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

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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⁻/⁻ T regulatory cells (CD4⁺CD25⁺CD127⁻/⁻ Tregs) and in healthy controls were measured using flow cytometry and immunohistochemistry. 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⁻/⁻ 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⁻/⁻ 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⁻/⁻ Tregs, and low cellular immunity of MM patients are related to clinical staging and progression of the disease. The quantity of CD4⁺CD25⁺CD127⁻/⁻ 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⁻/⁻ 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 gammapathy 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 immunosuppression 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 them, 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. The imbalance between Th17 and Treg cells has become a key factor in MM. 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.

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+CD127low 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 was studied by using flow cytometry to detect peripheral T-cell subsets and the CD4+CD25+CD127low 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 physical 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 plasma cell ≥ 5%; newly discovered bone injury or soft-tissue plasmacytoma or increase in the original size; and hypercalcaemia (>11.5mg/dL or > 2.65 mmol/L), can be taken as an independent factor reflecting plasma cell 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 Cedarlane 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 II Cell 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 ±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-
T-cells in MM patients.

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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\(^+\)CD127\(^{lo}\)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).

Levels of Tregs were changed in different clinical 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).

| Group                  | n    | CD4+CD25+ | CD4+CD25+CD127\(^{lo}\) |
|------------------------|------|-----------|-------------------------|
| MM patients            | 48   | 231.22±20.75* | 159.74±19.45*       |
| Control group          | 24   | 134.53±18.45  | 98.45±10.36           |

Compared with the control group, * \(p < 0.05\).

| 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, * \(p < 0.05\).
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+CD127low 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+ 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+CD127low 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+CD127low 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+CD127low Tregs. These changes are related to clinical staging and progression of the disease. The quantity of CD4+CD25+CD127low Tregs of peripheral blood of MM patients could be significantly increased through the inhibition of CD4+ and CD8+ T cell activity. CD4+CD25+CD127low 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-

| 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/mm³). |
| --- |
| 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/mm³). |
| --- |
| 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.
tients for publication of this paper.

Conflict of interest statement
None declared.

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