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Regulatory Cells and Multiple Myeloma

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1. Introduction

The concept of immune surveillance posits innate and adaptive immune cell mediated recognition and elimination of tumor cells, which express either tumor specific antigens or molecules by cellular stress. Both innate and adaptive immunity are important to inhibit tumor formation and rejection of transplanted tumors (Dunn et al., 2004; Pardoll, 2003). Despite immune surveillance, tumors do progress. Therefore, new concept immunoediting provides complete explanation of immune system in cancers. Immunoediting has three phases against tumors including elimination, equilibrium and escape (Swann & Smyth, 2007). Elimination phase is similar to immune surveillance, where the immune system detects tumor cells and kills them. In the equilibrium phase, tumor cells become dormant, and the immune system selectively destroys susceptible tumor clones and prevents tumor progression. In the escape phase, immune responses fail to suppress the tumor growth which leads to development of tumor variants that are resistant to anti-tumor responses. The major immune cells involved in targeting the tumor mass are CD8 T cells (MHC I dependent) and NK cells (MHC I independent/deficient) (Pardoll, 2003; Smyth et al., 2000, 2001). Perforin and Fas/FasL pathways constitute for contact-mediated cytotoxicity represented by NK and CD8 T cells (Lieberman, 2003; Russell & Ley, 2002). Also, other pathways play an important role in tumor elimination, such as IFN- γ and IFN- α/β . Regulatory T cells have been initially described in studies by Sakaguchi et al who proved the role of (CD4+CD25+) regulatory T (Treg) cells in maintaining the tolerance against self-antigens (Sakaguchi et al., 1995, 2004, 2008). Treg cells have been shown to contain distinct populations (Table 1) which are able to actively suppress the function of other immune cells, including CD4+CD25- T cells, CD8 T cells, dendritic cells, macrophages, B cells, NK cells and NKT cells (Azuma et al., 2003; Chen, 2006; Lim et al., 2005; Murakami et al., 2002; Romagnani et al., 2005; Trzonkowski et al., 2004). Several studies recently proved that Treg cells could induce tolerance against tumors (Nagai et al., 2004; von Boehmer, 2005; Yamaguchi & Sakaguchi, 2006). Also, studies addressed the expansion of Treg cells in various non-hematological and hematological malignancies (Beyer & Schultze, 2006b). Treg cells were also proved to

inversely correlate with outcome of various cancers, including gastric malignancies, ovarian and breast cancers (Curiel et al., 2004; Merlo et al., 2009; Sasada et al., 2003). However, it appears that in certain diseases, such as follicular lymphoma and Hodgkin's lymphoma, higher number of FoxP3+ cells correlate with better survival (Alvaro et al., 2005; Carreras et al., 2006).

Multiple myeloma is a plasma cell proliferative disorder and the second most common hematological malignancy standing next to lymphoma (Kyle & Rajkumar, 2008). Multiple myeloma is clinically characterized by $\geq 10\%$ of plasma cell infiltration in the bone marrow, $\geq 30\text{g/L}$ of monoclonal protein and presence of CRAB symptoms (hypercalcemia, renal insufficiency, anemia and bone lytic lesions) (Raja et al., 2010). It has been proved that B and T lymphocyte populations in multiple myeloma significantly associate with survival (Kay et al., 2001). Multiple myeloma patients commonly present with defects in numbers and function of various immune cells including dendritic cells, B cells, T cells and NK cells (Pratt et al., 2007). Concept of immunoediting also fits in multiple myeloma because of its several stages, starting from premalignant stage known as monoclonal gammopathy of undetermined significance to symptomatic stage (Swann & Smyth, 2007). Elimination phase of immunoediting has been explained in premalignant stage of myeloma, where strong T cell response was observed against the tumor clone compared to malignant stage (Dhodapkar, 2005). Followed by surveillance, T cells patrol the premalignant clone (equilibrium) and finally lose responses against malignant clones which lead to symptomatic myeloma (escape) (Dhodapkar et al., 2003). Recently, in multiple myeloma several studies showed elevated level of Treg cells; these cells were functionally active in suppressing the function of naïve T cells. In this chapter, we focus on describing general aspects of Treg cells, including subtypes, functions, migration and induction of tolerance at tumor microenvironment. Then, we discuss the role of CD4 Treg cells in multiple myeloma and their association with stages and survival, and influence of immunomodulatory drugs on multiple myeloma patient's Treg cells. Additionally, we discuss the role of Th17 cells, CD8 Treg cells and myeloid-derived suppressor cells (MDSCs) in multiple myeloma.

2. Phenotypic characterizations of regulatory T cells in humans

In mice, CD4 Treg cells can be easily characterized as CD4+FoxP3+ and high expression of CD25 (Sakaguchi et al., 1995). However, identification of human Treg cells is ambiguous because approximately 1-2 % of CD25 expressing T cells are functional Treg cells (Allan et al., 2005; Baecher-Allan et al., 2001). Isolation of CD25^{hi} T cells excludes FoxP3 low and CD25 low/intermediate Treg cells which are found to be naïve Treg cell population (CD45RA+/CD45RO-). Unclear identification of CD25 (intermediate and high) expression on CD4 Treg cells influence the reproducibility of results in various disorders. Several groups suggested that negative expression of CD127 in Treg cells might help in characterization. But it will not ensure the accurate identification of Treg cells because activated CD4 T cells are also CD127- (Liu et al., 2006; Seddiki et al., 2006). Alternatively, CD62L expression could differentiate the recently activated CD4 T cells (CD62L low) and Treg cells (CD25^{hi}+CD62L+CD127^{low}) but CD62L expression is not solely restricted to Treg cells (Hamann et al., 2000). Characterization of Treg cells using the CD127 and CD25

does not exclude the non-regulatory Foxp3 expressing T cells. These cells express CD45RO, lack suppressive ability and secrete pro-inflammatory cytokines IL-2, IFN- γ and IL-17 (Miyara et al., 2009). In conclusion, heterogenic expression of FoxP3 by non-regulatory and Treg cells precludes the inclusion of FoxP3 as a sole marker in humans to characterize the Treg cells.

3. Types and functions of T regulatory cells

The existence of Treg cells was uncovered more than four decades ago in studies showing that neonatally thymectomized mice developed autoimmunity, which could be prevented by reconstitution with CD4 T cells (Nishizuka & Sakakura, 1969; Sakaguchi et al., 1982). Further work characterized these Treg cells as CD4+ T cells expressing high levels of IL-2 receptor α chain (Sakuguchi et al., 1995). Fontenot et al determined that in mice, forkhead transcription factor FoxP3 is a specific marker of Treg cells and a master regulator in development and function of Treg cells (Fontenot et al., 2003). Types and functions of various Treg cells are summarized in Table 1 and Table 2.

Types	Origin	Functions	References
CD4+ natural regulatory T cells	Arise in thymus and disseminate to periphery; constitute about 10%-15% of CD4 cells	In mice, depletion of these cells leads to autoimmunity. In humans, mutation in FoxP3 gene located on X chromosome leads to fatal immune disorder IPEX syndrome (Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome)	Gambineri et al., 2003.
CD4+ Tr1 regulatory cells	Periphery	Induced in the periphery from naïve T cells in the presence of IL-10. These cells lack FoxP3 expression but secrete IL-10 and TGF- β .	Groux et al., 1997; Vieira et al., 2004
CD4+ Th3 cells	Periphery	Induced in the periphery from naïve T cells in the presence of TGF- β . Suppression is mediated by TGF- β . Rare Th3 cells express FoxP3 molecule due to induction by TGF- β .	Apostolou & von Boehmer, 2004; Chen et al., 2003.
Double negative Treg cells	Periphery	In mice and humans these cells constitute about 1%-3% and 1%, respectively. Double negative Treg cells inhibit T cell activation and proliferation in an antigen specific manner.	Fischer et al., 2005.
$\gamma\delta$ T cells	Infiltrate into tumor site (breast cancer)	Suppress naïve and effector T cell responses and inhibit maturation and function of dendritic cells.	Peng et al., 2007.

Types	Origin	Functions	References
CD8 regulatory T cells	Most of CD8 Treg cells are induced by antigen- specific manner	There are several subsets of CD8 Treg cells: Qa-1 specific CD8 Treg cells suppress autoreactive T cells expressing Qa-1 molecule associated with self peptide. CD8+CD28- Treg cells express FoxP3α molecule and suppress other cells via contact dependent mechanism. CD8+CD25+ Treg cells suppress both naïve CD4 and CD8 T cells by contact dependent or independent (IL-10) mechanisms. CD8+CD25+ Treg cells mostly accumulate in the tumor bed rather than peripheral tissues.	Filaci et al., 2007; Kiniwa et al., 2007; Sarantopoulos et al., 2004; Wang, 2008.

Table 1. Subsets of T regulatory cells

Functions of Treg cells	Mechanism of Suppression	References
Inhibitory cytokines	Mainly cytokines such as IL-10, TGF- β and IL-35 secreted by Treg cells are involved in inhibitory function. Chen et al proved in murine colon carcinoma IL-10 induced suppression of tumor specific CD8 T cell immunity. Peptide inhibitor targeted against the surface TGF-β on Treg cells abrogated their function and enhanced anti-tumor response. In mouse model of inflammatory bowel disease, it was shown that IL-35 played a role in severity of inflammatory bowel disease. In humans IL-35 is not expressed constitutively by Treg cells.	Bardel et al., 2008; Chen et al., 2005; Collison et al., 2007; Gil-Guerrero et al., 2008; Loser et al., 2007.
Cytotoxicity	Perforin/granzyme pathway is well known to be associated with CD8 T cells and NK cells for destruction of intracellular pathogens and tumor cells. Recent studies have shown Treg cells also use the perforin/granzyme pathway. An <i>in vitro</i> study demonstrated Treg cells activated with anti CD3 and anti CD46 antibodies expressed granzyme A and B. In murine Treg cells, it was shown that perforin lacking Treg cells also exhibit suppressive function. Cao et al reported in a tumor inoculation system the adoptively transferred Treg cells induced suppression of tumor immunity (CD8 T cells and NK cells) specifically by granzyme B pathway. Fas ligand utilizing Treg cells presence was reported in head and neck squamous cell carcinoma patients and found to suppress CD8 T cells.	Cao et al., 2007; Gondek et al., 2005; Grossman et al, 2004; Lieberman, 2003; Russell & Ley, 2002; Strauss et al., 2009.

Functions of Treg cells	Mechanism of Suppression	References
Inhibition of antigen presenting cells (APCs)	Expression of CTLA-4 under the control of FoxP3 facilitates Treg cells interaction with CD80 and CD86 on APCs and induces the suppression of T cell activation. CTLA-4 mediated interaction between Treg cells and APCs leads to upregulation of indoleamine 2, 3-dioxygenase (IDO) production by the APCs which leads to degradation of the essential amino acid tryptophan. Recently, it was proposed that IDO mediated depletion of tryptophan inhibited T cell proliferation and response against tumors.	Mellor & Munn, 2004; Munn et al., 1999; Oderup et al., 2006; Read et al., 2000.

Table 2. Functions of T regulatory cells

4. Mechanism of migration and induction of tolerance in the tumor bed by regulatory cells

There are several chemokine receptors and ligands are involved in the migration of Treg cells. Before going into detail, we would like to summarize the migration process of Treg cells from thymus to secondary lymphoid organ in normal physiological conditions. Preliminary switch occurs in chemokine receptors CCR8/CCR9 and CXCR4 to CCR7 on thymic precursors of Treg cells. These precursor Treg cells are a homogeneous population and express CD62L, CCR7 and CXCR4^{low} (Lee et al., 2007). After migration from thymus to the periphery, these Treg cells acquire complete expression of CXCR4. Thymic emigrant Treg cells most exclusively migrate to secondary lymphoid organ for antigen contact. After the antigen contact, the second switch occurs in the receptors where they downregulate the CCR7 and CXCR4 expression. Consequently, upregulation of effector/memory chemokine receptors (CCR2, CCR4, CCR6, CCR8, and CCR9) occurs to enhance the heterogenic property of Treg cell population (Lee et al., 2007). It was shown that CCR7 mediated migration of Treg cells into secondary lymphoid organ is an important process for encountering mature dendritic cells and consequent differentiation and proliferation (Schneider et al., 2007). All peripheral blood Treg cells express CCR4 receptor and show chemotactic response to ligands CCL22 and CCL17. In ovarian cancer, Treg cell migration and accumulation at the tumor site is attributed by CCL22 chemokine (Curiel et al., 2004). Secretion of CCL22 by lymphoma cells largely recruits the intratumoral Treg cells that express CCR4 but not CCR8 (Ishida et al., 2006; Yang et al., 2006b). In non-hematological malignancies, it was observed that increased frequency of CCR4 expressing Treg cells associated with CCL22 and CCL17 chemokines. An *in vitro* study in gastric cancer showed that migratory activity of Treg cells was induced by CCL22 and CCL17 chemokines (Mizukami et al., 2008).

Several molecules and receptors contribute the suppressive function of Treg cells in the tumor microenvironment. Suppression mechanism by Treg cells is not solely dependent on cell-cell contact rather it is also supported by soluble IL-10 and TGF- β molecules (Strauss et al., 2007). Treg cells do not depend always on direct inhibition of effector T cells; rather, it impedes function of dendritic cells via IL-10 and TGF- β . This mechanism downregulates NF- κ B and subsequently changes downstream molecules (CD80, CD86 and CD40) as well as

soluble factors, such as TNF- α , IL-12 and CCL5 (Larmonier et al., 2007). Moreover, Treg cells in tumor bed prevent CD4 T cell mediated generation of CD8 T cell cytotoxic responses (Chaput et al., 2007). Prostaglandin E2 is the effector molecule released by Treg cells; it is also important for activation of Treg cells. This molecule efficiently suppresses the effector T cell responses via COX2 induction. Prostaglandin E2 mediated suppression by Treg cells was observed in colorectal cancer patients (Mahic et al., 2006). Cell-cell contact dependent suppression is partly attributed to CTLA-4 expression by Treg cells (Read et al., 2000). Expression of CTLA-4 also facilitates the TGF- β mediated suppression via intensifying the TGF- β signals at the interaction point of Treg cell and target cell (Oida et al., 2006). Tumor infiltrating Treg cells express ICOS molecule but peripheral Treg cells do not express ICOS. ICOS receptor and its ligand are involved in Treg cell mediated suppression. Treg cells with ICOS low expression did not show strong suppressive function as compared to Treg cells with ICOS high expression (Strauss et al., 2008). Very recently, other novel

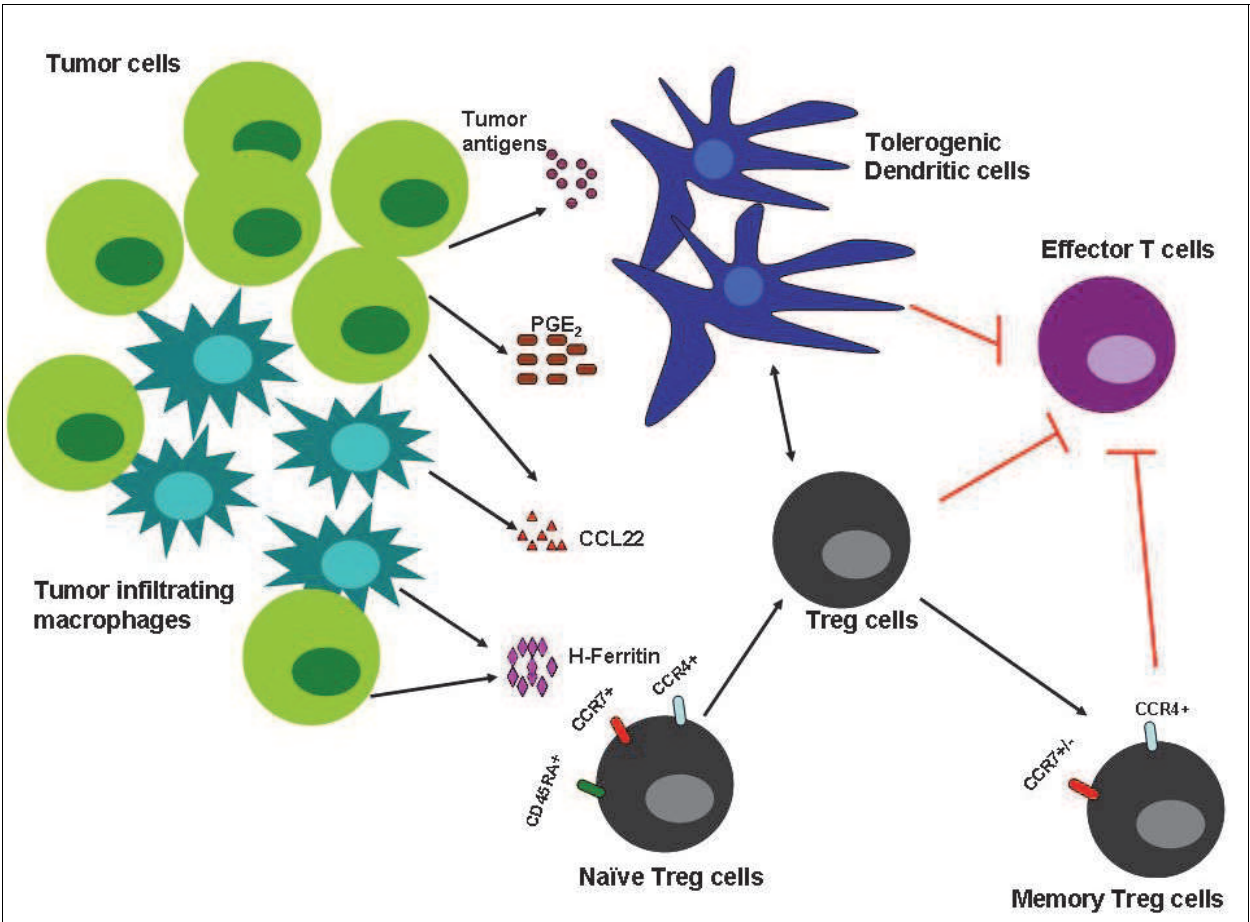


Fig. 1. Mechanism of accumulation and expansion of T regulatory cells in the tumor bed
This schematic diagram represents a mechanism of immune tolerance induced by Treg cells in the tumor microenvironment. CCL22 as well H-ferritin secretion by tumor cells and tumor infiltrating macrophages recruit naïve Treg cells (CCR4+) in the tumor bed. These accumulated naïve Treg cells differentiate and proliferate into memory Treg cells via interaction with prostaglandin E2 (PGE2) induced tolerogenic dendritic cells. These expanded memory Treg cells along with tolerogenic dendritic cells impede the functions of tumor effector T cells.

mechanisms of suppression by Treg cells were revealed. Expression of CD39 in conjunction with CD73 generates the adenosine molecule and its interaction with adenosine A2A receptor on activated T cells creates strong immunosuppressive loops; this suggests one of the important suppressive mechanisms of Treg cells (Deaglio et al., 2007). Transfer of cyclic adenosine monophosphate by Treg cells to effector T cells via cell-cell interaction induces suppression and impedes IL-2 secretion (Bopp et al., 2007). Also, recently it was proven that effector T cells are suppressed via apoptosis induced by deprivation of γ cytokines by Treg cells. Strong association was observed between Treg cell induced apoptosis and increased level of pro-apoptotic proteins Bim and Bad as well as decreased level of pro-survival protein kinase Akt (Pandiyan et al., 2007). Taking all these observations into consideration, the significance of chemokines and their receptors in migration of Treg cells could act as a suitable target for deprivation of Treg cells in the tumor microenvironment; this might also enhance anti-tumor responses. To add more flavors in impeding Treg cell migration, a chimeric monoclonal antibody targeting CCR4 receptor is already under clinical trial. All of the above mentioned mechanisms of suppression collectively work together to induce suppression of immune cells and tolerance in the tumor bed. In some circumstances, these mechanisms might independently act to enforce the suppression of anti-tumor responses.

5. Regulatory T cells and multiple myeloma

Recently, several research groups analyzed Treg cells in multiple myeloma. So far, Treg cells data in multiple myeloma are conflicting. Study from Prabhala et al and Gupta et al reported decreased frequency of peripheral blood Treg cells in multiple myeloma when compared to control group (Gupta et al., 2011; Prabhala et al., 2006). Both studies confirmed that FoxP3 expression was reduced in myeloma patients. In contrast to these studies, Feyler et al and Beyer et al reported increased frequencies of peripheral blood Treg cells in multiple myeloma patients (Feyler et al., 2009; Beyer et al., 2006a). Most of the studies confirmed that peripheral blood and bone marrow Treg cells frequencies were comparable. According to Beyer et al, Treg cells associated markers, such as CTLA-4, GITR, CD62L and OX40 were elevated in myeloma patients compared to healthy subjects (Beyer et al., 2006a). Contrasting to this observation, Prabhala et al showed decreased frequency of CTLA-4 expression on Treg cells of monoclonal gammopathy of undetermined significance and myeloma cohorts than healthy subjects (Prabhala et al., 2006). These opposing results may be due to Treg cells identification strategy. For instance, Prabhala et al identified Treg cells as CD4+FoxP3+, Gupta et al characterized Treg cells with the inclusion of CD127 in their gating, Feyler et al identified Treg cells as CD4+CD25hi+FoxP3+ and Beyer et al identified Treg cells using only CD4 and CD25 markers (Beyer et al., 2006a; Feyler et al., 2009; Gupta et al., 2011; Prabhala et al., 2006). Most of the studies in other cancers including hematological and non-hematological malignancies showed elevated level of Treg cells and these cells are associated with worse prognosis. To support the concept of tumor based expansion of Treg cells, studies clearly showed that established Treg cell clones recognized tumor antigen in MHC class II restricted manner (Wang et al., 2004). In multiple myeloma, no strong conclusions could be made due to the existence of equal number of conflicting results with regard to Treg cells frequency.

5.1 Immunosuppressive function of T regulatory cells in multiple myeloma

Most studies in myeloma agree that Treg cells efficiently suppress both autologous and allogeneic responder cells (CD4+CD25-) similarly to healthy subjects (Beyer et al., 2006a; Brimnes et al., 2010; Feyler et al., 2009; Gupta et al., 2011). Exclusively, Prabhala et al showed that multiple myeloma patients Treg cells lack suppressive function (Prabhala et al., 2006). This contrasting result by Prabhala et al might be due to the use of whole peripheral blood mononuclear cells depleted with CD25+ cells as responder cells (Prabhala et al., 2006). The suppressive nature of Treg cells could be well appreciated by the presence of intracellular cytokines TGF- β and IL-10. Beyer et al confirmed that myeloma patients Treg cells express increased level of TGF- β and IL-10 when compared to healthy subjects (Beyer et al., 2006a). *In vitro* matured dendritic cells using inflammatory cytokines generate functionally active FoxP3+ Treg cells from CD25- T cells. Treg cells derived from dendritic cells are functionally similar in between healthy subjects and myeloma patients. Also, an *in vivo* study showed administration of cytokine matured dendritic cells in myeloma subjects enhanced increase of Treg cell numbers (Banerjee et al., 2006). This study also proposed that use of human dendritic cell vaccination may affect the balance of effector T cells generation because of Treg cell enhancement (Banerjee et al., 2006). In allogeneic stem cell transplanted multiple myeloma patients, donor derived Treg cells reconstituted largely in the bone marrow compartment and prevented graft versus host disease (Atanackovic et al., 2008). This data suggest that *in vivo* inhibitory function of Treg cells and also *in vitro* assay showed reconstituted Treg cells possess complete inhibitory function. Moreover, Atanackovic et al proved that reconstitution of Treg cells in bone marrow positively correlates with time passed since transplantation. Donor derived Treg cells reconstituted in the bone marrow were found to be memory Treg cells, which indicates that Treg cells indeed expanded outside the thymus (Atanackovic et al., 2008). Most of the studies strongly suggest that myeloma patients Treg cells are functional in suppressing the conventional T cell proliferation, and this suppressive function encourages the immune impairments and dysfunctions. However, Treg cells suppressive function could be appreciated in the case of graft versus host disease where donor cells require engraftment to ensure the anti-tumor effects.

5.2 Association of international staging system and myeloma survival status with T regulatory cells

Based on the international staging system (ISS), Treg cells were increased in newly diagnosed multiple myeloma patients. This observation was noticed only in ISS 1 and ISS 2 (Feyler et al., 2009). On the other hand, Gupta et al found a trend of decrease in Treg cells in stages ISS 2 and ISS 3 (Gupta et al., 2011). Apart from ISS, paraprotein level of myeloma patients was positively correlated with frequencies of Treg cells (Feyler et al., 2009).

Giannopoulos et al demonstrated patients with higher level of Treg cells have significantly reduced survival time compared to patients with lower level of Treg cells (21 months vs. median not reached) (Giannopoulos et al., 2010). Our observation also showed that patients with increased peripheral blood Treg cells ($\geq 5\%$) have shorter progression free survival compared to patients with reduced Treg cells ($< 5\%$) cohort (13 months vs. median not reached) (Muthu Raja et al., 2011). These data showing that elevated level of Treg cells

correlated unfavorably with progression free survival and overall survival of myeloma patients, so Treg cells could be targeted along with the tumor cells in multiple myeloma.

6. Influence of immunomodulatory drugs on T regulatory cells of multiple myeloma patients

Immunomodulatory drugs are orally bioavailable agents, derived from thalidomide (first generation immunomodulatory drug). The second generation immunomodulatory drugs are lenalidomide and pomalidomide which share similar chemical structure with thalidomide (Galustian et al., 2004). Quach et al recently reviewed the functions of immunomodulatory drugs (Quach et al., 2010). The functions are:

6.1 Immune modulation

Co-stimulation of CD4 and CD8 T cells, activation of NK and NKT cells, production of Th1 cytokines, enhancement of antibody dependent cellular cytotoxicity and Treg cell suppression.

6.2 Hampering tumor microenvironment interactions

Anti-angiogenesis, inhibition of inflammatory effects, anti-osteoclastogenesis and downregulation of adhesion molecules on plasma cells.

6.3 Direct anti-tumor effects

Induction of cyclin dependent kinase inhibitors, such as p15, p21 and p27 which results in cell cycle arrest (G0/G1 phase), increases expression of early growth genes (1, 2), downregulation of NF- κ B with subsequent reduction in anti-apoptotic proteins FLIP and cIAP2.

Minnema et al showed that in relapsed myeloma patients (after allogeneic stem cell transplantation), lenalidomide increased frequencies of Treg cells during treatment (Minnema et al., 2009). In contrast, CD4 and CD8 T cells were decreased. This study also showed that ratio of FoxP3⁺ T cells to IFN- γ secreting T cells was significantly increased during treatment (Minnema et al., 2009). Increased Treg cells always favour allogeneic stem cell transplanted patients because these cells inhibit graft versus host disease (Rezvani et al., 2006). However, Minnema et al study did not show the advantage of increased Treg cells with relevance to graft versus host disease, probably due to small patient numbers (Minnema et al., 2009). Contrasting to this study, an *in vitro* observation demonstrated that lenalidomide and pomalidomide are able to inhibit the proliferation of Treg cells at very low concentrations (10 μ M and 1 μ M) but thalidomide failed to inhibit the proliferation even at maximum concentration of 200 μ M. This study reveals lenalidomide and pomalidomide inhibited the Treg cells mediated suppression of CD4⁺CD25⁻ cells and also proposes that FoxP3 molecule was targeted efficiently by lenalidomide and pomalidomide but no alterations to GITR were observed (Galustian et al., 2009). Lenalidomide and pomalidomide also inhibit the function of Treg cells by hindering the expression of OX-40 (CD134) molecule (Galustian et al., 2009; Valzasina et al., 2005). Carcinoma animal model study showed Treg cells were depleted after cyclophosphamide treatment; tumor growth was also

repressed due to depletion of Treg cells, and tumor clones were cleared followed by immunotherapy. Without administration of cyclophosphamide, no tumor regression or clearance was noticed in the tumor bearing animal (Ghiringhelli et al., 2004). Apart from sensitivity of Treg cells to immunomodulatory drugs and cyclophosphamide, a recent study showed that naturally occurring Treg cells were resistant to pro-apoptotic effect of proteasome inhibitor bortezomib. Long-term culturing of CD4 T cells in the presence of bortezomib promoted the emergence of Treg cells and significantly inhibited proliferation, IFN- γ production and CD40L expression by effector T cells (Blanco et al., 2009). Drug-induced apoptosis resistance by Treg cells is due to the increased expression of BCL-2 and inhibitor of apoptosis protein 1 (IAP1); the expression of IAP1 is in response to TNF induced apoptosis. These BCL-2 and IAP1 protein expression was significantly elevated in Treg cells of B cell leukemic patients than healthy volunteers (Jak et al., 2009). Gupta et al confirmed that multiple myeloma patients treated with immunomodulatory drug combination showed increased Treg cells in relation to irrespective of the response achieved (Gupta et al., 2011). They also showed patients with stable and progressive disease had decreased Treg cells. Taking all these observations into consideration, no strong conclusion can be forwarded because of contrasting results between *in vitro* and *in vivo* studies. However, *in vitro* studies showed promising effects of immunomodulatory drugs on Treg cells when compared to proteasome inhibitor but immunomodulatory drugs effects might be diluted by inclusion of corticosteroid during treatment.

7. Th17 cells in multiple myeloma

Th17 cells are one of the subsets of CD4 T cells. These cells are differentiated in the presence of IL-6, IL-1 β , IL-21 and IL-23 with or without TGF- β . Th17 cells secrete IL-17, IL-21, IL-22 and IL-26 cytokines. These cytokines are involved in anti-fungal and anti-parasite responses and participate in inflammatory and autoimmune reactions (Acosta-Rodriguez et al., 2007; Aujla et al., 2008; Bettelli et al., 2006; Ivanov et al., 2006; Veldhoen et al., 2008; Wilson et al., 2007; Zheng et al., 2008). Both Treg cells and Th17 cells originate from naïve CD4 T cells. For Treg cell differentiation, TGF- β is required whereas for Th17 cell differentiation, IL-6 and TGF- β are required (the role of TGF- β in human is unclear) (Bettelli et al., 2006; Veldhoen et al., 2006). Xu et al observed that mature Treg cells can be converted into Th17 cells in the presence of IL-6 (Xu et al., 2007). IL-6 and IL-21 might be involved in transition of Th17/Treg cells to Th17 cells. Some reports show switching of Th17 cells to Th1 cells, but reverse switching is not possible (Annunziato & Romagnani, 2009; Bending et al., 2009; Lee et al., 2009; Shi et al., 2008). Upregulated T-bet expression in Th17 cells resulted in a switch to Th1 cells in the presence of IL-12 or/and IL-23 (Annunziato et al., 2007; Lee et al., 2009).

Recently, a study reported increased Th17 cells and IL-17 in freshly isolated mononuclear cells and sera of myeloma patients compared to healthy subjects. *In vitro* polarization of CD4 T cells to Th17 cells showed increased Th17 cells in multiple myeloma patients than healthy subjects (Prabhala et al., 2010). Moreover, this study showed expression of IL-17 receptor on myeloma cells which promotes the growth of myeloma cells. IL-21 is a pro-inflammatory cytokine associated with Th17 cells, which is also capable of inducing STAT-3 mediated myeloma growth-promoting effects in synergism with insulin like growth factor-1 (Brenne et al., 2002). Most of the myeloma patients present with bone lytic disorder. In cell culture experiments, Dhodapkar et al showed dendritic cells were efficient inducers of Th17

cells, especially mature dendritic cells. Dendritic cells induced Th17 cells were multifunctional and secreted IL-17 and IFN- γ (Dhodapkar et al., 2008). In myeloma, tumor cells are infiltrated by dendritic cells; Dhodapkar et al demonstrated that Th17 cells were enriched in the tumor microenvironment (Dhodapkar et al., 2008). These IL-17 producing cells were found to enhance the activation of osteoclasts (Noonan et al., 2010). This study also reported significant association between extent of bone lytic lesions and IL-17 cytokine producing Th17 cells (Noonan et al., 2010). These findings suggest that Th17 cells enhance myeloma cell growth and development of bone lytic lesions.

8. CD8 T regulatory cells in multiple myeloma

T cells play an important role in immunosurveillance against cancers. Eventually, these T cells may become CD4/CD8 regulatory T cells due to stimulation by and interaction with tumor cells. Thus, these generated regulatory cells promote the growth of tumor rather than inhibition of tumor (Wang, 2008). Mechanisms of suppression and expansion are well documented for CD4 Treg cells but recent research has been directed to screen the presence of CD8 Treg cells in various tumors and inflammatory conditions.

8.1 Subtypes of CD8 T regulatory cells

8.1.1 Qa-1 restricted CD8 Treg cells

These CD8 Treg cells downregulate autoimmune T cell responses. They are Qa-1 (MHC class 1b molecule) restricted and specifically target self-reactive activated T cells which express Qa-1 molecules associated with self peptide (Jiang & Chess, 2004; Sarantopoulos et al., 2004).

8.1.2 CD8+CD28⁻ Treg cells

These cells are induced by MHC class I peptide antigens. CD8+CD28⁻ Treg cells were found to express FoxP3 α molecule and mediate suppression by cell-cell contact mechanism. CD8+CD28⁻ Treg cells also indirectly target CD4 T cells to become tolerogenic via dendritic cells and non-professional antigen presenting cells. These cells were identified in tumors as well as in the context of transplantation (Cortesini et al., 2001; Filaci & Suciu-Foca, 2002; Filaci et al., 2007; Suciu-Foca et al., 2005).

8.1.3 CD8+CD25^{hi} Treg cells

These cells express CD122, Foxp3 and GITR molecules typically associated with CD4 Treg cells (Cosmi et al., 2003; Kuniwa et al., 2007; Lee et al., 2008). They suppress naïve CD4 and CD8 T cells via contact dependent mechanism or soluble IL-10. These Treg cells are different from Qa-1 specific CD8 Treg cells but similar to CD4 Treg cells. CD8+CD25^{hi} Treg cells are induced by the tumor environment and require antigen stimulation to suppress naïve T cells (Wang, 2008).

8.2 Role of CD8 T regulatory cells

So far, only a few studies have documented the role of CD8 Treg cells (CD8+CD25^{hi}+FoxP3+) in cancers. In prostate and colorectal cancers, elevated levels of CD8

Treg cells have been reported (Chaput et al., 2009; Kiniwa et al., 2007). Both these studies showed that CD8 Treg cells are capable of suppressing naïve T cell proliferation. Additionally, Chaput et al demonstrated suppression of Th1 cytokine production. CD8 Treg cells from colorectal cancer patients were found to correlate with disease stage and micro-invasive status (Chaput et al., 2009). In multiple myeloma, data on CD8 Treg cells are lacking. Our recent observation showed that CD8 Treg cells were significantly elevated in monoclonal gammopathy of undetermined significance and multiple myeloma when compared to healthy subjects (Muthu Raja et al., 2010). However, functional data of CD8 Treg cells are still lacking.

9. Myeloid-derived suppressor cells (MDSCs) in multiple myeloma

Myeloid-derived suppressor cells (MDSCs) are activated immature myeloid cells that have been prevented from differentiation to mature cells. These cells are expanded in pathological conditions (Gabrilovich & Nagaraj, 2009). MDSCs lack the expression of cell surface markers that are specifically expressed by monocytes, macrophages or dendritic cells and comprise a mixture of myeloid cells that have the morphology of granulocytes or monocytes (Youn et al., 2008). They are potent suppressors of T cells. Human MDSCs can be characterized phenotypically as CD14-CD11b⁺ or by CD33 expression which is a common marker for myeloid cells. Moreover, MDSCs lack the expression of mature lymphoid and myeloid markers as well HLA-DR (MHC class II). Healthy individuals were found to have approximately 0.5% of immature myeloid cells from total peripheral blood mononuclear cells (Gabrilovich & Nagaraj, 2009). In cancer patients and tumor models, accumulation of MDSCs occurs due to release of soluble factors by tumor cells or cells in tumor environment (Almand et al., 2001, Diaz-Montero et al., 2009).

9.1 Mode of suppression

9.1.1 Arginase and inducible nitric oxide synthase

Arginase and inducible nitric oxide synthase enzymes are released by MDSCs. Arginase depletes the non-essential amino acid L-arginine and leads to inhibition of T cell proliferation (Rodriguez et al., 2002). Inducible nitric oxide synthase mediates nitric oxide production. Nitric oxide suppresses the T cell function via induction of apoptosis, inhibition of MHC II expression and inhibition of STAT-5 and JAK3 function in T cells (Gabrilovich & Nagaraj, 2009).

9.1.2 Reactive oxygen species

This is also an important factor from MDSCs that contributes to suppressive activity. Reactive oxygen species release was noticed in tumor bearing mice and cancer patients. Several tumor derived factors including TGF- β , IL-6, IL-3, IL-10 and granulocyte macrophage colony-stimulating factor induce reactive oxygen species synthesis by MDSCs (Gabrilovich & Nagaraj, 2009).

9.1.3 Peroxynitrite

Peroxynitrite induces MDSCs mediated suppression of T cell function. Accumulation of peroxynitrite is noticed where recruitment of MDSCs occurs at the site of inflammation or

immunological reactions. In addition, increased peroxynitrite levels associated with tumor progression in several cancers (Gabrilovich & Nagaraj, 2009). Interaction of peroxynitrite producing MDSCs and T cells leads to nitration of T cell receptors and alters specific peptide binding of T cells. This process leads to T cell unresponsiveness (Nagaraj et al., 2007).

9.1.4 Induction of Treg cells

In vivo studies showed that MDSCs can induce *de novo* generation of Treg cells (Huang et al., 2006; Yang et al., 2006a). Induction of Treg cells by MDSCs requires tumor specific T cells together with IFN- γ and IL-10, but independent of nitric oxide production (Huang et al., 2006). In murine models, MDSCs induce generation of Treg cells by CTLA-4 (ovarian tumor) and arginase 1 (lymphoma) molecules (Serafini et al., 2008; Yang et al., 2006a). However, in contrast, other studies report no association of Treg cell generation with MDSCs (Dugast et al., 2008; Movahedi et al., 2008).

9.2 Role of myeloid-derived suppressor cells

Accumulation of MDSCs in cancer patients is an immune evasion mechanism. MDSCs were found to be elevated in peripheral blood of solid tumors including breast, colon, prostate, hepatocellular and esophageal carcinomas. It was also shown that increase of MDSCs in solid tumors is stage-dependent. Stage IV solid tumor patients showed increased level of MDSCs which correlated with metastatic tumor burden (Diaz-Montero et al., 2009). Early stage breast cancer patients who received cyclophosphamide plus doxorubicin also had increased level of MDSCs. In multiple myeloma, information about MDSCs is lacking. However, a recent study showed significant increase of MDSCs in multiple myeloma patients (Brimnes et al., 2010). This study identified MDSCs as CD14+HLADR-/low, which is contradictory to other studies. In our study, we identified MDSCs as CD33+CD11b+CD14-HLADR-. Cells with this phenotype were elevated in multiple myeloma patients and also an increasing trend was showed in monoclonal gammopathy of undetermined significance patients compared to healthy subjects (Muthu Raja et al., 2011). Due to limited studies on MDSCs of myeloma patients, no strong conclusion could be forwarded. However, studies have shown increased level of MDSCs. Further studies are required to prove their functional activity.

10. Therapeutic targeting of regulatory and suppressor cells to enhance anti-tumor responses

Treg cells favored as a potential target in various cancers to enhance the anti-tumor responses. Chemotherapy agents such as fludarabine and cyclophosphamide were reported to reduce Treg cell numbers in animal models. Cyclophosphamide plus fludarabine and high dose IL-2 treatment was given to metastatic melanoma patients where transient decrease in Treg cells was observed (Powell et al., 2007). Use of chemotherapeutic agents to target Treg cells is relatively unspecific approach but targeting CD25 was found to be more selective in hitting Treg cells than chemotherapies. Various preclinical trials have shown that depletion of Treg cells via specific monoclonal antibodies targeting CD25 in combination with adoptive T cell transfer, denileukin diftitox (a fusion protein of diphtheria toxin and IL-2) and LMB-2 (a fusion protein of a single-chain Fv fragment of an anti-CD25

antibody and the bacterial pseudomonas exotoxin A or 3) enhanced the anti-tumor immunity (Attia et al., 1997; Knutson et al., 2006; Litzinger et al., 2007; Shimizu et al., 1999). Translation of denileukin diftitox and LMB-2 into clinical studies along with vaccination showed efficient improvement in anti-tumor response plus reduced frequency of Treg cells in various cancers, including metastatic renal cell carcinoma, melanoma and colorectal carcinoma. Targeting CTLA-4 molecule is not a precise approach because both effector T cells and Treg cells express CTLA-4. However, a recent animal model study showed CTLA-4 deficient mice lacked the Treg cell mediated immune suppression (Wing et al., 2008). Disadvantage in blockade or depletion of Treg cells is autoimmune toxicity, which was observed in murine models and cancer patients (Dougan & Dranoff, 2009). Recent understanding of MDSCs in cancers provokes to target these suppressor cells. All-trans retinoic acid (ATRA) administration in animal models and *in vitro* study showed decreased number of MDSCs, activated CD4 and CD8 T cells and delayed tumor progression (Kusmartsev et al., 2008). ATRA administration with granulocyte macrophage colony-stimulating factor helped in differentiation of MDSCs into myeloid dendritic cells in tumor-bearing mice (Gabrilovich et al., 2001). In the clinical study ATRA plus IL-2 combination did not have impact on MDSCs of renal cell carcinoma patients. Chemotherapeutic agents, such as gemcitabine and 5-fluorouracil, were reported to reduce the peripheral blood MDSCs in animal models as well as *in vitro* (Le et al., 2009; Vincent et al., 2010). Cyclophosphamide and doxorubicin have negative impact on MDSCs of breast cancer patients (Diaz-Montero et al., 2009). A recent study proposed combination of cyclophosphamide with IL-2 enhanced the clearance of intra-tumoral Treg cells and MDSCs, and enhanced the generation of myeloid inflammatory cells which lack the suppressive function (Medina-Echeverez et al., 2011). STAT-3 is a key regulatory molecule in MDSCs, and this molecule is constitutively expressed by malignant cells. There are several STAT-3 inhibitory molecules under investigation. Sunitinib is one of the STAT-3 inhibitors which influence the phosphorylation of STAT-3 via tyrosine kinase; additionally, it has anti-angiogenic property. Currently, Sunitinib is under investigation for its efficiency on MDSCs (Ko et al., 2009). These observations are showing the efficiency of various inhibitors and chemotherapy agents to hinder the regulatory and suppressor cells *in vitro* and in pre-clinical trials. Unfortunately, clinical trials did not show flourishing impact in all cancers. This might be due to autoimmune toxicities caused by depletion or targeting of Treg cells. Approach of hitting the Treg cells needs further investigation; it is essential to target specifically the tumor associated Treg cells not the global Treg cells which might cause imbalance in the immune homeostasis.

11. Conclusion

Large evidence is available in hematological malignancies and solid tumors for elevated level of various regulatory and suppressor cells which impede anti-tumor responses. Therefore, targeting the regulatory cells could be a useful strategy to enhance the anti-tumor immunity. Approaches of depletion or inhibition of regulatory cells showed countable benefits in pre-clinical and clinical studies of some cancers including renal cell carcinoma, metastatic melanoma and colorectal carcinoma. Targeting regulatory T cells in a non-specific approach might cause detrimental autoimmune toxicities which is the key issue. Further studies are necessary to identify tumor associated regulatory cells which will enhance the depletion of specific regulatory cells but not the global population of regulatory cells.

Moreover, characterization of regulatory cells in humans is an ambiguous aspect due to lack of precise markers. Studies are needed to disclose specific characterization marker for human Treg cells, so that results do not vary between groups. In multiple myeloma, immunotherapeutic targeting of tumor cells at the pre-clinical and clinical studies showed remarkable immunological as well clinical responses in some cohort of patients. When compared to non-hematological malignancies, there are no clinical trials performed to target regulatory cells in myeloma patients. Investigations are required with the inclusion of pre-clinical and clinical studies in myeloma via combinational approach of targeting tumor cells as well regulatory cells. This approach might overcome tumor induced immunosuppression in myeloma patients.

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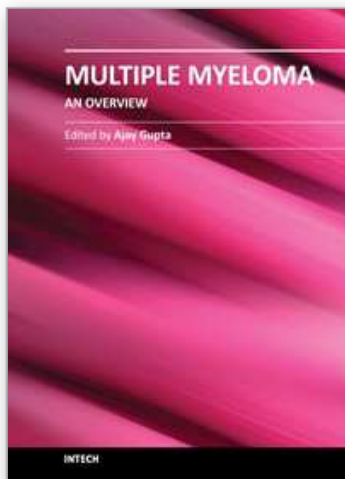
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Multiple myeloma is a malignant disorder characterized by the proliferation of plasma cells. Much insight has been gained into the molecular pathways that lead to myeloma and indeed much more remains to be done. The understanding of these pathways is closely linked to their therapeutic implications and is stressed upon in the initial chapters. Recently, the introduction of newer agents such as bortezomib, lenalidomide, thalidomide, liposomal doxorubicin, etc. has led to a flurry of trials aimed at testing various combinations in order to improve survival. Higher response rates observed with these agents have led to their integration into induction therapies. The role of various new therapies vis a vis transplantation has also been examined. Recent advances in the management of plasmacytomas, renal dysfunction, dentistry as well as mobilization of stem cells in the context of myeloma have also found exclusive mention. Since brevity is the soul of wit our attempt has been to present before the reader a comprehensive yet brief text on this important subject.

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