Nitric oxide blocks the development of the human parasite Schistosoma japonicum


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SI Materials and Methods

Determination of worm burdens and egg burdens. Parasites were harvested by perfusion from the portal system of infected animals at 7 weeks post-infection. Male and female worms were counted and photographed under a stereoscopic microscope (M205FA, Leica, Germany). The length and diameter of worms were measured from digital micrographs using the LAS imaging program (Leica, Germany). For this purpose, the male crosssections of the crescent-shaped (gynecophoral canal) were treated as hollow cylinders.

Egg burdens in tissues were determined as described previously (7). Briefly, liver tissues from the infected animals were weighed and completely digested overnight with 4% potassium hydroxide (KOH) at 37°C on a rocking platform. Released eggs were counted under a dissecting microscope.

Macrophages, isolation and nitric oxide analysis. Peritoneal macrophages were isolated as previously described (16). Briefly, rats and mice were killed by CO₂ asphyxiaton and injected intraperitoneally with 15 mL (rat) or 5 mL (mouse) ice-cold PBS. The injected PBS with peritoneal cavity fluid was recovered by a plastic syringe and transferred to a sterile centrifuge tube. The harvested suspended cells were centrifuged at 250 g, for 10 min at 4°C, and the cells were resuspended in RPMI-1640 medium (GIBCO, USA) with penicillin (100 U/mL) and streptomycin (100 mg/mL). Cells were counted and seeded into 24-well culture plates (5 × 10⁵ cells/well) for 2 hrs at 37°C with 5% CO₂. Then, the wells were washed with FBS-free RPMI-1640 medium supplementary with 10% FBS (GIBCO, USA) and penicillin (100 U/mL) and streptomycin (100 mg/mL). Macrophages were stimulated with lipopolysaccharide (LPS, 100 ng/ml; Sigma-Aldrich, USA), IFN-γ (50 ng/ml; Sigma-Aldrich, USA), or medium alone. Supernatants were collected 24 hrs post treatment and nitric oxide was determined using the Griess reagent as previously described (16). Briefly, 100 μl samples were mixed 1/1 with Griess reagent, and absorbance was detected at 550 nm using ELISA reader (Multiskan MK3, Thermo-fish, Finland). Sodium nitrite was used as a standard. Nitric oxide in serum from animals was determined as described above.

Scanning electron microscopy. Adult worms isolated from BALB/c mice, WT and iNOS⁻/⁻ rats were fixed individually with 0.2 M PBS containing 2.5% glutaraldehyde (pH 7.4) at 4°C for 24
The samples were washed three times with PBS and six times with distilled water before being dehydrated in gradient ethanol. Following ethanol exchange with acetone and isoamyl acetate, the samples were critical-point dried, and then coated with gold in an ion coater (Hitachi E-102, Japan). Worms were observed and photographed using a scanning electron microscope (Hitachi S-2500, Japan).

**Transmission electron microscopy analysis.** The samples were fixed, washed and dehydrated as described above, and then embedded in araldite. Ultrathin sections were cut and contrasted with 1% methanolic uranyl acetate and Reynold’s solution of lead citrate. The sections were observed under a Hitachi H-300 transmission electron microscope (Japan).

**Reproductive organ examination.** The worms were fixed in AFA (95% ethanol, 3% formalin and 2% glacial acetic acid) and stained with 2.5% hydrochloric carmine red (Merck, USA) for 1 hr, and destained in 70% acidic ethanol. Following dehydration in an ethanol gradient, worms were clarified in methyl salicylate and preserved as whole-mounts on glass slides. Confocal laser scanning microscopy images were taken using a Zeiss7 DUO NLO microscope (Germany) with a 488 nm laser and a 470 nm long-pass-filter under reflection mode.

**Isolation of Eggs.** *S. japonicum* eggs were isolated from liver tissues of infected rabbits, mice, WT and iNOS−/− rats at 45 days post-infection, respectively. After homogenization of the livers in 1.2% NaCl solution, the eggs were collected with a sedimentation glass, and then were centrifuged at 1500 g for 20 min on Percoll with a density of 1.070 (rabbits and mice) or 1.043-1.056 (rat). The pelleted eggs were stored in sterile 0.9% NaCl solution at 4°C until use.

**Acridine orange staining.** The method was followed as previously described (53, 54). Briefly, the purified eggs were mixed with 0.01% acridine orange in an Eppendorf tube and incubated for 2 hrs at 37°C. After washing with PBS, a 5 μl aliquot of suspension was placed on a slide and observed under a fluorescence microscope with 515 nm long-pass reading filter. Live eggs presented as a green and/or a red fluorescence showing abundant DNA and/or RNA while dead eggs exhibited poor staining with only a slight autofluorescence.

**Circumoval precipitation.** A total of 10 μl egg suspension containing 50-100 eggs was pipetted onto a slide, and one drop of anti-*S. japonicum* rabbit serum was added. After sealing with a petroleum jelly bordered cover slip, slides were incubated for 24 hrs at 37°C, and results were observed and recorded under a microscope.
Hatching test. Eggs were transferred to distilled water and distributed into 96-well culture plates. After counting the number of eggs in each well, the 96-well culture plates were placed under a lamp at room temperature and monitored for the hatching of miracidia in the first 2 hrs, with a dissecting microscope.

Induction of pulmonary granulomas. The induction of pulmonary granulomas was performed as previously described (55). Briefly, *S. japonicum* eggs were isolated and purified from the livers of infected animals including rabbits, WT rats and iNOS−/− rats. A total of 2,000 and 15,000 eggs were injected through the tail vein into mice and rats, respectively. Animals were killed on days 7 and 14 post inoculation and the left lung was removed for histological analysis.

Histopathology. Liver and lung tissues were fixed in 4% neutral buffered formalin, embedded in paraffin. Sections were dewaxed and stained with hematoxylin and eosin (H&E) for granuloma analysis. The size of granulomas was calculated as previously described (56). Granuloma volume density, defined as the volume of liver occupied by egg granulomas (57), was quantified by point counting stereology on tissue sections (57, 58).

Adoptive transfer experiments. In this work, 8–10 week old males of wild type SD rats were injected with 3 ml of 2% sterile starch solution (Sigma-Aldrich, USA), and 4 days later peritoneal macrophages were harvested as described above. Total of 1×10⁸ cells suspended in PBS were transferred into iNOS−/− rats through the tail vein on day 0 (before infection), 7, 14, 21, 28 and 35 post infected with *S. japonicum* (iNOS−/−+Mφ). A group of NOS−/− rats that received only PBS was used as control (iNOS−/−). The rats were killed on day 43 (6 weeks post-infection) to investigate the status of parasite development.

Immunohistochemistry. Prior to immunostaining, liver sections were boiled in 10 mmol/L citrate buffer for 20 min in a microwave oven for epitope retrieval. After slow cooling and washing with PBS, sections were treated with 3% hydrogen peroxide for 5 min, and incubated with 1:100 diluted anti-iNOS antibody (Abcam, USA) overnight at 4°C. Incubation with secondary antibody and visualization were done using UltraVision Quanto Detection System HRP DAB kit (Thermo-fisher, USA). Sections were counterstained with hematoxylin and examined under a microscope.

Assay for cytochrome c oxidase activity. Cytochrome c oxidase activity in isolated mitochondria was determined using the Cytochrome c Oxidase Assay Kit (Sigma-Aldrich, USA). Protein concentration was determined using the Pierce BCA Protein Assay Kit (Pierce, USA).
**Determination of mitochondrial gene expression by Real-time PCR.** After perfusion from infected animals, the harvested worms were immediately placed in 0.5 mL TRIzol (Invitrogen, USA) and mashed using a TissueLyser II (Qiagen, USA). Total RNA was isolated and further purified using RNeasy Mini Kit (Qiagen, USA) following manufacturer’s instructions. Purified RNA was quantified using a NanoDrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA). First strand cDNA was synthesized using isolated RNA, Superscript II reverse transcriptase (Invitrogen), and oligo dT as a primer. Mitochondrial cytochrome c oxidase subunit I (CcO I) (GenBank accession no. FN314248.1) and NADH dehydrogenase (GenBank accession no. FN317713.1) were analyzed by quantitative real-time PCR (qRT-PCR) using the LightCycler480 real-time PCR system (Roche, Switzerland) and SYBR green qPCR Master Mixes (Roche, USA). Expression levels of *S. japonicum* β-actin (GenBank accession no. AF223400.1) were used as endogenous controls within each sample. β-actin primers: forward 5’- AGCGTGGTTACAGCTTCACG-3’, reverse 5’- AACGCCCTCAGGACAACGGAA-3’. CcO I primers: forward 5’- TGGGTTCTATTGTGTGTTTGGG-3’, reverse 5’- CACGCAACCCACTACTCCCT-3’. NADH dehydrogenase primers: forward 5’- TCTGGAAGCCGCACTTGTTG-3’, reverse 5’- CGAACCGTCAACAGCAAAGGT-3’.

Relative expression was calculated using the $2^{-\Delta\Delta CT}$ method.

**Statistical analysis.** All statistical analyses were performed using SPSS 19.0 software. Significant differences between two groups were determined using a student’s unpaired *T*-test with Welch’s correction or one-way ANOVA. All data shown represent mean ± SEM, and $P$ values ≤0.05 were considered statistically significant. At least 3–6 animals were used per experimental group and all experiments were performed at least twice.
SI Table 1. Worm and egg burden in iNOS knockout rats (iNOS<sup>−/−</sup>) without macrophage transfer compared with macrophage transfer (iNOS<sup>−/−</sup> + Mφ) of iNOS<sup>−/−</sup> rats at 6 weeks post *S. japonicum* infection.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total</th>
<th>Male</th>
<th>Female</th>
<th>No. of eggs found in liver (/g)</th>
<th>Eggs / Female worm</th>
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<tr>
<td>iNOS&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>106±5</td>
<td>58±3.5</td>
<td>49±1.5</td>
<td>18568±3158</td>
<td>379±60</td>
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<tr>
<td>iNOS&lt;sup&gt;−/−&lt;/sup&gt; + Mφ</td>
<td>67±5.5*</td>
<td>50±2.2</td>
<td>17±3**</td>
<td>3034±354**</td>
<td>176±20*</td>
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iNOS<sup>−/−</sup> rats were infected percutaneously with 200 *S. japonicum* cercariae. Macrophages from WT rats were transferred into iNOS<sup>−/−</sup> recipients as described in the Materials and Methods. Data are expressed as the means ± SEM from two biological repeats. n =10. * P < 0.05, ** P < 0.01.
Fig. S1. Susceptibility to S. japonicum infection in mice and rats is correlated with their NO production levels in peritoneal macrophages. (A) Parasite burdens in BALB/c mice, SD rats and Lewis rats after infection with S. japonicum at 7 weeks. (B) Trapped eggs in liver were enumerated by microscopy at 7 weeks post-infection. (C) Fertility was calculated as numbers of eggs produced per female worm. (D - E) The lengths and diameters of parasites were measured from digital micrographs. (F) Size of liver granulomas at 7 weeks post-infection. (G) Representative granulomas at 7 weeks post-infection, as indicated by arrows. Liver sections were stained with H&E and observed at 10x magnification (scale bars 100um). (H) The levels of NO production in peritoneal macrophages from BALB/c mice, SD rats and Lewis rats, after 24h
stimulation with LPS (100ng/ml), IFN-γ (50ng/ml), or not stimulated. Data are expressed as the mean ± SEM of 5 rats or mice per group. * $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$. ND: not detectable.
Fig. S2. The difference in iNOS expression and NO production in wild type and iNOS−/− rats.

(A) iNOS protein expression in peritoneal macrophages from WT rats and iNOS−/− rats as analyzed by western blotting. (B) NO production in peritoneal macrophages from WT rats and iNOS−/− rats after induction with LPS (100 ng/ml) plus IFN-γ (50 ng/ml). Supernatants were collected and analyzed after 24h of induction. Non-induced cells were maintained in culture medium as controls. ND: not detectable. (C) NO concentration in serum of WT rats and iNOS−/− rats. The data are expressed as the mean ± SEM of 5 rats per group. * P < 0.05. Data are representative of three independent experiments.
Fig. S3. Transmission electron microscope analysis of the tegument of adult male worms obtained from a mouse, WT and iNOS<sup>−/−</sup> rats. Bar = 2 μm. R, ridge; P, pit.
Fig. S4. Vitelline glands and the uterus in female *S. japonicum* observed using confocal laser scanning microscopy. Worms collected from BALB/c mice, WT and iNOS<sup>−/−</sup> rats were stained with hydrochloric carmine and observed by confocal laser scanning microscopy. (A) Vitelline glands of female worms. (B) Eggs in the uterus of female worms. VG : Vitelline glands ; e : egg.
Fig. S5. Pulmonary granuloma formation in WT and iNOS−/− rats induced by schistosome eggs collected from rabbits. WT and iNOS−/− rats were injected intravenously with 15,000 eggs and lungs were removed for histological analysis after 7 and 14 days. (A) Representative images of pulmonary granuloma, bar = 100 μm. (B) The size of single granuloma. (C) Granuloma volume density in lung tissue (>7.8 mm³). The data are expressed as the mean ± SEM. ns, non-significant. Data are representative of three independent experiments.
Fig. S6. Adoptive transfer of wild-type macrophages into infected iNOS−/− rats. Adoptive transfer of wild type macrophages was performed in iNOS−/− rats, as described in the Materials and Methods (group iNOS−/− + Mφ), simultaneously with a group of infected iNOS−/− rats that did not receive macrophages but instead PBS was used as an additional control (group iNOS−/−). The rats were killed on day 43 (6 weeks post-infection). (A) The expression of iNOS in liver was identified by immunohistochemistry using an iNOS antibody. Arrows indicate the iNOS signal. (B) Quantitation of the positive area of fields of view showing iNOS expression. (C) NO concentration in the serum of infected iNOS−/− rats and iNOS−/− recipients at 6 weeks post-infection. (D) Representative micrographs showing parasites present. Arrows identify stunted parasites. (E–F) Worm lengths and diameters were measured from digital micrographs. Mean values are represented by horizontal bars. (G) H & E stain of representative hepatic granulomas. (H) Quantitation of hepatic granulomas sizes as measured from H & E stained slides. Data are expressed as the mean ± SEM from two biological repeats. n =10. **P<0.01, ***P<0.001.
Fig. S7. Damaged mitochondria from testicular tissue and ovarian cross-sections were counted using a transmission electron microscope, including 342 mitochondrial organelles from four worms from the mouse group, 254 mitochondrial organelles from four worms from the WT rat group, and 309 mitochondrial organelles from four worms from the iNOS−/− rat group. *P < 0.05; **P < 0.01; ***P < 0.001.
Fig. S8. The relative expression of the mitochondrial respiratory chain enzymes cytochrome c oxidase subunit I (CcO I) and NADH dehydrogenase in WT and iNOS−/− rats. The relative expression of each gene was normalized to the expression of β-Actin. Data are shown as mean ± SEM from two biological repeats, n = 10. Significant differences have been noted, * $P < 0.05$, **$P < 0.01$, ***$P < 0.001$. 