITC and mAb-Ly6G therapy prevents or reduces the fibrosis and other pulmonary alterations through down-regulation of genes coding for metalloproteinases and their inhibitors (MMP-1α, MMP-8, TIMP-1, TIMP-2, and Col-3α1), as well as for pro-inflammatory cytokines (IL-1β, IL-6, IL-17, TNF-α, TGF-β1, TGF-β3) and specific Th-related transcription factors [GATA-3 (Th2), RORc (Th17) and Ahr (Th22)], all of them associated with the development of an inflammatory response. Previous reports have described the importance of pro-inflammatory pattern for the granuloma formation and the development of fibrosis.[37–39]

Overall, these findings suggest that the combined therapy ITC plus the mAb anti-neutrophil reduce the fibrotic sequelae induced by *P. brasiliensis* infection through down-regulation of pro-inflammatory and pro-fibrotic genes in an additive manner. Furthermore, one of the most important issues in this study was the confirmation that ITC exert immunomodulatory effects *in vivo*. The present work open the door for implementing new therapy strategies using antimicrobials in combination with new biological agents such as mAbs or other molecules capable to enhance or modulate the immune response.

Supplementary material

Supplementary data are available at *MMYCOL* online.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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