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Original Article

Activation of RIG-I signaling in the early stage of *Paragonimus proliferus* infection causes lung injury via type I immune response in rat

Qing-Qing Wang[1 #], Guo-Zhong Zhou[2 #], Kun-Li Wu[1 #], Yong-Rui Yang[1 #], Hong-Juan Li[1], Jie Ding[1], Xing Liu[1], Chong-Xi Li[1], Lu Zhang[3], Sheng-Hao Li[1], Rui-Xian Zhang[4]

[1] Department of Hepatology 1, The Third People's Hospital of Kunming/Yunnan Clinical Center for Infectious Diseases, Kunming, China
[2] Office of Academic Research, The First People's Hospital of Anning, Anning, China
[3] School of Public Health, Dali University, Dali City, China
[4] Department of Disease Control and Prevention, The First People's Hospital of Yunnan Province / The Affiliated Hospital of Kunming University of Science and Technology, Kunming, China

# Authors contributed equally to this work.

Abstract

Paragonimiasis is a common zoonotic parasitic disease. The retinoic acid-inducible gene I (RIG-I) signaling is very important for the host to recognize invading pathogens (especially viruses and bacteria). However, the role of RIG-I signaling in the early stages of *P. proliferus* infection remains unclear. Therefore, in this study, Sprague–Dawley (SD) rat models with lung damage caused by *P. proliferus* were established. Experimental methods including Enzyme-linked Immuno Sorbent Assay (ELISA), real-time fluorescent quantitative polymerase chain reaction (PCR), western blotting, and hematoxylin and eosin (HE) staining were used to explore the mechanisms of lung injury caused by *P. proliferus*. As a result, the expression of the mRNA and proteins of RIG-I signal-related key target molecules, including RIG-I, tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6), interferon regulatory Factor 7 (IRF7), IPS-1, and downstream C-X-C chemokine ligand 10 (CXCL10), were significantly up-regulated immediately after infection, peaked at 3 or 7 days, and showed a downward trend on after 14 days. The levels of pro-inflammatory cytokines interleukin-1 (IL-1), interferon (IFN)-α, -β, and -γ, which represent type 1 immune response, gradually increased and reached a peak by 14 days, which was consistent with the changes in the degree of inflammatory damage observed under HE staining of lung tissues. In conclusion, RIG-I signaling is activated in the early stage (before 14 days) of *P. proliferus* infection, it is inferred that the lung injury of the host may be related to the activation of RIG-I like signaling to induce type I immune response.

Key words: *Paragonimus proliferus*; paragonimiasis; RIG-I signaling pathway; type I immune response.

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Introduction

Paragonimus is a highly evolved parasite and the causative agent of paragonimiasis, a common zoonotic, endemic, and systemic parasitic infectious disease [1,2]. In most cases, infection by *Paragonimus* leads to severe lung damage. The primary pathogenic mechanism of *Paragonimus* infection is the mechanical destruction of the host tissue, caused by migration by the worms and sensitization to its antigens through metabolic secretions in the host [3]. However, the specific mechanism underlying lung injury caused by *Paragonimus* remains largely unknown.

Our previous studies [4,5] revealed that, in the early stage of *P. proliferus* infection, host immunity was activated by exogenous double-stranded RNA (dsRNA), and a large number of genes or signaling associated with type I immune response, such as interferon, and some innate immune cells, such as NK cells, were regulated. It is well known that dsRNAs or ssRNAs of pathogens often induce type I immune responses through the activation of retinoic acid-inducible gene I (RIG-I)-like receptor (RLRs) signals [6,7]. RLRs, one of the types of pattern recognition receptors (PRRs), can recognize pathogenic invasions and the host's signaling factors in order to activate an immune response against parasites, bacteria, viruses, and fungi [8]. RLRs activate type I immune responses by recognizing viral invasions and activating immune cells to secrete type I interferons and other inflammatory factors [9], such as cytokines and chemokines. Once pathogen invasion is recognized by the host, RLRs bind to IPS1, which subsequently

activates two signal transduction events [10,11]: a) pro-inflammatory cytokine gene activation through NF-κB activation [12] and b) interferon regulatory factor (IRF3 and IRF7) phosphorylation through TBKI activation [12,13]. IPS-1 and TRAF6 are key molecules in RIG-I signaling and are closely related to signal transduction. Inhibition of IPS-1 or TRAF6 can inhibit RIG-I induced nuclear factor kappa-B (NF-κB) activation and reduce the levels of inflammatory cytokines induced by chemical hypoxia [14], and the viral effects of IPS-1 tend to be achieved through the release of type I interferons or the activation of IFN-inducible genes (such as IRF3, IRF7, NF-κB) [15]. Although PRR activation exhibits anti-viral effects [16], it can cause inflammatory damage to infected tissues.

Studies on viral infections have shown that viral replication is more pronounced in IRF3$^{-/-}$ mice, and these mice are more likely to develop diseases caused by viruses; however, in *Toxoplasma gondii* infections, knockout of IRF3 results in resistance towards parasite replication and pathogenicity [17], strongly suggesting that IRF may also play an important role in parasitic infections. IRF7 is also an important regulator of the type I interferon response, which is important for innate immunity against viral infection and is even involved in protection against a variety of pathogens, including parasites such as *Sarcocystis miescheriana* [18]. IFN-γ, CXCL10, and other inflammatory factors or chemokines are also closely related to parasitic infection, inflammation, or immunopathological damage [19]. Activation of RIG-I signaling allows the downstream transcription factors NF-κB and IRF7 to bind to the CXCL10 promoter, resulting in an increase in CXCL10 expression, which is highly correlated with disease severity [11].

However, the role of RIG-I signaling in the early stages of *P. proliferus* infection and its specific molecular mechanisms remain unclear. Thus, in this study, we established animal models of *P. proliferus* infection to detect the mRNA and protein expression levels of key factors in the RIG-I-like receptor signaling pathway in lung tissue. The results of these analyses were coupled with histopathological analysis to explore the specific mechanisms of RIG-I-like receptor signaling in the early stage of *P. proliferus* infection required for immune response activation.

Methodology

Establishment of rat models of P. proliferus infection

Sample collection was conducted at the endemic foci of *P. proliferus* in Jinghong City in Yunnan Province. *P. proliferus* metacercariae were isolated from freshwater crabs. Female Sprague–Dawley (SD) rats weighing 190 – 210 g were purchased from the Experimental Animal Center of Kunming Medical University. The rats in the infected (n = 22) and uninfected control (n = 5) groups (0 days) were housed in separate cages. Rats in the infected group were grouped based on the duration of infection, that is, 3, 7, and 14 days, and the weights of the rats were recorded at these time points. After 7 days of infection (In our previous study [5], by 7 days post infection of *P. proliferus* metacercariae to rat's abdominal wall, the worms had already reached the thorax or lungs), two rats were randomly selected to be anesthetized using chloral hydrate and then dissected; parasitic worms were found in the thorax or lung surfaces, indicating that the rat model of *P. proliferus* infection was successfully prepared.

The rats in each group were sacrificed according to the time of infection and various indicators were assessed. All experiments adhered to procedures consistent with the National Research Council Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Kunming Medical University (NO. KMMU2019047).

Hematoxylin-Eosin staining (HE) staining and semi-quantitative score of alveolitis

HE staining was used to evaluate lung histology in each group. We then quantitatively assessed the severity of lung tissue damage in the rats using a semi-quantitative score of alveolitis of the HE-stained slices [4]: 0 for no alveolitis and 1, 2, and 3 for mild, moderate, and severe alveolar inflammation, respectively.

ELISA Determination

The serum samples were obtained from peripheral blood after centrifugation at 3000 rpm for 10 minutes and stored at -20 °C for IL-1, -4, -5, and -13, IFN-α, -β and -γ determination by Dot Blot-ELISA.

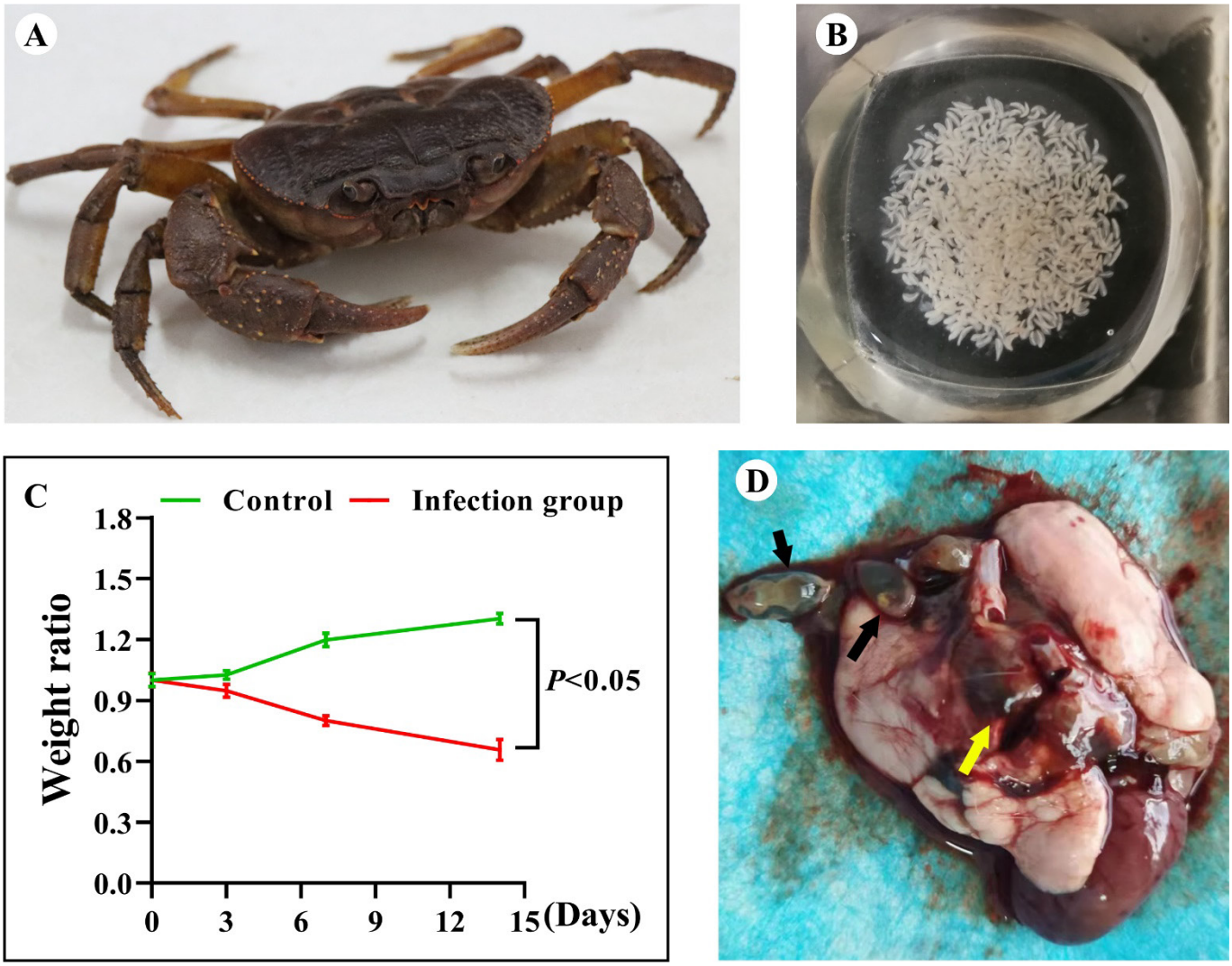
RNA extraction and quantitative real-time polymerase chain reaction

Quantitative real-time PCR (qPCR) was performed using SYBR Green Master Mix (TIANGEN, Beijing, China). Whole RNA was extracted with TRIzol™ Reagent (TIANGEN, Beijing, China) and quantitated by a UV spectrophotometer (Shimadzu, Shanghai, China).

Table 1. Sequences and other relevant information of primers.

	Sequence (5'->3')	Template strand	GC (%)	Length (bp)
RIG-I	Forward primer	TGTGCTCCTACTGGTTGTGG	55	20
	Reverse primer	CACATTATCAGCCGTTGCCC	55	20
	Product length	210 bp		
IPS-1	Forward primer	CAACCTATGGACCTGTGTCTCC	54.55	22
	Reverse primer	AAGTGGTCTTGGCAGATAGAGC	50	22
	Product length	114		
TRAF6	Forward primer	GCCCATGCCGTATGAAGAGA	55	20
	Reverse primer	CGTGACAGCCAAACACACTG	55	20
	Product length	170		
IRF7	Forward primer	ATACAGCCACCTACTGGACTCT	50	22
	Reverse primer	CATCAGAACTGGGTGTTGAAGC	50	22
	Product length	151		
CXCL10	Forward primer	ATGAACAGACGCTGAGACCC	55	20
	Reverse primer	GATCTCAACATGCGGACAGGA	52.38	21
	Product length	80		
GAPDH	Forward primer	ACATCAAGAAGGTGGTGAAGCA	45.45	22
	Reverse primer	TGGAAGAATGGGAGTTGCTGTT	45.45	22
	Product length	111		

Figure 1. A: Crab collected from endemic foci of *P. proliferus*; **B:** Metacercariae of *P. proliferus* isolated from crabs; **C:** Rat weight ratio change in control and *P. proliferus* infection group; **D:** Rat lung infected with *P. proliferus* after 14 days. Yellow clip: the cyst formed by *P. proliferus* in the lungs; Black clip: parasite slides out of the capsule after opening the capsule.



RNA was reverse transcribed to cDNA using a reverse transcription kit (TIANGEN, Beijing, China) according to the manufacturer's protocol. qRT-PCR was used to evaluate the gene expression levels. β-actin expression was used as an internal standard to determine the mRNA levels. The primers, whose sequences and other relevant information are shown in Table 1, were synthesized by Sangon Biotech in Shanghai.

Protein extraction and immunoblotting

Lung tissues were lysed using RIPA buffer and protein concentrations were determined using the BCA assay kit. Total protein (40 μg) was separated using SDS-PAGE and electrotransferred onto a nitrocellulose (NC) membrane. The NC membrane was then blocked with 5% nonfat skim milk and anti-RIG-I, anti-actin, anti-TRAF6, anti-IPS-1, and anti-IRF7 antibodies at 4 °C overnight. Membranes were then incubated with anti-rabbit or anti-mouse IgG secondary antibodies.

Data analysis

Each experiment was repeated thrice. Representative examples are One-way analysis of variance (ANOVA) (SPSS Version 25.0) was used to determine the significant differences between the treatment groups; $p < 0.05$ was considered statistically significant.

Results

Collection of stream crabs and isolation of metacercariae of P. proliferus

Freshwater crabs (Figure 1A) collected from natural foci were brought back to the laboratory, and *P. proliferus* metacercariae (Figure 1B) were successfully isolated. The isolated worms were all excysted cercariae, however, no encysted cercariae can be recovered from tissues of dissected crabs; the metacercariae had body lengths of 1-3 mm, body widths of 0.5-1 mm, and were highly refractile, and the worms were very active, stretching or writhing constantly. The above features were in accordance with the typical characteristics of the metacercariae of *P. proliferus*.

Rat body weight change and gross visual observation of lung tissues and parasite worms

We tracked the changes in the weights of the rats, and the results are shown in Figure 1C. Uninfected rats (0 days) gained weight gradually over the duration of the infection, while infected rats did not gain weight until 14 days after infection. Weight between the two groups reached a statistically significant difference at 14 days.

At 3 days after infection, the thorax and lungs of the rats appeared normal, no inflammation or hemorrhage was observed, no parasitic worms were found, and the parasite had not reached the thorax or lungs at this time point.

However, at 7 days of infection, parasite worms were found on the lung surface, which showed some inflammation, congestion, and even a few hemorrhagic spots.

At 14 days after infection, more areas of the lung surfaces were observed with congestion and hemorrhage, and in severe cases, even blackening. At this time point, several capsules containing parasitic worms had formed in the lungs of rats. The walls of these capsules were still thin, soft, and easily cut open. Following the incision of these capsules, a large amount of black fluid with one or two parasitic worms emerged from the incision (Figure 1D). To the naked eye, the body of the parasitic worm body appeared light grey, translucent, with a half-elliptical peanut shape with tips at both ends, with black intestinal branches visibly distributed on both sides of the body of the worms. The worms appeared lethargic and barely crawled or squirmed.

HE staining of lung tissues

HE staining was performed to assess the morphology, structure, and pathology of the lung tissues from SD rats infected with *P. proliferus* (Figure 2A–D). As with uninfected rats (0 days), the lung tissues of the 3 days infected rats were normal with no inflammatory injury; however, by 7 days there was mild inflammation and bleeding, with slight inflammatory cell infiltration in the lung tissues. At 14 days after *P. proliferus* infection, inflammation of the lung tissues was significantly aggravated, the alveolar wall was thickened, a large number of inflammatory cells were observed, the alveolar cavity shrank, and some collapsed. The semi-quantitative scores at 14 days were significantly higher than those at 0, 3, or 7 days (Figure 2E).

Dynamic expression of mRNA and proteins of the key target molecules in RIG-I signaling

Furthermore, to explore the role and molecular mechanism of RIG-I signaling in lung injury caused by *P. proliferus* infection, we quantified the mRNA (Figure 3) and protein (Figure 4) levels of RIG-I/IPS-1/TRAF6/IRF7 signal-related factors in the lung tissues at different time points (0, 3, 7, and 14 days).

Figure 2. A–D: HE stained pulmonary tissue sections from *P. proliferus* infected Sprague–Dawley rats on 0, 3, 7, and 14 days; **E:** Semi-quantitative score of alveolitis at each time point.

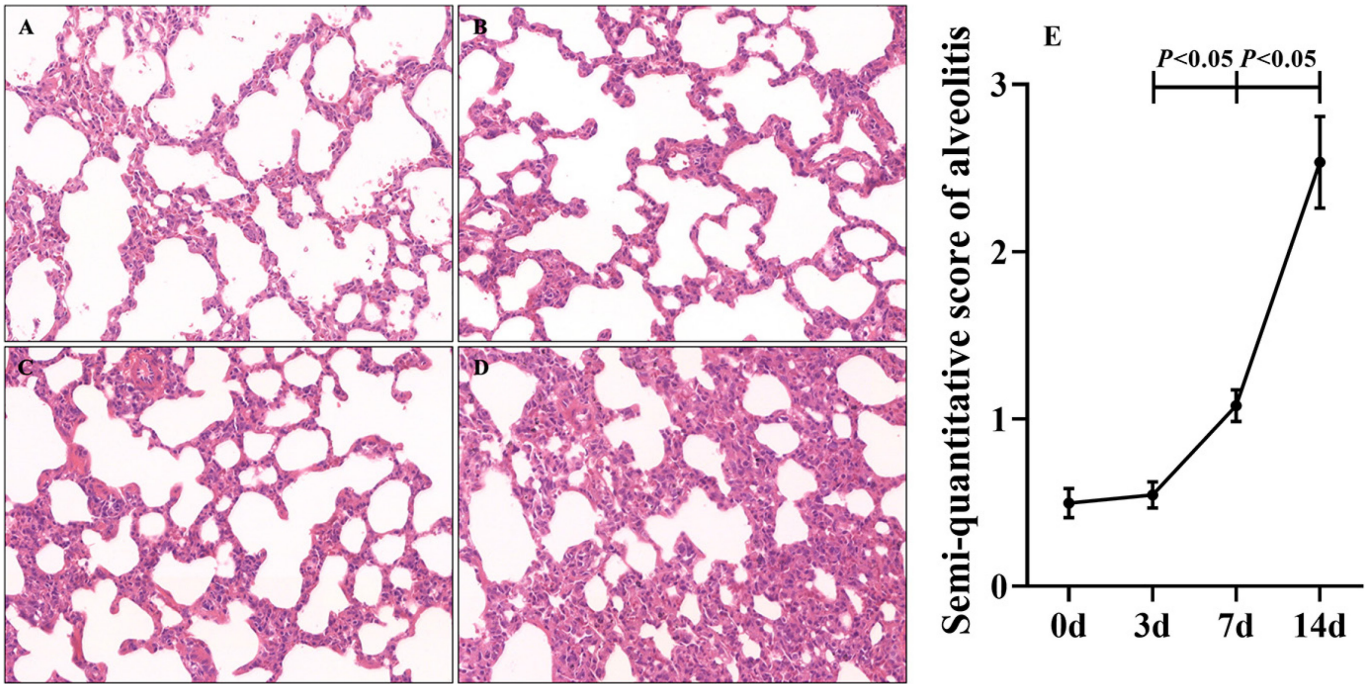
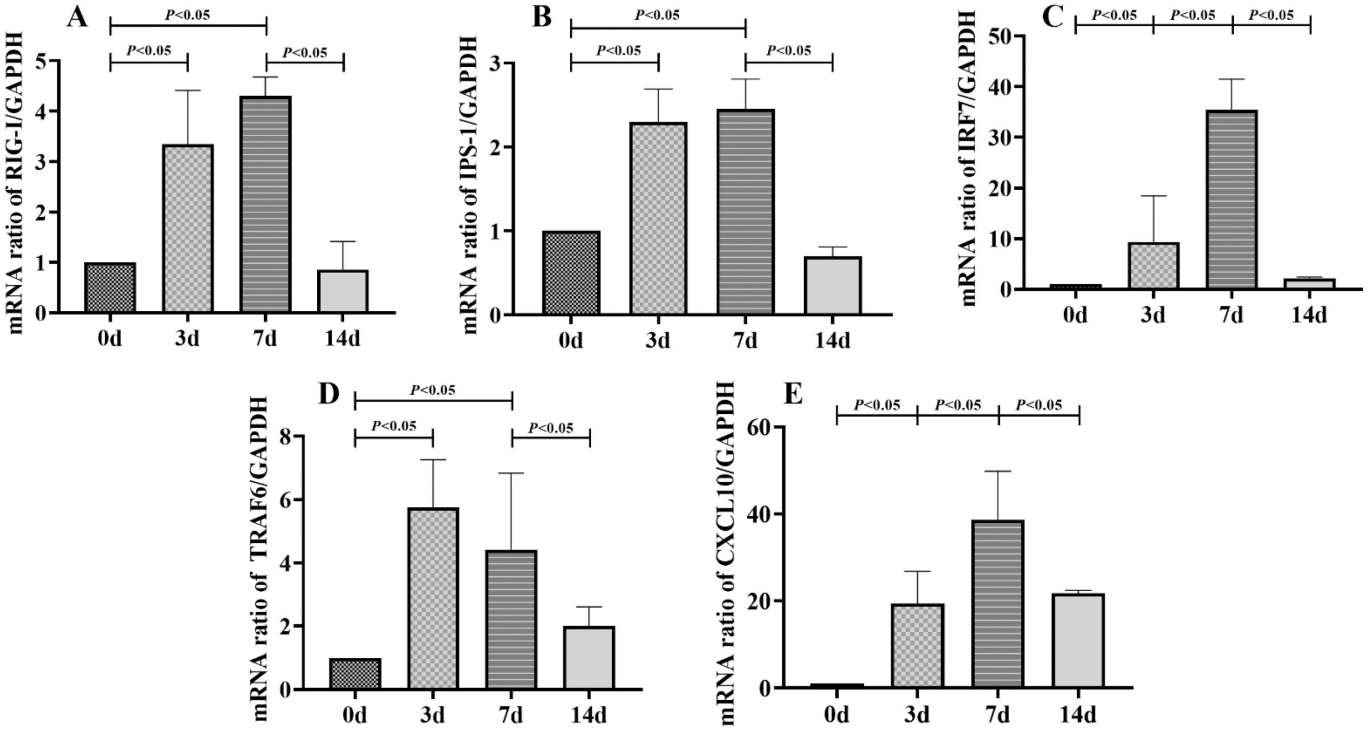


Figure 3. The mRNA expression of factors related to RIG-I/IPS-1 signaling pathway in lung tissues of *P. proliferus* infected Sprague–Dawley rats during the early and middle stages of infection (0 to 14 days).



As per the qPCR outcomes, RIG-I, IPS-1, IRF7, and TRAF6 were rapidly upregulated, peaked at 3 or 7 days, and returned to uninfected levels by 14 days. Meanwhile, the dynamic expression patterns of their proteins were highly consistent with those of qPCR, namely, protein expressions of RIG-I, IPS-1, IRF7, and TRAF6 were also rapidly upregulated, peaked on 3 or 7 days, and downregulated back to uninfected levels by 14 days.

These results indicate that the RIG-I receptor is activated in the early stages of *P. proliferus* infection in SD rats, which in turn activates downstream molecules. Notably, the activation of RIG-I signaling occurs even before the parasite reaches the thorax or lungs of rats (3 days). We speculated that this signaling pathway might play a crucial role in the inflammatory response during *P. proliferus* infection.

Dynamic expression patterns of mRNA and proteins of downstream CXCL10

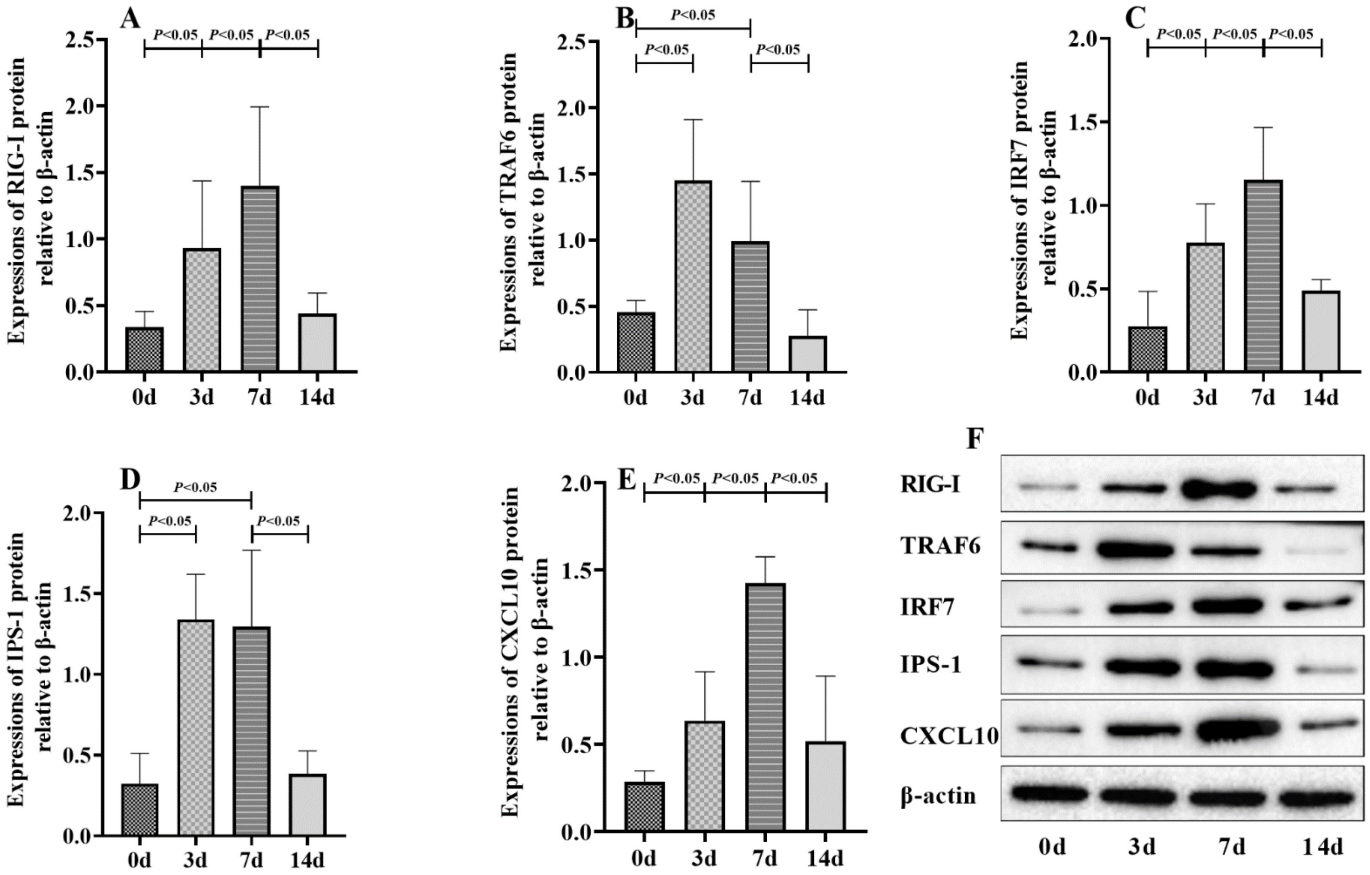
CXCL10 is an important downstream effector after activation of RIG-I signaling. To assess whether the activation of RIG-I signals produced chemotactic effects at the site of infection, we examined CXCL10 mRNA and protein expression in the lung tissues. As expected, expression of CXCL10 mRNA (Figure 3E) and proteins (Figure 4E and F) were rapidly upregulated after infection by *P. proliferus*, peaked at 3 or 7 days, and 14 days, and rapidly reduced to the levels seen at 0 days. Dynamic expression patterns of mRNA and proteins of CXCL10 were also in line with those of the key target factors of RIG-I signaling, such as RIG-I, IPS-1, IRF7, and TRAF6.

This suggests that RIG-I signaling regulates the chemotaxis of downstream CXCL10, further confirming that RIG-I signaling plays an important role in lung injury caused by *P. proliferus* infection.

ELISA Determination

To validate the role of RIG-I/IPS-1 in the inflammatory response, we evaluated the altered serum levels of immune response-related cytokines on 0, 3, 7, and 14 days in *P. proliferus*-infected SD rats. Compared with 0 days, serum pro-inflammatory cytokines related to type I immune response (IFN-α, -β and -γ, and IL-1) increased significantly in the *P. proliferus* infected SD

Figure 4. Altered protein expression levels of factors related to the RIG-I/IPS-1 signaling pathway in lung tissue of *P. proliferus* infected Sprague–Dawley rats during the early stage of infection (0 to 14 days).



rats, peaked on 7 or 14 days (Figure 5A–D). However, the levels of type II immune response-related cytokines (IL-4, 5, 13) did not significantly increase from 0 to 14 days (Figure 5E – G).

This indicates that at the early stages of *P. proliferus* infection (0 – 14 days), the type I immune response played a primary role in resisting pathogenic invasion, suggesting that *P. proliferus* infection in rats activates the RIG-I signaling pathway to induce type I interferon production, and activation of the type I immune response plays a crucial role in the elimination of the invading parasites.

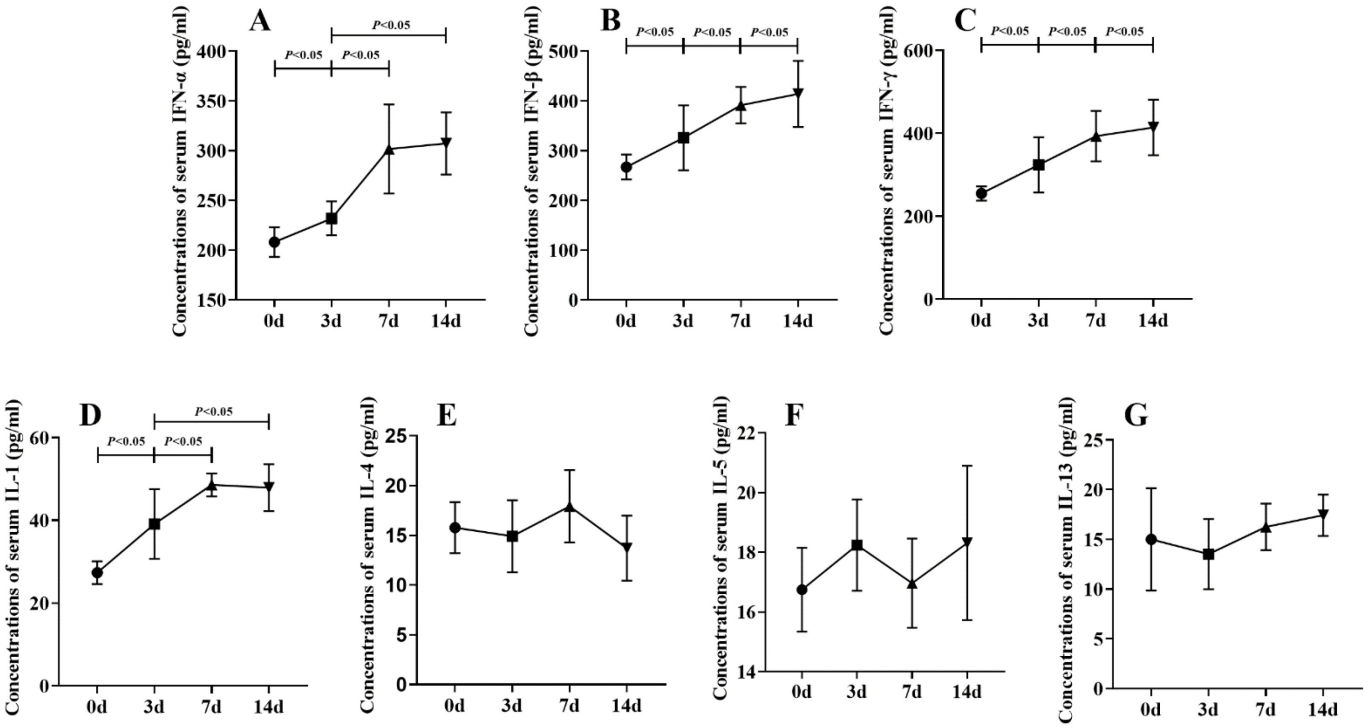
Discussion

Paragonimus mainly causes injuries to the host lungs, and acute inflammation is always the primary pathophysiological manifestation in the early stage of infection, such as signs and symptoms of pleural effusion, pneumonitis, bronchiectasis, or bronchopneumonia [20]. Our previous study found *P. proliferus* only took about 7 days to reach the rat thorax, after which the onset of paragonimiasis began, and the disease had begun to alleviate by about 14 days after infection, according to the transcriptomic cluster analysis, we defined this period (before 14 days) as the early stage of *P. proliferus* infection [4].

RIG-I, one of the primary PRRs [21], has been reported to be closely related to the development of parasitic diseases [22]. In the current study, the mRNA and protein expression of upstream and downstream target molecules of RIG-I signaling pathway (including RIG-I, IPS-1, TRAF6, IRF7, etc.) were up-regulated rapidly after *P. proliferus* infection and peaked on 3~7 days, when the parasites were migrating or just reached the hosts' lung or thorax, strongly suggesting that activation of RIG-I signaling in the early stage of *P. proliferus* infection is closely related to the host initiating an immune response in an attempt to clear the invading pathogen.

RIG-I activation can ultimately activate IRFs and NF-κB, which promote transcription of type I interferons while translocating to the nucleus, TRAFs, including TRAF1~6, are critical in this signaling transduction, and the interferon response will be blocked once the TRAF-binding motifs mutate [6,7,23]. The IRFs have coevolved with NF-κB family, both of them are activated by PRRs or IKKs, and they cooperate extensively in the regulation of target cytokines such as interferon [24]. IRF3 and 7, the two closest family members of IRFs, are key regulators of type I IFN responses and are central to both innate and adaptive immunity [25]. IRF3, constitutively expressed in cells, is critical for the initiation of IFNb and IFNa1 induction [26]. IRF7, with low levels of constitutive expressions in cells, also plays a crucial role in subsequent feedback amplification of both IFN-α and

Figure 5. Expression levels of inflammatory factors in *P. proliferus* -infected Sprague–Dawley rats during days 0, 3, 7, and 14 days.



IFN-β, especially when new IRF7 is synthesized but IRF3 is degraded [25]. Type I IFNs were severely impaired in IRF7$^{-/-}$ mice [27]. Thus, regulation of IRF7 expression is imperative for the production of type I IFNs [28]. In the current study, Type I IFNs products, including IFN-α, -β and -γ, were also rapidly elevated once the hosts were infected with *P. proliferus*, this upward trend continued until 14 days, such an interferon response was possibly caused by activation of RIG-I signaling. However, cytokines (IL-4, -5, and -13) associated with type II immune response did not show a significant increase nor a sustained upward trend. The trend of changes in serum IFN-α, -β and -γ levels were consistent with the severity of lung tissue injuries and the semiquantitative scores of alveolitis, indicating that the host initiates the interferon response in an attempt to clear the foreign invaders while also causing some degree of inflammation or immune damage to itself (lung tissues).

In addition to inducing the release of type I interferon products, activation of RIG-I signaling can also induce the production of the pro-inflammatory cytokine IL-1β [29] by activating NF-κB signaling [30]. In our study, the dynamic change pattern of serum IL-1β level was consistent with that of type I interferon products and semi-quantitative score of alveolitis, that is, the serum IL-1β increased rapidly in rats after infection and reached a peak at 14 days. This further confirms that, in the early stages of *P. proliferus* infection, the host initiated a type I immune response.

Moreover, NF-κB and IRF7, downstream of RIG-I signaling, can bind directly to the CXCL10 promoter, resulting in increased CXCL10 expression [11]. Our study also found, similar to other key target molecules upstream of RIG-I signaling (such as IPS-1, RIG-I, TRAF6, and IRF7), the mRNA and protein expressions of CXCL10 were rapidly upregulated in the early stage of *P. proliferus* infection, reaching a peak at 7 days. CXCL10 can recruit or activate a variety of immune cells, such as Th1, NK, or dendritic cells, as well as macrophages and B lymphocytes [31], among which Th1 cells are also one of the most important cells in the type I immune response. Therefore, the expression pattern of CXCL10 in this study confirms the important role of RIG-I signaling activation in lung injury caused by *P. proliferus* infection.

In a word, intervention of RIG-I signaling at an early stage may be one of the effective strategies for alleviating lung injury caused by *P. proliferus* infection, this work lays a theoretical and experimental foundation for the further exploration of strategies to control disease progression in the early stage of *P. proliferus* infection. However, we still need some experiments in vitro or by using inhibitors, agonists, or small molecule intervention techniques (such as gene knockout or knockdown) to further explore the immunological mechanism in the early stage of *P. proliferus* infection, which can make this work more conducive to the discovery of therapeutic candidate targets to *P. proliferus* paragonimiasis.

Conclusions

RIG-I signaling is activated in the early stage (before 14 days) of *P. proliferus* infection, meanwhile, levels of serum type I immune response-related products (such as IFN-α, -β, -γ, and IL-1) elevate rapidly, accompanied by a simultaneous inflammation or immune damage of lungs. We infer that the lung injury of the host in the early stage of *P. roliferus* infection may be related to the activation of RIG-I like signaling to induce type I immune response.

Funding

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Authors' contributions

Sheng-hao LI and Rui-xian ZHANG contributed to the study concepts and design, Jie DING and Kun-li WU performed the experiment studies, Yong-rui YANG performed the data acquisition, Hong-juan LI, Xing LIU, and Chong-xi LI contributed significantly to data, analysis. Qing-qing WANG and Lu ZHANG performed the manuscript preparation, Guo-zhong ZHOU contributed to the manuscript editing and literature research.

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Corresponding authors

Sheng-hao LI, PhD
No. 319 Wujing Road, Guandu District,
Kunming 650041, Yunnan, China
Tel: +8613698705323
Email: doctorlee3h@163.com

Rui-xian ZHANG, PhD
No. 157 Jinbi Road, Xishan District,
Kunming 650032, Yunnan, China
Tel: +8613987198917
Email: zhangrx2005@163.com

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