Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

Analysis of peripheral blood T-cell subsets and regulatory T-cells in multiple myeloma patients

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Received January 16, 2018; **Accepted** April 23, 2018; **Published** April 30, 2018

Doi: http://dx.doi.org/10.14715/cmb/2018.64.5.19

Abstract: To study the peripheral blood T-cell subsets and regulatory T-cells of multiple myeloma (MM) patients. 48 MM patients and 24 healthy controls were enrolled. Changes in peripheral blood T-cell subsets in the MM patients i.e. $CD4^+CD25^+$T cells and $CD4^+CD25^+CD127^{low}$T regulatory cells ($CD4^+CD25^+CD127^{low}$Tregs) and in healthy controls were measured using flow cytometry and immunohischemistry. The total T-cells ($CD3^+$) in peripheral blood lymphocyte and auxiliary/induced T-cells ($CD3^+CD4^+$ T cell) of the 48 MM patients showed no statistical significance when compared with those of the control group. Suppressor/cytotoxicity T-cells ($CD3^+CD8^+$ T cell) increased ($p < 0.05$). $CD4^+CD25^+$T cells and $CD4^+CD25^+CD127^{low}$ Tregs were significantly higher than corresponding values in the healthy group ($p < 0.05$). The $CD4^+/CD8^+$ T cell ratio of Stage III MM patients was significantly lower than that of the control group ($p < 0.05$). The $CD4^+CD25^+$T cells and $CD4^+CD25^+CD127^{low}$ Tregs of MM patients in the stable and the progressive stages were significantly higher than those of MM patients in the control group ($p < 0.05$). The abnormality of the peripheral blood T-cell subset, increased expression of $CD4^+CD25^+CD127^{low}$ Tregs, and low cellular immunity of MM patients are related to clinical staging and progression of the disease. The quantity of $CD4^+CD25^+CD127^{low}$Tregs of peripheral blood cells of MM patients could be significantly increased through the inhibition of $CD4^+$ and $CD8^+$T cell activities. $CD4^+CD25^+CD127^{low}$ Tregs promotes tumor growth through the inhibition of immunologic cell proliferation. Immunological dysfunction based on Tregs cells plays an important role in the pathogenic course.

Key words: Multiple myeloma; T cell subsets; Regulatory T cells; Flow cytometry.

Introduction

Multiple myeloma (MM), a clonal disease associated with plasma cell abnormality, results in renal damage and bone destruction. The incidence of MM is increasing globally. Statistics have shown that the incidence of MM exceeds that of leukemia; MM patients account for approximately 1% of all tumor patients, and about 10% of patients with malignant tumors of the hemopoietic system and is the second largest malignant tumor of the blood system (1-3). Given the abnormal proliferation of malignant plasma cell immune escape, monoclonal gammopathy of undetermined significance results in abnormalities in cellular immunity and humoral immunity function abnormalities. Therefore, lymphocytes involved in immune responses play an important role in MM incidence. T-regulatory cells (Tregs) are involved in the negative regulation of the immune system. They play an important role in tumor immuno-suppression and immune escape (4). The negative immune regulation mechanisms of Tregs occur through many routes such as the direct killing of effector T-cells by contacting sthem , or dendritic cells (DC), and activating the Fas/FasL signal pathway; inducing effector T-cell apoptosis through the competitive binding of high expression $CD25^+$ molecules (IL-2 receptor) and IL-2; and inhibiting multiple immune cells by secreting inhibitory cytokines (TGF-β and IL-10) (5 – 7). The most frequently studied Tregs is $CD4^+CD25^+$Tregs. Natural $CD4^+CD25^+$Tregs account for approximately 5 to 10% of all $CD4^+$T cells in peripheral blood and the spleen in normal people and mice. Recent studies indicate that the specific expression of $CD4^+CD25^+$ Tregs transcription factor Foxp3 is responsible for the development, activation, and functioning of Tregs, and is considered to be a specific marker of Tregs (8, 9). However, a recent study found a new Tregs indicator, CD127. The application of CD127, a cell-surface antigen, provides more convenient and effective means of detecting $CD4^+CD25^+$Tregs.

Numerous studies have proven that solid tumors (e.g., lung cancer, ovarian cancer, breast cancer) are associated with abnormalities in the quantity and function of Tregs, and that the progression and prognosis of some types of tumors are negatively correlated with the quantity of Tregs (10 - 12). In recent years, numerous studies have been conducted on Tregs in hematological tumors, finding that Tregs expression increases in local tumors and the peripheral blood of Hodgkin lymphoma patients (9). In addition, $CD4^+CD25^+$Tregs expression has been shown to increase in peripheral blood, reflecting the poor ability of acute myelocytic leukemia patients to resist apoptosis (13). Immunological T-cells have been shown to be involved in the pathogenesis of MM. Multiple myeloma (MM) involves B/T/NK/DC cell dysfunction, but the imbalance in Treg cells is a key factor

in its pathogenesis. Analysis of T-lymphocyte subsets in peripheral blood of MM patients indicated that the percentage of Th1 and Th17 cells were significantly higher, while that of Treg cells was lower when compared with healthy controls (14). The imbalance between Th17 and Treg cells has become a key factor in MM (14). It has also been shown that the amount of Tregs in the peripheral blood of newly diagnosed MM patients, relapsed MM patients, and MM patients in remission is significantly higher than that of the control group (15).

Multiple myeloma (MM) is an immunological effector cell, and immunological dysfunction is a significant cause and serious consequence of MM. The abnormal Tregs in MM patients is the key component of a tumor immunity tolerance network and may arguably be an important reason for immuno-suppression. In particular, Tregs play an important role in the case of less residual myeloma cells in the body post-treatment. Therefore, many scholars have studied $CD4^+CD25^+CD127^{low}$T cells as Tregs.

Although there are many reports on the relationship between Tregs and MM, there are few studies in Chinese patients. Moreover, these studies were not targeted at different clinical stages. In the present study, the MM immune micro- environment status were studied by using flow cytometry to detect peripheral T-cell subsets and the $CD4^+CD25^+CD127^{low}$ Tregs of 48 Chinese MM patients and 24 healthy volunteers. This aimed to provide a stronger basis for immune mechanisms, clinical treatment targets, and MM-related prognosis assessment.

Materials and Methods

Subjects

The study subjects were 48 MM patients seen in the Department of Hematology, Yijishan Hospital, The First Affiliated Hospital of Wannan Medical College from 2012 to 2015. They consisted of 25 male patients and 23 female patients, with a median age of onset being 63 years. All 24 control group patients were deemed to be healthy through physical examination from the physic al examination center of the hospital, and included 12 males and 12 females, with a median age of 61 years.

ISS-based disease staging: Stage I: serum beta-2 microglobulin < 3.5mg/L, serum albumin ≥ 3.5 g/dL, Stage II: No ISS Stages I or III; Stage III: serum beta-2 microglobulin ≥5.5 mg/L. There were 11, 12, and 25 patients in Stages I, II and III, respectively. Condition-based staging: (a) criterion for the stable stage: changes in M protein level and light chain secretion < 25% lasting for at least 3 months. (b) criteria for the progressive stage: at least one of the following conditions must be met: serum or protein electrophoresis or immuno-fixation electrophoresis M protein recurrence; bone marrow puncture or biopsy result indicating plasmacyte ≥ 5%; newly discovered bone injury or soft-tissue plasmacytoma or increase in the original size; and hypercalcemia (>11.5mg/dL or > 2.65 mmol/L), can be taken as an independent factor reflecting plasmacyte proliferation disorder. This group included 12 patients in the stable stage and 36 patients in the progressive stage which there were 30 patients in stage 3 and 6 patients in stage 2.

Reagent and materials

Lymphocyte separation medium was purchased from American Cedarlanem Company. CD3-FITC, CD4-FITC, CD4-APC, CD8-PE, CD25-PE, CD127-APC, and other antibodies were purchased from American BD Company. Cleaning solution, PBS containing 1% FBS, and 0.1% sodium azide were stored at 4°C. PBS containing 1% paraformaldehyde was also stored at 4°C. FACS Aria IICell Sorter flow cytometry (BD, USA) and CellQuest software were used for data analysis.

Determination of T cell sub-sets

Anticoagulated whole blood (50μL) was mixed evenly with 20μL of antibody (CD3, CD4, CD8, or any other antibody). Incubation was conducted at room temperature (25 °C) for 15 minutes away from light. Then, red blood cell lysis buffer (1.5mL) was added and mixed. The solution was incubated at room temperature (25 °C) for 15 minutes away from light, and centrifuged (1,000 rpm, 5min). The supernatant was discarded, and PBS (1.5mL) was added. The solution was mixed evenly and centrifuged (1,000 rpm, 5 minutes). The supernatant was discarded, and the solution was analyzed.

Assay of Tregs

Peripheral blood (2mL) was drawn and added to the EDTA anti-coagulation tube. The mononuclear cell (buffy coat) was extracted with a lymphocyte separation medium. PBS washing was undergone twice. The solution was centrifuged, and the supernatant was discarded. A single-cell suspension was made. Cell concentration was adjusted, and 100μl of the solution was added to the FCM detector tube containing 20μL of antibodies (i.e., CD3, CD4, CD25, and CD127), and the solution was mixed evenly. Incubation was conducted for 15 minutes in the dark, and the solution was washed twice with PBS containing 1% FBS and 0.1% sodium azide. The supernatant was discarded, and PBS re-suspension cells (300 mL) containing 1% paraformaldehyde were added. The solution was stored at 4°C in the dark prior to analysis.

Statistical analysis

All statistical data was analyzed using SPSS 15.0 statistical software. Measurement data of each group is expressed as $\bar{x}\pm s$. One-way analysis of variance (ANOVA) was used for comparison. Then, student t-test was used for two-group comparisons. Values of $p < 0.05$ were taken as indicators of statistically significant differences.

Results

A comparison of peripheral blood T cell subsets of MM patients and healthy people showed no statistically significant differences with respect to peripheral blood total $CD3^+$T cell level and $CD4^+$T cell level ($p > 0.05$). However, the $CD8^+$T cell level of MM patients was significantly higher than that of the control group ($p < 0.05$) and the $CD4^+/CD8^+$T ratio in MM patients was significantly lower than that of the healthy control group ($p < 0.05$). These results are shown in Table 1.

A comparison of peripheral blood Tregs of MM patients and Tregs in the control group indica-

Table 1. Comparison of peripheral blood T cell subsets of MM patients and the control group (cells/mm3).

Group	N	CD3+	CD4+	CD8+	CD4+/CD8+
MM patients	48	672.51±91.54	459.47±87.41	364.66±87.66*	1.26±0.29*
Control group	24	657.19±106.24	468.21±91.22	310.07±76.85	1.51±0.36

Comparison with the control group* P<0.05.

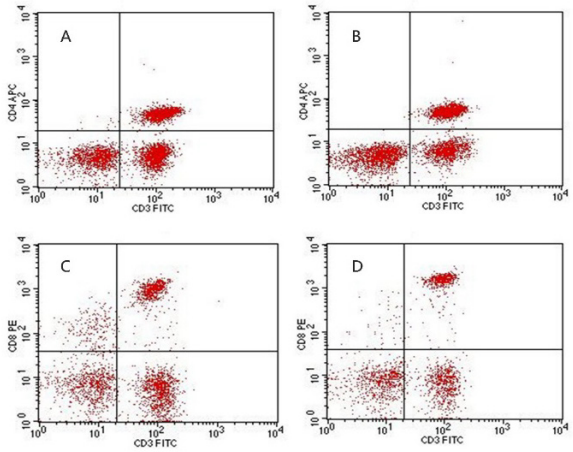

Figure 1. Characterization of peripheral blood T-cell subsets of MM patients relative to healthy donors. A comparison of peripheral blood T-cell subsets of MM patients and the healthy control group showed that the $CD4^+$T cell percentage of MM patients and the healthy control group had no statistically significant difference ($p > 0.05$), while the $CD8^+$T cell level of MM patients was significantly higher than that of the control group ($p < 0.05$). (A) = $CD4^+$T cells in MM patients; (B) = $CD4^+$T cells in healthy control; (C) = $CD8^+$T cells in MM patients; (D) = $CD8^+$T cells in healthy control.

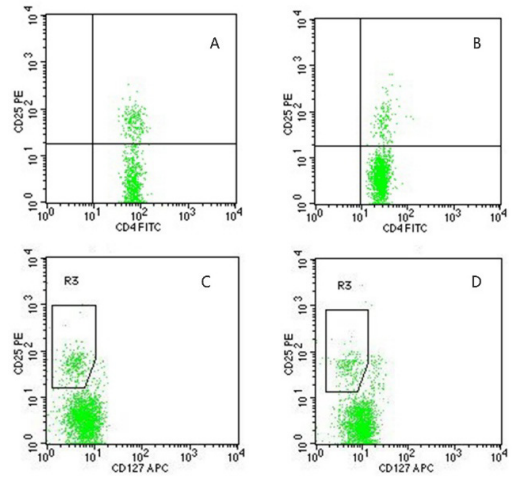

Figure 2. Characterization of peripheral blood $CD4^+CD25^+$ T-cells and $CD4^+CD25^+$ $CD127^{low}$Tregs of MM patients and healthy donors. A comparison of peripheral blood Treg of MM patients and the control group showed that peripheral blood $CD4^+CD25^+$T cells and $CD4^+CD25^+CD127^{low}$T cells of MM patients were significantly higher than those of the control group ($p < 0.05$). (A) = $CD4^+CD25^+$ T cells in MM patients; (B) = $CD4^+CD25^+$ T cells in healthy controls; (C) = $CD4^+CD25^+$ $CD127^{low}$ T cells in MM patients; (D) = $CD4^+CD25^+$ $CD127^{low}$ T cells in healthy controls.

ted that peripheral blood $CD4^+CD25^+$T cells and $CD4^+CD25^+CD127^{low}$Tregs of MM patients were significantly higher than those of the control group ($p < 0.05$; Figure 2, Table 2)

Changes in T-cell subsets of MM patients in different clinical stages indicated that the percentage of $CD3^+$T cell of MM patients showed no statistically significant differences with each other and the control group ($p > 0.05$). In addition, the total $CD4^+$T cell, $CD8^+$T cell, and $CD4^+/CD8^+$T cell ratio of MM patients at different stages showed no statistical differences ($p > 0.05$). However, a comparison of MM patients in Stage III and the control group showed a statistical difference with respect to these parameters ($p < 0.05$, Table 3).

Levels of Tregs were changed in different clinical stages of MM patients. The $CD4^+CD25^+$T cells in MM patients in Stage III and $CD4^+CD25^+$T cells in MM patients in Stage II showed no statistical significance ($p > 0.05$) but were significantly higher than the corresponding levels of the control group ($p < 0.05$). The $CD4^+CD25^+CD127^{low}$ Tregs of MM patients in Stage III were significantly higher than that of MM patients in Stage I ($p < 0.05$), and that of the control group ($p < 0.01$) (Table 4).

Tregs of MM patients were changed in different stages. In the stable and the progressive stages, Tregs were higher than those of the control group ($p < 0.05$). Further , Tregs of MM patients in the progressive stage were higher than those of MM patients in the stable stage ($p < 0.05$, Table 5).

Table 2. Comparison of peripheral blood CD4+CD25+T cells and CD4+CD25+CD127lowTreg of MM patients and the control group (cells/mm3).

Group	n	CD4+CD25+	CD4+CD25+CD127low
MM patients	48	231.22±20.75*	159.74±19.45*
Control group	24	134.53±18.45	98.45±10.36

Comparison with the control group * P<0.05.

Table 3. Comparison of peripheral blood T-cell subset of MM patients in different clinical stages and the control group(cells/mm^3).

Group	N	CD3+	CD4+	CD8+	CD4+/CD8+
Stage I	11	659.77±89.41	412.33±56.14	429.51±53.16	0.96±0.31
Stage II	12	648.21±90.32	423.16±54.62	475.46±62.12	0.89±0.56
Stage III	25	661.36±85.71	397.95±48.69	765.29±98.41*	0.52±0.72*
Control group	24	655.94±77.69	406.55±51.37	269.24±38.45	1.51±0.36

Compared with the control group, * p < 0.05.

Table 4. Comparison of levels of CD4+CD25+T cells and Treg cells of MM patients at different clinical stages and the control group(cells/mm^3).

Group	N	CD4+CD25+	CD4+CD25+CD127low
Stage I	11	263.74±35.41	103.52±13.64
Stage II	12	271.34±34.16*	122.27±15.11*Δ
Stage III	25	351.66±38.15	203.18±20.33
Control group	24	232.32±32.22	95.67±10.39

Compared with the control group, * $p < 0.01$; compared with Stage I Δ $p < 0.05$.

Table 5. Comparison of CD4+CD25+T cells and Treg cells levels of MM patients in different stages and the control group(cells/mm^3).

Group	N	CD4+CD25+	CD4+CD25+CD127low
Stable stage	12	287.55±26.41*	190.27±15.74*
Progressive stage	36	368.65±32.17*Δ	285.42±20.76*Δ
Control group	24	203.33±19.52	103.45±9.69

Compared with the control group, * $p < 0.05$; compared with the stable stage, Δ $p < 0.05$.

Discussion

With the continuous developments in molecular biology and immunology, mechanisms related to immunological dysfunction have been gradually revealed in recent years. Anti-tumor responses in the body are mainly implemented by T-cells, in which $CD4^+$T cells are classified into three types of effector cells, namely Th1, Th2 and Th17. Th1 and Th2 function in cellular and humoral immunities respectively, while Th17 is involved in innate immunity. $CD8^+$T cells are composed of cytotoxic and inhibitory T-cells. Therefore, lymphocytes involved in immune responses play an important role in the occurrence of MM. Tregs play a negative regulatory role in the body's immune system, in immunosuppression and escape (18). The traditional analytical method for $CD4^+CD25^+$ Tregs uses the markers CD4 and CD25(IL-2Ra) for detection. Yet, as research in this area deepened, it was found that defining Tregs in terms of $CD4^+CD25^+$ alone was no longer appropriate. Foxp3 is a member of the fork-like transcription factor family and a specific marker of $CD4^+CD25^+$Tregs. However, as an intra-nuclear protein, it requires a membrane rupture marker, and its *in vitro* assay is rather complicated. A study in 2006 revealed that the Tregs cell marker CD127, a cell-surface antigen, could be used to achieve high-purity Tregs. Recent studies have proven that CD127 (IL-7Ra) expression is negatively correlated with Foxp3, which means that $CD4^+CD25^+CD127^{low}$ marker Tregs and $CD4^+CD25^+Foxp3^+$ marker cells are of the same cell mass, and that flow cytometry is convenient, fast, stable, and more suitable for clinical applications (8).

Multiple myeloma (MM) is a malignant tumor of the immune lymphatic system, the cause of which remains unidentified . The MM patients had dysfunctional B, T, NK, and DC cells, and abnormal Tregs. The current study found that the peripheral blood $CD3^+CD8^+$ of MM patients at the first visit was significantly higher than that of the healthy control group, which implies an imbalance in the $CD4^+/CD8^+$ T cell ratio, consistent with previous reports (19). The probable mechanism is that the decrease in CD4+ T cells in MM may be mediated by the activation of CD8+ T cell-derived cytokine IL-16 (21, 22). The findings in this study indicate that disorders in percentages of T-cell subsets are closely related to the occurrence and progression of MM. These patients showed abnormal increases in $CD8^+$T. The traditional $CD8^+$ T cells are unlikely to have normal antineoplastic functions and are in the deactivation or even tolerance state for tumor cells; they play an immunosuppressive role. Further studies involving patients at different clinical stages indicated that that $CD4^+CD25^+$T cells and $CD4^+CD25^+CD127^{low}$Tregs in MM patients of Stages II, and Stage III were significantly higher than those of the control group, and those of MM patients in Stage I (22). In the current study, it was found that Tregs also changed in different clinical stages of MM, but although $CD4^+CD25^+$T cells of MM patients in Stage III and those in Stages I and II showed no statistical significance, they were significantly higher than corresponding values in the control group. Moreover, $CD4^+CD25^+CD127^{low}$ Tregs of MM patients in Stage III were significantly higher than those of MM patients in Stage I, and those of the control group. Previous research showed that the peripheral blood Tregs increases with disease progression. However, the results of the present study on the analysis of $CD4^+/CD8^+$T cells of MM patients at different clinical stages showed that only patients in Stage III had statistically significant differences to the control group, and those in Stages I and II did not. This finding might be related to the fact that there were more MM patients in Stage III than in Stages I and II. It also indicates that the cellular immunity function of MM patients should not be judged based on $CD4^+$ and $CD8^+$T alone. The quantity of $CD4^+CD25^+$Tregs requires more specific analysis.

Multiple myeloma patients have reduced $CD4^+/CD8^+$T cell ratio and increased percentage of $CD4^+CD25^+$ $CD127^{low}$Tregs. These changes are related to clinical staging and progression of the disease. The quantity of $CD4^+CD25^+CD127^{low}$Tregs of peripheral blood of MM patients could be significantly increased through the inhibition of $CD4^+$ and $CD8^+$T cell activity. $CD4^+CD25^+CD127^{low}$ Tregs promotes tumor growth through the inhibition of immunologic cell proliferation. Immunological dysfunction based on Tregs cells plays an important role in the pathogenic course of MM. However, the specific mechanism is not known and requires further study.

Patients' consent

Written informed consent was obtained from the pa-

tients for publication of this paper.

Conflict of interest statement

None declared.

Acknowledgments

We give sincere thanks to Jun Zhang, Guangxi Li and Xiaoke Jin for assistance with bone marrow immunohistochemistry. We thank Lanfang Zhang for assistance with the flow cytometry.

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