Expression of Th17 and $CD4^+$ $CD25^+$ T regulatory cells in peripheral blood of acute leukemia patients and their prognostic significance

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Abstract: To discuss the expression of T helper cell 17 (Th17) cells and $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells (Treg) in peripheral blood (PB) of patients with acute leukemia (AL), and to explore the relationship between them and disease prognosis. 40 patients diagnosed with acute leukemia in The First Affiliated Hospital of Zhengzhou University from July 2012 to August 2014 were selected as the observation group. Meanwhile, 40 healthy people were taken as the control group. Flow Cytometry Method (FCM) was used to detect the level of Th17 cells and $CD4^+$ $CD25^+$ $Foxp3^+$ cells in peripheral blood of the two groups, and enzyme-linked immuno sorbent assay (ELISA) method was used to test the level of IL17 and TGF-β in peripheral blood of two groups; reverse transcription-polymerase chain reaction (RT-PCR) was adopted to analyze the mRNA levels of RORγT and Foxp3 in peripheral blood. In addition, we examined the levels of Th17 and $CD4^+$ $CD25^+$ $Foxp3^+$ cells and associated factor levels in patients with remission after AL chemotherapy. the Th17 cells ($CD3^+$ $CD4^+$ $IL\text{-}17^+$) in acute leukemia patients accounted for (1.51±0.27)%, which was significantly higher than that of control group (0.36±0.23)%, with statistical significance (t=20.51, P<0.001); the percentage of $CD4^+$ $CD25^+$ $Foxp3^+$ cells in AL patients was (3.37±0.48)%, which was significantly higher than that of control group of (1.26±0.27)%, with statistical significance (t=24.23, P<0.001); the serum levels of IL-17 and TGF-β in AL patients were (28.12±6.33) pg/ml and (38.41±8.44) pg/ml respectively, which were all significantly higher than that of control group of (14.41±6.21) pg/ml and (24.49±7.42) pg/ml, with statistical significance (t=7.83, P<0.001; t=7.83, P<0.001); the RORγT mRNA and Foxp3 mRNA levels in AL patients were all significantly higher than that of control group, with statistical significance (t=12.27, P<0.001; t=7.89, P<0.001). In addition, compared with before chemotherapy, the levels of Th17 cells and $CD4^+$ $CD25^+$ $Foxp3^+$ cells, and the serum levels of IL-17 and TGF-β in acute leukemia patients all decreased significantly after 6 months of chemotherapy, and the difference was statistically significant (P<0.001). Th17 cells, $CD4^+$ $CD25^+$ $Foxp3^+$ cells and their secretory proteins IL-17, TGF-β and transcription factors were significantly increased in AL patients. Therefore, regular detection of peripheral blood Th17 and Treg cells, as well as their secretory proteins are useful for monitoring the immune status and prognosis of patients.

Keywords: Th17 cells, Treg cells, IL-17, TGF-β, acute leukemia.

INTRODUCTION

Acute Leukemia (AL) is a common serious and sudden onset blood disease, including Acute Lymphocytic Leukemia (ALL) and Acute Nonlymphocyticl Eukemia (ANL). It has various clinical manifestations, and bleeding is the most common one, with the locations spread all over the body such as mouth cavity, nasal cavity as well as subcutaneous tissues. Thrombocytopenia is the crucial cause of bleeding, while cerebral hemorrhage often leads to death. If the rescue is not timely, the patient is likely to lose their lives. Therefore, its pathogenesis and clinical diagnosis are very important.

Cluster of Differentiation 4 ($CD4^+$) T cells are the important immune cells in human immune system, which can differentiate into Th1, Th2, Th17 and Treg cells in the presence of antigen and antigen presenting cells (APC) (Zanetti, 2015; Antignano and Zaph, 2015; Protti *et al*., 2014; Assudani *et al*., 2007). Among them, Th17 is a subgroup stimulated by T cell receptor (TCR) pathway and interleukin 6 (IL-6) as well as Transforming growth factor beta (TGF-β) (Bettelli *et al*., 2006). Treg is a class of cells regulating the immune function of the body, which can maintainthe immune system tolerance to their own components, so as to maintain the immune homeostasis of the body, can also express forkhead box P3 (Foxp3), CD25 CD4. Previous researches have confirmed that the balance of Th17 and Treg cells have an inhibitory effect on the pathophysiologic immune response of various pathways in infections, tumors, organ transplants, and allogeneic fetal immune-related diseases (Gratz and Campbell, 2014; Morikawa and Sakaguchi, 2014; Wang *et al*, 2013; Duan *et al*, 2014; Kleinewietfeld and Hafler, 2013). However, there were less researches on the relationship between Th17, $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells and AL prognosis. Therefore, in this study, the levels of Th17 and Treg cells and secretion factors in peripheral blood of patients with acute leukemia and normal people were compared, and the changes of the levels of Th17 and Treg cells in the peripheral blood were detected before and after chemotherapy, to explore the relationship between these two cells and disease prognosis.

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MATERIALS AND METHODS

Clinical information

40 acute leukemia patients admitted to our the Hematology Department of The First Affiliated Hospital of Zhengzhou University from July 2012 to August 2014 were selected in this study as the observation group, with 21 males and 19 females, aging 6-14 years (average of 10.17±3.36 years). Among them, 15 cases were diagnosed with acute non-lymphocytic leukemia (ANLL) and 6 cases with acute lymphocytic leukemia (ALL). And 17 patients (6 males and 11 females, aged 6-13 years, mean aged (9.57±3.19) years old) had received complete remission. There was 40 people (23 males and 17 females, aged 6-14 years old, mean aged (10.84±3.61) years old) in the control group who had received health examination in our hospital, without important organ disease such as heart, liver, lung and kidney and with normal blood routine and hepatorenal function. In addition, there was no significant difference in sex and age between the two groups (P>0.005). All people in both groups voluntarily joined this study with informed consents and the study had been approved by the Medical Ethics Committee of The First Affiliated Hospital of Zhengzhou University.

Inclusion criteria: the patients who conformed to *Blood disease diagnostic and curative standard (second edition)*; patients who had completed remission induction chemotherapy for 1 or more than 1 courses; patients who insisted on follow-up.

Exclusion criteria: Patients who failed to complete remission induction therapy, including patients who gave up treatment or died within 48 hours of treatment; patients who combined with severe heart, liver and renal disease; patients who had serum creatinine and urea nitrogen exceeded the normal range.

Main regent

RNA of was extracted by Trizol Kit (Shanghai Yingjun Company); cDNA reverse transcriptase kit (Femantes Company, US) was used to reverse transcription of RNA; PCR kit was purchased from NanJing SunShine Biotechnology Co., LTD; Mouse anti-human streaming antibody against CD3-APC, CD4-FITC, IL-17-PE, CD25-APC, FoxP3-PE were all purchased from US eBioscience Company; Red Blood Cell Lysis Buffer were bought from Wuhan Boster Company. Fix/Perm Cell Permeabilization Kit was purchased from US Invitrogen Company. GolgiStop was purchased from BD Company; Incomplete 1640 culture media, fetal bovine serum and penicillin-streptomycin (P-S double-antibody) were all made from Shanghai Maicang Biological Technology Co., LTD., and the complete 1640 culture media was prepared according to the ratio of 89:10:1.

Treatment

The chemotherapy regimens of AL patients were: ANLL patients adopted with DA (daunorubicin-arabinoside) or HA (Homoharringtonine-arabinoside); ALL patients with VCDP (vincristine-cyclophosphamide-daunorubicin-prednison).

Methods

Th17 cell and Treg cell flow cytometry: The patients in two groups were extracted anticoagulant and non-anticoagulant fasting venous blood, with 2 ml in each group, adding Red Blood Cell Lysis Buffer to split red blood cell, and to culture them in the 1640 complete media with concentration of $1*10^6$ cell/ml. Th17 cells dyeing: Th17 cells were centrifuged and suspended in 100 μl phosphate buffer saline (PBS), adding antibody against CD3-APC (0.2mg/ml) and CD4-FITC (0.25mg/ml), mixing them up then incubating for 40 min at room temperature. After washed up by PBS again, 1ml fixative solution was added to incubate for 40min at 4^{o}C preventing from lightness. After centrifugation, it was resuspend into 100ul permeabilization buffer, added IL-17-PE (0.25mg/ml) to incubate at 4^{o}C for 30 min without lightness, after washing up with PBS, 400μl buffer solution was used to suspend the cells and then detected with FCM (FACS Calibur, US BD company). Treg cells dyeing: the Treg cells were centrifuged and suspended into 100μl PBS, adding the antibody of CD25-APC (0.25mg/ml) and CD4-FITC (0.25mg/ml), mixing up and incubating for 40 min at room temperature. And then washing with PBS and adding fixative solution for 1 ml to incubate them for 40 min without light. After centrifugation, it was resuspended into the 100ul permeabilization buffer, adding 2ul FC receptor blocking pharmacon and incubating for 20 min at 4^{o}C, adding anti-FoxP3-PE (0.15mg/ml) and incubating for 40 min at 4^{o}C. After washing up with PBS, 400μl buffer solution was used to resuspend the cells and then detected by FCM.

Detection of the concentration of IL-17 and TGF-β in serum by ELISA method

All the kits were bought from Dakewe Biotech Co., LTD., and the detection process in strict accordance with the kit instructions.

Detection of the mRNA level of RORγT and Foxp3 in peripheral blood by RT-PCR method

After centrifugation of the peripheral blood, 200μl upper plasma was extracted and added into 1ml Trizol homogenate. The extraction of total RNA was strictly in accordance with the instruction, and the acquired RNA was dissolved into 20μl DEPC. The extracted total RNA were quantitatively analyzed by ultraviolet spectrophotometer. Then the RNA were reversed into cDNA with Femantes RT-PCR kit, and kept in -20^{o}C. The PCR condition: 95^{o}C, 20 s; 60^{o}C, 20 s; 70^{o}C, 1 s for 40 cycles. The primer sequences of RORγT: F: 5'-

gcaatggaagtggtgctggtt-3', R: 5'-aggatgctttggcgatgagtc-3'; the primer sequences of Foxp3: F: 5'-cacgcatgtt-tgccttcttcaga-3', R: 5'-gtagggttggaacacctgctggg-3'; the primer sequence of reduced glyceraldehyde-phosphate dehydrogenase (GAPDH): F: 5'-atctggcaccacaccttc-3', R: 5'-agccaggtccagacgca-3'. All of them were expanded by 7300 type Real-Time PCR instrument (ABI company). The results were quantitatively analyzed by $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2011).

STATISTICALLY ANALYSIS

SPSS18.0 software was used to analyze all the data obtained in this study and measurement data were expressed by Mean Standard Deviation (MSD) (x±s). *t* test was used for the comparison between groups, and $P<0.05$ meant the difference was statistically significant.

RESULTS

Comparison of Th17 and Treg cells between the two groups

The FCM detection results showed that the proportion of Th17 cells in peripheral blood of healthy control group was (0.36+0.23)%, which was significantly lower than that of the observation group of (1.51±0.27)%, with statistical significance (t=20.51, P<0.001); while the proportion of Treg cells in peripheral blood of healthy control group was (1.26+0.27)%, which was significantly lower than that of the observation group of (3.37±0.48)%, with statistical significance (t=24.23, P<0.001). The results suggested that the abnormal expression of Th17 and Treg cells in peripheral blood of the AL patients might be closely related to the pathogenesis of AL. As shown in fig. 1.

Comparison of IL-17and TGF-β levels between the two groups

According to the ELISA detection results, the level of IL-17 in serum of patients with acute leukemia was (28.12 + 6.33) pg/ml, significantly higher than that of the control group of (14.41±6.21) pg/ml,with statistical significance (t=9.78, P<0.001); in addition, the TGF-β level of the observation group was (38.41±8.44) pg/ml, which was significantly higher than that of the control group of (24.49±7.42) pg/ml, and the difference was statistically significant (t=7.83, P<0.001). The study results showed that Th17 and Treg cells might be involved in the pathopoiesis of AL through the production of specific cytokines IL-17 and TGF-β. As shown in fig. 2.

Comparison of transcription factor RORγT and Foxp3 between the two groups

Transcription factor RORγT mRNA and Foxp3 mRNA not only could be used as a marker of Th17 and Treg cells, but also determine the key genes of Th17 and Treg cell function (Barbi *et al*, 2013). Therefore, this study made a further examination on the levels of Th17 and Treg cell specific transcription factors in peripheral blood cells of two groups by RT-PCR, and the results showed that the expression level of RORγT mRNA and Foxp3 mRNA in observation group was significantly higher than that in the control group, the difference was statistically significant (t=12.27, P<0.001; t=7.89, P<0.001), which further proved that Th17 and Treg cells played an important role in the AL patients. Seen in fig.3.

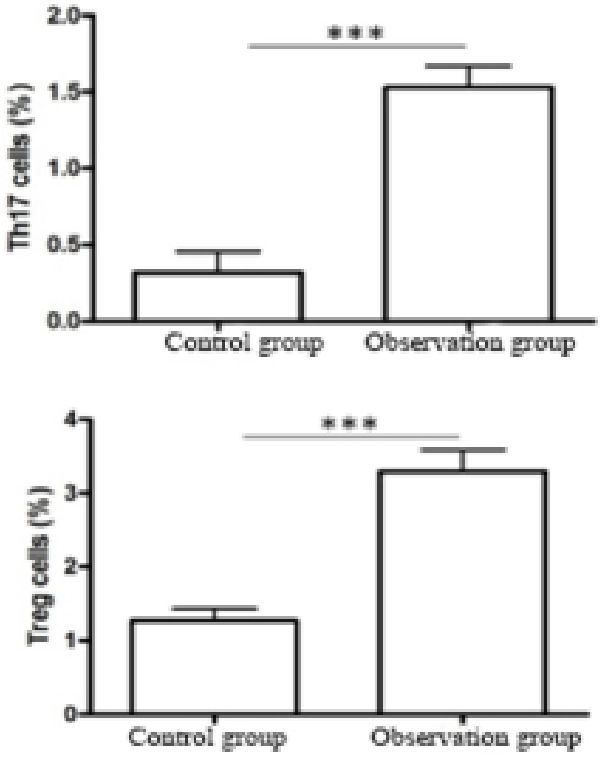

Fig. 1: The ratio of Th17 and Treg cells in the two groups

Variation of Th17 and Treg cells in AL patients after receiving chemotherapy

After chemotherapy, there was 17 patients with significant results among 40 acute leukemia patients. By comparing the levels of Th17 and Treg cells before and after treatment in these 17 patients with remission, the results showed that the proportion of Th17 after treatment was significantly lower compared with before treatment, and the diffidence was statistically significant (t=4.90, P<0.001; t=7.02, P<0.001). Moreover, the percentage of Treg cells was significantly decreased after treatment, with statistical significance (table 1). In addition, the serum levels of IL-17 and TGF-β were significantly decreased after treatment compared with before treatment (t=2.32, P<0.05; t=5.26, P<0.001) (table 2). Besides, the mRNA levels of RORγT and Foxp3 in the peripheral blood were significantly decreased compared with before treatment, and the difference was statistically significant (t=7.00, P<0.001; t=9.41, P<0.001) (table 3). These results suggested that the prognosis of acute leukemia after chemotherapy was closely related to the Th17 and Treg cells in patients and related factors.

Table 1: The ratio of Th17 and Treg cells in AL patients after receiving chemotherapy

Effect	Groups	Cases	Th17 (%)	Treg (%)
Remission group	Before treatment	17	1.45±0.23	3.21±0.34
	After treatment		1.01±0.29△	2.08±0.57*
Non-remission group	Before treatment	23	1.65±0.26	3.47±0.51
	After treatment		1.71±0.31	3.61±0.77

Note: ◆compared with the survival group, △t=4.90，P<0.001; ★t=7.02，P<0.001

Table 2: The level of Th17 and Treg cells in AL patients after receiving chemotherapy (pg/ml)

Effect	Groups	Cases	IL-17 (pg/ml)	TGF-β (pg/ml)
Remission group	Before treatment	17	25.43±7.12	36.17±7.35
	After treatment		20.08±6.28△	22.41±7.89*
Non-remission group	Before treatment	23	31.13±8.12	38.41±8.44
	After treatment		32.21±9.67	36.78±9.16

Note: ◆compared with the survival group, △t=4.90, P<0.001; ★t=7.02，P<0.001.

Table 3: The RORγT and Foxp3 levels in the PB of AL patients after receiving chemotherapy

Effect	Groups	Cases	RORγT	Foxp3
Remission group	Before treatment	17	1.25±0.35	1.21±0.31
	After treatment		0.51±0.26△	0.38±0.19*
Non-remission group	Before treatment	23	1.31±0.31	1.28±0.25
	After treatment		1.27±0.24△	1.33±0.35*

Note: ◆compared with the survival group, △t=4.90，P<0.001; ★t=7.02，P<0.001.

DISCUSSION

AL is a kind of malignant clonal disease of hematopoietic stem cells, its occurrence and development is closely related to the abnormality of immune level, especially the abnormality of cellular immunity. Th17 and CD4$^+$ CD25$^+$ Tregs (Treg) cells are the two major classes of CD4$^+$T lymphocytes induced by antigen presenting cells and their important immune cell subsets. Among them, Th17 is group of cell subset induced by TGF-β and IL-6, which plays an important role in autoimmune disease and defense reaction (Kleinewietfeld and Hafler, 2013). Treg cells are a type of immunosuppressive cells in recent years, which are induced by persistent antigen stimulation in thymus peripheral blood and IL-10 or TGF-β and other cytokines, with important immune modulating function, and involving in various physiological and pathological immune modulation (Wang *et al*, 2013; Kleinewietfeld and Hafler, 2013; Hou *et al*, 2013).

In recent years, Th17 and Treg cells in the tumor-related diseases is getting more and more concerned by people. Minchao Duan *et al* hold the idea that the Th17 and Treg cells level were significantly increased in the lung cancer, and they were correlated (Duan *et al*, 2014). Zhifang Chen *et al* found that the Th17 and Treg cells were significantly increased in Uygur patients with cervical cancer and the ratio of Th17/Treg changed, which breaking the balance of Th17/Treg in the human body (Chen *et al*, 2013). However, so far, there are still less researches on the clinical expression of Th17 and Treg cells and their changes after clinical chemotherapy in the AL clinical data.

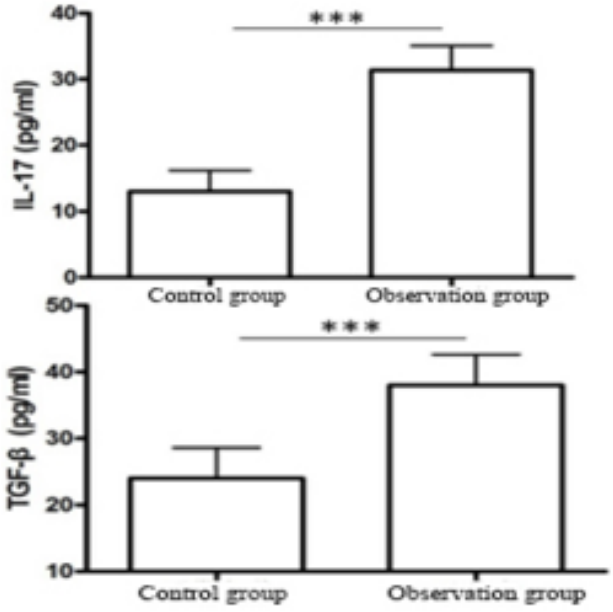

Fig. 2: The levels of IL-17 and TGF-β in the PB between the two groups

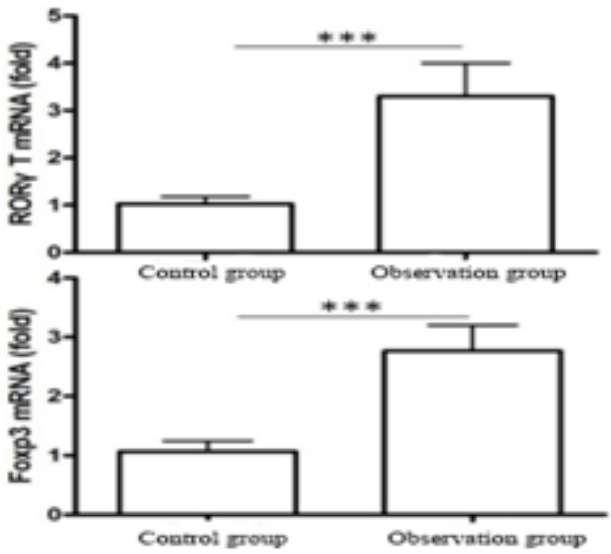

Fig. 3: The levels of RORγT mRNA and Foxp3 mRNA in the PB of the two groups

In this study, we detected the expression of Th17 and Treg cells and their related factors in AL patients by a verity of methods and discussed the significance of Th17 and Treg cells in the diagnosis and treatment of acute leukemia patients. The results showed that the levels of Th17 and Treg cells in patients with acute leukemia were significantly higher than those in the normal people, indicating that they were involved in the immune response in AL patients and might played an important regulatory effect on the immune function of the patients. IL-17 and TGF-β were the functional factors secreted specifically by Th17 and Treg cells respectively. We detected the IL-17 and TGF-β level in both groups and found that the expression level of IL-17 and TGF-β of the AL patients was higher than that of the normal people, suggesting that Th17 and Treg cells in acute leukemia patients were likely to participate in the pathogenesis of leukemia through the secretion of the corresponding functional factors. In addition, RORγT and Foxp3 were not only the characteristic indicator of Th17 and Treg cells, but also the key transcription genes (Barbi *et al*, 2013). Therefore, this study detected RORγT and Foxp3 mRNA levels in peripheral blood cells of the two groups and found that mRNA level of RORγT and Foxp3 in the serum of AL patients were much higher than those of normal people, which further proved the above results. Qiaoxia Li *et al* found that the Th17 and Treg cells in the PB of the patients with gastric cancer were significantly increased, and the high level of IL-17 and TGF-β were also expressed, relating to the clinicopathological parameters (Li *et al*, 2013), which supported this study indirectly.

To investigate whether Th17 and Treg cells participate in the prognosis of acute leukemia, we analyzed 17 patients with leukemia who were relieved after chemotherapy in our study. The results showed that the Th17, Treg cells, related factors and transcription factors were all significantly decreased compared with before treatment; while no significant changes were observed in the non-remission patients, which suggested that the therapeutic effect and prognosis of acute leukemia were largely related to the expression level of Th17 and Treg in immune cells and the functional factors in the patients with acute leukemia. Such results was consistent with the findings in the study by Verma, Chandan on breast cancer (Verma *et al*, 2013).

CONCLUSION

All in all, Th17 and Treg cells and related cytokines play an important role in the pathopoiesis of AL. The increase of Th17 and Treg cells and their functional factors may be an important reason for the complications and death of acute leukemia in clinic, and they are closely related to the prognosis. Therefore, Th17 and Treg cells have a significant guidance in the clinical treatment and prognosis.

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