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TGF-β/Smad signaling pathway positively up-regulates the differentiation of Interleukin-9-producing CD4⁺ T cells in human *Echinococcus granulosus* infection

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ABSTRACT

Objectives: Cystic echinococcosis (CE) is a zoonotic disease caused by Echinococcus granulosus (Eg) infection. Th9 cells are reported to be involved in the immune responses in CE patients. This study aims to investigate the role of TGF-β/Smad pathway in the regulation of Th9 cells in CE patients.
Methods: Using Western blot analysis, flow cytometry, qPCR, immunohistochemistry, ELISA and MTT assay, we measured the expression levels of TGF-β/Smad, PU.1, IRF-4, and IL-9 in CE patients.
Results: The levels of TGF-β, p-Samd3, PU.1 and IL-9 were elevated in the liver of CE patients. IL-9 and IL-9R expressions were also elevated in the infected liver tissue, and IL-9 level was positively correlated with the liver inflammation. The levels of IL-9, IL-4, TGF-β and IL-10 in the supernatant were also significantly increased after stimulating hepatic lymphocytes of CE patients with Eg antigen B. After blocking the TGF-β pathway signaling in vitro, PU.1 and IL-9 were obviously reduced.
Conclusions: IL-9 may aggravate the inflammatory response in the liver of CE patients. The TGF-β/Smad signaling pathway is activated, and the signaling pathway may promote the differentiation of Th9 cells and IL-9 expression in active CE patients.

Introduction

Cystic echinococcosis (CE) is a worldwide zoonotic parasitic disease, which is caused by the infection of *Echinococcus granulosus* (Eg).[1] Eg infection is more common in the countries and regions with animal husbandry.[1,2] Xinjiang Uygur Autonomous Region of China is one of the most prevalent areas of CE. According to one survey, the infection rate of CE disease is around 3.1% -31.5%, and the prevalence rate is 0.5% -5.0%.[1]

The immune responses are changed in CE patients. P. Licona-Limón et al.[3] have suggested that the cytokine-regulated cellular immunity played a leading role in regulating the development of hydatid infections. Both Th1 and Th2 responses are involved in chronic CE.[4] It has been reported that Th1 cytokines can defend against Eg infection, however, Th2 cytokines are related to chronic Eg infection by impairing Th1 responses, which further results in Eg persistent infection.[5] In recent years, it has been found that Th17 and Treg cells and the related IL-17, IL-10 and TGF-β cytokines were involved in the chronic Eg infection.[6,7] The imbalance between Th1/Th2 or Treg/Th17 leads to the Eg immune escape and chronic infection in the host.[6,7] In a recent study, we found that the follicular helper CD4⁺ T and IL-21 were closely related to the active stage of CE.[8] Indeed, one study performed in humans has reported that patients with an inactive cyst had a Th1 profile whereas the T-cell lines derived from patients with active and transitional cysts had mixed Th1/Th2 and Th0 profiles.[4] Moreover, several studies imply a Th2 response in CE active stages.[9–11]

Th9 cell, which is a new group of Th-cell subsets, can secret IL-9 and is differentiated from the initial CD4⁺ T cells under the co-stimulations of TGF-β and IL-4.[12,13] In addition, Th9 cells can also

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be directly differentiated from Th2 cells in the presence of TGF-β.[12] PU.1 is an important transcription factor in the differentiation and development of Th9 cells,[14] which is linked to the promoter of IL-9 gene in Th9 cells and thus promotes the secretion of IL-9. Th9 cells are reported to be involved in the allergic diseases,[15] autoimmune diseases,[16,17] and the development of infectious diseases.[18,19] We have reported that the levels of PU.1 and IL-9 were up-regulated in CE patients and that the levels of IL-9 and TGF-β in the peripheral blood of CE patients were significantly higher.[19] However, the Th9 cells, IL-9 and TGF-β gradually decreased to normal levels after surgery and albendazole therapy, suggesting that Th9/IL-9 may be involved in the immune response in CE patients and may be beneficial to the long-term survival of the Eg in the host.[19] By using an animal model, we found that TGF-β and Smad proteins were highly expressed in Echinococcus multilocularis infected lesions, and both could regulate the differentiation of $CD4^+$ T cell subsets (Treg/Th17) after Eg infection.[6]

TGF-β is an important factor mediating cellular processes, including cell growth, cell differentiation, cell apoptosis, and cellular homeostasis.[20] TGF-β is thought to function through the Smad mediated downstream signal transduction.[21] In Schistosoma mansoni infection, TGF-β/Smad pathways are thought to transduce signals to the cell nuclei to regulate gene expression.[22] One recent study has found that TGF-β could activate Smad2, Smad3, and Smad4 and play an important role in the differentiation of Th9 cells.[23] It had been demonstrated that Smad2 or Smad4 deficiency in T cells could affect both IL-9 expression and Th9 cells differentiation and development.[23,24] However, the peculiar feature of Th9/IL-9 in CE patients with active and inactive CE cysts is still unknown. Understanding the relationship of Th9 and TGF-β/Smad signaling pathway in active CE may facilitate to clarify mechanism and development of the disease. In this study, we have investigated the difference of Th9/IL-9 in active and inactive CE. Further, the potential role TGF-β/Smads signal in regulation of the Th9 differentiation in CE patients was analyzed and discussed.

Materials and methods

Patients

A total of 52 newly diagnosed CE patients were enrolled in the First Affiliated Hospital of Xinjiang Medical University from October 2015 to October 2016. The 52 CE patients were classified into stages of CE1 (12 cases), CE2 (18 cases), CE3 (12 cases, including 3 cases of CE3a and 9 cases of CE3b) and CE4-5 (10 cases) according to WHO-IWGE Classification.[25] CE diagnosis was confirmed by surgery and postoperative pathology. Based on the ultrasound image and morphological changes in the structure of hepatic cysts, CE is classified into CE1-2, 3b (active cysts), CE3a (transitional cysts), and CE4-5 (inactive cysts) types.[25,26] In CE1, CE2, and CE3b, it is likely that cysts contain viable Protoscolices, thus, they are considered as active cysts. The CE3a cysts show the collapse or detachment of the parent cyst wall and are considered as transitional cysts.[27] In this study, CE patients were further classified into the categories of "active CE" group (active cysts of CE1, 2, 3b; n = 39) and "inactive CE" group (inactive cysts of CE4, 5; n = 10). The control samples were from 40 patients with liver hemangioma (LH) from the Department of Gastroenterology. Patients with malignant tumors, autoimmune diseases, HIV, viral hepatitis, tuberculosis or other chronic infectious diseases were excluded. Prior written and informed consent were obtained from every patient. This study conformed to the approved institutional guidelines and was approved by the Ethical Committee of Xinjiang Medical University (20120220-126). The clinical characteristics of study cohort were shown in Table 1.

Table 1
The clinical characteristics of study cohort.

Characteristics	CE (n = 52)	Control (n = 40)
Age (years)	37 ± 8	42.8 ± 8
Sex (male/female)	27/25	23/17
Liver localization (right lobe/left lobe)	40/12	22/18
Diameter of lesions (cm)	11.5 ± 4.9	10.4 ± 3.5
Stages of CE	CE1: 12 CE2 : 18 CE3a : 3 CE3b : 9 CE4 : 6 CE5 : 4	—

Note: Values are expressed as median ± SD or number. Control: Liver Hemangioma patients, CE: cystic echinococcosis. "—": not determined.

Sample collection

The peripheral bloods were collected from CE patients and the control group in the morning with EDTA anticoagulant. The peripheral blood mononuclear cells (PBMCs) were then separated. In CE patients, the liver tissues were taken at 0.1–0.2 cm away from the parasitic cyst by surgery. In the control group, the normal liver tissues surrounding the hemangioma were reseated during surgery.

HE staining and immunohistochemistry

The liver tissues were fixed by 4% formalin, then embedded in paraffin and cut into 4 μM sections. The slices were dewaxed with xylene, followed by gradient hydration by ethanol. For HE staining, the samples were stained with hematoxylin for 3–5 minutes and then stained with eosin for 1–4 minutes. After dehydration and differentiation in ethanol, sections were mounted and observed under microscopy (Olympus BX50, Olympus, Tokyo, Japan). The inflammation grade of liver tissues was analyzed based on HE staining results and as previously described. G0: no inflammation; G1: portal area inflammation, lobular degeneration and a few points, focal necrosis; G2: mild portal area debris necrosis; G3: moderate portal area debris necrosis.

For immunohistochemical staining of IL-9 (Serotec Ltd, Kidlington, UK) and IL-9R (Abcam, USA), the sections were incubated overnight at 4 °C with primary antibodies. Then the sections were incubated with the anti-IgG secondary antibodies (Beijing Zhong Shan-Golden Bridge Biological Technology CO., Ltd., Beijing, China) at 37 °C for 20 min. Finally, the sections were developed with DAB reagents (Beijing Zhong Shan-Golden Bridge Biological Technology CO., Ltd., Beijing, China). Five views were randomly selected at 400 × magnification, and the positive cells were counted.

FACS

The PBMCs and liver mononuclear cells were washed with PBS and then the cell concentration was adjusted to 1×10^6 cells/mL. Phorbolmyristate acetate (50 ng/ml), ionomycin (1 μM) and monensin (500 ng/mL) were added to cells and stimulated for 6 h in a 37 °C. Then, the cells were collected, and 5 μL of anti-CD3-FITC and anti-CD4-APC was added and incubated for 15 min in the dark. After surface staining, the cells were fixed and permeabilized. Intracellular IL-9 was stained by adding 5 μL of anti-IL-9-PE and incubated for 30 min. Isotype-matched antibodies were used as negative controls. Then, cells were analyzed with BD Calibur flow cytometry. All of the antibodies and reagents were purchased from eBioscience (San Diego, CA, USA). After gating on SSC and FSC, cells with positive expression of CD3 and CD4 were further gated. The percentage of $CD4^+IL\text{-}9^+$ (Th9 cells) in $CD4^+$ T cells was then analyzed.