RESEARCH ARTICLE

GdCl$_3$ Attenuates Schistosomiasis japonicum Egg-Induced Granulomatosis Accompanied by Decreased Macrophage Infiltration in Murine Liver

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Abstract

Early-stage hepatic granuloma and advanced-stage fibrosis are important characteristics of schistosomiasis. The direct consequences of gadolinium chloride (GdCl$_3$) in egg-induced granuloma formation have not been reported, although GdCl$_3$ is known to block the macrophages. In present study, mice were infected with 15 Schistosoma japonicum (S. japonicum) cercariae and treated with GdCl$_3$ (10 mg/kg body weight) twice weekly from day 21 to day 42 post-infection during the onset of egg-laying towards early granuloma formation. Histochemical staining showed that repeated injection of GdCl$_3$ decreased macrophages infiltration in liver of mice infected with S. japonicum. Macrophage depletion by GdCl$_3$ during the initial phase attenuated liver pathological injury characterized by smaller granuloma size and decreased immune inflammation as well as less fibrogenesis. In addition, IL-13Rα2 expression was reduced by GdCl$_3$ in liver of mice infected with S. japonicum. The results suggest that GdCl$_3$ depleted macrophages, which attenuated helminth infected immune responses involving with IL-13Rα2 signal. These findings would highlight a therapeutic potential via manipulating IL-13Rα2+ macrophage in schistosomiasis.

Introduction

Schistosomiasis is one of the most important poverty-related health problems, and more than 200 million people are currently infected worldwide [1,2]. In the tropical and subtropical regions, it ranks second among human parasitic diseases [3,4]. The social health and economic burdens for affected populations are poorly measured, despite the incidence of acute and advanced schistosomiasis is significantly reduced in China [5–7]. The major cause of mortality is caused by liver granuloma and progressive fibrosis, which often lead to portal hypertension. However, the key cellular and molecular factors that triggered pathological cascade in

schistosomiasis are not well understood, which prevent the therapeutic development that targets for reversing hepatic granulomatosis [8–10].

Schistosome infection induces an increase in the levels of Th2 cytokines such as interleukin (IL)-4, IL-5, and IL-13, among which IL-13 is the dominant effector cytokine of liver fibrogenesis. Kupffer cells as the first macrophage population of the liver [9,11–12]. Macrophages at the boundary of granuloma during schistosoma infection are indispensable to the generation of the Th2 response [13–17]. IL-13 can signal through the IL-13 receptor (R) α2 and type II IL-4 receptor, which both regulate the development of fibrosis. IL-13 and IL-13 receptor complex were critical regulators of disease progression in schistosomiasis [16,18–19]. Moreover, macrophages can produce a pro-fibrogenic transforming growth factor (TGF)-β1 via IL-13Rα2 that is the 'decoy' IL-13 receptor as a key life sustaining 'off' switch for tissue damaging inflammation [20–22]. Macrophages are able to produce a variety of enzymes, cytokines, and mediators that could initiate and/or maintain the inflammatory and immune responses. In this way, we hypothesize that IL-13Rα2 expressing macrophages contribute to the immunopathological development in schistosomiasis.

It is well known that intravenous injection of GdCl₃, a rare earth metal salt, is able to not only block the phagocytosis of macrophages in liver and spleen, but also eliminate them [23,24]. GdCl₃ selectively depleted macrophage and used to be a tool for macrophage function research. In the present study, we determined whether GdCl₃ administration attenuates hepatic immunopathological injury in *S. japonicum* murine model.

Materials and Methods

Ethics statement

All animal protocols were approved by the Animal Research Committee of the Anhui Medical University at Hefei, China.

Animals and mice attacked with *S. japonicum*

Female BALB/c mice, 6 weeks old, approximately 25 g, were obtained from the Experimental Animal Center of the University of Science and Technology of China (Hefei, China), and housed with free access to food and water. Cercariae of *S. japonicum* were released from the Oncomelania hupensis snails (Wuxi, China). The mice were randomly assigned into four groups (n = 6 in each group). Mice were percutaneously infected through abdomen with 15 cercariae of *S. japonicum* with GdCl₃- or saline-injection as described previously [22,25]. Non-infected animals of the same sex and age with GdCl₃- or saline-injection were used as controls.

Treatment of mice with GdCl₃ in vivo

GdCl₃ solution at a concentration of 2 mg/mL was prepared. Briefly, 0.056 g of GdCl₃·6H₂O (Sigma Aldrich; St. Louis, MO, USA) was weighed, and dissolved in 20 mL of saline. The solution was filtered through a 0.22 μm filter, aliquoted, stored at 4°C, and used within a week. Mice were injected with GdCl₃ 10 mg/kg body weight or saline every 3 days from day 21 to day 42 post-infection by the tail vein as described previously [26]. Twenty-four hours prior to sacrifice, reinforced injection once was performed, then mice were sacrificed and the liver samples were harvested for the following analysis. The whole experiment was repeated twice.

Histological examination, immunohistochemistry and verification of macrophage depletion in murine liver tissues

The liver tissues were fixed in 4% paraformaldehyde for overnight, embedded in paraffin, and sectioned (3 μm), which sections were used for hematoxylin and eosin (H&E), Masson trichrome

and immunohistochemistry staining following the standard protocols. After H&E staining, the single-egg granulomas were counted, and their sizes were calculated in each section. The following formulae were employed for calculation: size = the maximum transverse diameter × the maximum longitudinal diameter; and the mean size of the egg granulomas = the sum of the size of all egg granulomas/the total number of egg granulomas in each section. Eight to ten images per liver section were photographed under an inverted microscope (Nikon 80I, Japan).

Immunohistochemistry studies was performed on paraffin-embedded tissues sections using primary antibodies against F4/80 (1:400; eBioscience, San Diego, CA,), collagen I, collagen III (both 1:200, Bioworld Technology, USA), monoclonal alpha-smooth muscle actin (α-SMA) (1:400; Dako, Carpinteria, CA), and a horseradish peroxidase-labeled secondary antibody. Immunofluorescent staining in liver sections was performed using the primary antibodies against both IL-13Rα2 (1:200; R&D Systems; Minneapolis, USA) and CD68 (1:100; ED-1; AbD Serotec, Oxford, UK) after the antigen retrieval with citric acid buffer. Secondary antibody of Cy3-conjugated donkey anti-goat IgG (Abcam, Cambridge, UK) and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (Sigma) were applied at a 1:500 dilution, respectively. Nuclei detection was performed with DAPI (Vector Laboratories), and the staining was visualized under a fluorescence microscope (Nikon 80I; Japan). Goat anti-mouse IgG (1:200; Jackson, USA) were used for each primary antibody.

Real-time PCR for IL-13Rα2 and collagen I mRNA of murine liver tissues

RNA was extracted from whole liver tissue using the RNA extraction kits (Qiagen) according to the manufacturer's instructions. Complementary DNA was generated from 1 μg of RNA using the Superscript II kit (Invitrogen). The primers and probe were designed by the Shanghai Shinegene Molecular Biotechnology Co., Ltd. (Shanghai, China), and the primers are as follows: IL-13Rα2 sense, 5′-ATG GCT TTT GTG CAT ATC AGA TGC T-3′; antisense, 5′-CAG GTG TGC TCC ATT TCA TTC TAA T-3′. Collagen I sense, 5′-GCC CGG AAG AAT ACG-3′; antisense, 5′-ACA TCT GGG AAG CAA A-3′. GAPDH sense, 5′-GAG GGG CCA TCC ACA GTC TTC-3′; antisense, 5′-CAT CAC CAT CTT CCA GGA GCG-3′[22]. The cycle threshold (Ct) value of the GAPDH gene served as the housekeeping, and the IL-13Rα2 and collagen I expression was normalized to obtain the $^\Delta$Ct value. The $^\Delta$Ct values for IL-13Rα2 and collagen I were calculated using the $^\Delta$Ct value of the mice in the non-infected mice injected with saline as the reference, and the difference in the expression of the IL-13Rα2 and collagen I genes in the other groups was expressed as $2^{-\Delta\Delta Ct}$ [22,27]. All reactions were performed in triplicate. Levels are expressed relative to matched control samples from the same time points.

Statistical analysis

Data are presented as mean ± standard deviation of the mean. For the data fitting of the approximate normal distribution, one-way analysis of variance was used to compare the differences between groups, while a *q* test (Newman-Keuls test) was performed to compare the pairwise difference between group means. All tests performed were two-sided, with $P < 0.05$ being considered statistically significant.

Results

GdCl$_3$ treatment decreases F4/80- or CD68-positive signal expression in egg-induced hepatic granuloma

Anti-mouse F4/80 antibody was used to detect macrophages by the immunohistochemistry staining. As shown in Fig 1, F4/80-positive cells were significantly reduced in GdCl$_3$-infected
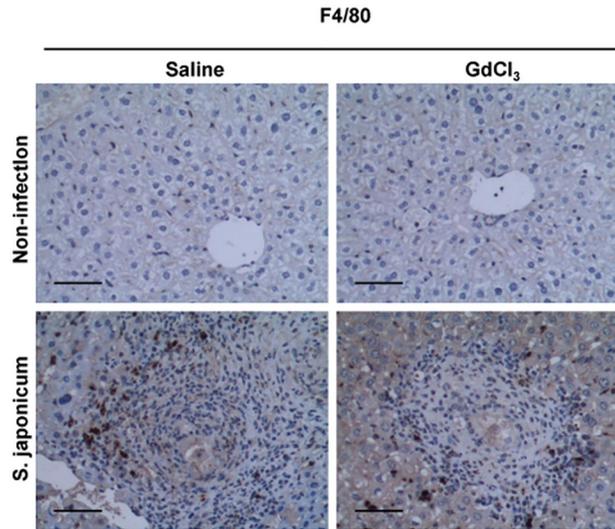
F4/80

Fig 1. GdCl₃ injection reduces F4/80 expression in *S. japonicum* egg-induced hepatic granuloma.
Mice infected with 15 *S. japonicum* cercariae and treated with GdCl₃ (10 mg/kg body weight) or saline twice weekly for 8 times. F4/80 immunohistochemical staining on liver sections prepared from saline-treated mice (control, left column) or GdCl₃-treated mice (right column) at day 42 post-infection was shown. Original magnification ×200, bar scale 50 µm. Experiments were repeated at least twice with comparable results.

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mice, comparing to saline-infected mice. Consistent with the findings that under a fluorescence microscope, many spots highlighted with green fluorescence were observed in saline-infected liver sections (middle row, Fig 2A). Nevertheless, CD68 positive signal (green) were
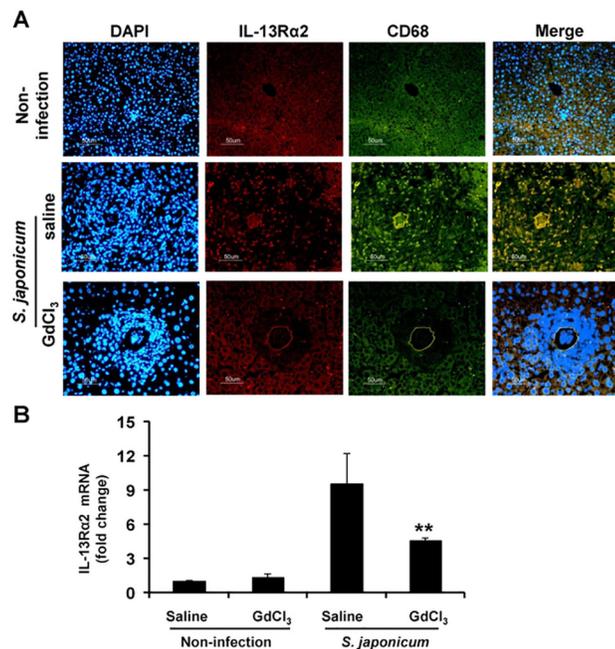
Fig 2. Effect of GdCl₃ treatment on expression of IL-13Rα2 and CD68 in egg-induced hepatic granuloma. (A) Immunofluorescence double labeled staining was performed to detect IL-13Rα2 (red) and CD68 (macrophages marker, green) in liver sections, with DAPI (blue) conterstain for the nuclei. Merged images were shown in the right panels. Levels of IL-13Rα2 mRNA in livers tissues were measured by real-time PCR (B). **$P<0.01$, vs infected mice treated with saline. Magnification ×400, bar scale 50 µm.

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occasionally noticed in GdCl$_3$-infected liver sections (bottom row, in Fig 2A). These results indicate that repeated GdCl$_3$ injection decreased macrophages infiltration in hepatic granulomatosis.

GdCl$_3$ administration attenuates egg-induced hepatic granuloma inflammation

To investigate the effect of GdCl$_3$ on early egg-induced granuloma formation, the mice were sacrificed after 8 times injection of GdCl$_3$ by tail vein. Histochemical staining revealed that liver sections were indistinguishable between GdCl$_3$- and saline-treated control non-infected mice by H&E staining (Fig 3A). While most of the cells around egg-miracidia granuloma were eosinophils in infected liver sections treated with saline or GdCl$_3$ (red arrow, Fig 3A). More importantly, the granuloma size (Fig 3B) in GdCl$_3$-infected mice (10.25±2.1 μm^2× 10^3) was significantly smaller than that in saline-infected mice (23.87± 3.86 μm^2× 10^3) ($P < 0.05$). Therefore, depletion of macrophages of GdCl$_3$ attenuates hepatic granuloma inflammation.

GdCl$_3$ injection attenuates hepatic fibrogenesis

Hepatic fibrogenesis is one of the features of chronic schistosomiasis [10]. Next to investigate the effect of GdCl$_3$ on hepatic fibrosis, collagen deposition were stained with Masson trichrome, and expression of collagen isoforms of collagens I, III and α-SMA were examined by immunohistochemical staining shown in Fig 4. The large, thick, and flame-like fibers surrounding the granulomas and extending outward was observed in saline-infected mice, which was markedly reduced in GdCl$_3$-infected mice (Fig 4A). The positive area of collagen I, III, and α-SMA was significantly reduced in GdCl$_3$-infected mice, comparing to saline-infected mice, respectively (Fig 4B). Consistently, the level of collagen I mRNA was significantly decreased in GdCl$_3$-infected mice compared with control mice (Fig 4C). Hence, GdCl$_3$ reduces egg-induced hepatic fibrogenesis in granulomas.

GdCl$_3$ treatment has no effect on worm load, egg burden

Worm pairs, total worms, and total parasite eggs contained similar mature miracidia in the livers of GdCl$_3$- and saline-infected mice, which were not significantly different (Table 1). Hence
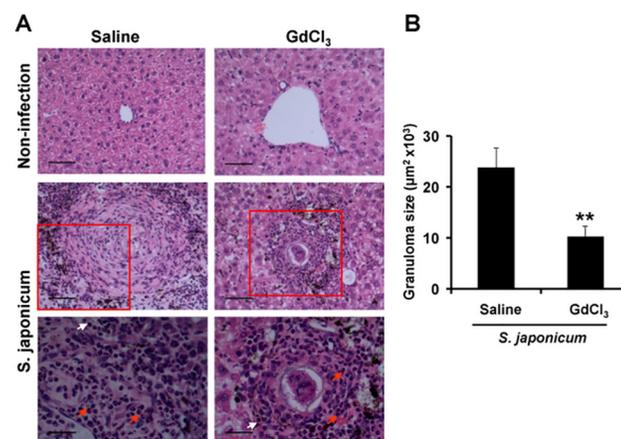
Fig 3. Repeated injection of GdCl$_3$ attenuates *S. japonicum* egg-induced hepatic granuloma inflammation. Liver sections were stained with H&E (A) for eosinophils (red arrow) and lymphocytes (white arrow) (Original magnification, top and middle row ×200,bar scale 50 μm, bottom row ×400,bar scale 25 μm) or average granuloma sizes (B). Means and SD are shown. $^{**}P<0.01$.
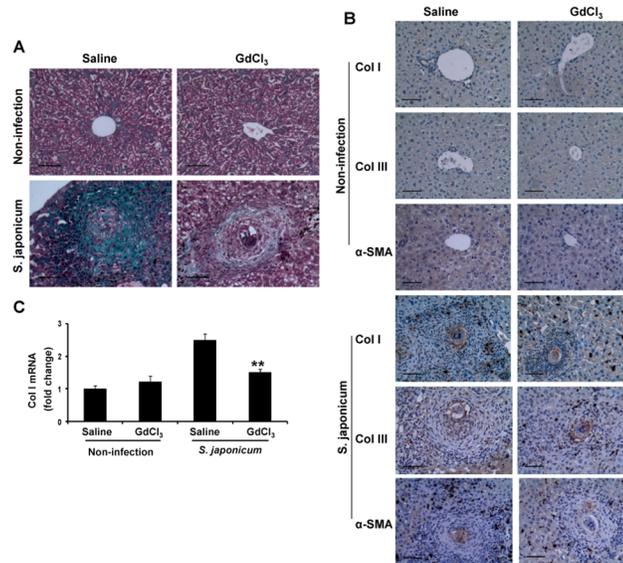
Fig 4. Repeated injection of GdCl₃ reduced egg-induced hepatic fibrogenesis. Liver sections of saline-treated mice (control, left column) or GdCl₃-treated mice (right column) were used for Masson trichrome staining for collagen deposition (A). Immunohistochemical staining was performed for collagen I (top row), collagen III (middle row), α-SMA (bottom row) (B). Collagen I mRNA in livers tissue was measured by real-time PCR (C). $^{**}P < 0.01$, vs infected mice treated with saline. Original magnification ×200, bar scale 50 μm.

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decreased hepatic granulomatous inflammatory and fibrogenesis by GdCl₃ were not resulted from differences in worm load and egg burden.

Reduction of IL-13Rα2 expression in GdCl₃-infected liver tissues

Our previous study shows enhanced IL-13Rα2 expression in primary macrophages of murine schistosomiasis [22]. Then, we further determined whether depleted macrophages of GdCl₃ resulted in any differences in IL-13Rα2 expression. Liver sections were detected by double labeled staining of IL-13Rα2 and CD68. In saline-infected mice (middle row, Fig 2A), there was some scattered red coffee bean-like staining, indicating specific IL-13Rα2 positive signals (red). Green coffee bean-like staining was donated to CD68 positive signals (green). Merge in white were demonstrated co-expression of IL-13Rα2 and CD68 at the same position of egg-induced hepatic granuloma (white, in Fig 2A). Surprisingly, the co-expression of the CD68⁺ (green) and IL-13Rα2⁺ (red) signal diminished simultaneously in GdCl₃-infected mice (bottom row, Fig 2A). Further, the hepatic IL-13Rα2 mRNA expression was analyses by TaqMan PCR. IL-13Rα2 mRNA expression was not significantly changed between GdCl₃- and saline-treated normal/non-infected mice ($P > 0.05$). However, level of IL-13Rα2 mRNA in saline-infected mice was significantly augmented (9-fold), which was reduced in GdCl₃-infected mice (5-fold) ($P < 0.05$) (Fig 2B). Overall, depletion of macrophages by injection of GdCl₃ reduced IL-13Rα2 expression in murine *S. japonicum* liver.

Table 1. Parasitological measurements in Schistosomiasis japonicum mice treated with GdCl₃.

Group	No	Total warms	Warms pairs	Total liver eggs (×10³)	Size of granulomas (μm²×10³)
Saline	6	10.0±1.26	5.2±0.49	38.25±4.72	23.87± 3.86
GdCl₃	6	10.0±2.08	5.0±1.01	38.27±5.89	10.25±2.1

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Discussion

Accumulating evidence has shown that macrophages are able to promote, restrict, or resolve inflammation and fibrosis [28–30]. It has been shown that monocytes/macrophages are not only responsible for fibrosis progression, but also for the resolution of hepatic inflammation and fibrosis (for fibrosis regression) [31,32]. For example, the findings using mice genetically defective in macrophage function have confirmed that these cells are essential to normal wound healing, because their depletion results in retarded and abnormal repair [33]. This may due to the function of macrophages to produce anti-inflammatory mediators and matrix metalloproteinases. However, the signaling pathway resulted from changes in gene expression pattern mechanisms of macrophage in the regulation of hepatic pathological development in schistosomiasis is complex [31,32].

GdCl₃ is nontoxic to other cells and used as a magnetic resonance imaging contrast agent in clinical medicine. GdCl₃ can induce macrophage inactivation or dormancy as well as macrophage apoptosis [34–36]. To explore the role of IL-13Rα2-expressing macrophages, the current study was in search of GdCl₃ to selectively deplete macrophages following with effect on IL-13Rα2 expression. Helminth worms live in the portal venous system, and begin to lay eggs after 4 weeks post-infection. In this way, we have designed to administer GdCl₃ on day 21 post-infection before the initial egg-induced immune responses. Macrophage depletion during the initial phase attenuated liver pathological injury, which is reflected on smaller granuloma size and decreased immune inflammation as well as less fibrogenesis. This is in agreement with the findings that targeting Kupffer cells by GdCl₃ ameliorates carbon tetrachloride-induced liver fibrosis [37,38], and that pharmacological inhibition of the chemokine CCL2 diminishes liver macrophage infiltration thereby attenuating steatohepatitis during chronic hepatic injury [39]. Thus, macrophage depletion in the initial stage protects against *S. japonicum* egg-induced hepatic granuloma formation and collagen deposition. Further study is required to determine the effect of macrophage depletion on the granulomatosis and collagen deposition during the resolution phase.

In chronic stage, schistosomes down-regulate host immune response, which promotes their survival as well as limits the pathological changes in hosts [40,41]. A mixed Th1/Th2 response or slightly biased Th1 response appear to be beneficial by minimizing fibrosis and protecting the host against intestinal and hepatic damage during chronic *S. mansoni* infection [19]. IL-13 is a potential therapeutic target for various diseases, such as asthma and ulcerative colitis [42]. IL-13 can also directly induces expression of collagen I and other critical fibrosis-associated genes, e,g, α-SMA and connective tissue growth factor, in hepatic stellate cells [43–45]. IL-13 binds to a receptor complex of IL-4Rα and two IL-13-binding proteins (IL-13Rα1 and IL-13Rα2). These receptors have different affinities to IL-13, participate in different signaling pathways in different contexts [46]. In general, IL-13Rα1 pairs with IL-4α forming a functional receptor for IL-13 that signals and activates the downstream JAK/Stat6 pathway [47]. In contrast, IL-13Rα2 acts as a decoy receptor and has a short cytoplasmic tail that binds IL-13 with 100-fold higher affinity than IL-13Rα1, which inhibits the biological action of IL-13 [48]. Interestingly, the mice with genetic deletion of IL-4Rα in macrophages die due to severe intestinal and liver pathology during acute *S. mansoni* infection [49]. Intravenous injection of exogenous soluble IL-13Rα2 protein significantly reduces the volume of granulomas in IL-13Rα2 knock-out mice with schistosomiasis [50]. It is interesting to note that IL-13Rα2 gene silencing or IL-13Rα2 signal pathway blockade leads to marked down-regulation of TGF-β1 production and collagen deposition in lung fibrogenesis and allograft fibrosis [21,51]. This is corroborated well with our findings that the expression of IL-13Rα2 was significantly increased in liver macrophages in response to *S. japonicum* cercariae infection [22]. The protection against hepatic

granulomatosis and collagen deposition by GdCl$_3$ is associated with reduced expression of IL-13Rα2 in macrophages. These findings suggest that increased expression of IL-13Rα2 in macrophage plays an important role in triggering hepatic fibrogenesis in response to *S. japonicum* cercariae infection. However, it remains unknown if IL-13Rα2 reduction in macrophage decreases TGF-β1 expression. The role of IL-13Rα2 positive macrophages need to be intensively studied. The experiments using mice with macrophage specific knockout of IL-13Rα2 will help to unravel the causal role of macrophage IL-13Rα2 in *S. japonicum* egg-induced liver injury, despite global knockout of IL-13Rα2 aggravates granulomatous inflammation and reduces host survival in schistosomiasis [50].

Acknowledgments

Author Contributions

Conceived and designed the experiments: SZ JS WW. Performed the experiments: SZ QL YX XW WW. Analyzed the data: SZ QL YX XW WW. Wrote the paper: SZ WW.

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