Dysfunctional T regulatory cells in multiple myeloma

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Abstract

Multiple myeloma (MM) is characterized by production of monoclonal immunoglobulin, associated with suppressed uninvolved immunoglobulins and dysfunctional T cell responses. The biological basis of this dysfunction remains ill defined. Since T regulatory (T_{reg}) cells play an important role in suppressing normal immune responses, we have here evaluated the potential role of T_{reg} cells in immune dysfunction in MM. We observed a significant increase in CD4^{+}CD25^{+} T cells in individuals with monoclonal gammopathy of undetermined significance (MGUS) and patients with MM compared to normal donors (25% and 26% versus 14%, respectively); however, T_{reg} cells as measured by Foxp3 expression are significantly decreased in both MGUS and MM compared to normal donors. Moreover, T_{reg} cells in MM and MGUS even when added in higher proportions are unable to suppress anti-CD3-mediated T cell proliferation. This decreased number and function of T_{reg} cells in MGUS and in MM may account, at least in part, for the non-specific increase in CD4^{+}CD25^{+} T cells, thereby contributing to dysfunctional T cell responses.
Introduction

T regulatory (T_{reg}) cells play an important role in the maintenance of self-tolerance, control of auto-immunity, and regulation of T cell homeostasis as well as modulate overall immune responses against infectious agents and tumor cells\(^1\). Natural T_{reg} cells develop during normal T cell maturation in the thymus and represent 5-10% of the CD4\(^+\) cell compartment in the peripheral blood\(^2\). These cells express CD4 and CD25 surface antigens as well as CTLA-4, GITR, CD103, CD62L, CD69, CD134, CD71, CD54, and CD45RA\(^3\). The suppressive activity of T_{reg} cells is associated with over-expression of Foxp3, a member of the forkhead/winged helix family, which acts as a transcriptional repressor\(^4\). T_{reg} cells suppress CD25\(^+\)CD4\(^+\) T cell proliferation on the basis of cell-cell contact and also suppress immune responses by secreting immunosuppressive cytokines like IL10 and TGF-\(\beta\)\(^5\).

A significant impairment of T cell function is observed in both patients with multiple myeloma (MM) and in individuals with monoclonal gammopathy of undetermined significance (MGUS). Although both phenotypic and functional aberrations in CD4 and CD8 cells have been described in MM and MGUS\(^6\text{-}9\), the biological basis for these abnormalities remains unclear. Since T_{reg} cells play an important role in modulating normal immune responses, characterization of T_{reg} cell activity in myeloma patients could contribute to immune dysfunction in MM, as well as provide a new target to enhance immune responses. Therefore, in this study we have evaluated natural T_{reg} cell number and function in MGUS and MM as compared with normal donors.
Study Design

**Phenotypic Characterization:** CD4⁺CD25⁺ T_{reg} cell numbers were analyzed by flow cytometric analyses in peripheral blood mononuclear cells (PBMC) collected from normal healthy volunteers, individuals with MGUS, and newly diagnosed MM patients. Approval was obtained from the DFC and VABHS, Harvard Medical School institutional review board for these studies. Informed consent was provided according to the Declaration of Helsinki.

**Measurement of Foxp3 expression:** Since Foxp3 is specifically expressed by T_{reg} cells and is required for its suppressive activity, we have analyzed the proportion of PBMCs expressing intracellular Foxp3 using anti-Foxp3 antibody (eBiosciences, CA) using both dual color flow cytometry and multiphoton microscopy. Level of protein expression was quantitated by western blotting and by real time RT-PCR, using previously described methods^{10}.

**Suppressive activity of T regulatory cells:** In order to evaluate the function of regulatory T cells, PBMC were first depleted of CD25⁺ T cells (which contain T_{reg} cells) by positive selection using anti-CD25-coated micro beads (Miltenyi Biotec, CA), according manufacture’s instructions^{11}. PBMC depleted of CD25⁺ cells as well as control PBMC containing CD25⁺ cells, were stimulated with anti-CD3 antibody for three days and proliferation was measured by ³H thymidine uptake
during the last 8h of culture. In a separate study purified CD25+ cells were added in various proportions to PBMC depleted of CD25+ cells in order to assess their effects on anti-CD3-induced T cell proliferation.

**Results and Discussion**

We evaluated proportion of CD4+CD25+ cells in peripheral blood of normal donors and patients with MGUS and MM. As seen in Figure 1A, the proportion of these cells in PBMCs was significantly elevated in MGUS (Mean 25±1.8; range 20 to 29) and MM (Mean 26±3.6; range 6-51) compared to normal donors (mean 14±2.3%; range 4-28%) p<0.01. As both T\textsubscript{reg} cells and activated CD4 cells express both CD4 and CD25\textsuperscript{12}, we next evaluated the proportion of cells expressing high levels of CD25, characteristic of cells with regulatory function. As seen in Figure 1B and 1C, we did not observe significant differences in the proportion of CD4+CD25\textsuperscript{high} cells in PBMC from MGUS and MM compared to normal donors.

T\textsubscript{reg} cells express Foxp3, a transcriptional factor required for regulatory as well as suppressive function\textsuperscript{13}. Therefore, we next used flow cytometry to define the proportion of cells expressing both CD4 and Foxp3 in normal donor, MGUS, and MM. As seen in Figure 1D and E, whereas 6.0±0.8% PBMC from normal donors express Foxp3; significantly reduced numbers of Foxp3+ PBMC were detected in MGUS (1.6±0.5%, p<0.01) and MM (0.9±0.4%, p<0.01). This reduction in Foxp3 expressing cells observed by flow cytometry was further
confirmed by immuno-histochemistry using multiphoton microscopy (Figure 1F); as well as by western blot analysis and with quantitative RT-PCR (data not shown). The proportion of PBMCs expressing CTLA-4, a cell surface molecule expressed by T_{reg} cells, is also significantly reduced in MGUS (0.9±0.5%; p<0.01) and MM (1±0.6%; p<0.01) compared to normal donors (6.8±0.6%) (Data not shown). This data therefore show a significantly reduced numbers of T_{reg} in both MGUS and MM compared to normal donors.

Next, we evaluated the regulatory function of T_{reg} cells in MGUS and MM compared to normal donors. To assess function, we measured the ability of T_{reg} cells to suppress T cell proliferation induced by soluble anti-CD3 antibody. PBMC were activated by anti-CD3 antibody in the presence or absence of T_{reg} cells (depleted using anti-CD25 coated micro beads), and proliferation was measured by $^3$H thymidine uptake. In normal donor PBMC, proliferation was significantly suppressed in the presence of (71,770±8,010) versus absence of (115,753±10,113; p<0.05) CD25$^+$ cells (Figure 2A). In contrast, CD25$^+$ cells failed to significantly suppress PBMC proliferation in MGUS and MM (29,813±8,396 vs 39,437±7,463; p=Not Significant, NS and 62,223±10,175 vs 51,893±12,361; p=NS, respectively) (Figure 2A). To account for the reduced frequency of Foxp3$^+$ T_{reg} cells in MGUS and MM, we evaluated their activity following addition of proportionally higher number of T_{reg} cells to CD25-depleted PBMC. As can be seen in Figure 2B, addition of even 10 fold greater number of T_{reg} cells was unable to suppress soluble anti-CD3-mediated T cell proliferation in both MGUS and MM.
These results highlight important T\textsubscript{reg} cell abnormality in MM and MGUS; since natural T\textsubscript{reg} cells are both significantly reduced; and dysfunctional. The biological basis for the reduction and dysfunction of T\textsubscript{reg} cells in MM and MGUS remains undefined. However, in a murine model of asthma, it has been demonstrated that IL-6 and soluble IL-6 receptor (sIL-6R) together decrease T\textsubscript{reg} cell number and function in the lungs. Interestingly, interactions between MM cells and bone marrow stromal cells trigger production of IL-6, as well as number of cytokines and chemokines including TNF-\textalpha, VGEF, IGF-1, SDF-1\textalpha, IL-1\beta, TGF-\beta, and MIP-1\alpha/\beta with immunomodulatory activity \textsuperscript{14,15}. In MM and MGUS, serum levels of both IL-6 and sIL-6R are highly elevated and may therefore play an important role in the T\textsubscript{reg} cell development and function\textsuperscript{16}. Moreover, mice defective in TGF-\beta receptor II expression and signaling also have low numbers of T\textsubscript{reg} cells\textsuperscript{17}. TGF-\beta has also been shown to inhibit IL-2 dependent T-cell proliferation\textsuperscript{18}. In myeloma, TGF-\beta is induced by interactions between MM cells and the bone marrow stromal cells, which may modulate peripheral expansion and maintenance of T\textsubscript{reg} cells.

A second unresolved issue is how dysfunctional T\textsubscript{reg} cells affect immune function in MGUS and MM. The significantly elevated numbers of CD4\textsuperscript{+}CD25\textsuperscript{+} cells, which are predominantly Foxp3\textsuperscript{+}, suggest an increase in activated T cells in MGUS and MM. Low T\textsubscript{reg} cell numbers and/or function generate non-specific immune responses or autoimmunity\textsuperscript{5,19}. If T\textsubscript{reg} cells are so low that immune responses are not suppressed, particularly at the terminal stages of an immune
response, an increase in hyper-reactive T cells may be seen as in myeloma patients\textsuperscript{7}. This hyperactivity of T cells has been associated with defective of TCR-signaling\textsuperscript{20} and increased sensitivity to co-stimulatory signals\textsuperscript{7}. In contrast, recent studies in patients with breast and gastro-esophageal cancers, metastatic melanoma, and Hodgkin lymphoma/CLL have reported increased number of T regulatory cells that may suppress immune responses leading to ineffective anti-tumor immune responses\textsuperscript{21-26}.

These results identify potentially important mediators of immune dysfunction in myeloma. The presence of $T_{\text{reg}}$ cell dysfunction in MGUS suggests a role for $T_{\text{reg}}$ cells even at an earlier stage in the development of disease. Evaluation of $T_{\text{reg}}$ cells at various stages in MGUS may provide further insight into the development and progression of MGUS to MM. Additionally, MM is reported to be associated with number of autoimmune disorders such as thyroid abnormalities\textsuperscript{27}, rheumatoid arthritis\textsuperscript{28}, and renal complications\textsuperscript{29}. It will be intriguing to study whether these conditions are cause or effect, in regards to dysfunctional $T_{\text{reg}}$ cells in MM. If the hyperactive T cells are source of immune dysfunction in myeloma, improving $T_{\text{reg}}$ cell number and function may provide an ideal setting for enhancing immune function and vaccination strategies in future.

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References


Figure Legends

Figure 1. Characterization of T_{reg} cells in MGUS and MM compared with normal donors. (A) PBMC were isolated, incubated with anti-CD4 and CD25 antibodies, and then analyzed by flow cytometry. Results are expressed as % of lymphocytes. Number of samples analyzed in each category is given in parentheses. *Indicates statistical significance (p≤0.01) by Student ‘t’ test analysis. MGUS and MM patients have significantly greater number of CD4^{+}CD25^{+} T cells compared to normal donors. (B) CD4 and CD25 expressing T cells were isolated as above, and cells expressing high levels of CD25 were then analyzed. Results are expressed as % of lymphocytes expressing CD4 and CD25^{high}. Number of samples analyzed in each category is given in parentheses. No significant increase in CD4^{+}CD25^{high} T cells was observed in MGUS and MM patients compared to normal donors. (C) Representative example of flow cytometry data shown above, using normal donor cells, and cells from patients with MGUS and MM. Frequency of CD25 positive cells in the lymphocyte gates was analyzed using anti-CD25 PE antibody along with anti-CD4 FITC antibody. Quadrants were established using isotype controls, and stained cells were analyzed using Cytomics FC 500 (Beckman-Coulter, FA) and CXP software. (D) PBMC were isolated and incubated with anti-Foxp3
antibodies (eBiosciences, CA) for intra-cellular staining, and then analyzed by flow cytometry. Cells in the lymphocyte gates were used for the analysis. Results are expressed as percent of lymphocytes expressing Foxp3, and number of samples analyzed in each category is given in parentheses. A significantly decreased number of Foxp3+ (p<0.01) T<sub>reg</sub> cells were observed in MGUS and MM patients compared to normal donors. (E) Frequency of Foxp3 positive cells in the lymphocyte gates was analyzed using anti-Foxp3 PE antibody. Dual color analysis (PE-Foxp3 and Pc5-CD4) was optimized and used in these studies. Quadrants were established using isotype controls, and stained cells were analyzed using Cytomics FC 500 (Beckman-Coulter, FA) and CXP software. Data is representative of five separate experiments. (F) PBMCs were stained with anti-Foxp3 antibodies, and then analyzed using multiphoton microscopy. Higher frequencies of stained cells were observed in PBMC from normal donors compared to PBMC from MGUS and MM patients.

**Figure 2. Lack of suppression of T cell proliferation by T<sub>reg</sub> cells in MGUS and myeloma.** (A) Anti-CD3-mediated T cell proliferation of PBMCs incubated with or without CD25+ T cells for 72 hours was measured by 3H-thymidine uptake during last 8h of culture. Results are mean±SEM CPM, and number of samples analyzed is in parentheses. *Indicates statistical significance (p<0.05) by Student ‘t’ test analysis. Compared to normal donor cells, T<sub>reg</sub> cells from MGUS and MM patients were unable to significantly suppress T cell proliferation. (B) CD25+ T cell depleted PBMC were co-cultured with increasing number of CD25+ T cells at
different ratios, and anti-CD3-mediated proliferation was then measured at 72 hours by $^3$H-thymidine uptake during last 8h of culture. Addition of excess CD25$^+$ cells failed to suppress T cell proliferation in both MGUS and MM, compared with normal donors. Results are one representative of seven experiments.
A

B

Thymidine Incorporation (×1000 cpm)

-  +  -  +  -  +  CD25+

Control (9)  MGUS (9)  MM (9)

Thymidine Incorporation (×1000 cpm)

Ratio of CD25+ T cells

Control  MGUS  MM
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