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Original Article

Influence of type 2 macrophages (M2) in echinococcosis

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Abstract: Echinococcosis is a zoonotic parasitic disease of human and mammalians mainly caused by the larval stages of Echinococcus. To protect the integrity of the host, macrophages as a nonspecific immune response cells and the antigen presenting cell of specific immune response play an important role in the development of hydatid diseases. In the processing of defense, macrophages polarize gradually into two different subtypes, which suggested that there was a close bond between macrophages polarization and the outcomes of different diseases. Characterized by expressing $CD206^+$, $CD68^+$, $CDl63^+$, $FR\beta^+$, IL-10 and Arg-1, M2 had a weak ability of presenting, which reduced the specificity immune response. Meantime low expression level of IL-12 and high expression level of Arg-1 and IL-10 may induce Echinococcus escape of immune system. In this research, immunohistochemistry and RT-PCR would be used to investigate the M2 relative cytokines of mice infected with Echinococcosis in different stages. The date suggested that M2 appeared a peak in the late stage, which indicated that it was probably a way to immune escape.

Keywords: M2, macrophage, polarization, echinococcosis

Introduction

Echinococcosis (hydatid diseases) is a zoonotic parasitic disease of human and mammalians mainly caused by the larval stages of Echinococcus. Cystic echinococcosis (CE) and alveolar echinococcosis (AE) respectively caused by Echinococcus granulosus and E. multilocularis [1]. Many immune regulations were involved in Cysticechinococcosis (CE) Macrophage as a nonspecific immune response cell and the antigen presenting cell of specific immune response play an important role in the development of disease. According to arginine metabolic mechanism, Th cells presented different antigenic types and the inherent attributes of inflammatory reactions, macrophages could be divided into two different types, M1 and M2 [2]. Characterized by expressing $CD16/32^+$, $CD68^+$, $CD86^+$, MHC-II, IL-12 and iNOS, M1 activated Th1 cell to make anti-inflammatory effects. Characterized by expressing $CD206^+$, $CD68^+$, $CDl63^+$, $FR\beta^+$, IL-10 and Arg-1, M2 had a weak ability of presenting, which reduced the specificity immune response. By lowing expression level of IL-12 and increasing expression level of Arg-1 and IL-10, M2 inhibited Th1 immune response and repaired the injured tissues, grew blood vessel, decreased inflammation, meantime M2 also involved in pathophysiologic processes such as immune tolerance and damage repair [3]. Studies had shown that the type of macrophages polarization have an influence on the outcome of disease, however the mechanism of M2 in Cystic Echinococcosis was not clear. Therefore, this research would investigate the role in the process of Cystic Echinococcosis via the detection of M2 changes in infected mice in different stages.

Materials and methods

Animal and echinococess granulosus

6-8 week-old, 16-22 g, female BLAB/C mice were purchased from The Center of Experimental Animals of Xinjiang Medical University (Urumqi,

Table 1. Primer and length of fragment

Primer	Gene sequence	Temperature (°C)	Fragment length (bp)
GADPH	F: 5'AACTTTGGCATTGTGGAAGG3'	60	222
	R: 5'CACATTGGGGGTAGGAACAC3'		
Arg-1	F: 5'CAGAAGAATGGAAGAGTCAG3'	60	233
	F: 5'CAGATATGCAGGGAGTCACC3'		
IL-10	F: 5'TGCTATGCTGCCTGCTCTTA3'	60	243
	F: 5'TCATTTCCGATAAGGCTTGG3'		

China). Echinococcsis granuulosus was provided by the infected sheep from slaughterhouse.

Main reagent

Trizol was purchased from Invitrogen, etc. RevertAid™ First strand cDNA Synthesis Kit was purchased from Thermo Fisher scientific Inc. 2×Taq PCR Master Mix was purchased from Invitrogen. Maxima SYBR Green/ROX qPCR Master Mix was purchased from Invitrogen. Rabbit anti Arg-1 antibody were purchased from Beijing Boosen Biotechnology Co. Rabbit anti IL-10 antibody were purchased from Beijing Boosen Biotechnology Co.

Establishment of animal model and the collection of samples infected with Echinococcus granulosus

Collected the fresh sheep liver infected with Echinococcus granulosus. Capsule fluid was extracted using Sterile syringe and transferred to 50 ml sterile centrifuge tube, protoscolex was separated by static precipitation, Rinse several times using 0.9% normal saline. Counting of Coloring rate and morphology through 0.5% Red Dyes, Protoscolices of survival rate of >90%. The injection for experimental mice was prepared with 0.9% normal saline which contained 500 U/ml penicillin, streptomycin 100 U/ml and 2000 protoscolex/ml suspension. Each mouse in the experimental group was injected with 0.2 ml of the above suspension, the control group was injected with equal volume of 0.9% normal saline per mouse.

Grouping

Mice were randomly divided into 7 experimental groups and 7 control groups, 7 mice per group. Experimental contained 1-month-group (1 m), 2-month-group (2 m), 3-month-group (3 m), 5-month-group (5 m), 6-month-group (6 m) and 10-month-group (10 m). The control groups were injected 0.9% normal saline (saline group) and the experimental groups were injected Echinococcus granulosus (group infected with Echinococcus granulosus) and sacrificed at different infected stages (at 1, 2, 3, 5, 6 and 10 months after infection). The mice were killed by cervical dislocation and collected the liver and spleen of the mice. Part of the liver was routinely placed 10% formaldehyde solution for more than 24 hours for HE staining and ELISA measurement. The remaining liver was immediately frozen in liquid nitrogen and kept in -80°C refrigerators for real-time fluorescence quantitative PCR (QRT-PCR).

Hematoxylin and eosin staining (H&E staining)

The liver samples of experimental mice were preserved in 4% paraformaldehyde for 48 hours at room temperature. Dehydrated and embedded in paraffin following routine methods. The paraffin sections were removed paraffin, and then immersed in the distilled water following routine methods. Rinsing the paraffin sections (3×5 min) with PBS (0.01M PBS pH 7.4: KH_2PO_4 0.02%, N_2HPO_4 0.29%, KCl 0.02%, 0.8% NaCl). Staining for 8 minutes with hematoxylin and eosin.

Immunohistochemistry

Gradient dehydration of paraffin sections. Blocked with 3% peroxide-methanol at room temperature for endogenous peroxidase ablation for 15 minutes. Incubate overnight at 4°C with the appropriate antibody and biotinylated secondary antibody for 30 minutes. Following manual of the two step immunohistochemical kit, DAB color was performed, under the high magnification. Counterstain for 1 minute with hematoxylin. Randomly counted positive cells were of five visual images.

Quantitative real-time PCR (qRT-PCR)

The gene sequence of Arg-1, IL-10 and GAPDH came from PubMed Genebank and primers were designed by Yingjie Bio Co. Extracted total RNA using Trizol, RNA was reversed to cDNA by reverse transcription kit. Fluorescence dye method was used for qRT-PCR, SYBR Green/ROX qPCR Master Mix real-time fluorescence

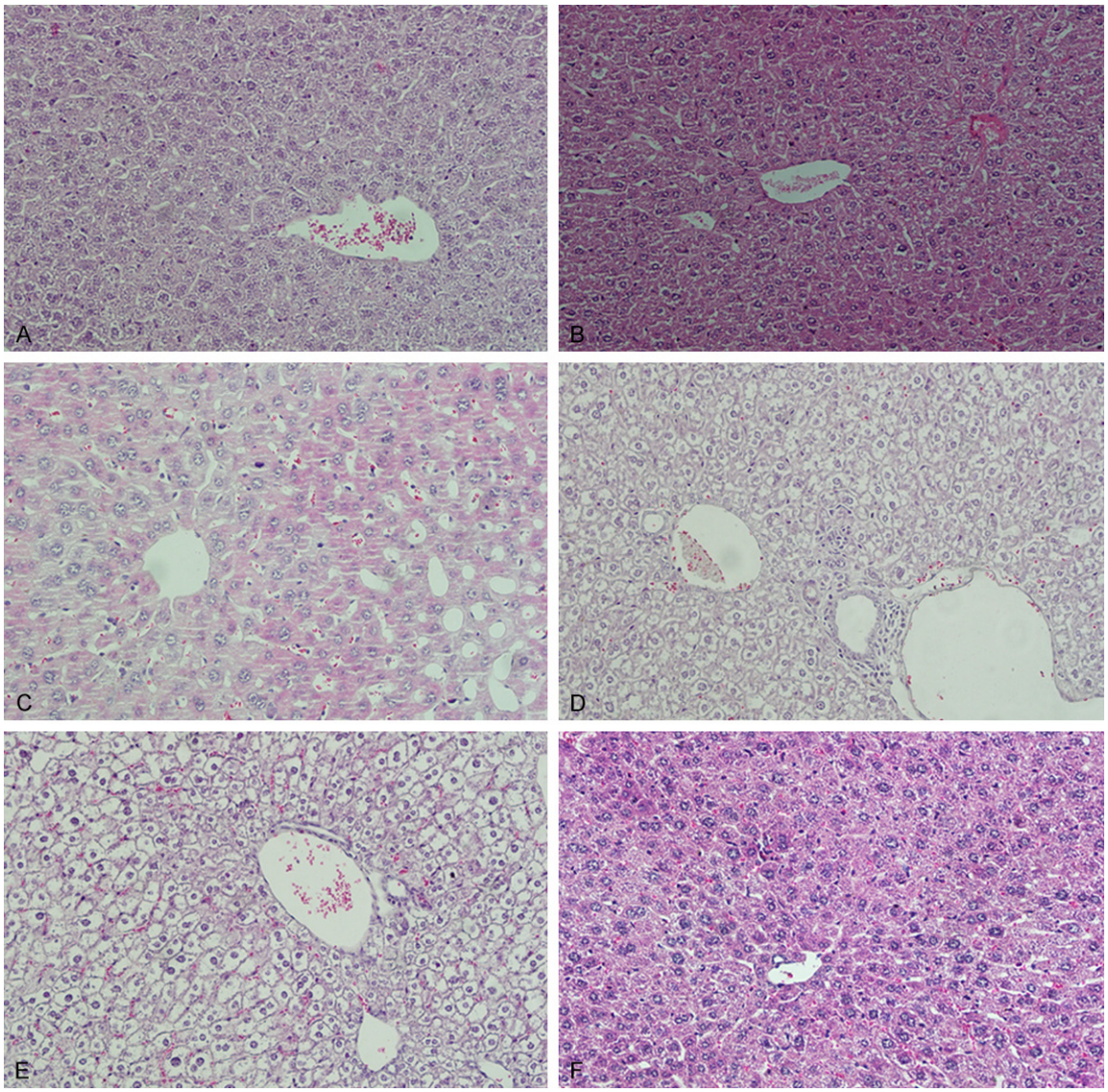

Figure 1. The H&E staining result of infected mice in different periods. (A) Control group, (B) After 1 month infected, (C) After 2 months infected, (D) After 3 months infected, (E) After 5 months infected, (F) After 10 months infected.

quantitative PCR was used for quantitative analysis of Arg-1, IL-10 and GAPDH mRNA objective gene. The primer details were shown in **Table 1**. Reactions were performed using 2 μl of cDNA in a 20 μl reaction volume and the following thermal cycle profile: 10 minutes of denaturation at 95°C, 40 cycles of denaturation at 95°C for 15 seconds and then 60 seconds of extension at 60°C. PCR amplification of GADPH was performed to allow normalization between samples.

Statistical analysis

The experimental data were statistically analyzed by SPSS17.0 software, Data were represented by mean ± SE. ($\bar{x}$ ± s). Date was compared using 1-way ANOVA with Tukey-Kramer multiple comparisons and the group t-test. Significant difference was set at $P<0.05$.

Results

Pathological changes

There were no changes in the mice in the control groups. Compared to control group, 1 month after infection with Echinococcus granulosus, hepatocyte appeared edema gradually and inflammatory cells invasion, the cellular structure was disordered. After 2 months infection, hepatocyte edema got worse compared to

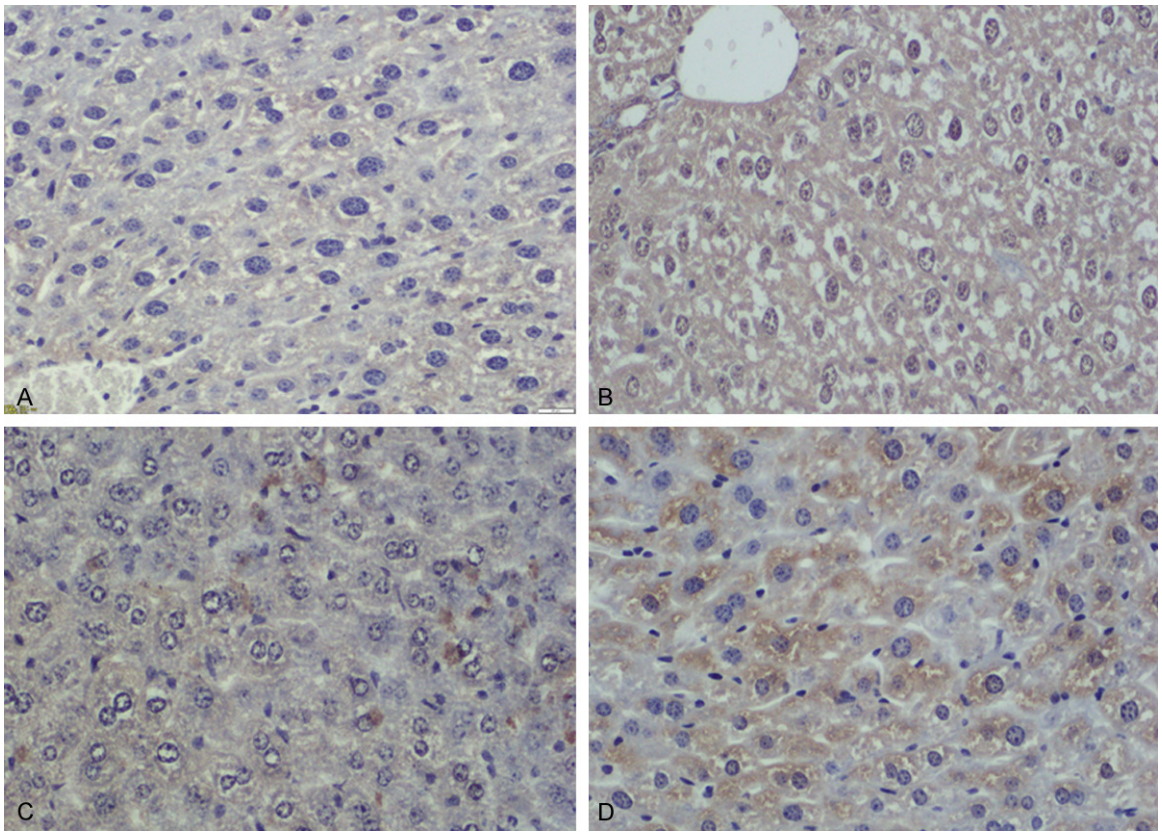

Figure 2. The Immunohistochemistry expression of Arg-1 in infected mice. (A) Control group, (B) After 1 month infected, (C) After 5 months infected, (D) After 10 months infected.

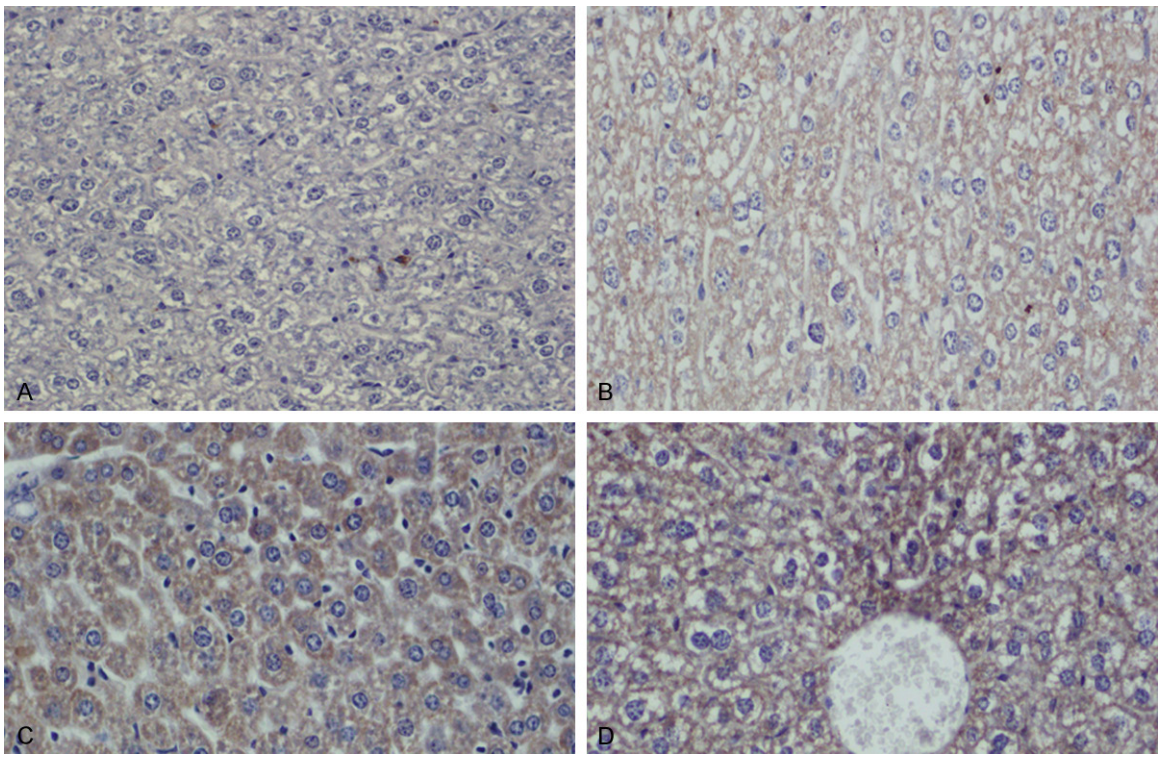

Figure 3. The immunohistochemistry expression of IL-10 in infected mice. (A) Control group, (B) After 1 month infected, (C) After 5 months infected, (D) After 10 months infected.

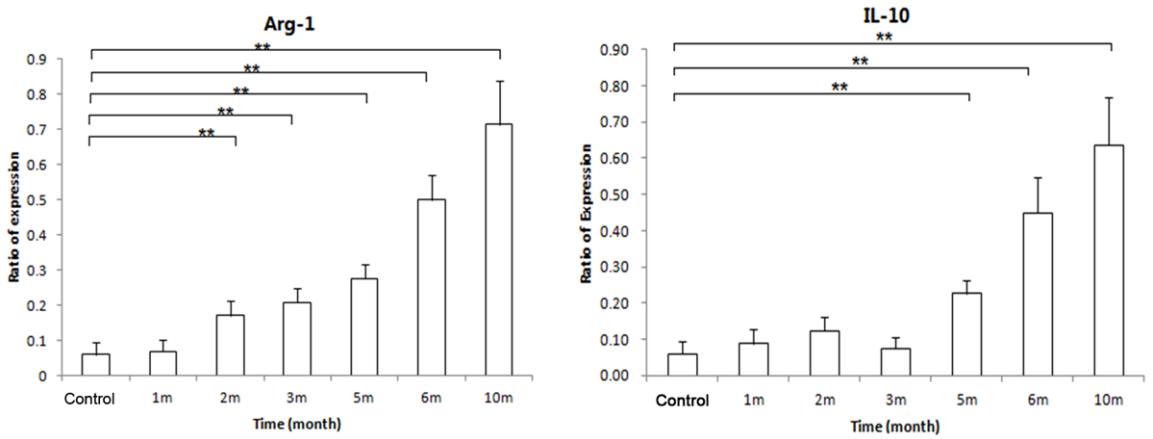

Figure 4. The change of Arg-1 and IL-10 in immunohistochemistry.

Table 2. The immunohistochemistry expression of Arg-1and IL-10 ($\bar{x} \pm s$)

Time (month)	N	Relative expression of IHC	
		Arg-1	IL-10
Control	7	0.06±0.04	0.06±0.04
1 m	7	0.07±0.03	0.09±0.04
2 m	7	0.17±0.04	0.12±0.04
3 m	7	0.21±0.04	0.07±0.03
5 m	7	0.28±0.04	0.23±0.04
6 m	7	0.50±0.07	0.45±0.10
10 m	7	0.70±0.12	0.64±0.13

Table 3. The amount of mRNA expression of Arg-1 in infected mice by QRT-PCR ($\bar{x} \pm s$)

Time (month)	N	Relative expression of mRNA	
		Arg-1	IL-10
Control group	10	1.00±0.06	1.00±0.10
1 m	10	0.99±0.13	1.41±0.14
2 m	10	1.19±0.13	1.57±0.21
3 m	10	1.54±0.05	2.27±0.21
5 m	10	2.65±0.12	2.95±0.42
6 m	10	5.05±0.51	4.53±0.46
10 m	10	9.51±0.70	8.46±0.61

1 month injection. After 3 months infection, hepatocytes of the biliary duct around hepatic lobule had presented hyperplasia and necrosis, meanwhile presented liver cells proliferation. 5 months after infection, hepatocyte presented vacuolar degeneration and hard to form hepatic lobule. 6 months later after, hepatocyte could be formed Fibrous capsule wall of Echinococcus granulosus, fibroblast cells started to proliferate and infiltration of inflammatory cells around the capsule wall, pseudolobuli appeared in structural damage of hepatic. After 10 months infection, hepatic portal area diffused fibrous hyperplasia, fibrosis, and fiber tissue around the liver cells. Necrosis of liver tissue and Echinococcus granulosus cyst wall could be found, the cellar structure disappeared while the lymphocyte infiltrating (**Figure 1**).

Immunohistochemical expression of Arg-1 in infected mice

Arg-1 expression level was lower in the nucleus of the hepatocytes of mice without infection with Echinococcus granulosus. Compared to control group, Arg-1 still appeared the low expression level after inoculation for 1 month (t_{1m}=*-0.017,* P_{1m}=*0.069*), however the expression level increased greatly after 2 months (t_{2m}=*-1.117,* P_{2m}=*0.001*). The expression of Arg-1 was mainly in the cytoplasm of the most hepatocytes in the infected mice, the germinal layer of Echinococcus granulosus and fibrous hyperplasia section around the wall. With the time of mice infected with Echinococcus granulosus, a continued increasing in Arg-1 expression, the peak expression level occurred at 10 months after injection (t_{10m}=*-0.646,* P_{10m}=*0.000*) (**Figures 2** and **4**; **Table 2**).

Immunohistochemistry expression of IL-10 in infected mice

IL-10 expression level was lower in the cytoplasm or sinus hepaticus of the hepatocytes in the liver tissues of control group mice. Compared to control group, the same with Arg-1, the IL-10 still at the low expression level after inoculation for 1 month, and 2 months and 3 months later, the expression started to increase

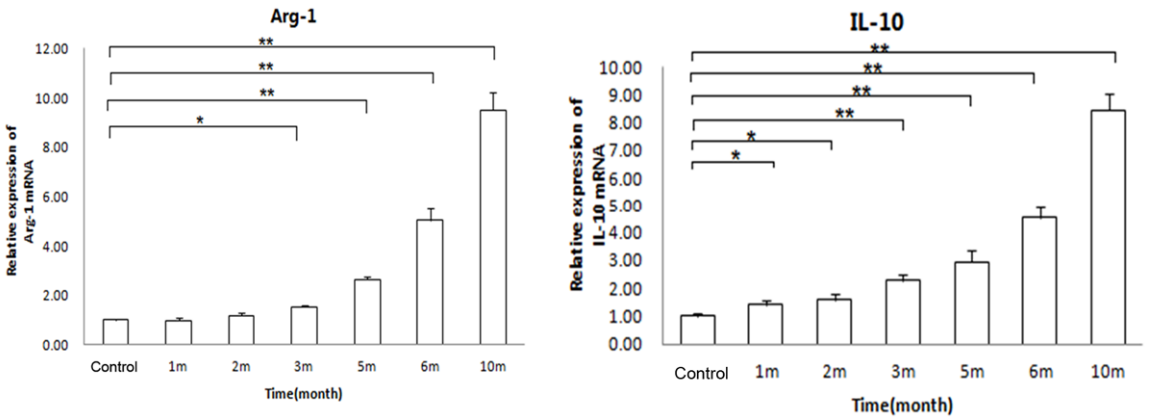

Figure 5. The changes of Arg-1 and IL-10 of RT-PCR.

(t_{1m}=-0.0290, P_{1m}=0.443, t_{2m}=-0.066, P_{2m}=0.082; t_{3m}=-0.013, P_{3m}=0.729) and after 5 months injection, IL-10 increased significantly (t_{5m}=-0.170, P_{5m}=0.000, t_{6m}=-0.390, P_{6m}=0.000; t_{10m}=-0.587, P_{10m}=0.000) (**Figures 3** and **4**; **Table 2**).

mRNA expression of Arg-1: Compared to the control group, the mRNA expression of Arg-1 in the liver was not significantly changed at 1 month and 2 months after infection (t_{1m}=0.009, P_{1m}=0.962; t_{2m}=-0.191, P_{2m}=0.289); After 2 months, Arg-1 was increased continually. The expression of Arg-1 mRNA increased significantly after 10 months infection (t_{10m}=-8.514, P_{10m}=0.000); (**Table 3**; **Figure 5**).

mRNA expression of IL-10: Compared to the control group, the expression of IL-10 mRNA in the liver was changed from 1 month after infection and increased continually (t_{1m}=-0.410, P_{1m}=0.036; t_{2m}=-0.570, P_{2m}=0.004; t_{3m}=-1.276, P_{3m}=0.000; t_{5m}=-1.950, P_{2m}=0.000; t_{6m}=-3.539, P_{6m}=0.000; t_{10m}=-7.460, P_{10m}=0.000). The mRNA expression of Arg-1 reached maximal level after 10 months infection (t_{10m}=-7.460, P_{10m}=0.000); (**Table 3**; **Figure 5**).

Discussion

As known, parasitic diseases are associated with many immune regulations. In our study, with the extension of time, parasite escaped from the immune system regulation. The infected mice with Echinococcus granulosus had a heavy burden because of growth and reproduction. Therefore, the immune system transferred to Th2 reaction to avoid the excessive pathological damage.

Macrophages as a nonspecific immune response cells and the antigen presenting cell of specific immune response play an important role in the development of disease. M2 had a weak ability of presenting, which reduced the specificity immune response. Through decreasing expression of IL-12 and increasing expression of Arg-1 and IL-10, M2 could inhibit Th1 immune response, and repaired injured tissue, grew blood vessel, decreased inflammation reaction and involved in pathophysiologic processes such as immune tolerance and damage repair [3]. It happened in many diseases. Yuanling et al. [4] had found that the patients of diffuse large B-cell lymphoma (DLBCL) had a relationship between M2 and the outcome of DLBCL. According to the study of Ohlsson et al. [5], we knew that M2c from patients of systemic vasculitis were more than normal people and Lu J [6] had detected M2c possessed a ability of vessel regeneration and sclerosis reduction.

Arginase is a primordial hydrolytic enzyme responsible for converting arginine to ornithine and urea, widely distributed in the biosphere. Type-1 arginase plays a critical role in the hepatic metabolism as a cardinal component of the urea cycle. Arginine has diverse functions in mammalian physiology and plays an important role in host immunity. Arginine is a key element to product Nitric Oxide (NO) by Nitric Oxide Synthase 2 (iNOS). As a toxicant, NO removes many pathogens. Meanwhile, arginine is the only amino acid substrate for NO production [7]. Therefore, the consumption of arginine as a way of limiting NO production to help many pathogenic organisms survived [8, 9]. In this research, we detected that the level of Arg-1 had a maximal expression after 10

months infection via the RT-PCR and immunohistochemistry measurement.

Interleukin 10 (IL-10) as a multi-function negative regular factor mainly originated from mononuclear macrophages and T helper cell. STAT is a crucial element of M2 macrophages polarization. STAT6 is the key transcription factor in IL-4 and IL-13 mediated M2 polarization. STAT6 results in recruitment of IRF4 and activation of target gene promoters to initiate transcription. Many of the genes associated with mouse M2 macrophages are regulated by STAT6, including Arg-1, macrophage mannose receptor 1 (CD206). STAT3 is the key transcription regulator of IL-10, a major anti-inflammatory mediator. Through the JAK1-mediated activation of STAT3, it repressed pro-inflammatory cytokines, including TNFα, IL-1β, IL-12, and IFNγ. Murine studies of STAT3 knockout in macrophages highlight the anti-inflammatory role of STAT3 [10]. Therefore, STAT3 has an ability of driving M2 polarization in mice to suppress inflammatory cytokine expression. Meanwhile, IL-10 could be the specific production to reflect the expression of M2 [11]. The same result appeared in Specht's research, which found IL-10 secreted from macrophages could weaken the immunity of defeating the filariasis [12].

In conclusion, we observed the related cytokine factors of M2 to reflect the dynamic changes in this research. The results showed that Arg-1 and IL-10 had increased in later period, which had a close bond with escape of immunity. The host could not clean up pathogene and assisted them alive in vivo. However, the inhibitory mechanism of M2 in E. granulosus was not clear yet.

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Disclosure of conflict of interest

None.

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