Transforming Growth Factor-β Signaling Promotes Pulmonary Hypertension Caused by Schistosoma Mansoni

Brian B. Graham, MD; Jacob Chabon, BS; Liya Gebreab; Jennifer Poole, MD; Elias Debella; Laura Davis, BS; Takeshi Tanaka, MD; Linda Sanders, BS; Nina Drocko; Angela Bandeira, MD; R. William Vandivier, MD; Hunter C. Champion, MD; Ghazwan Butrous, MB, ChB, PhD; Xiao-Jing Wang, MD; Thomas A. Wynne, PhD; Rubin M. Tuder, MD

Background—The pathogenetic mechanisms underlying pulmonary arterial hypertension resulting from schistosomiasis, one of the most common causes of pulmonary hypertension worldwide, remain unknown. We hypothesized that transforming growth factor-β (TGF-β) signaling as a consequence of Th2 inflammation is critical for the pathogenesis of this disease.

Methods and Results—Mice sensitized and subsequently challenged with Schistosoma mansoni eggs developed pulmonary hypertension associated with an increase in right ventricular systolic pressure, thickening of the pulmonary artery media, and right ventricular hypertrophy. Rho-kinase–dependent vasoconstriction accounted for ≈60% of the increase in right ventricular systolic pressure. The pulmonary vascular remodeling and pulmonary hypertension were dependent on increased TGF-β signaling, as pharmacological blockade of the TGF-β ligand and receptor, and mice lacking Smad3 were significantly protected from Schistosoma–induced pulmonary hypertension. Blockade of TGF-β signaling also led to a decrease in interleukin-4 and interleukin-13 concentrations, which drive the Th2 responses characteristic of schistosomiasis lung pathology. Lungs of patients with schistosomiasis-associated pulmonary arterial hypertension have evidence of TGF-β signaling in their remodeled pulmonary arteries.

Conclusion—Experimental S mansoni–induced pulmonary vascular disease relies on canonical TGF-β signaling. (Circulation. 2013;128:1354-1364.)

Key Words: hypertension, pulmonary ▪ schistosomiasis ▪ transforming growth factor beta

Pulmonary hypertension (PH) is a significant cause of morbidity and mortality in the developing world, largely as a result of schistosomiasis or long-term high-altitude exposure, which together affect ≈1 in 350 individuals. The trematode Schistosoma is the third most common parasitic infection worldwide, affecting >200 million people; ≈6.1% of individuals with recurrent chronic infection develop pulmonary arterial hypertension (PAH). Pathologically, pulmonary vascular disease caused by schistosomiasis shares the pulmonary vascular histopathology of other forms of World Health Organization group 1 PAH, including idiopathic PAH and connective tissue disease–associated PAH. This remodeling includes thickening of the media, neoplastic-like proliferation of endothelial cells in the intima, and inflammatory infiltrates in the adventitia. The shared pulmonary vascular remodeling in PAH suggests that, despite differences in origin, common aspects of pathogenesis promote schistosomiasis–induced PAH, idiopathic PAH, and connective tissue disease–associated PAH. Thus, insights into the pathogenesis of schistosomiasis–induced PAH not only may have profound impact on the disease worldwide but also may instruct the clinical management of PAH as whole.

The most prevalent species and the one particularly associated with PAH is Schistosoma mansoni. Schistosoma have an obligate 2-host lifecycle. The snail intermediate host releases cercariae into water, which penetrate the skin of susceptible animals (eg, humans, mice, and birds). After infecting individuals, the parasites mate in the vasculature of the gastrointestinal tract, releasing eggs that reach the intestinal lumen. A large percentage of the eggs are retained within the host, many of which are carried via the portal venous system into the portal triads. The initial reaction to Schistosoma is Th1 dominated as a result of worm-derived antigens, with eosinophils and neutrophils secreting tumor necrosis factor-α, interleukin (IL)-1, IL6, and interferon-γ. The chronic infection is characterized

Received April 5, 2013; accepted July 19, 2013.

From the Program in Translational Lung Research, Division of Pulmonary Sciences and Critical Care Medicine (B.B.G., J.C., L.G., J.P., E.D., L.D., T.T., L.S., N.D., R.W.V.) and Department of Pathology (X.-J.W.), Anschutz Medical Campus, Aurora, CO; Pulmonary Vascular Research Institute (B.B.G., A.B., H.C.C., G.B., R.M.T.); Memorial S. Jose Hospital, Universidade de Pernambuco in Recife, Recife, Brazil (A.B.); Division of Cardiology, University of Pittsburgh, Pittsburgh, PA (H.C.C.); School of Pharmacy, University of Kent, Kent, UK (G.B.); and Immunopathogenesis Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD (T.A.W.).

The online-only Data Supplement is available with this article at http://circ.ahajournals.orglookup/suppl/doi:10.1161/CIRCULATIONAHA.113.003072/-/DC1.

Correspondence to Brian B. Graham, MD, Division of Pulmonary Sciences and Critical Care Medicine, University of Colorado Denver, 12700 E 19th Ave, C-272, Aurora, CO 80045. E-mail brian.graham@ucdenver.edu

© 2013 American Heart Association, Inc.

Circulation is available at http://circ.ahajournals.org DOI: 10.1161/CIRCULATIONAHA.113.003072

1354
by Th2 inflammation, directed primarily against egg-derived antigens; in this context, cytokines, including IL4, IL5, IL10, and IL13, drive the formation of granulomas containing lymphocytes, fibrocytes, and macrophages. Once induced by Th2-CD4+ T cells, macrophages stimulated by Schistosoma infection become alternatively activated (M2 phenotype).

We hypothesized that transforming growth factor-β (TGF-β) signaling is necessary for the pathogenesis of experimental PH resulting from Schistosoma. We used pharmacological blockade of TGF-β signaling and mice lacking the canonical intracellular TGF-β signaling molecules Smad2 or Smad3 exposed to S mansoni, and we validated our findings with human tissue collected at autopsy from individuals who died exposed to S mansoni β intracellular TGF-β signaling molecules Smad2 or Smad3 blockade of TGF-β signaling and mice lacking the canonical.Treatment of schistosomiasis-associated PAH. In contrast to the lack of human tissue collected at autopsy from individuals who died of schistosomiasis-associated PAH. In contrast to the lack of pathogenic role of TGF-β in hepatic Schistosoma inflammation and fibrosis, our study documents that TGF-β signaling is involved in PH in mice and possibly humans resulting from exposure to S mansoni antigens.

Methods

Animals

Smad2−/− and Smad3−/− mice on an ICR×B129 background were provided by Chuxia Deng (National Institutes of Health). RELM-α−/− mice on a Balb/c background were provided by Regeneron Pharmaceuticals (Tarrytown, NY). The phenotypes of these transgenic models have previously been described.9–11 Because of the complex background of these genotypes, wild-type littermates of wild-type C57Bl6/J mice (and Balb-c mice to serve as controls to RELM-α−/− mice) were purchased from Taconic (Hudson, NY). All experimental procedures in rodents were approved by the Animal Care and Use committees at the University of Colorado Denver.

Treatments

S mansoni eggs were obtained from homogenized and purified livers of Swiss-Webster mice infected with cercariae, provided by the Biomedical Research Institute (Rockville, MD). Experimental mice were intraperitoneally and intravenously challenged with S mansoni eggs at the time points indicated at a dose of 240 eggs per 1 g body weight. SB431542 (Sigma-Aldrich), and SB203580 (LCLabs, Woburn, MA) were purposed intraperitoneally at the time points indicated in the figures at the following doses: SB431542, 4.2 µg·g−1·d−1; LY364947 (Sigma-Aldrich), and SB203580 (LCLabs, Woburn, MA) were purchased, reconstituted in dimethyl sulfoxide, diluted in PBS, and administered intraperitoneally at the time points indicated in the figures at the following doses: SB431542, 4.2 µg·g−1·d−1; LY364947, 1 µg·g−1·d−1; and SB203580, 20 µg·g−1·d−1. Control mice received the same volume of dimethyl sulfoxide diluted in PBS without the active drug. Monolonal mouse anti–pan-TGF-β antibody (clone 1D11) was purchased from American Type Culture Collection (Manassas, VA) and grown in a bioreactor by the University of Colorado Denver Tissue Culture Core Facility. 1D11 or control IgG (R&D Systems, Minneapolis, MN) was administered intraperitoneally at a dose of 0.5 µg/g every 3 days, as indicated in the figures. Mice were placed in a hypoxia chamber and administered 10% FiO₂ for 3 weeks, controlled by an oxygen sensor feedback loop (Pnoox 110, BioSpherix, Lacona, NY).

Assessment of PH

Measurement of the right ventricular pressure was performed as previously described.9 Mice were anesthetized with ketamine/xylazine and ventilated through a tracheal catheter. The abdominal and thoracic cavities were opened, and a 1F pressure-volume catheter (PVR-1035, Millar Instruments, Houston, TX) was placed through the right ventricle apex to transduce the pressure. Fasudil (LC Laboratories, Woburn, MA) was acutely administered intravenously at 30 mg/kg during right ventricular pressure tracing, and the posttreatment right ventricular systolic pressure (RVSP) was recorded 5 minutes later.

Protein and RNA Assessment

Immunostaining was performed on mouse and human tissue with the reagents in Tables I and II in the online-only Data Supplement, respectively. Protein from mouse whole-lung lysates was used for Western blot and ELISA with the reagents in Tables III and IV in the online-only Data Supplement, respectively. mRNA was retrieved from mouse whole-lung tissue by Qiagen RNAeasy kit (Hilden, Germany). mRNA expression levels were quantified with an Illumina (San Diego, CA) HiSeq 2000 RNA sequencing system, and the reads were mapped to the mouse NCBI Build 37/mm9 genome with CASAVA version 1.7 (Illumina). The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE49116.

Statistics

Statistical analyses were performed in SigmaStat version 2.03 (IBM, Armonk, NY). Data are presented as mean±SE. Differences between 2 groups were assessed with the rank-sum test and for ≥3 groups with the Kruskal-Wallis test/ANOVA on ranks followed by the Dunn post hoc test. Values of P<0.05 were considered to be statistically significant. Additional details are given in the Methods section in the online-only Data Supplement.

Role of Pulmonary Vascular Remodeling in Schistosoma-Induced PH

We used a mouse model of Schistosoma-induced pulmonary vascular disease triggered by sequential sensitization with S mansoni parasite egg antigens (by intraperitoneal injection) followed by intravenous challenge with S mansoni eggs (delivered by tail vein injection). This protocol allowed us to use a relevant mouse model while shortening the natural life cycle, which requires 2 to 3 months for mature worms to deposit eggs that reach the liver and cause lung disease.9 Indeed, our experimental protocol caused pulmonary interstitial and vascular disease as seen in the natural life cycle. Consistent with our prior observations, we confirmed that this mode of Schistosoma challenge leads to a significant increase in RVSP and pulmonary artery media thickness (by both h−μm and myosin heavy chain staining) but not intima thickness in mice (denoted hereafter as Schisto-PH; Figure 1A–1D and Figure I in the online-only Data Supplement). The S mansoni–exposed mice also developed a trend toward right ventricular hypertrophy (P=0.052) without significant fibrosis or myocyte hypertrophy (Figure 1E and Figure II in the online-only Data Supplement). There were no differences in left ventricular systolic pressure, right or left ventricular diastolic pressure, heart rate, or body weight between S mansoni–challenged and unchallenged mice (Figure III in the online-only Data Supplement).

We next sought to clarify the cause of the increased media thickness after S mansoni egg exposure. Pulsing with intraperitoneally injected BrdU early (3 days) or late (6 days) after intravenous egg augmentation allowed us to identify proliferating cells in the intima, media, and adventitia of pulmonary arteries, particularly at early time points of vascular remodeling (Figure IIA–IID in the online-only Data Supplement). There was significant BrdU incorporation in cells surrounding egg-centered granulomas (Figure IVA–IVE in the online-only Data Supplement). In both vessels and peri-egg granulomas,
BrdU+ cells colocalized with phospho-Smad2/3 nuclear activity, consistent with signaling resulting from TGF-β (Figure 2E and Figure IVD and IVE in the online-only Data Supplement), although the fraction of cells double-positive for BrdU and phospho-Smad2/3 among all BrdU+ cells did not significantly change with S mansoni egg exposure (Figure IVF and IVG in the online-only Data Supplement). A subpopulation of macrophages (Mac3+ cells) was also BrdU positive, indicative of macrophage proliferation in situ (Figure 2F).

Bone marrow progenitors may contribute to the increased pulmonary vascular wall thickness in other forms of human PAH and experimental models of PH.11 We interrogated for the presence of bone marrow–derived cells infiltrating the vasculature in Schisto-PH using mice with ubiquitous green fluorescent protein (GFP)–positive bone marrow adoptively transferred into lethally irradiated wild-type recipient mice, which were then challenged with S mansoni. Mice treated in this manner had significant GFP signal likely in inflammatory cells in the adventitia layer and peri-egg granulomas but not in structural cells in the vascular intima or media (Figure 2G and Figure V in the online-only Data Supplement). In aggregate, these data suggest that the lung inflammation caused by S mansoni involves both recruitment of bone marrow cells and local cell proliferation.

Role of Pulmonary Vascular Vasoconstriction in Schisto-PH

There is evidence that human schistosomiasis-associated PAH involves both pulmonary vasoconstriction and vascular remodeling.4,15 We sought to identify the selective contribution of the cellular components of pulmonary vascular remodeling and pulmonary artery vasoconstriction to the increase in RVSP observed in Schisto-PH. We found a 6-fold increase (P<0.05) in the concentration of activated (GTP-bound) RhoA, which has been implicated in pathological vasoconstriction in other models of experimental PH,16 in whole-lung lysates of mice exposed to S mansoni compared with unexposed mice (Figure 3A). Short-term intravenous administration of fasudil, an inhibitor of Rho-activated kinase, in S mansoni–exposed mice resulted in a significant decrease in RVSP toward the baseline pressures of unexposed mice 5 minutes after treatment, although the RVSP remained elevated after fasudil administration compared with unexposed mice (P=0.052; representative RVSP tracings in Figure 3B; statistical analysis of the effect of fasudil in multiple mice in each group in Figure 3C and CD). Although the fasudil–treated mouse lungs showed persistent media thickening as in untreated Schisto-PH mice, the intima thickness was now also found to be significantly increased (Figure 3E and 3F), possibly as a result of thinning of the intima in control arteries after vasodilation.

A potential inducer of pulmonary vasoconstriction and Rho–Rho-activated kinase activation is hypoxia, which might contribute to pulmonary vascular remodeling after S mansoni egg exposure. We found that unanesthetized mice exposed to S mansoni had normal tail oxygen saturation measured noninvasively (Figure VIA in the online-only Data Supplement). Furthermore, intraperitoneal administration of pimonidazole hydrochloride (Hypoxyprobe) followed by staining for 2-nitroimidazole adducts to amino acid thiol groups (which occurs when the Po2 is <10 mm Hg)12 failed to demonstrate significant tissue hypoxia in the pulmonary vasculature, whereas small regions of hypoxia were detected in the center of very large peri-egg granulomas (Figure VIB–VIG in the online-only Data Supplement). Accordingly, we found no evidence of increased vascular hypoxia-inducible factor-1α activity because the expression levels of hypoxia-inducible factor-1α targets glucose transporter 1 and hexokinase 2 were similar in S mansoni–exposed mouse lung tissue compared with the pulmonary vasculature of control mice (Figure VII and VIII in the online-only Data Supplement). In line with these findings, no significant changes suggestive of increased hypoxia-inducible factor-1α activity in the pulmonary vascular compartment were observed for either of these proteins in the lung tissue of 4 subjects who died of RVSP observed in Schisto-PH. We found a 6-fold increase in the concentration of activated (GTP-bound) RhoA, which has been implicated in pathological vasoconstriction in other models of experimental PH,16 in whole-lung lysates of mice exposed to S mansoni compared with unexposed mice. A. Mice intraperitoneally sensitized to S mansoni eggs followed by intravenous augmentation have an increase in right ventricular (RV) systolic pressure (RVSP; mean±SE; n=5–6 mice per group; rank-sum test, ****P<0.001; this experiment was repeated 3 times with similar results). B. Representative hematoxylin and eosin (H&E) and immunofluorescence staining for α-smooth muscle actin (α-SMA) and thrombomodulin of unexposed and intraperitoneal/intravenous egg–exposed mouse lungs (scale bars, 50 μm). C and D. Quantitative fractional thickness of the pulmonary vascular media and intima in unexposed and intraperitoneal/intravenous egg–exposed mice (mean±SE; n=5–6 mice per group; rank-sum test, **P<0.01; P=0.66 for intima thickness). E. Fulton index [RV/(LV+S)] of unexposed and intraperitoneal/intravenous egg–exposed mice (mean±SE; n=5–6 mice per group; rank-sum test, P=0.052).
schistosomiasis-associated PAH compared with 2 samples of control (unsuccessful donor) lung tissue. Specifically, 1 of the 4 subjects with schistosomiasis-associated PAH had an increase in intimal glucose transporter 1, and 3 of the 4 subjects had decreased hexokinase 2 in the intima and, to a lesser extent, decreased hexokinase 2 in the media compared with the control tissue (representative images are shown in Figures IX and X in the online-only Data Supplement).

TGF-β Signaling in Schistosoma-Induced PH
Increased TGF-β family signaling has been implicated in multiple forms of human and experimental PH, including mice infected with *S mansoni* and patients with schistosomiasis-associated PAH. We observed a significant (1.75-fold; *P*<0.05) increase in the levels of TGF-β1 mRNA as measured by RNA sequencing in *S mansoni*-exposed mice compared with unexposed mice; there was no change in TGF-β2 or TGF-β3 expression with *S mansoni* exposure (Figure 4A). The increase in TGF-β1 was dependent on the schistosomiasis-induced Th2 cytokines IL4 and IL13 because mice lacking both did not have increased TGF-β1 after *S mansoni* exposure (Figure 4B).

Macrophages in the vascular adventitia potentially represented the main cellular source of TGF-β in mice infected with *S mansoni* on the basis of immunodetection with a
TGF-β1–specific antibody that preferentially binds active TGF-β1 (Figure 4C). Within the adventitia compartment, the Mac3–positive area increased 4-fold, the TGF-β1–positive area increased 10-fold, and the cellular profiles positive for both Mac3 and TGF-β1 increased 8-fold after S mansoni exposure (Figure 4D–4F). Furthermore, the average pixel intensity of the anti–TGF-β1 stain within the adventitia increased 2-fold in all TGF-β1–positive areas (Figure 4H). Overall, 70% of the TGF-β1–positive area costained for Mac3, suggesting that the majority of TGF-β1 in the adventitia is produced by macrophages (Figure 4I). There was a similar increase in the Mac3, TGF-β1, and Mac3-TGF-β1 colocalizing areas and in TGF-β1 intensity in both the lung parenchyma (excluding vessels and granulomas) and peri-egg granulomas after S mansoni exposure (Figure X in the online-only Data Supplement).

We have previously documented increased TGF-β signaling in pulmonary arteries of mice exposed to S mansoni and lungs of patients with schistosomiasis-associated PAH. To quantitatively complement these data, we observed a more extensive expression of phospho-Smad2/3 in the pulmonary vascular intimal, medial, and adventitial regions in experimental Schisto-PH lungs compared with control mice; moreover, the average intensity of the phospho-Smad2/3 stain was equally and significantly increased (≈1.5-fold) in all 3 pulmonary vascular compartments (Figure 5) and in the lung parenchyma from infected mice (Figure XII in the online-only Data Supplement). These changes are relevant to the human disease in that we detected increased cellular profiles expressing phospho-Smad2/3 staining within the pulmonary vascular media compartment in subjects who died of this disease compared with control human lungs (Figure 6).

Differential Protection by Inhibition of TGF-β and TGF-β Signaling in Schisto-PH

To address whether TGF-β–induced signaling might contribute to the pathology of Schisto-PH, we tested the impact of neutralization of all TGF-β isoforms after S mansoni exposure. Treatment with the anti–pan-TGF-β neutralizing antibody 1D11 resulted in partial protection against the increases in RVSP and media thickness induced by S mansoni exposure (Figure 7A and 7B); no significant changes were seen in intima thickness, right ventricular hypertrophy, peri-egg granuloma volume, or clearance of eggs by the host (measured by quantifying the remaining egg burden after 4% KOH digest of the lung tissue) with 1D11 treatment (Figure XIII in the online-only Data Supplement).

IL4 and IL13 are highly upregulated by S mansoni infection, and activation of the Th2 immune response by an inhaled antigenic stimulus has been shown to induce pulmonary vascular remodeling. We sought to determine whether the prevention of PH by blocking the TGF-β signaling pathway after S mansoni exposure correlated with decreases in IL4 or IL13. As anticipated, the amounts of IL4 and IL13 mRNA and protein were significantly increased after exposure to S mansoni (Figure 7C–7F). These increases trended toward suppression by treatment with 1D11. Taken together with our prior report of increased TGF-β signaling in schistosomiasis–infected mice lacking the decoy IL13 receptor IL13Rα2, these data indicate that IL4/IL13 and TGF-β form a mutually amplifying feed-forward loop.

A potential mechanism for a decrease in IL4 and IL13 with TGF-β blockade would be a shift from a Th2 toward a Th1 immune response, which would be characterized by an increase in interferon-γ. However, we observed a mild (although significant) increase in interferon-γ RNA and protein levels after exposure to S mansoni, which was not significantly altered by treatment with 1D11 or the TGF-β receptor 1 (ALK5) inhibitor SB431542 (Figure IX in the online-only Data Supplement). We also investigated the IL4/IL13 target RELM-α, which has been reported to correlate with Th2-induced vascular remodeling. RELM-α mRNA and protein increased with S mansoni exposure but were not modulated in mice protected by 1D11 (Figure XVA–XVC in the online-only Data Supplement). Furthermore, RELM-α knockout mice were not protected against the increase in RVSP induced by S mansoni exposure (Figure XVD in the online-only Data Supplement).
We then tested the effect of blocking TGF-β receptor 1 (ALK5) kinase activity in *S. mansoni*-exposed mice using the 2 small-molecule inhibitors SB431542 (which also blocks ALK4 and ALK7) and LY364947 (which also blocks TGF-β receptor 2 and p38 mitogen-activated protein kinase). Both of these inhibitors partially prevented the increase in RVSP induced by *S. mansoni* exposure (Figure 8A and 8B). Surprisingly, there was no significant effect on the increase in media thickness induced by *S. mansoni* exposure (Figure 8C and 8D). Similarly, there were no consistent trends in alterations in intima thickness, Fulton index, peri-egg granuloma volume, or clearance of eggs by the host with either treatment, although the Fulton index was slightly increased with LY364947 treatment and egg clearance decreased with SB431542 treatment (Figure XVI in the online-only Data Supplement). These results suggest that the effectiveness of ALK5 blockade, particularly in regard to vascular remodeling, may be less than blockade at the level of the TGF-β ligand, potentially as a result of incomplete ALK5 inhibition or non-ALK5 TGF-β receptor signaling (such as via ALK1).

We sought to characterize the individual roles of Smad2 and Smad3, which relay canonical intracellular TGF-β signaling, in *Schistosoma*-induced pulmonary vascular disease. We used Smad2+/−, Smad3+/−, Smad3−/−, and Smad2+/−Smad3+/− (double heterozygous) mice; Smad2 homozygous deletion is embryonically lethal. We found that Smad3−/− mice had substantial protection against *Schistosoma*-induced PH (Figure 8E), although similar to the SB431542- and LY364947-treated mice, there was no effect of Smad3 deletion on the increase in medial thickness after *S. mansoni* exposure (Figure 8F). There was no significant benefit from Smad2+/−, Smad3+/−, or Smad2+/−Smad3+/− genotype, although there was a trend toward less medial remodeling in the Smad2+/− mice (Figure XVIIA and XVIIIB in the online-only Data Supplement). We then interrogated whether Smad3+/+ bone marrow transplantation into lethally ablated Smad3−/− recipients conferred sensitivity to pulmonary vascular disease resulting from *S. mansoni*. We observed that these mice were protected from *Schistosoma*-induced PH to a degree comparable to Smad3−/− mice (Figure 8E), suggesting that the relevant Smad3 signaling involved the non–bone marrow–derived cells and...
potentially the components of the pulmonary vasculature. There were no consistent changes in intima thickness, Fulton index, peri-egg granuloma volumes, or clearance of eggs by the host after *S. mansoni* egg exposure with these genetic modifications (Figure XVIIC–XVIIF in the online-only Data Supplement).

We also studied the role of Smad signaling in a less inflammatory stimulus of PH, chronic hypoxia exposure. We found the Smad3+/− but not the Smad2+/− genotype was protected (at least partially) against the chronic hypoxia–induced increase in RVSP by exposure to 3 weeks of normobaric (Denver, CO, altitude) room air or 10% FiO₂, whereas neither genotype was significantly protected against the development of hypoxia-induced right ventricular hypertrophy (Figure XVIIA and XVIIIB in the online-only Data Supplement). Interestingly, we found that Smad2+/−Smad3+/− mice trended toward more severe disease than Smad2+/−Smad3+/− mice (Figure XVIIIA in the online-only Data Supplement), suggesting a protective role for Smad2 in chronic hypoxia exposure (Figure XVIIIIC and XVIIIID in the online-only Data Supplement).

We suspected that the decrease in RVSP in the setting of persistent medial thickness with TGF-β signaling blockade was accounted for by a vasodilation effect. Fasudil administration to Smad2+/−, Smad3+/−, and Smad2+/−Smad3+/− mice after exposure to *S. mansoni* eggs led to reductions in RVSP comparable to those in wild-type mice (Figure 8G and Figure XIX in the online-only Data Supplement). To test whether noncanonical TGF-β signaling mediated by p38 mitogen–activated protein kinase, implicated in models of familial (BMPR2 mutation) and hypoxic PH,22,23 may be relevant in Schisto-PH, we treated *Schistosoma*-exposed mice with the p38 mitogen–activated protein kinase inhibitor SB203580. We found inhibiting p38 mitogen–activated protein kinase had no effect on RVSP, degree of vasoconstriction as determined by response to fasudil treatment, media or intima remodeling, Fulton index, peri-egg granuloma volume, or egg clearance after *S. mansoni* egg exposure (Figure XX in the online-only Data Supplement).
Discussion

Our findings reveal a pathogenic role of TGF-β1 and Smad3-dependent TGF-β signaling in Schisto-PH. Relying on neutralizing antibodies, pharmacological blockade of TGF-β receptor signaling, and mice with a reduction in copy numbers of Smad2 and Smad3, we demonstrate that TGF-β contributes to Schistosoma-induced PH (Figure 8H). We found the Th2 cytokines IL4 and IL13 to be necessary for TGF-β activation; previously, we observed IL13 gain of function to be sufficient for TGF-β activation.12 Coupled with the finding of IL4 and IL13 suppression by TGF-β signaling blockade, there may be a positive feedback loop of IL4/IL13 and TGF-β propagating the disease. Importantly, Schisto-PH does not represent a form of hypoxia-induced PH because we found that mice with this condition lacked perivascular and intravascular hypoxia. The relevance of these experimental data is supported by identification of TGF-β signaling in lungs of patients with Schistosoma, like that documented for idiopathic PAH and connective tissue disease–associated PAH.15 The aggregate of these findings indicates that therapeutic targeting of TGF-β holds promise in the treatment of this highly prevalent disease.

A combination of portopulmonary hypertension and inflammation triggered by Schistosoma egg antigens likely contributes to PAH in patients infected with schistosomiasis.3 Preportal fibrosis resulting from chronic trematode infection and egg deposition can result in portal hypertension and portosystemic shunts, facilitating embolization of Schistosoma ova to the lung. However, patients who currently die of schistosomiasis-associated PAH do not have a significant burden of intrapulmonary egg antigens,25 probably as a result of the modern widespread use of anthelmintics, often after egg translocation to the lungs. Thus, PAH caused by schistosomiasis may result not only from egg embolization but also from the host response to localized deposition of egg antigens. Accordingly, we have experimentally observed that Schistosoma-induced PH requires intraperitoneal sensitization with egg antigens before intravenous egg augmentation, likely contributing to a robust Th2 immune response.9 As demonstrated above, in the mouse, hepatic disease is not required for Schisto-PH.

Our findings suggest a mechanistic link between the pathogenesis of schistosomiasis-associated PAH and other forms of World Health Organization group 1 PAH. Alterations in TGF-β family signaling have been implicated in heritable PAH from mutations in TGF-β family receptors, including BMPR2, ALK1, and endoglin, leading to enhanced TGF-β–induced growth in pulmonary artery smooth muscle cells; these findings complement the evidence of enhanced intracellular TGF-β signaling in idiopathic PAH.15,16 However, no role for the loss of BMPR2 in the development of pulmonary vascular disease resulting from S mansoni has been detected in BMPR2 heterozygous mice.17 In line with our findings, inhibition of TGF-β signaling also prevents experimental PH induced by hypoxia or exposure to the alkaloid toxin monocrotaline.18,19 The Schistosoma-induced PH model therefore explicitly links host-induced inflammation and TGF-β with pulmonary vascular disease, which is likely relevant to the development of all forms of group 1 PAH, including idiopathic PAH and connective tissue disease–associated PAH.20
We observed a Rho-kinase–dependent vasoconstriction resulting in elevation in RVSP of ≈30% in our mouse model of Schistosoma-induced PH, comparable to that in previously demonstrated rat models of PH.21,22 Vasoreactivity also is present in a subset of patients with schistosomiasis-associated PAH.23 Even after fasudil treatment, we observed a significant increase in the thickness of the media and intima in mice exposed to S mansoni. We found a disconnect between RVSP and the degree of vascular remodeling after blocking the TGF-β signaling pathway in that mice had a substantial decrease in RVSP but only a borderline decrease in vascular remodeling with TGF-β1 ligand blockade and no change in vascular remodeling with inhibition of the ALK5 receptor or Smad2/3 loss of function. We suspected that much of the decrease in RVSP may be attributable to alterations in vascular tone, but treatment with the Rho kinase inhibitor fasudil did not reveal a difference in Rho kinase–mediated vasoconstriction after inhibition of TGF-β signaling. We did not observe a significant effect of TGF-β signaling blockade on the right ventricle as determined by right ventricular hypertrophy (Fulton index) or cardiac output (data not shown). Alternative causes for the greater decrease in RVSP than vascular remodeling may include signaling via ALK1, an effect on intima thickness (an increase in intima thickness was seen only after quantifying fasudil-treated mice), or non–Rho kinase–mediated vasoconstriction. It is also possible that the doses of the TGF-β signaling inhibitors used were inadequate to fully protect against S mansoni–induced vascular remodeling, although effects on RVSP were observed for all compounds, and the phenotype induced was comparable to genetic ablation of the canonical TGF-β signaling mediator Smad3.

Our studies interrogated for the first time the relative contributions of the canonical mediators Smad2 and Smad3 in PH. Smad2 and Smad3 have differential actions in cancers, with Smad2 suppressing tumor growth and Smad3 promoting tumor growth.24 We found that in Schistosoma–induced PH, Smad3, specifically within the peripheral non–bone marrow–derived compartment, may be largely responsible for PH and may contribute to vascular remodeling. Of note, complete loss of Smad3 may be required on the basis of the protection seen in Smad3−/− mice compared with the heterozygous mouse or the compounded Smad2+/−Smad3+/− mouse. In contrast, the loss of a single copy of Smad3 partially protected against

Figure 7. Treatment with the pan–transforming growth factor-β (TGF-β) neutralizing antibody 1D11 prevents the increase in pressure from intraperitoneal/intravenous Schistosoma mansoni egg exposure. A, Right ventricular (RV) systolic pressure (RVSP) in mice treated with 1D11 or IgG control and unexposed or exposed to eggs (means±SE; n=3–6 mice per group; ANOVA on ranks, P<0.001; *P<0.05 by post hoc Dunn test). B, Quantitative fractional thickness of the pulmonary vascular media in mice treated with 1D11 or IgG control and unexposed or exposed to eggs (means±SE; n=3–6 mice per group; ANOVA on ranks, P=0.002; *P<0.05 by post hoc Dunn test). C through F, Interleukin (IL)-4 and IL13 RNA and protein quantity as measured by RNA sequencing and ELISA in mice treated with 1D11, IgG, or no treatment (NT) unexposed or intraperitoneally/intravenously exposed to S mansoni eggs. RPKM indicates reads per kilobase of exon model per million mapped reads (means±SE; n=3–5 mice per group; ANOVA on ranks, P=0.008 for IL4 RNA data, P=0.025 for IL4 protein data, P=0.010 for IL13 RNA data, P=0.027 for IL13 protein data; *P<0.05 by post hoc Dunn test).
hypoxia-induced PH. We did not observe a significant effect of suppressing Smad2 in Schisto-PH or hypoxia-induced PH.

Despite the availability of effective single-dose anthelmintics such as praziquantel,25 significant social and economic hurdles prevent adequate epidemiological control of *Schistosoma*, which is compounded by restricted access to available treatment and cultural hurdles to proper sanitation and exposure.26 Given the worldwide burden of schistosomiasis-associated PAH, with potentially >10 million affected individuals, elucidation of the disease pathogenesis remains a key to developing specific treatments. Thus, our study not only provides novel insights into the pathogenic role of TGF-β signaling in Schisto-PH but also expands the scope of therapeutic targets. Interruption of the positive feedback interaction between IL4/IL13 and TGF-β signaling with biologicals targeted to the former or Smad3 inhibitors to the latter may provide viable candidates to treat schistosomiasis-induced PAH.

**Acknowledgments**

Smad2−/− and Smad3−/− mice were provided by Chuxia Deng, National Institutes of Health. RELM-α−/− mice were provided by Regeneron Pharmaceuticals.

**Sources of Funding**

This study was supported by Parker B. Francis, National Institutes of Health K08HL105536, and Pfizer Advancing Science Through...
Disclosures

None.

References


Clinical Perspective

One of the most common causes of pulmonary arterial hypertension worldwide is infection with Schistosoma, the parasite that causes schistosomiasis, but how this infection results in pulmonary vascular disease is unknown. We developed an animal model of this disease by exposing mice to Schistosoma eggs, which recapitulates several key aspects of the human pathology, including elevated right ventricular pressures and pulmonary vascular remodeling. We observed an increase in the cytokine transforming growth factor-β (TGF-β) after exposing the mice to Schistosoma, along with an increase in the TGF-β signaling mediator phospho-Smad2/3, and an increase in phospho-Smad2/3 was seen in the lung tissue of patients who died of this condition in Brazil. When we blocked TGF-β signaling in our animal model at the level of the TGF-β ligand using a neutralizing antibody, at the level of the receptor using small-molecule inhibitors, or at the level of intracellular signaling using Smad3 knockout mice, we prevented pulmonary hypertension. We thus suspect that the vascular remodeling in human schistosomiasis-associated pulmonary arterial hypertension and possibly other inflammatory forms of pulmonary arterial hypertension such as idiopathic disease or connective tissue-associated disease may be mediated by TGF-β, and targeting TGF-β signaling in the lung blood vessels may be of benefit for patients with this condition.
Transforming Growth Factor-β Signaling Promotes Pulmonary Hypertension Caused by Schistosoma Mansoni

Brian B. Graham, Jacob Chabon, Liya Gebreab, Jennifer Poole, Elias Debella, Laura Davis, Takeshi Tanaka, Linda Sanders, Nina Dropcho, Angela Bandeira, R. William Vandivier, Hunter C. Champion, Ghazwan Butrous, Xiao-Jing Wang, Thomas A. Wynn and Rubin M. Tuder

_Circulation_. 2013;128:1354-1364; originally published online August 19, 2013;
doi: 10.1161/CIRCULATIONAHA.113.003072

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/128/12/1354

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2013/08/19/CIRCULATIONAHA.113.003072.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/
SUPPLEMENTAL MATERIAL

TGF-β signaling promotes pulmonary hypertension caused by *Schistosoma mansoni*
Supplemental Methods

Animals

All mice were bred and housed under specific pathogen-free conditions in an American Association for the Accreditation of Laboratory Animal Care-approved facility. All experiments were performed in a coded format, with the investigators lacking knowledge of the specific experimental group identifiers prior to final data reporting. Bone marrow recipients were maintained on diet containing trimethoprim-sulfa antibiotic for 4 weeks after transplant, but otherwise all mice were maintained on normal rodent diet.

Bone Marrow Transplantation

For bone marrow transplantation, recipient mice were irradiated with 10 Gy split in 2 fractions, delivered 4 hours apart, and 0.5-2.0x10^6 bone marrow cells isolated from the thigh of donor mice were administered by tail vein injection shortly thereafter; mice were allowed to recover for at least 8 weeks before undergoing additional treatments as described above.

Assessment of Oxygen Saturation

Pimonidazole hydrochloride (Hypoxyprobe; HPI Inc., Burlington, MA) was purchased and a single 60µg/g dose administered IP 2 hours before sacrifice. Oxygen saturation was measured non-invasively in non-sedated mice by tail probe (MouseOx, Starr Life Sciences, Pittsburgh, PA).

Egg Burden Quantification

The number of S. mansoni ova present in mouse lung tissue was determined as previously described (1). Briefly, 10 to 20 mg of frozen right lung tissue was digested in 4% potassium hydroxide for 18 hours at 33degC, and the number of eggs in aliquots of the digest was counted.
Protein and RNA Assessment

After the right ventricular catheterization was complete, the blood was flushed out of the lungs, the right bronchus sutured, and 2% agarose instilled into the left lung through the transtracheal catheter. The left lung was removed, formalin-fixed, and processed for paraffin embedding. The right lung was removed and divided into lobes which were frozen or placed in RNAlater (Life Technologies, Carlsbad, CA). The right ventricle free wall was dissected off of the heart, weighed relative to the septum and left ventricle, and formalin-fixed and paraffin-embedded.

A sample of the right lung frozen tissue was macerated and sonicated in PBS containing anti-proteases, protein concentration determined by Bradford assay (BioRad), and 50 µg of protein from each sample was used to detect specific proteins by Western blot using the reagents listed in the Supplemental Table 3. ELISA, including GLISA for GTP-RhoA, was performed to determine the concentration of specific proteins in samples using the kits in Supplemental Table 4. mRNA expression levels quantified using an Illumina (San Diego, CA) HiSeq 2000 RNA sequencing (RNA-seq) system were expressed as reads per kilobase of exon model per million mapped reads (RPKM: the Illumina standard).

Image Analysis

Images were acquired using a Nikon Eclipse E800 microscope with 10x, 20x and 40x air objectives, at room temperature, using either a color camera (Nikon, Melville, NY) or a black and white CCD camera (Photometrics, Tuscon, AZ), with Nikon NIS Elements Software v3.2. Quantification of media and intima thickness in mouse lung tissue was determined as previously described (2). Briefly, 10 to 12 images of vessels at 40x magnification were randomly acquired from masked paraffin-embedded samples immunofluorescence stained for α-smooth muscle actin and thrombomodulin, or smooth muscle myosin heavy chain, as described above. Image
processing software (Image Pro Plus v4.5.1, Media Cybernetics, Bethesda, MD) was used to identify the cross-sectional areas contained by the external perimeter of the media, the internal perimeter of the media, and the internal perimeter of the intima. The radius \( r_i \) for each of the three vessel layers \( i \) enclosing an area \( A_i \) was calculated using the equation \( r_i = \sqrt{A_i/\pi} \). The thicknesses of the media and intima were calculated as the differences between the respective radii, and expressed as a fraction of the external media radius. Peri-egg granuloma volumes were measured using the optical rotator stereologic method (3). Briefly, paraffin-embedded tissue was stained with hematoxalin and eosin, and 8-10 images of granulomas with a single visible ova were acquired for each sample. The rotator method for object volume estimation was then applied using the ova as the central reference point with image processing software (Image Pro Plus). Co-localization and pixel intensity analysis of Mac3 with TGF-\( \beta_1 \) and pSmad2/3 with \( \alpha \)-smooth muscle actin and thrombomodulin was performed by acquiring 10 to 12 images of vessels, parenchyma and granulomas from each sample. Image processing software (Metamorph, Molecular Devices LLC, Sunnyvale, CA) was used to threshold each signal for positive cells, and the area occupied by the thresholded area for each signal (and co-localized areas) as well as the average pixel intensity within thresholded areas was determined; the adventitia was taken as the space outside the media and inside twice the diameter of the external media layer. Picrosirius red was used to quantify right ventricular fibrosis in unexposed and IP/IV egg-exposed mice. Ten images of each mouse were acquired at 10x magnification and superimposed on a 1700 point grid using MetaMorph software. Points intersecting with collagen (positive polarization with picrosirius red staining) were counted and used to determine the volume fraction of RV free wall fibrosis. 10 images of fluorescein-labeled wheat germ agglutinin-stained (see above) RV free wall tissue were acquired at 40x magnification. These images were analyzed using MetaMorph to determine the volume density of myocytes (using the same point intersection as for picrosirius red staining above) and myocyte cross sectional
area. To exclude longitudinally sectioned myocytes, a length to breadth ratio of 2:1 was implemented. Likewise, a size exclusion selected myocyte area between 30µm and 550µm for quantification.
## Supplemental Tables

### Table S1. Reagents for immunostaining mouse tissue.

<table>
<thead>
<tr>
<th>Immunostain</th>
<th>Antigen Retrieval</th>
<th>Block</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
<th>Tertiary Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexokinase</td>
<td>Citrate Buffer 30 min in steamer (Vector H-3300)</td>
<td>Peroxidase block (Dako S2003) 10min at RT, 3% H2O2 10min at RTx3 each, Avidin 10 min, Biotin 10 min, 1.5% Goat Serum in PBS</td>
<td>1:50 30min at RT (Cell Signaling #2106)</td>
<td>1:200 Biotinylated Goat anti-Rabbit (Vector BA1000) 1hr at RT</td>
<td>Streptavidin-HRP (Vector #SA-5704), then DAB 5min, then Hematoxalyn</td>
</tr>
<tr>
<td>GLUT1</td>
<td></td>
<td></td>
<td>1:100 1hr at RT (Thermo Scientific #RB-9052)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoxyprobe</td>
<td>Serum free protein block (Dako X0909)</td>
<td></td>
<td>1:200 Biotinylated Goat anti-Mouse (Dako E0464)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGF-β1</td>
<td></td>
<td>10% Goat Serum in PBS</td>
<td>1:25 O/N at 4degC (R&amp;D Systems AF-101-NA)</td>
<td>AF488 Goat anti-Chicken IgY (Invitrogen A11039)</td>
<td>Vectashield with DAPI (Vector H-1200)</td>
</tr>
<tr>
<td>BrdU</td>
<td></td>
<td>10% Donkey Serum in PBS, Avidin 10 min, Biotin 10 min</td>
<td>1:50 O/N at 4degC (Abcam #1893)</td>
<td>1:200 FITC-labeled Donkey anti-Sheep (Abcam ab6896-1)</td>
<td></td>
</tr>
<tr>
<td>Thrombo-</td>
<td></td>
<td>10% Horse Serum in 1:1 5% BSA : Superblock (ScyTek AAA5000) 1hr at RT</td>
<td>1:1000 1hr at RT (R&amp;D Systems #AF3894)</td>
<td>1:200 AF594 Donkey anti-Goat (Invitrogen #A11058) 1hr at RT</td>
<td></td>
</tr>
<tr>
<td>modulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(CD141)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Smad2/3</td>
<td></td>
<td>5% Horse Serum in PBS 1hr at RT</td>
<td>1:100 overnight at 4degC (Cell Signaling #3101)</td>
<td>1:100 AF488 Donkey anti-Rabbit (Invitrogen #A21206) 1hr at RT</td>
<td></td>
</tr>
</tbody>
</table>
Table S1 (continued). Reagents for immunostaining mouse tissue.

<table>
<thead>
<tr>
<th>Immunostain</th>
<th>Antigen Retrieval</th>
<th>Block</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
<th>Tertiary Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>αSM-actin</td>
<td>Citrate Buffer 30 min in steamer (Vector H-3300)</td>
<td>Avidin 10 min, Biotin 10 min, Mouse on Mouse (MOM) kit blocking solution (Vector BMK-2202) 1hr at RT</td>
<td>1:100 30min at RT (Dako #M0851)</td>
<td>MOM Biotinylated anti-Mouse Reagent (Vector BMK-2202) 10min at RT</td>
<td>Texas Red-Strepavidin 1:2000 (Invitrogen #S872), Vectashield with DAPI (Vector H-1200)</td>
</tr>
<tr>
<td>Smooth Muscle Myosin Heavy Chain</td>
<td>Citrate Buffer 30 min in steamer (Vector H-3300)</td>
<td>10% Goat Serum in PBS</td>
<td>1:100 O/N at 4degC (Biomedical Technologies #5620305)</td>
<td>1:200 Texas Red-Goat anti-Rabbit (Invitrogen #T2767) 1hr at RT</td>
<td>Vectashield with DAPI (Vector H-1200)</td>
</tr>
<tr>
<td>Mac-3</td>
<td>Borg Buffer 30 min in steamer (Biocare #BD1000G1)</td>
<td>10% Goat Serum in 1:1 5% BSA: Superblock (ScyTek AAA5000) 1hr at RT</td>
<td>1:50 1hr at RT (BD Pharmingen #550292)</td>
<td>1:300 AF488 Goat anti-Rat (Invitrogen #A11006) 1hr at RT</td>
<td>Vectashield with DAPI (Vector H-1200)</td>
</tr>
<tr>
<td>Fluorescein-Labeled Wheat Germ Agglutinin</td>
<td>Citrate Buffer 20 min in steamer (Vector H-3300)</td>
<td>None</td>
<td>1:500 O/N at 4degC (Vector FL-1021)</td>
<td>None</td>
<td>Vectashield with DAPI (Vector H-1200)</td>
</tr>
<tr>
<td>Picrosirius Red</td>
<td>0.1% (w/v) Sirius red (Direct Red 80, Sigma #365548) in saturated aqueous solution of picric acid, 1hr at RT, followed by 2 rinses of 0.5% acetic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table S2. Reagents for immunostaining human tissue.

<table>
<thead>
<tr>
<th>Immunostain</th>
<th>Antigen Retrieval</th>
<th>Block</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
<th>Tertiary Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexokinase</td>
<td>Citrate Buffer 30 min in steamer (Vector H-3300)</td>
<td>Peroxidase block (Dako S2003) 10min at RT, 3% H2O2 10min at RTx3 each, Avidin 10 min, Biotin 10 min, 1.5% Goat Serum in PBS</td>
<td>1:50 30min at RT (Cell Signaling #2106)</td>
<td>1:200 Biotinylated Goat anti-Rabbit (Vector BA1000) 1hr at RT</td>
<td>Streptavidin-HRP (Vector #SA-5704), then DAB 5min, then Hematox-alyn</td>
</tr>
<tr>
<td>GLUT1</td>
<td>Citrate Buffer 30 min in steamer (Vector H-3300)</td>
<td>5% Horse Serum in PBS 1hr at RT</td>
<td>1:2000 overnight at 4degC (Cell Signaling #3101)</td>
<td>1:100 AF594 Goat anti-Mouse (Invitrogen #A11005) 1hr at RT</td>
<td>Vectashield with DAPI (Vector H-1200)</td>
</tr>
<tr>
<td>p-Smad2/3</td>
<td>Citrate Buffer 30 min in steamer (Vector H-3300)</td>
<td>5% Goat Serum in PBS 1hr at RT</td>
<td>1:200 1hr at RT (Dako #M0851)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-SMactin</td>
<td>Citrate Buffer 30 min in steamer (Vector H-3300)</td>
<td>5% Goat Serum in PBS 1hr at RT</td>
<td>1:200 1hr at RT (Dako #M0851)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table S3. Reagents for immunoblotting mouse protein.

<table>
<thead>
<tr>
<th>Immunoblot</th>
<th>Block</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
<th>Tertiary Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>RELM-α</td>
<td>StartingBlock 2hr at RT (Thermo Scientific, #37542)</td>
<td>1:50,000 Abcam #39626 O/N at 4degC</td>
<td>HRP-bound Goat anti-Rabbit 1:5000 1hr at RT (Vector PI-1000)</td>
<td>ECL Detection 5min at RT (GE Healthcare, RPN2106)</td>
</tr>
<tr>
<td>β-actin</td>
<td>Scientific, #37542</td>
<td>1:20,000 Cell Signaling #4967 O/N at 4degC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table S4. Reagents for quantifying mouse protein.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL4</td>
<td>R&amp;D Systems M4000B</td>
</tr>
<tr>
<td>IL13</td>
<td>R&amp;D Systems M1300CB</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>R&amp;D Systems MIF00</td>
</tr>
<tr>
<td>GTP-RhoA</td>
<td>Cytoskeleton Inc. BK124</td>
</tr>
</tbody>
</table>
Figure S1.
Antibodies against smooth muscle myosin heavy chain (SMMHC) and α-smooth muscle actin (α-SMA) identify comparable vascular remodeling of the pulmonary media after *S. mansoni* egg exposure. (A) Representative immunofluorescence staining for SMMHC of unexposed and IP/IV egg exposed mouse lungs (Scale bars: 100µm). (B) Quantitative fractional thickness of the pulmonary vascular media assessed by antibodies against α-SMA and SMMHC in unexposed and IP/IV egg exposed mice (mean ± SE; n = 6-7 mice per group; ANOVA on ranks *P* < 0.001; *P* < 0.05 by post-hoc Dunn’s test).
Figure S2.
Exposure to IP/IV S. mansoni eggs does not result in RV free wall fibrosis or myocyte hypertrophy. (A) Representative picrosirius red and wheat germ agglutinin staining to detect fibrosis (picrosirius red stain, imaged using cross-polarized lenses; arrows show areas of fibrosis) and myocyte membranes in the right ventricle of unexposed or IP/IV egg-exposed mice (Scale bars: 50µm). (B) Volume fraction of tissue that is polarized positive with picrosirius red stain, related to collagen volume density (see Supplemental Methods, mean ± SE; n = 6-7 mice per group; rank-sum test $P=0.63$). (C) Volume fraction of myocytes (see Supplemental Methods, mean ± SE; n = 6-7 mice per group; rank-sum test $P=0.84$). (D) Average myocyte cross-sectional area (mean ± SE; n = 6-7 mice per group; rank-sum test $P=0.53$).
Figure S3.
Exposure to IP/IV S. mansoni eggs does not affect systemic hemodynamics or body weight. (A) Left ventricular systolic pressure (mean ± SE; n = 3-5 mice per group; rank-sum test P=0.79). (B) Right ventricular diastolic pressure (mean ± SE; n = 5-6 mice per group; rank-sum test P=0.43). (C) Right ventricular diastolic pressure (mean ± SE; n = 3-5 mice per group; rank sum test P=0.79). (D) Heart rate (mean ± SE; n = 5-6 mice per group; rank-sum test P=0.80). (E) Body weight (mean ± SE; n = 5-6 mice per group; rank-sum test P=0.93).
Figure S4.
Additional images of BrdU-treated and GFP bone marrow-recipient mice. (A-C) Intestine of mice treated with BrdU (A is stained with isotype control antibody; LP: lamina propria; Scale bars: 100 µm). (D and E) Peri-egg granulomas in BrdU-treated S. mansoni egg IP/IV exposed mice co-stained for BrdU and phospho-Smad2/3 (Egg: S. mansoni egg; Scale bars: 50 µm). (F) Fraction of BrdU-positive pixels that are also phospho-Smad2/3-positive in vessels (includes all 3 layers: intima, media, and adventitia) in unexposed and S. mansoni egg IP/IV exposed mice (mean ± SE; ANOVA on ranks P=0.59). (G) Fraction of BrdU-positive pixels that are also phospho-Smad2/3-positive in peri-egg granulomas in S. mansoni egg IP/IV exposed mice (mean ± SE; rank-sum test P=0.25).
Figure S5.
Additional images of GFP bone marrow-recipient mice. (A and B) Peri-egg granuloma and peri-vascular GFP signal in wildtype recipient mice with GFP positive bone marrow donor exposed to IP/IV *S. mansoni* eggs (arrowheads mark positive cells; Egg: *S. mansoni* egg; *: vessel lumen; Scale bar: 100 µm; G is the same image as Figure 2G without the dotted lines).
Figure S6.
Absence of significant hypoxia after IP/IV S. mansoni egg exposure. (A) Non-invasive tail oxygen saturation (mean ± SE; n = 5-6 mice per group; rank-sum test P=0.54). (B-D) Immunostain for pimonazole (hypoxyprobe) of vessels of IP/IV S. mansoni egg exposed mice (* vessel lumen; Scale bars: 100 µm). (E-G) Immunostain for pimonazole (hypoxyprobe) of peri-egg granulomas of IP/IV S. mansoni egg exposed mice (Egg: S. mansoni egg; Scale bars: 100 µm).
Figure S7.
Immunohistochemistry for glucose transporter 1 (GLUT1) in mouse tissue. (A and B) Positive control: angiomyolipoma from von Hippel-Lindau disease. (C and D) Unexposed mouse vessel. (E and F) Peri-egg granuloma in mice exposed to IP and IV *S. mansoni* eggs. (G and H) Vessel in mice exposed to IP and IV *S. mansoni* eggs. (* vessel lumen; Egg: *S. mansoni* egg; Scale bars: 100 µm.)
Figure S8.
Immunohistochemistry for hexokinase 2 (HK2) in mouse tissue. (A and B) Positive control: angiomyolipoma from von Hippel-Lindau disease. (C and D) Unexposed mouse vessel. (E and F) Peri-egg granuloma in mice exposed to IP and IV S. mansoni eggs. (G and H) Vessel in mice exposed to IP and IV S. mansoni eggs. (* vessel lumen; Egg: S. mansoni egg; Scale bars: 100 µm.)
Figure S9.
Immunohistochemistry for glucose transporter 1 (GLUT1) in human tissue. (A and B) Normal control tissue from a failed lung donor. (C - H) Lung tissue from three autopsy cases of patients who died of schistosomiasis associated-PAH. (Scale bars: 100 µm.)
Figure S10.
Immunohistochemistry for hexokinase 2 (HK2) in human tissue. (A and B) Normal control tissue from a failed lung donor. (C - H) Lung tissue from three autopsy cases of patients who died of schistosomiasis associated-PAH. (Scale bars: 100 µm.)
Figure S11.
Quantification of Mac3 (macrophage marker) and TGF-β1 immunostaining and co-localization in the parenchyma and peri-egg granulomas of unexposed and mice exposed to IP and IV S. mansoni eggs. (A and B) Representative images showing Mac3 and TGF-β1 co-localization in the parenchyma and peri-egg granuloma of an IP/IV S. mansoni egg-exposed mouse (Scale bars: 50 µm). (C) Quantification of Mac3+ area in the parenchyma and peri-egg granulomas (mean ± SE; n = 5 mice per group; ANOVA on ranks P=0.002; *P<0.05 by post-hoc Dunn’s test). (D) Quantification of TGF-β1+ area in the parenchyma and peri-egg granulomas (mean ± SE; n = 5 mice per group; ANOVA on ranks P=0.002; *P<0.05 by post-hoc Dunn’s test). (E) Quantification of Mac3+ and TGF-β1+ co-localized area in the parenchyma and peri-egg granulomas (mean ± SE; n = 5 mice per group; ANOVA on ranks P=0.002; *: P<0.05 by post-hoc Dunn’s test). (F) Mean fluorescent intensity of Mac3+ pixels (arbitrary units; normalized to average of uninfected parenchyma = 1; mean ± SE; n = 5 mice per group; ANOVA on ranks P=0.008; *P<0.05 by post-hoc Dunn’s test). (G) Mean fluorescent intensity of TGF-β1+ pixels (arbitrary units; normalized to average of uninfected parenchyma = 1; mean ± SE; n = 5 mice per group; ANOVA on ranks P=0.005; *P<0.05 by post-hoc Dunn’s test). (H) Ratio of areas of co-localized Mac3+ and TGF-β1+ area to all TGF-β1+ area in the parenchyma and peri-egg granulomas (mean ± SE; n = 5 mice per group; ANOVA on ranks P=0.005; *: P<0.05 by post-hoc Dunn’s test).
Figure S12.
Quantification of phospho-Smad2/3 quantity and co-localization with parenchyma and peri-egg granuloma compartments in unexposed and mice exposed to IP and IV *S. mansoni* eggs. (A) Representative image showing phospho-Smad2/3 in the parenchyma of an IP/IV *S. mansoni* egg-exposed mouse (Scale bars: 50 µm). (B) Representative image showing phospho-Smad2/3 in the peri-egg granuloma of an IP/IV *S. mansoni* egg-exposed mouse (Scale bars: 50 µm). (C) Quantification of phospho-Smad2/3+ area in the parenchyma and granuloma (mean ± SE; n = 5 mice per group; ANOVA on ranks P=0.009; *P<0.05 by post-hoc Dunn’s test). (D) Mean fluorescent intensity of phospho-Smad2/3+ pixels in the parenchyma and granuloma (arbitrary units; normalized to average of uninfected parenchyma = 1; mean ± SE; n = 5 mice per group; ANOVA on ranks P=0.007; *P<0.05 by post-hoc Dunn’s test).
Exposure to 1D11 does not affect intima thickness, RV hypertrophy, per-egg granuloma volumes or eggs cleared from the lung tissue. (A) Quantitative intima fractional thickness (mean ± SE; n = 3-6 mice per group; ANOVA on ranks P=0.087). (B) Fulton index (mean ± SE; n = 3-6 mice per group; ANOVA on ranks P=0.56). (C) Peri-egg granuloma volume (mean ± SE; n = 3-6 mice per group; rank-sum test P=0.18). (D) Egg counts after 4% KOH digest (mean ± SE; n = 3-6 mice per group; rank-sum test P=0.59).
Figure S14.
Effect of 1D11 on IFN-γ levels. (A and B) IFN-γ RNA and protein quantity as measured by RNA-seq and ELISA in mice treated with SB431542, 1D11, DMSO, or no treatment (NT), unexposed or IP/IV exposed to *S. mansoni* eggs (mean ± SE; n = 3-5 mice per group; ANOVA on ranks *P*=0.005 for RNA data, *P*=0.007 for protein data; *P*<0.05 by post-hoc Dunn’s test).
Figure S15.
Effect of TGF-β signaling blockade on RELM-α. (A) RELM-α RNA quantity as measured by RNA-seq in mice treated with 1D11 or no treatment (NT), unexposed or IP/IV exposed to *S. mansoni* eggs (RPKM: reads per kilobase of exon model per million mapped reads; mean ± SE; n = 3-5 mice per group; ANOVA on ranks *P*=0.010). (B and C) Western blot and densitometry quantification of whole lung lysates from mice treated with 1D11 or IgG, unexposed or IP/IV exposed to *S. mansoni* eggs, and probed for RELM-α and β-actin (relative units; ratio normalized to average of unexposed IgG treated = 1; mean ± SE; n = 3-5 mice per group; ANOVA on ranks *P*=0.008; *P*<0.05 by post-hoc Dunn’s test). (D) RVSP in RELM-α+/+ and
RELM-α/- mice (both on a Balb-c background) IP/IV exposed to *S. mansoni* eggs (mean ± SE; n = 5-7 mice per group; rank-sum test *P*=0.34).
Figure S16.
Treatment with either of two TGF-β receptor type 1 (ALK5) inhibitors SB431542 or LY364947 prevents the increase in pressure from IP/IV egg exposure. (A-B) Quantitative fractional thickness of the pulmonary vascular intima in mice treated with SB431542, LY364947 or DMSO vehicle, and unexposed or IP/IV exposed to *S. mansoni* eggs (mean ± SE; n = 5-6 mice per group; ANOVA on ranks $P=0.303$ for SB431542 treatment, $P=0.016$ for LY364947 treatment). (C-D) Fulton index (mean ± SE; n = 5-6 mice per group; ANOVA $P=0.018$ for SB431542 treatment; ANOVA $P=0.017$ for LY364947 treatment; *$P < 0.05$ by post-hoc Tukey test). (E-F) Granuloma volume (mean ± SE; n = 5-6 mice per group; rank-sum test $P=0.093$ for SB431542 treatment, $P=0.18$ for LY364947 treatment). (G-H) Egg counts after 4% KOH digest (mean ± SE; n = 5-6 mice per group; rank-sum test *$P<0.05$ for SB531542 treatment, $P=0.82$ for LY364947 treatment).
Figure S17 (Continued on next page).
Figure S17.
Smad2+/-, Smad3+/-, Smad2+/-Smad3+/- and Smad3-/- genotypes with IP/IV egg exposure. (A) RVSP in wildtype, Smad2+/-, Smad3+/- and Smad2+/-Smad3+/- mice unexposed or IP/IV exposed to *S. mansoni* eggs (mean ± SE; n = 4-12 mice per group (note data for 6 wildtype uninfected and 6 wildtype infected mice presented in Figure 11A are also included; ANOVA on ranks $P<0.001$; *$P<0.05$ by post-hoc Dunn’s test). (B) Quantitative fractional thickness of the pulmonary vascular media in wildtype, Smad2+/-, Smad3+/- and Smad2+/-Smad3+/- mice unexposed or IP/IV exposed to *S. mansoni* eggs (mean ± SE; n = 4-13 mice per group; note data for 6 wildtype uninfected and 6 wildtype infected mice presented in Figure 11A are also included; ANOVA on ranks $P<0.001$; *$P<0.05$ by post-hoc Dunn’s test). (C) Quantitative
fractional thickness of the pulmonary vascular media in wildtype, Smad2+/−, Smad3+/−, Smad2+/−Smad3+/− and Smad3−/− mice unexposed or IP/IV exposed to S. mansoni eggs (mean ± SE; n = 4-13 mice per group; ANOVA on ranks P<0.001; *P < 0.05 by post-hoc Dunn’s test).

(D) Fulton index in wildtype, Smad2+/−, Smad3+/−, Smad2+/−Smad3+/− and Smad3−/− mice unexposed or IP/IV exposed to S. mansoni eggs (mean ± SE; n = 5-13 mice per group; ANOVA on ranks P=0.070).

(E) Granuloma volume in wildtype, Smad2+/−, Smad3+/−, Smad2+/−Smad3+/− and Smad3−/− mice unexposed or IP/IV exposed to S. mansoni eggs (mean ± SE; n = 4-13 mice per group; ANOVA on ranks P=0.16). (F) Egg counts after 4% KOH digest in wildtype, Smad2+/−, Smad3+/−, Smad2+/−Smad3+/− and Smad3−/− mice unexposed or IP/IV exposed to S. mansoni eggs (mean ± SE; n = 4-13 mice per group; ANOVA on ranks P=0.053).
Figure S18 (Continued on next page).
Figure S18.
Effect of Smad2+/- and Smad3+/- genotypes on hypoxia-induced pulmonary hypertension. (A) RVSP (mean ± SE; n = 5-12 mice per group; ANOVA on ranks $P<0.001$; *$P < 0.05$ by post-hoc Dunn’s test). (B) Fulton index (mean ± SE; n = 5-13 mice per group; ANOVA on ranks $P<0.001$; *$P < 0.05$ by post-hoc Dunn’s test). (C and D) Quantitative fractional thickness of the pulmonary vascular media and intima (mean ± SE; n = 5-13 mice per group; ANOVA on ranks $P=0.006$ for media, $P=0.004$ for intima; *$P < 0.05$ by post-hoc Dunn’s test).
Figure S19.
Effect of the Rho kinase inhibitor fasudil in mice lacking Smad2 and/or Smad3. (A and B) RVSP in unexposed and IP/IV egg exposed Smad2+/-, Smad3+/-, and Smad2+/-Smad3+/- mice before and 5 minutes after acute fasudil administration (mean ± SE; n = 5-6 mice per group; ANOVA on ranks $P=0.53$ for pre-fasudil, $P=0.60$ for post-fasudil). The decrease in pressure is shown in Figure 8G.
Figure S20.
Treatment with the p38 kinase inhibitor SB203580 has no additional effect on *S. mansoni*-induced pulmonary hypertension. (A-C) RVSP in unexposed and IP/IV egg exposed mice treated with SB203580 or vehicle control, before and after fasudil administration, and the decrease in pressure (mean ± SE; n = 5-7 mice per group; ANOVA on ranks $P=0.005$ for pre-fasudil, $P=0.024$ for post-fasudil, $P=0.038$ for change in pressure; *$P < 0.05$ by post-hoc Dunn's test). (D and E) Quantitative fractional thickness of the pulmonary vascular media and intima (mean ± SE; n = 6-7 mice per group; ANOVA on ranks $P<0.001$ for media, $P=0.007$ for intima;
*P < 0.05 by post-hoc Dunn’s test). (F) Fulton index (mean ± SE; n = 6-7 mice per group; ANOVA on ranks *P*=0.24). (G) Peri-egg granuloma volume (mean ± SE; n = 6-7 mice per group; rank-sum test *P*=0.84). (H) Egg counts after 4% KOH digest (mean ± SE; n = 7 mice per group; rank-sum test *P*=0.54).
Supplemental References

