Nitric oxide blocks the development of the human parasite *Schistosoma japonicum*

Jia Shen[1,2]†, De-Hua Lai[1]†, R. Alan Wilson[3], Yun-Fu Chen[1], Li-Fu Wang[2], Zi-Long Yu[2], Mei-Yu Li[2], Ping He[2], Geoff Hide[4], Xi Sun[2], Ting-Bao Yang[1], Zhong-Dao Wu[2]*, Francisco J. Ayala[5]*, Zhao-Rong Lun[1,2,4]*

1, State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, P.R. China

2, Department of Parasitology and Key Laboratory of Tropical Disease Control (SYSU), Ministry of Education, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou 510080, P.R. China

3, Centre for Immunology and Infection, Department of Biology, University of York, Heslington, York, YO10 5DD, UK

4, Ecosystems and Environment Research Centre and Biomedical Research Centre, School of Environment and Life Sciences, University of Salford, UK

5, Department of Ecology and Evolutionary Biology, Ayala School of Biological Sciences, University of California, Irvine, USA

†These authors contributed equally to this work

*Corresponding authors.

Abstract

Human schistosomiasis, caused by *Schistosoma* species, is a major public health problem affecting more than 700 million people in 78 countries, with over 40 mammalian host reservoir species complicating the transmission ecosystem. The primary cause of morbidity is considered to be granulomas induced by fertilized eggs of schistosomes in the liver and intestines. Some host species like rats (*Rattus norvegicus*) are naturally intolerant to *S. japonicum* infection, and do not produce granulomas or pose a threat to transmission, while others like mice and hamsters are highly susceptible. The reasons behind these differences are still a mystery. Using inducible nitric oxide synthase knockout (iNOS$^{-/-}$) Sprague Dawley rats, we found that inherent high expression levels of iNOS in wild type rats play an important role in blocking growth, reproductive organ formation and egg development in *S. japonicum* resulting in production of non-fertilized eggs. Granuloma formation, induced by fertilized eggs in the liver, was considerably exacerbated in the iNOS$^{-/-}$ rats compared to the wild type. This inhibition, by NO, acts by affecting mitochondrial respiration and energy production in the parasite. Our work not only elucidates the innate mechanism that blocks the development and production of fertilized eggs in *S. japonicum*, but also offers new insights into a better understanding of host-parasite interactions and novel drug development strategies against schistosomiasis.

Key words: **Rat; *Schistosoma japonicum*; Schistosomiasis; granuloma formation; mitochondria**

Author Contributions

J.S., D.-H.L., Y.-F.C., Z.-L.Y., Z.-D.W., and Z.-R.L. designed research; J.S., Y.-F.C., L.-F.W., Z.-L.Y., M.-Y.L., P.H., and X.S. performed research; J.S., D.-H.L., R.A.W., M.-Y.L., G.H., T.-B.Y., Z.-D.W., F.J.A., and Z.-R.L. analyzed data; and J.S., D.-H.L., R.A.W., G.H., F.J.A., and Z.-R.L. wrote the paper.

Significance Statement

Viable egg production by *Schistosoma* is the key pathogenic process causing granuloma formation in permissive hosts (e.g., mice) while non-permissive hosts [e.g,. Norway rats (*Rattus norvegicus*)] avoid such sequelae. Using inducible nitric oxide synthase knockout (iNOS$^{-/-}$) rats, we demonstrate that high expression levels of iNOS in rats play an important role in blocking the egg-induced granuloma formation of *Schistosoma japonicum*. The Nitric Oxide (NO), produced by iNOS, inhibits parasite growth, reproductive organ development, egg production and viability by interfering with mitochondrial function. This study solves the puzzle as to why rats are naturally resistant to *S. japonicum* infection and provides new insights for understanding the pathogenesis of human schistosomiasis and the interactions between host and parasite.

/body

Schistosomiasis caused by *Schistosoma* species, is the second most important parasitic disease for public health after malaria. In 2015, it was estimated that 700 million people were at the risk of this disease and 218 million people required treatment in 78 countries (1). *Schistosoma japonicum* is widely distributed in South China, Indonesia and the Philippines (1) with 170,438 patients being treated in China in 2015 (2). It is well-known that viable egg production is the key for both transmission and pathogenesis (egg-induced granulomas in liver and intestinal tissues) of this parasite. The female *S. japonicum* produces around 3000 eggs per day, 10-fold more than the related species *S. mansoni*, and this has been proposed to cause a more severe pathology to patients (3). *S. japonicum* is one of the most difficult parasites to control, since more than 46 non-human mammals can be naturally infected, especially cattle, goats, dogs, pigs and mice, and these play an important role in the transmission of this disease in endemic regions (4, 5). However, it is well known that some experimental animals such as Norway rats (*Rattus norvegicus*) show an innate resistance to infection, in which *Schistosoma* spp. cannot develop well and do not cause typical granuloma formation in the liver (6-8). These phenomena are described as susceptible or “permissive” and resistant or “nonpermissive” based on the capacity of the host species to allow development of sexual maturation and oviposition by the parasite (7). Although such natural characteristics have been investigated for several decades, little direct evidence has been obtained to fully explain these phenomena, even despite the publication of the genome of *S. japonicum* and the great benefits provided by it (9). We are interested to know why such huge differences in resistance occur between mice and rats when they are infected with *S. japonicum*. What are the host factors that relate to resistance? Obviously, a better understanding of the mechanism of resistance would provide a better understanding of the pathogenesis of human schistosomiasis and the host-parasite interactions.

In recent decades, experiments have been carried out to investigate potential mechanisms that could mediate natural resistance to *Schistosoma* infection in rats.

Capron and Caprom (6) and Capron et al. (10), primarily using in vitro assays, argued that humoral immunity, particularly antibody-dependent cell-mediated cytotoxicity played a critical role in the resistance of the rat host. In addition, the anaphylactic antibodies (IgG2a and IgE) and effector cells, including eosinophils, macrophages, platelets and mast cells could mediate cytotoxicity, which might act directly against schistosomula in vivo (10, 11). However, some contrasting results have indicated that cell-mediated responses appeared to be insignificant in rat schistosomiasis (12). Moreover, some results also suggested that a Th2 type response was involved in such resistance, based on the observations of preferential expression of Th2 cytokines before rejection of worms in infected rats (12-14). Endocrine gland removal studies revealed that hormones from the pituitary and thyroid/parathyoid glands were required for resistance in rats (15), but no specific hormones were identified due to technical limitations at the time. Nevertheless, none of these proposed mechanisms could definitely and satisfactorily fully explain the resistance.

A comparison between mice and rats has clearly shown that the expression levels of inducible nitric oxide synthase (iNOS or NOS2) and the production of nitric oxide (NO) are barely detectable in naïve mice but significantly higher in naïve rats (16). This production of NO is typically dependent on L-arginine metabolism by iNOS in activated macrophages and other immunocytes in response to microbial compounds and/or cytokines (*e.g*., IFN-γ and IL-1) (17, 18). NO has been identified as participating in macrophage-mediated killing or cytostasis of various extracellular or intracellular parasitic protozoans, such as *Toxoplasma*, *Leishmania*, *Plasmodium* and *Trypanosoma* (17). In fact, some studies on NO have also been carried out on *Schistosoma* in a mouse model. For example, it was reported that macrophage and endothelial cell-mediated cytotoxicity against schistosomula of *S. mansoni* in mice might be involved in the production of NO (19, 20). In addition, Wynn et al. (21) found that worm burdens were increased when mouse NO synthase activity was inhibited by aminoguanidine, a selective inhibitor of iNOS. Nevertheless, all of these studies were based on mouse models and the role of NO in rats on infection by *S. japonicum* remains a mystery. We hypothesized, therefore, that the mechanism of

natural resistance/intolerance to *S. japonicum* infection in rats could be related to inherent high expression levels of iNOS.

To test this hypothesis, iNOS knockout ($iNOS^{-/-}$) Sprague-Dawley (SD) rats were used. We found that inherent high expression levels of iNOS in wild type (WT) rats play a key role in blocking *S. japonicum* growth, reproductive organ development, egg production and the ability to lay fertilized eggs. The consequences of this were to limit granuloma formation in the liver. We show that this inhibition by NO acts by affecting mitochondrial respiration and energy production in the worm. These findings not only provide direct evidence to demonstrate that NO is the key factor for natural resistance to *S. japonicum* infection in rats, but also provide knowledge for a better understanding of the pathogenesis of schistosomiasis. They also inform potentially novel strategies to design new compounds and drugs to control schistosomiasis.

Results

Nitric oxide is a key molecule in rats that hampers the development of *S. japonicum*

To test the hypothesis that NO plays an important role in the natural resistance/ intolerance to *S. japonicum* infection in rats, initial studies were carried out to compare the status of NO production in BALB/c mice, Sprague Dawley (SD) and Lewis rats post-infection with *S. japonicum*. As expected, based on previous studies, development and fecundity levels of the parasite, parasite loads and the size of granulomas in the tested animals were negatively correlated with their capacity to produce NO (Fig. S1), implying the inhibitory effect of NO in *S. japonicum* growth, maturation, fecundity and pathogenesis.

Furthermore, iNOS$^{-/-}$ SD knockout rats were generated with undetectable NO production in peritoneal macrophages and lower levels of NO in sera (Fig. S2). Following infection, iNOS$^{-/-}$ rats showed a significant increase in worm burden (iNOS$^{-/-}$ rat, 82±4; WT rat, 21±2; P<0.001) and egg deposition in the liver (eggs / gram liver tissue: iNOS$^{-/-}$ rat, 106334±19955; WT rat, 4903±1239; P<0.001; Table 1). Notably, the worm fecundity in the iNOS$^{-/-}$ rats, defined as the average egg production per female, was found to be nearly fivefold higher than that found in WT rats (Table 1).

To better understand the effects on *S. japonicum* in the iNOS$^{-/-}$ rat, detailed biological characteristics of the worms were examined. The lengths and diameters of male and female worms collected from iNOS$^{-/-}$ rats at 7 weeks post-infection were significantly greater than those obtained from WT rats (Fig. 1A). The tegument of *S. japonicum* from the infected iNOS$^{-/-}$ rats were covered with well-arranged ridges, abundant pits, as well as sensory papillae with setae, and were similar to that of worms collected from mice (Fig. 1B and Fig. S3). However, in contrast these characteristics disappeared or were rudimentary in the worms from WT rats (Fig. 1B and Fig. S3). In addition, a large number of spines and several sensory papillae were found in the tegument of oral suckers of *S. japonicum* from iNOS$^{-/-}$ rats and mice but were not observed in the WT rat group (Fig. 1B).

Most importantly, we also found a huge difference between the reproductive

systems of *S. japonicum* collected from iNOS$^{-/-}$ and WT rats. The testes of adult male schistosomes from iNOS$^{-/-}$ rats were composed of 6-8 testicular lobes containing large amounts of spermatogonia and spermatocytes while the seminal vesicle was filled with thousands of mature sperm (Fig. 1C). In the control mice, *S. japonicum* had the similar phenotype as in the iNOS$^{-/-}$ rats. In contrast, in WT rats *S. japonicum* displayed a significant reduction in the number and size of testicular lobes, accompanied by a remarkable decrease in cell density in the testes plus a lack of mature sperm (Fig. 1C - E). Furthermore, in female worms, drastic differences were observed in the size of ovaries, vitellaria and numbers of non-excreted eggs in the uterus of *S. japonicum* collected from the iNOS$^{-/-}$ and WT rats (Fig. 1C, F, G and Fig. S4). Analogous to the worms observed in mice, we found that the ovaries of mature female worms collected from the iNOS$^{-/-}$ rats were composed of abundant oogonia, immature and primary oocytes (Fig. 1C and F), while the uteri were filled with eggs (Fig. 1G and Fig. S4B), and the vitelline lobes were clustered with closely arranged vitelline cells (Fig. S4A). In contrast, there were significant reductions in the diameters of ovaries which contained only a few oocytes in the female worms collected from the WT rats (Fig. 1C and F). The occurrence of eggs in uteri was rare and those present were not properly formed; the vitelline lobes had scantily organized vitelline cells (Fig. 1G and Fig. S4). These results obtained from the WT rats are consistent with those previously described (8), but show a clear difference to phenotypes observed in the iNOS$^{-/-}$ rats.

S. japonicum produced viable eggs in the infected iNOS$^{-/-}$ rats

To investigate the hypothesis that *S. japonicum* should produce non-fertilized eggs and under-developed embryos in the WT rats. Acridine orange fluorescence staining was used as a detection system to measure viable egg production. We found a lower percentage (21.05%) of live eggs of *S. japonicum* from the WT rats, compared with much higher percentage (86.28%) of viable eggs from the iNOS$^{-/-}$ rats (Fig. 2A, $P<0.001$). Furthermore, results from the circumoval precipitin reaction (CPR), a specific indicator of the secretion activity of viable mature eggs, showed that a

characteristic and dense reaction product surrounded 29.58% of 2000 eggs collected from the iNOS$^{-/-}$ rats, while only weak CPR activity was observed in 5.42% of 1200 eggs collected from the WT rats (Fig. 2B). Moreover, we found that much more severe pulmonary granulomas were induced by eggs collected from the livers of iNOS$^{-/-}$ rats than those from the WT rats, when injected intravenously into naïve mice (Fig. 2C and D).

To test the developmental status of *S. japonicum* eggs from the infected iNOS$^{-/-}$ and WT rats, a hatching test was carried out. Parasite eggs recovered from the iNOS$^{-/-}$ rats were capable of hatching to miracidia (25.3 %) in a similar proportion to those recovered from mice (Fig. 2E). However, in contrast to the results from the iNOS$^{-/-}$ rats, eggs collected from the WT rats were unable to hatch, thus demonstrating that NO has a specific role in affecting egg viability.

Exacerbated granuloma formation in the iNOS$^{-/-}$ rats was attributed only to the full development of parasites, not to other host factors.

It is well known that viable eggs of schistosomes are a key factor for the formation of granulomas in their hosts (22). Indeed, as we predicted, rare and small granulomas were found in the liver of WT rats infected with *S. japonicum* at 7 and 12 weeks post infection, while both the number and size of granulomas were dramatically increased in the infected iNOS$^{-/-}$ rats (Fig. 3A-C). The size of hepatic granulomas in iNOS$^{-/-}$ rats infected with *S. japonicum* was 20.97±1.87 (×10^{-3} mm^3) at 7 weeks post-infection, nearly 8-fold larger than those found in the infected WT rats (2.56±0.42 (×10^{-3} mm^3), P<0.001). In a follow-up at 12 weeks post-infection, the size, remarkably, increased to 201.18±25.91 (×10^{-3} mm^3) in iNOS$^{-/-}$ rats infected with *S. japonicum*, over 22-fold larger than those found in the infected WT rats (8.79±0.83 (×10^{-3} mm^3)) (P<0.001) (Fig. 3B). Furthermore, comparison of the granuloma density of iNOS$^{-/-}$ and WT rats showed an increase of 30 and 20 fold in liver tissue in the knockout rats at 7 (WT: 0.09±0.01%; iNOS$^{-/-}$: 2.73±0.19%; P<0.001) and 12 weeks (WT: 0.43±0.14%; iNOS$^{-/-}$: 8.84±0.40%; P<0.001) post infection (Fig. 3C). The marked increase in hepatic granulomatous inflammation in the infected iNOS$^{-/-}$ rats was

largely dependent on the increased egg production of *S. japonicum* and maturation of eggs (Table 1 and Fig. 2B).

To exclude the possibility that the changes in host immunity factors post-NO deficiency may contribute to the hepatic granuloma, pulmonary granulomas were compared in the WT and iNOS$^{-/-}$ rats after injection of eggs obtained from rabbits infected with *S. japonicum*. To our surprise, similar sizes and volume density of pulmonary granulomas were observed in both the WT and iNOS$^{-/-}$ rats after injection of the same dose of viable mature eggs (Fig. S5). Thus, our results clearly demonstrated that the exacerbation of hepatic granulomas in the iNOS$^{-/-}$ rats was not attributed to host factors, but to the viability of *Schistosoma* eggs.

Adoptive transfer of wild-type macrophages into the iNOS $^{-/-}$ rats could partially restore the inhibition against *S. japonicum*

To provide further evidence of the role of NO on the inhibition of development of *S. japonicum*, adoptive transfer of wild type rat macrophages into iNOS$^{-/-}$ rats was performed. Macrophages were used as they are considered to be the best characterized source of NO (18). After transfer, the iNOS$^{-/-}$ recipient rats were able to express iNOS (Fig. S6A and B) and elevated the production of NO *in vivo* (Fig. S6C). As seen in Table. S1, in contrast to the status in the iNOS$^{-/-}$ rats, the worm burden and egg production, together with worm fecundity were significantly reduced in the recipient group of animals (iNOS$^{-/-}$ + Mϕ). Furthermore, we found that the adoptively transferred macrophages could partially inhibit the parasite growth, which resulted in a decrease in length and diameter (Fig. S6D-F). As a consequence, the size of granulomas in livers displayed a marked reduction in the iNOS$^{-/-}$ + Mϕ group (Fig. S6G and H). Thus, these data further demonstrated that NO is a key factor involved in blocking the development of *S. japonicum* in rats.

NO inhibits the mitochondrial respiration of *S. japonicum*

In this study, we speculated that the mechanisms of NO blocking the development of *Schistosoma* might be linked to the inhibition of mitochondrial

respiration, resulting in inhibition of mitochondrial energy production and lethal metabolic interference. To test this hypothesis, the mitochondrial morphology and structure of *S. japonicum* were compared. Ultrastructural observations revealed that worms from the mice had typical eukaryotic mitochondria with well-defined outer membranes and a clear cristae structure. In contrast, clusters of damaged mitochondria, exhibiting mitochondrial swelling and distortion, loss of intact internal membranes and disruption of mitochondrial cristae with vacuolization were observed in worms from the WT rats. However, mitochondrial alterations were considerably diminished in the worms from the iNOS$^{-/-}$ rats (Fig. 4A and Fig. S7). In addition, the relative mRNA expression of the mitochondrial respiratory chain enzymes, cytochrome c oxidase (CcO, complex IV) subunit I and NADH dehydrogenase (complex I), in worms from the WT rats were significantly decreased (Fig. S8). CcO activity was also significantly decreased in worms from the WT rats, compared with those from the iNOS$^{-/-}$ rats and mice (Fig. 4B). These results strongly suggest that the mechanisms of NO blocking the development of *S. japonicum* in rats act by affecting mitochondrial respiration in the parasite.

Discussion

Understanding defense mechanisms against parasites is a key aspect of elucidating host-parasite interactions. *Schistosoma japonicum* is a zoonotic parasite with a naturally wide permissive host range; however, some hosts, including the brown rat, are non-permissive hosts. This provides a good model system for investigating the host-parasite interactions that control and limit infection. In permissive hosts, such as mice and hamsters, the parasites are able to reach sexual maturation, deposit eggs which then trigger the formation of granulomas which ultimately are responsible for mortality. However, in non-permissive hosts, such as rats, the parasites struggle to survive and do not fully develop into mature stages (7, 8, 15). Scientists have long been puzzled by these biological differences among different mammalian species and the causative mechanism(s) remained unclear;

many hypotheses have been proposed to account for this (6, 12, 14).

In early studies based on the mouse model, evidence indicated the effect of NO on killing *Schistosoma* (17), but the mechanism was not clarified. Based on our results from the rat models (WT *vs* iNOS$^{-/-}$, and adoptive transfer of macrophages), we have demonstrated that high expression of iNOS with a higher amount of NO in rats is strongly linked to the inhibition of development of *S. japonicum*, and is a key factor contributing to their resistance against the parasite. The huge differences in development of *S. japonicum* between the WT and iNOS$^{-/-}$ rats clearly showed that NO could significantly influence the tegument structures, body size and the development of the reproductive organs in *S. japonicum*. The tegument is known to be required as essential protection for parasite survival during host immune attacks (23) and a key structure for driving nutrient absorption and cholesterol metabolism (24, 25). The modified structure of the tegument of *S. japonicum* in WT rats causes significant problems for the absorption of nutrients and the development of the parasite. Importantly, we found that the reproductive organs of the female worms were not properly formed in the infected WT rats, represented as significant decreases in the size of ovaries and the number of vitelline cells and non-excreted eggs, compared with those found in iNOS$^{-/-}$ rats and mice groups. These deformities led to a significant decrease in both egg production and deposition in the tissues of the host. This, in turn, alleviated the pathogenesis caused by egg deposition. In fact, early evidence obtained from the mouse model system indicated similar effects of NO on *S. mansoni* when NO production was elevated by chemical compounds (26) or vaccination (27). Interestingly, the inhibition of development and fecundity by NO was also found in *Cooperia oncophora,* a parasitic nematode in cattle, in which elevated expression of iNOS was observed in acquired resistance during reinfection of this parasite (28). Perhaps this represents a generic effect of NO in helminths. Alongside effects in females, we also found that NO could cause notable reductions in testicular lobe formation (both in size and quantity) and lack of production of mature sperm in the males of *S. japonicum*. The most significant effect of NO on the inhibition of *S. japonicum*, in WT rats, was the production of non-fertilized eggs. This

was manifested as a significant decrease in the proportion of viable eggs, showing a weak circumoval precipitation reaction (CPR), and inability to lead to hatching of the important miracidial stages which are required for transmission to new hosts. Interestingly, removal of the pituitary gland and thyroid/parathyroid glands from rats prior to infection with *S. mansoni* resulted in increasing in worm burdens, worm development, oviposition and miracidial development (15). Indeed, growth hormone and thyroid hormones have been demonstrated to directly induce iNOS expression and increase iNOS activity by influencing the maturation and function of immune cells, such as macrophages (29-33). These results strongly support the important role of NO on the development of *Schistosoma*.

Egg granuloma formation in the liver and intestinal tissues of many permissive mammalian hosts, such as mice, have long been considered to be the primary cause of morbidity of schistosomiasis (34). This is reported to be caused by antigens secreted by the mature viable eggs (22) followed by induction of inflammatory cells surrounding the eggs (34). In fact, rare egg granulomas have also been reportedly found in non-permissive hosts (7, 8). In our work, we found that *S. japonicum* worms developing in WT rats laid 20-fold fewer eggs than those developing in the iNOS$^{-/-}$ rats. Surprisingly, the magnitude of change in egg granuloma production (volume density) between WT and iNOS$^{-/-}$ rats was more than 30 fold. This clearly indicated that the viability of eggs contributed to the difference observed. This result is consistent with the traditional concept that only viable eggs of schistosomes are able to induce granuloma formation in their hosts (22). However, it was still unclear in previous studies why such obvious differences occur between permissive and non-permissive hosts during infection with *S. japonicum*. This was largely attributed to host specificity although detailed mechanisms were not then forthcoming.

There was some evidence suggesting that NO could play a direct role in limiting granulomatous inflammation in iNOS$^{-/-}$ mice infected with *S. mansoni* (35) and in the *in vitro* granuloma reaction with the iNOS inhibitor *N* ω -nitro-L-arginine methyl ester (L-NAME) (36). However, this inhibition effect was not observed when aminoguanidine was administered to mice infected with *S. mansoni* (37), and it was

not observed when the inhibitors L-N6-(iminoethyl)-lysine and L-NAME were used in a model where hepatic granulomas were induced by implanting *S. japonicum* eggs (38); however the toxicity of these inhibitors to the parasites had not been clarified. In our study, we were able to exclude the effect of host immunity factors, post-NO deficiency, on egg granuloma formation and attribute it solely to the quantity and viability of parasite eggs. This was confirmed by observing a similar volume density and size of pulmonary granulomas formed in both the WT and iNOS$^{-/-}$ rats following injection with the same dose of viable mature eggs.

NO is an unusual effector molecule because of its ability to diffuse freely across cell membranes, allowing it to diffuse into the worm (39), and directly influence its physiology (e.g. toxic peroxynitrite anion) (17, 40) or indirectly (e.g. via *S*-nitrosylation) target inactivation and degradation of iron-containing enzymes (17, 40, 41), which were essential for parasite metabolism (42, 43). For example, earlier studies suggested that NO/nitrite could mediate *in vitro* schistosomula killing by causing mitochondrial lesions and inhibition of mitochondrial respiration (19, 44). In fact, mitochondrial metabolism, especially the tricarboxylic acid cycle, has been shown to have an essential function in *S. japonicum* (43). This was demonstrated by using fluoroacetate, an inhibitor of aconitase, and showing that it could cause a separation and hepatic shift of paired worms, a significant fall both in glycogen and protein content, and consequently, a considerable loss of worm body weight. Other studies demonstrated that the mitochondrial respiratory chain also plays an important role in egg production (45), and biosynthetic processes in *Schistosoma* (46), important in rebuilding the surface membrane complex to protect schistosomes from immune attack (23, 46). In addition to the detailed knowledge in *Schistosoma*, mitochondrial respiration is also known to be required for development in other parasitic nematodes (47) suggesting a general role in helminth survival and transmission. Our data clearly show damaged mitochondria in the surviving *S. japonicum* worms collected from infected WT rats while observing typical normal mitochondrial structures in the worms collected from the iNOS$^{-/-}$ rats infected with the same parasite. Respiration chain impairment was confirmed by significant

decreases in expression levels of complex I and IV (CcO), and CcO activity, which usually is responsible for 90% of oxygen consumption (48, 49). A large number of pioneering studies have documented that NO can inhibit CcO in competition with oxygen (48, 50, 51). Such suppression of CcO activity is reversible (48, 52). In fact, a parasite transfer study which was carried out in rats showed results consistent with this notion of reversible inhibition of the development and fecundity of *Schistosoma* (7). By analysis of the function of mitochondrial of *S. japonicum* collected from the WT and iNOS$^{-/-}$ rats, our results strongly suggest that the mechanisms of NO blocking of the development of *S. japonicum* in rats acts by affecting the mitochondrial respiration.

Taken together, our results demonstrate unequivocally that the key role of NO in blocking the development of *S. japonicum*, may be strongly linked to the inhibition of parasite mitochondrial respiration, which, in turn, leads to decreases in worm survival, egg production and quantity of fertilized eggs. This consequently limits granuloma formation in the liver and subsequent pathogenesis. By studying the reproductive biology of schistosomes in this way, our results not only solve the long-term puzzle as to why rats are naturally resistant/intolerant to *S. japonicum* infection, but also offer new insights into possible control. The knowledge that the interaction and evolution of host and parasite is functionally driven by host NO production suggests new strategies for the design of new compounds and drugs for the control and prevention of human schistosomiasis. We also propose that this iNOS$^{-/-}$ rat model will be a highly beneficial and generic model for determining the role of NO in resistance/intolerance to other pathogen infections.

Materials and Methods

Animals. Six to eight week old male Bagg albino (BALB/c) mice and Sprague Dawley (SD) rats were purchased from the Laboratory Animal Center of Sun Yat-Sen University. Six to eight week old male Lewis rats were purchased from Vital River Laboratories. The iNOS-deficient rats were

generated by TALENs technology and breeding at the SPF house of Sun Yat-Sen University. The mutant rats are viable, fertile and do not display any obvious appearance or physical abnormalities. All animals were housed under specific-pathogen-free conditions and work was approved by the Laboratory Animal Use and Care Committee of Sun Yat-Sen University under the license number 2012CB53000.

Parasite infection. Cercariae of *S. japonicum* (Chinese mainland strain) were obtained from infected *Oncomelania hupensis* snails purchased from Jiangsu Institute of Parasitic Diseases (China). Each rat or mouse was percutaneously infected with 200 or 20 cercariae, respectively. Parasites were harvested by perfusion from the portal system.

Other methods used in this paper can be found in SI Materials and Methods

Acknowledgments We thank the members of our laboratorieswho provided great help during the work. This work was supported by National Research and Development Plan of China (No.2016YFC1200500) and National Science Foundation of China (31472058, 31402029 and 31672276).

References

1. WHO (2016) Schistosomiasis. (http://www.who.int/mediacentre/factsheets/fs115/en/).
2. Zhang L*, et al.* (2016) Endemic status of schistosomiasis in People's Republic of China in 2015. *Chin J Schisto Control* 28(6):611-617.
3. Roberts. LS & Janovy J (2005) *Foundations of Parasitology* (McGraw-Hill, Boston) 7th Ed p 702 p.
4. He YX, Salafsky B, & Ramaswamy K (2001) Host--parasite relationships of *Schistosoma japonicum* in mammalian hosts. *Trends Parasitol* 17(7):320-324.
5. Minggang C & Zheng F (1999) Schistosomiasis control in China. *Parasitol Int* 48(1):11-19.
6. Capron M & Capron A (1986) Rats, mice and men - models for immune effector mechanisms against schistosomiasis. *Parasitol Today* 2(3):69-75.
7. Cioli D, Knopf PM, & Senft AW (1977) A study of *Schistosoma mansoni* transferred into permissive and nonpermissive hosts. *Int J Parasitol* 7(4):293-297.
8. Hu Y*, et al.* (2012) Immune changes of *Schistosoma japonicum* infections in various rodent disease models. *Exp Parasitol* 131(2):180-189.
9. *Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium (2009) The *Schistosoma japonicum* genome reveals features of host-parasite interplay. *Nature* 460(7253):345-351.
10. Capron M, Nogueira-Queiroz JA, Papin JP, & Capron A (1984) Interactions between eosinophils and antibodies: in vivo protective role against rat schistosomiasis. *Cell Immunol*

83(1):60-72.
11. Capron M*, et al.* (1983) Immunologic response of athymic rats to *Schistosoma mansoni* infection. II. Antibody-dependent mechanisms of resistance. *J Immunol* 131(3):1475-1480.
12. Khalife J, Cetre C, Pierrot C, & Capron M (2000) Mechanisms of resistance to *S. mansoni* infection: the rat model. *Parasitol Int* 49(4):339-345.
13. Cetre C*, et al.* (1998) In vivo expression of cytokine mRNA in rats infected with *Schistosoma mansoni*. *Parasite Immunol* 20(3):135-142.
14. Cetre C*, et al.* (1999) Profiles of Th1 and Th2 cytokines after primary and secondary infection by *Schistosoma mansoni* in the semipermissive rat host. *Infect Immun* 67(6):2713-2719.
15. Knopf PM & Soliman M (1980) Effects of host endocrine gland removal on the permissive status of laboratory rodents to infection by *Schistosoma mansoni*. *Int J Parasitol* 10(3):197-204.
16. Li Z*, et al.* (2012) Differences in iNOS and arginase expression and activity in the macrophages of rats are responsible for the resistance against *T. gondii* infection. *PLoS One* 7(4):e35834.
17. Oswald IP & James SL (1996) Nitrogen Oxide in Host Defense against Parasites. *Methods* 10(1):8-14.
18. MacMicking J, Xie QW, & Nathan C (1997) Nitric oxide and macrophage function. *Annu Rev Immunol* 15:323-350.
19. James SL & Glaven J (1989) Macrophage cytotoxicity against schistosomula of *Schistosoma mansoni* involves arginine-dependent production of reactive nitrogen intermediates. *J Immunol* 143(12):4208-4212.
20. Oswald IP EI, Wynn TA, Schwartz B, Caspar P, Paulin D, Sher A, James SL (1994) Endothelial cells are activated by cytokine treatment to kill an intravascular parasite, *Schistosoma mansoni,* through the production of nitric oxide. *Proceedings of the National Academy of Sciences of the United States of America* 91(3):999-1003.
21. Wynn TA*, et al.* (1994) Elevated expression of Th1 cytokines and nitric oxide synthase in the lungs of vaccinated mice after challenge infection with *Schistosoma mansoni*. *J Immunol* 153(11):5200-5209.
22. Hang LM, Warren KS, & Boros DL (1974) *Schistosoma mansoni*: antigenic secretions and the etiology of egg granulomas in mice. *Exp Parasitol* 35(2):288-298.
23. Abath FG & Werkhauser RC (1996) The tegument of *Schistosoma mansoni:* functional and immunological features. *Parasite Immunol* 18(1):15-20.
24. Faghiri Z & Skelly PJ (2009) The role of tegumental aquaporin from the human parasitic worm, *Schistosoma mansoni,* in osmoregulation and drug uptake. *FASEB J* 23(8):2780-2789.
25. Pereira AS, Padilha RJ, Lima-Filho JL, & Chaves ME (2011) Scanning electron microscopy of the human low-density lipoprotein interaction with the tegument of *Schistosoma mansoni*. *Parasitology research* 109(5):1395-1402.
26. Sayed AA*, et al.* (2008) Identification of oxadiazoles as new drug leads for the control of schistosomiasis. *Nat Med* 14(4):407-412.
27. Zhang R*, et al.* (2001) Vaccination with calpain induces a Th1-biased protective immune response against *Schistosoma japonicum*. *Infect Immun* 69(1):386-391.
28. Li RW, Li C, & Gasbarre LC (2011) The vitamin D receptor and inducible nitric oxide synthase associated pathways in acquired resistance to *Cooperia oncophora* infection in cattle. *Vet Res* 42:48.

29. Kumar P & Menon R (2013) New insights into growth hormone's actions on the macrophage: Implications for non-growth-related actions of growth hormone. *OA Biochemistry* 1(2):1-10.
30. De Vito P*, et al.* (2011) Thyroid hormones as modulators of immune activities at the cellular level. *Thyroid* 21(8):879-890.
31. Fernandez V, Tapia G, Varela P, & Videla LA (2005) Redox regulation of thyroid hormone-induced Kupffer cell-dependent IkappaB-alpha phosphorylation in relation to inducible nitric oxide synthase expression. *Free Radic Res* 39(4):411-418.
32. van der Spek AH, Fliers E, & Boelen A (2017) Thyroid hormone metabolism in innate immune cells. *J Endocrinol* 232(2):R67-R81.
33. Chen Y*, et al.* (2012) Thyroid hormone enhances nitric oxide-mediated bacterial clearance and promotes survival after meningococcal infection. *PLoS One* 7(7):e41445.
34. Wynn TA, Thompson RW, Cheever AW, & Mentink-Kane MM (2004) Immunopathogenesis of schistosomiasis. *Immunological reviews* 201:156-167.
35. Hesse M, Cheever AW, Jankovic D, & Wynn TA (2000) NOS-2 mediates the protective anti-inflammatory and antifibrotic effects of the Th1-inducing adjuvant, IL-12, in a Th2 model of granulomatous disease. *Am J Pathol* 157(3):945-955.
36. Oliveira DM, Silva-Teixeira DN, Carmo SA, & Goes AM (1998) Role of nitric oxide on human *schistosomiasis mansoni:* upregulation of in vitro granuloma formation by N omega-nitro-L-arginine methyl ester. *Nitric Oxide* 2(1):57-65.
37. Brunet LR, Beall M, Dunne DW, & Pearce EJ (1999) Nitric oxide and the Th2 response combine to prevent severe hepatic damage during *Schistosoma mansoni* infection. *J Immunol* 163(9):4976-4984.
38. Hirata M*, et al.* (2001) Effect of nitric oxide synthase inhibition on *Schistosoma japonicum* egg-induced granuloma formation in the mouse liver. *Parasite Immunol* 23(6):281-289.
39. Gusarov I*, et al.* (2013) Bacterial nitric oxide extends the lifespan of *C. elegans*. *Cell* 152(4):818-830.
40. Thomas DD*, et al.* (2008) The chemical biology of nitric oxide: implications in cellular signaling. *Free Radic Biol Med* 45(1):18-31.
41. Stadler J*, et al.* (1994) Inhibition of cytochromes P4501A by nitric oxide. *Proceedings of the National Academy of Sciences of the United States of America* 91(9):3559-3563.
42. Ziniel PD*, et al.* (2015) The *Schistosoma mansoni* Cytochrome P450 (CYP3050A1) Is Essential for Worm Survival and Egg Development. *PLoS Negl Trop Dis* 9(12):e0004279.
43. Huang TY (1980) The energy metabolism of *Schistosoma japonicum*. *Int J Biochem* 12(3):457-464.
44. McLaren DJ & James SL (1985) Ultrastructural studies of the killing of schistosomula of *Schistosoma mansoni* by activated macrophages in vitro. *Parasite Immunol* 7(3):315-331.
45. Huang SC*, et al.* (2012) Fatty acid oxidation is essential for egg production by the parasitic flatworm *Schistosoma mansoni*. *PLoS Pathog* 8(10):e1002996.
46. Bennet E-M, Behm CA, & Bryant C (1989) *Comparative biochemistry of parasitic helminths* (New York : Chapman and Hall, London ;) p 180 p.
47. Fry M & Jenkins DC (1984) *Nippostrongylus brasiliensis*: the effect of mitochondrial inhibitors on life-cycle stages. *Parasitology* 88 (Pt 1):163-177.
48. Brown GC (2001) Regulation of mitochondrial respiration by nitric oxide inhibition of cytochrome c oxidase. *Biochim Biophys Acta* 1504(1):46-57.

49. Xu W, Charles IG, & Moncada S (2005) Nitric oxide: orchestrating hypoxia regulation through mitochondrial respiration and the endoplasmic reticulum stress response. *Cell Res* 15(1):63-65.
50. Sarti P, Forte E, Mastronicola D, Giuffre A, & Arese M (2012) Cytochrome c oxidase and nitric oxide in action: molecular mechanisms and pathophysiological implications. *Biochim Biophys Acta* 1817(4):610-619.
51. Sen S, Kawahara B, & Chaudhuri G (2013) Mitochondrial-associated nitric oxide synthase activity inhibits cytochrome c oxidase: implications for breast cancer. *Free Radic Biol Med* 57:210-220.
52. Aguirre E, Rodriguez-Juarez F, Bellelli A, Gnaiger E, & Cadenas S (2010) Kinetic model of the inhibition of respiration by endogenous nitric oxide in intact cells. *Biochim Biophys Acta* 1797(5):557-565.
53. Zhong ZR, Xu YX, Gong JY, Luo QL. (2012) Isolation and identification of viability of *Schistosoma japonicum* eggs with high purity. Chinese Journal of Zoonoses 28(3):209–212.
54. Sarvel AK, Kusel JR, Araujo N, Coelho P, Katz N (2006) Comparison between morphological and staining characteristics of live and dead eggs of *Schistosoma mansoni*. Memorias do Instituto Oswaldo Cruz 101 Suppl 1:289–292.
55. Wynn TA, Eltoum I, Oswald IP, Cheever AW, Sher A (1994) 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 179(5):1551–1561.
56. Amiri P*, et al.* (1992) Tumour necrosis factor alpha restores granulomas and induces parasite egg-laying in schistosome-infected SCID mice. *Nature* 356(6370):604-607.
57. Bartley PB, et al. (2006) A contributory role for activated hepatic stellate cells in the dynamics of *Schistosoma japonicum* egg-induced fibrosis. *Int J Parasitol* 36:993–1001.
58. Burke ML*, et al.* (2010) Temporal expression of chemokines dictates the hepatic inflammatory infiltrate in a murine model of schistosomiasis. *PLoS Negl Trop Dis* 4(2):e598.

Figure legends

Fig. 1. The development of adult *S. japonicum* in WT and iNOS$^{-/-}$ rats. BALB/c mice, WT and iNOS$^{-/-}$ rats were infected with *S. japonicum* and parasites were harvested at 7 weeks post-infection. (A) The length and diameter of male and female worms were measured from digital micrographs. (B) Scanning electron microscopy (SEM) analysis of the body tegument (upper panel) and tegument in the oral sucker (lower panel) of adult male worms, bar = 10 μm. R, ridge; P, pit; SP, sensory papillae; S, spine. (C) Morphological analysis of reproductive organs of worms. The worms were stained with hydrochloric carmine and observed under a light microscope (left) and confocal laser scanning microscopy (right), bar = 100 μm. t, testicular lobules; so, spermatocytes; sv, seminal vesicle; s, sperm; o, ovary; io, immature oocytes; mo, mature oocytes; ot, ootype; e, egg. (D - G) Quantitative analysis of data from Fig.1C. Mean values are represented by horizontal bars. $^{**}P < 0.01$, $^{***}P < 0.001$. Groups of 6-10 rats or mice were used for each experimental condition. Data are representative of at least three independent experiments.

Fig. 2. Characteristics of *S. japonicum* eggs collected from WT and iNOS$^{-/-}$ rats. (A) Acridine orange staining of *S. japonicum* eggs. White arrows indicate the dead eggs; magenta arrows indicate the live eggs. Bar = 100 μm. (B) Circumoval precipitation (red arrows) surrounding eggs indicated the secretion activity of live mature eggs. The percent of positive and negative eggs with the circumoval reaction were noted. Bar = 20 μm. (C) Pulmonary granuloma formation in BALB/c mice induced by schistosome eggs collected from the livers of WT and iNOS$^{-/-}$ rats, respectively. Histological analysis of lungs by H&E staining after 7 and 14 days. Bar = 50 μm. (D) Size of pulmonary granulomas from Fig.2C. (E) Hatching of the eggs of *S. japonicum*. nd, non-detectable. The data are expressed as the mean ± SEM of 5 animals per group. $^{**}P < 0.01$, $^{***}P < 0.001$. Data are representative of three independent experiments.

Fig. 3. Egg-induced granulomatous inflammation in livers and lungs in WT and iNOS$^{-/-}$ rats. (A) Representative H&E staining images of hepatic granulomas at 7 weeks and 12 weeks post-infection with 200 *S. japonicum* cercariae. Bar = 100 μm. Arrows identify egg-induced granulomas. (B) The size range of liver granulomas (WT group, n=49 and 69; iNOS$^{-/-}$ group,

n=138 and 90). (C) Granuloma volume density in liver tissue. Granulomas were measured in tissue section (8.2 >mm^3) in 5 individual rats per group. The data are expressed as the mean ± SEM. $^{*}P$ ＜0.05, $^{***}P$＜0.001. Data are representative of three independent experiments.

Fig. 4. Mitochondrial respiration was inhibited in worms collected from WT rats. *S. japonicum* were harvested from infected animals at 7 weeks post-infection. (A) Ultrastructural analysis of mitochondria in worms. Arrows indicate mitochondria. Bar = 200 nm. (B) Respiratory chain enzyme cytochrome c oxidase activity from isolated mitochondria of adult worms. The data are expressed as the mean ± SEM. $^{***}P$＜0.001. Data are representative of three independent experiments.