ORIGINAL RESEARCH

Th2 CD4$^+$ T Cells Are Necessary and Sufficient for *Schistosoma*-Pulmonary Hypertension

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***Background*—**Inflammation underlies many forms of pulmonary hypertension (PH), including that resulting from *Schistosoma* infection, a major cause of PH worldwide. Schistosomiasis-associated PH is proximately triggered by embolization of parasite eggs into the lungs, resulting in localized type 2 inflammation. However, the role of CD4$^+$ T cells in this disease is not well defined.

***Methods and Results*—**We used a mouse model of schistosomiasis-associated PH, induced by intraperitoneal egg sensitization followed by intravenous egg challenge, with outcomes including right ventricle systolic pressure measured by cardiac catheterization, and cell density and phenotype assessed by flow cytometry. We identified that embolization of *Schistosoma* eggs into lungs of egg-sensitized mice increased the perivascular density of T-helper 2 (Th2) CD4$^+$ T cells by recruitment of cells from the circulation and triggered type 2 inflammation. Parabiosis confirmed that egg embolization is required for localized type 2 immunity. We found Th2 CD4$^+$ T cells were necessary for *Schistosoma*-induced PH, given that deletion of CD4$^+$ T cells or inhibiting their Th2 function protected against type 2 inflammation and PH following *Schistosoma* exposure. We also observed that adoptive transfer of *Schistosoma*-sensitized CD4$^+$ Th2 cells was sufficient to drive type 2 inflammation and PH.

***Conclusions*—**Th2 CD4$^+$ T cells are a necessary and sufficient component for the type 2 inflammation-induced PH following *Schistosoma* exposure. **(*J Am Heart Assoc*. 2019;8:e013111. DOI: 10.1161/JAHA.119.013111.)**

Key Words: CD4 T cell • pulmonary hypertension • schistosomiasis • type 2 immunity

Inflammation can trigger the development of pulmonary vascular disease in humans and experimental models, resulting in pulmonary hypertension (PH). Furthermore, there is a significant expansion of B and T cells in the pulmonary vascular adventitia in PH.[1,2] These lymphocytes drive adaptive immunity, but earlier studies in this field have primarily used triggers of innate immunity, such as hypoxia, leaving the roles of these cells unclear.

Schistosomiasis, resulting from infection with parasitic helminth *Schistosoma*, has a worldwide prevalence of >200 million.[3] Schistosomiasis is a major cause of World Health Organization Group 1 pulmonary arterial hypertension (PAH) worldwide, affecting around 6% of those chronically and recurrently infected.[4,5] Despite the availability of effective antihelminthic treatment, numerous social, economic, and political barriers have prevented widespread treatment of schistosomiasis, leading to its characterization as a "neglected tropical disease." Once significant schistosomiasis-associated PAH has developed, it is clinically no longer responsive to antihelminthic therapy; vasodilators can improve symptoms and prolong life, but the condition remains fatal.

Schistosomiasis-associated PAH is thought to be triggered by embolization of *Schistosoma mansoni* eggs into the lungs (the *Schistosoma* species particularly associated with PAH), resulting in inflammation and associated severe vascular remodeling. The type 2 immune response triggered by *Schistosoma* egg antigens is characterized by T cells, macrophages, eosinophils, and basophils, and the release of inflammatory cytokines, including interleukin (IL)-4, -5, -10,

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Accompanying Tables S1 through S7 and Figures S1 through S6 are available at https://www.ahajournals.org/doi/suppl/10.1161/JAHA.119.013111

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Clinical Perspective

What Is New?

- Inflammation contributes to the pathogenesis of many forms of pulmonary hypertension (PH), but how it does this is unknown.
- Schistosomiasis is a major cause of PH worldwide.
- We found that embolization of *Schistosoma mansoni* eggs is critical to experimental schistosomiasis-triggered PH, and also found that T-helper 2 CD4 T cells are necessary and sufficient for the type 2 inflammation and PH that is triggered by experimental *Schistosoma* exposure in mice.

What Are the Clinical Implications?

- Targeting T cells may be a future way to prevent or treat inflammatory forms of PH, such as that induced by schistosomiasis.

and -13.[6,7] The pulmonary pathology includes peri-egg granulomas and vascular remodeling with perivascular infiltrates and thickening of the vessel walls. We and others have previously shown that adaptive immunity and type 2 inflammation is required for the pulmonary phenotype.[8–10] These cytokines trigger a cascade of inflammation, including the recruitment of Ly6C$^+$ monocytes, into the lung interstitium, where these cells express thrombospondin-1 resulting in local activation of transforming growth factor beta, which drives the vascular disease.[11]

In this study, we hypothesized that T-helper 2 (Th2) CD4$^+$ T cells are both necessary and sufficient to drive *Schistosoma*-induced type 2 inflammation and PH. We found that embolization of *Schistosoma* eggs in egg-sensitized mice induces the recruitment and proliferation of CD4$^+$ T cells in the lungs, and blockade of either T cells or, more specifically, their Th2 phenotype inhibited the pulmonary vascular pathology. In addition, adoptive transfer of primed CD4$^+$ Th2 cells from egg-sensitized mice was sufficient to convey the adaptive immunity which drives the PH phenotype. These results reveal that egg-antigen–specific Th2 CD4$^+$ effector T cells are the critical immunological driver of *Schistosoma*-PH.

Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Animals

C57Bl6/J wild-type and *Rag1*$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). *Il4*$^{-/-}$*Il13*$^{-/-}$ mice were obtained from Dr Thomas Wynn (NIAID, NIH). *Il4*$^{-/-}$*Il13*$^{-/-}$*Rag1*$^{-/-}$ mice were generated by crossing these 2 lines. The mice used were female and between 6 and 8 weeks of age at the start of the experiment. All animals were housed under specific pathogen-free conditions in an American Association for the Accreditation of Laboratory Animal Care–approved facility of University of Colorado (Aurora, CO). All animal studies were approved by the University of Colorado Institutional Animal Care and Use Committee. The procedures followed were in accord with institutional guidelines.

Schistosoma-Induced PH Model

Live *S. mansoni* eggs were obtained by homogenizing and purifying livers of Swiss–Webster mice preinfected with *S. mansoni* cercariae, from the Biomedical Research Institute (Rockville, MD). Similar to earlier publications,[8,11,12] experimental mice were intraperitoneally sensitized to 240 *S. mansoni* eggs/g body weight and then 2 weeks later intravenously challenged with 175 *S. mansoni* eggs/g body weight. Control mice were unexposed to *S. mansoni* eggs.

Shared Circulatory System (Parabiosis)

Parabiosis was performed using published techniques.[13] In brief, mice were anesthetized with isoflurane, carprofen, and buprenorphine, incisions made on opposing flanks, and skin flaps sutured together. Postoperatively, carprofen and buprenorphine were administered for pain and sulfamethoxazole-trimethoprim antibiotic administered in rodent chow for 10 days. Two weeks after surgery, to allow time for recovery, mice were used for subsequent experiments.

Adoptive Transfer of CD4 Cells or Splenocytes

Some of the mice were used as donors for CD4$^+$ T cells or splenocytes. These mice were euthanized and spleens removed, homogenized, and CD4$^+$ cells purified by negative selection column (BD Biosciences, San Jose, CA). CD4 cells ($3–8\times10^6$) were administered by intraperitoneal injection to each recipient. Recipients of splenocytes received 3.8×10^7 cells from homogenized donor spleens by intraperitoneal injection.

Assessment of PH

At the conclusion of the experiments, mice underwent terminal right heart catheterization and tissue harvest, as described previously.[8,11,12] Briefly, mice were sedated with intraperitoneal ketamine-xylazine and a tracheostomy placed and mechanical ventilation initiated at 6 cc/kg. Sharp dissection was used to open the abdomen and diaphragm,

and a 1-Fr pressure-volume catheter (PVR-1035; Millar ADInstruments, Houston, TX) was placed directly into the right ventricular and then left ventricular chambers through the free walls. Lungs were then flushed with PBS, the right bronchus sutured, and the left lung inflated with 1% low-melt agarose for formalin fixation and paraffin embedding for histology, and the right lung divided for snap freezing for protein quantification or placed in RNAlater (Life Technologies, Carlsbad, CA) for RNA quantification.

Flow Cytometry

Three days after intravenous egg augmentation, mouse lungs were flushed with PBS and digested for flow cytometry analysis as previously reported.[11] Briefly, lungs were digested with Liberase (Roche, Waiblingen, Germany) and dissolved in RPMI medium (Mediatech, Corning, NY); tissue was disrupted by passing it 5 times through a 16-Gg needle followed by 5 times through an 18-Gg needle. Cells were then filtered using a 100-μm cell strainer (Thermo Fisher Scientific, Waltham, MA) and centrifuged for 5 minutes at 300*g*. Red blood cells were lysed with ACK lysis buffer (Gibco, Grand Island, NY), and cells were resuspended in RPMI, filtered again, centrifuged, and resuspended into flow wash buffer (5% BSA in PBS with EDTA). Blocking of nonspecific Fc-receptormediated antibody binding was performed (CD16/CD32; BD Biosciences), and cells were stained (extracellularly for all antibodies except IL-4) using the antibodies listed in Table S1. Data were acquired using a BD Biosciences LSRII flow cytometer with a BD Biosciences Facs DIVA software. Compensation was calculated using DIVA software based on compensation controls for each fluorochrome used in the experiment. The raw data were then analyzed using FlowJo (version 7.6; BD Biosciences). RT-PCR for IL-4 and IL-13 mRNA was performed on sorted cells using primers from Applied Biosystems (Foster City, CA), with the $2^{-\Delta Ct}$ method, using β-actin and glyceraldehyde-3-phosphate dehydrogenase as reference genes.

Protein Quantification

Samples of frozen right lung tissue were macerated and sonicated in RIPA buffer containing antiproteases. Total protein concentration was determined by Bradford assay, and IL-4 and IL-13 protein concentrations were quantified by ELISA in mouse lung lysates using the kits listed in Table S2.

Histopathology Assessment

Immunostaining for CD3 and α-smooth muscle actin (α-SMA) was performed on formalin fixation and paraffin embedding mouse lung tissue, using the reagents listed in Table S3. For quantification of perivascular CD3$^+$ density, slides were scanned using an Aperio VERSA slide scanner (Leica Biosystems, Buffalo Grove, IL), the number of CD3$^+$ T cells in the perivascular region was counted, and the cross-sectional area of the adventitial space (defined as the space between the outside of the medial layer and inside of the adjacent alveoli) and vessel radius (calculated from the measured cross-sectional area encompassed by the outside of the medial layer) was determined for 10 to 21 vessels per specimen. For quantification of fractional media thickness, images of 10 to 12 vessels of each specimen from the α-SMA–stained slides were acquired using a Nikon Eclipse E800 microscope (Nikon, Melville, NY) with a black and white charge-coupled device camera (Photometrics, Tuscon, AZ). External and internal perimeters of the media were identified using image-processing software (Image Pro Plus v4.5.1; Media Cybernetics, Bethesda, MD), the radii of each layer calculated (r=sqrt(A/pi)), and the fractional thickness calculated from the difference between the 2 radii divided by the external radius. Peri-egg granuloma volumes were measured using a stereological method, termed the optical rotator, with formalin fixation and paraffin embedding tissue was stained with hematoxylin and eosin, 8 to 10 images of granulomas with single visible egg acquired for each sample, and the rotator method for object volume estimation applied using the egg as the central point (Image Pro Plus v4.5.1; Media Cybernetics).[8,14]

Egg Burden Quantification

The number of residual *S. mansoni* eggs in mouse lung tissue was determined by digesting 20 to 30 mg of frozen tissue in 4% KOH for 18 hours at 33°C and counting the number of eggs in the digest.[15]

Statistical Analysis

ProStat (Poly Software International, Pearl River, NY) and SigmaPlot (Systat Software, San Jose, CA) were used to perform statistical analyses and graphs. Differences between 2 groups were assessed by t test; for ≥3 groups, differences were assessed by ANOVA followed by Tukey's post hoc test. Non-normally distributed data or groups with samples <5 were analyzed by nonparametric analysis. P values <0.05 were considered statistically significant.

Results

There Is an Increased Number of Th2 CD4$^+$ T Cells in Lungs of Mice Exposed to *Schistosoma*

We used an established experimental murine model of *S. mansoni*–induced PH, which is triggered by intraperitoneal egg injection (sensitization) followed by intravenous

embolization of egg antigens into the pulmonary circulation 2 weeks later (challenge; Figure 1A).[8,10,11] This model mimics the natural form of infection with *S. mansoni*, a helminth parasite that migrates to the portal vasculature and lays hundreds of eggs per day, which promote development of a systemic type 2 adaptive immune response to the egg antigens. Some patients with chronic and severe hepatosplenic disease develop portal hypertension, resulting in opening of portocaval shunts that facilitate the embolization of *Schistosoma* eggs from the portal venous system to the pulmonary circulation. This experimental model thus focuses on the immunological trigger of intrapulmonary vascular disease, while avoiding the confounding role of portal hypertension that could also contribute to PH development. We have shown previously that mice must be sensitized to parasite eggs to develop PH after egg embolization, suggesting a role for egg-antigen–specific adaptive immune cells in the pathogenesis of PH.[8]

Flow cytometry of cell-dispersed murine lungs showed that the absolute number of CD4$^+$ T cells increased in *Schistosoma*-exposed mice (Figure 1B and Figure S1), with a large increase in the percent of CD4$^+$ T cells expressing intracellular IL-4, consistent with a Th2 phenotype (Figure 1C). To confirm the Th2 phenotype of recruited cells, we sorted CD3$^+$CD4$^+$ cells and observed that both IL-4 and IL-13 mRNA were measurable in cells from *Schistosoma*-exposed mice, but neither was detectable in cells from unexposed mice (Figure S2).

By immunostaining, we quantified CD3$^+$ T cells in the perivascular adventitia specifically, a position most likely to interact with the vasculature. We determined that density was significantly higher in *Schistosoma*-exposed wild-type mice as compared with unexposed mice, around both small and large pulmonary vessels (Figure 1D and Figure S3). This observation in experimentally treated animals parallels the reported increase in perivascular T cells in lung tissue of patients who died of schistosomiasis-associated PAH by Mauad et al.[16]

There could also be an increase in circulating CD4 T cells, potentially preceding the observed increase in lung tissue, and we thus assessed the time course of CD4 T-cell density in the blood and in the lung tissue. We observed that there was evidence of an increased density of CD4 T cells in both the lung and in the blood, starting 3 days after intravenous challenge, but did not find that there was an earlier change in 1 compartment compared with the other (Figure S4).

Eosinophils and basophils are also present in *Schistosoma*-exposed mouse lungs and could contribute to the PH pathology. Flow cytometry of lung digest samples for these cells revealed a 20-fold increase in the number of eosinophils and a 10-fold increase in the number of basophils, both peaking 3 days after intravenous *Schistosoma* egg challenge (Figures S1 and S5).

Schistosoma Egg Embolization Into the Lungs Triggers CD4$^+$ T-Cell Recruitment From the Circulation and Th2 Phenotype

To test whether *Schistosoma* egg embolization into the pulmonary vasculature drives localized T-cell activation and type 2 immunity, we performed parabiosis surgery, linking the circulatory systems of 2 mice by creation of anastomoses in the subcutaneous tissue in the flanks. We administered intraperitoneal egg sensitization to both, but then intravenous egg challenge to only 1 parabiont (Figure 2A): *Schistosoma* eggs have a short axis diameter of $\approx$50 μm, preventing further migration distal to the pulmonary vasculature, such as to the noninjected partner. This revealed, interestingly, a similar density of CD4$^+$ T cells in lungs of both parabionts (Figure 2B), which was as high as the density in an individually challenged mouse (mean of 4×10^5; Figure 1B). These data indicate that intravenous antigen challenge can induce a lung-specific homing mechanism, such as has been reported for inhaled antigens.[17] We observed there was a higher number of proliferating (Ki67$^+$) CD4$^+$ T cells in lungs of the parabiont that received the intravenous eggs as compared with the unchallenged parabiont: Here, Ki67$^+$ as a marker of proliferation indicates CD4 T-cell activation (Figure 2C). We also observed more IL-4 expression in lung tissue and a trend toward increased IL-13 levels in the parabiont that received the intravenous eggs as opposed to the unchallenged parabiont (Figure 2D and 2E), results further consistent with egg embolization triggering localized type 2 immunity.

Deficiency of CD4$^+$ T Cells Protects Against *Schistosoma*-PH

To understand the functional role of CD4$^+$ T cells in *Schistosoma*-PH, we studied the phenotype of mice with an absence of functional T and B cells using recombinase 1–deficient (hereafter designated *Rag*$^{-/-}$) mice, which prevents T- and B-cell receptor maturation.[18] In wild-type mice, *Schistosoma* egg embolization following earlier egg sensitization results in a significant increase in right ventricular systolic pressure (RVSP), vascular media thickness, and IL-4 and IL-13 cytokine concentrations in lung tissue (Figure 3). However, upon *Schistosoma* egg embolization, previously egg-sensitized *Rag*$^{-/-}$ mice had no increase in RVSP, change in vascular media thickness, or a change in concentration of pulmonary IL-4 and IL-13 as compared with unexposed *Rag*$^{-/-}$ mice. The volume of the peri-egg granulomas—which consist of macrophages, fibroblasts, eosinophils, and T cells—can be used as another readout of total type 2 inflammation in lung.[8,19] Similar to the lower IL-4/IL-13 cytokine levels, we observed that granulomas were also much smaller in *Schistosoma*-challenged *Rag*$^{-/-}$ mice compared with wild-type mice challenged with *Schistosoma*.

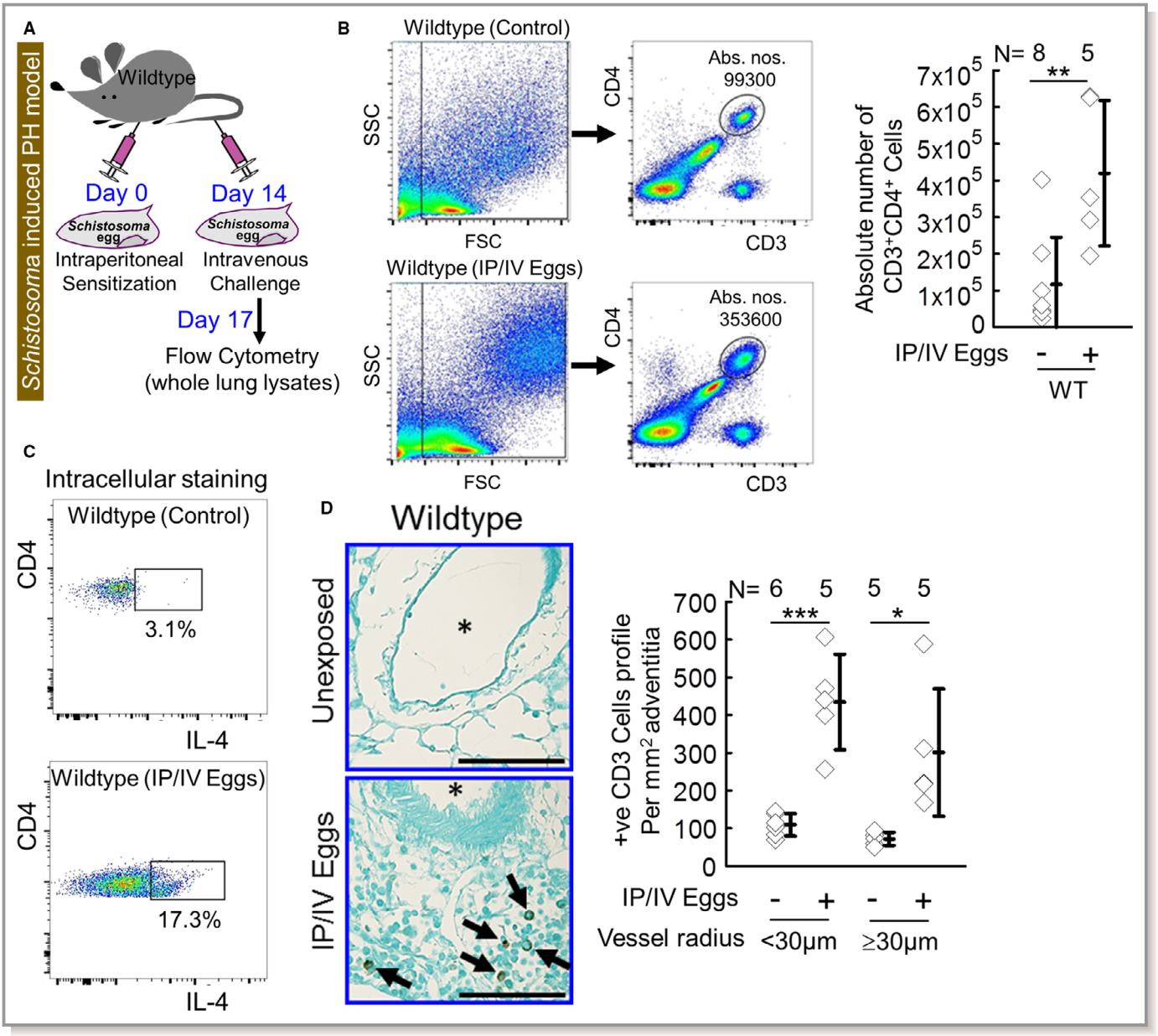

Figure 1. Intrapulmonary Th2 CD4$^+$ T cells are increased following *Schistosoma* exposure. **A**, Experiment outline. **B**, Representative sorting of CD3$^+$CD4$^+$ singlets from whole lung digest from unexposed or *Schistosoma*-exposed mice and the absolute number of CD4$^+$ cells per mouse lung. **C**, Intracellular IL-4 staining of sorted cells (not further stimulated; representative of 2 independent experiments). **D**, Representative immunostaining and quantification of the CD3$^+$ T-cell profile density in vessel adventitia (asterisk, vessel lumen; arrows, representative positive CD3 cells; scale bars, 100 μm). Mean±SD plotted; *t* test, **P*<0.05; ***P*<0.01; ****P*<0.005. FSC indicates forward scatter; IL-4, interleukin-4; SSC, side scatter; WT, wild type.

We observed no significant differences in left ventricular systolic pressure, right or left ventricular diastolic pressures, heart rate, or body weight between *Schistosoma*-exposed and unexposed *Rag*$^{-/-}$ mice (Table S4). Of note, the otherwise unchallenged *Rag*$^{-/-}$ mice had a higher RVSP than unchallenged wild-type mice, suggesting that deficiency of B and T cells can impair normal lung vascular development, but there was no evidence of a baseline change in pulmonary vessel media thickness or type 2 immune activation.

Reconstitution of *Rag*$^{-/-}$ Mice With CD4$^+$ T Cells Restores *Schistosoma*-PH

The *Rag*$^{-/-}$ phenotype could be confounded by B-cell deficiency: B cells can present antigen to effector and memory T cells and can modulate Th2 immunity in schistosomiasis.[20] To confirm that CD4$^+$ T cells specifically are the subset of lymphocytes necessary for the *Schistosoma*-PH phenotype, we adoptively transferred CD4$^+$ T cells, isolated by

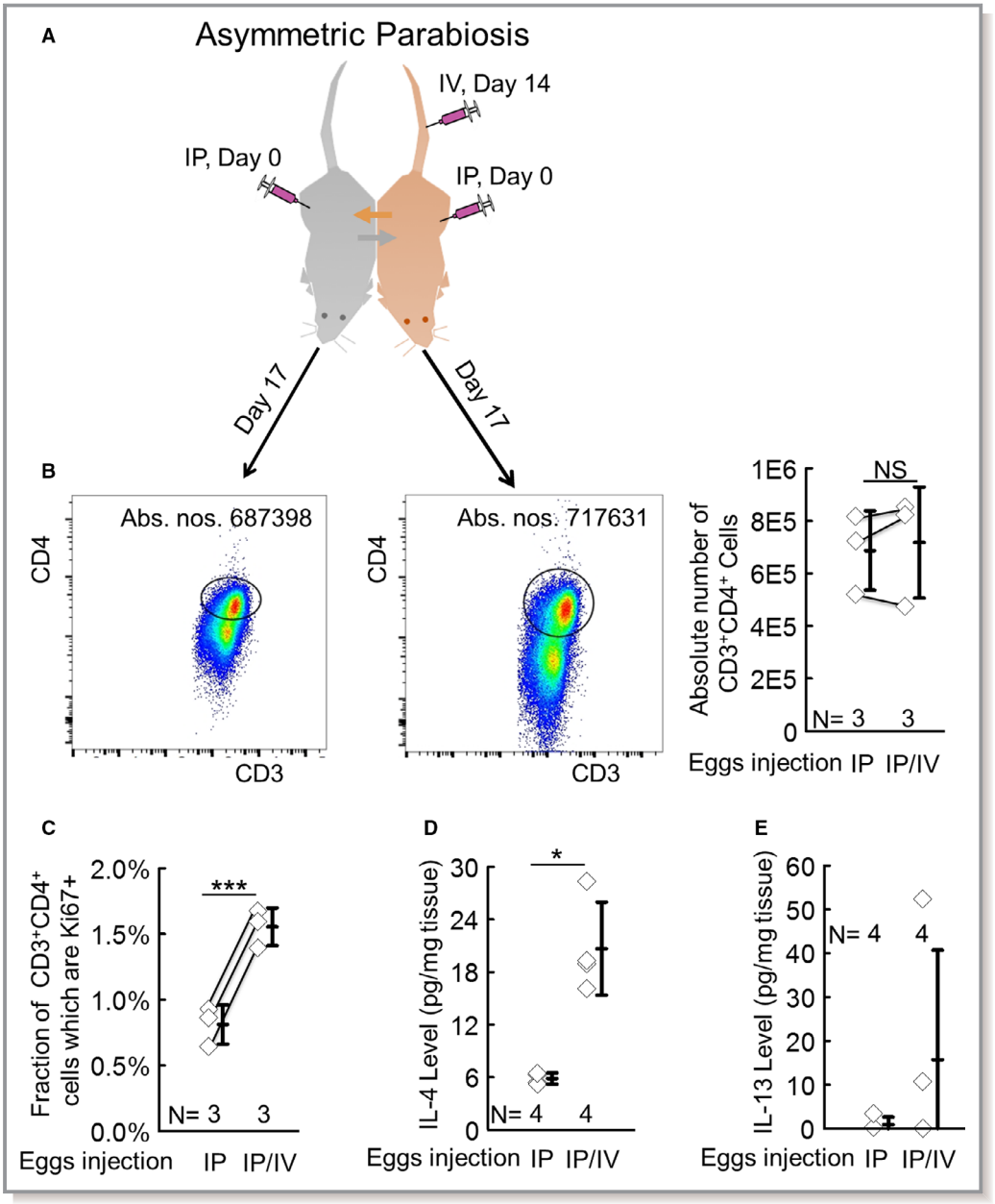

Figure 2. Intravenous eggs are necessary for CD4$^+$ T-cell activation and type 2 immunity. **A**, Design of the experiment. **B**, Absolute number (Abs. nos.) of CD3$^+$CD4$^+$ cells in lungs of *Schistosoma*-exposed and unexposed parabionts (representative of 3 parabiont pairs per experimental group). **C**, Fraction of CD3$^+$CD4$^+$ cells, which are Ki67$^+$ in the lungs of each parabiont. **D**, IL-4 and **E**, IL-13 protein concentrations (N=4/group; however, the IL-13 was undetected in 2 and 1 samples from IP only and IP/IV groups, respectively) in whole lung lysates of each parabiont (N=number of animals/group; mean±SD plotted; *t* test for [**B** and **C**], rank sum test for all [**D**]; **P*<0.05; ****P*<0.005). IL-4 indicates interleukin-4; IL-13, interleukin-13; IP, intraperitoneal sensitization alone; IP/IV, intraperitoneal sensitization and intravenous challenge with *Schistosoma mansoni* eggs; NS, nonsignificant.

negative selection from the spleens of naïve wild-type mice, into *Rag*$^{-/-}$ mice and then sensitized and challenged them with *Schistosoma* eggs (Figure 4A). Adoptive transfer of wild-type CD4$^+$ T cells was sufficient to induce PH as evidenced by higher RVSP and increased media thickness, as compared with CD4$^+$ T-cell–reconstituted *Rag*$^{-/-}$ mice that remained unchallenged (Figure 4B and 4C). These gain-of-function mice also had significant increases in the concentration of IL-4 and IL-13 following *Schistosoma* challenge (Figure 4D and 4E). There was also a modest trend (*P*=0.1, with correction for multiple comparisons) toward an increase in the estimated volume of the peri-egg granulomas between *Schistosoma*-

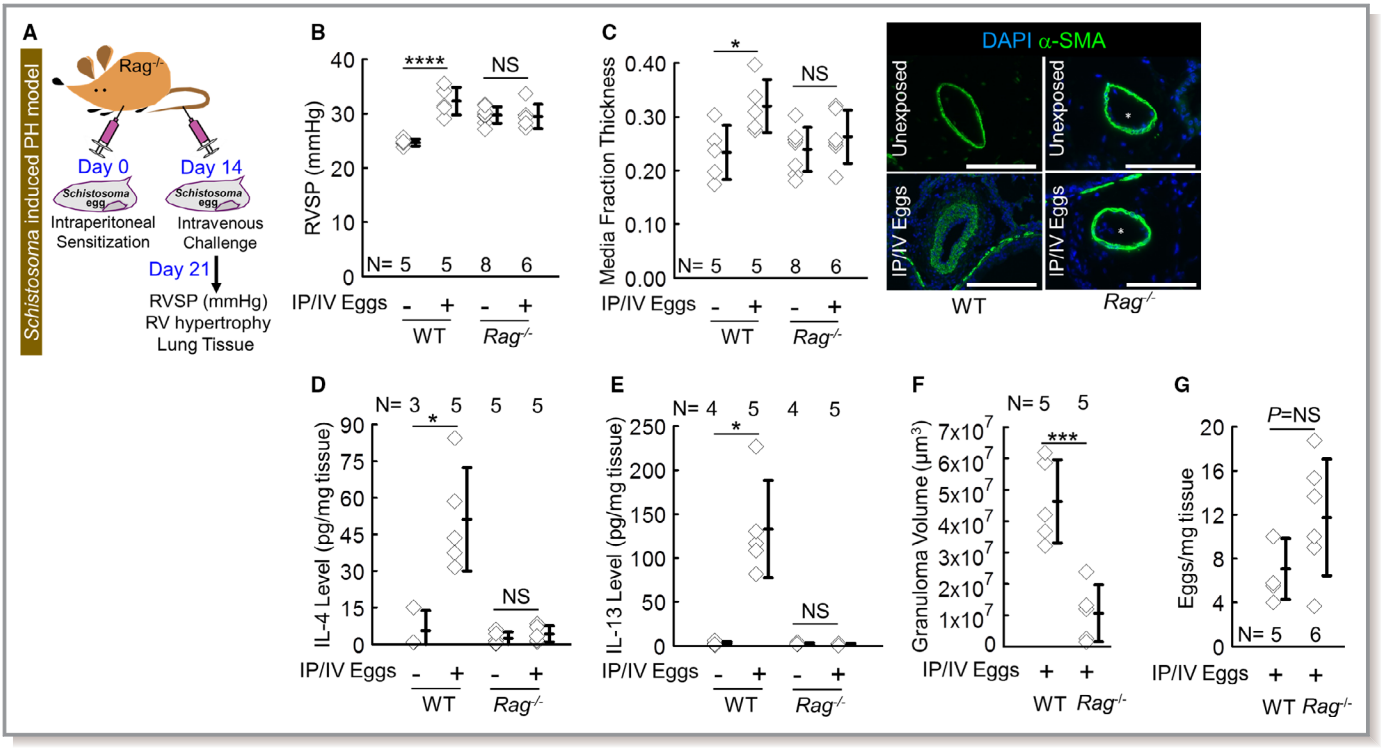

Figure 3. Compared with wild-type (WT) mice, *Rag*$^{-/-}$ mice, which lack B and T cells, do not develop PH or type 2 inflammation following *Schistosoma* exposure. **A**, Experimental outline. **B**, Right ventricular systolic pressure (RVSP); (**C**) media thickness (asterisks, vessel lumen; scale bars, 50 μm); (**D**) IL-4 and (**E**) IL-13 protein concentrations; (**F**) estimated granuloma volumes, as measured by stereology; (**G**) number of *Schistosoma mansoni* eggs recovered from lung digests in whole lung lysates of unexposed or *Schistosoma*-exposed WT or *Rag*$^{-/-}$ mice (mean±SD plotted; *t* test for [**B**, **C**, **F**, and **G**], rank-sum test for [**D** and **E**]; **P*<0.05; ****P*<0.005; *****P*<0.001). DAPI indicates 4′,6-diamidino-2-phenylindole; IL-4, interleukin-4; IL-13, interleukin-13; IP/IV, intraperitoneal/intravenous *S. mansoni* eggs; NS, nonsignificant; PH, pulmonary hypertension; RV, right ventricular; α-SMA, alpha smooth muscle actin.

exposed *Rag*$^{-/-}$ and *Rag*$^{-/-}$ mice reconstituted with wild-type CD4$^+$ T cells (Figure S6). We considered the possibility that the immune system impairment in *Rag*$^{-/-}$ mice may block the clearance of schistosome eggs, which could confound the PH phenotype. However, we observed no difference in the residual egg burden in lungs measured 1 week after intravenous challenge. There were no significant differences in systemic pressures, heart rate, or body weight between *Schistosoma*-exposed and unexposed *Rag*$^{-/-}$ mice reconstituted with wild-type CD4$^+$ T cells (Table S5).

Egg Antigen-Specific Th2 Cells Are Required for *Schistosoma*-PH

IL-4/IL-13–deficient mice do not develop *Schistosoma*-PH,[10] suggesting that cytokine production by CD4$^+$ T cells may be required to drive type 2 inflammation and PH. To determine the role of Th2 cells in *Schistosoma*-PH, we reconstituted *Rag*$^{-/-}$ mice with CD4$^+$ T cells harvested from *Il4*$^{-/-}$*Il13*$^{-/-}$ donors. We observed that these mice were protected from PH following *Schistosoma* sensitization and intravenous challenge, as evidenced by lower RVSP, less vascular remodeling, and less intrapulmonary IL-4 and IL-13, as compared with *Schistosoma*-exposed *Rag*$^{-/-}$ mice reconstituted with wild-type CD4$^+$ T cells (Figure 5). Furthermore, the *Schistosoma*-exposed *Rag*$^{-/-}$ mice reconstituted with *Il4*$^{-/-}$*Il13*$^{-/-}$ CD4$^+$ T cells had significantly smaller peri-egg granulomas compared with recipients of wild-type CD4$^+$ T cells, consistent with reduced type 2 inflammation. There were no significant differences in systemic pressures, heart rate, or body weight between *Schistosoma*-exposed *Rag*$^{-/-}$ mice reconstituted with wild-type or *Il4*$^{-/-}$*Il13*$^{-/-}$ CD4$^+$ T cells (Table S6).

Primed CD4$^+$ T Cells Are Sufficient to Drive *Schistosoma*-PH in Mice Lacking an Adaptive Immune System

Adaptive immunity is required for the *Schistosoma*-PH phenotype, given that a single intravenous challenge of *S. mansoni* eggs alone is inadequate to trigger PH.[10] We suspected that the key cells which need to be activated in this adaptive immune response are the CD4$^+$ T cells. To test this hypothesis, we first sensitized wild-type mice with *Schistosoma* eggs, and then transferred CD4$^+$ T cells isolated from the spleens of these primed mice into *Rag*$^{-/-}$ mice, followed by intravenous challenge with *Schistosoma* eggs alone (without further sensitization). The control animals for this experiment

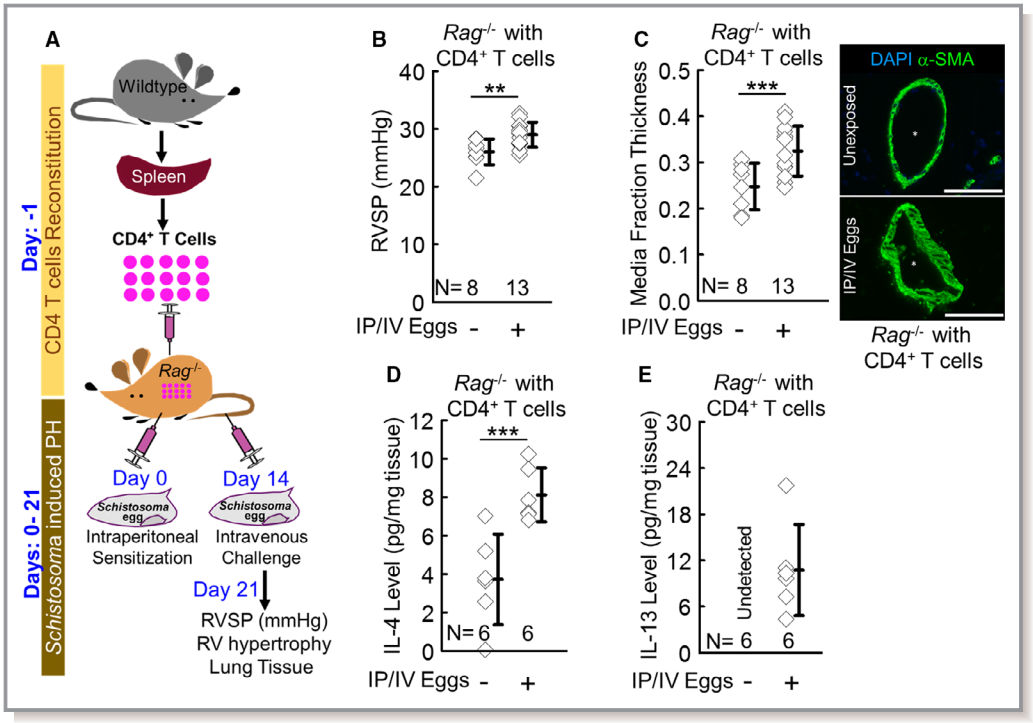

Figure 4. Adoptive transfer of wild-type CD4$^+$ T cells into *Rag*$^{-/-}$ mice restores *Schistosoma*-induced PH and type 2 immunity. **A**, Experimental outline of wild-type CD4$^+$ T-cell reconstitution into *Rag*$^{-/-}$ mice. **B**, Right ventricular systolic pressure (RVSP); (**C**) media thickness; (**D**) IL-4 and (**E**) IL-13 protein concentrations in whole lung lysates in unexposed and *Schistosoma*-exposed *Rag*$^{-/-}$ mice reconstituted with wild-type CD4$^+$ T cells (mean±SD plotted; *t* test for all panels: **P*<0.05; ***P*<0.01; ****P*<0.005; asterisks, vessel lumen; scale bars, 50 μm). DAPI indicates 4′,6-diamidino-2-phenylindole; IL-4, interleukin-4; IL-13, interleukin-13; IP/IV, intraperitoneal/intravenous *Schistosoma mansoni* eggs; PH, pulmonary hypertension; RV, right ventricular; α-SMA, alpha smooth muscle actin.

were *Rag*$^{-/-}$ mice reconstituted with CD4$^+$ T cells from unsensitized donors and challenged with *Schistosoma* eggs. Interestingly, we observed significantly higher RVSP and vascular remodeling in the challenged *Rag*$^{-/-}$ recipients of primed CD4$^+$ T cells, as compared with challenged recipients of unprimed CD4$^+$ T cells, accompanied by significantly higher IL-4 and IL-13 concentrations in lung (Figure 6). There was also a trend toward increased granuloma volumes in the *Rag*$^{-/-}$ recipients of primed CD4$^+$ T cells, as compared with recipients of unprimed CD4$^+$ T cells (P=0.079). These results suggest that egg-antigen–specific CD4$^+$ T cells are the only component of the adaptive immune response required for *Schistosoma*-PH. There were no significant differences in systemic pressures, heart rate, or body weight between *Rag*$^{-/-}$ recipients of *Schistosoma*-sensitized or unsensitized wild-type CD4$^+$ T cells followed by *Schistosoma* challenge (Table S7).

IL-4/IL-13 Expression by Parenchymal Cells Is Also Required for *Schistosoma*-PH

Type 2 immunity by cells other than CD4 T cells may also contribute to *Schistosoma*-induced PH. We crossed B-/T-cell–deficient mice with IL-4/IL-13–deficient mice to generate mice which lack B cells, T cells, and expression of IL-4 and IL-13 by all other cells (*Il4*$^{-/-}$*Il13*$^{-/-}$*Rag*$^{-/-}$). When sensitized and challenged with *Schistosoma* eggs, these mice also did not develop pulmonary hypertension (Figure 7A). We then reconstituted the mice with CD4$^+$ T cells from wild-type donors and observed that these mice had only a nonsignificant trend toward mildly increased RVSP, with a mean increase of only 2 mm Hg compared with nonreconstituted mice. Similarly, there were nonsignificant differences in the Fulton index, vascular remodeling, and granuloma volumes following *Schistosoma* sensitization and challenge (Figure 7A through 7D). There were also no significant changes in IL-4 or IL-13 whole lung expression (Figure 7E and 7F). Furthermore, we found that reconstituting these mice with splenocytes from a wild-type mouse (which includes CD4 T cells as well as many additional cells, including ILC2s, eosinophils, basophils, and others) did not alter the PH or type 2 immunity phenotype compared with CD4 T cells alone (Figure 7). These results indicate that there is an additional requirement for IL-4/IL-13 expression by cells other than CD4 T cells to trigger the type 2 immunity and PH phenotype in response to *Schistosoma* exposure, and that these additional cells likely have an extravascular, parenchymal location.

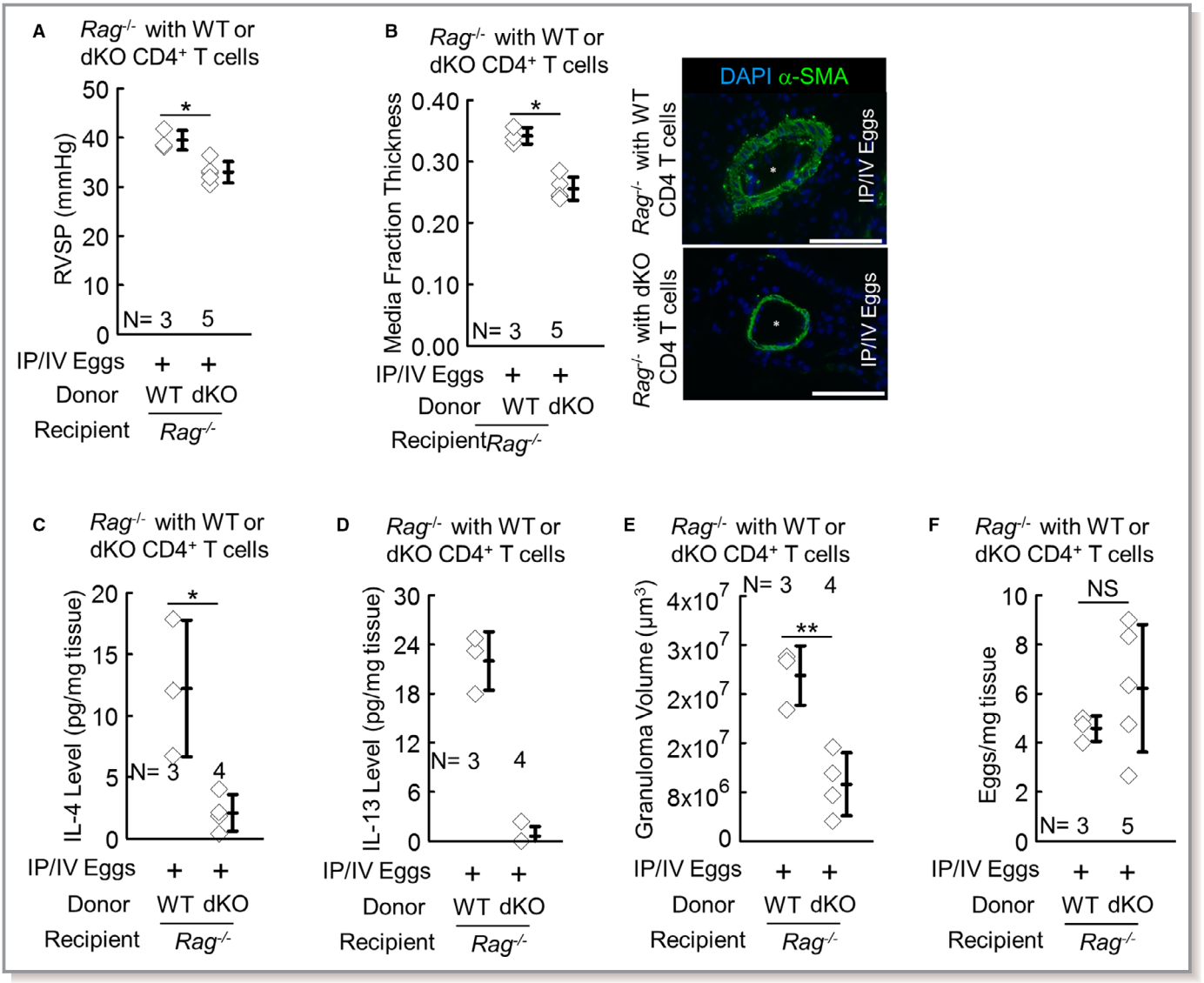

Figure 5. Adoptive transfer of Th2-deficient CD4+ T cells into *Rag*$^{-/-}$ mice blocks *Schistosoma*-induced PH and type 2 immunity. **A**, Right ventricular systolic pressure (RVSP); (**B**) media thickness; (**C**) IL-4 and (**D**) IL-13 protein concentrations (in *Rag*$^{-/-}$ recipients of dKO BM, IL-13 levels were undetectable in 3 of 4 samples); (**E**) estimated granuloma volumes, as measured by stereology; (**F**) number of *Schistosoma mansoni* eggs recovered from lung digests in whole lung lysates in unexposed and *Schistosoma*-exposed *Rag*$^{-/-}$ mice reconstituted with *Il4*$^{+/+}$*Il13*$^{+/+}$ (WT) or *Il4*$^{-/-}$*Il13*$^{-/-}$ (dKO) CD4 T cells mean±SD plotted; rank-sum test for all panels, **P*<0.05; ***P*<0.01; asterisks, vessel lumen; scale bars, 50 μm. BM indicates bone marrow; DAPI, 4′,6-diamidino-2-phenylindole; dKO, double knockout; IL-4, interleukin-4; IL-13, interleukin-13; IP/IV, intraperitoneal/intravenous *Schistosoma mansoni* eggs; NS, nonsignificant; PH, pulmonary hypertension; α-SMA, alpha smooth muscle actin; WT, wild type.

Discussion

We observed Th2 CD4+ T cells recruited from the circulation of *Schistosoma*-egg–sensitized mice are both necessary and sufficient for orchestrating the type 2 inflammation and experimental PH triggered by the pulmonary embolization of *S. mansoni* eggs. The significance of these data is reinforced by previous findings of type 2 inflammation in the lungs and peripheral blood of patients with schistosomiasis-associated PAH[8,21] and in lungs and peripheral blood of mice exposed to *Schistosoma*.[8,22] Furthermore, an increased density of perivascular CD4+ T cells has been previously reported in human schistosomiasis-associated PAH,[16] which closely parallels the observations here in *Schistosoma*-PH mice. Perivascular CD4 T cells have also been observed in idiopathic PAH,[1,2] but the role for these cells in the pathogenesis of this particular disease etiology is unclear. The clear role of antigen-specific adaptive immunity in *Schistosoma*-PH facilitates mechanistic analysis of the connection between antigen and vascular remodeling, in

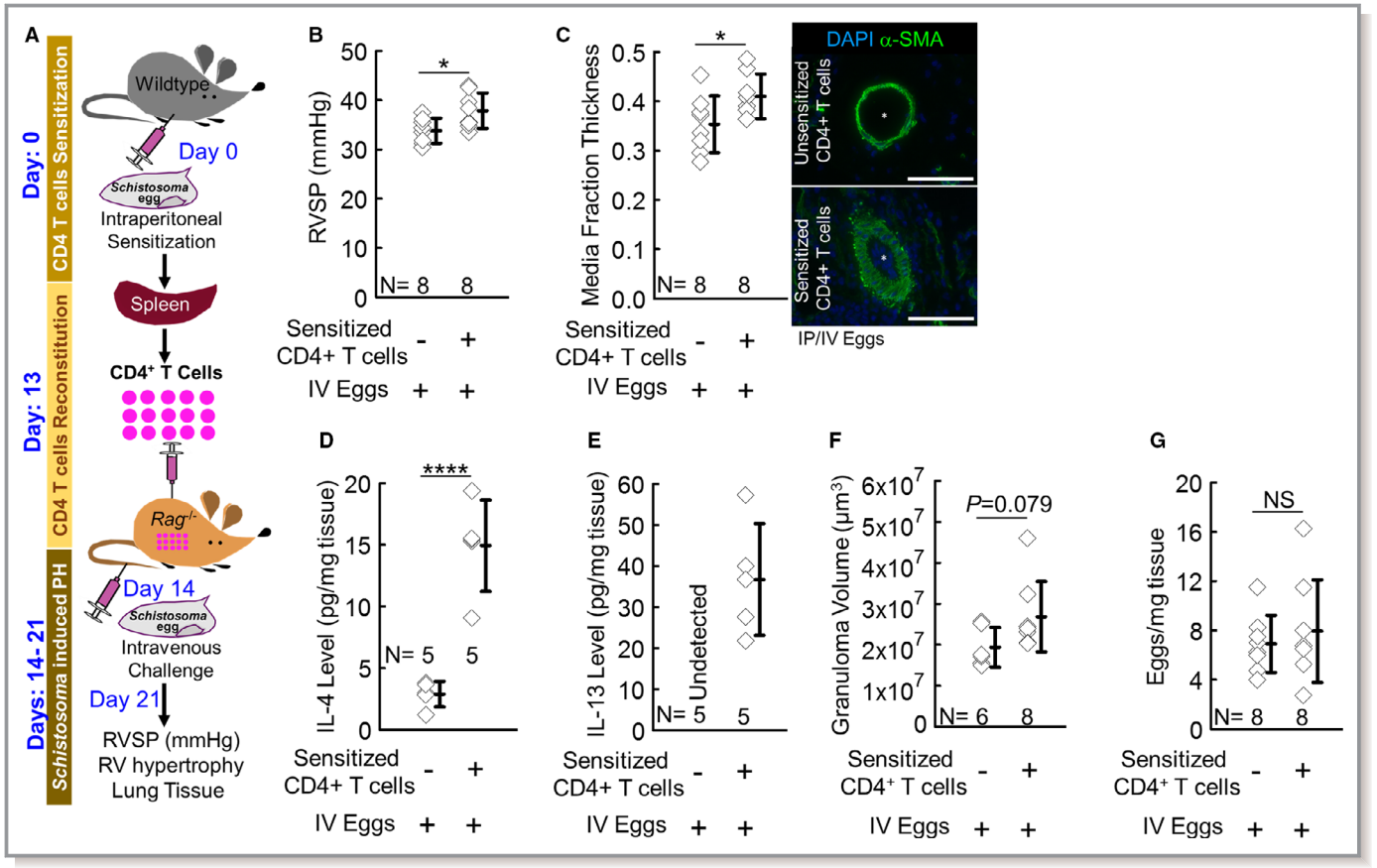

Figure 6. Reconstitution of *Rag*$^{-/-}$ mice with CD4$^+$ T cells from egg-sensitized mice is sufficient to drive type 2 inflammation and PH following *Schistosoma* egg challenge alone. **A**, Experimental outline. **B**, Right ventricular systolic pressure (RVSP); (**C**) media thickness; (**D**) IL-4 and (**E**) IL-13 protein concentration; (**F**) estimated granuloma volumes, as measured by stereology; (**G**) number of *Schistosoma mansoni* eggs recovered from lung digests in whole lung lysates in intravenously challenged *Rag*$^{-/-}$ mice reconstituted with unprimed or primed CD4$^+$ T cells mean±SD plotted; *t* test, **P*<0.05; *****P*<0.001; asterisks, vessel lumen; scale bars, 50 μm. DAPI indicates 4′,6-diamidino-2-phenylindole; IL-4, interleukin-4; IL-13, interleukin-13; IP/IV, intraperitoneal/intravenous *S. mansoni* eggs; NS, nonsignificant; PH, pulmonary hypertension; RV, right ventricular; α-SMA, alpha smooth muscle actin.

contrast to the use of hypoxia or nonspecific inflammation following monocrotaline treatment or vascular endothelial growth factor receptor 2 blockade commonly used in other PH animal models.

Previous studies have implicated other CD4$^+$ T-cell phenotypes in other forms of experimental PH. In athymic, T-cell–deficient rats, vascular endothelial growth factor receptor 2 blockade induces a severe PH phenotype, whereas restoring FoxP3$^+$ regulatory T cells is protective.[23] Mice challenged with inhaled ovalbumin can develop type 2 immunity and some degree of PH, and the PH phenotype is inhibited by anti-CD4 antibody administration, or blocking IL-4 or IL-13 cytokines.[24] In contrast, Th17 T cells contribute to the development of hypoxia-induced PH.[25,26] Interestingly, mice challenged with a combination of hypoxia and inhaled ovalbumin develop PH, which is mediated predominantly by type 2 immunity.[27] In this model, the PH phenotype is inhibited by deletion of the Th2 promoting receptor, CRTH2, with the phenotype restored by supplementing the mice with wildtype CD4 T cells, and reversed by IL-4/IL-13 dual neutralization.[27] An open question is whether inflammation contributes to the ongoing propagation of PH after the disease has been initiated. The potential for targeted immune blockade to reverse established PH needs to be tested in future studies: The clinical relevance is the observed general absence of clinical benefit of broad immunosuppressant therapy in PH patients, except in particularly inflammatory conditions such as lupus-associated disease.[28]

Our use of *Rag*$^{-/-}$ mice could be confounded by a phenotype resulting from B-cell deficiency: B cells can present antigen to effector and memory T cells and are reported to modulate Th2 immunity in schistosomiasis.[29] CD8$^+$ T cells are also depleted in *Rag*$^{-/-}$ mice and could contribute to the PH phenotype. However, the regained *Schistosoma*-PH phenotype in *Rag*$^{-/-}$ mice following adoptive transfer with CD4$^+$ T cells alone suggests that B cells and CD8 T cells are not required for *Schistosoma*-PH. We found that *Rag*$^{-/-}$ mice at baseline have PH, potentially attributed to abnormal perinatal lung

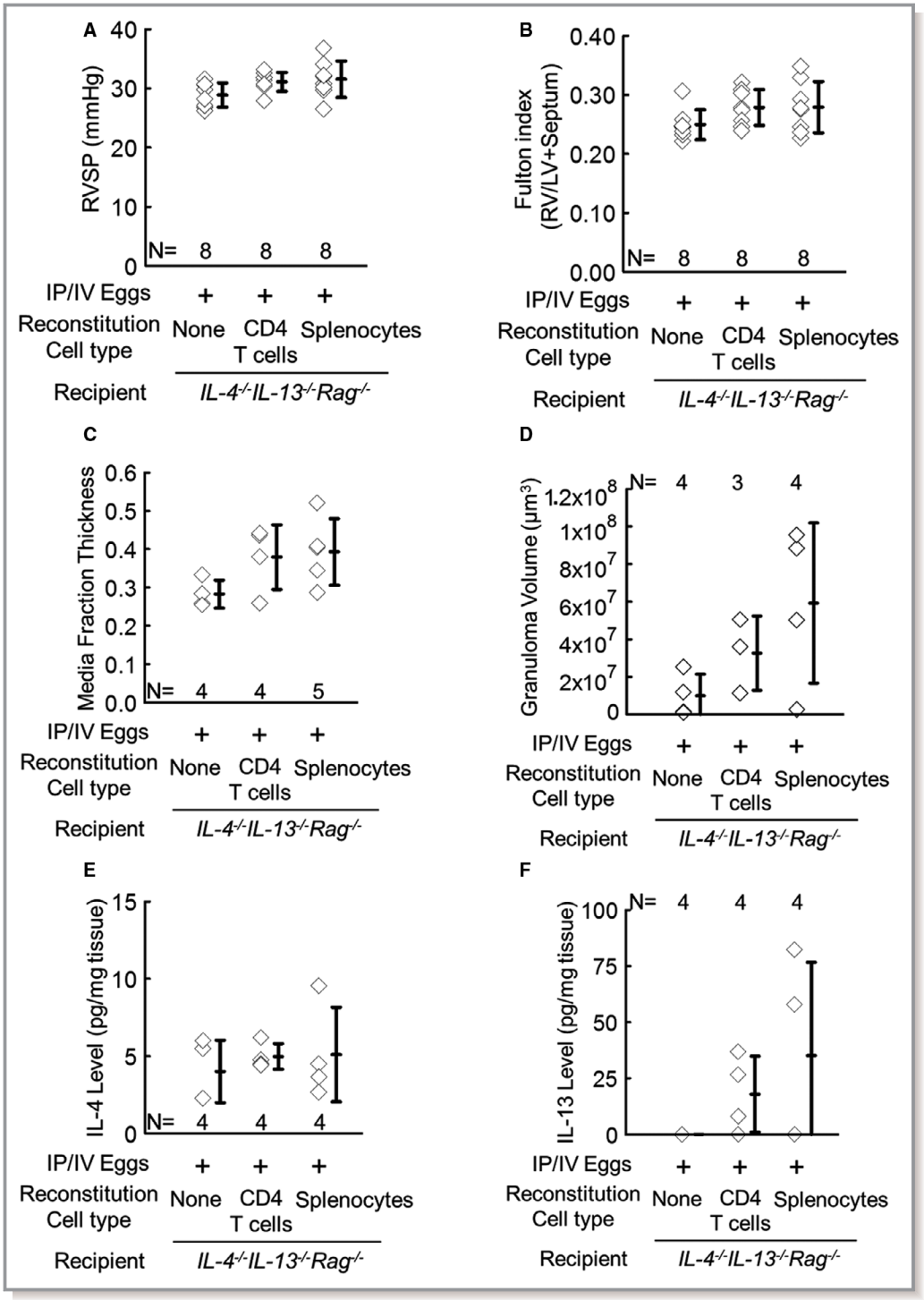

Figure 7. PH phenotype of Il4$^{-/-}$Il13$^{-/-}$Rag$^{-/-}$ mice exposed to IP/IV *Schistosoma* eggs is not rescued by reconstitution with either CD4 T cells or splenocytes. **A**, Right ventricular systolic pressure (RVSP); (**B**) Fulton index; (**C**) media thickness; (**D**) granuloma volumes; and (**E**) IL-4 and (**F**) IL-13 protein concentration in whole lung lysates in *Schistosoma*-sensitized and intravenously challenged Il4$^{-/-}$Il13$^{-/-}$Rag$^{-/-}$ mice reconstituted with no cells, CD4^{+} T cells, or splenocytes from a wild-type mouse mean±SD plotted; ANOVA *P* values: 0.07, 0.16, 0.11, 0.11, 0.81, and 0.33, respectively; *t*-test values are shown in all panels; IL-13 was undetectable in 4 of 4, 1 of 4, and 2 of 5 samples in the no cells, CD4 T cells, and splenocytes groups, respectively. IL-4 indicates interleukin-4; IL-13, interleukin-13; IP/IV, intraperitoneal/intravenous *S. mansoni* eggs; LV, left ventricular; RV, right ventricular.

development with this germline absence of mature T and B cells. It has been reported that primary immunodeficiency in humans can be associated with the spontaneous occurrence of PH.[30,31]

We investigated a potential role for other non-B/T cells in modulating type 2 immunity in *Schistosoma*-PH. We found that expression of IL-4/IL-13 by parenchymal cells was required for the proximate activation of type 2 immunity (IL-4 expression in particular) and PH: We suspect that these are dendritic cells which guide the activation of CD4 T cells, also called "signal 3."[32] Consistent with these data, we previously observed that IL-4 knockout mice had less right ventricular hypertrophy and smaller granuloma volumes compared with wild-type mice following *Schistosoma* challenge.[8] Our data do not exclude the possibility that other non-B/T cells, such as ILC2s, eosinophils, or basophils, could amplify the type 2 immunity or drive PH downstream of Th2 CD4$^+$ T cells, and indeed we found an increase in density of eosinophils and basophils in *Schistosoma*-exposed mouse lungs. However, in models of liver fibrosis resulting from *Schistosoma* infection, both eosinophils and basophils have been shown to be dispensable to granuloma formation and fibrosis.[33,34] We suspect that circulating monocytes and interstitial macrophages are critical targets of Th2 signaling, because we recently reported that recruitment of CCR2$^+$Ly6c$^+$ monocytes to the vascular adventitia is a necessary step for transforming growth factor beta activation and the PH phenotype in *Schistosoma*-exposed mice.[11]

We observed evidence of lung-specific homing of activated CD4 T cells, given that a parabiosis experiment in which only 1 parabiont received intravenous egg challenge resulted in a comparable increase in density of CD4$^+$ T cells in both parabionts. Lung-specific CD4$^+$ T-cell homing has been previously described in mycobacterium infection[35,36] and in response to inhaled ovalbumin or influenza infection by a C-C chemokine receptor type 4 (CCR4)-dependent mechanism.[17]

A highly relevant modulation of CD4 T cells in *Schistosoma*-PAH will occur in the estimated 6M individuals worldwide who are simultaneously infected with human immunodeficiency virus.[37] Given that both human immunodeficiency virus and *Schistosoma* are triggers of PAH, it is possible that dually infected individuals will have a higher prevalence of PAH, and in line with this hypothesis is the observation that there is a higher prevalence of liver disease complicating schistosomiasis in dually infected individuals, as compared with those infected with *Schistosoma* alone.[38] Alternatively, it is possible that the immune suppression resulting from human immunodeficiency virus infection could block the type 2 immunity required for *Schistosoma*-induced PH as shown here.

In summary, we found Th2 CD4$^+$ T cells are necessary and sufficient for the type 2 immunity that drives PH following *Schistosoma* exposure. Depleting CD4$^+$ T cells, blocking the Th2 phenotype, or switching the cells to a different phenotype could be a potential therapeutic target in inflammation-triggered forms of PAH, including that following *Schistosoma* infection.

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Disclosures

None.

References

1. Savai R, Pullamsetti SS, Kolbe J, Bieniek E, Voswinckel R, Fink L, Scheed A, Ritter C, Dahal BK, Vater A, Klussmann S, Ghofrani HA, Weissmann N, Klepetko W, Banat GA, Seeger W, Grimminger F, Schermuly RT. Immune and inflammatory cell involvement in the pathology of idiopathic pulmonary arterial hypertension. *Am J Respir Crit Care Med*. 2012;186:897–908.
2. Marsh LM, Jandl K, Grünig G, Foris V, Bashir M, Ghanim B, Klepetko W, Olschewski H, Olschewski A, Kwapiszewska G. The inflammatory cell landscape in the lungs of patients with idiopathic pulmonary arterial hypertension. *Eur Respir J*. 2018;51:1701214.
3. Schistosomiasis: World Health Organization Fact sheet No 115. Updated March 2013. Available at: http://www.who.int/mediacentre/factsheets/fs115/en/index.html. Accessed January 15, 2018.
4. Ward TJC, Fenwick A, Butrous G. The prevalence of pulmonary hypertension in schistosomiasis: a systematic review. *PVRI Rev*. 2011;3:12–21.
5. Gavilanes F, Fernandes CJC, Souza R. Pulmonary arterial hypertension in schistosomiasis. *Curr Opin Pulm Med*. 2016;22:408–414.
6. Graham BB, Bandeira AP, Morrell NW, Butrous G, Tuder RM. Schistosomiasis-associated pulmonary hypertension: pulmonary vascular disease: the global perspective. *Chest*. 2010;137(6 Suppl):20S–29S.
7. Chiaramonte MG, Mentink-Kane M, Jacobson BA, Cheever AW, Whitters MJ, Goad ME, Wong A, Collins M, Donaldson DD, Grusby MJ, Wynn TA. Regulation and function of the interleukin 13 receptor alpha 2 during a T helper cell type 2-dominant immune response. *J Exp Med*. 2003;197:687–701.
8. Kumar R, Mickael C, Chabon J, Gebreab L, Rutebemberwa A, Rodriguez Garcia A, Koyanagi DE, Sanders L, Gandjeva A, Kearns MT, Barthel L, Janssen WJ, Mauad T, Bandeira A, Schmidt E, Tuder RM, Graham BB. The causal role of IL-4 and IL-13 in *Schistosoma mansoni* pulmonary hypertension. *Am J Respir Crit Care Med*. 2015;192:998–1008.
9. Chiaramonte MG, Schopf LR, Neben TY, Cheever AW, Donaldson DD, Wynn TA. IL-13 is a key regulatory cytokine for Th2 cell-mediated pulmonary granuloma formation and IgE responses induced by *Schistosoma mansoni* eggs. *J Immunol*. 1999;162:920–930.

10. Graham BB, Mentink-Kane MM, El-Haddad H, Purnell S, Zhang L, Zaiman A, Redente EF, Riches DWH, Hassoun PM, Bandeira A, Champion HC, Butrous G, Wynn TA, Tuder RM. Schistosomiasis-induced experimental pulmonary hypertension: role of interleukin-13 signaling. *Am J Pathol*. 2010;177:1549–1561.
11. Kumar R, Mickael C, Kassa B, Gebreab L, Robinson JC, Koyanagi DE, Sanders L, Barthel L, Meadows C, Fox D, Irwin D, Li M, McKeon BA, Riddle S, Dale Brown R, Morgan LE, Evans CM, Hernandez-Saavedra D, Bandeira A, Maloney JP, Bull TM, Janssen WJ, Stenmark KR, Tuder RM, Graham BB. TGF-β activation by bone marrow-derived thrombospondin-1 causes Schistosoma- and hypoxia-induced pulmonary hypertension. *Nat Commun*. 2017;8:15494.
12. Graham BB, Chabon J, Gebreab L, Poole J, Debella E, Davis L, Tanaka T, Sanders L, Dropcho N, Bandeira A, Vandivier RW, Champion HC, Butrous G, Wang XJ, Wynn TA, Tuder RM. Transforming growth factor-β signaling promotes pulmonary hypertension caused by *Schistosoma mansoni*. *Circulation*. 2013;128:1354–1364.
13. Kamran P, Sereti KI, Zhao P, Ali SR, Weissman IL, Ardehali R. Parabiosis in mice: a detailed protocol. *J Vis Exp*. 2013;80:50556.
14. Tandrup T, Gundersen HJ, Jensen EB. The optical rotator. *J Microsc*. 1997;186:108–120.
15. Cheever AW. Conditions affecting the accuracy of potassium hydroxide digestion techniques for counting *Schistosoma mansoni* eggs in tissues. *Bull World Health Organ*. 1968;39:328–331.
16. Mauad T, Pozzan G, Lancas T, Overbeek MJ, Souza R, Jardim C, Dolhnikoff M, Mello G, Pires-Neto RC, Bernardi FC, Grunberg K. Immunopathological aspects of schistosomiasis-associated pulmonary arterial hypertension. *J Infect*. 2014;68:90–98.
17. Mikhak Z, Strassner JP, Luster AD. Lung dendritic cells imprint T cell lung homing and promote lung immunity through the chemokine receptor CCR17. *J Exp Med*. 2013;210:1855–1869.
18. Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell*. 1992;68:869–877.
19. Wynn TA, Eltoum I, Oswald IP, Cheever AW, Sher A. Endogenous interleukin 12 (IL-12) regulates granuloma formation induced by eggs of *Schistosoma mansoni* and exogenous IL-12 both inhibits and prophylactically immunizes against egg pathology. *J Exp Med*. 1994;179:1551–1561.
20. Ferru I, Roye O, Delacre M, Auriault C, Wolowczuk I. Infection of B-cell-deficient mice by the parasite *Schistosoma mansoni*: demonstration of the participation of B cells in granuloma modulation. *Scand J Immunol*. 1998;48:233–240.
21. Ferreira RC, Montenegro SM, Domingues AL, Bandeira AP, Silveira CA, Leite LA, Pereira CA, Fernandes IM, Mertens AB, Almeida MO. TGF beta and IL13 in schistosomiasis mansoni associated pulmonary arterial hypertension; a descriptive study with comparative groups. *BMC Infect Dis*. 2014;14:282.
22. Crosby A, Jones FM, Southwood M, Stewart S, Schermuly R, Butrous G, Dunne DW, Morrell NW. Pulmonary vascular remodeling correlates with lung eggs and cytokines in murine schistosomiasis. *Am J Respir Crit Care Med*. 2010;181:279–288.
23. Tamosiuniene R, Manouvakhova O, Mesange P, Saito T, Qian J, Sanyal M, Lin Y-C, Nguyen LP, Luria A, Tu AB, Sante JM, Rabinovitch M, Fitzgerald DJ, Graham BB, Habtezion A, Voelkel NF, Aurelian L, Nicolls MR. A dominant role for regulatory T cells in protecting females against pulmonary hypertension. *Circ Res*. 2018;122:1689–1702.
24. Daley E, Emson C, Guignabert C, de Waal MR, Louten J, Kurup VP, Hogaboam C, Taraseviciene-Stewart L, Voelkel NF, Rabinovitch M, Grunig E, Grunig G. Pulmonary arterial remodeling induced by a Th2 immune response. *J Exp Med*. 2008;205:361–372.
25. Hashimoto-Kataoka T, Hosen N, Sonobe T, Arita Y, Yasui T, Masaki T, Minami M, Inagaki T, Miyagawa S, Sawa Y, Murakami M, Kumanogoh A, Yamauchi-Takihara K, Okumura M, Kishimoto T, Komuro I, Shirai M, Sakata Y, Nakaoka Y. Interleukin-6/interleukin-21 signaling axis is critical in the pathogenesis of pulmonary arterial hypertension. *Proc Natl Acad Sci USA*. 2015;112:E2677–E2686.
26. Maston LD, Jones DT, Giermakowska W, Howard TA, Cannon JL, Wang W, Wei Y, Xuan W, Resta TC, Gonzalez Bosc LV. Central role of T helper 17 cells in chronic hypoxia-induced pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol*. 2017;312:L609–L624.
27. Chen G, Zuo S, Tang J, Zuo C, Jia D, Liu Q, Liu G, Zhu Q, Wang Y, Zhang J, Shen Y, Chen D, Yuan P, Qin Z, Ruan C, Ye J, Wang XJ, Zhou Y, Gao P, Zhang P, Liu J, Jing ZC, Lu A, Yu Y. Inhibition of CRTH2-mediated Th2 activation attenuates pulmonary hypertension in mice. *J Exp Med*. 2018;215:2175–2195.
28. Jais X, Launay D, Yaici A, Le PJ, Tcherakian C, Sitbon O, Simonneau G, Humbert M. Immunosuppressive therapy in lupus- and mixed connective tissue disease-associated pulmonary arterial hypertension: a retrospective analysis of twenty-three cases. *Arthritis Rheum*. 2008;58:521–531.
29. Ji F, Liu Z, Cao J, Li N, Liu Z, Zuo J, Chen Y, Wang X, Sun J. B cell response is required for granuloma formation in the early infection of *Schistosoma japonicum*. *PLoS One*. 2008;3:e1724.
30. Boïkova NV. [Primary immunodeficiency state in a child with the pulmonary hypertension syndrome]. [Article in Russian]. *Arkh Patol*. 1985;47:63–66.
31. Jesenak M, Banovcin P, Jesenakova B, Babusikova E. Pulmonary manifestations of primary immunodeficiency disorders in children. *Front Pediatr*. 2014;2:77.
32. Curtsinger JM, Schmidt CS, Mondino A, Lins DC, Kedl RM, Jenkins MK, Mescher MF. Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. *J Immunol*. 1999;162:3256–3262.
33. Swartz JM, Dyer KD, Cheever AW, Ramalingam T, Pesnicak L, Domachowske JB, Lee JJ, Lee NA, Foster PS, Wynn TA, Rosenberg HF. *Schistosoma mansoni* infection in eosinophil lineage-ablated mice. *Blood*. 2006;108:2420–2427.
34. Schwartz C, Oeser K, Prazeres da Costa C, Layland LE, Voehringer D. T cell-derived IL-4/IL-13 protects mice against fatal *Schistosoma mansoni* infection independently of basophils. *J Immunol*. 1950;2014(193):3590–3599.
35. Sakai S, Kauffman KD, Schenkel JM, McBerry CC, Mayer-Barber KD, Masopust D, Barber DL. Cutting edge: control of *Mycobacterium tuberculosis* infection by a subset of lung parenchyma-homing CD4 T cells. *J Immunol*. 1950;2014 (192):2965–2969.
36. Kauffman KD, Sallin MA, Sakai S, Kamenyeva O, Kabat J, Weiner D, Sutphin M, Schimel D, Via L, Barry CE, Wilder-Kofie T, Moore I, Moore R, Barber DL. Defective positioning in granulomas but not lung-homing limits CD4 T-cell interactions with *Mycobacterium tuberculosis*-infected macrophages in rhesus macaques. *Mucosal Immunol*. 2018;11:462–473.
37. Colombe S, Machemba R, Mtenga B, Lutonja P, Kalluvya SE, de Dood CJ, Hoekstra PT, van Dam GJ, Corstjens PLAM, Urassa M, Changalucha JM, Todd J, Downs JA. Impact of schistosome infection on long-term HIV/AIDS outcomes. *PLoS Negl Trop Dis*. 2018;12:e0006613.
38. Marti AI, Colombe S, Masikini PJ, Kalluvya SE, Smart LR, Wajanga BM, Jaka H, Peck RN, Downs JA. Increased hepatotoxicity among HIV-infected adults co-infected with *Schistosoma mansoni* in Tanzania: a cross-sectional study. *PLoS Negl Trop Dis*. 2017;11:e0005867.

SUPPLEMENTAL MATERIAL

Table S1. Flow cytometry reagents.

Antibody Specificity	Fluoro-chrome	Isotype	Clone	Final Concentration	Manufacturer
CD3	AF700	IgG2b	17A2	0.1µg/2x10^6 cells	eBioscience
CD4	BV510	IgG2a	RM4-5	0.1µg/2x10^6 cells	Biolegend
CD45.1	APC	IgG2a	A20	0.1µg/2x10^6 cells	BD Biosciences
CD45.2	FITC	IgG2a	104	0.1µg/2x10^6 cells	eBioscience
IL-4	AF488	IgG1	11B11	0.2µg/2x10^6 cells	BD Pharmingen
Ki67	PE	IgG2a, kappa	SolA15	0.1µg/2x10^6 cells	eBioscience
CD49b	FITC	IgM, kappa	DX5	0.1µg/2x10^6 cells	Biolegend
CD200R3	PE	Rat IgG2a, κ	Ba13	0.1µg/2x10^6 cells	Biolegend
FceRI-α	APC	Armenian Hamster IgG	MAR-1	0.1µg/2x10^6 cells	Biolegend
CD193	PE/Cy7	J073E5	Rat IgG2a, κ	0.1µg/2x10^6 cells	Biolegend
CD45	BV570	Rat IgG2b, κ	30-F11	0.1µg/2x10^6 cells	Biolegend
CD11c	PerCP	Armenian Hamster IgG	N418	0.1µg/2x10^6 cells	Biolegend
CD11b	BV650	Rat IgG2b, κ	M1/70	0.1µg/2x10^6 cells	Biolegend
MHC-II	APC/Cy7	Rat IgG2b, κ	M5/114.15.2	0.1µg/2x10^6 cells	Biolegend

Table S2. Protein quantification reagents.

Protein	Kit
Total protein conc.	Bradford assay (5000201, BioRad, Hercules, CA)
IL-4	M4000B (R&D Systems, Minneapolis, MN).
IL-13	M1300CB (R&D Systems, Minneapolis, MN).

Table S3. Immunostaining reagents.

Immuno-stain	Antigen Retrieval	Block	Primary Antibody	Secondary Antibody	Tertiary Reagent
CD3	Borg Buffer 20 min in steamer (Biocare # BD1000G1) (TBST rinses throughout)	Dako S2003 5min@RT, 3% H2O2 5min@RT, Avidin 10 min, Biotin 10 min, 10% goat serum in TBS 1hr at RT	1:200 in TBS 1hr at RT (Bio-Rad MCA-1477T)	1:200 in TBS Biotinylated Goat anti-Rat (Vector BA9400)	SA-HRP (Vector SA5704) 30min@RT, DAB (Dako K3568) 10min@RT, light green counterstain (American MasterTech STLGC100)
αSM-actin		Avidin 10 min, Biotin 10 min, Mouse on Mouse (MOM) kit blocking solution (Vector BMK-2202) 1hr at RT	1:100 30min at RT (Dako M0851)	MOM Biotinylated anti-Mouse Reagent (Vector BMK-2202) 10min at RT	Fluorescein-Strepavidin 1:2000 (Invitrogen #S869), Vectashield with DAPI (Vector H-1500)

Table S4. Left ventricular systolic pressure (LVSP), right and left ventricular diastolic pressures (RVDP/LVDP), heart rate (HR), and body weight (Bd. wt.) of *Schistosoma* exposed (IP/IV eggs) and unexposed *Rag*$^{-/-}$ mice Mean±SD. N=6-8 mice per group.

Genotype	Experimental conditions	LVSP (mmHg)	RVDP (mmHg)	LVDP (mmHg)	HR (bpm)	Bd. wt. (g)
Rag$^{-/-}$	Unexposed	84.9±7.9	1.2±0.6	1.3±0.5	316±90.4	22.5±1.9
Rag$^{-/-}$	IP/IV eggs	89.2±16.5	1.4±0.5	2.9±1.3	286.7±100.6	23.2±2.5
t test		NS	NS	NS	NS	NS

IP/IV, Intraperitoneal/intravenous.

Table S5. Left ventricular systolic pressure (LVSP), right and left ventricular diastolic pressures (RVDP/LVDP), heart rate (HR), and body weight (Bd. wt.) of *Schistosoma* (IP/IV) exposed and unexposed *Rag*$^{-/-}$ mice reconstituted with wildtype CD4^{+} T cells Mean±SD. N=3-13 mice per group.

CD4^{+} T cells Recipient	CD4^{+} T cells Donor	Experimental conditions	LVSP (mmHg)	RVDP (mmHg)	LVDP (mmHg)	HR (bpm)	Bd. wt. (g)
Rag$^{-/-}$	WT	Unexposed	80.9±6.3	0.9±0.6	1.0±0.7	301.2±39.3	19.7±5.9
Rag$^{-/-}$	WT	IP/IV eggs	74.7±9.4	0.9±0.6	1.0±0.4	305±63.8	21.8±2.8
t test			NS	NS	NS	NS	NS

IP/IV, Intraperitoneal/intravenous.

Table S6. Left ventricular systolic pressure (LVSP), right and left ventricular diastolic pressures (RVDP/LVDP), heart rate (HR), and body weight (Bd. wt.) of *Schistosoma* sensitized and unsensitized wildtype $CD4^+$ T cells reconstituted into *Rag*$^{-/-}$ mice. Mean±SD. N=8 mice per group.

CD4 T cells Recipient	WT $CD4^+$ T cells	Expet. conditions	LVSP (mmHg)	RVDP (mmHg)	LVDP (mmHg)	HR (bpm)	Bd. wt. (g)
Rag$^{-/-}$	Unsens-itized	IP/IV eggs	74.7±9.6	1.9±2.5	3.8±7.4	284.7±114.3	21.8±3.5
Rag$^{-/-}$	Sensitized	IP/IV eggs	75.5±10.6	1.1±0.7	0.8±0.4	363.7±30.4	21.7±2.3
t test			NS	NS	NS	NS	NS

IP/IV, Intraperitoneal/intravenous.

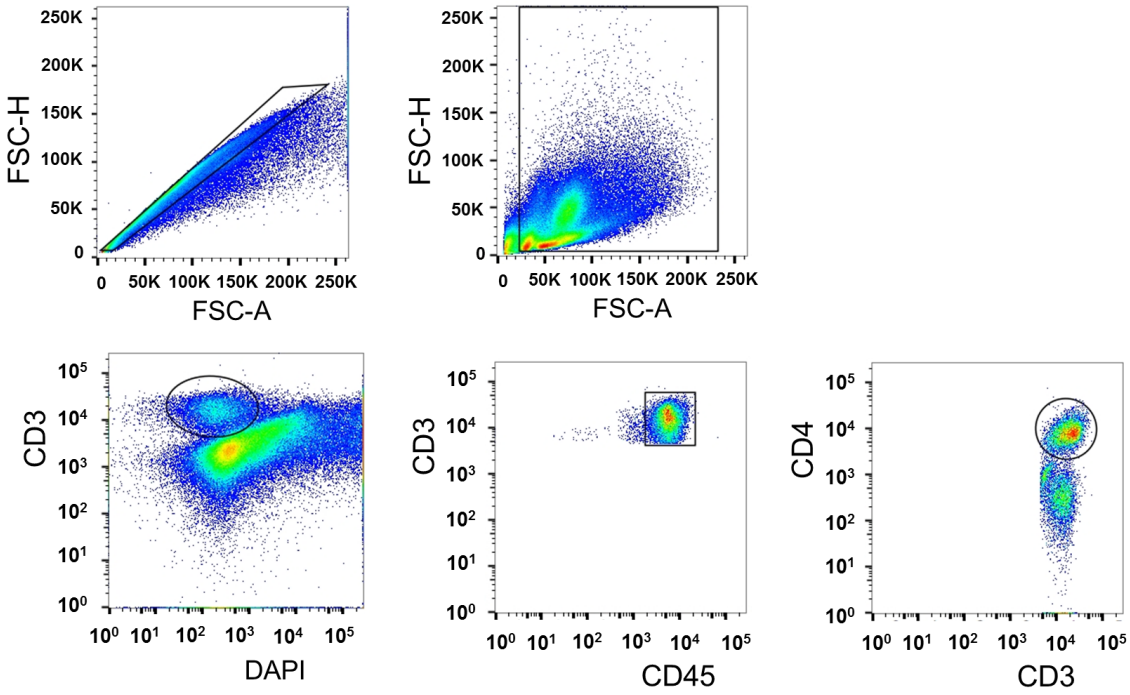
Table S7. Left ventricular systolic pressure (LVSP), right and left ventricular diastolic pressures (RVDP/LVDP), heart rate (HR), and body weight (Bd. wt.) of *Schistosoma*-exposed *Rag*$^{-/-}$ mice reconstituted with *Il4*$^{+/+}$*Il13*$^{+/+}$CD4+ T cells and *Il4*$^{-/-}$*Il13*$^{-/-}$ CD4+ T cells.

CD4^{+} T cells Recipient	CD4^{+} T cells Donor	Experimental conditions	LVSP (mmHg)	RVDP (mmHg)	LVDP (mmHg)	HR (bpm)	Bd. wt. (g)
Rag$^{-/-}$	*Il4*$^{+/+}$*Il13*$^{+/+}$	IP/IV eggs	96.6±18.6	0.7±0.1	0.8±0.4	283±72.6	27.2±2.5
Rag$^{-/-}$	*Il4*$^{-/-}$*Il13*$^{-/-}$	IP/IV eggs	89.5±8.2	1.1±0.5	1.0±0.4	320±40.7	22.8±3.1
t test			NS	NS	NS	NS	NS

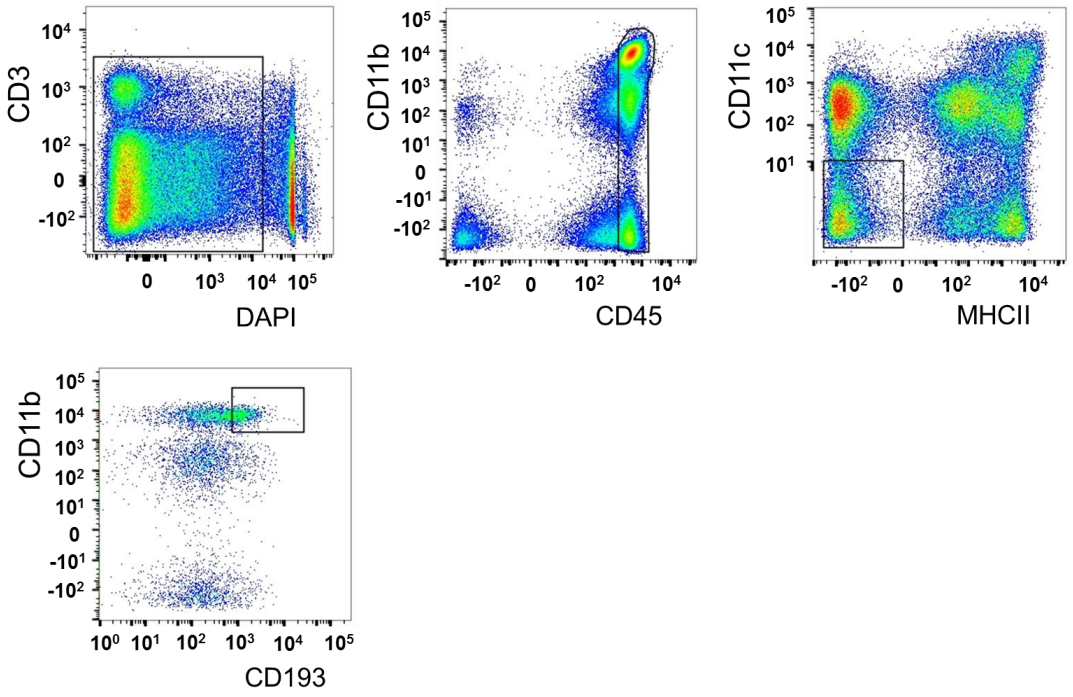
Mean±SD. N=3-5 mice per group. IP/IV, Intraperitoneal/intravenous.

Figure S1. Flow gating strategies for CD4 T cells, eosinophils and basophils.

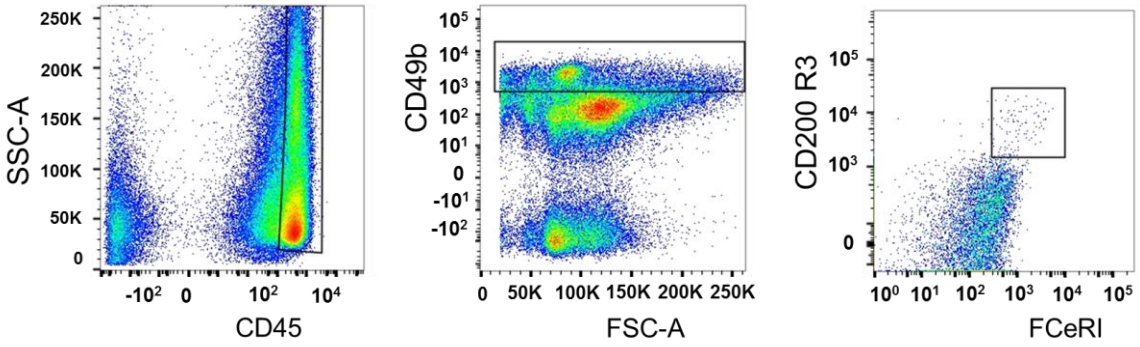
A. CD4 T cells gating.



B. Eosinophils gating.

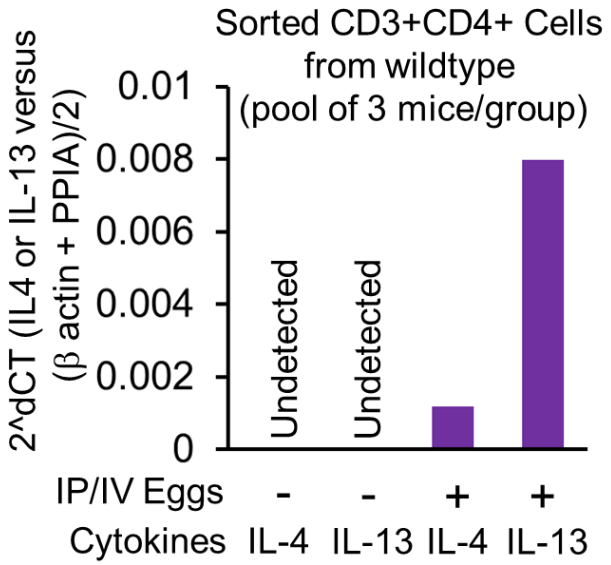


C. Basophils gating.



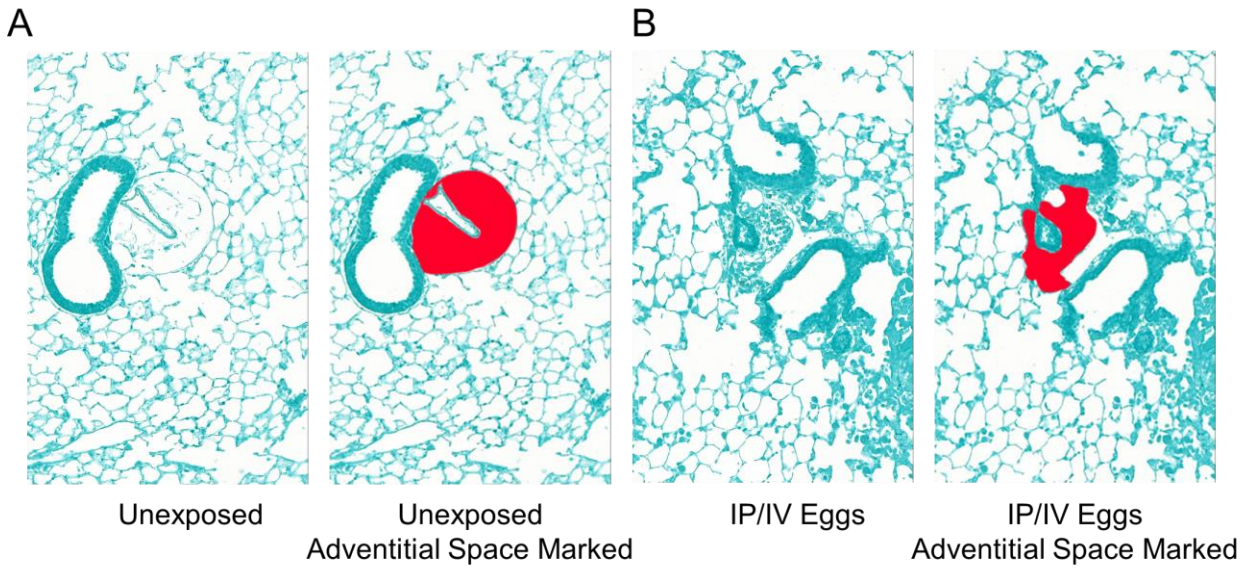
A. Flow gating strategy for CD4 T cells. Singlets are identified, and then debris is removed. Next $CD3^+$ cells which are negative for the viability dye (DAPI) are identified. Then $CD45^+$ cells are identified, and finally $CD4^+$ cells. **B.** Flow gating strategy for eosinophils. After removing debris and identifying singlets (first 2 panels in A), viable cells are identified, then CD45+ cells, then cells negative for both CD11c and MHCII, and then cells positive for both CD11b and CD193. **C.** Flow gating strategy for basophils. After removing debris and identifying singlets (first 2 panels in A), CD45+ cells are identified, then CD49b+ cells, and then cells positive for both CD200 and FCeRI.

Figure S2. IL-4 and IL-13 mRNA expression in CD4⁺ T cells from control and *Schistosoma*-exposed mice.



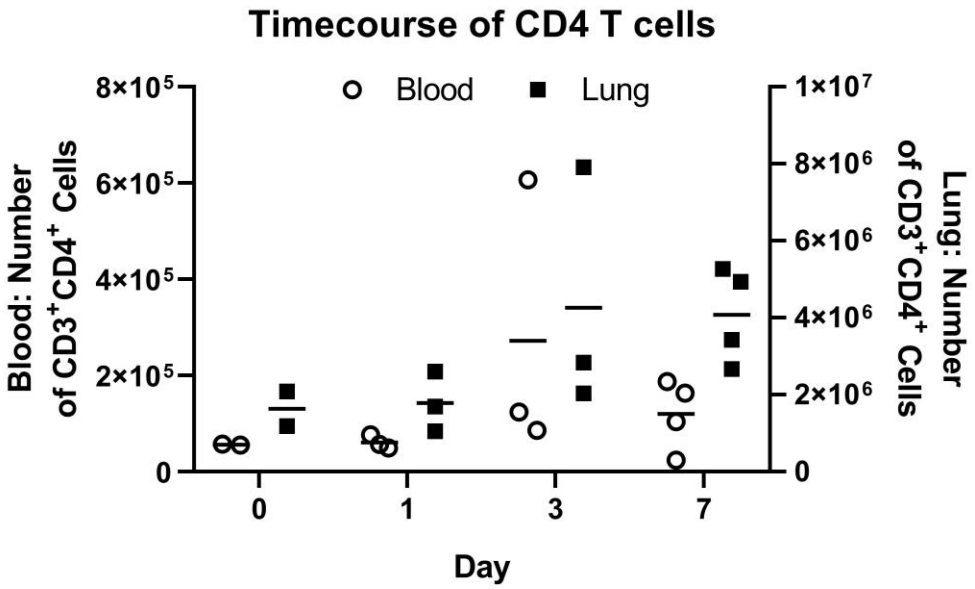
IL-4 and IL-13 mRNA content of the sorted CD4⁺ T cell populations, assessed by RT-PCR. (IP/IV=intraperitoneal and intravenous *S. mansoni* eggs; cells were grouped from 3 mice per experimental group.)

Figure S3. Representative images of adventitial space.



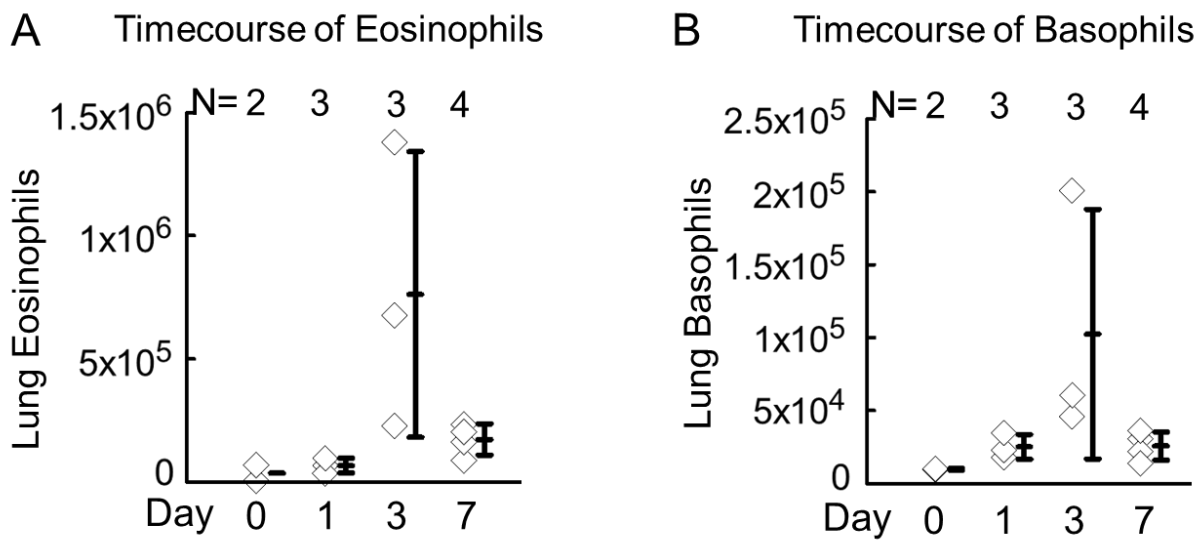
For CD3 T cells density analysis (**Figure 1D**), we quantified CD3 cell profiles in the adventitial space; representative images of the perivascular adventitial space are marked red in wildtype (A) unexposed and (B) *Schistosoma mansoni*-exposed mice.

Figure S4. Timecourse of CD4 T cells in lung and blood.



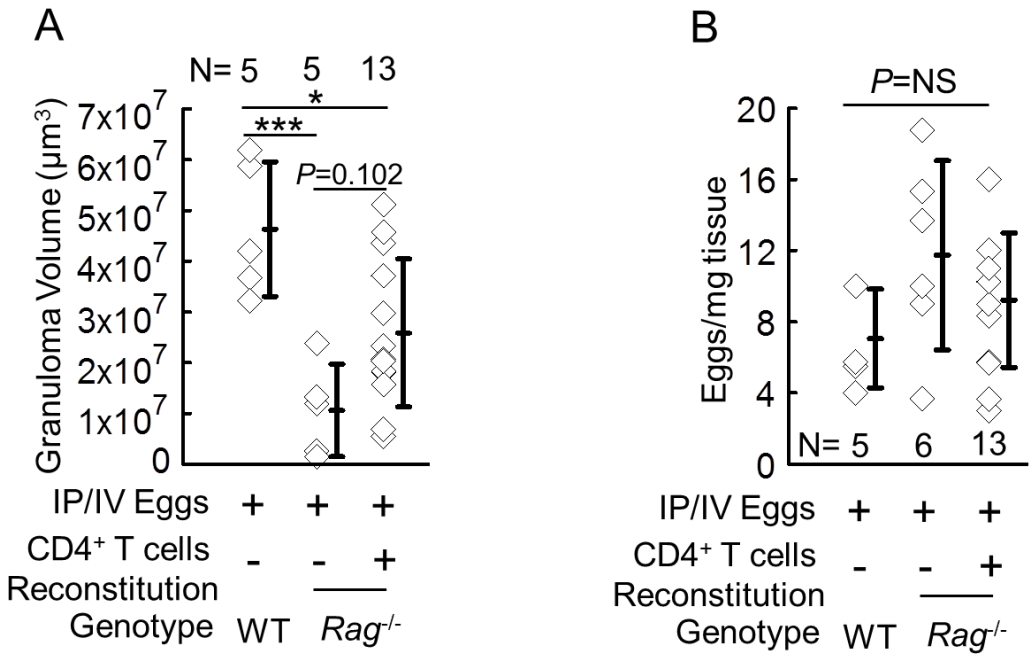
Flow cytometry of CD4 T cells from lung and blood samples (see gating in Figure S1A) prior to IV egg challenge (Day 0) and at Days 1, 3 and 7 after IV egg challenge identifies an increase in both the lung and blood compartment starting at day 3 after IV challenge (N=2-4 animals per group; mean shown).

Figure S5. Timecourse of eosinophils and basophils in the lung.



Absolute number of (**A**) eosinophils and (**B**) basophils in the lung as assessed by flow cytometry (see gating in Figures S1B and SC, respectively) prior to IV egg challenge (Day 0) and at Days 1, 3 and 7 after IV egg challenge identifies an increase in the lung compartment starting at day 3 after IV challenge (N=2-4 animals per group; mean shown).

Figure S6. Granuloma volumes and residual egg density in *Schistosoma*-exposed wildtype, *Rag*$^{-/-}$, and *Rag*$^{-/-}$ mice reconstituted with wildtype CD4^{+} T cells.



(A) Estimated granuloma volumes, as measured by stereology (ANOVA *P*=0.002; post-hoc Tukey test statistics shown). (B) Number of *S. mansoni* eggs recovered from lung digests (ANOVA *P*=NS). (N=Number of animals / group; mean ± SD plotted; **P*<0.05, ****P*<0.005; IP/IV=intraperitoneal and intravenous *S. mansoni* eggs.)