Monocyte-derived Macrophages: the Supplements of Hepatic Macrophage in *Echinococcus Multilocularis* Infected Mice

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Abstract

Background Alveolar echinococcosis (AE) is a potentially lethal zoonosis caused by the cestode *Echinococcus multilocularis*. The aim of this study is to study the dynamic changes of monocytes, macrophages and related cytokines in animal models of persistent infection of *Echinococcus multilocularis*. To explore the possible connection between them and the persistent infection of *Echinococcus multilocularis*.

Methods An infection model was established by intraperitoneal injection of a protoscolex suspension. The pathological changes of mice liver were observed by HE staining and the score scale of infection was established. The ratio of Ly6C^{hi} and Ly6C^{lo} Monocytes in peripheral blood of mice was detected by flow cytometry. The distribution and expression of CX3CL1, CX3CR1, iNOS, CD163 and CD11b in mouse liver were detected by immunohistochemistry. The mRNA expression levels of TNF-α and Arg1 in mouse liver were detected by qRT-PCR. The expression levels of INF-γ, IL-17, IL-4 and IL-10 in peripheral blood of mice were detected by ELISA.

Results The results of HE staining showed that in the later stages of infection, significant lesions appear in the liver. The results of the infection degree score scale show that with the extension of the infection time, the severity score value increases. The results of flow cytometry showed that the proportion of Ly6C^{hi} monocytes in the peripheral blood of the experimental group mice was decreased after a brief rise, Ly6C^{lo} monocytes decreased first and then increased.. The results of immunohistochemistry showed that the expression of CX3CL1, CX3CR1, CD11b, CD163 and iNOS in the mice liver of the experimental group was increased. The results of qRT-PCR showed that the expression level of TNF-α and Arg1 mRNA in the liver of the experimental group mice were increased. The results of ELISA showed that the expression level of IFN-γ, IL-17, IL-4 and IL-10 increased with the duration of infection.

Conclusions Monocytes as a supplement to hepatic macrophage, it and kupffer cells (KCs) participate in Th1 and Th2 immune responses of the body by differentiating into M1 or M2 at different stages of *Echinococcus multilocularis* infection.

Background

Echinococcosis refers principally to two serious zoonosis caused by *Echinococcus granulosus* and *Echinococcus multilocularis*, Cystic echinococcosis (CE), and Alveolar echinococcosis (AE). Both diseases are transmitted through ingestion of parasite eggs in the feces of the ultimate host, and humans are not necessary intermediate hosts in the life cycles of the two parasites. The life cycle of *Echinococcus multilocularis* takes place between canids as the definitive hosts and their prey, small mammals such as rodents, which act as intermediate hosts. Annually there are estimated more than 16,000 new cases worldwide of AE, with 90% of those occurring in China[1]. In AE patients, 98% of infections occur primarily in the liver, and the mortality rate of untreated or poorly treated patients after diagnosis is about 90% within 10 to 15 years[2].

Macrophages play a leading role in regulating liver homeostasis under physiological and pathological conditions. Resident macrophages of the liver, kupffer cells, represent a unique cell population. Most of the KCs belong to the self-sustaining macrophage cell population, whose origin is not bone marrow[3]. Blood monocyte-derived macrophages are a small fraction of macrophages in the liver. According to different estimates, they account for 5–30% of the total number of liver macrophages[4, 5]. Chemokines are cytokines with chemotaxis functions, which are specifically used to regulate the migration of immune cells into damaged or diseased organs. A variety of chemokines and receptors are expressed on the surface of monocytes and macrophages. These chemokines and receptors are involved in the migration of them. The CX3CL1 / CX3CR1 pathway affects the recruitment of leukocytes to target areas by affecting the expression of cytokines and chemokines in the active phase of the disease. These leukocytes include monocytes and macrophages[6]. kupffer cells and monocyte-derived macrophages play different but crucial roles in the inflammatory response caused by persistent infection of the liver[7]. Different types of macrophages synthesize and secrete cytokines such as TNF- α , IL-1 β , IL-6, IL-17, IL-4, IL-12, IL-18, IL-10 and IFN- γ when they are stimulated. Then they will participate fully in the immune response[3, 8, 9]. The M1 and M2 phenotypes are currently considered two extremes in the type of macrophage function. M1 macrophages are mainly involved in Th1 type immune response, promote inflammatory response, enhance the body's ability to kill pathogens, and are functional cells that are generally understood to play a positive role. M2 macrophages are mainly involved in Th2 type immune response and humoral immunity, which can cause relatively weak lethality to the pathogens in the cell, and at the same time strengthen the ability to remove body residual materials and tissue repair[10, 11].

In this study, we used flow cytometry, immunohistochemistry, qRT-PCR and other techniques to detect the dynamic changes of KCs and monocytes in mice during continuous infection of *Echinococcus multilocularis*. It was proved that monocyte-derived macrophages and KCs secreted Th1 cytokines in the form of M1 to participate in the immune response in the early stage of infection, and secreted Th2 cytokines in the form of M2 to participate in the process of infection in the late stage of infection.

Methods

Mice

SPF grade Balb / c mice, female, 80, 6–8 weeks old, provided by the First Affiliated Hospital of Xinjiang Medical University Experimental Animal Science Research Department. All experimental procedures were conducted under strict guidelines and approval of Institutional Animal Use and Care Committee at Xinjiang Medical University (Approving Number: 20170214-106).

Grouping and establishment of *Echinococcus multilocularis* infection model

80 Balb / c mice are randomly divided into 2 groups. The experimental group has 40 mic, and the control group has 40 mice. Take Balb / c mice that were inoculated intraperitoneally with *Echinococcus multilocularis*, sacrifice the mice by cervical dislocation, remove the vesicles fluid aseptically with a 50 ml syringe and remove the infected tissue. After the tissue is shredded, it was ground in a sterile mortar. The

ground tissue was sifted through a 200-mesh nylon mesh using prepared normal saline solution containing 500 U / ml penicillin and 100 U / ml streptomycin. Putting the filtrate into 50 ml sterile centrifuge tube to make protoscolex deposit. Finally, the sediments were resuspended in PBS to prepare a 2,000 protoscolexes / ml protoscolex suspension. The mice in the experimental group were injected intraperitoneally with 1 ml of protoscolex suspension, and the mice in the control group were injected intraperitoneally with the same amount of saline. They were maintained in an air-conditioned animal room with a 12-hour light/dark cycle, and provided with rodent chow and water.

Specimen collection

Specimens were taken and preserved 2d, 8d, 30d, 90d, 180d after infection respectively. 1000 µl blood was collected in 1.5 ml EP tube by picking eyes' blood. Approximately 500 µl was transferred to a blood collection tube containing EDTA-2K and awaited detections by flow cytometry. The rest was left at room temperature for 1 hour, centrifuged at 4°C, 3000 r / min for 5 min, and the upper serum obtained after centrifugation was transferred to a new EP tube. Label the specimens and freeze them in a refrigerator at -20°C until serological detections. Immediately after the blood collection, the mice were sacrificed by cervical dislocation, and then immersed in 75% alcohol for 5 min. The mice were dissected and observed for intra-abdominal infection and photographed. The livers of the mice were stripped aseptically and photographed. Part of the livers were fixed in 4% paraformaldehyde fixing solution for 7 days, waiting for subsequent histopathological and special staining detections. The remaining part of the liver was placed in a cryopreservation tube and immediately frozen in liquid nitrogen, and then transferred to a -80°C refrigerator for storage, waiting for subsequent qRT-PCR experiments.

Histopathological examination

The Paraformaldehyde-fixed samples were dehydrated in ethanol, cleared with xylene and embedded in paraffin wax. Sections (4 µm thick) were prepared and stained with hematoxylin and eosin.

Flow cytometry

500 µl of blood was collected from mice by tube with EDTA-K2, red cells lysed using RBC lysis buffer (Biolegend, San Diego, USA), the remaining cells stained with antibodies at 4°C for 30 min., and then the cells were washed with 1% BSA wash solution. The cells were suspended in 300 µl of PBS solution and then sorted using a BD LSR II system. FITC Rat Anti-CD11b (Becton, Dickinson and Company) and PerCP-Cy™5.5 Rat Anti-Mouse Ly-6C (Becton, Dickinson and Company) were used. All the data were processed using the FlowJo (Tree Star, Ashland, OR, USA).

Immunohistochemical detection

The sections were routinely dewaxed to water, incubated with 3% hydrogen peroxide at room temperature for 10 min, placed in citrate buffer at 95°C for 15 min, cooled to room temperature. The sections were blocked in goat serum for 30 min and incubated overnight at 4°C with the following primary antibodies: rabbit anti-CX3CL1 (1:500, Bioss, China), rabbit anti-CX3CR1 (1:500, Bioss, China), rabbit anti-iNOS

(1:400, Bioss, China), rabbit anti-CD163 (1:500, Bioss, China) and rabbit anti-CD11b (1:4000, Abcam, USA). After being washed in PBS solution, the sections were incubated with the secondary antibodies for 1 h. The following secondary antibodies were used: goat anti-rabbit IgG HRP conjugate (ZSGB-Bio, China). After being washed in PBS solution, develop color at room temperature with DAB. The nuclei were stained with hematoxylin. Positive area were counted by an unbiased, blinded investigator using ImageJ software.

RNA isolation and qRT-PCR

Total RNA of liver samples was isolated using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions and reverse transcribed using Prime Script RT Master Mix (RR036A, Takara, Japan).Real-time PCR was performed using SYBR Premix Ex Taq II (RR820A, Takara, Japan) in Thermal Cycler Dice™ Real Time System II (BIO-RAD, IQ5, USA). All PCRs were performed at least twice. The comparative method of relative quantification ($2^{-\Delta\Delta Ct}$) was used to calculate the expression level of the target gene normalised to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used for qRT-PCR were listed in Table 1.

Table 1 qRT-PCR using gene primers

Gene	Primers (5'→3')
TNF-α	Forward: CCCCTTTATCGTCTACTCCTC
	Reverse: GCTGGTAGTTTAGCTCCGTTT
Arg1	Forward: CTCCAAGCCAAAGTCCTTAGAG
	Reverse: AGGAGCTGTCATTAGGGACATC
GAPDH	Forward: AACTTTGGCATTGTGGAAGG
	Reverse: CACATTGGGGGTAGGAACAC

ELISA for quantitative detection of INF-γ and other cytokines

50 µl samples (serum) and diluted standards were added into wells, and then, 50 µl of biotin conjugate was added into each well. The plates were covered with adhesive film and incubated at room temperature for 2 h on a microplate shaker. Then, the wells were washed six times with wash buffer. Next, 100 µl of diluted streptavidin-HRP was added to wells and then incubated at room temperature for 1 h. After six washes, 100 µl of TMB substrate solution was added into each well, incubated at room temperature for 30 min, and then stop solution was added. The optical density in each well was measured by microplate reader (Multiskan Spectrum, Thermo Scientific, USA). Cytokine concentrations were calculated by referring to standard curves.

Statistical Analysis

Results were analyzed using SPSS software (version 17). Comparisons between groups were made using chi-square test / one way ANOVA. Differences were considered significant when $P\leq 0.05$.

Results

General condition of mice

The mice of normal control group showed no abnormal changes in the liver and abdominal cavity after dissection. The liver texture was soft, smooth, ruddy in color, no obvious adhesion, the position was normal. After dissecting the mice in the experimental group, it was found that as the infection continued, the liver of the mice gradually hardened, the color gradually faded, and the position gradually deviated from the normal position. At 30d after infection, the vesicles formed by the infection were seen on the liver, then the vesicles gradually increased. The boundary between vesicles and tissue was blurred. The vesicles were also formed in the abdominal cavity, and at the late infection, abdominal cavity was almost full of vesicles (Fig.1, Fig.2).

Histological analysis of the liver tissue

The normal control group's mice in all periods had normal liver tissue structure and complete hepatic lobules. Hepatocytes had clear and complete borders and were arranged neatly. No cell edema, necrosis, and ballooning degeneration of liver cell were seen. After observing the mice in the experimental group, we found that as the infection continued, the hepatocytes gradually developed from initial edema to ballooning degeneration and steatosis. At 8d of infection, the mild inflammation in the portal area began to appear, and it became worse with the extension of the infection time. At the same time, a small number of focal necrosis can be seen in the hepatic lobules. With the extension of infection time, it gradually increased, developed into fusion necrosis or bridging necrosis, and involved many hepatic lobules. In the late stage of infection, a large number of fibroblasts around the lesion proliferated to form a fibrous layer, and a large number of inflammatory cells infiltrated. Normal hepatic lobule structures were no longer visible (Fig.3).

We according to the pathological changes in HE stained hepatic lobules of liver tissue of mice infected with *Echinococcus multilocularis*, lesions in the manifold area, other abnormal structures and substances, combined with chronic hepatitis GS classification, a score scale for the degree of *Echinococcus multilocularis* infection in mice (Table 2). Tissue samples scored according to Table 2. After summing up the scores, the higher the score, the more severe the infection (Fig.4).

Table 2 Liver infection of *Echinococcus multilocularis* severity score (mouse)

Degree of inflammation		Vesicles* number	Abnormal structures and substances	Score
Hepatic lobule	Portal area and surrounding			
No inflammation	No inflammation	0	No obvious change	0
Degeneration, small amount spotted and focal necrosis	Mild inflammation (Inflammatory cells occupy less than 1/3 of the portal area)	1-3	Granuloma	1
Degeneration, large amount spotted and focal necrosis		4-6		2
Confluent necrosis or bridging necrosis	Moderate inflammation (Inflammatory cells occupy 1/3~2/3 of the portal area)	7-9	Laminated layer and germinal layer	3
Extensive bridging of necrosis and involved many hepatic lobules	Severe inflammation (Inflammatory cells occupy more than 2/3 of the portal area)	≥10	Brood capsule and protoscolex	4

* Note: the average number of vesicle counts was observed in at least 3 different fields under low power microscope (magnification, ×100).

Detection results of monocyte subsets in peripheral blood

Ly6C is currently the most ideal marker for distinguishing monocyte subpopulations in mice[12]. Then we used flow cytometry to detect the proportion of monocyte subsets in the peripheral blood of mice in the control and experimental groups in different periods. We found that little change in the proportion of two kinds of monocytes in mice of control group. In the experimental group, the proportion of Ly6C^{hi} type monocytes gradually increased in the early stage of infection, reached a peak at 8 days after infection. Then it began to show a downward trend. At the later stage of infection, it had been significantly lower than the control group at the same time (Fig.5 G). For Ly6C^{lo} type monocytes, its proportion rapidly decreases to a minimum after a brief rise in the early stages of infection. Then its proportion began to rise, and at the later stage of the infection, its proportion was already significantly larger than that of the control group during the same period (Fig.5 H). The above results showed that in the continuous infection of *Echinococcus multilocularis*, the mainly monocyte subsets in peripheral blood transition from Ly6C^{hi} to Ly6C^{lo}.

Immunohistochemical results of liver tissue of *Echinococcus multilocularis* infection mice

Earlier, we have observed changes in the proportion of peripheral blood mononuclear cell subsets in infected mice. So did these cells reach the lesion? Subsequently, we used immunohistochemistry to detect monocyte markers, CD11b, related chemokines and their receptors CX3CL1 and CX3CR1, different types of macrophages markers, iNOS and CD163. The results showed that all indicators were low and stable in the control group. The expression of chemokine CX3CL1 increased with the extension of the infection time. Although the expression level decreased in the late stage of infection, it remained at a high level (Fig.7 A). Most of its expression locations were concentrated in inflammatory cell clusters or around lesions. CX3CR1 had a higher expression level than the control group at the early stage of infection. Although the expression level decreased briefly at 30d after infection, it was still significantly higher than that of the control group in the later period (Fig.7 B). The location of its expression gradually concentrated around the lesion with the extension of the infection time. For CD11b, its expression increased with the extension of infection time (Fig.7 C), and almost all of the expression sites were at the inflammatory cell aggregation site. The expression of iNOS, one of the markers of M1 macrophages, increased with the extension of the infection time, but the expression level in the late stage of infection is no longer than the intermediate stage (Fig.7 D). CD163 is one of the markers of M2 macrophages. And its expression also increased with the extension of infection time. But unlike iNOS, the expression of CD163 in the late stage of infection was the highest during the entire infection process (Fig.6, Fig.7 E). In summary, our results illustrate the following conclusion, under the action of chemokines and their receptors, monocytes expressing CD11b were recruited into the liver and concentrated around the lesion at the late stage of infection. Macrophages around the lesion were predominantly M1 and M2 in the early and late stages of infection, respectively.

Detection of different subsets macrophage related genes

After that, in order to verify the previous conclusions, we carried out the detections of liver macrophage-related gene. The results of qRT-PCR showed that TNF- α , a marker gene of M1 macrophages, began to increase significantly at 30d after infection. Its expression reached its peak at 90d. Although it decreased in the late period, it was still significantly higher than the control group in the same period (Fig.8 A). Arg1, one of the marker genes of M2 macrophages, also began to increase significantly at 30d after infection. But the difference was that with the extension of the infection time, it reached a peak in the late stage of infection (Fig.8 B). The experimental results supported our previous conclusions that macrophages in the liver underwent a continuous transition from M1 to M2 in the continuous infection of *Echinococcus multilocularis*. So, how do these macrophages that have been polarized participate in the mice's immune response at different stages of continuous infection in *Echinococcus multilocularis*?

Results of detection of macrophages related cytokines

In order to explore the immune function of macrophages that have been polarized at different stages of infection, we detected several cytokines associated with different types of macrophages. First, we detected changes in the expression of IFN- γ in peripheral blood. Its expression trend is similar to that of M1-type macrophages (Fig.9 A). Both of them increase with the duration of infection, and begin to decline in the later stages of infection. Then, we detected changes in the expression of IL-17. During the entire infection process, its expression continued to increase with the extension of the infection time, reached a peak in the late stage (Fig.9 B). Finally, we continuously detected two cytokines, IL-4 and IL-10, and their expression trends were similar. When the infection progressed to 90d, their expression reached a peak and gradually decreased in the late stage of infection. The difference was that IL-10 was significantly higher than the control group at 30d after infection, but IL-4 was not (Fig.9 C, D). This is the difference between the two cytokines. Our detect results indicate that in the early stage of persistent infection with *Echinococcus multilocularis* infection, Th1 immune response represented by IFN- γ may play a major and important role. In the late stage of infection, it may be that Th2 cytokines with higher expression are playing a major role. Therefore, no matter in the early or late stage of infection, macrophages of different subsets participate in the mice's immune regulation by synthesizing and secreting relevant cytokines, and play an indispensable role.

Discussion

Echinococcosis is a zoonosis caused by cestodes of the genus *Echinococcus* (family Taeniidae). This serious and near-cosmopolitan disease continues to be a significant public health issue, with western China being the area of highest endemicity for AE[13]. The onset of AE is hidden, with a long course, with only atypical signs such as abdominal distension, abdominal pain, loss of appetite, and weight loss. When *Echinococcus multilocularis* resides in the liver for a certain period of time, it can have a direct effect on the liver. It can cause an immune response to the liver and its surrounding tissues through mechanical compression and continuous overflow of small vesicle fluids. In severe cases can cause hepatic encephalopathy or portal hypertension[14, 15]. Although the outcomes of AE patients include the above, they are not the only ones. In clinically inactive AE patients, the lesions were prone to deposit calcium salts, forming irregular liquefied necrotic lesions, with a milder degree, and the phenomenon of self-healing may occur. In active AE patients, the lesions gradually increase in the liver and spread by invasive growth. Not only are the surrounding tissues easy to be invaded, but also extrahepatic metastases or intra-abdominal vesicular bulbous plantar metastasis can be difficult to cure. As a result, local recurrence or secondary infections often develop. The difference in the host's different immune states is an important factor influencing different outcomes. Therefore, in actual clinical work, the main direction is still to adjust the host's immune status in a timely manner, thereby affecting or even changing the occurrence and development of the disease.

As an important part of the body's immune defense mechanism, the function and phenotype of macrophages are highly heterogeneous, depending on its source and type of polarization. It has been reported that bone marrow-derived macrophages adapt to the needs and burdens of the liver under inflammatory conditions, while kupffer cells, liver macrophages that reside in tissues, adapt to the normal

function of the liver[16]. So, how do monocytes in peripheral blood participate in the immune response? When the liver is damaged, KCs and other liver intrinsic cells will recruit a large number of mature monocyte in the blood circulation to migrate to the damaged site through the action of chemokines and their receptors, and differentiate into different types of macrophages according to different liver microenvironments. For example, Ly6C^{lo} expressing recovery macrophages observed during the resolution of mouse liver fibrosis are derived from Ly6C^{hi} peripheral blood monocytes and have the characteristics of M1 and M2 macrophages[17]. Monocyte-derived macrophages will differentiate into the types that play a positive role in the inflammatory response and aggravate liver damage. At the same time, they will also develop toward a phenotype that promotes tissue repair after liver damage. After selective depletion of KCs with clodronate liposomes or inhibition of monocyte infiltration with CCR2^{-/-} mice, KCs and inflammatory Ly6C^{hi} monocytes were found to cause liver during amoebic liver abscess Destruction of the main effector cells[18]. In inflammatory response syndrome of liver injury caused by African trypanosome infection, Ly6C^{lo} macrophages can inhibit the pro-inflammatory function of Ly6C^{hi} monocytes, and differentiate Ly6C^{hi} monocytes into anti-inflammatory macrophages[19]. But the similar results have not been reported in AE. Therefore, we conducted related research.

In this study, mice were injected intraperitoneally with Protoscolexes to make a model. After the mice were sacrificed, the infection situation in the abdominal cavity of the mice was first observed. We found that with the prolongation of the modeling time, the number and volume of vesicles scattered in the abdominal cavity of mice and the liver increased, the amount of sac fluid increased, the adhesion of vesicles to surrounding tissues gradually increased. Squeezing of giant vesicles disengages the tissue from its normal position and affects its normal function. The results of HE staining showed that with the progress of *Echinococcus multilocularis* infection, the initial focal necrosis in the hepatic lobules gradually developed into fusion or even bridging necrosis. The infiltration of inflammatory cells in the mesial area gradually worsened, and in the late stage of infection, no obvious hepatic lobular structure has been seen, and even part of the lobular structure has disappeared. After that, the infection status of the liver tissues of mice infected with *Echinococcus multilocularis* was scored, and the results showed that the total score increased with the increase of the infection time. However, the scoring criteria developed in this study are semi-quantitative rather than quantitative. The scores represent only the type of certain pathological patterns and are not true measurements. Therefore, different observers use the same scoring criteria can lead to different conclusions because of their experience or differences in prejudice. Therefore a more detailed description of the histological morphology can help improve the scoring criteria to play a more detailed role.

After determining the infection status of the mice, we immediately detected the change in the proportion of monocytes in different subpopulations of peripheral blood by flow cytometry. The results showed that the monocytes in the peripheral blood in the early stage of infection were mainly Ly6C^{hi} type, but in the late stage, they were mainly Ly6C^{lo} type. These monocytes which differentiate into different subpopulations at different times, were affected by chemokines and their receptors and were recruited around the lesions of liver tissue. KCs and these monocytes recruited to the liver mainly exerted their

immune function in the form of M1 macrophages in the early stages of infection, but in the later stages, most of them had differentiated into M2 macrophages involved in subsequent immune responses. But there is an interesting phenomenon in our test results, the expression trends of chemokines and their receptors are not exactly the same. The early expression of CX3CR1 in the liver of the experimental group of mice was scattered, while the late stage was concentrated around the lesions or inflammatory cell clusters. That may indicate that CX3CR1 positive cells were recruited throughout the liver in the early and middle stages of infection, and that in the later stages of infection, these effector cells were concentrated in obvious lesions to exert corresponding immune functions. The expression trends of chemokines and their receptors are not exactly the same, the reason may be that CX3CL1 has two types: soluble and bound. The expression trends of chemokines and their receptors are not exactly the same. The reason may be that the two at different stages of infection is caused by the expression of different positive cells. First, CX3CL1 has two types: soluble and bound. Second, CX3CL1 positive cells include not only monocytes and macrophages, but also myofibroblasts and endothelial cells. However CX3CR1 positive cells in mouse liver are mainly macrophages[20].

The results of immunohistochemistry and qRT-PCR of our detections on macrophages in different subsets indicate that in the early stage of *Echinococcus multilocularis* infection, macrophages in the liver will mostly participate in the development of the disease in the form of M1. In the late stage of infection, the differentiation direction of liver macrophages is mainly M2 type. There are evidences that Ly6C^{hi} monocytes can be differentiated into M1 macrophages and Ly6C^{lo} monocytes can be differentiated into M2 macrophages[21], the results of peripheral blood flow cytometry in this study also support this phenomenon. Therefore, in combination with the previous results of this study, we have reason to believe that in the early stage of *Echinococcus multilocularis* infection, a large number of Ly6C^{hi} monocytes in peripheral blood were recruited to the liver, and together with KCs played an immune role in the form of M1 macrophages. In the late stage of infection, a predominant number of Ly6C^{lo} monocytes were recruited into the liver tissue, and they acted as M2 macrophages together with KCs.

Finally, we preliminary explored how different subsets of macrophages are involved in the entire process of *Echinococcus multilocularis* infection. We selected several cytokines synthesized by macrophages, IFN- γ , IL-17, IL-4, IL-10. IFN- γ was one of the most representative cytokines of Th1 immune response. In contrast, as the most effective antagonist of IFN- γ , IL-4 was also selected by us. Then we added one of the representative factors of Th2 immune response, IL-10. IL-17 was chosen because it was a recently discovered new type of pro-inflammatory cytokine. The change in IFN- γ in the serum of the experimental group mice further proves that during the process of *Echinococcus multilocularis* infection, the body's immune type trend is dominated by the early Th1 type and gradually transitions to the late Th2 type which plays the main function. When the early expression of IFN- γ is increased, KCs can be activated to promote them to inflammatory sites or pathogenic sites, upregulate the secretion of inflammatory molecules, and enhance the inflammatory response[22]. However, when Th1 type immune cells had an excessively strong inflammatory response, a higher concentration of IFN- γ could induce CD4⁺ CD25⁺ T cells to differentiate into CD4⁺ CD25⁺ Tregs, to inhibit the inflammatory response of disorders and reduce

body damage[23]. This may be the reason why IFN- γ is still maintained at a higher concentration in the late stage of infection. Throughout the infection process, IL-17 had maintained an upward trend. In the early stages of infection, it may be due to its synergy with other pro-inflammatory cytokines such as IFN- γ . However, at the late stage of infection, high levels of IL-17 may be closely related to liver fibrosis and cirrhosis that during chronic liver disease[24]. IL-4, as an important anti-inflammatory factor, can down-regulate the effects of IFN- γ on pathogen killing and tissue damage, down-regulate the secretion of Th1 cytokines, inhibit Th1 type immune function, strengthen Th2 cytokine expression, and promote the formation of immune tolerance. This is why high concentrations of IL-4 appear and produce effects in the later stages of infection[25]. As one of the cytokines overexpressed by M2 macrophages, IL-10 also has a higher concentration in the late stage of infection. Although high concentrations of IL-10 helped to suppress inflammatory reactions and repair damaged tissues, they also promoted the formation of immune tolerance in the body and helped pathogens escape the body's immune response[26, 27].

Echinococcus multilocularis infection was a long process, and as the infection gradually became a chronic disease, the treatment method would become single, the disease would easily recur, and it would be difficult to cure completely. Therefore, finding a way to prevent chronic infection will be a feasible idea for treating AE. The immune tolerance mechanism formed by the body at the late stage of infection is closely related to the long-term coexistence of *Echinococcus multilocularis* in the host and causes chronic infection. The imbalance of cellular immunity and humoral immunity caused by the imbalance of Th1 and Th2 is of vital importance role in this progress. Due to the strong plasticity and functional heterogeneity of macrophages, monocyte-derived macrophages and KCs were keeping to participate in the development of Th1 and Th2 immune functions at the same time. However, there were no clear conclusions about specific functions and mechanisms. Just like we don't know what kind of cell mainly produces which kind of cytokine. But we have reasons to believe that in the future, monocyte-derived macrophages to supplement depleted KCs to help to treat AE will be a feasible idea.

Conclusions

In a summary, our research indicate that monocytes as a supplement to hepatic macrophage, it and kupffer cells (KCs) participate in Th1 and Th2 immune responses of the body by differentiating into M1 or M2 at different stages of *Echinococcus multilocularis* infection.

Abbreviations

AE: Alveolar echinococcosis; HE: Hematoxylin-eosin; qRT-PCR: Quantitative real time polymerase chain reaction; TNF- α : Tumor necrosis factor α ; Arg1: Arginase-1; ELISA: Enzyme linked immunosorbent assay; IFN- γ : Interferon- γ ; IL-17: Interleukin-17; IL-4: Interleukin-4; IL-10: Interleukin-10; KCs: Kupffer cells; CE: Cystic echinococcosis;

Declarations

Acknowledgements

Not Applicable

Authors' contributions

Prof. MXM and LL conceived and designed the study. LB and QXW drafted the manuscript, MXM and LB critically revised manuscript. LB, LYM, YY, SJY, CXL, conducted experimental work. QXW, LJ, ZX interpreted data. All the authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All experimental procedures were conducted under strict guidelines and approval of Institutional Animal Use and Care Committee at Xinjiang Medical University (Approving Number: 20170214-106).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Deplazes P, Rinaldi L, Alvarez Rojas CA, Torgerson PR, Harandi MF, Romig T, Antolova D, Schurer JM, Lahmar S, Cringoli G *et al*: Global Distribution of Alveolar and Cystic Echinococcosis. *Adv Parasitol* 2017, 95:315-493.
2. McManus DP, Gray DJ, Zhang W, Yang Y: Diagnosis, treatment, and management of echinococcosis. *BMJ* 2012, 344:e3866.
3. Elchaninov AV, Fatkhudinov TK, Vishnyakova PA, Lokhonina AV, Sukhikh GT: Phenotypical and Functional Polymorphism of Liver Resident Macrophages. *Cells* 2019, 8(9).
4. Chazaud B: Macrophages: supportive cells for tissue repair and regeneration. *Immunobiology* 2014, 219(3):172-178.
5. Nishiyama K, Nakashima H, Ikarashi M, Kinoshita M, Nakashima M, Aosasa S, Seki S, Yamamoto J: Mouse CD11b+Kupffer Cells Recruited from Bone Marrow Accelerate Liver Regeneration after Partial Hepatectomy. *PLoS One* 2015, 10(9):e0136774.
6. Wang L, Sun Z, Liu L, Peng B: Expression of CX3CL1 and its receptor, CX3CR1, in the development of periapical lesions. *Int Endod J* 2014, 47(3):271-279.
7. Zigmond E, Samia-Grinberg S, Pasmanik-Chor M, Brazowski E, Shibolet O, Halpern Z, Varol C: Infiltrating monocyte-derived macrophages and resident kupffer cells display different ontogeny and functions in acute liver injury. *J Immunol* 2014, 193(1):344-353.
8. Isailovic N, Daigo K, Mantovani A, Selmi C: Interleukin-17 and innate immunity in infections and chronic inflammation. *J Autoimmun* 2015, 60:1-11.
9. Wynn TA: Type 2 cytokines: mechanisms and therapeutic strategies. *Nat Rev Immunol* 2015, 15(5):271-282.
10. Murray PJ, Wynn TA: Obstacles and opportunities for understanding macrophage polarization. *J Leukoc Biol* 2011, 89(4):557-563.
11. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdts S, Gordon S, Hamilton JA, Ivashkiv LB, Lawrence T *et al*: Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity* 2014, 41(1):14-20.
12. Mildner A, Marinkovic G, Jung S: Murine Monocytes: Origins, Subsets, Fates, and Functions. *Microbiol Spectr* 2016, 4(5).
13. Wen H, Vuitton L, Tuxun T, Li J, Vuitton DA, Zhang W, McManus DP: Echinococcosis: Advances in the 21st Century. *Clin Microbiol Rev* 2019, 32(2).
14. Vuitton DA, Zhang SL, Yang Y, Godot V, Beurton I, Mantion G, Bresson-Hadni S: Survival strategy of *Echinococcus multilocularis* in the human host. *Parasitol Int* 2006, 55 Suppl:S51-55.
15. Vuitton DA: The ambiguous role of immunity in echinococcosis: protection of the host or of the parasite? *Acta Trop* 2003, 85(2):119-132.
16. Guillemins M, Scott CL: Does niche competition determine the origin of tissue-resident macrophages? *Nat Rev Immunol* 2017, 17(7):451-460.

17. Ramachandran P, Pellicoro A, Vernon MA, Boulter L, Aucott RL, Ali A, Hartland SN, Snowdon VK, Cappon A, Gordon-Walker TT *et al*: Differential Ly-6C expression identifies the recruited macrophage phenotype, which orchestrates the regression of murine liver fibrosis. *Proc Natl Acad Sci U S A* 2012, 109(46):E3186-3195.
18. Helk E, Bernin H, Ernst T, Ittrich H, Jacobs T, Heeren J, Tacke F, Tannich E, Lotter H: TNFalpha-mediated liver destruction by Kupffer cells and Ly6Chi monocytes during *Entamoeba histolytica* infection. *PLoS Pathog* 2013, 9(1):e1003096.
19. Morias Y, Abels C, Laoui D, Van Overmeire E, Guilliams M, Schouppe E, Tacke F, deVries CJ, De Baetselier P, Beschin A: Ly6C- Monocytes Regulate Parasite-Induced Liver Inflammation by Inducing the Differentiation of Pathogenic Ly6C+ Monocytes into Macrophages. *PLoS Pathog* 2015, 11(5):e1004873.
20. Lee YS, Kim MH, Yi HS, Kim SY, Kim HH, Kim JH, Yeon JE, Byun KS, Byun JS, Jeong WI: CX3CR1 differentiates F4/80(low) monocytes into pro-inflammatory F4/80(high) macrophages in the liver. *Sci Rep* 2018, 8(1):15076.
21. Laskin DL, Sunil VR, Gardner CR, Laskin JD: Macrophages and tissue injury: agents of defense or destruction? *Annu Rev Pharmacol Toxicol* 2011, 51:267-288.
22. Tay SS, McCormack A, Lawson C, Rose ML: IFN-gamma reverses the stop signal allowing migration of antigen-specific T cells into inflammatory sites. *J Immunol* 2003, 170(6):3315-3322.
23. Sakaguchi S: Regulatory T cells: history and perspective. *Methods Mol Biol* 2011, 707:3-17.
24. Fabre T, Molina MF, Soucy G, Goulet JP, Willems B, Villeneuve JP, Bilodeau M, Shoukry NH: Type 3 cytokines IL-17A and IL-22 drive TGF-beta-dependent liver fibrosis. *Sci Immunol* 2018, 3(28).
25. Balato A, Unutmaz D, Gaspari AA: Natural killer T cells: an unconventional T-cell subset with diverse effector and regulatory functions. *J Invest Dermatol* 2009, 129(7):1628-1642.
26. Stout RD, Jiang C, Matta B, Tietzel I, Watkins SK, Suttles J: Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. *J Immunol* 2005, 175(1):342-349.
27. Li M, Sun R, Xu L, Yin W, Chen Y, Zheng X, Lian Z, Wei H, Tian Z: Kupffer Cells Support Hepatitis B Virus-Mediated CD8+ T Cell Exhaustion via Hepatitis B Core Antigen-TLR2 Interactions in Mice. *J Immunol* 2015, 195(7):3100-3109.

Figures

Figure 1

General condition of mice infected with *Echinococcus multilocularis* A. Control group B. Infected after 2d C. Infected after 8d D. Infected after 30d E. Infected after 90d F. Infected after 180d

Figure 2

HE staining of liver in mice infected with *Echinococcus multilocularis* (×400) A. Control group B. Infected after 2d C. Infected after 8d D. Infected after 30d E. Infected after 90d F. Infected after 180d

Figure 3

General condition of liver in mice infected with *Echinococcus multilocularis* A. Control group B. Infected after 2d C. Infected after 8d D. Infected after 30d E. Infected after 90d F. Infected after 180d

Figure 4

Total score of liver tissue in mice infected with *Echinococcus multilocularis* in different periods

Figure 5

Dynamic changes of the proportion of Ly6Chi and Ly6Clo type monocytes in peripheral blood of mice infected with *Echinococcus multilocularis*. * $P \leq 0.05$, ** $P \leq 0.01$ A. Control group B. Infected after 2d C. Infected after 8d D. Infected after 30d E. Infected after 90d F. Infected after 180d G. Dynamic change of Ly6Chi type monocyte Proportion H. Dynamic change of Ly6Clo type monocyte Proportion

Figure 6

Immunohistochemical results in liver of mice infected with *Echinococcus multilocularis* at different periods×400

Figure 7

Immunohistochemical positive cells area of liver tissue in mice infected with *Echinococcus multilocularis*.

* $P \leq 0.05$, ** $P \leq 0.01$ A. CX3CL1 B. CX3CR1 C. CD11b D. iNOS E. CD163

Figure 8

mRNA expression of TNF- α and Arg1 in liver of mice infected with *Echinococcus multilocularis* at different periods by qRT-PCR. * $P \leq 0.05$, ** $P \leq 0.01$ A. TNF- α B. Arg1

Figure 9

The dynamic changes of mice serum concentration of cytokines infected with *Echinococcus multilocularis*. * $P < 0.05$, ** $P < 0.01$ A. IFN- γ B. IL-17 C. IL-4 D. IL-10

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