Breaking Tolerance in a Mouse Model of Multiple Myeloma by Chemoimmunotherapy

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A unique mouse model of multiple myeloma (MM), namely 5T2MM-bearing mouse, was useful for elucidating the pathophysiological mechanisms underlying the disease. Increased accumulation of suppressive CD4⁺CD25HiFoxp3⁺ regulatory T cells (Tregs) was observed in the thymus and lymphoid peripheral organs during disease progression. Adoptive transfer of Tregs, but not other thymocytes, from 5T2MM-bearing mice led to increased progression of disease manifestations in young syngeneic mice. Depletion of Tregs, a proposed strategy in cancer immunotherapy, was tested using cyclophosphamide (CYC), an alkylating agent with selective cytotoxicity. Both low- and high-dose CYC, administered to sick mice with hind limb paralysis, caused the paralysis to disappear, the plasma tumor cells in the bone marrow (BM) cavity to be replaced by normal cell populations, and the survival of the mice to be significantly prolonged. Low-dose CYC, which selectively depletes Tregs, decreased MM incidence, in contrast to high-dose CYC, which was generally cytotoxic, and did not reduce MM incidence. In contrast, low-dose CYC induced Tregs to become susceptible to apoptosis by down-regulating Bcl-xL and CTLA-4 in these cells, and by decreasing the production of IL-2 by...
effector CD4 cells. This treatment consequently triggered the recovery of IFN-γ-producing natural killer T cells and the maturation of dendritic cells. Transient gradual depletion of Tregs in low-dose CYC-treated 5T2MM mice was maintained beyond 45 days. Thus, less frequent injections of low-dose CYC enabled us to recruit compatible immune-derived cells that would reduce tumor load and delay or prevent tumor recurrence, hence breaking immune tolerance toward MM tumor cells. © 2010 Elsevier Inc.

I. INTRODUCTION

Multiple myeloma (MM) is a progressive B-lineage neoplasia characterized by proliferation of clonal malignant plasma cells in the bone marrow (BM). The tumor cells secrete an immunoglobulin, usually monoclonal IgE or IgA in the serum and/or light chains in the urine. The progression of the disease may include anemia, lytic bone lesions, renal dysfunction, hypercalcemia, hypogammaglobulinemia, and peripheral neuropathy. Immune dysfunction is an important feature of the disease and leads to infections that are a major cause of morbidity and mortality. Moreover, it may promote tumor growth and resistance to chemotherapy. MM is characterized by numerous defects in the immune system including impaired lymphocyte functions, steroid-related immunosuppression, and neutropenia secondary to chemotherapy (Bergsagel and Kuehl, 2005). A reduced level of polyclonal immunoglobulins is a consistent feature of active MM, reflecting the suppression of CD19+ B lymphocytes that correlate inversely with the disease stage (Rawstron et al., 1998). The relationship between myeloma plasma cells and the BM microenvironment is critical for maintaining the disease. Tumor cells and stromal cells interact via adhesion molecules and cytokine networks to simultaneously promote tumor cell survival, drug resistance, angiogenesis, and disordered bone metabolism. In addition, a number of immunologically active compounds are increased including transforming growth factor (TGF)-β, interleukin (IL)-10, IL-6, vascular endothelial growth factor (VEGF), Fas ligand, Mucin 1 (MUC-1), Cyclooxygenase (COX)-2, and related prostanoids and metalloproteinases (Pratt et al., 2007).

Various drugs having immunomodulatory effects have been used in MM treatment. Thalidomide, shown to have potent anti-inflammatory, antiangiogenic, and immunomodulatory properties, was reported to have anti-MM activity as well (Bartlett et al., 2004; Rajkumar et al., 2002; Singhal et al., 1999). Lenalidomine is another immunomodulatory drug used recently (Richardson et al., 2006) in a NKT cell target combinatorial immunotherapy approach (Chang et al., 2006).

Animal models mimicking human MM are useful for better understanding the pathophysiological mechanisms involved in the progression of the disease and for developing new therapeutic strategies. A series of murine
models were described by Radl et al. (1988), in which MM arose spontaneously in aging mice of the C57BL/KaLwRij strain with a frequency of 0.5%. A series of tumors have been propagated in vivo by intravenous transfer of the diseased BM into young syngeneic mice. This series of MM tumors represents the human form of the disease since their clinical characteristics involve selective localization in the BM, serum M component, angiogenesis, and adhesion and chemokine profiles that are similar to human myeloma (Asosingh et al., 2000; Vanderkerken et al., 1997). The BM microenvironment consists of extracellular matrix protein and BM stromal cells, osteoblasts, and osteoclasts that play a crucial role in the pathogenesis of MM cell growth and survival (Hideshima et al., 2007).

T cell tolerance to tumor-associated antigens plays a significant role in immune evasion by tumors (Drake et al., 2006; Zou, 2006). Naturally occurring and adaptive regulatory T cells (Tregs) are anergic cells with suppressive capabilities that constitute 5–10% of CD4 cells. These cells are induced early during tumor development and were shown to contribute to tumor tolerance (Peng et al., 2002; Zhou and Levitsky, 2007). The mechanisms underlying these effects include inhibiting the activity of a variety of immune cells that are tumor specific such as effector CD4 cells, CD8 cells, dendritic cells (DCs), natural killer (NK) cells, natural killer T (NKT) cells, and B cells (Chen et al., 2005; Ghiringhelli et al., 2006; Lim et al., 2005; Nishikawa et al., 2005; Piccirillo and Shevach, 2001; Thornton and Shevach, 1998; Turk et al., 2004). Phenotypically, these suppressor cells are characterized by their expression of certain surface and intracellular molecules, which include the following: the IL-2 receptor alpha chain (e.g., CD25), cytotoxic T lymphocyte-associated protein 4 (CTLA-4), and glucocorticoid-induced TNFR-related protein (GITR). Recently, the lack of CD127 expression was shown to predict functional Tregs in normal humans (Liu et al., 2006), but it is relatively unstudied in tumor Tregs. The transcription factor Forkhead-box-p3 (Foxp3) is a more specific marker of Tregs (Hori et al., 2003). Recently, it was demonstrated that Bcl-xL plays a role in the induction and suppressive function of Tregs, in addition to its antiapoptotic effect (Sharabi et al., 2009).

The presence of Tregs in tumors is associated with a poor prognosis (Curiel et al., 2004). Patients with many different types of cancers had increased numbers of Tregs in their blood, tumor mass, and draining lymph nodes. Increased numbers of Tregs in lung and ovarian cancers were first reported by Woo et al. (2001). Later it was demonstrated that high frequencies of Tregs are allocated not only at the proximity of tumors but also in peripheral blood, thus suggesting that an increased number of Tregs is a generalized phenomenon (Liyanage et al., 2002). It is thought that active proliferation of Tregs rather than redistribution from other compartments is responsible for the tumor-associated increase in the numbers of Tregs (Wolf et al., 2006).
Increased numbers of Tregs were found in patients with MM as well (Beyer and Schultze, 2006; Beyer et al., 2006; Feyler et al., 2009). Interestingly, \textit{in vitro} expansion of Tregs could be induced in the presence of MM-specific antigens (Han et al., 2008). The increased number of Tregs was associated with reduced immune effector functions (Han et al., 2008), and was suggestive of the progression of malignant transformation (Beyer et al., 2006).

Therapeutic approaches for breaking tolerance to tumor cells have been tried; the depletion of Tregs is the most studied strategy (Ercolini et al., 2005; Ghiringhelli et al., 2004; Shimizu et al., 1999). Specific depletion of Tregs by anti-CD25 antibodies improved endogenous immune-mediated tumor rejection (Shimizu et al., 1999) by enabling the development of tumor-specific CD8 cells and NK cells that reacted against tumors (Shimizu et al., 1999). Nevertheless, despite the tumor antigen-specific immunity (Tanaka et al., 2002), the tumors were not completely rejected (Jones et al., 2002). Cyclophosphamide (CYC) was found to have specific effects on T cells, with tumor-inhibiting properties (Proietti et al., 1998). This alkylating agent was shown to have beneficial effects in the treatment of MM, and to be associated with increased survival rates (Rivers et al., 1963). It was reported that the beneficial effects of CYC were due to the removal of suppressor T cells rather than to the reduction in tumor burden (McCune et al., 1998).

The use of various doses of CYC for depleting Tregs in different types of solid tumors has been reported. In this regard, low doses of CYC had a specific effect in depleting Tregs (Awwad and North, 1989; Ghiringhelli et al., 2004). High-dose CYC also depleted Tregs but was less effective than the low-dose CYC in rejecting the tumor (Castano et al., 2008). Thus, apparently the beneficial effects of low-dose CYC on tumor rejection may predominantly be immune mediated and less cytotoxic mediated. Indeed, the resulting depletion of Tregs by low-dose CYC augmented the immune response to cancer immunotherapy (Machiels et al., 2001), unlike the high-dose CYC, which caused general immune cell depletion, and as a consequence, the concomitant depletion of CD4 cells and CD8 effector T cells that are required for developing an effective antitumor immunity (Castano et al., 2008). Further, low-dose CYC inhibited angiogenesis and vasculogenesis (Kerbel and Kamen, 2004), and impeded tumor cell repopulation kinetics (Wu and Tannock, 2003). In agreement, mathematical analysis of the evolutionary dynamics of tumor populations predicted that the control of tumors by chemotherapy could be achieved using progressively lower doses and increasingly long intervals between doses (Gatenby et al., 2009). Hence, it is suggested that a desirable effect of a chemotherapeutic compound would result in a tumor volume that is either stable or slowly increases for a prolonged period of time.
II. INCREASED TREGS IN MOUSE MODELS OF MM

In recent years, the role of Tregs in tumor development has been extensively studied. CD4⁺CD25⁺Foxp3⁺ Tregs suppress T cell proliferation, downregulate proinflammatory cytokines, and are involved in tumor tolerance, which is one of the main obstacles to overcome for improving antitumor immunity. Elevated Treg levels in rodents and humans with solid tumors and hematological malignancies, including human MM have been observed (Beyer and Schultze, 2006; Curiel et al., 2004; Liyanage et al., 2002; Marshall et al., 2004; Ormandy et al., 2005), and their functional role in reducing antitumor responses has been demonstrated in rodents (Onizuka et al., 1999, Shimizu et al., 1999; Sutmuller et al., 2001; Turk et al., 2004). In humans, the contribution of Tregs to tumor tolerance was strongly suggested by the significant correlation between Treg levels and the poor survival of ovarian cancer patients as well as tumor relapse in other malignancies such as breast cancer and non-small lung cancers (Bates et al., 2006; Curiel et al., 2004; Petersen et al., 2006). Onizuka et al. (1999) were the first to suggest that CD4⁺CD8⁺ T cells played an important role in inhibiting tumor immunity, causing regression induced by CD25⁺ cell depletion; similar conclusions were presented by Shimizu et al. (1999). These studies on depletion of CD4⁺CD25⁺ T cells and adoptive transfer of CD4⁺CD25⁺ T cells strongly suggest that the effectiveness of an antitumor therapy is greatly enhanced by removal of CD4⁺CD25⁺ T cell suppression activity. Sutmuller et al. (2001) were able to demonstrate that antibody-mediated depletion of CD25⁺ T cells followed by vaccination with the GM-CSF-transfused melanocyte cell line resulted in enhanced tumor rejection. Experiments with adoptively transfused Tregs provided a direct link between Treg cells and reduced tumor immunity (Antony et al., 2005). Thus, it is essential to reveal the mechanism leading to Treg expansion for developing strategies to eliminate them and to improve the results of cancer immunotherapy (Zou, 2005).

Several mechanisms describing Treg induction or recruitment to the tumor site have been described in the literature. It has been suggested that Tregs are induced at the tumor site and further affect the tumor microenvironment and draining lymph nodes (Kim et al., 2006; Zou, 2005). Indeed, it was recently shown that Tregs were induced at the tumor site as a result of IL-10 and TGF-β secretion by tumor cells (Jarnicki et al., 2006; Larmonier et al., 2007; Liyanage et al., 2006). Additionally, Tregs were shown to specifically recruit to the tumor by chemotaxis that was mediated by the release of CCL22 and CCL17 by the tumor cells (Mizukami et al., 2008). The thymus is recognized as the main site of Treg development (Itoh et al., 1999; Kim et al., 2007; Sakaguchi, 2005; Shevach, 2000). Treg development in the thymus has been
discussed as a possible mechanism contributing to Treg accumulation in malignancy (Beyer and Schultze, 2006) and thymus output was indirectly tested in human MM patients (Beyer et al., 2006). However, Treg development in the thymus during malignancy has not been directly explored. Unique mouse models (5TM series) that mimic human MM disease served as a tool to examine levels in the periphery as well as developmental processes that may occur in the thymus to increase Treg ratios in MM development. The 5T33 (IgG2bκ)MM and 5T2MM (IgG2aκ) are the best characterized tumors (Radl et al., 1988; Vanderkerken et al., 1997). The 5T2MM model closely resembles the most common form of human MM in its selective localization to the BM, the presence of serum M component, the development of osteolytic bone disease, and the moderate progressive course of the disease. 5T2MM cells grow exclusively in vivo and can only be maintained in vitro for a very short period when coculture with BM stromal cells. In contrast, the 5T33 MM model represents an aggressive, rapidly progressive variant and cells can easily be maintained in vitro (Manning et al., 1992).

Accumulation of suppressive functional CD4⁺CD25↑Foxp3↑ Tregs was observed in peripheral organs during disease progression in both 5T2MM and 5T33MM mouse models. Treg levels were tested in spleen, lymph nodes, BM, and peripheral blood at different time points (28, 42, 66, 90, and 104 days) following 5T2MM cell injection. At the first two time points, 5T2MM-bearing mice were asymptomatic; the clinical phase involving hind limb paralysis appeared around 60 days and became more severe with increased latency. Treg frequency significantly increased at an early stage (28 d) in spleen and lymph nodes and remained constant during disease progression. Treg ratios increased similarly in lymph nodes surrounding the main sites of tumor infiltration (inguinal, caudal, and lumbar nodes) and lymph nodes distal to the main tumor site (superficial nodes, auxiliary nodes, and branchial nodes). In contrast, Treg frequency in BM remained normal in early stages but increased markedly only in the more progressive phases of the disease, about 90 days onwards after tumor cell challenge. In peripheral blood, a mild but significant increase was observed before paralysis onset (at 42 days) and remained constant during disease progression (Laronne-Bar-On et al., 2008). These observations concerning elevated Treg levels during MM progression coincide with similar findings in MM patients (Beyer et al., 2006; Feyler et al., 2009).

A. Changes in Thymus Structure and Composition

Since Tregs normally develop in the thymus (Itoh et al., 1999; Kim et al., 2007; Sakaguchi, 2005; Shevach, 2000), it was essential to examine whether thymic processes were involved in increased Treg frequency in the periphery.
of MM-bearing mice. Thymus atrophy, manifested by a significant reduction in thymus weight and cellularity (~5.5), was observed in both ST33M and ST2MM mouse models during the disease’s progression (Laronne-Bar-On et al., 2008). A distortion of the normal distinction between cortical and medullary areas was observed. No thymus atrophy was observed in MM-bearing mice during the asymptomatic phase (40 days after tumor cell injection). The atrophy was correlated with the clinical phase of hind limb paralysis caused by spinal cord compression (from 60 days onwards post-tumor cell challenge) and further increased with disease progression and/or severity. Only thymus atrophy that occurred in paralyzed mice was associated with increased Treg-to-effector T cell proportions in MM-diseased mice. Although thymus cellularity was reduced, Treg numbers were not severely decreased in MM-bearing mice whereas numbers of effector T cells were dramatically reduced. The CD4+CD8+ double positive (DP) population, normally the largest thymocyte subset, significantly decreased, whereas the CD4−CD8− double negative (DN) population increased. The proportion of the most mature population of CD4+CD8+ single positive (SP) cells significantly increased in the thymus, suggesting that a change in kinetics rather than a developmental block at the DN stage was responsible for changes in the subpopulation proportions.

Thymus atrophy was reported in cancer patients and tumor-bearing animals (Thomas et al., 1985; Zhang, 1989). In a mouse mammary tumor model, thymic atrophy progressed with tumor growth; in mice with a large tumor mass, the thymus became involuted to less than a 10th of its normal size and its architecture was totally disrupted. Phenotypic analysis of the thymus from tumor bearers revealed a dramatic decrease in the percentage of DP immature thymocytes compared with those in normal controls. Severely altered levels of subpopulations of the CD4−CD8− precursors suggested an early block in the maturation of DN cells. An impaired thymic stromal microenvironment in tumor-bearing mice, increasingly disorganized and altered, coincided with tumor growth (Adkins et al., 2000; Lopez et al., 2002). Similar observations were described in the thymus in mice bearing Lewis lung carcinoma and in ascitic growth of a spontaneous transplantable T cell lymphoma (Kaiserlian et al., 1984; Shanker et al., 2000).

Prolonged infusion of recombinant VEGF, a factor secreted by various tumors including MM, caused profound thymic atrophy (Ohm et al., 2003). A dramatic reduction in CD4+CD8+ thymocytes and a decreased number of the earliest occurring progenitors in the thymus was observed. Thus, pathophysiologically relevant concentrations of VEGF may block the differentiation and/or migration of these progenitors, resulting in thymic atrophy. Cessation of VEGF infusion resulted in the restoration of the normal composition and cellularity of the thymus. Thus, continuous administration of recombinant VEGF mimics the profound thymic atrophy observed in
tumor-bearing mice, and inhibits the production of T cells. VEGF acts on thymic progenitors rather than directly on the thymus itself. VEGF infusion results in defective seeding of the thymus by BM-derived progenitors. These earliest thymocytes fail to replace maturing T cells emigrating to the periphery and consequently, all thymocytes are depleted.

B. Increased Frequency of Treg Development in the Thymus of MM-Bearing Mice

The thymus is normally the main site of Treg development. Following the observation of significant changes in thymus characteristics in MM-bearing mice, it was important to determine whether Treg development was altered in the thymus of MM-bearing mice. The frequency of mature CD4⁺ thymocytes expressing CD25 significantly increased in 5T2MM-bearing mice approximately twofold, and most of the CD4⁺ cells that expressed CD25, coexpressed Foxp3. Foxp3 is a transcription factor that identifies functional Tregs (Hori et al., 2003). There was no significant difference in the percentage of Foxp3⁺ among CD4⁺ SP CD25⁺ cells in controls or in 5T2MM-bearing mice. Interestingly, CD25 expression was increased already at the DP stage. Although most CD25⁺ DP cells did not express Foxp3, the frequency of CD25⁺ Foxp3⁺ cells significantly increased during this stage, and this increase was accompanied by a decrease in the ratios of CD25⁺ Foxp3⁻ DP cells (Laronne-Bar-On et al., 2008). These results are in accordance with previous data suggesting the commitment of the Treg lineage as early as the DP stage (Bayer et al., 2007; Cabarrocas et al., 2006; Pennington et al., 2006).

Increased CD25⁺ Foxp3⁺ expression in the DP stage implies that increased Treg ratios among mature thymocytes result from changes in the developmental processes in the thymus of MM-bearing mice. To exclude the possibility that increased Treg ratios reflect Treg recirculation from the periphery to the thymus (Bosco et al., 2006; Zhan et al., 2007), we compared the naïve phenotype of Tregs in the thymus and in the periphery of 5T2MM-bearing mice. The mouse naïve T cells can be distinguished by the marked expression of CD62L. Tregs in the periphery might have been activated, thereby losing their naïve phenotype. Actually, the percentage of peripheral CD62Lhigh Tregs significantly decreased in the 5T2MM-diseased mice compared with controls, indicating loss of the naïve phenotype. Tregs in the thymus of the same mice retained a naïve phenotype and a statistically insignificant increase was observed (compared with the controls). Effector T cells in the periphery and thymus of 5T2MM-bearing mice exhibited similar trends. The Treg memory phenotype was also tested in the thymus and periphery using the memory marker CD44. The percentage of Tregs expressing CD44 did not change in the periphery (spleen and lymph nodes)
or in the thymus of 5T2MM-bearing mice. Analysis of CD44 and CD62L coexpression revealed a similar decrease in CD62L$^{\text{high}}$ expressing cells as was found in the total Treg population, suggesting a shift from a CD44$^{\text{high}}$ CD62L$^{\text{high}}$ (central memory) to CD44$^{\text{high}}$ CD62L$^{\text{low}}$ (effector memory) phenotype among peripheral Tregs (Laronne-Bar-On et al., 2008). These results indicate that Tregs have a distinct naïve phenotype in the thymus of 5T2MM mice and suggest that Treg recirculation from the periphery is not the cause of increased ratios of Tregs in the thymus.

Since atrophy in MM mice was associated with reduced cellularity, it was interesting to follow Treg levels and their physiological activity in the involuted thymuses. Treg absolute numbers in the thymus of diseased mice were not altered when compared with controls. In contrast, the number of effector T CD25$^-$ cells was reduced ~2.5-fold. Treg numbers decreased only in the severely atrophied thymuses up to ~3-fold, compared with a more dramatic ~9.5-fold reduction of effector T cell numbers (Laronne-Bar-On et al., 2008).

The effect of thymus atrophy on peripheral effector T cell numbers and function in MM are largely unknown (Raitakari et al., 2003). Low effector T cell frequency occurring in the peripheral blood of 5T2MM mice may be associated with thymus atrophy. Effector T cell depletion but not activation or proliferation in the periphery is significantly correlated with thymus atrophy. However, since thymus atrophy is associated with diseased severity, it cannot be concluded that effector T cell depletion results from thymus atrophy or from other processes associated with the disease. Accumulating data suggest that increased Treg-to-effector T cell ratios in the thymus of MM-diseased mice did not result from altered thymocyte survival or increased Treg proliferation. Importantly, the balance between Tregs and effectors has been stressed as critical for deciding between immune response and suppression (Belkaid and Rouse, 2005; Pennington et al., 2006). The data showing increased Treg to effector T cell proportions among immature thymocytes suggest that an effector immune balance exists in the periphery of MM mice. The reviewed data suggest a thymic contribution to increased Treg ratios among CD4$^+$ cells, as was found in the mouse MM models and in human MM patients (Beyer et al., 2006), in addition to peripheral mechanisms reported to contribute to Treg accumulation at the tumor site.

C. Adoptive Transfer of Thymocytes from 5T2MM-Diseased Mice Affects the Severity of MM Manifestations in 5T2MM-Injected Mice

Patients with MM commonly develop bone disease, including bone pain, osteolytic lesions, pathologic fractures, and hypercalcemia. Bone destruction in MM results from asynchronous bone turnover. Normal osteoclasts
are induced by osteoclast-activating factors produced by myeloma cells or the cells in the microenvironment; however, the process is not accompanied by increased bone formation by osteoblasts (Callander and Roodman, 2001; Terpos et al., 2007; Yeh and Berenson, 2006). The 5T2MM mouse model also involves bone lesions as a primary sign of the disease (Dingli and Russell, 2007; Vanderkerken et al., 1997). The 5T2MM cells localize primarily to the BM, replacing the normal BM cells and causing bone lesions. The mice develop hind limb paralysis as a result of spinal cord compression. An adoptive transfer assay (Deng et al., 2006) was carried out to determine whether thymocytes from 5T2MM-diseased mice could support in vivo tumor progression. Mice challenged with 5T2MM cells, still in the asymptomatic phase (42 days after 5T2MM cell challenge), received thymocytes from paralyzed 5T2MM-bearing mice, or from healthy mice. The severity of disease manifestations was apparent. Eighty percent of mice injected with thymocytes from diseased mice developed severe bone destruction and massive tumor growth around the spine, and had infiltration into the surrounding muscles in contrast to 20% in mice injected with control thymocytes, which developed less severe bone destruction. Adoptive transfer of Treg thymocytes from thymus of 5T2MM-diseased mice and thymocytes excluding Tregs (Treg depleted) presented an early onset of disease only following the transfer of Treg thymocytes. Thus, Tregs alone, but not other thymocyte populations, could account for the tumor progressive effect of 5T2MM-derived thymocytes (Laronne-Bar-On et al., 2008).

III. TREG DEPLETION BY CYC IMPROVES ANTITUMOR IMMUNITY

The role of Tregs in tumor development has been extensively studied in recent years. Tregs suppress T cell proliferation, downregulate proinflammatory cytokines, and are involved in tumor tolerance to self-antigens. In addition, Tregs are thought to dampen T cell immunity to tumor-associated antigens and to be the main obstacle to successful immunotherapy. Much data suggest that early-stage cancers are eliminated by immune surveillance, whereas established tumors are more likely to induce immune tolerance (Pardoll, 2003). A multitude of tumor-derived factors contribute to tumor microenvironmental immune tolerance and to immunosuppression; this helps elucidate the lack of effective immune surveillance in later stages of tumor development. Functional Tregs are increased in peripheral blood and in the tumor microenvironment of patients suffering from different types of cancer. A correlation between increased rates of Tregs and disease
progression was observed in cancer patients and in rodent models of solid tumors and hematological malignancies. In humans, the contribution of Tregs to tumor tolerance was strongly suggested by the significant correlation between Treg levels and the poor survival of ovarian cancer patients, progression of pancreatic ductal adenocarcinoma, and tumor relapse in patients with breast cancer and non-small cell lung cancer (Bates et al., 2006; Curiel et al., 2004; Hiraoka et al., 2006; Petersen et al., 2006). Thus, Treg-mediated immunosuppression could be a crucial evasion mechanism that prevents the elimination of cancerous cells by the immune system. Experiments with adoptively transferred Tregs provided a direct link between Treg cells and reduced tumor immunity. Hence, new strategies in cancer immunotherapy, aimed at reducing Tregs, have been proposed (Ruter et al., 2009). Five general strategies to reduce Treg functions have been used: (1) depletion of Tregs; (2) blockade of Treg functions; (3) blockade of Treg trafficking; (4) blockade of Treg differentiation; and (5) combining depletion of Tregs with tumor vaccines.

In our previous studies concerned with the effect of erythropoietin on MM development, using the 5T33MM mouse model, we found that erythropoietin acted as an immunomodulating agent, promoting specific T cell-dependent immune response (Mittelman et al., 2001). The 5T33MM mouse model represents an aggressive rapidly progressive variant that survives for about 4 weeks. Since we were interested in following the pathophysiological mechanism involved in MM development and prevention, for our further studies we chose the 5T2MM mouse model, which has a moderate, progressive course of disease, lasting about 3 months. We observed a correlation between increased ratios of CD4⁺CD25HighFoxp3⁺ Tregs and disease progression (Laronne-Bar-On et al., 2008). The obvious next phase was to study the effect of CD4⁺CD25High Foxp3⁺ Treg depletion on the progression of the disease. CYC was used to deplete Tregs.

CYC is an alkylating agent widely used in chemotherapeutic regimes because of its broad antitumor spectrum and its selective cytotoxicity (Brode and Cooke, 2008). CYC is known to reverse immunological tolerance and to facilitate adoptive immunotherapy through inhibition of suppressor T cell activity. High doses of CYC are required for effective tumor chemotherapy, which might lead to immunosuppression. Strikingly, low-dose CYC can selectively decrease Tregs; therefore, it can be useful for immunomodulation. CYC was also shown to increase the production of inflammatory cytokines (IL-1, TNF-α, IFN-γ), and tumor-induced immuno-suppressive factors (TGF-β, IL-10, VEGF).

Low-dose CYC was shown to decrease Treg numbers and to inhibit their suppressive function (Ikezawa et al., 2005; Lutsiak et al., 2005), as well as to enhance apoptosis and decrease Treg homeostatic proliferation (Lutsiak et al., 2005). A single administration of low-dose CYC was shown to
deplete Tregs in colon carcinoma-bearing rats, thereby delaying tumor growth. In rats bearing established tumors, treatment with a single dose of CYC, followed by an immunotherapy strategy, restored antitumor activity of effector T cells (Ghiringhelli et al., 2004). Inhibitory effects of low-dose CYC on tumor were determined in mice that spontaneously develop prostate carcinoma also through the depletion of Tregs (Wada et al., 2009). Treatment of a mammary tumor model in the neu-N line with immunomodulating doses of CYC in sequence with neu-targeted vaccine revealed high avidity-specific CD8\(^+\) T cell activity associated with more effective eradication of neu-expressing tumors in vivo (Ercolini et al., 2005). The mechanism by which CYC chemotherapy enhances the vaccine-induced specific T cells is through depletion of Tregs. Adoptive transfer of CD4\(^+\)CD25\(^+\) Tregs was shown to inhibit the antitumor immune response induced by CYC administered with vaccine. This is the first report demonstrating the unmasking of high-avidity CD8\(^+\) T cell responses against a naturally expressed tissue-specific tumor antigen in a murine model of tolerance.

Another model showed a direct functional link between the transfer of CD4\(^+\)CD25\(^+\) T cells and reduced therapeutic efficiency of adoptively transferred tumor-antigen-specific effector T cells in a mouse melanoma model. Thus, the optimal vaccine effect against melanoma antigen could be achieved only when CD4\(^+\)CD25\(^+\) Tregs were depleted by CYC treatment (Antony et al., 2005). Single administration of low-dose CYC was shown to potentiate the antitumor effect of DC vaccine in mice bearing B16 melanoma or C26 colon carcinoma. Increased proportions of IFN-\(\gamma\) by removing suppressor T cells induced a bystander effect (Gorelik et al., 1994; Liu et al., 2007; Machiels et al., 2001). Schiavoni et al. (2000) showed that CYC acts by removing suppressor T cells followed by production of type I IFN, thus increasing CD44\(^{hi}\)CD4\(^+\) and CD44\(^{hi}\)CD8\(^+\) T cells (memory phenotype). CYC was also shown to have an antiangiogenic component. Scheduled CYC administration for shorter intervals without interruption (defined metronomic regime; Kerbel and Kamen, 2004) resulted in apoptosis of vascular endothelial cells within the tumor bed. The therapeutic advantage of slowing or suppressing the growth of tumors was demonstrated in mice bearing Lewis lung carcinoma cells or L1210 leukemia cells (Browder et al., 2000).

The metronomic low-dose CYC regime used in advanced cancer patients was shown to induce a profound and selective reduction of circulating Tregs and the reduction of tumor-induced tolerance. CYC treatment led to the restoration of peripheral T cell proliferation and innate killing activities, favoring a better control of tumor progression. This metronomic CYC regime dramatically enhanced T and NK cell effector function through its suppressive effect on Treg number and function (Ghiringhelli et al., 2007).
A. Effects of a Single Low- and High-Dose CYC on 5T2MM Progression

Norths’ pioneering studies in the 1980s suggested that suppressive T cell function could be selectively inhibited in tumor hosts receiving low-dose CYC treatment (North, 1982). Extensive studies on Treg biology presented evidence that different mechanisms govern the antitumor effect of low- and high-dose CYC (Brode and Cooke, 2008; Lutsiak et al., 2005; Motoyoshi et al., 2006). A single injection of different doses of CYC (50, 100, and 200 mg/kg body weight) administered to 5T2MM-bearing mice in the early clinical phase (70 days after cell challenge) prolonged their survival very significantly in comparison with the control group (5T2MM with diluent treatment). The tumor load at the timing of CYC treatment, reflected in the serum protein level (using a standard electrophoretic technique), was 0.95–1.52 g/dl and administering CYC reduced it to the control level (0.13–0.2 g/dl) within 2 days after injecting CYC. The hind limb paralysis involving a nerve compression syndrome such as spinal cord compression, observed in the 70-day clinical phase of 5T2MM-bearing mice, disappeared 14 days after administering the three different CYC doses. The tumor cells in the hind limbs were replaced by normal BM cell populations for several months. The prolonged survival of cell populations following a single CYC injection, irrespective of its dose level, might be related to the CYC-induced disappearance of plasma tumor cells from the BM. Homing of MM cells in the BM is important for their interaction with stromal cells, which induce a microenvironment for their survival as well as growth signals (Hideshima et al., 2007). The main difference between administering low- and high-doses of CYC to the 5T2MM mice lies in the ultimate development of disease (Fig. 1). A high incidence of diseased mice (80%) was observed in those 5T2MM mice treated with a high CYC dose (200 mg/kg). Since the cytotoxic high-dose CYC is less selective to all lymphocytes, including populations with antitumor properties, the residual 5T2MM cells apparently recovered during their prolonged latency, ultimately yielding a high MM incidence. Both groups treated with a low CYC dose (50 or 100 mg/kg) developed a similar lower MM incidence (53% and 59%). Low-dose CYC treatment is associated with selective transient depletion of Tregs in the diseased mice, leading to restoration of peripheral T cell proliferation and immune functions.

The clinical effect of a single injection of low-dose CYC was shown to depend on tumor load. Administering CYC at different intervals of the 5T2MM tumor cell injection affected the final MM incidence, though prolonged survival was observed irrespective of the tumor load level. The single CYC treatment was given to mice harboring 5T2MM cells for 47, 70, or 94 days. The levels of M paraproteins associated with MM development
were 0.57–0.98 g/dl in mice in the asymptomatic phase (47 d), 1.1–1.52 g/dl in the early clinical phase (70 d), (all mice with early hind limb paralysis), and 2.55–2.99 g/dl in very sick mice (94 days). In the control group (injected with diluent) all mice (100%) developed MM at a mean latency of 73 days.

In mice carrying the lowest tumor load (at 47 d), reduced tumor incidence was observed (66%); a further tumor increase (83%) was noted in the early clinical phase (70 d) and 100% in the very sick mice (94 days post MM cell challenge). The efficiency of a single low-dose of CYC in reducing MM progression depended on the tumor load, as reflected in the serum paraprotein level at the time of drug administration. There was a 40–60% MM incidence with a lower tumor burden, and 80–100% incidence with a further increased tumor burden. Nevertheless, substantially prolonged survival was observed.
among 5T2MM mice treated with CYC in comparison with those injected with the diluent, irrespective of the tumor burden.

B. Cellular Component of the Immune System in MM

The number and function of T cell subsets were reported to be abnormal in patients with MM. The CD4:CD8 ratio is inverted, and the Th1:Th2 ratio among CD4$^+$ cells is abnormal (Mills and Cawley, 1983; Ogawara et al., 2005). T cells from MM patients were shown to function aberrantly (Brown et al., 1998; Frassanito et al., 2001). In addition, the levels of expression of CD28 and CTLA-4 costimulatory molecules required for T cell activation and inhibition, respectively, were downregulated in T cells derived from MM patients (Mozaffari et al., 2004). B cell activity was suppressed in patients with an active stage of MM because the cells secreted reduced levels (hypogammaglobulinemia) of polyclonal immunoglobulin, which was inversely correlated with the disease stage (Rawstron et al., 1998). The elevated levels of TGF-β (Urashima et al., 1996), in addition to the impaired accessory signals from Th cells, contributed to dysfunctional B cells. Defective NK cells have also been noted in patients with MM (Jarahian et al., 2007). This is of major importance since NK cells have antymyeloma activity (Carbone et al., 2005; Frohn et al., 2002). Circulating DCs from MM patients were shown to be dysfunctional because the cells failed to upregulate costimulatory molecules required for activation (Brimnes et al., 2006; Brown et al., 2001). It was suggested that reduced function of DCs indicates the progression of the disease (Brown et al., 2001). Further, DCs from MM patients had reduced phagocytic capacity (Ratta et al., 2002). In addition, monocyte-derived DCs exhibited downregulated expression of activation markers and impaired presentation capacity to T cells (Wang et al., 2006). Impaired activity of DCs may be linked to the upregulation of Tregs (Onishi et al., 2008). Cytokines such as IL-6, TGF-β, IL-10, and VEGF, which were actively produced by myeloma cells (Brown et al., 2001), and were found to be in the tumor microenvironment as well as in the serum (Wang et al., 2006), played a role in preventing the development of functional DCs.

C. CYC Effects on Molecules Essential for the Survival and Function of Tregs

There are several molecules that phenotypically characterize Tregs and enable their suppressive function. Foxp3 is a master gene that identifies functional Tregs (Hori et al., 2003). It was reported that injecting a low-dose
CYC results in downregulated expression of Foxp3 in Tregs, which could cause a loss of suppressive activity (Lutsiak et al., 2005). It is possible that CYC downregulate the expression of Foxp3 in Tregs because CYC was shown to result in the upregulation of OX40 (CD134) primarily in Tregs (Hirschhorn-Cymerman et al., 2009) and OX40 engagement on Tregs can reduce Foxp3 levels (Kitamura et al., 2009; Vu et al., 2007). TGF-β is elevated in patients with MM (Cook et al., 1999), and this immunosuppressive cytokine plays a significant role in many aspects of Treg activity. For example, it can maintain the expression of Foxp3 in Tregs (Marie et al., 2005), it induces responder T cells to be sensitive to suppression (Fahlén et al., 2005), and when it is membrane-bound, it may mediate suppression (Nakamura et al., 2001). Although treatment with CYC may result in the upregulation of TGF-β and enhance the induction of functional Tregs, plasma cells from MM patients were resistant to the inhibitory effects of TGF-β on B cell proliferation and immunoglobulin production (Urashima et al., 1996).

Bcl-xL is an antiapoptotic molecule known to play a role in the development, differentiation, and clonal selection of B cells (Amann et al., 2003; Takahashi et al., 1999). Upregulation of Bcl-xL expression was demonstrated in patients with MM (Gauthier et al., 1996; Tu et al., 1998). Further, the expression of Bcl-xL was associated with the progression of MM and impaired the response to treatment in those patients with elevated levels of this antiapoptotic molecule (Tu et al., 1998). Recently, we showed that Bcl-xL plays a role in the induction of Tregs (Sharabi et al., 2010a). Bcl-xL was involved in the induction of Foxp3 in Tregs and in enabling their suppressive function. We also found that the reduced numbers of Tregs in 5T2MM-bearing mice following treatment with a low-dose CYC could be accomplished by downregulating the expression of Bcl-xL in Tregs and increasing their apoptosis.

CTLA-4 is an inhibitory T cell molecule essential for T cell homeostasis and tolerance induction (Chambers, 2001; Salomon and Bluestone, 2001). It is constitutively expressed in Tregs. We found that administration of a low-dose CYC to 5T2MM-bearing mice resulted in a significant reduction of CTLA-4 expression in Tregs. Recently, it was demonstrated that deficient expression of CTLA-4 may hinder the in vivo development and suppressive function of Tregs (Wing et al., 2008). In addition, the downregulation of CTLA-4 may decrease the expression of Bcl-xL (Sharabi et al., 2010a), thus interfering further the development of functional Tregs.

Tregs highly consume IL-2 for their homeostasis and since they cannot produce this cytokine, they depend on effector T cell production (Fontenot et al., 2005). We showed that treatment of 5T2MM-bearing mice with a low-dose CYC resulted in a significant decreased production of IL-2 in CD4 effector cells (Sharabi et al., 2010b). Therefore, it is possible that deficient expression of IL-2 might interrupt Treg maintenance.
D. Adoptive Transfer of Tregs Shortly After Administering CYC to 5T2MM-Bearing Mice

The involvement of Tregs in the pathogenesis of MM has been frequently manifested by the increased number of Tregs associated with the progression of MM, and also by improved disease manifestations after depletion of Tregs. Since specific downregulation of Tregs can be accomplished by injection of low-dose CYC, we conducted a series of experiments in 5T2MM-bearing mice aimed at highlighting other beneficial aspects of CYC, in addition to depletion of Tregs, which might explain its ameliorative effects on MM. In these experiments, 5T2MM-bearing mice with full-blown MM were treated with a single injection of low-dose CYC, and 24 h later, when the cytotoxic effects of CYC were substantially diminished (Sladek et al., 1984), these mice were injected by means of adoptive transfer of two types of cells, for example, the treated mice received either Tregs or effector T cells. Thus, we found that amelioration of MM manifestations, observed in diseased mice in response to low-dose CYC, was abrogated when the mice were injected with Tregs. In contrast, CYC-treated mice that were adoptively transferred with effector T cells preserved the ameliorative effects of CYC on MM.

E. The Effect of CYC on NKT Cells and DCs

Patients with MM have reduced numbers of NKT cells, and IFN-γ production by freshly isolated NKT cells was deficient in patients with progressive myeloma (Dhodapkar et al., 2003). In agreement with previous reports showing that a reciprocal relationship exists between NKT and Tregs (Smyth and Godfrey, 2000) and that Tregs could suppress the function of NKT cells (Azuma et al., 2003; Nishikawa et al., 2003), we noted that depletion of Tregs in 5T2MM-bearing mice that were treated with low-dose CYC was accompanied by significantly upregulated numbers of IFN-γ-producing NKT cells. The main role of NKT cells is to protect against tumors and pathogens (Kronenberg, 2005; Smyth et al., 2002). It has been well documented that NKT cells produce large amounts of IFN-γ upon activation (Arase et al., 1992), and that the antitumor properties of NKT cells are linked to this capability (Liu et al., 2005; Smyth et al., 2002).

Using 5T2MM-bearing mice, we demonstrated the reversibility of aberrant differentiation and function of DCs, observed in patients with MM (Brown et al., 2001; Ratta et al., 2002). DCs from mice with MM and treated with low-dose CYC did not expand but instead differentiated further and acquired a mature phenotype, for example, the DCs upregulated the
expression of MHC class II and costimulatory molecules (Cederbom et al., 2000; Höltl et al., 2005; Larmonier et al., 2007; Misra et al., 2004). The latter effect is of great importance since tumor cells may evade immune responses by losing the expression of HLA molecules (Seliger et al., 2000). It is possible that the elevated production of IFN-γ in the treated mice contributed to the differentiation process of DCs (Beatty and Paterson, 2001). Remarkably, treatment of 5T2MM-bearing mice with high-dose CYC, as oppose to treatment with low-dose CYC, neither affected substantially the number of NKT cells nor the production of IFN-γ by these cells, and did not result in maturation of DCs (Sharabi et al., 2010b).

F. A Window of Opportunity

Because patients with MM are considered to have competent immune systems, it is reasonable to speculate that each patient’s system would be capable of dealing with the disease by generating an antitumor immune response, provided that the inhibitory and regulatory pathways of the immune system are removed or at least put on hold. Tregs are major suppressors of the immune response; therefore, these cells may serve as a convenient target through which the development of MM can be manipulated. In this review, we focused on CYC and showed that using low doses of this drug may, on the one hand, result in depletion of Tregs, and on the other hand, still maintain functional immune-derived cells that would contribute to the amelioration of MM. Hence, the number and function of NKT cells could be recovered, the production of IFN-γ was enhanced, and DCs could continue their differentiation and become mature and ready for activation. Once the concept of low-dose CYC was proven feasible for potentially enabling an effective immune response against myeloma cells, it was essential to find the most effective protocol of treatment that would optimally achieve satisfactory and durable antitymoma effects.

IV. OPTIMAL TIME SCHEDULES OF CYC TREATMENT AFFECTING MM PROGRESSION

Studies in animal models of cancer showed that tumor rejection can be facilitated by inhibiting the function of Tregs, which play a key role in tumor-induced tolerance. Administration of either low- or high-dose CYC to 5T2MM-bearing mice in their early clinical phase of the disease prolonged dramatically their survival. The main difference between the single injection of low- or high-dose CYC was the ultimate high MM incidence
following high-dose CYC treatment in comparison with low-dose CYC. Since the cytotoxic effect of high-dose CYC was substantially less selective and without resulting in the recovery of immune-derived cells with anticancer properties, it may have enabled the growth of residual tumor cells, yielding ultimately a high MM incidence. Treatment with low-dose CYC was associated with selective transient depletion of Treg in the diseased mice, leading to restoration of peripheral T cell proliferation and immune functions. It seemed of interest to test whether reduced MM development during prolonged latency could be accomplished by repeated injections of low-dose CYC at intervals that would coincide with the timing before Treg restoration occurred.

A. The Clinical Effect of a Single Injection Versus Repeated Injections of Low-Dose CYC at Different Time Intervals

The kinetics of suppressor T cell depletion following low-dose CYC administration was described in several studies (summarized in Table I). The results differ according to whether normal mice, tumor-bearing rodents, or advanced cancer patients were tested. In normal mice Treg reduction began after 1–2 days, with the lowest decrease at 4–6 days, and this was restored to a normal level at 10–14 days (in one study the levels were monitored for 4 weeks and were still low). In colon cancer-bearing rats the decrease in Tregs began at day 1, the lowest level was at 7 days and it was restored to normal at 28 days. In 5T2MM-bearing mice a gradual decrease (tested at 14, 25, and 42 days post CYC administration) was observed at all testing points, including 42 days. In advanced cancer patients, 1 month after administering CYC, a dramatic selective Treg depletion was observed and 2 months after starting treatment, pretreatment Treg levels were observed.

Our studies involving the kinetics of CD4^+CD25^{High}Foxp3^+ Tregs following administration of low-dose CYC to 5T2MM-bearing mice showed that Treg depletion was maintained beyond 45 days. Populations involved in antitumor immune responses could effectively be recruited during this period, before renewal of Tregs. Thus, it was of interest to test the possible influence of the “timing window” period on MM progression involving repeated CYC treatments at 45-day intervals. To this end, mice bearing 5T2MM cells for 70 days were treated with three repeated CYC injections at 21- or 45-day intervals. Results are shown in Fig. 2. All treated mice had hind limb paralysis and their paraprotein level was 1.1–1.75 g/dl; the control group developed 100% MM (75± 8 days mean survival) versus 71% MM (188±14 days mean survival) at 21-day interval CYC treatments and
Table I  Kinetics of Tregs Following the Single Administration of CYC

<table>
<thead>
<tr>
<th>Animal model/Human patients</th>
<th>Dose of i.p. CYC (mg/Kg)</th>
<th>First day of Treg reduction</th>
<th>Peak day of Treg depletion</th>
<th>Day of Treg normalization</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal C57BL/6</td>
<td>100</td>
<td>1</td>
<td>4</td>
<td>10</td>
<td>Lutsiak et al. (2005)</td>
</tr>
<tr>
<td>Naïve neu-N</td>
<td>100</td>
<td>2</td>
<td>n.d.</td>
<td>14</td>
<td>Ercolini et al. (2005)</td>
</tr>
<tr>
<td>Normal C3H/HeN</td>
<td>20</td>
<td>1</td>
<td>4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Motoyoshi et al. (2006)</td>
</tr>
<tr>
<td>Normal C3H/HeN</td>
<td>200</td>
<td>1</td>
<td>4</td>
<td>28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Motoyoshi et al. (2006)</td>
</tr>
<tr>
<td>5T2MM-bearing mice</td>
<td>100</td>
<td>n.d.</td>
<td>14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Sharabi et al. (2010)</td>
</tr>
<tr>
<td>5T2MM-bearing mice</td>
<td>200</td>
<td>n.d.</td>
<td>14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Sharabi et al. (2010)</td>
</tr>
<tr>
<td>Patients with advanced cancer</td>
<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
<td>n.d.</td>
<td>30&lt;sup&gt;d&lt;/sup&gt;</td>
<td>60&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Ghiringhelli et al. (2007)</td>
</tr>
<tr>
<td>Patients with advanced cancer</td>
<td>200&lt;sup&gt;c&lt;/sup&gt;</td>
<td>n.d.</td>
<td>30&lt;sup&gt;e&lt;/sup&gt;</td>
<td>n.d</td>
<td>Ghiringhelli et al. (2007)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Depletion of 50% of baseline levels.
<sup>b</sup>Levels of Tregs remained reduced.
<sup>c</sup>Administered orally, daily, every 2 weeks, for a month.
<sup>d</sup>Selective reduction in Tregs number and function.
<sup>e</sup>Nonselective cell reduction.
<sup>f</sup>Days after treatment cessation.
25% MM (228±8 days mean latency) when the interval between the repeated treatments was prolonged to 45 days (Fig. 2A). CYC administration markedly prolonged the survival of the CYC-treated mice, but the repeated treatments often (21 days vs. 45 days) did not improve the effectiveness of the drug, since MM incidence was much higher in spite of more frequent CYC treatment.

A similar experiment involving four repeated CYC treatments at intervals of 7 or 45 days also did not improve CYC effectiveness. The four weekly treatments at 7-day intervals resulted in 90% MM development at a 191±11 day mean latency versus 16% (2/12) at 45-day intervals (sick at 201 and 240 days).

**Fig. 2** Effect of repeated treatments of low-dose CYC (100 mg/kg). (A) Sixty days after 5T2MM cell injection, the mice were treated with 3 CYC injections administered at intervals of 21 days (n=12) or 45 days (n=12). The control group (n=16) was injected with the diluent at intervals of 21 days. All mice (16/16) in the control group developed MM at 75±8 days mean latency. MM incidence in mice receiving repeated injections of CYC at intervals of 21 days was 75% (9/12) at 188±14 days latency versus 25% (3/12) at 228±9 days mean survival at 45-day repeated treatments. (B) 5T2MM-bearing mice were treated 60 days after tumor cell challenge with four CYC injections either at 7-day intervals (n=10) or at 45-day intervals (n=12): 100% (12/12) in the control group developed MM at 76±9 days mean survival; 90% (9/10) that received four weekly injections developed MM at a mean latency of 191±11 days versus 16% (2/12) at 45-day intervals (sick at 201 and 240 days).
following repeated injection of low doses of CYC might enhance the reduction of MM incidence by tipping the balance toward effector T cells for a durable period coinciding with previous observations that depletion of Tregs promoted anti-T-cell responses (O’Garra and Vieira, 2004; Piccirillo and Thornton, 2004). The latter effect is of major importance since the number of Tregs is increased in patients and in mice with MM progression (Curiel et al., 2004; Hiraoka et al., 2006; Laronne-Bar-On et al., 2008; Liyanage et al., 2002; Marshall et al., 2004; Ormandy et al., 2005).

B. Prolonged Maintenance of Treg Depletion

The effect of chemotherapy by administering CYC depends on the timing and dose of CYC, while considering the transient depletion of Tregs. Our observations concerning the efficacy of a long time interval between repeated low-dose CYC treatments served as the basis for testing the prolonged maintenance of Treg depletion (thereby increasing immune antitumor responses) for developing MM. The protocol for this experiment is presented in Fig. 3A.

The initial antitumor treatment involved the administration of a cytotoxic high-dose CYC (200 mg/kg body weight) to mice that received 5T2MM cells 70 days earlier. The tumor load was eradicated (indicated by the normalization of serum preparation level), and hind limb paralysis disappeared within 14 days following CYC treatment. MM incidence in the treated mouse group B was 71% (15/21) within a mean latency of 157±17 days. In the control group A, of the mice bearing 5T2MM cells injected with diluent, 100% (23/23) developed the disease within 82±17 days of mean latency. Bone lesions mostly in femur and/or tibia developed in 43% (10/23) of the control group in about 80–114 days following tumor cell injection. No bone lesions were observed in mice treated with CYC. In mice treated with a high dose of CYC, this chemotherapeutic administration kills both tumor cells but also induces systemic immune suppression, thereby damping the therapeutic efficacy of immunotherapy. To further control the proliferation of 5T2MM residual cells (escaping the high-dose CYC cytotoxic effect), repeated low doses of CYC (100 mg/kg) were administered at 45-day intervals. We tested the effect of three different time schedules, 80-, 60-, and 45-day intervals following the administration of the initial high-dose CYC. In group C, 80 days following high-dose CYC treatment, two additional low doses of CYC were administered at 45-day intervals, yielding 30% (3/10) MM development at a mean latency of 200±27 days. In group D, 60 days after the initial treatment, two additional repeated low-dose CYC injections at 45-day intervals resulted in 20% (2/10) MM development at 191±4 days mean latency. Mice in group E were treated with three repeated low doses of
Fig. 3  Prolonged maintenance of transient Treg depletion for myeloma prevention by administering a single high-dose CYC followed by repeated low-doses of CYC. Seventy days after 5T2MM cell injection, 74 mice were divided into five groups. The control group A (n=23) was injected with diluent—all mice (23/23) developed MM at 82±17 days latency. Group B (n=21) administered with 200 mg/kg CYC yielded 71% MM (15/21) at 157±17 days mean survival.
CYC at 45-day intervals, starting 45 days after the high-dose CYC initial treatment, resulting in 10% (1/10) MM development at 220 days (Fig. 3). Thus, durable transient depletion of Treg cells in 5T2MM-bearing mice with low-dose CYC enhances the function of Treg depletion by tipping the balance toward effector T cells, thereby reducing tumor load to minimal residual disease during prolonged latency.

The prevention of bone lesions developing in CYC-treated mice was remarkable. Summing up results from several experiments involving 150 mice treated with high or low doses of CYC indicated that only 6% (9/150) bone lesions were observed. In 5 mice from these groups, CYC was administered quite late, around 80–92 days after the 5T2MM cell challenge. In control mice challenged with 5T2MM cells, hind limb paralysis was observed at about 60–70 days but bone lesions, due to uncontrolled osteoblast bone resorption, appeared later, from about 80 days onwards in 90% of mice surviving for 90–120 days. The development of lytic bone disease is due to an imbalance, with increased osteoclasts and decreased osteoblasts. MM cells trigger osteoclast activity by secreting an osteoblast stimulating factor and an angiogenesis factor, which result in the development of osteolytic lesions involving bone resorption and the formation of new blood cells (Yaccoby et al., 2002).

Bone disease in MM patients is a major cause of morbidity. Bisphosphonates are potent inhibitors of osteolytic bone resorption. They were found to reduce the incidence of skeletal-related events, thus preventing the development of MM bone disease in vivo. Angiogenesis is also an active and important process in MM disease progression since the BM is richly vascularized. An important open question is whether treatment with bisphosphonates would influence the tumor burden and MM progression. Dallas et al. (1999) used the 5T3MM mouse model to examine the effect of a potent bisphosphonate ibandronate on myeloma-associated bone destruction. Treatment with ibandronate significantly reduced the development of osteolytic lesions in myeloma-bearing mice, but it was not effective in preventing mice from developing hind limb paralysis and did not prolong the survival of myeloma-bearing mice. Treatment of 5T2MM-bearing mice with another potent heterocyclic bisphosphonate, zoledronic acid, prevented the development of lytic bone lesions. A moderate decrease in tumor burden (a 31–35% decrease in serum paraprotein), angiogenesis, and prolonged survival (about 15 days) was also observed (Croucher et al., 2003).

At different intervals after the initial high-dose CYC treatment (80, 60, or 45 days), low-dose CYC (100 mg/kg) was administered repeatedly at 45-day intervals (protocol schedules are presented in Fig. 3A). In group C (n=10), 30% (3/10) developed MM at 200±27 days. In group D (n=10), 20% (2/10) developed MM at 191±4 days and in group E (n=10), 10% (1/10) developed MM at 220 days.
C. Residual Tumor Cells

Initial antitumor treatment may reduce the tumor mass to minimal resid-
ual disease, thereby altering the balance of the disease. We evaluated the
therapeutic efficacy of immunotherapy involving prolonged Treg depletion,
by recruiting antitumor immune response expressed in tumor load size. 5T2MM-bearing mice were reduced to minimal residual disease by injecting
a cytotoxic high dose of CYC followed by prolonged administration of low
doses of CYC at long intervals. The CYC-induced immunomodulation
resulted in remarkably low MM incidence and prolonged survival. An
important question was whether prolonged CYC treatment eradicated all
tumor cells. We approached this enigma by transferring BM from CYC-
treated mice that did not develop overt disease for a prolonged period to
young normal syngeneic recipients and followed MM development in these
BM recipients for 220 days. BM (2×10^7 cells) was transferred i.v. from one
donor to one recipient. The results are summarized in Table II.

Experiment I involved three groups. Group A, a control group, provided
evidence that the transferred BM collected from sick mice not receiving any
additional CYC treatment reflects the tumor load in these 5T2MM-bearing
mice. BM was collected individually from five mice 80 days after 5T2MM
cell injection and was transferred to normal recipients. All five BM recipi-
ents developed overt disease at a mean latency of 61±5 days. Group B—
low-dose CYC (100 mg/kg) was administered to 5T2MM-bearing mice 60
days after tumor cell injection (all mice had hind limb paralysis) and after
170 days the BM of mice grossly normal were transferred to syngeneic
young recipients. None of these recipients (0/10) developed MM within a
220-day follow-up period. Group C: 5T2MM-bearing mice (for 60 days)
were treated with a high dose of CYC (200 mg/kg) and 170 days later, BM
from grossly normal mice was transferred to young normal recipients. All
BM recipients (10/10) developed MM at a mean latency of 111±14 days.
The high-dose CYC reduced the tumor load only transiently (similar results
are shown in Fig. 1), but during their prolonged survival the cells regained
their tumor growth potential. In the control group (injected with 3×10^5
BM cells from sick mice) bearing only MM cells without any further CYC
treatment, their BM activity was replaced by tumor cells and therefore
transferring their BM included a high tumor load and all BM recipients
developed the disease within a short period of 61±5 days. This situation
represents the acute phase of the disease. CYC administration irrespective
of CYC dose levels triggered the disappearance of plasma tumor cells from
the BM area (replaced by the normal BM population) and markedly
prolonged their survival (150–220 days vs. 61–95 days survival of the
controls), thereby reverting the disease development to a chronic phase.
<table>
<thead>
<tr>
<th>Number of experiment</th>
<th>Timing of CYC injection of donor mice (days post 5T2MM injection)</th>
<th>Dose of i.p. CYC (mg/Kg)</th>
<th>Timing of BM transfer from donor mice (days post CYC injection)</th>
<th>MM incidence in BM of recipient mice (n/n, %)</th>
<th>Mean (± SD) latency of recipient mice (days)</th>
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</thead>
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<tr>
<td>Experiment I</td>
<td>60</td>
<td>0</td>
<td>80</td>
<td>5/5, 100%</td>
<td>61±5</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>100</td>
<td>170</td>
<td>0/10, 0%</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>200</td>
<td>170</td>
<td>10/10, 100%</td>
<td>111±14</td>
</tr>
<tr>
<td>Experiment II</td>
<td>66</td>
<td>100</td>
<td>196</td>
<td>4/10, 40%</td>
<td>170±20</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>100</td>
<td>240</td>
<td>1/10, 10%</td>
<td>142</td>
</tr>
</tbody>
</table>

*BM (2×10^7/mouse) from 5T2MM injected mice treated with CYC that did not develop overt disease for a prolonged period was transferred to young normal syngeneic recipients (from one donor to one recipient) and followed for MM development in the BM recipient for 220 days.*
The high CYC dose destroys all T lymphocyte populations, in contrast with the low CYC dose that transiently depletes Treg cells and thereby facilitates antitumor immune responses as long as Treg cells are blocked. BM collected from mice 170 days after being treated with low doses of CYC might therefore have a decreased tumor load.

Experiment II involved two groups of 5T2MM-bearing mice treated either at 66 or 70 days after a tumor cell challenge (at the clinical phase) with a single low dose (100 mg/kg) of CYC, and BM was collected at 196 or 240 days afterwards, when the mice looked grossly normal. The development of MM manifestations occurred in 4/10 recipients in Group A at 170±20 days mean latency and in 1/10 recipients in Group B at 142 days. Thus, the residual tumor load after a long latency following the repeated low-dose CYC treatment seems to be very much reduced, thereby delaying or preventing tumor recurrence. The BM donors might still carry dormant solitary tumor cells that are quiescent and/or in growth arrest (G0/G1 phase) or as small avascular foci. Among the prolonged surviving mice (200–250 days following the initial 5T2MM cell challenge), 30 mice developed undifferentiated lymphoid tumors. These tumors would also grow after subcutaneous grafts (in contrast to 5T2MM tumor cells that grow only following i.v. cell transfer). The spleen was always the main site of lymphoma development, usually involving an enlarged spleen (two- to eightfold weight): the involvement of lymph nodes was observed in 50% of these sick mice and sometimes small foci were observed in the liver; however, their BM was always normal. In several mice (9/30) besides the lymphoma, small foci of plasma tumor cells were observed in the spleen and lymph nodes.

V. CONCLUDING REMARKS

A major impediment to cancer immunotherapy is tumor-induced suppression and tumor evasion of antitumor immune response, which ultimately render the host tolerant to tumor-associated antigens. In recent years, the role of Tregs in tumor development has been extensively studied. A direct link between Tregs and reduced immunity has been demonstrated, strongly suggesting that the effectiveness of antitumor therapy could be greatly enhanced by removal of Treg suppressive activity. A mouse model mimicking human MM was useful to perceive those mechanisms involved in the progression and prevention of the disease. The clinical phase of the disease in 5T2MM-bearing mice involves hind limb paralysis coinciding with increased tumor load and initiation of bone lesions. Suppressive functional Tregs accumulate in the spleen, LNs, BM, peripheral blood, and thymus of sick mice, and contribute
to the development of MM. Eradication of Tregs in this context is therefore desired. The use of CYC may be beneficial for treating MM, since it may selectively deplete Tregs depending on timing and dose. High-dose CYC is cytotoxic and causes general lymphodepletion, whereas low-dose CYC selectively depletes Tregs, induces immunostimulation and antiangiogenesis, and enhances effector cell functions. A single low- or high-dose CYC administered to 5T2MM-bearing mice in their early clinical phase prolonged their survival very significantly in comparison with the control group. More specifically, the tumor load was eradicated, hind limb paralysis disappeared, and the tumor cells homing in the BM cavity were replaced by normal BM cell populations. Thus, this treatment changed the acute phase (100% control mice challenged with 5T2MM cells, with a surviving rate of 80–120 days) into a chronic phase (surviving rate of 160–240 days). Administering a single low-dose CYC reduced the disease incidence (38–60%) in contrast with the high-dose CYC, which resulted in higher incidence rates (70–85%). The efficiency of a single low-dose CYC in reducing MM progression was found to depend on tumor load. Kinetic studies on transient Treg depletion showed that low-dose CYC injected in 5T2MM-bearing mice maintained Treg depletion beyond 45 days. Cell populations with antitumor activity could be recovered while Treg renewal was still blocked. More frequent injections of low-dose CYC at 7- or 21-day intervals did not improve the therapeutic effect since these treated mice developed a high incidence of MM. In contrast, mice treated at 45-day intervals developed a significantly lower MM incidence, thus tipping the balance toward effector T cells for a more prolonged period of time. To further control the proliferation of residual tumor cells that escaped the cytotoxic high-dose CYC, we injected additional low-dose CYC at 45-day intervals. These repeated CYC treatments prolonged the transient Treg depletion, thereby facilitating antitumor immune responses to decrease tumor load to minimal residual disease. The low incidence of bone lesions following CYC injection might be due to the disappearance of plasma tumor cells from the BM. More specifically, they detach from the BM microenvironment, which leads to bone resorption and bone lesions.

In summary, the data presented here and supported by evidence from previous studies indicate that beneficial treatment of mice affected with MM may be accomplished by repeated injections of low-dose CYC at long time intervals corresponding to the transient Treg depletion. Consequently, compatible immune cells such as effector T cells, NKT cells, and DCs may possibly be recovered and play a role in breaking immune tolerance against the tumor cells. We believe this approach should be translated to a clinical setting in future therapy for MM in humans.
ACKNOWLEDGMENTS

We are grateful to Dr. Jay A. Levy of the University of California, San Francisco, for his continuous support, valuable discussions and helpful suggestions. Special thanks to Mrs. Tania Meri for patient assistance in the preparation of the manuscript.

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