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Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Original article http://dx.doi.org/10.1016/j.apjtb.2015.07.013

Role of Th9 cells and Th17 cells in the pathogenesis of malignant ascites

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ARTICLE INFO

Article history:
Received 16 Jun 2015
Received in revised form 26 Jun 2015
Accepted 4 Jul 2015
Available online 7 Aug 2015

Keywords:
Th9 cells
Th17 cells
Malignant ascites

ABSTRACT

Objective: To assess the role of Th9 and Th17 cells in malignant ascites (MA).
Methods: MA from 30 hepatic carcinoma patients and benign ascites from 30 cirrhotic patients were collected. Corresponding peripheral blood samples from these hepatic carcinoma and cirrhotic patients as well as 30 healthy subjects were collected. The frequency of Th9 and Th17 cells was tested by flow cytometry. Serum levels of interleukin (IL)-9 and IL-17 were examined by ELISA.
Results: The observed frequency of Th9 and Th17 cells, and the IL-9 and IL-17 serum levels were significantly higher in MA patients than those in cirrhotic patients and healthy control samples ($P < 0.05$). Moreover, the Th9 cells demonstrated positive correlation with Th17 cells as well as IL-9 in MA patients; however, this positive correlation was not observed in the cirrhotic patients or healthy control samples. The frequency of Th9 and Th17 cells was distinctly higher in MA patients presenting with stage III or IV malignancy and with lymph node or distant metastasis than those in patients in stage I or II and without distant metastasis ($P < 0.05$).
Conclusions: The increased frequency of Th9 and Th17 cells in MA patients suggests that these two T cell subsets play a synergistic role in MA pathogenesis. This study also demonstrated that Th9 and Th17 cells may perform their biological functions in conjunction with IL-9 production.

1. Introduction

Malignant ascites (MA) is a pathological condition caused by a variety of primary abdominal and extra-abdominal neoplasms. MA is often associated with gastric, ovarian, and peritoneal malignancies. Up to 15% of all patients with gastrointestinal cancer develop ascites at an advanced stage of the cancer [1]. $CD4^+$ T cell subsets play an important role in autoimmune diseases. The Th subset of Th9 cells is characterized by the production of interleukin (IL)-9 and IL-10. They develop from naive $CD4^+$ precursors, which are driven by the combined effects of transforming growth factor-β and IL-4 and are distinct from Th1, Th2, or Th17 cells [2,3]. In recent years, a new IL-17-producing T cell subset, termed Th17, has been described [4]. It has been established that Th17 cells play a critical role in several animal models of autoimmunity, such as experimental allergic encephalomyelitis and murine arthritis models [5,6]. In addition, Th17 cells have been found in some human cancers and are considered to be involved in many human inflammatory diseases, including multiple sclerosis, psoriasis and inflammatory arthritis [7–9].

IL-9 is a T cell-derived factor and serves as a critical molecule during the differentiation of Th17 cells and the function of regulatory T cells (Tregs). IL-9 can be secreted by both Th2 cells and Tregs [2]. Recently, evidence indicates that IL-9 can also be produced by Th17 cells and this process is regulated by IL-23. It has been established that IL-9 increases the suppressive effect of $FoxP3^+$ $CD4^+$ Tregs *in vitro*, while the absence of IL-9 decreases the function of Tregs *in vivo*, which leads to an increase in effector cells and worsening of experimental allergic encephalomyelitis, respectively [10]. Both signal transducer and activator of transcription (STAT) 3 and STAT5 have been reported to mediate the IL-9 signaling in Th17 cells [10].

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Peer review under responsibility of Hainan Medical University.
Foundation Project: Supported by grants from the National Natural Scientific Foundation of China (81260083; 31360221), Guangxi Natural Science Foundation (2014GXNSFAA118203) and Innovation Project of Guangxi Graduate Education (YCBZ2015025).

However, their roles are different; STAT3 promotes the differentiation of Th17 cells, while STAT5 negatively regulates Th17 differentiation *in vitro* and *in vivo* [11].

Although the role of Th9 cells has been described in mice [2,3], knowledge of them in humans is limited. Some studies have demonstrated that Th9 cells may stimulate inflammation and contribute to the development of allergic diseases [12,13], but the role of Th9 cells in the immune response is largely unknown and requires further examination. A study has found that the levels of tumor-infiltrating Th17 cells and levels of ascites IL-17 are reduced in more advanced ovarian cancer patients and positively predict patient outcome [14]. The role of Th9 cells in MA has not yet been investigated. The biological function of IL-9 and IL-17 in MA remains to be established. Therefore, the present study aims to investigate the role of Th9 and Th17 cells in the pathogenesis of MA by examining the frequency of Th9 and Th17 cells in MA caused by hepatic carcinoma (HCC), as well as serum levels of IL-9 and IL-17. Our results will provide a preliminary insight into the role of Th9 and Th17 cells in MA patients.

2. Materials and methods

2.1. Patients and controls

The study protocol was approved by the review board in Guangxi Medical University for human studies, and the written informed consent was obtained from each participant. All patients were either hospitalized or treated at the First Affiliated Hospital of Guangxi Medical University between October 2012 and April 2014. MA and peripheral blood samples were collected from 30 HCC patients. The diagnosis of MA was established by the presence of malignant cells in peritoneal effusion and/or in the closed peritoneal biopsy specimen, and the diagnosis of cancer was established by histological testing. Benign ascites (BA) and peripheral blood samples were collected from 30 patients newly diagnosed with hepatitis B virus-associated cirrhosis. Peripheral blood samples from 30 healthy subjects were collected as controls. The demographic and clinical information of the HCC patients, cirrhotic patients, and the healthy controls is summarized in Table 1.

Patients were excluded if they had been treated with any invasive procedures directed into the peritoneal cavity or if any abdominal trauma had occurred within 3 months prior to their hospitalization. Patients with an existing peritoneal effusion of unknown origin were also excluded.

At the time of sample collection, none of the patients had received any anticancer therapy, corticosteroids, or other nonsteroidal anti-inflammatory drugs.

Table 1
Characteristics of the study population.

Characteristics		Values
HCC patients (n = 30)	Sex (male/female)	25/5
	Age (year)	61.7 ± 2.8
	Protein (g/mL)	4.1 ± 0.4
	Lactate dehydrogenase (IU/L)	747.3 ± 125.6
Cirrhotic patients (n = 30)	Sex (male/female)	25/5
	Age (year)	58.7 ± 2.1
	Protein (g/mL)	3.50 ± 0.35
	Lactate dehydrogenase (IU/L)	448.5 ± 67.2
Healthy controls (n = 30)	Sex (male/female)	25/5
	Age (year)	57.9 ± 2.5

2.2. Sample collection and processing

MA and BA samples from each patient were collected in volumes of 500 mL to 1 000 mL heparin-treated tubes, via a standard thoracentesis technique within 24 h after hospitalization. At the same time, 10 mL of peripheral blood was drawn. The specimens were immediately placed on ice and then centrifuged at 2 500 r/min for 5 min. The clarified supernatants of MA and BA samples, as well as the serum samples were frozen at −80 °C immediately after centrifugation, for subsequent determination of cytokine and chemokine concentrations. The cellular pellets of MA and BA samples were resuspended in phosphate buffer solution, and within 1 h, mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation (Solarbio) to determine the T cell subsets.

2.3. Flow cytometry

Intracellular cytokines were studied by flow cytometry to reflex the cytokine-producing cells. The expression of markers on T cells from the MA and BA and peripheral blood samples was determined by flow cytometry, as previously described [15], after surface or intracellular staining with specific anti-human antibodies, conjugated with PerCP-Cy5.5, phycoerythrin, or AlexaFluor 647. These human antibodies included anti-CD4, anti-IL-9, and anti-IL-17 monoclonal antibodies, which were purchased from BD Biosciences (Franklin Lakes, NJ) and eBioscience (San Diego, CA). The T cell subsets were incubated in Roswell Park Memorial Institute 1640 medium for 5 h at 37 °C in the presence of 5% CO_2 and stimulated with phorbol myristate acetate (50 ng/mL) (Sigma–Aldrich, St. Louis, MO), ionomycin (1 μg/mL) (Sigma–Aldrich) and Golgi-Stop (1.7 μg/mL) (BD Biosciences). Phorbol myristate acetate and ionomycin are pharmacological T-cell-activating agents that mimic signals generated by the T-cell receptor complex and have the advantage of stimulating T-cells of any antigen specificity. Monensin was used to block intracellular transport mechanisms, thereby leading to accumulation of cytokines in the cells [15,16]. After incubation, the cells were stained at room temperature in the dark for 30 min with PerCP-Cy5.5-conjugated anti-CD4 monoclonal antibodies (Catalog No. 560650). The cells were then stained with Alexa Fluor 647-conjugated anti-IL-9 monoclonal antibodies (Catalog No. 560813) and PE-conjugated anti-IL-17A monoclonal antibodies (Catalog No. 560436) at 4 °C for 30 min after fixation and permeabilization. All the antibodies were from BD Biosciences PharMingen. Isotype controls were analyzed to ensure correct compensation and confirm antibody specificity. Stained cells were analyzed by flow cytometry by using a FACScan cytometer equipped with the Cell Quest software (BD Biosciences PharMingen).

2.4. IL-9 and IL-17 ELISA

The ascites and peripheral blood samples were collected in heparin-treated tubes. The cell-free supernatants of ascites and serum from all subjects were obtained by centrifugation and immediately stored at −80 °C for cytokine determination. The IL-9 (Catalog No. 434707) and IL-17 (Catalog No. BMS2017) levels were determined by a quantitative sandwich ELISA in accordance with the manufacturer's recommendations (lower detection limit: 1 pg/mL) (eBioscience).

2.5. Statistical analysis

Data were expressed as mean ± SD. The statistical significance of Th9, Th17, IL-9, and IL-17 among MA patients, BA patients, and the healthy controls was determined by ANOVA, and the difference between the groups was determined by the Newman–Keuls multiple comparison test (q test) unless the data were not normally distributed, in which case the Kruskal–Wallis test (H test) and the Nemenyi test were used. The Pearson or Spearman correlation test was used for correlation analysis depending on the data distribution. Analysis was completed with SPSS version 16.0 by statistical software (Chicago, IL, USA), and P values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Frequency of Th9 and Th17 cells

The expression of a typical dot plot of Th9 and Th17 cells in representative MA and BA and their corresponding blood samples, as well as blood from the healthy participants, is represented in Figure 1. The frequency of Th9 cells in MA (3.16% ± 0.34%),

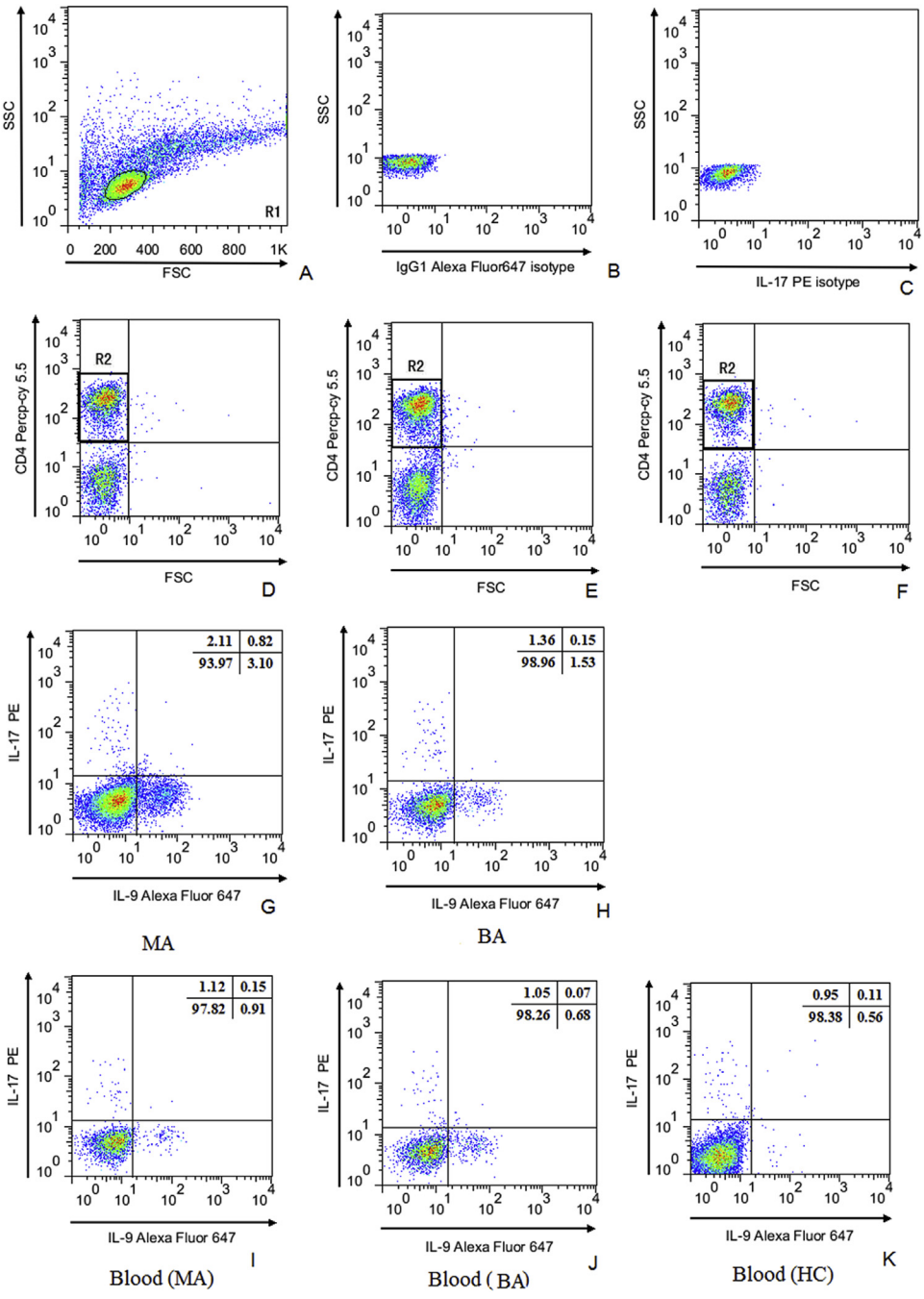

Figure 1. Significant higher levels of circulating Th9 cells and Th17 cells in MA from HCC patients than those in both BA from cirrhotic patients and healthy control samples.

A: Lymphocytes were gated by flow cytometry; B, C: Isotype controls (IL-9, IL-17) were analyzed to ensure correct composition and confirm antibody specificity; D, E, F: $CD4^+$ T lymphocytes were gated by flow cytometry from MA, BA, and healthy controls; G, H: Frequency of Th9 and Th17 cells from MA, BA; I, J, K: Frequency of Th9 and Th17 cells in blood (from MA patients, BA patients, and healthy controls, respectively). FSC: Forward Scatter; SSC: Side scatter; IgG: Immunoglobulin G; PE: Phycoerythrin; HC: Healthy control.

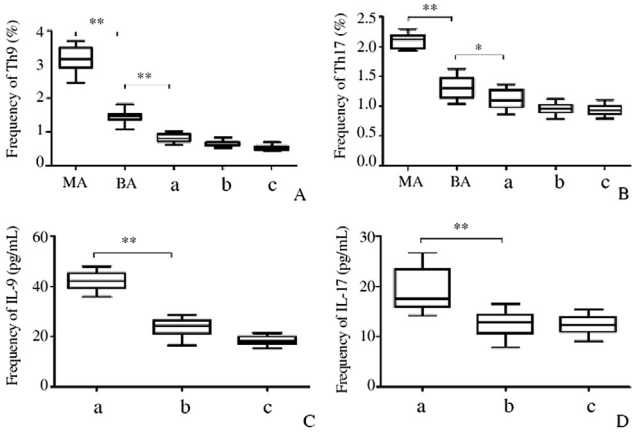

Figure 2. Frequency of Th9 cells, Th17 cells, IL-9 and IL-17.
A: Comparison of frequency of Th9 in MA, BA and blood from MA and BA patients and healthy controls; B: Comparison of frequency of Th17 in MA, BA and blood from MA and BA patients and healthy controls; C: Comparison of frequency of IL-9 in blood from MA patients, BA patients and healthy controls; D: Comparison of frequency of IL-17 in blood from MA patients, BA patients and healthy controls. a: MA patient blood; b: BA patient blood; c: Control blood. *: $P < 0.05$; **: $P < 0.01$.

was significantly higher than that in the corresponding blood samples (0.83% ± 0.12%), while the frequency of Th9 cells in BA samples was (1.53% ± 0.24%). No significant increase of Th9 cells was observed among any of the blood samples obtained from MA patients, BA patients, or healthy participants (Figure 2A). The frequency of Th17 acells in MA (2.10% ± 0.12%), was significantly higher than that in BA (1.31% ± 0.19%). The Th17 cell frequency in BA was significantly higher than that in blood samples from MA patients, BA patients, and healthy participants. There was no significant difference among blood samples from any of the participants with respect to the frequency of Th17 cells (Figure 2B).

3.2. Serum expression levels of IL-9 and IL-17

The serum IL-9 levels of MA patients [(42.20 ± 3.46) pg/mL] were significantly higher than those of BA patients [(22.38 ± 2.50) pg/mL] and healthy controls [(18.36 ± 1.63) pg/mL]; no significant difference was observed between the levels for the BA patients and healthy controls ($P > 0.05$) (Figure 2C). The IL-17 levels in the blood from MA patients [(19.25 ± 3.84) pg/mL] were significantly higher than those in the blood from BA patients and healthy controls [(12.32 ± 1.88) and (12.27 ± 0.94) pg/mL, respectively]; there was no significant difference between the levels for the BA patients and healthy controls ($P > 0.05$) (Figure 2D).

3.3. Correlation between Th9 and Th17 cells

A significant positive correlation was observed between the frequencies of Th9 and Th17 cells in MA (Figure 3A). This correlation was not observed between Th9 cells and Th17 cells in BA ($P = 0.534$), blood from BA patients ($P = 0.372$), or the healthy controls ($P = 0.467$). A positive correlation was found between MA levels of IL-9 and Th9 cells (Figure 3B), as well as IL-9 and Th17 cells (Figure 3C) in MA patients. However, this same correlation was not observed between serum levels of IL-9 and Th17 cells ($P = 0.125$) in MA patients, and no correlation was observed between Th9 cells and IL-9 cells in blood from BA patients ($P = 0.802$) and healthy controls ($P = 0.731$).

3.4. Association of Th9 and Th17 cells with clinical parameters in MA

The frequency of Th9 and Th17 cells was distinctly elevated in patients with stage III or IV cancers as compared with those in stage I or II ($P < 0.05$). Patients with distant metastasis or lymph node metastasis also showed higher frequency of Th9 and Th17 cells than those without metastasis ($P < 0.05$). However, no significant difference was observed with regard to the patient's gender, hepatitis Be antigen (HBeAg) status and tumor size ($P > 0.05$), as displayed in Table 2.

Table 2
Association of Th9 and Th17 cells (%) in MA with clinical parameters.

Clinical parameters		*n*	Th9 cells	*P* value	Th17 cells	*P* value
Gender	Male	22	3.22 ± 0.37	0.333	1.20 ± 0.24	0.094
	Female	8	3.08 ± 0.25		1.13 ± 0.07	
Tumor size	≥5 cm	15	3.23 ± 0.39	0.125	1.18 ± 0.20	0.078
	<5 cm	15	3.05 ± 0.18		1.05 ± 0.07	
HBeAg	Positive	12	3.31 ± 0.41	0.126	1.22 ± 0.28	0.107
	Negative	18	3.06 ± 0.16		1.08 ± 0.06	
Stage	I + II	13	2.99 ± 0.20	0.001	0.97 ± 0.08	0.001
	III + IV	17	3.52 ± 0.33		1.31 ± 0.47	
Lymph node metastasis	Yes	16	3.34 ± 0.33	0.024	1.29 ± 0.17	0.032
	No	14	3.06 ± 0.19		1.02 ± 0.04	
Distant metastasis	Yes	7	3.47 ± 0.31	0.002	1.29 ± 0.35	0.044
	No	23	2.89 ± 0.19		0.88 ± 0.05	

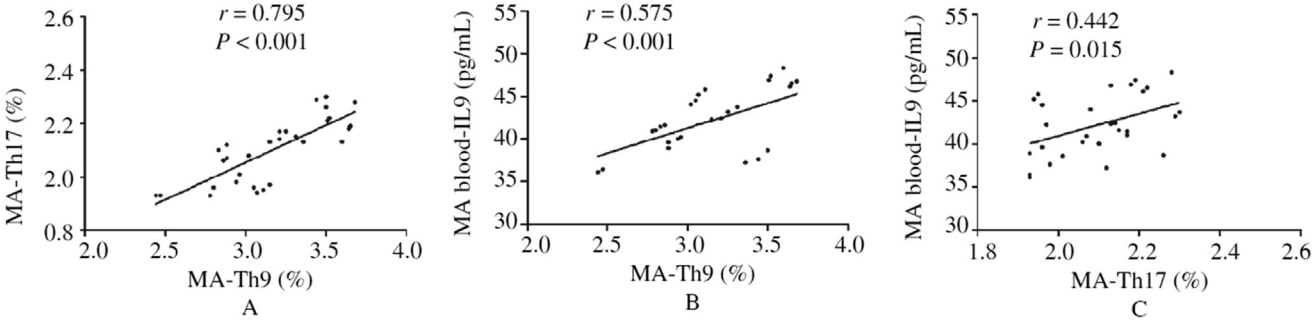

Figure 3. Correlation of Th9 cells, Th17 cells, and IL-9.
A: Positive correlation between Th9 and Th17 in MA patients; B: Positive correlation between Th9 and serum IL-9 in MA patients; C: Positive correlation between Th17 and serum IL-9 in MA patients.

4. Discussion

Th17 cells are enriched in the pleural effusion of lung cancer patients and express significantly high levels of the chemokine receptors C–C chemokine receptor (CCR) 6 and CCR4 [16]. In addition, high levels of chemokine (C–C motif) ligand (CCL) 20 and CCL22, the ligands of CCR6 and CCR4, have been observed in the malignant pleural effusion of patients with lung cancer [15,16]. Thus, these markers indicate that the interaction between receptor and ligand may play an important role in attracting Th17 cells to the malignant pleural effusion of cancer patients. Similar to Th17 cells, Th9 cells also co-express high levels of the chemokine receptors CCR6, CCR4, CCL20, and CCL22 in the pleural effusions of patients with tuberculosis. Therefore, it can be presumed that Th9 cells might selectively migrate to a peritoneal effusion in a similar way. Moreover, it is speculated that Th9 cells might be increased in the peritoneal effusions of MA patients, which could play a pathogenic role in the MA.

The present study explored the role of Th9 cells and Th17 cells in peritoneal effusions of MA from HCC patients and of BA from cirrhotic patients and found that the frequency of Th9 cells and Th17 cells was significantly higher in MA than that in the corresponding blood samples of BA patients.

Moreover, the frequency of these cells in the blood of MA patients was significantly higher than that in the blood of BA patients and the healthy controls, indicating that Th9 cells and Th17 cells may contribute to the pathogenesis of MA. These results were similar to those of other studies that have reported increased Th9 cells in the pleural effusions of patients with tuberculosis and increased Th17 cells in the malignant pleural effusions of patients with lung cancer [15,16].

In this study, we found a positive correlation between the frequency of Th9 and Th17 cells in MA. This result suggests that these two T cell subsets may play a synergistic role in the pathogenesis of MA; however, the specific link between them remains unclear and needs further investigation. The frequency of Th17 cells has been found to increase in some cancers [14,17], indicating that the Th17 cells may be involved in the pathogenesis of certain cancers. It has been suggested that Th17 cells play an indirect role in antitumor immunity by promoting dendritic cell, cytotoxic T cell, and natural killer cell to traffick to, and remain within, the tumor microenvironment [18]. In a study of malignant pleural effusion, both Th17 cells and Tregs were increased; these cells were generated and regulated by cytokines secreted from pleural cells. In addition, the accumulation of Th17 cells in malignant pleural effusion predicts improved patient survival, implying a new role of the proinflammatory response in regulating tumor progression in humans [15].

IL-9 can be secreted from various cell types, including Th9 cells [3], Th17 cells [19], and Tregs [20]. It has been associated with the regulation of macrophage recruitment and their effector functions [21,22]. However, IL-9 has also been shown to participate in peripheral tolerance, either through direct production by Tregs or by increasing the survival and activity of Tregs [11,20]. Considerable evidence suggests that IL-9 is involved in the pathogenesis of MA, but its pathophysiologic function is not well known. IL-17, the main cytokine of Th17 cells, has been reported to contribute to the pathogenesis of arthritis in many arthritis models [23], but the role of IL-17 has not been reported in MA. In the present study, elevated levels of serum IL-9 and IL-17 levels were detected in MA patients, with higher levels in MA patients than that in BA patients, indicating that IL-9 and IL-17 may be involved in the pathogenesis of MA.

In this study, MA IL-9 levels positively correlated with Th9 cells in MA patients. In addition, correlation between MA IL-9 levels and Th17 cells was also observed in MA patients. In contrast, serum IL-9 levels were not associated with Th17 cells in MA patients. The correlation between Th17 cells and IL-9 in MA supports the theory that the Th17 subset is an important T cell subset secreting IL-9 in the peritoneal effusions of MA patients. These observations indicate that Th9 cells and Th17 cells perform their biological function in the pathogenesis of MA through production of IL-9 other than IL-17. These results agree with those of a study which suggested that IL-9 was produced by both Th9 and Th17 cells, while IL-17 was not produced by Th9 cells [3].

Analysis of the association between Th9 cells and Th17 cells in MA patients with the clinical parameters revealed that the frequency of both Th9 cells and Th17 cells was significantly elevated in patients in stages III and IV, and with distant metastasis. These results suggest that Th9 cells and Th17 cells are also associated with progression in MA patients.

However, no significant difference was found in terms of tumor size and HBeAg status, suggesting that tumor size may not influence the frequency of Th9 cells and Th17 cells. The reasons for these results are unknown and require further study.

Generally, HBeAg-positive status indicates more active inflammation in the liver; however, in this study, no distinct difference was observed with respect to the Th9 cells and Th17 cells in HBeAg-positive patients. We speculated that this phenomenon may indicate that, in addition to inflammatory factors, other factors such as immune cytokines are also involved in the production of Th9 cells and Th17 cells.

This study has some limitations. Firstly, the number of MA patients was small, which may lead to a relatively lower statistical power, and some significant differences cannot be detected. Additional studies including larger patient population are necessary to confirm this study. Secondly, the MA was exclusively collected from HCC patients, while several other cancers were also associated with MA, such as gastric cancer and ovarian cancer. The pathogenesis of MA in these cancers is different; therefore, these results should be interpreted with caution if extrapolated for other causes of MA. Thirdly, this study is a descriptive study measuring the frequency of Th9 cells and Th17 cells; the mechanisms and underlying effects of Th9 cells and Th17 cells and their association with MA should be studied further in experimental research.

In conclusion, this study suggests that increased occurrence of Th9 cells and Th17 cells may synergistically contribute to the pathogenesis and progression of MA. Th9 cells and Th17 cells may perform their biological function through IL-9 production. Thus, Th9 cells and Th17 cells may be potential therapeutic targets for MA. Further studies are necessary to clarify the pathophysiologic role of Th9 cells and Th17 cells in MA.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

This work was supported by grants from the National Natural Scientific Foundation of China (81260083; 31360221), Guangxi

Natural Science Foundation (2014GXNSFAA118203) and Innovation Project of Guangxi Graduate Education (YCBZ2015025).

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