

The Association between Peripheral Th17, Th1, IL-17, and IFN- γ Levels and TACE Response in Patients with Unresectable Hepatocellular Carcinoma with or without Cirrhosis

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ABSTRAK

Latar belakang: Sel Th17, yang memiliki kemampuan memproduksi IL-17, dilaporkan memiliki efek pro-tumor dan anti-tumor. Sel Th1 diketahui dapat mengeliminasi sel tumor melalui IFN- γ yang dihasilkannya. Kemoembolisasi transarterial (TACE) merupakan pilihan terapi untuk pasien karsinoma sel hati (KSH) yang tidak dapat dilakukan reseksi. Penelitian ini bertujuan untuk menentukan hubungan antara kadar Th1, Th17, IL-17, dan IFN- γ pada sirkulasi perifer dengan respons TACE pada pasien KSH sirotik maupun non-sirotik yang pertama kali menjalani TACE. **Metode:** penelitian ini menggunakan desain studi kohort prospektif yang dilakukan di Rumah Sakit Cipto Mangunkusumo dan beberapa rumah sakit jejaring dari Juni 2015 hingga Januari 2019. Pasien KSH dengan atau tanpa sirosis hati yang memenuhi kriteria inklusi diikutsertakan dalam penelitian ini. Pengambilan sampel darah dilakukan sesaat sebelum TACE dan 30 hari setelah TACE. Sel Th1 dan Th17 dianalisis menggunakan flowcytometry, sedangkan sitokin IL-17 dan IFN- γ diperiksa menggunakan metode ELISA. Respons terhadap TACE dinilai menggunakan mRECIST. **Hasil:** sebanyak 41 pasien KSH diikutsertakan dalam penelitian ini. Berdasarkan kriteria mRECIST, 12 pasien dinilai sebagai kelompok respons (respons komplet dan parsial) dan 29 pasien dinilai sebagai kelompok nonrespons (lesi stabil dan progresif). Kadar Th1 dan Th17 meningkat secara bermakna setelah TACE pada kelompok respons. Di sisi lain, kadar IL-17 dan IFN- γ menurun setelah TACE pada kedua kelompok, walaupun tidak bermakna secara statistik. Yang menarik, pada kelompok respons didapatkan peningkatan signifikan pada kadar sel T yang mengekspresikan baik IFN- γ maupun IL-17 pada permukaannya, yaitu sel sel T CD4+/IFN- γ +/IL-17+. **Kesimpulan:** peningkatan kadar sel Th1, Th17, dan sel T CD4+/IFN- γ +/IL-17+ ditemukan pada pasien KSH dengan respons komplet atau parsial terhadap TACE.

Kata kunci: karsinoma sel hati, kemoembolisasi transarterial, Th1, Th17.

ABSTRACT

Background: Th17 cells, a subset of CD4+ T cells with the capacity to produce IL-17, were reported to have pro-tumor and anti-tumor effects. Th1 cells are known for their capacity to eliminate tumor cells by producing IFN- γ . Transarterial chemoembolization (TACE) is a treatment of choice for patients with unresectable hepatocellular carcinoma (HCC). Therefore, this study aimed to determine the association between peripheral

*Th17, Th1, IL-17, and IFN- γ levels and TACE response in patients with unresectable HCC with or without cirrhosis. **Methods:** a prospective cohort study was conducted in Cipto Mangunkusumo Hospital and several affiliated hospitals from June 2015 to January 2019. HCC patients with or without cirrhosis who met the inclusion criteria were included in this study. Blood samples were obtained immediately before TACE and 30 days after TACE. Th1 and Th17 cells were analyzed by flowcytometry, while IL-17 and IFN- γ were examined with ELISA method. TACE response was assessed with mRECIST. **Results:** forty-one HCC patients were enrolled in this study. According to mRECIST, 12 patients were assessed as response group (complete and partial response) and 29 patients were assessed as nonresponse group (stable and progressive disease). Levels of Th1 and Th17 increased significantly after TACE in the response group. On the other hand, IL-17 and IFN- γ decreased after TACE in both groups, although not statistically significant. Interestingly, in the response group, a significant increase was found in the number of T cells subset showing both IFN- γ and IL-17 markers on their surfaces, i.e. CD4+/IFN- γ +/IL-17+ T cells. **Conclusion:** increased circulating Th1, Th17, and CD4+/IFN- γ +/IL-17+ T cells were observed in HCC patients with complete or partial response to TACE.*

Keywords: hepatocellular carcinoma, Th1, Th17, transarterial chemoembolization.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fourth leading cause of cancer-related deaths globally and a major health problem in South-East Asia,¹ including Indonesia. Nearly 85% of HCC patients were diagnosed in the intermediate or advanced stage, when curative treatment is no longer eligible.² In the intermediate stage, transarterial chemoembolization (TACE) is the recommended treatment modality for HCC patients with good performance status and preserved liver function.³ However, the outcome of TACE is not satisfactory with reported 3-year overall survival (OS) rate as low as 10-40%.⁴

The immune cells in the tumor microenvironment play important role in modulating liver fibrosis, hepatocarcinogenesis, tumor invasion, and metastasis.⁵ T helper 17 (Th17) cells, are among the many immune cells that play a major role in tumor microenvironment by producing proinflammatory cytokines capable of promoting or inhibiting tumor growth.⁶ Several studies reported that high levels of Th17 cells, either in peripheral blood or intratumoral, were associated with poor prognosis of HCC.⁷⁻⁹ These findings were supported by the negative correlation of increased intratumoral interleukin-17 (IL-17), one of the cytokines that is secreted by Th17, with survival rate.¹⁰ On the other hand, Liao et al¹¹ found that increased circulating Th17 level 30 days after TACE was associated with better

OS and time-to-progression (TTP) in stage III HCC patients. These results are representative of Th17 controversies in HCC.

Meanwhile, Th1 cells are known for their anti-tumor effect through IFN- γ production.¹² Yan et al.¹³ reported that high ratio of Th17/Th1 in peripheral blood was associated with poor prognosis in HCC patients and emphasized the role of Th1 in tumor elimination. To date, data on the association between Th17, Th1, IL-17, and IFN- γ levels and response to TACE are limited. Most studies only reported the effects of Th17 or IL-17 on survival. Therefore, this study aims to determine the association of peripheral Th17, Th1, IL-17, and IFN- γ levels and TACE response in patients with unresectable HCC with or without cirrhosis.

METHODS

Study Design and Patient Selection

A prospective cohort study was conducted on HCC patients who were hospitalized to undergo TACE procedure in Cipto Mangunkusumo National General Hospital, Premier Jatinegara Hospital, and Metropolitan Medical Centre Hospital between June 2015 and January 2019. Diagnosis of HCC was based on the criteria published by the Indonesian Association for the Study of the Liver.¹⁴ Tumor staging was based on the Barcelona Clinic Liver Cancer (BCLC) staging system.¹⁵

Inclusion criteria were unresectable HCC patients with or without cirrhosis who were admitted for their first TACE procedures. All patients who were willing to participate in this study were asked to sign informed consent forms. HCC patients with contraindications for TACE (Child-Pugh class C liver cirrhosis, diffuse multiple liver nodules, extrahepatic metastasis, and presence of intrahepatic arteriovenous fistula), history of autoimmune disease, positive anti-HIV serology, and/or history of consuming sorafenib during the study period, were excluded from this study.

Information about demographic data, baseline laboratory, and blood examination for levels of Th17, Th1, IL-17 and IFN- γ before and 30 days after TACE were obtained. Response of TACE was evaluated 30 days after TACE. The level of peripheral Th17, Th1, IL-17, and IFN- γ before and 30-days after TACE were evaluated in overall general samples and in two comparison subgroup (response vs nonresponse).

TACE Procedure and Evaluation of TACE Response

Conventional TACE (cTACE) procedures were performed by interventional radiology specialists and team. Tumor blood vessels and tumor-supplying arteries were identified by hepatic arteriography. The combination of doxorubicin and lipiodol was used to perform chemoembolization, with dose being adjusted to tumor size, liver function, and tumor vascularization. This was followed by embolization with gelatin sponge particle (Gelfoam; Pharmacia-Upjohn, Kalamazoo, MI) until stasis in the tumor-feeding arteries.

Three-phase abdominal CT or MRI was performed 30 days after TACE procedure. TACE response was assessed with the modified Response Evaluation Criteria in Solid Tumors (mRECIST).¹⁶ Complete response (CR) and partial response (PR) were classified into "response group", meanwhile stable disease (SD) and progressive disease (PD) were classified into "nonresponse group".

Blood Collection and Peripheral Blood Mononuclear Cell Isolation (PBMC)

20 mL of blood sample was obtained from HCC patients (immediately before TACE

and 30 days after TACE procedure). Blood sample collection was postponed until seven days if patient had symptoms of sepsis. For the examination of Th17 and Th1, PBMC were isolated from fresh EDTA whole blood by Ficoll density gradient centrifugation. The aliquot of plasma was stored at temperature -80°C for IL-17 and IFN- γ analysis.

Th1 and Th17 Flowcytometry Analysis

PBMC were stained with the following antibodies: PerCP/Cyanine5.5 anti-human CD4, PE anti-human IL-17A, and FITC anti-human IFN- γ (BD Biosciences). Sorted cells were stimulated with PMA/ionomycin (Sigma-Aldrich), stained with surface markers, fixed with stain buffer and BD cytofix fixation buffer. Cells were permeabilized with BD Perm/Wash Buffer and finally stained with anti-IL-17A and anti-IFN- γ . Data were acquired using FACSCanto Flowcytometer BD Biosciences. Th1 and Th17 cells were identified as CD4 subsets expressing intracellular IFN- γ and IL-17A, respectively. CD4⁺/IFN- γ ⁺/IL-17⁻ T cells only expressed IFN- γ , meanwhile CD4⁺/IFN- γ ⁻/IL-17⁺ T cells only expressed IL-17A and CD4⁺/IFN- γ ⁺/IL-17⁺ T cells expressed both IFN- γ and IL-17A.

IL-17 and IFN- γ Cytokine Analysis

Plasma cytokine levels of IL-17 and IFN- γ were analyzed using the sandwich enzyme-linked immune-sorbent assay (ELISA) technique with Human IL-17 ELISA (Elabscience E-EL-H0105) and Human IFN- γ ELISA (Elabscience E-EL-H0108) kit according to the manufacturer's instructions.

Statistical Analysis

All data were analyzed using statistical software. Continuous variables were presented as mean (standard deviation) if data were normally distributed and median (range) if data were not normally distributed. Shapiro-Wilk test was conducted to assess for normality of data distribution. Categorical variables were presented as frequency (proportion). Statistical analysis of paired samples, i.e. before and 30-days after TACE, within two subgroups (response vs. non-response) were determined using paired T-test if the data was normally

distributed or Wilcoxon signed-rank test if the data was not normally distributed. The paired parameters was considered significant if the p-value was less than 0.05.

Statement of Ethics

This study protocol has been approved by the Ethics Committee of Faculty of Medicine Universitas Indonesia (Ref. no. 546/UN2.F1/ETIK/2015). All subjects who participated in this study signed written informed consents.

RESULTS

Baseline Characteristics

Majority of subjects in this study were male with mean age of 54.14 (SD 12.18) years. Chronic HBV infection was the most common etiology. Most of the patients had liver function of class A or B based on the Child-Pugh classification. Demographic and laboratory baseline characteristics of the study population is shown in **Table 1**.

Peripheral Th17, Th1, IL-17, and IFN- γ levels before and 30 days after TACE

In the response group, Th17 and Th1 levels were significantly elevated after TACE. In the non-response group, only the CD4+/IFN- γ + /IL-17- T cells level was significantly elevated ($p = 0.042$). Levels of IL-17 and IFN- γ were decreased in both groups after TACE, but were not statistically significant (**Table 2**). Interestingly, in the response group, a significant increase was found in the number of T cells subset showing both IFN- γ and IL-17 markers on their surfaces, i.e. CD4+/IFN- γ +/IL-17+ T cells. In the response group, AFP level and tumor size were also significantly reduced.

DISCUSSION

In the past decade, the role of Th17 and IL-17 in tumor immunity has been widely studied in various types of tumors, such as breast cancer,¹⁷ ovarian cancer,¹⁸ myeloma,¹⁹ melanoma,²⁰ and hepatocellular carcinoma.^{9,11} The results are conflicting and most studies only focus on the role of Th17 in tumor growth and survival.²¹ Considering the fact that the immune system has substantial contributions in tumor development,

Table 1. Demographic and Laboratory Baseline Characteristics of Study Population.

Baseline characteristics	Values
Demographic characteristics	
Sex, n (%)	
- Female	9 (22)
- Male	32 (78)
Age, mean (SD)	54.41 (12.18)
Etiology, n (%)	
- Hepatitis B	35 (85.4)
- Hepatitis C	6 (14.6)
Child Pugh class, n (%)	
- A	34 (82.9)
- B	7 (17.1)
- C	0 (0)
Laboratory characteristics	
- Hemoglobin, mean (SD) (g/dL)	12.43 (1.95)
- White blood cells (WBC) count, median (range) (x103 cells/ μ L)	5.9 (2.42–16.1)
- Platelet count, median (range) (x103 cells/ μ L)	205 (74–686)
- AST, median (range) (U/L)	46 (18–190)
- ALT, median (range) (U/L)	29 (9–454)
- Albumin, mean (SD) (mg/dl)	3.57 (0.51)
Levels of Th17, Th1, IL-17 and IFN- γ	
- CD4, mean (SD) (%)	23.98 (14.02)
- Th17, median (range) (%)	38.0 (12.2–90.1)
- Th1, median (range) (%)	8.9 (1.8–31.5)
- IL-17, median (range), (pg/mL)	326.94 (136.44–683.78)
- IFN- γ , median (range), (pg/mL)	80.00 (58.96–245.47)

HCC: hepatocellular carcinoma, SD: standard deviation
AST: aspartate aminotransferase, ALT: alanine aminotransferase, HCC: hepatocellular carcinoma, CD4: cluster of differentiation 4, IFN- γ : interferon- γ , IL-17: interleukin-17, Th1: T helper 1, Th17: T Helper 17

any treatment modality would undoubtedly cause inflammatory reaction in tumor site and; thus, affect the immune system, as well. However, studies that focused on the association between the immune system, especially Th17 cells, and response to treatment, are very limited. Tumor response is the assessment of treatment efficacy. Studying the association between tumor response

Table 2. Levels of Th17, Th1, IL-17 and IFN- γ before and after TACE.

Parameters	Response (n=12)			Nonresponse (n=29)		
	Before TACE	After TACE	p	Before TACE	After TACE	p
CD4, mean (SD)	21.93 (13.90)	20.30 (9.69)	*0.690	24.82 (14.23)	22.31 (12.92)	*0.380
Th17, mean (SD)	39.72 (17.15)	50.43 (21.76)	*0.04	43.87 (22.91)	44.18 (11.75)	*0.927
Th1, median (range)	9.4 (1.8 – 25.5)	11.75 (2.9–34.3)	†0.037	8.9 (2.2–31.5)	9.5 (2.5–26.4)	†0.665
CD4+/IFN- γ +/IL-17- (%), median (range)	0.1 (0.0–1.3)	0.2 (0.0–4.0)	†0.096	0.1 (0.0–1.1)	0.2 (0.0–4.8)	†0.042
CD4+/IFN- γ -/IL-17+ (%), mean (SD)	29.15 (12)	36.15 (15.67)	*0.079	32.57 (15.54)	34.66 (8.56)	*0.398
CD4+/IFN- γ +/IL-17+ (%), median (range)	9.3 (1.7–24.2)	11.5 (2.9–34.3)	†0.041	8.9 (2.1–30.5)	9.2 (2.3–24.4)	†0.531
IL-17 (pg/mL), median (range)	325.64 (136.4–577.2)	225.42 (163.56–551.19)	†0.388	326.94 (144.8–683.8)	241.81 (136.7–662.5)	†0.157
IFN- γ (pg/mL), median (range)	79.23 (60.06–223.48)	71.25 (59.43–208.46)	†0.695	80.00 (58.96–245.47)	77.30 (49.77–219.21)	†0.256
AFP (ng/mL), median (range)	274.4 (2.4–12.147)	19.9 (1.3–646.98)	†0.012	311.1 (2.3–716,780)	400 (2.18–400,000)	†0.903
Largest tumor diameter (cm), mean (SB)	7.29 (2.60)	4.68 (3.58)	*0.001	11.38 (4.66)	11.42 (5.04)	*0.916

*T-test dependent, †Wilcoxon test AFP: alpha fetoprotein, CD4: cluster of differentiation 4, HCC: hepatocellular carcinoma, IFN- γ : interferon- γ , IL-17: interleukin-17, TACE: transarterial chemoembolization, Th1: T helper 1, Th17: T Helper 17

and immune system should be considered in the era of precision medicine.

This study showed that significantly elevated levels of peripheral Th17 and Th1 cells were found in HCC patients with complete or partial response to TACE. The elevation of Th17 levels in patients with complete or partial response to TACE was in contrary with the findings that high levels of Th17 cells, either in peripheral blood or intratumoral, were associated with poor prognosis of HCC.^{7–9} Interestingly, in patients with partial or complete response to TACE, there was a significant increase in the number of T cells subset showing both IFN- γ and IL-17 markers on their surfaces, i.e. CD4+/IFN- γ +/IL-17+ T cells. We suggest that the source of elevated Th17 and Th1 cells was the increase in Th17/Th1-like cells. In line with our results, Liao et al¹¹ reported that highest OS and TTP were observed in high Th17 with high IFN- γ +IL-17+ group.

Th17 cells had unique capacity to transform themselves into Th17/Th1-like cells that produce both IL-17 and IFN- γ . Plasticity of Th17 cells in the HCC milieu is determined by the local environment. Th17 cells are derived from CD4+ cells in the presence of TGF- β , IL-6, IL-1 β , and are maintained long-term in

the presence of IL-21 and IL-23.²² These Th17 cells are unstable and are readily converted into Th17/Th1-like cells in the presence of IL-12 or IL-23 and in the absence or low amount of TGF- β , whereas high level of TGF- β preserved a Th17 phenotype.²³ TGF- β is a growth factor excessively expressed in tumor cells and secreted by tumor associated macrophages (TAMs).²⁴ TGF- β can induce the expression of angiogenic factors by macrophages²⁵ or dendritic cells²⁶ in hypoxic condition. Furthermore, TGF- β increases vascular endothelial growth factor (VEGF) mRNA in an AP-1/HIF-1 (activator protein-1/hypoxia-inducible factor-1) dependent mechanism and may potentiate the hypoxic response.²⁷

In patients with small tumor volume (less than 7 cm), perfect embolization of the whole tumor could be achieved and thus expression of TGF- β by tumor cells reduced significantly after TACE. On the other hand, if the tumor is not perfectly embolized, expression of TGF- β remains high. In line with this, there is a dynamic relationship between HIF-1 α and VEGF levels in patients undergoing TACE. Immediately after TACE, there is also an increase in IL-6, as described by Kim et al.²⁸ reflecting acute-

phase responses and partly associated with post-treatment hepatitis. IL-6 and TGF- β 1 promote differentiation of Th17 cells from naïve T cells^{29,30} by inducing transcription factors orphan nuclear receptors, retinoid-related orphan receptor (ROR) γ t, and ROR α .^{31,32} IL-23 is needed for the expansion and survival of Th17 lineage and for maintaining its phenotype.³³ An in vitro study showed that absence of TGF- β , IL-12 and IL-23 cytokines promotes differentiation of Th17 toward Th17/Th1-like cells with suppressed IL-17 and enhanced IFN- γ production.³⁴

In accordance with our results, we suggest that patients with complete or partial response after TACE had high levels of IL-6 with low expression of TGF- β in their tumor microenvironment, which supported the differentiation of Th17 into Th17/Th1-like cells. These Th17/Th1-like cells were detected as CD4 T cells showing both IFN- γ and IL-17 markers on their surfaces. Th17/Th1-like cells are known to be capable of producing IFN- γ with less IL-17A,³⁵ thus promoting antitumor effect. At low concentrations, TGF- β with IL-6 and IL-21 promote IL-23R expression, favoring Th17 cell differentiation from naïve CD4 T cells.^{36,37} This is also in accordance with our results, which shows that Th17 cells increased significantly in patients with complete or partial response (**Table 2**).

To our surprise, there is a significant elevation of CD4+/IFN- γ +/IL-17- T cells, expressing only IFN- γ , in non-response group. The most destructive antitumor response is known to come from Th1.^{38,39} In the absence of Th1 cells or their cytokines, it is difficult for immune cells to eradicate tumor cells. A strong antigenic interaction with APC is needed to maintain Th1 response. Death of tumor cells have strong immunostimulatory activity for activation and maturation of APC, which become a strong signal for Th1 activation and differentiation.⁴⁰ Thermal ablation in HCC can also stimulate APC maturation.⁴¹ However, after chronic specific antigen exposure, T cell lymphocyte could fall into functional hyporesponsiveness and fail to destroy cancer cells.^{42,43} In our study, we could not determine whether elevation of Th1 cells

was accompanied with good functional potential or not.

IL-17, one of the cytokines secreted by Th17 cells, was found lower after TACE in both response and nonresponse group but not statistically significant. Wu et al.⁴⁴ reported that tumor size > 5 cm and higher baseline serum IL-17 had higher risk of early HCC recurrence in patients who underwent liver resection. The presence of Th17 in remnant liver tissue was expected to stimulate cytokine associated with tumor progression. Another study also reported that increased expression of intratumoral IL-17 and IL-17 RE were associated with lower survival rate. Only Kim et al.²⁸ reported the changes of serum IL-17 levels in HCC patients after TACE. The levels of IL-17A decreased over time until statistically significant compared to baseline levels in two months after TACE. However, this study did not analyze the reduction of serum IL-17 with therapy outcome or OS. IFN- γ secreted by Th1 cells was also lower after TACE in both groups with p-value > 0.05. Kim et al.²⁸ did not report changes of IFN- γ after TACE procedure. The decrease in IL-17 and IFN- γ was expected to be associated with the nature of cytokines. Cytokines are only secreted in the presence of ongoing inflammation. Kim et al.²⁸ showed that the concentration of serum IL-6 increased 3 days after TACE and reached baseline seven days after TACE. This finding showed that inflammation due to TACE lasted for seven days. The decrease in levels of IL-17 and IFN- γ 30 days after TACE occurred because the cytokines were not actively secreted into peripheral blood as a result of reduction in inflammation. Only cells secreting these cytokines could be detected.

Finally, our data suggest that tumor size and completeness of embolization play a major part in determining immune response after TACE. Tumor expression of TGF- β , hypoxia, and inflammatory cytokines produced after TACE, were all important factors that determine differentiation of Th17 cells into favourable Th17/Th1-like cells which have antitumor phenotype. To the best of our knowledge, this is the first study that determines plasticity of Th17 in response to TACE.

CONCLUSION

HCC patients with complete or partial response to TACE had a significant increase of peripheral Th1, Th17, and CD4+/IFN- γ /IL-17+ T cells levels.

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