

Flow Cytometric Phenotyping and Analysis of T Regulatory Cells in Multiple Myeloma Patients

Fenotypizace a kvantifikace T regulačních lymfocytů u pacientů s mnohočetným myelomem pomocí průtokové cytometrie

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Summary

Multiple myeloma (MM) is a plasma cell (PC) disorder and associated with immune impairments. Flow cytometry based phenotyping and quantification of regulatory T cells (Tregs) enable to monitor the immune status of myeloma patients. Apart from enumeration of Tregs, functional characterization using proliferation or suppression assay adds key value in demonstrating the functional value of Tregs. Our study revealed that in MM patients Tregs are elevated compared to healthy subjects, which demonstrate the immune deregulation in MM.

Key words

Tregs – flow cytometry – multiple myeloma – thalidomide

Souhrn

Mnohočetný myelom (MM) je onemocnění plazmatických buněk (PC), které bývá často spojeno s poruchami imunity. Fenotypizace a stanovení počtu regulačních T lymfocytů (Tregs) pomocí průtokové cytometrie může být využito k monitorování stavu imunity u myelomových pacientů. Charakterizace funkčního stavu Tregs pomocí proliferačních či inhibičních testů pak může odhalit jejich možnou poruchu. V naší studii bylo zjištěno, že u pacientů s MM jsou počty Tregs zvýšeny oproti zdravým kontrolám, což u těchto pacientů svědčí o deregulaci imunity.

Klíčová slova

Treg buňky – flow cytometrie – mnohočetný myelom – thalidomid

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Introduction

Multiple myeloma (MM) is a malignant plasma cell (PC) disorder characterized by higher ($\geq 10\%$) PC infiltrations and ≥ 30 g/L of monoclonal protein (M-protein), whereas monoclonal gammopathy of undetermined significance (MGUS) characterized by $< 10\%$ of PC infiltrations and < 30 g/L of M-protein [1,2]. There are evidences available in MM for impaired T cells counts and functional abnormalities [3]. Recently, a study proved increased number of regulatory T cells (Tregs) in cancer patients [4]. Tregs plays active role in establishing and maintaining immunological unresponsiveness to self antigens and negative control of various immune responses to non-self antigens [5]. Regulatory function for Tregs is provided by a master molecule FoxP3. At present, several studies proved that Tregs were expanded both in hematological malignancies and solid tumors [6,7].

Subtypes of T regulatory cells:

Natural Tregs – Arise from thymus and disseminate to periphery; these cells constitute about 10%–15% of CD4 cells. Destruction in the development or function of natural Tregs leads to autoimmune diseases [8].

Tr1 regulatory cells – Induced from the peripheral naïve T cells in the presence of IL-10. These cells lack FoxP3 expression but secrete IL-10 and TGF (transforming growth factor)- β [9].

Th3 cells – Induced from the peripheral naïve T cells in the presence of TGF- β . Secrete mostly TGF- β for suppression. Rare Th3 cells express FoxP3 molecule due to induction by TGF- β [10].

Double negative (DN) Tregs – In mice and humans, these cells constitute about 1–3% and 1%, respectively. DN Tregs inhibit T cell activation and proliferation in antigen-specific manner [11].

$\gamma\delta$ T cells – Suppress naïve and effector T cell responses and inhibit maturation and function of dendritic cells [12].

NKT (natural killer T) regulatory cells – CD1d dependent/restricted type II NKT cells are able to suppress tumor immune surveillance, but type I NKT cells lack suppressive function [13].

Functions of T regulatory cells:

Release of inhibitory cytokines – Mainly cytokines such as IL-10, TGF- β and IL-35 are secreted by Tregs that are involved in inhibitory function. Peptide inhibitor targeted against the surface TGF- β on Tregs abrogates their function and enhances the anti-tumour response [14].

Cytotoxicity – Perforin/granzyme pathway is well-known to be associated with CD8 T cells and NK cells for destruction of intracellular pathogens and tumour cells. Recent studies have shown Tregs also use perforin/granzyme pathway [15,16].

Inhibition of antigen presenting cells (APCs) – Expression of cytotoxic T

lymphocyte antigen-4 (CTLA-4) under the control of FoxP3 by Tregs facilitates the interaction with APCs co-stimulatory molecules CD80 and CD86 and induces suppression of T cell activation [17].

Recommended Methodology for Identification and Characterization of T Regulatory Cells

Flow Cytometry Method for Identification of T Regulatory Cells

Flow cytometry based identification of Tregs is a feasible method. Globally, a simple three-color flow cytometric analysis including CD4, CD25 and FoxP3 is able to characterize and quantify the Tregs in MM. To characterize the Tregs more precisely we use four-color cytometry with the inclusion of additional marker CD127 and the protocol is summarized here. One-two millions of erythrocytes lysed peripheral blood (PB) cells are labeled with the following fluorochrome conjugated monoclonal antibodies: phycoerythrin-cyanin 7 (PE-Cy7)-CD4, allophycocyanin (APC)-CD25 and phycoerythrin (PE)-CD127, and incubated at 4°C for 20–30 min (all monoclonal antibodies are obtained from BD Biosciences). Then, cells are fixed and permeabilized according to eBioscience recommendations (eBioscience, San Diego, CA), and finally, cells are labeled with FoxP3 antibody conjugated with fluorescein isothiocyanate (FITC) from eBioscience and incubated at 4°C for 30–60 min. All prepared samples are measured on BD FACSCanto II™ and approximately 300–400 thousands of events are acquired to enumerate the Tregs. For negative control, FITC conjugated isotype antibody is used. We usually identify and quantify Tregs by the phenotype CD4+CD25hi+FoxP3+ along with presence or absence of CD127 (Fig. 1). Inclusion of CD127 in the analysis will add value in defining the Tregs because these cells usually have dim/negative expression for CD127.

Methods for Functional Characterization of T Regulatory Cells

Two methods such as 5, 6-carboxyfluorescein-diacetate succinimidyl-ester (CFSE) and 3 H-thymidine could be

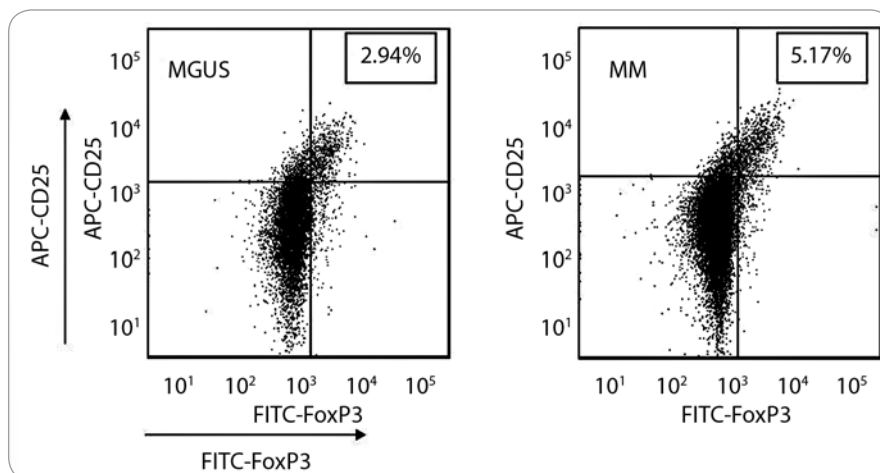


Fig. 1. Phenotypic feature of T regulatory cells. These two dot plots represent the typical phenotypic nature of Tregs (CD4+CD25hi+FoxP3+). Dot plot in the left (MGUS) demonstrates decrease in the frequency of Tregs when compared to MM dot plot in the right.

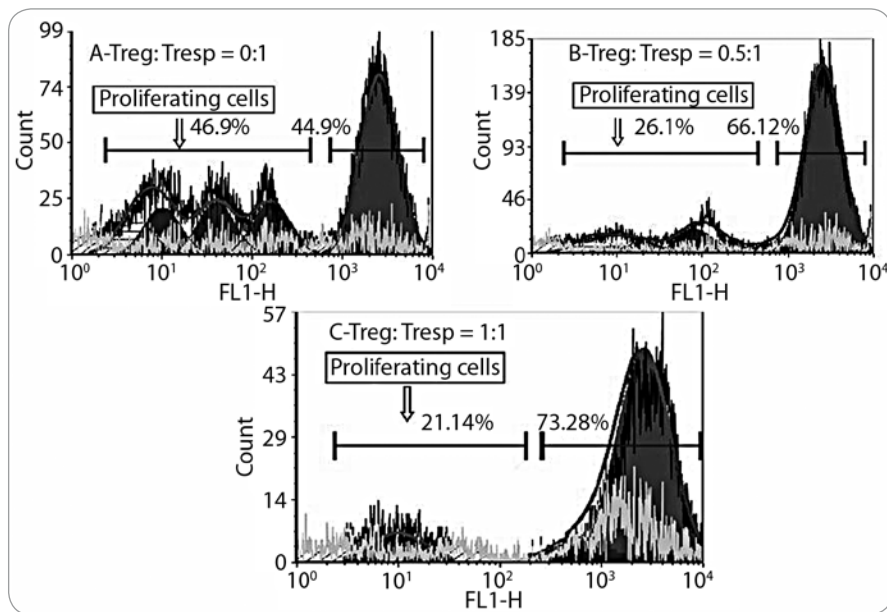


Fig. 2. Functional characterization of multiple myeloma T regulatory cells. These histograms demonstrate that on the basis of increasing concentration of Tregs, the proliferation of responder or naïve T cells (CD4+CD25-) decreases [21].

used for assessing the suppressive function of Tregs according to literature. The assay includes the separation of Tregs (CD4+CD25hi+) and responder cells (CD4+CD25-) either by magnetic bead separation or flow cytometry based se-

paration. Tregs are mixed in different proportions to stimulated responder cells with anti-CD3/anti-CD28 beads or irradiated allogenic peripheral blood mononuclear cells (PBMNCs) along with CFSE labeling. When there will be pro-

liferation of responder cells CFSE intensity will decrease, the duration of assay period is 4–6 days (Fig. 2) [21]. In ³H-thymidine assay after incubation of Tregs and stimulated responder cells together for 3 days. The proliferation of responder cells is measured by incorporation of ³H-thymidine for the last 18 hours during the incubation period [19,20].

Our Study Experience of T Regulatory Cells in Multiple Myeloma Patients

We analyzed Tregs in PB of 69 monoclonal gammopathy (MG) patients including MGUS- (11/69), SMM- (5/69), newly diagnosed MM- (40/69) and relapsed MM- (13/69). For comparison, 12 healthy volunteers PB was also analyzed (Tab. 1). A cohort of 20 newly diagnosed MM patients were followed-up to determine the PB Tregs number after 4 treatment cycles with thalidomide plus cyclophosphamide and dexamethasone (CTD). To analyze the difference between two groups Mann-Whitney U test was used and P value of ≤ 0.05 was considered as statistically significant.

Results and Discussion

In consistent with other studies, our study also showed PB Tregs were increased in MM patients (Tab. 1) [18,19]. In contrast to Beyer et al and Feyler et al studies we did not observe any significant expansion of PB Tregs in MGUS and SMM cohorts compared to healthy volunteers [18,19]. Prabhala et al study showed significantly reduced FoxP3 expressing CD4 T cells in MGUS and MM patients which is contrasting to our observation and other studies [18–20]. This conflicting result might be due to the use of different identification strategy. For instance, Prabhala et al and Brimnes et al studies used only CD4 and FoxP3 molecules for identification [20,22]. British study characterized and quantified the Tregs as CD4+CD25hi+FoxP3+, which is the globally accepted phenotypic feature of Tregs [19]. In contrast, German study identified Tregs as CD4+CD25hi+ [18]. This identification will give the information about 80–90% of Tregs but with out combination of FoxP3 one might not completely characterize Tregs. Gupta

Tab. 1. Comparison of peripheral blood Tregs frequencies between healthy volunteers and MM patients.

| Healthy volunteers (n = 12) | Patient cohort (n) | median % (range %) | P value |
|-----------------------------|--------------------|--------------------|--------------|
| 4.62 (3.40–6.71) | MGUS (11) | 5.02 (2.11–7.34) | 0.15 |
| | SMM (5) | 3.99 (3.03–7.48) | 0.29 |
| | MM (40) | 5.66 (2.74–11.73) | 0.056 |
| | Relapsed MM (13) | 6.80 (4.80–8.75) | 0.008 |

MGUS – Monoclonal gammopathy of undetermined significance, SMM – Smoldering multiple myeloma, MM – Multiple myeloma

Tab. 2. Assessment of Tregs frequencies between pre treatment versus post treatment cycles (CTD).

| Pre treatment (n = 20) | Post treatment (n) | median % (range %) | P value |
|------------------------|--------------------|--------------------|---------|
| 5.39 (2.74–11.73) | 1st cycle (17) | 4.97 (2.48–10.58) | 0.76 |
| | 2nd cycle (17) | 5.94 (3.64–26.98) | 0.35 |
| | 3rd cycle (13) | 6.14 (2.70–17.89) | 0.083 |
| | 4th cycle (9) | 7.03 (3.41–9.09) | 0.069 |

CTD – Cyclophosphamide, thalidomide and dexamethasone

et al used CD127 along with CD25 to identify Tregs [21].

Tregs in MM patients were proved to be functionally active as similar to healthy volunteers Tregs [18,19,22]. Exclusively, Prabhala et al study showed MM patients Tregs failed to suppress the proliferation of responder cells when compared to healthy subjects Tregs [20]. This contrasting result by Prabhala et al study might be due to the use of PBMCs as responder cells [20]. In concordance with *in vitro* findings in MM, an *in vivo* study showed, after allogeneic stem cell transplantation the donor-derived Tregs reconstituted in the bone marrow (BM) were functional and also enhanced the survival of transplant without graft versus host disease [23]. As similarly to MM, dysfunctional and increased number of Tregs were also documented in various hematological malignancies including B-cell chronic lymphocytic leukemia (B-CLL), acute myeloid leukemia and non-Hodgkins lymphoma [7,24,25].

Our observation showed a trend of increase in Tregs number (Tab. 2) after treatment with thalidomide combination (CTD), but in B-CLL patient's substantial decrease in Tregs number was reported after thalidomide plus fludarabine treatment [26]. The possible reason behind the observation of increased Tregs after thalidomide is IL-6 has the ability to decrease the number of Tregs, which was proved in murine models. Well known functions of thalidomide in MM are downregulation of various adhesion molecules on PCs and cytokine molecules such as IL-6, TNF- α and RANKL [27]. From this point we could able to clarify that after thalidomide treatment the level of IL-6 reduces and as a cascade the Tregs increase in MM patients. Unprecedentedly, our data showed positive association between Tregs and BMPC infiltrations ($r = 0.25$; $P = 0.034$).

In summary flow cytometry based analysis of Tregs is a useful method which facilitates to understand the immune status in MM patients. This technique is also feasible to monitor the MM patients before and after treatment to screen the changes of regulatory and immune cells. Using flow cytometry, several studies and our observation proved that Tregs were elevated in MM patients. This observation should be taken into consideration to improve the immune status in myeloma patients by following different treatment approaches.

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