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Research Article

Schistosomiasis causes remodeling of pulmonary vessels in the lung in a heterogeneous localized manner: Detailed study

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ABSTRACT

Schistosomiasis is a global parasitic disease with high impact on public health in tropical areas. Schistosomiasis is a well-described cause of pulmonary arterial hypertension (PAH). The exact pathogenesis is still unclear, though inflammatory mechanisms are suspected. Another unknown is whether the changes in the pulmonary vasculature are generalized or localized. We studied 13 mice infected with cercariae for 12 weeks compared with 10 control mice. In our model, we observed that the liver was a target during infection and was enlarged more than two-fold after infection. However, right heart hypertrophy as measured by RV/(LV + S) ratio was not observed at this time point. Moreover, we noticed that 72% of the sampled lobes (92% of the lungs) harvested from these animals costained evidence of granulomatous changes, secondary to egg deposition. We systemically mapped the distribution of granulomatous lesions in right lung lobes (n = 43) of infected mice. We observed that the distribution of the granulomatous lesions was heterogeneous. Remodeled pulmonary vessels were seen in 26% of the lobes (46% of the lungs) and were observed only in close proximity to the granuloma. No remodeling was observed in the absence of granulomas. These findings support the view that pulmonary vascular remodeling is caused by the local presence of granulomas in PAH associated with schistosomiasis. The heterogeneous nature of the remodeling partly explains why many patients with schistosomiasis do not develop pulmonary hypertension.

Key Words: schistosomiasis, pulmonary hypertension, inflammation, experimental models

Schistosomiasis is a disease caused by trematode worms of genus Schistosoma. The main species that infect humans are S. mansoni, S. japonicum, and S. hematobium.[1-3] The disease mostly occurs in poor or rural populations. An estimated 200 million people are believed to be infected, with 85% of these people in sub-Saharan Africa. Additionally, more than 600 million are believed to be at risk in other subtropical countries in Latin America and Asia.[4-6] The pathology of schistosomiasis includes an inflammatory and immunological response followed by granuloma formation. Granulomas are a result of antigenic reaction generated mainly by the Schistosoma egg.[7,8-14] These inflammatory changes cause periportal fibrosis in the liver and may result in portal hypertension.[3,13,15-18]

Pulmonary arterial hypertension (PAH) is a rare progressive pulmonary disorder usually associated with right heart failure and vascular remodeling. PAH

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is characterized by elevation in the mean pulmonary arterial pressure above 25 mmHg at rest, which may be the result of pulmonary vasoconstriction, remodeling, and/or thrombosis of small pulmonary arteries.[19-22] Inflammation is one of the hallmarks of PAH[22-25] and causes activation of circulating monocytes, neutrophils, and macrophages, but is also observed in pulmonary vascular fibroblasts and endothelial and smooth muscle cells (SMCs). Those cells release inflammatory mediators such as cytokines, chemokines, and growth factors which activate a number of inflammatory pathways leading to cell proliferation and migration as well as extracellular matrix deposition.[26-28]

Among the many models of PAH which have been described[29-33] is the Schistosoma-infected murine model.[34-36] However, while the monocrotaline rat and hypoxic mice models are well-established, the Schistosoma mice models and the associated vascular changes appear to be completely different. Initial observation suggested that the pattern of pulmonary hypertension was not severe in some models. We hypothesized that this may due to the patchy nature and unequal distribution of granulomas and inflammation caused by Schistosoma eggs in the lung.

In this study, we explore this phenomenon at 12 weeks postinfection with S. mansoni by assessing the distribution of granulomas and their relationship to the pulmonary vasculature.

MATERIALS AND METHODS

Animals

A Belo Horizonte strain of S. mansoni was used in all experiments. Female standard outbred BKW adult mice were infected with a suspension containing 100 cercariae (maintained at the Natural History Museum, London, UK). Briefly, mice were paddled in warm snail water for approximately 30 minutes. All the animals were sacrificed after 12 weeks postinfection including the control noninfected mice. All protocols and surgical procedures were approved and carried out by the qualified personnel at the Natural History Museum, London, UK.

Tissue preparation

Lung. The right lungs (all four lobes: superior, middle, and postcaval) were washed with saline followed by 4% formaldehyde infusion into the trachea. Next, lungs were removed and placed in 4% formaldehyde for 24 hours. After this time, they were placed in 1 × PBS (phosphate buffered saline) for 24 hours, followed by dehydration and paraffin embedding. The left lung was washed with saline and frozen at -80°C for future investigations.

Liver. The liver was removed, washed with saline, weighed to determine the degree of liver enlargement, and frozen at -80°C.

Heart. The heart was removed, washed with saline, and the right ventricle free wall was dissected from the left ventricle and septum for right ventricular hypertrophy index measurements. They were weighed separately and the ratio was determined. The right ventricular index was determined by measuring the ratio RV/(LV+S), where RV is right ventricle, LV is left ventricle, and S is septum. Afterward, the hearts were stored at -80°C.

Immunohistochemistry

Right lung lobes were dehydrated (in increasing ethanol concentration starting from 20% up to 100% and followed by xylol). The four lobes were carefully separated and each lobe was embedded in paraffin (as described previously[36,37]). The lobes were cut into slices (5 µm thickness). The orientation of each lobe was considered during each slicing process, and the slices were organized sequentially. Every 5th section was selected, labelled, and stained (Fig. 1). Sections were costained with anti-α smooth muscle antibody (α-SMA) for SMC detection (Sigma Aldrich, UK; 1:500 dilution) and anti-von Willebrand factor (vWF) antibody for endothelial cell detection (Dako Cytomation, UK; 1:1250 dilution). Staining was developed using a MOM Immunodetection Kit (Vector Laboratories, USA) for α-SMA and an ImmPRESS reagent anti-rabbit (Vector Laboratories, USA) for vWF, according to the manufacturer's instructions.

Lung mapping and pulmonary vascular morphometry

Every 5th slice was examined thoroughly for the presence of granulomas, eggs, worms, and changes in the pulmonary vessels (Fig. 1). The location and size of each granuloma was carefully located and measured for size and thickness. Likewise, the vessel changes were located and measured for size and muscularization. As previously described,[29] for morphometrical analysis of pulmonary vessels, a computerized morphometric analyzing system was used (Leica QWin Standard Analyzing Software; Leica Microsystems GmbH). Slides were analyzed by light microscopy in a blinded manner. Extension of smooth muscle cells into normally nonmuscular arterioles of the alveolar wall and alveolar duct was assessed. Briefly, pulmonary vessels of each animal were counted and noted as muscular, partially muscular, or nonmuscular. To assess the degree of muscularization, the amount of α-SMA positive-vessel wall area was determined. Nonmuscular arterioles were detected by the endothelial anti-vWF. Arteries that contained 70% α-actin positive-vessel wall area were set as muscular; arteries with 4% of α-actin positive-vessel area were set as nonmuscular. Arteries

that contained 4-70% of α-actin positive-vessel area were defined as partially muscularized.

Statistical analysis

Data are presented as the mean ± standard error of the mean. Statistical comparisons between two populations were performed using paired and unpaired Student's *t*-tests where appropriate with a probability value of $P < 0.05$ considered to be significant.

RESULTS

A total of 10 control animals and 13 infected animals were investigated. A total of 43 lobes of the right lung of infected animals were available for full evaluation, and the remaining nine lobes were not available for the study due to damage during processing or the orientation could not be determined.

The changes in the liver and heart after schistosoma infection

There was a significant increase in liver weight in 12 weeks postinfection. The hepatic weight increased from 1,437 ± 532 mg in control to 4,606 ± 566 mg in infected livers ($P < 0.0001$; Fig. 2A).

There was no significant difference in the heart weight ratio (RV/[LV+S]). The ratio was 0.276 ± 0.032 in control versus 0.297 ± 0.017 ([P = NS] not significant) in 12 weeks postinfected animals (Fig. 2B).

Granulomas, eggs, and adult worms presence in the lungs

In the lungs of 12-week infected animals, eggs were detected in 24 of 43 lobes (56%) and 10 of 13 lungs (76%). We detected a total of 136 granulomas but we did not detect eggs in every granuloma. However, eggs appeared to be trapped in the very distal portion or the small vessels and around which the granulomas developed. Interestingly, in the 12-week infected animal worms (most likely matured worms), eggs were seen in 8 of 43 lobes (4 of 13 lungs) (Fig. 3), although no granuloma or inflammatory cells were observed around the worm (Fig. 3). Most of the granulomas were located at the periphery of the lobes (Figures 1B and 3B and see later Figures 4C, D, and F). We investigated the deposition of granulomas in different lobes and did not see any differences in the number of granulomas in the different lobes (Fig. 5C). In the lungs of 12-week infected animals, granulomas were present in 92% of lungs studied (12 of 13 lungs) and 72% of the lobes (31 of 43 lobes) in 12-week postinfection (Fig. 5A). They were centered around eggs but not in close proximity to the adult worms. The average diameter of the granuloma was 279 ± 86 µm and ranged from 72 to 680 µm. A total of 77% of all

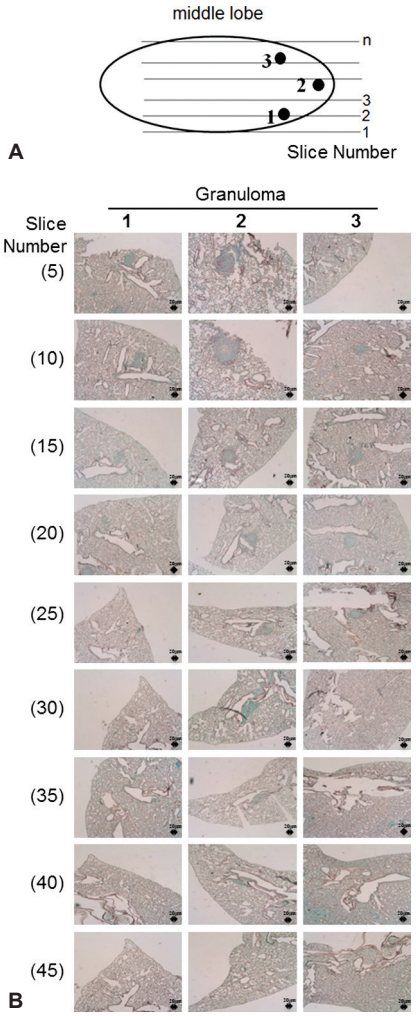

Figure 1: Lung mapping in 12 weeks *Schistosoma* infected mice. (**A**) Example of granuloma deposition and numbering in the single lobe and direction of cutting of the lobe. (**B**) Representative mapping of granulomas (labelled as 1, 2, and 3, respectively) and associated vessel changes in the single lobe of a single mouse. Scale bar = 20 µm.

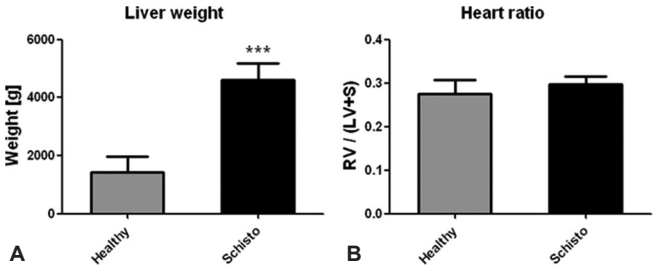

Figure 2: Effect of 12 weeks Schistosoma infection on liver weight and right heart hypertrophy. (**A**) Liver weight in the lungs 12 weeks postinfection. (**B**) The right ventricular index in the lungs 12 weeks postinfection as analyzed by measuring the ratio RV/(LV+S), where RV is right ventricle, LV is left ventricle, and S is septum. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus control.

detected eggs were present near medium-sized granulomas (200-400 µm diameter), whereas 12% were present near granulomas 0–200 µm in diameter. 11% were found near large granulomas (> 400 µm diameter; Fig. 4B).

Lung mapping

All lobes from the right lung (43 lobes from inferior, superior, middle, and postcaval) of 13 animals were mapped in respect to changes in granuloma formation, egg, and adult worm deposition, as well as vessel remodeling (Figures 1 and 6). In all of the lobes, granulomas were carefully labelled in each slide and their position was determined accordingly to the lobe orientation (Fig. 1). The presence of eggs and the status of the vessels were labelled carefully and systematically. Next, mapping was performed and examples displaying four representative lungs are shown in Figure 6. Results show that although granulomas were seen in all lobes, there was a heterogeneous distribution (Fig. 5C).

Remodeling of the vessels

Remodeling of pulmonary vessels (increased muscular thickness; Fig. 7) was seen in 28% of the lobes (10 of 43 lobes; 46% of the infected mice) and 6 of 13 lungs (Fig. 7A). Remodeled vessels were heterogeneous and in close proximity to granuloma formations.

We assessed the pulmonary vessels in each section. Remodeled vessels were assessed and muscularization was measured. No remodeling was observed in the small vessels (0-70 μm). In the medium vessels (70-150 μm), 16% were not remodeled, whereas 42% were partially muscularized, and 42% were fully muscularized. In the group of large vessels (> 150 μm), 40% were partially muscularized and 60% were fully muscularized (Fig. 7B). It was observed that all muscularized vessels were inside or in close proximity to the granuloma. No remodeled vessels were seen distant to granulomas.

Interestingly, in the 12-week infected animals, we found no relation between the size of the granuloma and the degree of associated vascular remodeling. However, there was a trend observed that remodeled vessels were mostly present near granulomas of 200-400 μm diameter (60% of all remodeled vessels observed), whereas only 34% could be found near small granulomas (0-200 μm diameter). Six percent were seen near large granulomas (> 400 μm diameter; Fig. 7C).

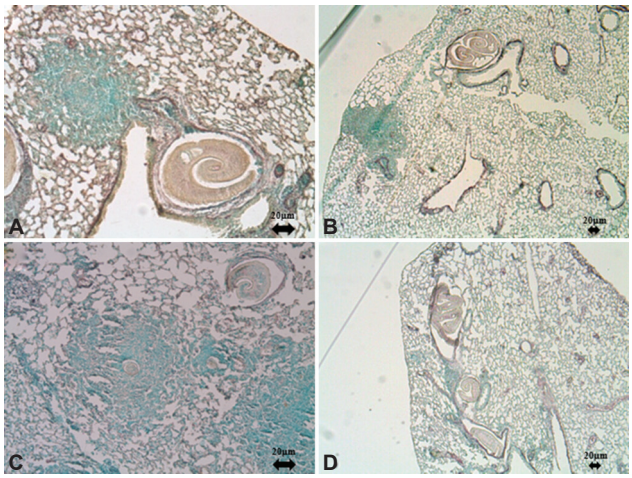

Figure 3: Adult worms in 12 weeks *Schistosoma* infected mice. Representative photomicrographs of lung sections. (**A, B**) Adult worms were localized near granuloma (brown stain), (**C**) egg surrounded with granuloma (green stain), (**D**) as well as alone. **B and D** are represented at original magnification 10×. A and C are represented at original magnification 20×. Scale bar = 20 μm.

DISCUSSION

Pulmonary hypertension remains a fatal disease with a poor prognosis.[6] Contemporary control of schistosomiasis is based upon the delivery of praziquantel (PZQ).[38,39] However, despite treatment with PZQ, which eliminates adult worms from patients, schistosomiasis remains a serious condition.[25,37,40-43] Therefore, it is imperative to establish an effective treatment for schistosomiasis and related pulmonary conditions. In schistosomiasis, the real incidence of pulmonary vascular diseases is not known, nor is the prevalence of pulmonary hypertension.[44,45] In Brazil, it was estimated that 30% of patients with symptoms of PH have associated schistosomiasis.[25] Moreover, it was recently found that the incidence of pulmonary

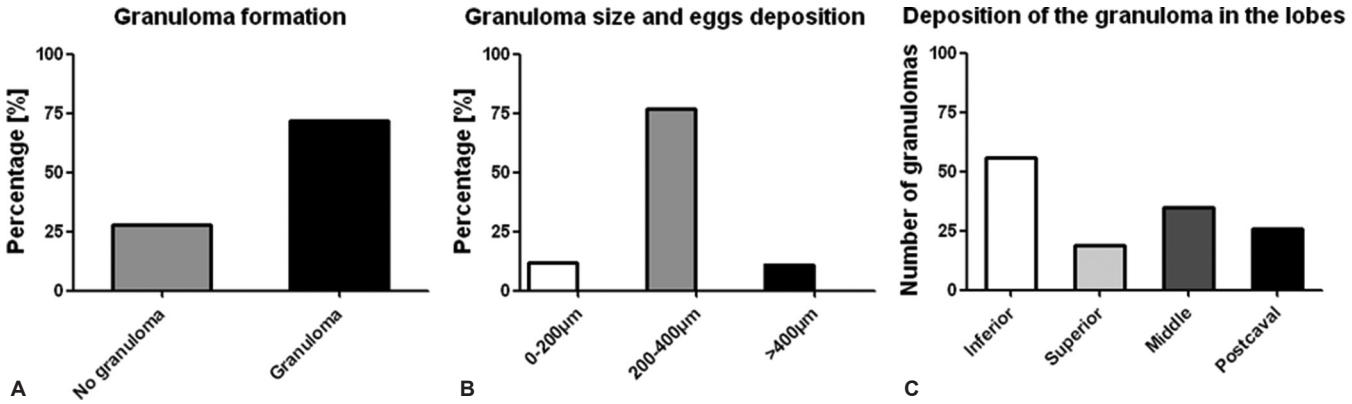

Figure 4: Granuloma formation, effect on eggs deposition and deposition in the lung in 12 weeks *Schistosoma* infected mice. **(A)** Incidence of granuloma formation by lung lobe, **(B)** Percentage of eggs deposition according to the size of granulomas, **(C)** Deposition of granuloma in the lobes of the right lung.

hypertension is about 10% among the patients with portal hypertension in Brazil.[25,46,47] It is also observed that schistosomiasis-associated PH is not severe in the majority of patients.[25] These observations lead us to question of the extent of pulmonary vascular remodeling due to Schistosoma infection.

Schistosomiasis-induced pulmonary hypertension has been observed in both patient and animal models.[16,34,35,37,41,43-45,48,49] Unlike other studies,[34,35] we used a natural way of infection by infecting via cercariae only, as we believe it most closely models human infection. Exposure of mice to S. mansoni cercariae for 12 weeks resulted in severe changes in those animals. Livers of the animals after the infection were significantly affected and enlarged more than two times the original size (Fig. 2A). The time frame required for these changes was only 12 weeks, which is similar to other reports.[34,50] Right heart hypertrophy was not observed as there was no change in heart weight ratio (RV/(LV+S)) during the 12-week time point (Fig. 2B), which was in line with reports by others.[34] We were not able to measure portal and pulmonary pressures in our experiments. However, previous studies have shown that mice infected with cercariae do develop portal hypertension. Moreover, in the natural environment of human infection with Schistosoma, liver changes are found along with lung involvement. In our study, we noticed that granulomas, the result of the inflammatory reaction induced by eggs, were present in 72% of the lungs studied, but remodeled pulmonary vessels were found in only

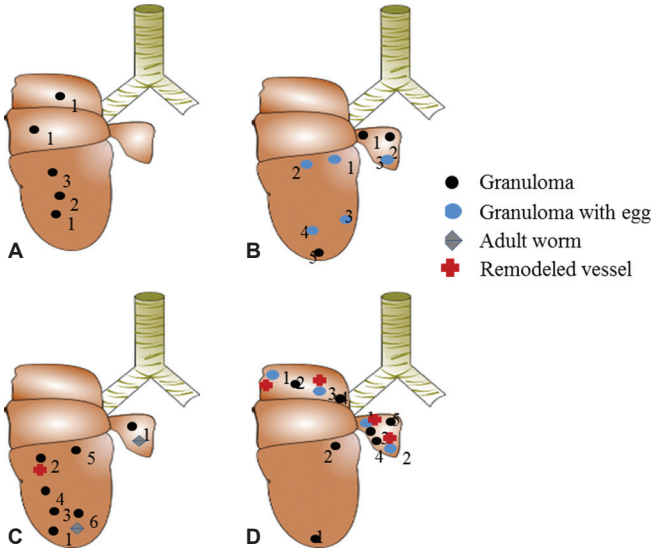

Figure 5: Anatomy of the mouse lung, granuloma deposition, adult worms and vessel remodelling in 2 example lungs in 12 weeks *Schistosoma* infected mice. The diagrams show there was a heterogeneous distribution of the granulomas.

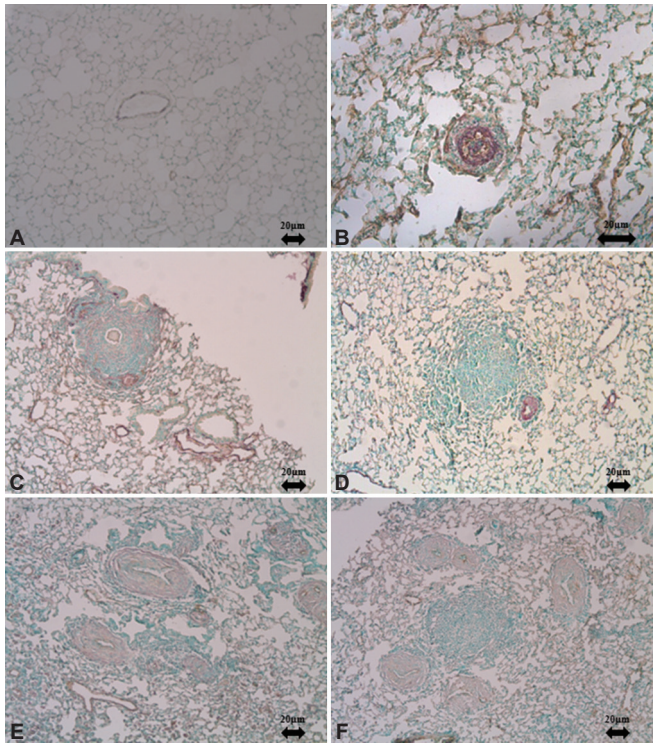

Figure 6: Vascular remodeling and inflammation in 12 weeks Schistosoma infected mice. Representative photomicrographs of lungs sections from **(A)** control mouse and **(B, C, D, E, F)** *Schistosoma* infected mice co-stained with alpha-smooth muscle actin and von Willebrand Factor. Negative staining (no primary antibody) revealed no signal (data not shown). **(A, C, D)** are presented at original magnification 10x, **(B, E, F)** are represented at original magnification 20x. Scale bar = 20

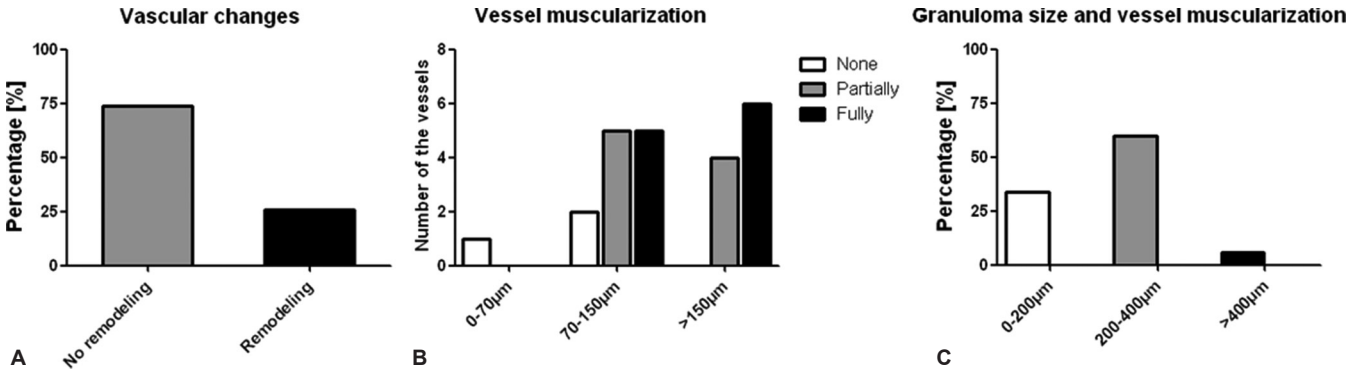

Figure 7: Vascular remodeling and effect of granuloma on vascular remodeling in 12 weeks *Schistosoma* infected mice. **(A)** Vascular remodeling, **(B)** muscularization of the vessels localized near granulomas, **(C)** correlation between granuloma size and vessel muscularization.

less than half of these lungs (28% of the lobes). Furthermore, remodeled vessels with various degrees of remodeling (Fig. 7B) were only seen adjacent to the granuloma and not in the rest of the lungs. In addition, we noticed that the granulomas were heterogeneously scattered throughout all lobes (Figures 4C and 5).

The inflammatory response in schistosomiasis is principally Th2-driven, characterized by an initial eosinophilia and granuloma formation with the involvement of macrophages and neutrophils. There is then subsequent downmodulation of the inflammatory response and a more fibrotic reaction predominates, with involution of older lesions and accumulation of fibrotic scars. In the case of S. mansoni, the eggs are laid in the mesenteric vessels. The eggs may be excreted, retained in the local tissue, or may enter into the hepatic-portal system. Within the host tissue, a granulomatous reaction is induced around the eggs. Granulomas consist primarily of macrophages, eosinophils, lymphocytes, and mast cells, with the proportion of cells differing in different organs. The cytokines that are stimulated include interleukin IL-13, IL-5, IL-4, IL-10, and RELM-*a* (resistin-like molecule alpha).[36,51] We believe that this inflammatory reaction can enhance the remodeling of pulmonary vessels adjacent to it. It was reported that inflammation has an effect on obliteration of the arteries.[34] The direct effect of inflammation on obliteration of the arteries was reported previously,[17,25,34,35,43,44,45,49] but none of these investigated whether the size of the adjacent granulomas had an effect.

Possible differences between humans and mouse models relate to exposure to cercariae. Usually, humans are exposed repeatedly during life, whereas our mouse models are exposed only once. This could influence degree and response to inflammation caused by the parasite and by the eggs.

We localized and detected remodeled vessels in close proximity to inflammatory response. Data suggest that this effect depends on granuloma presence itself, rather then the distance. We observed vascular changes in 5 of 13 lungs (38%) and 10 of 43 lobes (23%; Fig. 7A). Our observations revealed that no changes were detected when granulomas were not nearby. Moreover, the role of the size of the granuloma was not taken into consideration in any previous studies. Our more detailed study shows a trend between granuloma size and vascular changes. We observed that medium-sized granulomas (200-400 μm diameter) were more frequently present near remodeled vessels as compared to small granulomas (0-200 μm diameter), whereas no remodeled vessels were detected near large granulomas (> 400 μm diameter; Fig. 7C). Out of all 23 detected vessels, we did not observe remodeling in the small vessels (0-70 μm). While findings presented in our study confirm that the vascular remodeling is due to the presence of granuloma, it is conceivable that these vascular obstructions, resulting in increased blood pressure, could promote secondary vascular remodeling events.[30]

Interestingly, adult worms were found in the lungs of infected animals. To our knowledge, this is the first report demonstrating adult worm presence in the lung. However, presence of the worms was not correlated with granuloma formation, egg presence, or vessel changes. Potentially, the portal hypertension due to chronic preportal liver disease allowed the worms to migrate from the portal circulation to the systemic circulation by portosystemic shunts, and thus were observed in the lungs.

This study has three salient findings. First, cercariae infection and resulting inflammation cause remodeling of the vessels and this remodeling always occurs in close proximity to granulomas. Second, there was a trend in the size of granuloma and vessel changes, where remodeled vessels were most often observed to be located near medium sized granulomas. Third, adult worms can be present in the lungs of infected animals but are not associated with granuloma formation or vessel changes.

This study supports the major role of inflammation in vessel remodeling and to our knowledge it is the first study demonstrating mapping of the lobes in respect to granuloma and changed vessel localization.

In conclusion, the current study provides evidence for a physiologically important role for inflammation in a Schistosoma-induced model of PAH. We demonstrated that vascular remodeling occurs in our mouse model of S. mansoni infection, which we believe is closest to human infection. Distribution of pulmonary changes was not uniform and depends on the presence of granulomas. Egg deposition causing granuloma as well as granuloma localization and size appear to be crucial for vascular remodeling and may, therefore, be considered a potential main focus for treatment of the disease. The latter also potentially explains the lower incidence of clinical pulmonary hypertension in recent reports since the introduction of antihelminthic treatments such as PZQ.[44,45,49]

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