Nikhil C. Munshi
Kenneth C. Anderson *Editors*

Advances in Biology and Therapy of Multiple Myeloma

Volume 2: Translational and Clinical Research

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ISBN 978-1-4614-5259-1 ISBN 978-1-4614-5260-7 (eBook)
DOI 10.1007/978-1-4614-5260-7
Springer New York Heidelberg Dordrecht London

Library of Congress Control Number: 2012951132

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To my parents Gulab and Chandravadan Munshi who have inspired me and taught me what I know

and

To my wife Medha and sons Vidit and Manit whose love, support, and sacrifi ce helped me become who I am

Nikhil C. Munshi

To my mentors who sparked my scientific and clinical interest in myeloma
To my fellow researchers and caregivers, with whom I have been privileged to work
To my patients, who are my true heroes and inspire all that I do
And to Cynthia, Emily, David, and Peter for their loving support

Kenneth C. Anderson

Preface

Multiple myeloma has evolved from an incurable disease with no therapeutic options five decades ago to a readily treatable disease, based upon increased understanding of its biology and pathogenesis. Nonetheless, myeloma remains a complex disease driven by both genomic and epigenetic alterations. Moreover, interaction of tumor cells with the bone marrow microenvironment confers additional tumor cell growth, and survival advantage, and drug resistance. Advances in our understanding of the pathobiology of the disease have also translated to improved diagnostic and prognostic methods including high-throughput genomics, serum-free light chain, MRI, and PET scanning. Notably, proteasome inhibitors, immunomodulatory agents, as well as other targeted agents, when used singly or in combination, have transformed myeloma therapy and now achieve unprecedented frequency and extent of response. These rapid advances highlight the need for a state-of-the-art resource focused on the biology of myeloma and its clinical application. Our book describes the basic advances in our understanding of the disease biology and delineates molecular mechanisms mediating tumor growth and progression, as well as bone disease and organ dysfunction. Importantly, it provides the preclinical rationale for and clinical efficacy of single and combination targeted therapies directed at the tumor cell in its bone marrow milieu. With an eye toward the future, we update the recent advances using high-density, high-throughput genomic technologies to integrate both DNA and transcriptional changes for improved molecular classification and personalized therapeutic options. Finally, since studies are already reporting prolonged disease-free survival in myeloma, our book highlights the fact that we are now at the threshold of curative outcome in this disease.

Boston, MA, USA

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Part I
Myeloma Immune and Antibody Targets

Chapter 1
T Cell Responses in Myeloma

Ross Brown, P. Joy Ho, John Gibson, and Douglas Joshua

1.1 Significance of T Cells in Patients with Myeloma

Although multiple myeloma is a neoplasm of the most differentiated cells of the B lineage, a complex range of numerical, phenotypic and functional abnormalities within the T cell compartment of patients with this disease is well recognised. Numerous attempts have been made to identify and understand the clinical significance of these changes; however, the complex interrelationships between cells and soluble factors and a realisation that novel T cell subpopulations exist, but have not yet been fully characterised, have hindered efforts to adequately define the clinical significance of any of these changes. Of major importance are the immunoregulatory role of various T cell subpopulations and the possibility of an immune response against tumour cells. So far the major clinical strategies have been to utilise cytotoxic T cells by adoptive T cell infusion post-allotransplantation or to generate anti-myeloma cytotoxic T cells by either *ex vivo* expansion or a variety of tumour vaccination strategies. Whilst a number of immunotherapy protocols have been attempted in patients with myeloma, in general, these have not had any significant success [1, 2], but the recent FDA approval of a commercial immunotherapy for prostate cancer [3] and the availability of immunostimulatory therapeutic agents has again renewed interest in all forms of cancer immunotherapy.

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N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 2: Translational and Clinical Research*,
DOI 10.1007/978-1-4614-5260-7_1, © Springer Science+Business Media New York 2013

1.1.1 T Cell Numbers in the Peripheral Blood of Patients with Myeloma

The absolute number of both lymphocytes and T cells are often deficient in the peripheral blood of patients with myeloma and is dependent on stage of the disease and recent therapy. The lymphopenia is primarily due to a reduction in the absolute number of $CD4^+$ cells, causing a relative increase in the number of $CD8^+$ T cells [4, 5] and a significantly reduced CD4/CD8 ratio [6] which is often more obvious in patients with progressive disease [7–9]. There is a selective loss of cells in the naive $CD4^+$ $CD45R^+$ subset [10, 11], suggesting that there is a relative enrichment of memory $CD4^+$ cells and a failure to give rise to new naive $CD4^+$ T cells [10]. Conversely, in patients with myeloma at diagnosis, a reduction in the total and activated $CD4^+$ T cells but not naive CD4 subsets has also been reported [12]. A persistence of $CD8^+$ and NK cells has been reported after therapy [13]. These various changes in T cell subsets may provide one explanation for the defect in the primary immune response in this disease; however, murine studies have suggested that the primary immune functional defect is not caused by T cells but rather by antigen-presenting cells [14]. Changes to T cell number and function post-therapy may be due to a reduction of tumour size, changes to the microenvironment, maintenance therapy or the cytotoxic effects of previous chemotherapy.

1.1.2 Activity of T Cells in Patients with Myeloma

T cells in patients with myeloma have a significantly increased activity as demonstrated by an increase in the expression of the activation markers CD38 and HLA-DR [15], serum neopterin [16], serum thymidine kinase [17] and Ki67 [18]. These "hyperactive" T cells produce high levels of IL-2 and interferon-γ [15]. A high expression of CD95 (Fas) and a low expression of bcl-2 on $HLA\text{-}DR^+$ T cells in patients with myeloma suggests a state of chronic activation which is associated with an enhanced susceptibility to apoptosis [19].

Although the T cells in patients with myeloma express various markers of activation, the generation of cytotoxic T lymphocytes and IL-2 induction of lymphokine-activated killer (LAK) cells from patients with myeloma is defective, and this defect correlates with disease status [20, 21]. Low T cell receptor excision circles, a marker of thymic output, were significantly associated with a higher incidence of infections and a shorter survival [22]. Other studies have demonstrated that T cells from patients with myeloma display an impaired response to mitogens Ullrich and Zolla-Pazner [23]), abnormal immunoregulatory functions, such as T suppressor cell dysfunction [24], suppression of polyclonal immunoglobulin synthesis [25, 26] and a decreased cloning efficiency of $CD8^+$ T cells [27]. *In vitro* there is a poor response to idiotype and tumour lysates [28]. These observations suggest that if myeloma-specific T cells exist, they are likely to be functionally defective.

1.2 Specific T Cell Subtypes in Myeloma

1.2.1 Clonal T Cells in Myeloma

Expansions of T cells and T cell subsets associated with a good prognosis have been reported in a number of haematological disorders including myeloma [29–34], chronic myeloid leukaemia [35, 36], myelodysplasia [37, 38] and Waldenstrom's macroglobulinemia [39, 40]. Expanded T cell populations in the blood of patients with myeloma were originally detected by Southern blot [29, 34] and more recently by an abnormal repertoire of expression of the T cell receptor (TCR) variable regions [4, 41–43]. TCR CDR3 fragment length analysis, determination of V beta gene usage and nucleotide sequencing [33, 39, 40, 44] have confirmed the presence and clonal nature of these expansions. The most significant observation concerning expanded T cell clones in patients with myeloma is that their presence is associated with a prolonged overall survival [29, 30, 45] which raises the suggestion that these T cells have some limited anti-tumour activity, though inadequate for disease control. The incidence of clonal T cells is higher in patients with progressive compared to stable disease [29, 30]. In addition it was demonstrated that thalidomide stimulated new T cell clones were associated with an additional survival [45].

Expanded T cell populations in patients with myeloma have been shown by flow cytometry to have the phenotype of cytotoxic T cells, i.e. $CD8^{+}$ $CD45RA^{+}$ $CD57^{+}$ $CD28^{-}$ and perforin positive [43]. Recently it has been demonstrated by TCR CDR3 fragment length analysis and nucleotide sequencing that it is the $CD8^{+}$ $CD57^{+}$ cells within the expanded TCRVβ family that are clonal [33, 39, 40]. Whilst age-matched normal controls may also contain expanded T cell populations, these are almost exclusively $CD4^{+}$ T cells [33]. CD8 expansions with virus specificity may exist (e.g. CMV-specific T cells) but have been shown to represent less than 10% of the expansions reported [29, 33, 43]. In a confirmatory study, Mileshkin et al. [44] reported that the presence of a low number of $CD8^{+}$ $CD57^{+}$ cytotoxic T cells was associated with a poor prognosis. The functional capacity of $CD8^{+}$ T cell expansions in patients with myeloma and their specificity to malignant plasma cells is a key issue that requires further study. These cells have been shown to respond poorly to proliferation stimuli [39, 40] suggesting that they exist either in or near a state of anergy. Overcoming the anergy of these cells may be an important mechanism to produce an anti-tumour response. Trials with anti CD137 (4-1BB) [47, 48], IL-15 [49] and rhIL-12 [50] may provide some opportunities and add to the responses achieved by IMiDs.

1.2.2 T Cells with Regulatory Control

1.2.2.1 Treg Cells

$CD4^{+}$ T cells (T helper cells) are essential in regulating the immune response and coordinate the function of other immune cell types. For many years it has been

recognised that T helper cells can be subdivided into Th1 or Th2 subsets. Th1 cells produce interferon-γ and are involved with autoimmune diseases and immunity against pathogens whereas Th2 cells produce interleukin-4 and participate in humoral immunity against parasites and allergic reactions. In more recent years, it has been shown that T helper cells can also develop into T regulatory (Treg) cells which are characterised by high CD25 and intracellular forkhead P3 (FOXP3) expression. Other workers have suggested that Treg cells can be more clearly defined by low CD127 surface expression [51]. Treg cells inhibit the immune response and maintain tolerance to self-antigens either by contact or the release of cytokines like transforming growth factor β (TGFβ) [52].

Tregs may play an important role in decreasing the host response to tumours as they have been reported to be increased in many malignancies [14], tend to be more common as tumour-infiltrating cells than in the peripheral circulation and their rate of infiltration correlates with tumour progression [53]. An exception is that they may be decreased in patients with chronic lymphocytic leukaemia treated with nucleoside analogues [54].

1.2.2.2 Th17 Cells

Another novel T helper subset, Th17 cells, has also been described [55]. Th17 cells produce interleukin 17 and are found to be increased in inflammation and autoimmune disease. There appears to be a balance between Treg and Th17 cells in normal individuals. Whether Th17 cells promote or inhibit tumour cells is still controversial [56]. Inozume et al. [57] have demonstrated that Th17 cells act as an angiogenic factor and may promote tumour neovascularisation; however, Th17 cells have reportedly "eradicated" melanoma [58], enhance the immune response in lymphoma vaccines [59] and limit tumour progression after immunotherapy [60]. Th17 cells may induce Th1-type chemokines to stimulate CXCL9 and CXCL10 to recruit effector cells to the tumour microenvironment. Evidence of increased interferon-γ release from these cells supports this view [61, 62]. Martin-Orozco et al. [63] demonstrated that Th17 cells promote cytotoxic T cell activation by recruiting dendritic cells, and Van Euw et al. [64] showed that CTLA4 blockade with anti-cytotoxic T lymphocyte antigen antibody treatment increased Th17 cell numbers in patients with metastatic melanoma.

1.2.2.3 Treg and Th17 Cells in Multiple Myeloma

The number and function of Treg and Th17 cells in patients with myeloma is controversial [14]. The conflicting results reported by various workers is summarised in Table 1.1. The number of Tregs has been variously reported as being reduced [65, 67, 69] or increased [66, 68, 70]. In addition the Treg function has been variously reported as reduced [65], normal [68] or increased [66]. Similarly Th17 cell numbers have been reported to be increased [65] and reduced (Brown et al. 2010 unpublished

Table 1.1 Summary of the conflicting reports of Treg and Th17 cell number and function in the peripheral blood of patients with myeloma

Reference	Treg number	Treg function	Th17
Joshua and Brown *Unpublished*	↑↑	↓	↓
Prabhala et al. [65]	↓	↓	↑
Beyer et al. [66]		↑	
Quach et al. [67]	↓		
Feyler et al. [68]	↑	**N**	
Chiarenza et al. [69]	↓		
Raja et al. [70]	↑		

observations). [71] suggested that Th17 cells are increased in the blood of patients with myeloma and that they promote myeloma cell growth and dysregulate immune function. It has also been reported that Th17 and not Treg cells mediate the bone marrow infiltrating lymphocytes of patients with myeloma [72]. However, as shown in Table 1.1, there is clearly no consensus on Treg and Th17 cell assays, and thus, the relative importance of Treg and Th17 cells is still not clear.

The conflicting results for Treg cells in myeloma may be due to both laboratory technical differences and the effect of recent immunomodulatory therapies. The FOXP3 assay is a technically difficult intracellular assay which relies on a subjective gating strategy. More importantly, there are several different antibody clones for FOXP3 which have been shown to produce significantly different results [73, 74]. As mentioned other workers have used the lack of CD127 expression as the final marker [51]. The correlation between FOXP3 and lack of CD127 expression is often poor [74].

Several therapeutic agents can also effect T cells and Treg numbers. The immunomodulatory drugs thalidomide, lenalidomide and probably also pomalidomide appear to stimulate T cells via the B7-CD28 pathway [75] and can also increase Treg numbers *in vivo* [67, 69, 76, 77].

1.2.2.4 T Cells with Acquired Regulatory Capacity

It has been suggested that in patients with myeloma, T cells can acquire antigens which change their function. Thus, although both CD4 and CD8 T cells expressing HLA-G are present in human peripheral blood under normal physiologic conditions, these cells are increased in patients with myeloma and can possess a potent suppressive function [78, 79]. This suppression is due to either upregulation of HLA-G or by T cells acquiring HLA-G by trogocytosis [79]. T cells of patients with myeloma can also acquire CD80 and CD86 [80, 81]. Lymphocytes that have acquired B7 molecules may be involved with inefficient antigen presentation to effector cells leading to anergy and/or apoptosis [80–82]. The impact of altered function due to the juxtaposition of acquired antigens has not been adequately addressed.

1.3 Anti-myeloma Activity of T Cells

1.3.1 General Observations on the Host Versus Myeloma Effect by T Cells in Myeloma

There is considerable clinical and circumstantial evidence for the presence of host control of the malignant cells in patients with myeloma. Conditions such as asymptomatic myeloma and long-standing plateau phase in the presence of an obvious but a hypoproliferative tumour state infer that some degree of "host control" exists. Scientific evidence for the role of immuno-editing and immunosurveillance of myeloma is also available from studies on the T cell graft-versus-myeloma effect seen after allogeneic transplantation, HLA-G induced immune tolerance [83], the abnormal regulatory T cells and dendritic cells [84] and the protective effects of the presence of T cell clones both in the peripheral blood and in the bone marrow of patients with myeloma [29, 45]. In clinical studies infusion of $CD3^+$ T cells induced a graft-versus-myeloma effect, although not without the risk of exacerbating graft-versus-host disease [85, 86].

1.3.2 Anti-myeloma Activity by T Cells in Mice

In murine models, T cells with a specificity for myeloma proteins have been detected, and anti-tumour responses involving T cell proliferation and cytotoxicity have been demonstrated [87–89]. Studies with the murine plasmacytomas MOPC-315 and MOPC-460 have demonstrated that tumour rejection can be elicited by immunising BALB/c mice with myeloma proteins [88–90]. Tumour immunity was shown to be ablated by post-immunisation thymectomy, suggesting a short-lived regulatory effector cell rather than a conventional cytotoxic T cell as the tumour suppressor cell [91]. Anti-idiotypic antibodies to MOPC-315 tumour cells were shown to mediate a reduction of surface membrane expression of M315 but did not influence M315 secretion or MOPC-315 growth. In contrast, anti-idiotypic T cells blocked the secretion of the M315 protein by the tumour cells without effects on cell growth, viability or surface membrane M315 expression [92]. The suppressive effect by anti-idiotypic T cells has been suggested to be mediated via a diffusible product that results in a selective inhibition of intracellular M315 biosynthesis [92]. These studies suggest that the T cell mediated protection may be a cytostatic effect rather than a cytotoxic effect [89]. The murine model has demonstrated that idiotype-specific T cells recognise the CDR3 region of the hypervariable region non-germline peptide produced due to somatic mutation [93]. These idiotype-specific T cells demonstrate T cell receptor diversity, suggesting that there is more than one T cell clone with tumour specificity [94]. It was demonstrated that secretion of tumour-specific antigen is required for immunosurveillance by $CD4^+$ T cells [95] and that deletion of idiotype-specific T cells occurs when idiotype levels exceeded 50 mg/L [96].

Using a 5 T murine myeloma model, Hong et al. [97] generated T cell clones of different subsets and examined their function in the context of myeloma cells. Idiotype-specific CTLs specifically lysed myeloma cells via MHC class I, perforin and Fas ligand (FasL). Th1, but not Th2, cells lysed the myeloma cells by FasL–Fas interaction. Cytotoxic and Th1 cells also suppressed the growth and function of myeloma cells, whereas Th2 cells promoted the proliferation and enhanced the secretion of idiotype protein and cytokines by myeloma cells. Th1 and cytotoxic T cells but not Th2 cells were able to eradicate established myeloma *in vivo* after adoptive transfer. These results showed that idiotype-specific cytotoxic T cells and Th1 are promising effector cells, whereas Th2 provide no protection and may even promote tumour progression *in vivo* [97]. Murine studies have also shown that an IL-21-based tumour vaccine caused a cytotoxic T cell response, tumour regression and increased overall survival in BALB/C mice [98].

1.3.3 Anti-myeloma Activity in Humans

1.3.3.1 T Cell Activity in Humans

In human studies, T cells have shown to have suppressive effects on polyclonal immunoglobulin production in patients with myeloma [25, 26, 99, 100]. Peripheral blood lymphocytes from patients with myeloma have demonstrated direct anti-myeloma activity by proliferative and cytotoxic responses to autologous and allogeneic myeloma plasma cells [101]. T cell mediated plasma cell killing was also shown in studies by [102], where cell-to-cell contact was required for CD3-induced killing of a plasma cell line with the complementary role of soluble factors, such as IFN-γ. Despite the current poor understanding of the nature of T cells in myeloma and the possibility that tumour vaccination may actually induce further T cell tolerance, many groups have performed immunotherapy trials based on either adoptive immunity or idiotype vaccination [1].

1.3.3.2 Idiotype Reactivity in Humans

When peripheral blood T cells were stimulated with $F(ab')_2$ fragments of autologous idiotype, responses within specific T cell subsets were observed using both proliferation and cytokine secretion assays [103, 104]. This mainly Th_1-type response (IFN-γ and IL-2 secreting T helper cells) [105] was inhibited by an anti-HLA-DR antibody suggesting that the idiotype-induced T cell stimulation is MHC class II restricted [106]. In addition, idiotype-induced T cell stimulation was shown to require the presence of antigen-presenting cells, such as B cells or monocytes [106], indicating that the idiotype alone is not sufficient to mount a T cell proliferative response. Plasma cells in myeloma are poor antigen-presenting cells, but the idiotype can be transferred from myeloma cells to other types of antigen-presenting cells for MHC class II presentation to $CD4^+$ T cells [107].

Evidence that idiotypic protein can bind to T cell subpopulations comes from panning experiments [108] and from incubation with fluorescent labelled $F(ab')_2$ fragments [29]. Although binding of the heavy chain class of the myeloma protein to Fc receptors on T cells has been demonstrated [109], the use of $F(ab')_2$ fragments and allogeneic M-protein as a control suggest that this binding is idiotype- and not merely isotype-specific [108]. Panning experiments showed a more marked adherence of activated and suppressor T cell subsets on plates coated with the patient related M-protein compared to the unrelated M-protein, whereas helper/inducer subpopulations showed no changes. There was also a direct correlation between the number of activated T cells and idiotype-reactive adherent cells in individual patients [108]. In the study using fluorescent labelled $F(ab')_2$ and flow cytometry, a close correlation was observed between the idiotype-binding T cells and T cell clonality in peripheral blood, as determined by Southern blotting [29]. However, when idiotype-induced reactivity was studied in patients with restricted TCRVβ expansions, idiotype recognition was not confined to the expanded populations [44, 103, 104]. It has been suggested that that idiotype-specific T cells are tumouricidal if they are Th1; however, Th2 idiotype-specific cells may even promote tumour growth [97].

1.3.3.3 Immunodominant Peptides in the Idiotype

The identification of immunodominant peptides is an important consideration if tumour specific peptides are to be used in idiotype vaccination strategies. The strength of the T cell response depends on the binding affinity of the peptide to the HLA molecule, the stability of the HLA-bound peptide and the avidity of the T cell receptor to the peptide-HLA complex. Bioinformatics can be used to predict which human immunoglobulin-derived peptides are capable of inducing a T cell response [110]. This process has also demonstrated that a subset of $CD8^+$ cells can recognise immunoglobulin-derived peptide sequences common to several patients [111]. Thus, it may be possible to develop a small set of shared peptides capable of inducing a T cell response in a range of patients. Certainly the ability to predict immunodominant peptides has significant implications for vaccination strategies in the treatment of all B cell malignancies.

Bioinformatics was used to predict immunodominant peptides from the sequence of the CDR3 region of the IgH gene of patients with myeloma [31, 32]. CDR3 peptides from most patients failed to achieve a high score, suggesting that the poor affinity between the unique peptides and the patient's HLA would fail to generate a significant T cell response. As most immunodominant peptides in other B cell malignancies are found outside the CDR3 region [111, 112] and more often in framework regions, future studies in patients with myeloma should not expect that immunodominant peptides with the potential to stimulate anti-tumour T cell activity will only be found in the CDR3 region. Such peptides would have a broader application than patient-specific sequences.

1.3.4 *Tumour-Specific T Cells Other than Idiotype-Specific T Cells*

The use of tumour lyses has the appeal of utilising all possible tumour antigens present that may be recognised by T cells and has been reviewed [113]. Dendritic cells loaded with autologous tumour cell antigen have been used to demonstrate the presence of human tumour-reactive T cells *in vitro* [114, 115]. CD8+ cells tumour-specific cells have been reported to have *in vitro* cytotoxic effects against autologous tumour cells (median 39.6% at an effector:target ratio of 40:1) [115].

There have been a range of non-idiotype-specific tumour antigens considered as potential targets to generate a tumour-specific T cell response. Melan-A/Mart peptide can trigger anti-myeloma T cells through cross reactivity with the myeloma cell surface antigen HM1.24 (CD317) [116]. The HM1.24 antigen has been shown to generate T cell responses in other studies [117, 118].

A range of cancer-testis (CT) antigens can be expressed by myeloma cells [119] have reported an antigen incidence of 56% (MAGEC2), 55% (MAGEA3), 35% (SSX1), 20% (SSX4, SSX5), 16% (SSX2), 15% (BAGE), 7% (NY-ESO-1) and 6% (ADAM2, LIPI) in patients with myeloma. In this study there was a strong antibody response against CT antigens preferentially in patients who had received allogeneic stem cell transplantation (alloSCT). These antibody responses correlated with a T cell response at least for NY-ESO-1. Goodyear et al. [120] used tetramer technology to identify CT peptide-specific T cells in the blood of a range of patients with myeloma and demonstrated a correlation with disease burden. However the frequency levels of 0.0004–0.01% of the total $CD8^+$ pool were lower than might be expected for a significant clinical impact. The NY-ESO-1 antigen appears to generate significant responses [121], but its low incidence on myeloma cells may be an issue [119]. SPAN-XB is another CT antigen which has demonstrated the potential to generate T cell response in myeloma patients [122].

In a single patient, MAGE-3 specific cells generated in a normal twin by immunisation with MAGE-3 protein prior to allotransplantation resulted in a significant T cell and antibody response [123].

Other tumour-associated antigens which have demonstrated potential to generate a T cell response are survivin [124], Dickkopf-1 [125], WT-1 [126, 127], RHAMM-R3 [128], hTERT [129] and the heat shock proteins [130].

1.4 T Cell Therapy and Immunotherapy in Myeloma

Current approaches to immunotherapy aim to either prime and expand tumour-reactive lymphocytes (e.g. by idiotype vaccination) or use adoptive transfer strategies based on the infusion of preformed immune effectors such as antibodies or lymphocytes. The immunogenicity of the idiotype protein in myeloma has been investigated for more than three decades. Vaccination with the idiotype protein is

attractive because it provides for patient-specific tumour epitopes which can be readily purified from the peripheral blood of p atients with myeloma. However, customised vaccinations are very expensive, and the poor response to a variety of idiotype vaccination approaches [1] suggests that either the idiotype is not an effective tumour antigen or that an effective mode of presentation for generating an immune response has not been used. Even with current adoptive transfer strategies, neither the optimal tumour target cell for antibody therapy (e.g. $CD20^+$, $CD138^+$, $CD38^+$, $CD138^+$ and $CD45^+$) nor the means to overcome graft-versus-host effect caused by donor lymphocytes after allogeneic transplantation have been identified.

Clinical immunotherapy trials have mainly used a relatively crude mononuclear cell preparation to act as antigen-presenting cells. Antigen can be presented by a number of different cell types. CD40 activation of B cells loaded with tumour lysate antigens has been used as an alternative basis for immunotherapy to traditional antigen-presenting cells and has shown potential to polarise naïve T cells into Th1 subsets and induced a strong target-specific cytotoxic lymphocyte response [131]. Others have argued that only high-potency dendritic cells should be used to present tumour antigen [132] to avoid the development of energy or apoptosis [82]. Interestingly even osteoclasts can function as antigen-presenting cells and activate T cells [39, 40].

1.4.1 *Adoptive Immunotherapy*

Adoptive immunotherapy, i.e. the transfer of immunocompetent cells [133], such as donor-derived lymphocytes, has been shown to be effective after allogeneic bone marrow transplantation in some patients whose disease has relapsed [85, 134–136]. Infusion of $CD3^+$ T cells induced a graft-versus-myeloma effect, although not without the risk of exacerbating graft-versus-host disease [85, 86]. Zeiser et al. [137] reported a 40–52% response rate to donor lymphocyte infusions.

Donor immunisation has been explored. In one report a healthy sibling donor was immunised with myeloma immunoglobulin before marrow transplantation [136]. Two years after transplantation, the monoclonal protein remained low in the recipient [136]. In another study, the donor was immunised against the recipient monoclonal protein before the infusion of donor T lymphocytes was used to treat relapse, post bone marrow transplantation [134]. Nineteen months after donor lymphocyte infusion, the patient remained in remission [134]. Immunisation of donors with idiotype is a viable option but has rarely been reported [138]. Another approach was to administer IL-2 with GM-CSF during the period of lympho-depletion. This resulted in a marked increase in the number and function of early cytotoxic effector T cells post transplantation, without suppression of engraftment [139].

Donor T cells have been shown to kill myeloma cells by a number of different mechanisms. These include recovery of a recipient $CD4^+$ T cell line with specificity for myeloma idiotype [136] and $CD8^+$ allospecific T cells that mediate the cytotoxicity through the perforin-mediated pathway [140]. Recent studies showing that

the T cell repertoire of graft-versus-myeloma differs from that of graft-versus-host disease have encouraged investigation into strategies that will stimulate a graft-versus-tumour effect without graft-versus-host disease [141]. Alternatively the expansion of autologous tumour-specific cytotoxic T cells *ex vivo* was achieved when plasma cells had a high B7-1 and/or 4-1BBL expression [142].

1.4.2 Clinical Trials of Idiotype Vaccination

A variety of idiotype vaccination strategies have been used in clinical trials. Ruffini et al. [1] and Yi [2] provide reviews of the major trials reported. Most protocols include idiotype-pulsed autologous dendritic cells or idiotype-specific proteins conjugated to keyhole limpet hemocyanin as immunogens, followed by granulocyte-macrophage colony-stimulating factor or interleukin-2 as immunoadjuvants [143–150]. In general these procedures have been well tolerated, and investigators have reported idiotype-specific T cell proliferative responses in 16–100% of patients [143, 145–147, 149, 151, 152]. Idiotype-specific cytotoxic T cell responses have been less frequent [144, 148]. Wen et al. [150] have been able to show that, using idiotype-pulsed dendritic cells as antigen-presenting cells, autologous idiotype-specific cytotoxic T cell lines could be generated that were able to lyse autologous idiotype-pulsed dendritic cells as well as autologous primary myeloma plasma cells [150]. Furthermore, using selective inhibitors of perforin-mediated and Fas-mediated cytotoxicity, it was also shown that the cytotoxic function of idiotype-specific cytotoxic lymphocytes was mediated mainly by the perforin-dependent pathway [150]. It may be significant that expanded T cell populations in patients with myeloma have the phenotype of cytotoxic T cells and, more specifically, have a higher expression of perforin than non-expanded T cell populations [43]. Myelovenge, a large commercially based effort to provide patient idiotype-specific immunotherapy demonstrated a significant survival advantage (median overall survival 5.3 vs. 3.4 years) in a 10-year follow-up study although the use of the vaccine did not prolong progression-free survival post transplant [153, 154].

Identification and monitoring of clinically relevant tumour-specific immune responses is an important part of any vaccination trial [146]. However, to date, it has been difficult to demonstrate the presence of idiotype-specific cytotoxic lymphocytes in patients with myeloma either before or after immunotherapy. However, most frequently, an enzyme-linked immunospot assay [149, 150] has been used to demonstrate the cytokine response and a tritiated thymidine assay [146, 148] has been used to measure T cell proliferation. In addition, delayed-type hypersensitivity skin tests and humoral responses to the idiotype or the immunoadjuvant have been utilised [144–146, 148, 149]. Abdalla et al. [155] reported a decrease in peripheral blood myeloma cells after idiotype vaccination. Curti et al. [156] reported that subcutaneous injection of cryopreserved idiotype-pulsed dendritic cells was safe and, in contrast with intravenous administrations, induced anti-MM T-cell responses. [157] demonstrated that a clinical response to immunotherapy required dendritic cell presentation and not just a KLH-idiotype vaccination.

1.4.3 Dendritic Cell Therapy

Vaccination protocols may require new therapeutic strategies with a more complex and multi-faceted approach to optimise antigen and antigen-presenting cells and to overcome T cell tolerance. Factors relating to antigen-presenting cells may include the generation of an increased number of high potency, functionally normal dendritic cells [80, 81], enhanced recruitment of dendritic cells with Flt3L [158], affinity purification of dendritic cells and an optimisation of the loading of dendritic cells with antigen.

The dendritic cells of patients with myeloma are defective [84], and the antigen processing machinery is more defective in patients with myeloma than in those with monoclonal gammopathy of undetermined significance [159]. This appears to be at least partially due to tumour-derived TGFβ and IL-10 [84] and can be neutralised with IL-12 [80, 81]. In the clinic, rhIL-12 has demonstrated some immunomodulatory properties especially when used in combination with GM-CSF, but there have been some significant side effects [50, 153, 154, 160]. One concern is that there is some evidence that bortezomib will impair the function of dendritic cells [161], an observation that may impact on the design of immunotherapy trials in patients treated with this agent.

Exposure to myeloma cells can affect host immunity by priming dendritic cells towards a maturation state favouring the generation of T cells with a regulatory rather than an effector phenotype [162]. If idiotypic peptides are to be used as antigen, bioinformatics could be used to predict the most appropriate immunodominant epitopes [31, 32, 111]. Upregulation of the expression of costimulatory molecules on the malignant cell population with a biological modifier such as CD40L may be necessary to induce plasma cells to function as antigen-presenting cells and also to induce the differentiation of high-potency dendritic cells [80, 81, 163]. Finally, it will be necessary to overcome T cell tolerance. This may require at least the addition of exogenous cytokines [164].

Several groups have begun to investigate immunotherapy with gene-modified dendritic cells and T cells. A phase I immunotherapy study of T cells manufactured under good manufacturing practice conditions for patients with Le^{Y}-positive myeloma may provide some interesting results [165]. As the infusion of dendritic cells can cause an increase in Tregs [166], it is likely that many forms of adoptive therapy may stimulate a host suppressive response.

1.5 Tumour-Derived Suppression/Inhibition of T Cells

A simplistic view of tumour immunology has utilised the same principles as the immunology of microorganisms. Thus, the paradigm has been that tumours possess some antigens which are “non-self” and that these foreign antigens could stimulate an appropriate humoral and cellular response. This view of tumour immunology

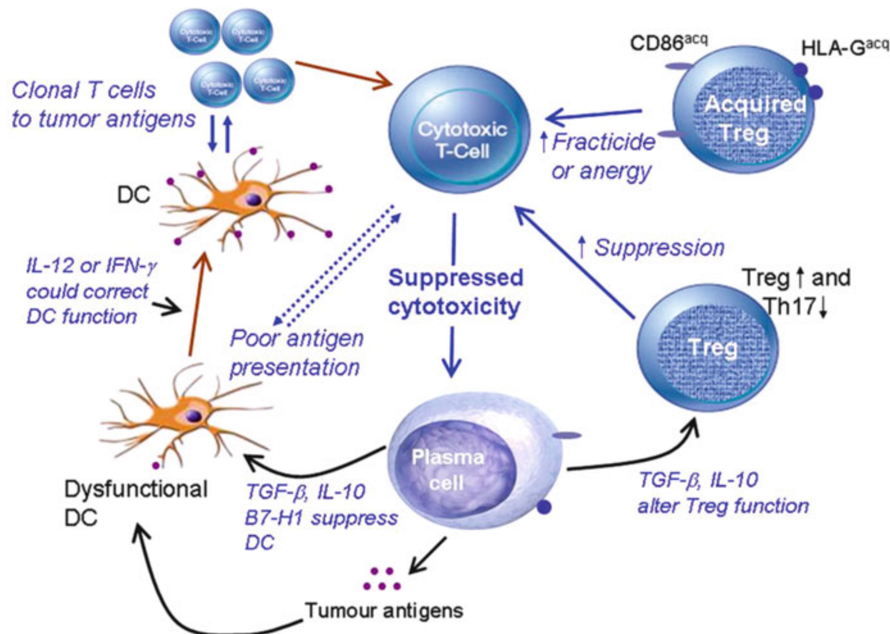

Fig. 1.1 Mechanisms associated with tumour-induced suppression of cytotoxic T cells in multiple myeloma include dysfunctional DC due to plasma cell-derived TGF-β or IL-10, fratricide or anergy induction by acquired regulatory cells and imbalance of Treg and Th17 cells causing suppression of T cell proliferation

fails to recognise the presence of tumour-derived inhibitors and immune tolerance. The fact that tumour-specific T cells exist but are not able to remove tumour cells suggests that there are other factors which inhibit the function of cytotoxic T cells. This may involve either cellular interactions or soluble factors, including cytokines. Spontaneous rejection of established tumours by an immune-mediated rejection is rare. There is good evidence that tumours actively avoid rejection and defeat host immunity. Even therapies which actively increase the number of anti-tumour cells may never be successful *in vivo* unless it is possible to also remove tumour-derived immune suppression [167]. Thus, a more complex paradigm which includes tumour-induced immune tolerance and tumour escape is required [167].

Tumour cells may interfere with the immune response by secreting suppressive factors or by promoting apoptosis in the immunoregulatory cells (Fig. 1.1). Transforming growth factor β1 (TGF-β1) produced by myeloma cell lines has been shown to suppress not only dendritic cell function [84] but also T-cell proliferation by inhibiting responses to IL-2 in stimulated peripheral blood T lymphocytes [164]. FasLigand, which induces programmed cell death in Fas-positive and Fas-sensitive target cells, was shown to be expressed on myeloma cell lines indicating a possible mechanism for the tumour to escape from immune surveillance [168]. Even though myeloma cells express Fas antigen, not all myeloma cells undergo apoptosis in

response to anti-Fas antibodies [169]. In line with these data, mutations in the Fas antigen have also been reported from patients with myeloma suggesting a loss of death control in these cells rather than a lack of growth control [170].

The key to successful cancer immunotherapy is to not only generate a significant humoral and cellular response but also to overcome the acquired cancer-specific immune tolerance and to correct the cytokine imbalance. Only a few studies have demonstrated the possibilities of this dual approach. Inactivation of T cells by IL-15 renders T cells resistant to suppression by TGFβ1-producing tumour cells and rTGFβ1 [49], whilst supraphysiological expression of calnexin (CNX) using lentiviral (LV) vectors in dendritic cells of myeloma patients overcame the immune suppression and enhanced MM-specific CD4 and CD8 T-cell responses [171]. The combination of Treg depletion and chemotherapy may also be a suitable approach to break tolerance [172].

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Chapter 2
Novel Antigenic Targets for Immunotherapy in Myeloma

Qing Yi

2.1 Introduction

The American Cancer Society estimates that 20,180 patients will have been diagnosed with multiple myeloma (MM) in year 2010 and 10,650 will die of this disease. These statistics indicate that MM is the second most commonly diagnosed hematologic malignancy after non-Hodgkin lymphoma. Moreover, over the past 25 years, the number of new cases has increased by more than twofold, supporting the importance of this disease as a public health concern [1]. Over the last decade, MM has emerged as a paradigm within the hematologic malignancies for the success of translational medicine. With the bench-to-bedside approaches used by the leaders of this field, four novel drugs have been approved for this disease in the past 5 years, including bortezomib, thalidomide, pegylated liposomal doxorubicin, and lenalidomide. These agents initially were used in the relapsed/refractory setting, and are now being adopted as part of front-line therapy [2], where they appear likely to have even greater benefits. Despite these advances, however, MM remains incurable, and the vast majority of patients eventually relapse with disease that is typically more resistant to therapy than in prior lines of treatment. This indicates that there is a greater need than ever to focus on this disease and to develop more effective therapies. Immunotherapy is an appealing option for this purpose [3].

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N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 2: Translational and Clinical Research*,
DOI 10.1007/978-1-4614-5260-7_2, © Springer Science+Business Media New York 2013

There is ample evidence to indicate that myeloma cells are susceptible to T cell-mediated cytolysis. In the post-allograft relapse setting, in which myeloma patients are chemotherapy refractory, long-lasting disease remission has been achieved after infusion of donor lymphocytes, a phenomenon termed graft-versus-myeloma effect [4, 5]. This graft-versus-myeloma effect is closely associated with graft-versus-host disease, and donor-derived alloreactive and tumor-specific T cells are believed to mediate these effects [6]. These observations strongly suggest that chemotherapy and immunotherapy kill myeloma cells by different modes of action that are non-cross-resistant; therefore, they should work synergistically.

2.2 Myeloma-Specific Antigen: Idiotype Proteins

Idiotype proteins are derived from monoclonal myeloma cells and are considered tumor-specific antigen. Active immunization against idiotypic determinants on malignant B cells has produced resistance to tumor growth in transplantable murine B-cell lymphoma and plasmacytoma [7–11]. The presence of idiotype-specific T cells in the peripheral blood of patients with MM or with the benign form of the disease, monoclonal gammopathy of undetermined significance (MGUS), has been studied by detecting idiotype-induced T-cell proliferation and cytokine secretion by using the enzyme-linked immunospot (ELISPOT) assay [12].

Idiotype-specific T cells at a low frequency were detected in 90% of patients with MM or MGUS [13–15]. Consistent with these results, we and others have shown that T cells in myeloma patients responded to peptides corresponding to complementarity-determining region I–III of heavy and light chains of the autologous M-component [16–19]. We found that idiotype-induced T-cell stimulation was mainly confined to the $CD4^+$ subset in most of the patients examined and was MHC class II-restricted. Idiotype-specific $CD8^+$ T cells were also demonstrated, but at a lower frequency. Idiotype-specific $CD4^+$ and $CD8^+$ T cells were mainly of the type-1 subsets, as judged by their secretion of interferon (IFN)-γ and interleukin (IL)-2 [20, 21]. Moreover, the proportion of individuals who had an idiotype-specific response of the T helper-1 (Th1)-type (IFN-γ- and/or IL-2-secreting cells) [22, 23] was significantly higher in patients with indolent disease (MGUS and MM stage I) compared with those with advanced MM (stage II/III). In contrast, cells secreting the Th2-subtype cytokine profile (IL-4 only) [22, 23] were seen more frequently in patients with advanced MM (stage II/III) [15]. A similar pattern of cytokine secretion was also reported by others [24]. Collectively, these findings indicate that the existing idiotype-specific immune response is too weak to control the growth of myeloma cells in vivo and that a shift from an idiotype-specific type-1 response, i.e., Th1 and T cytotoxic-1 (Tc1) [25], in early MM to a type-2 response (Th2 and probably Tc2; [25] in advanced disease may have occurred. These studies provide indirect evidence that idiotype-specific T cells may have a regulatory impact on human tumor B cells. Indeed, our recent study using a myeloma murine model clearly showed that idiotype-specific Th1 and TC1 are cytolytic to myeloma cells, while Th2 cells promote myeloma growth [26].

To examine whether idiotype-specific T cells can recognize and kill myeloma cells, we generated idiotype-specific cytotoxic T lymphocyte (CTL) lines from myeloma patients [27]. To enhance the immunogenicity of idiotype proteins, we used dendritic cells (DCs) as antigen-presenting cells. After repeated rounds of in vitro T-cell stimulation with idiotype-pulsed autologous DCs, idiotype-specific T-cell lines, which consisted of both $CD4^+$ and $CD8^+$ T cells, were generated and propagated from the peripheral blood mononuclear cells (PBMCs) of myeloma patients. Idiotype-specific proliferative responses were observed when these T cells were rechallenged with the autologous, but not allogeneic, idiotype-pulsed DCs. By using a standard ^{51}chromium-release assay, our results showed that idiotype-specific CTLs not only recognized and lysed autologous idiotype-pulsed DCs but also significantly killed autologous primary myeloma cells. The cytotoxicity was MHC class I- and, to a lesser extent, class II-restricted, suggesting that myeloma cells could process idiotype protein and present idiotype peptides in the context of their surface MHC molecules. Taken together, these findings provide direct evidence that myeloma plasma cells express idiotype peptides-MHC molecules on their surface and are susceptible to idiotype-specific T cell-mediated lysis.

Idiotype proteins have been used as myeloma antigens for immunotherapies of MM for the past 14 years [3]. Our group at the Karolinska Institutet, Stockholm, Sweden, was the first to introduce active immunization of myeloma patients with Id proteins [28, 29]. In our first pilot study, we recruited and immunized five previously untreated patients with stages I–III MM with the autologous Id protein precipitated in an aluminum phosphate suspension [28]. In three patients, an anti-Id T-cell response amplified 1.9- to 5-fold during the immunization. However, the induced T-cell response was transient and was eliminated during repeated immunization. The disease was stable in all patients, and no side effects or clinical responses were noted. In our second series of the study, immunization was performed by subcutaneous or intradermal injection of Id protein and granulocyte-monocyte colony-stimulating factor (GM-CSF) [29]. Five patients with IgG myeloma were treated, and an Id-specific type-1 T-cell response developed in all of them. One patient had a clinical response, defined by a significant decrease in serum Id protein (from 20 g/L to 7 g/L) and normalization of serum Ig levels. Although these studies involved a limited number of patients, the results clearly indicated that Id protein vaccination, particularly in combination with GM-CSF, was able to induce specific anti-Id cellular and humoral immune responses, which were occasionally accompanied by a clinical response in treated patients.

Other clinical settings for immunotherapy could be minimal residual disease status achieved by high-dose chemotherapy and early host immunologic recovery following stem cell transplantation. These are supported by a study from Massaia and coworkers [30] showing that Id vaccination of myeloma patients with minimal residual disease was able to induce a strong Id-specific cellular immunity in many of the patients. In their study, 12 patients who had been treated with high-dose chemotherapy followed by stem cell support received Id–keyhole limpet hemocyanin (KLH) vaccines and a low dose of GM-CSF or IL-2. Generation of Id-specific T-cell proliferative responses was documented in only two cases; however, a positive, Id-specific, delayed-type hypersensitivity (DTH) skin test reaction was observed in

eight out of the ten patients studied. The induction of humoral and cellular immune responses to KLH was observed in 100% and 80% of the patients, respectively, suggesting that the majority of patients were already able to mount immune responses to KLH shortly after high-dose therapy and stem cell transplantation. Collectively, these results indicate that immunization of myeloma patients with the autologous Id protein, together with GM-CSF, might be a promising method of immunotherapy [31].

Since the renewed interest in using myeloid DCs as tumor vaccine, several groups published their results of idiotype-pulsed DC vaccination studies in MM. Wen and coworkers [19] reported vaccinating an MM patient with autologous Id protein-pulsed DCs generated from blood adherent cells. Enhanced Id-specific cellular and humoral responses were observed in the patient. The immune responses were associated with a transient minor decrease in the serum Id protein level. In their subsequent study, six additional patients were treated according to the same protocol [32]. An immune response against Id was demonstrated in many of the patients. A minor clinical response (25% reduction in the M-component) was observed in one patient and stable disease in the remaining patients. Reichardt and coworkers [33] reported their experience with Id-pulsed DC vaccination in 12 myeloma patients after autologous peripheral blood stem cell transplantation. Their results were less compelling because only 2 out of 12 patients mounted cellular Id-specific proliferative responses as the sole evidence for effective vaccination. Nevertheless, all myeloma patients could mount a strong anti-KLH response despite recent high-dose therapy. Similar results were also obtained in their subsequent study involving 26 patients treated on the same protocol [34]. Although 24 out of 26 patients generated a KLH-specific cellular proliferative immune response, an Id-specific proliferative immune response developed in only four patients. No clinical benefit was observed. These results suggest that DC-based Id vaccination is feasible after transplantation and can induce an Id-specific T-cell response in certain patients.

Other clinical trials of Id-pulsed DC vaccination in myeloma patients have been reported. Cull and coworkers [35] reported on their experience of vaccinating two patients with advanced refractory MM with Id-pulsed DCs combined with GM-CSF. An anti-Id T-cell proliferative response was detected in both patients, which was associated with IFN-γ production by the T cells. One patient also had an anti-Id humoral response. Titzer and coworkers [36] treated 11 patients with advanced MM with Id-pulsed, $CD34^+$ stem cell–derived DCs and GM-CSF. After vaccination, three out of ten analyzed patients showed an increased anti-Id antibody titer, and four out of the ten patients had an Id-specific T-cell response measured by ELISPOT assay.

To improve the efficacy of DC vaccination in myeloma, we investigated the use of Id-pulsed mature DCs administered subcutaneously. Five patients with stable partial remission following high-dose chemotherapy were vaccinated at least 4 months posttransplantation [37]. After four DC vaccinations, Id-specific T-cell responses were elicited in four patients and anti-Id B-cell responses in all five patients. A 50% reduction in serum Id protein was observed in one immunologically responding patient and persisted for more than 1 year; stable disease was noted in the other three patients. The remaining patient without an immune response to the

vaccination experienced disease relapse. Similar results were recently reported by Curti and coworkers [38]. In their study, 15 patients received DCs pulsed with Id proteins or their peptides, and an Id-specific IFN-γ response was seen in eight patients. Clinically, 7 out of the 15 patients had stable disease after a median follow-up of 26 months, one patient achieved durable partial remission after 40 months, and seven patients progressed. Alternatively, Id-pulsed allogeneic DCs could also be used to vaccinate myeloma patients [39]. Taken together, these results indicate that subcutaneous DC vaccination indeed induces better antimyeloma responses than intravenous DC vaccination.

Recently we investigated the use of idiotype- and KLH-pulsed, CD40 ligand-matured DCs administered intranodally. Nine patients with smoldering or stable myeloma without treatment were enrolled, and DC vaccines were administered at weekly intervals for a total of four doses. Following vaccination, all patients mounted Id-specific IFN-γ T-cell response. IL-4 response was elicited in two and skin DTH reaction in seven patients. More importantly, idiotype-specific CTL responses were also detected in five patients. Most if not all patients mounted a positive T-cell response to KLH following vaccination. At 1-year follow-up, six of the nine patients had stable disease, while three patients had slowly progressive disease even during the vaccination period. At 5-year follow-up, four of the six patients continued with stable disease. No major side effects were noted. These results suggest that intranodal administration of Id-pulsed CD40 ligand-matured DCs was able to induce idiotype-specific T and B cell and perhaps clinical responses in patients [40]. In line with these results, Lacy and coworkers reported that idiotype-pulsed DCs following autologous transplantation for MM may be associated with prolonged survival [41].

2.3 Novel Antigenic Targets for Immune Targeting

2.3.1 Dickkopf-1 (DKK1)

DKK1 is a secreted protein that specifically inhibits the Wnt/β-catenin signaling by interacting with the co-receptor Lrp-6 [42, 43]. Previous studies have shown that the *DKK1* gene has restricted expression in placenta and mesenchymal stem cells (MSCs) and not in other normal tissues [44, 45]. Recent studies demonstrated that DKK1 in myeloma patients was associated with the presence of lytic bone lesions [46]. Immunohistochemical analysis of bone marrow biopsy specimens showed that only myeloma cells contain detectable DKK1. Recombinant human DKK1 or bone marrow serum containing an elevated level of DKK1 inhibited the differentiation of osteoblast precursor cells in vitro. Furthermore, anti-DKK1 antibody treatment was associated with reduced tumor growth in myeloma mouse models [47–49]. These results indicate that DKK1 is an important player in myeloma bone disease.

The identification of novel tumor-associated antigens, particularly those shared among patients, is urgently needed to improve the efficacy of immunotherapy for MM. For this purpose, we examined whether DKK1 could be a good candidate.

We identified and synthesized DKK1 peptides for HLA-A*0201 and confirmed their immunogenicity by in vivo immunization of HLA-A*0201 transgenic mice. We detected low frequencies of DKK1 peptide-specific $CD8^+$ T cells in myeloma patients by using peptide tetramers and generated peptide-specific T-cell lines and clones from HLA-A*0201^+ blood donors and myeloma patients. These T cells efficiently lysed peptide-pulsed but not unpulsed T2 or autologous DCs, $DKK1^+$/HLA-A*0201^+ myeloma cell lines U266 and IM-9, and more importantly, HLA-A*0201^+ primary myeloma cells from patients. No killing was observed on $DKK1^+$/HLA-A*0201^- myeloma cell lines and primary myeloma cells or HLA-A*0201^+ normal lymphocytes, including B cells [50]. These T cells were also therapeutic in vivo against established myeloma in SCID-hu mice after adoptive transfer. These results indicate that these T cells were potent CTLs and recognized DKK1 peptides naturally presented by myeloma cells in the context of HLA-A*0201 molecules. Hence, our study identified DKK1 as a potentially important antigen for immunotherapy in MM.

Inhibiting DKK1 activity by using specific monoclonal antibodies (mAbs) to treat MM and myeloma-associated bone disease is also a novel approach because DKK1 has been shown to contribute to osteolytic bone disease in MM by inhibiting the differentiation of osteoblasts [46]. A humanized DKK1-neutralizing mAb, BHQ880 has been developed by Novartis and tested in preclinical studies [47–49]. In both murine [48] and xenograft human [47, 49] myeloma mouse models, this mAb was shown to sustain or increase the numbers of osteoblasts, protect myeloma-induced bone loss, and reduce the development of osteolytic bone lesions. Furthermore, the mAb was also shown to inhibit the growth of xenografted human myeloma cells in SCID-hu [47] or SCID-rab [49] mouse models. These results provide the rationale for clinical evaluation of BHQ880 to improve bone disease and to inhibit myeloma growth.

2.3.2 β_2-Microglobulin (β_2M)

β_2M is an 11.6-kDa non-glycosylated polypeptide composed of 100 amino acids. It is part of the MHC class I molecule on the cell surface of nucleated cells. Its best characterized function is to interact with and stabilize the tertiary structure of the MHC class I α-chain [51]. Because it is non-covalently associated with the α-chain and has no direct attachment to the cell membrane, β_2M on the cell surface can exchange with free β_2M present in serum-containing medium [52]. Free β_2M is found in body fluids under physiological conditions as a result of intracellular release. Elevated levels of serum β_2M are present in hematological malignancies, including lymphomas [53], leukemias [54, 55], and MM [56, 57] and correlate with a poor prognosis regardless of a patient's renal function [57, 58]. This observation suggests an important, yet unidentified, role of this protein in these malignancies.

While examining the effects of β_2M on myeloma cells, we made a novel and exciting discovery, namely, that mAbs against β_2M have a remarkably strong apoptotic effect on myeloma cells and on other hematological tumor cells [59]. Anti-β_2M mAbs induced apoptosis in up to 90% of cells in a 48-h culture in all tested human myeloma cell lines ($n=8$) and primary myeloma cells from patients ($n=10$). The mAbs also kill β_2M/MHC class I-bearing lymphoma and leukemia cells. Anti-MHC class I mAbs (LY5.1, IgG1 or W6/32, IgG2a), purified mouse IgG and IgG1 had no effect. Cell death occurred rapidly, without the need for exogenous immunological effector mechanisms (e.g., complement or NK cells) or secondary cross-linking. Anti-β_2M mAb-induced apoptosis in myeloma cells was not blocked by soluble β_2M (10–100 μg/mL, 3- to 30-fold higher than the levels in most MM patients), IL-6, or other myeloma growth and survival factors and was stronger than apoptosis observed with chemotherapy drugs currently used to treat MM (e.g., dexamethasone).

Although the expression of β_2M on normal hematopoietic cells is a potential safety concern, the mAbs were selective to tumor-transformed cells and did not induce apoptosis of normal cells, including T and B lymphocytes, plasma cells, and purified CD34$^+$ stem cells. Furthermore, the mAbs selectively and effectively killed myeloma cells without damaging osteoclasts (OCs) or PBMCs in their cocultures with myeloma cells. More importantly, anti-β_2M mAbs are therapeutic in vivo in xenograft SCID and SCID-hu mouse models [59], and in the HLA-A2-transgenic NOD-SCID (A2-NOD-SCID) models of myeloma, in which every mouse tissue expresses human MHC class I/β_2M molecules and circulating human β_2M could reach the levels seen in most myeloma patients without causing damage to normal human hematopoiesis or murine organs [60]. Interestingly, following our publication, others have reported similar results using anti-MHC class single-chain Fv diabody or anti-β_2M antibodies, respectively, in human myeloma [61], renal cell carcinoma [62], and prostate cancer [63]. Therefore, such mAbs offer the potential for a therapeutic approach to hematological malignancies.

The mAbs induced apoptosis in myeloma cells by recruiting MHC class I to lipid rafts, activated JNK, and inhibited PI3K/Akt and ERK pathways [59]. Growth and survival cytokines such as IL-6 and IGF-I, which could protect myeloma cells from dexamethasone-induced apoptosis, did not affect mAb-mediated cell death. We elucidated the mechanisms underlying anti-β_2M mAb-induced PI3K/Akt and ERK inhibition and the inability of IL-6 and IGF-I to protect myeloma cells from mAb-induced apoptosis. We focused on lipid rafts and confirmed that these membrane microdomains are required for IL-6 and IGF-I signaling. By recruiting MHC class I into lipid rafts, anti-β_2M mAbs excluded IL-6 and IGF-I receptors and their substrates from the rafts. The mAbs were not only redistributed to the receptors in cell membrane, but also abrogated IL-6- or IGF-I-mediated JAK/STAT3, PI3K/Akt, and Ras/Raf/ERK pathway signaling, which are otherwise constitutively activated in myeloma cells [64]. Thus, our study further defines the tumoricidal mechanism of the mAbs and provides strong evidence to support the potential of these mAbs as therapeutic agents for myeloma.

2.3.3 CS1

CS1, a glycoprotein and a member of the immunoglobulin gene superfamily, has been found to be highly expressed on tumor cells from myeloma patients, and soluble serum CS1 correlates with active disease in myeloma patients [65]. However, CS1 is also expressed by NK cells, NKT cells, and $CD8^+$ T cells [65].

As the above data suggest that CS1 could be a novel target for therapy, a humanized mAb against CS1, HuLuc63, was generated [65]. HuLuc63 inhibited myeloma cell binding to bone marrow stromal cells and induced antibody-dependent cell-mediated cytotoxicity (ADCC) against myeloma cells in dose-dependent and CS1-specific manners. Furthermore, the mAb mediated autologous ADCC against primary myeloma cells resistant to conventional or novel therapies, and pretreatment with conventional or novel antimyeloma drugs markedly enhanced HuLuc63-induced myeloma cell lysis. In vivo injection of the mAb significantly induced tumor regression in xenograft myeloma mouse models [66]. In addition, a recent study showed that HuLuc63 (elotuzumab) in combination with bortezomib exhibited significantly enhanced in vivo antimyeloma activity in human myeloma-xenografted mouse model [67]. Based on these results, phase-I clinical trials are underway to evaluate the safety and toxicity of the mAb in myeloma patients.

2.3.4 C-Reactive Protein

C-reactive protein (CRP), the first acute-phase protein described and an ancient and highly conserved protein of the pentraxin family, has five identical subunits forming a planar ring that confers very high stability to the protein. In healthy young adults, the median concentration of CRP is 0.8 mg/L, but following an acute-phase stimulus, values may increase by 10,000-fold, from less than 50 μg/L to more than 500 mg/L [68, 69]. Plasma CRP is produced primarily in the liver, synthesized by hepatocytes in response to intermediary inflammatory cytokines such as IL-1 and IL-6. CRP has been shown to bind to a variety of ligands, including pneumococcal polysaccharides, membrane phospholipids, apoptotic cells, fibronectin, and ribonuclear particles [69]. CRP also binds C1q and activates the classical complement cascade and binds Fcγ receptors (FcγRs) leading to indirect (via classical complement) and direct opsonization (via FcγRs) [69]. Through these mechanisms, CRP can play a direct role in a wide range of inflammatory processes and contributes to innate host immunity.

CRP is a sensitive systemic marker of inflammation and tissue damage. Elevated levels of CRP are present in patients with infections, inflammatory diseases, necrosis such as myocardial infarction [70], or malignancies including MM [71, 72], lymphoma [73, 74], and carcinoma [75]. Accumulating evidence has strongly suggested that in cardiovascular disease CRP is not only a marker of inflammation but also contributes to pathogenesis of the disease [76]. Evidence includes the results that CRP directly activated various vascular cells to secrete cytokines, enhanced their expression of adhesion molecules, increased monocyte/macrophage chemotaxis and

adhesion, facilitated extracellular matrix remodeling, enhanced endothelial dysfunction, and activated coagulation [77, 78]. Furthermore, human CRP has been shown to increase myocardial and cerebral infarct size in rats subjected to coronary or cerebral artery ligation, respectively, and this drastic enhancement of infarct size by human CRP was completely abrogated by in vivo complement depletion of the rats using cobra venom factor [79, 80].

These findings led to our hypothesis that CRP may also have a functional role in tumor cells since elevated levels of CRP are present in cancer patients [71–74]. We discovered that addition of CRP to cultures at levels seen in patients with MM or other tumors promoted myeloma cell proliferation under stressed conditions and protected myeloma cells from chemotherapy drug-, IL-6 withdrawal-, or serum deprivation-induced apoptosis in vitro. The protective effect was verified in vivo in myeloma SCID and SCID-hu mouse models. These phenomena may be clinically relevant since CRP was found accumulating on the surface of bone marrow myeloma cells from patients with MM. Although myeloma cells expressed all three types of FcγR, we identified FcγRII, more specifically, FcγRIIA and FcγRIIC as the primary receptors for CRP on the tumor cells. Our results demonstrated that CRP activated PI3K/Akt, ERK, and NF-κB in treated cells via binding to these receptors, which led to inhibited activation of caspase cascades induced by chemotherapy drugs such as dexamethasone and undermined the therapeutic efficacy of chemotherapy in the myeloma mouse models [81]. Thus, our study demonstrates that CRP plays an active role in regulating tumor cell growth and survival and suggests that targeting CRP by CRP-neutralizing antibodies or FcγRII-blocking antibodies may sensitize myeloma cells to chemotherapy drug-induced apoptosis.

2.3.5 Cancer-Testis Antigens

Numerous studies have shown that the Cancer-Testis (CT) antigens, such as MAGE-A3 and NY-ESO-1, may be expressed by myeloma cells [82–84]. DNA microarray analysis of gene expression of >95% pure myeloma cells from more than 300 patients showed that the genes of these antigens were expressed in the tumor cells, particularly from patients with relapsed disease or abnormal cytogenetics (in 7–20% of MGUS and newly diagnosed MM and in 40–50% of relapsed patients or in patients with cytogenetic abnormalities) [85, 86]. With the use of specific mAbs against MAGE-A3 or NY-ESO-1, it was evident that the proteins of these antigens were also expressed in the tumor cells of patients with positive gene expression. Moreover, cellular immune responses against MAGE-C1/CT7 and humoral responses against other CT antigens, such as MAGE-A1 and SSX-1, can be detected in MM patients [87].

Recent studies indicated that the expression of CT antigens on myeloma cells may represent a predictor of outcome of myeloma patients. Among CT angens examined, MAGE-C1/CT-7 is the most prevalent CT antigen, expressed in about 60% of myeloma cells of patients [88, 89]. This CT antigen was more frequently expressed

in myeloma cells with an elevated proliferation rate compared with myeloma cells with a low proliferation rate and correlated well with overall survival [89, 90]. In another study, the expression of MAGE-C1 gene represented an important indicator of early relapse and dramatically reduced survival of patients after allogeneic stem cell tranplantation [91].

Van Rhee and his colleague reported their study of immunization of a sibling donor with recombinant CT protein for allogeneic/syngeneic transplantation [92]. As MAGE-A3 is frequently expressed in high-risk MM, they immunized a healthy donor with MAGE-A3 protein formulated in AS02B to transfer immunity to her identical twin, diagnosed with MAGE-A3-positive MM. After a melphalan 200 mg/m syngeneic peripheral blood stem cell transplant, primed donor cells collected after immunizations were transferred and followed by repeated patient immunizations. Strong MAGE-A3-specific antibody, CTL, and T-helper responses were induced in both twins. A humoral response was transferred to the patient with the donor peripheral blood stem cells and increased by booster immunization. The CTL response targeted a previously undescribed HLA-A*6801 binding MAGE-A3115-123 peptide. MAGE-A3115-123 CTLs were detected in the patient more than 1 year after the last immunization. Multiple T-helper cellular responses were detected with the dominant response to an HLA-DR11-restricted MAGE-A3 epitope. The patient remained in remission 2.5 years after the second transplant. These results show that immunization of a healthy donor with a defined cancer-testis protein can induce immune responses that can be transferred and expanded posttransplant in the recipient.

2.3.6 Other Potential Targets

Another potential target is CD40, which is expressed on B-cell tumors including MM. Two humanized anti-CD40 mAbs, SGN-40 and HCD122, have been developed and tested in preclinical studies [93, 94]. These mAb induced modest cytotoxicity in myeloma cell lines and primary myeloma cells from patients, but can effectively kill myeloma cell via mediating ADCC. Further, the immunomodulatory drug lenalidomide further augmented anti-CD40 mAb-induced cytotoxicity in human myeloma cells [95]. In addition to anti-CD40 mAbs, other mAbs currently in clinical trials include anti-CD74, anti-CD56, and anti-HM1.24 [96].

Furthermore, other antigens, such as MUC-1 [97–99], sperm protein 17 (Sp17) [100, 101], and HM1.24 [102–104], may also be expressed on myeloma cells, and MHC-restricted antigens MUC-1 [105] and Sp17 [106]-specific CTLs have been generated from myeloma patients that were able to lyse myeloma cells. Recently, a phase-I/II clinical trial has been initiated to examine the safety and efficacy of Sp17-pulsed DC vaccination in myeloma patients [100]. However, there is evidence that Sp17 is also expressed on normal T and B cells [107]; hence, although these antigens may be potential targets, further research is warranted to examine their applicability for immunotherapy in MM.

2.4 Conclusion

Immunotherapy has become an important part of therapeutic strategies for hematological malignancies including MM. Passive immunotherapies using mAbs directed against tumor-associated surface antigens, such as CD20 (rituximab, Rituxan), CD22 (epratuzumab, LymphoCide), CD52 (alemtuzumab, Campath), and major histocompatibility complex (MHC) class II (Hu1D10, Remitogen), have been approved by the US Food and Drug Administration and are in widespread use either alone or in combination with chemotherapy or with other biological agents. These reagents can be applied as conjugates with toxins or isotopes as means to deliver a toxic compound or radioactivity to tumor cells, or as unlabeled antibodies to cause direct anticancer effects or induce a secondary immune response against tumor cells via a number of mechanisms. Thus far, encouraging results have been obtained in the treatment of various hematological malignancies, including non-Hodgkin's lymphomas, chronic lymphocytic leukemia, Waldenström's macroglobulinemia, and MM [108–110]. Active immunotherapy, in which the patients are induced to generate a specific immune response against the tumor cells, has long been a goal of tumor immunologists. Idiotype proteins have been used as the only tumor antigen for clinical immunotherapies for the past 14 years. Although tumor-specific, idiotype proteins are weak tumor antigen and need to be prepared from each patient [111]. Idiotype-based vaccines have been shown to induce or enhance idiotype-specific immunity, indicating that the vaccines are able to elicit a specific immune response [112]. However, clinical response is still a rare event, occurring only in a minority of treated patients, suggesting that the elicited or enhanced immunity is still too weak to cause significant tumor destruction. Thus far, although no active immunotherapy maneuver has yet proven to be effective in the clinic, intensive efforts are underway to develop such an approach. Experiments in animal models have shown that vaccination against actively growing tumors is much more difficult to accomplish [113, 114]. It is therefore not surprising that clinical trials in patients with gross disease will be the most difficult setting in which to demonstrate efficacy. Thus, it is conceivable that immunotherapy may work better in patients in remission or with minimal residual disease, who are more likely to be able to generate a robust immune response against the tumor and to derive therapeutic benefit. Nevertheless, with a better understanding of the immune system and tumor microenvironment, as well as identification and development of many novel targets and methods for immune targeting, there is a realistic hope that immunotherapies will soon be a part of conventional treatment modalities in MM and help control or even cure the disease.

Conflict-of-interest disclosure: The author declares no competing financial interests.

Acknowledgements This work was supported by institutional start-up funds from the University of Texas M. D. Anderson Cancer Center, the Center for Targeted Therapy of The University of Texas M. D. Anderson Cancer Center, grants from the National Cancer Institute (R01 CA96569, R01 CA103978, and R01 CA138402), the Leukemia & Lymphoma Society, Multiple Myeloma Research Foundation, and Commonwealth Foundation for Cancer Research. I thank Mrs. Kimberly Jensen for providing editorial assistance.

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Chapter 3
Antibody-Based Therapies in Multiple Myeloma

Yu-Tzu Tai

3.1 Introduction

The unmet need for improved multiple myeloma (MM) therapy has stimulated clinical development of mAbs targeting either MM cells or cells of the bone marrow (BM) microenvironment. In contrast to small-molecule inhibitors, therapeutic monoclonal antibodies (mAbs) present the potential to specifically target tumor cells and directly induce an immune response to lyse tumor cells. Unique immune-effector mechanisms are only triggered by therapeutic mAbs but not small molecule targeting agents. Although therapeutic murine mAbs or chimeric mAbs can cause immunogenicity, the advancement of genetic recombination for humanizing rodent mAbs has allowed large-scale production and designation of mAbs with better affinities, efficient selection, decreasing immunogenicity, and improved effector functions. Tremendous advancement of antibody engineering technologies has largely overcome the critical obstacle of antibody immunogenicity and enabled the development and subsequent Food and Drug Administration approval of therapeutic Abs for cancer and other diseases.

Despite the landmark approval of the anti-CD20 mAb rituximab for the treatment of B-cell malignancies, to date, no mAb-based therapy has been approved for MM treatment. The development of effective cytotoxic mAb therapies in MM has been hindered by the lack of uniquely and constitutively expressed target molecules on all MM cells. Indeed, studies in early 2000 demonstrated only minimal activity of anti-CD20 rituximab and antibodies against plasma cell-specific CD38 antibodies in MM [1–4]. However, numerous efforts to identify new targets on MM cells including gene expression profiling and oncogenomic studies are under way. Derived mAbs [e.g., against CD40, HM1.24, IGF-1R, CD56, CS1, CD138, CD74,

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N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 2: Translational and Clinical Research*,
DOI 10.1007/978-1-4614-5260-7_3, © Springer Science+Business Media New York 2013

IL-6R, CD38, TRAIL-R1, and the activin receptor type IIA (ActRIIA)] have already demonstrated promising preclinical as well as early clinical activity (Table 3.1).

Given the importance of the bone marrow (BM) microenvironment for MM cell growth, survival, and drug resistance, mAbs have been additionally designed to functionally block both autocrine- and paracrine-secreted cytokines and growth factors as well as molecules mediating MM–stromal cell interaction. For example, mAbs targeting interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), receptor activator of NFκB ligand (RANKL) [also known as osteoprotegerin ligand (OPGL)], and dickkopf homolog 1 (DKK1) are among those under clinical evaluation. Specifically, targeting bone–MM cell interactions via bone biology modulating factors such as DKK1 and RANKL are likely to not only trigger anti-MM effects but also improve bone disease thereby improving both patient survival as well as patient's quality of life.

In the coming years, the preclinical progress in defining novel MM markers will be continued and subsequently advance the clinical development of therapeutic mAbs, alone or in combination with other anti-MM agents, to improve patient outcome in MM.

3.2 Classification of Therapeutic Monoclonal Antibodies for Cancer Therapies

The advancement of antibody engineering technologies has largely overcome the critical obstacle of antibody immunogenicity and enabled the development and subsequent Food and Drug Administration (FDA) approval of therapeutic Abs for cancer and other diseases. Early clinical trials with murine mAbs failed owing to their short half-life, xenogenicity, and limited activity. The application of genetic recombination for humanizing rodent mAbs allowed large-scale production and designation of mAbs with better affinities, efficient selection, decreasing immunogenicity, and improved effector functions. Subsequent advancement in proteomics and genomics further combined with bacteriophage display to make the rapid selection of high-affinity mAb feasible. The generation of the first chimeric mouse-human mAb, the anti-CD20 mAb rituximab (Rituxan), has led to revolutionize lymphoma treatments (Fig. 3.1) and stimulated development of unconjugated mAbs targeting a variety of cell-surface proteins expressed on tumor cells. Furthermore, trastuzumab (Herceptin), the first humanized and clinically approved mAb targeting erbB-2 receptor, has provided new prospects for the treatment of breast cancer. It not only blocks breast cancer cell growth and survival but also exhibits excellent antitumor activity, when combined with cytotoxic agents doxorubicin and paclitaxel. SGN-40 (CD40) and elotuzumab (CS1) are among the first humanized mAbs targeting MM surface protein that are currently in clinical evaluation, either alone or combined with bortezomib or lenalidomide (Fig. 3.1 and Table 3.1). Most recently, the development of specific transgenic mouse, such as XenoMouse® or HuMax-Mouse®, has made it possible to design and efficiently to produce fully human mAbs. In these

Table 3.1 Antigens targeted by antibodies in multiple myeloma in different stages of preclinical/clinical development

Target	Brand name	Company/sponsor	Type of mAb (conjugate)	Phase	Remarks
CD138	B-B4-DM1	ImmunoGen	The maytansinoid immunoconjugate mouse IgG1 mAb B-B4	Preclincial	Tassone Blood 2004,104:3688–3696
HM1.24	Humanized HM1.24	Chugai Pharmaceutical	Humanized	Preclincial	Ozaki Blood 1999,93:3922–3930
	Humanized HM1.24	Xencor Inc	Fc-engineered humanized IgG_1	Preclincial	
IL-6	OP-R003-1, 1339 Elsilimomab, Azintrel®	OPi EUSA Pharma; Vaccinex licensed to Glaxo Smith Kline	Human IgG1	Preclincial	Fulciniti Clin Cancer Res 2009,15:7144–7152
HLA-DR	1D09C3	GPC Biotech, AG	Human IgG1	Preclincial	Carlo-Stella Cancer Res 2007
Kininogen	C11C1	Temple University School of Medicine	Mouse	Preclincial	Sainz Cancer Immunol Immunother 2006 C11C1 mAb inhibits its own tumor growth in vivo, slows down B38-MM growth rate when both MM are implanted together and when mAb C11C1 is injected intraperitoneally. MAb C11C1-treated-MM showed decreased MVD and kininogen binding in vivo without FGF-2, B1R or B2R expression changes
HLA class I	2D7-DB	Chugai Pharmaceutical Co. Ltd.	Converted from mouse IgG2b,single-chain Fv diabody	Preclincial	Sekimoto Cancer Res 2007; 67:1184–1192. A recombinant single-chain Fv diabody 2D7-DB specifically induces multiple myeloma cell death in the bone marrow environment

(continued)

Table 3.1 (continued)

Target	Brand name	Company/sponsor	Type of mAb (conjugate)	Phase	Remarks
β2-Micro globulin	Anti-b2M mAbs	MD Anderson Cancer Center	Mouse	Preclincial	Yang Blood 2007;110:3028–3035 and Yang Clin Cancer Res 2009;15:951–959. Strong apoptotic effect on myeloma cells and low toxicity in the mice suppports potential use as therapeutic agents
CD38	MOR202	MorphoSys AG	Human IgG1	Preclincial	Tesar et al. J. Clin Oncol 2007, 25(18S): 8106
CD32B	MGA321(2B6)	MacroGenics	Humanized IgG1	Preclincial	Zhou Blood 2008;111:549–557. Humanized 2B6 MoAb may target in patients with systemic AL-amyloidosis. It blocks Fc engagement of CD32B may improve the performance of other cancer Mabs when combined with them during administration
FGFR3	PRO-001	Prochon Biotech Ltd.	Human IgG1	Preclincial	Trudel Blood 2006;2:4908–4915. The inhibitory anti-FGFR3 antibody, PRO-001, is cytotoxic to t(4;14) MM cells and deserves further study for the treatment of FGFR3-expressing myeloma
ICAM-1	cUV3	Abiogen	Chimeric IgG1	Preclincial	Smallshaw J Immunother 2004; Coleman J Immunother 2006 cUV3 significantly prolongs the survival of SCID/ARH-77 mice
BLyS	BLyS/rGel	Targa Therapeutics	Fusion protein of an antibody tethered to a toxin	Preclincial	Lyu et al. Mol Cancer Ther 2007;6:460–470
TACI	Atacicept (TACI-Ig)	ZymoGenetics Inc.	Fusion protein	Preclincial	Yaccoby Leukemia 2008;22:406–413
CD70	SGN-70	Seatle Genetics	Humanized IgG1	Preclincial	McEarchern Clin Cancer Res 2008;14:7763–7772
TRAIL-R2(DR5)	Lexatumumab	Human Genome Sciences	Human	Preclincial	Menoret et al. Blood 2006;132:1356–1362

IL-6R	NRI (engineered Tocilizumab)	Roche Pharmaceuticals	A single-chain fragment format dimerized by fusing to the Fc portion of human immunoglobulin G1	Preclincial	Yoshio-Hoshino Cancer Res 2007;67:871–875. The NRI gene introduction combined with adenovirus gene delivery inhibited the in vivo S6B45 cell growth significantly
BCMA	SG1	Seatle Genetics	Auristatin- BCMA mAb	Preclincial	Ryan et. al. Mol Cancer Ther 2007;6:3009-18
Matriptase	M24-DOX	UMDNJ-The Cancer Inst. of New Jersey	immunoconjugate with doxorubicin	Preclincial	Bertino et al. 2010 AACR abtract#2596. M24-DOX is as potent as free doxorubicin to inhibit the growth of MM cells. But target delivery of doxorubicin by the matriptase antibody significantly reduced the toxicity toward cardiomyocytes that lack maptriptase expression
IL-1beta	XOMA 052	XOMA (US) LLC	Human Engineered™ IgG2	Preclincial	Lust 2010 AACR abstract #2449. XOMA 052 is highly effective at inhibiting IL-1 induced IL-6 production in myeloma patients in vitro
CD20	Rituxan	NCI & Memorial Sloan-Kettering Cancer Center	Chimeric with a human IgG1 Fc	II (ongoing)	NCT00258206 (with cyclophosphamide); NCT00505895. High-dose cyclophosphamide in combination with rituximab in patients with primary refractory, high-risk, or relapsed myeloma; also being studied for the treatment of peripheral neuropathy in patients with MGUS
CD20	Zevalin (yttrium Y 90 ibritumomab tiuxetan)	NCI	Mouse IgG1	I (ongoing)	NCT00477815 Zevalin radioimmunotherapy with high-dose melphalan and stem cell transplant for MM

(continued)

Table 3.1 (continued)

Target	Brand name	Company/sponsor	Type of mAb (conjugate)	Phase	Remarks
CD40	SGN-40 (Dacetuzumab)	Seattle Genetics/ Genentech	Humanized IgG1	I b (ongoing)	NCT00664898, Safety and pharmacology of SGN-40 administered in combination with Bortezomib (Velcade®, PS-341) in patients with relapsed or refractory MM. NCT00525447 is the study of SGN40, lenalidodmide, and dex in MM patients
CD40	HCD122 (Lucatumumab)	Norvatis	Human IgG1	I (ongoing)	NCT00231166 Dose-finding trial of HCD122 in MM patients that is relapsed or has not responded to prior therapy
CD20	Bexxar (131- tositu-momab)	GlaxoSmithKline	Radioactive iodine 131 attaching to anti-CD20;muIgG2a (131)	II (ongoing)	NCT00135200 To see whether the treatment with Bexxar will decrease and possibly eliminate residual myeloma cells resistant to chemotherapy
CD56	BB-10901 (IMGN901)	ImmunoGen, Inc.	Humanized (maytansine DM1 conjugation)	I (ongoing)	NCT00346255: Given as an intravenous infusion weekly for two consecutive weeks every three weeks to relapsed and relapsed refractory CD56-positive MM; NCT00991562: IMGN901 in combination with lenalidomide and dexamethasone
RANKL	Denosumab	Amgen	Human IgG2	II/III (ongoing)	NCT00259740: To determine if denosumab is effective in the treatment of relapsed or plateau-phase MM; NCT00104650: to determine the effectiveness of AMG 162 in reducing urinary N-telopeptide in advanced cancer subjects with bone metastases; NCT00330759: Phase III Study of Denosumab Compared With Zoledronic Acid (Zometa) in the Treatment of Bone Metastases in Subjects With Advanced Cancer (Excluding Breast and Prostate Cancer) or MM

VEGF	Avastin becacizumab	Genentech	Humanized	II (ongoing)	NCT00428545 (in combination with bort-ezomib); NCT00410605 (added with lenalidomide and dexamathasone)
CD52	Campath-1H (alemtuzumab)	NCI; Fred Hutchinson Cancer Research Institute	Humanized	II (ongoing)	NCT00625144 Studying the side effects of giving fludarabine and busulfan together with alemtuzumab followed by donor stem cell transplant and to see how well it works in treating patients with hematological cancer or other disease
IL-6	CNTO 328	Centocor, Inc.	Chimerized IgG1	I/II (ongoing)	NCT00401843 (in combination with bort-ezomib); NCT00911859 (added with Velcade-Melphalan-Prednisone); NCT00402181 (in combination with dexamethason)
IL-6	B-E8 (Elsilimomab)	Orphan Pharma International and Diaclone SA	Murine	II	Preliminary efficacy was seen but there is a limitation for the clinical use of a murine monoclonal antibody since it frequently induces human anti-mouse antibodies (HAMA)
IL-6R	MRA (Tocilzumab)	Roche Pharmaceuticals	Humanized	II	
TRAIL-R1(DR4)	Mapatumumab (TRM-1)	Human Genome Sciences	Human	II (ongoing)	NCT00315757 (in combination with bortezomib)
EGFR	Erbitux(EMMA-1)	Imclone; Bristol Meyers-Squibb	Chimerized	II (ongoing)	NCT00368121 (in combination with dexamethasone)
CS1	Elotuzumab/ HuLuc63	Facet Biotech; Bristol-Myers Squibb	Humanized	I/II (ongoing)	NCT00742560 & NCT00726869 (in combina-tion with bortezomib)

(continued)

Table 3.1 (continued)

Target	Brand name	Company/sponsor	Type of mAb (conjugate)	Phase	Remarks
CD38	HuMax-CD38	Genmab	Human IgG1	I/II (ongoing)	NCT00574288: To establish safety profile of HuMax-CD38, given as monotherapy in patients with MM relapsed or refractory to at least 2 different cytoreductive therapies and without further established treatment options
CD38	SAR650984	Sanofi-Aventis; ImmunoGen	Humanized IgG1	I (not yet open for patient recruitment)	NCT01084252: Dose escalation safety and pharmacokinetic study in patients with selected CD38+ hematological malignancies
DKK	BHQ880	Novartis	Human IgG1	I/II (ongoing)	NCT00741377: in combination with Zoledronic Acid in relapsed/refractory myeloma
CD138	BT062	Biotest; ImmunoGen	Chimeric (B-B4-maytansinoid DM4)	I (ongoing)	NCT00723359
The activin receptor type IIA (ActRIIA)	ACE-011	Acceleron Pharma, Inc.	Human IgG1	I/IIa (ongoing)	NCT00747123 (in patients with osteolytic lesions with MM)
IGF-1R	AVE1642	Sanofi-Aventis	Humanized	I/II (ongoing)	Descamps et al. (B J Cancer 2009; 100:366) Anti-IGF-1R Monoclonal Antibody combined with bortezomib for patients with rel/ref MM
Ganglioside GM2	BIW-8962	BioWa, Incorporated	Humanized	I/II (ongoing)	Dosing study of anti-GM-2 ganglioside (expressed at high levels on the surface of MM cells) followed by efficacy study

CD74 (variant MHC II)	Milatuzumab (hLL1, IMMU-110)	Immunomedics, Inc.	Humanized IgG1 or humanized IgG1 doxorubucin conjugate	I/II (ongoing)	NCT00421525, in patients with recurrent or refractory multiple myeloma who have failed at least two prior standard systemic treatments. Its isotope, drug, and toxin conjugates have high antitumor activity in non-Hodgkin's lymphoma and multiple myeloma in vitro and in tumor xenograft models. Stein et al. 2007 & 2009
Alpha-4 integrin	Natalizumab (Tysabri®)	Biogen Idec	Humanized IgG4	I/II (ongoing)	NCT00675428, patients with relapsed or refractory multiple myeloma
MHC II (HLA-DR)	1D09C3	GPC Biotech	Human IgG4	I	Carlo-Stella et al. 2007 showed that IFN-gamma-induced up-regulation of HLA-DR results in a potent enhancement of the in vivo antimyeloma activity of 1D09C3 in mice. Initial clinical testing with 1D09C3 has not raised any unexpected or unacceptable safety concerns and the maximum tolerated dose has not yet been reached. GPC Biotech has decided to not put further internal resources into developing 1D09C3 due to potential swapping of IgG4 antibody one half of its Y-shaped structure with the half of a different antibody, thus resulting in a new molecule whose properties are unknown. However, the Company will seek a partner for the intellectual property relating to this program

(continued)

Table 3.1 (continued)

Target	Brand name	Company/sponsor	Type of mAb (conjugate)	Phase	Remarks
IGF-1R	CP-751, 871/figitumumab	Pfizer	Human IgG2	I	Lacy et al. (J. Clin Onclo 26:3196) reported that CP-751,871 is well tolerated and may constitute a novel agent in the treatment of multiple myeloma
KIR	IPH 2101	Innate Pharma	Human IgG4	I/IIa (ongoing)	NCT00552396 (ASCO May 30 2009 abstract 09-AB-3032) safety and tolerability study for patients with relapsed/refractory MM. Pre-clinical characterization of 1-7F9, a novel human anti-KIR therapeutic antibody that augments NK-mediated killing of tumor cells (Romagne et al. 2009)

Every effort has been made to obtain reliable data from multiple sources including http://clinicaltrials.gov, company, and other web sites, but accuracy cannot be guaranteed

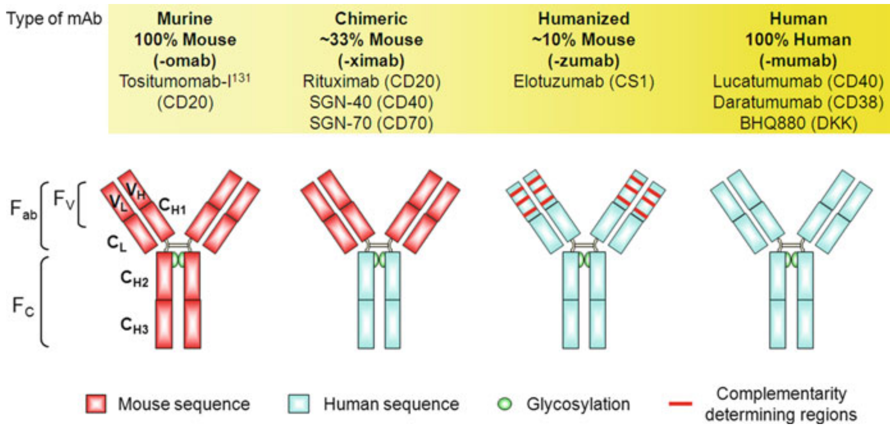

Fig. 3.1 *The classification of therapeutic monoclonal antibodies (mAbs) by the different antibody types*. The fundamental structure of an intact, immunoglobulin G (IgG) molecule has a pair of light chains and a pair of heavy chains. Light chains are composed of two separate regions, one variable region (V_L) and one constant region (C_L), whereas heavy chains are composed of four regions (V_H, C_{H1}, C_{H2}, and C_{H3}). A chimeric antibody splices the variable light (V_L) and variable heavy (V_H) portions of the murine IgG to a human IgG. A humanized Ab splices only the complementarity determining regions (CDRs) from the murine mAb, along with some of the adjacent framework regions to help maintain the conformational structure of the CDRs. A fully human IgG can be isolated from specialized phage display method or in transgenic mice (HuMAb-Mouse®). In HuMAb-Mouse®, the mouse genes for creating antibodies have been inactivated and replaced by human antibody genes, thus generating both the heavy and light chains of human antibodies. Examples of each type of potential therapeutic mAbs under clinical development for MM were listed

genetically engineered transgenic strains, key gene sequences from unrearranged human antibody genes that code for both the heavy and light chains of human antibodies have been introduced into the germ line of mice with inactivated mouse antibody machinery; fully human Ab proteins are thus produced. The first approved fully human mAb generated by such mice panitumumab (anti-EGFR) in 2006 has demonstrated significant clinical benefits to patients with metastatic colorectal cancers. Although EGFR may not be a suitable target for MM, lucatumumab (anti-CD40), daratumumab (anti-CD38), and 1-7F9 (anti-KIR), products of XenoMouse® or HuMax-Mouse®, are currently in preclinical and clinical trials for MM and other hematological malignancies. In addition, human combinatorial antibody library (HuCAL) platform, an antibody production method based on phage display [5], has been used to produce fully human immunoglobulin for MOR202(anti-CD38) [6] and BHQ880(anti-DKK) [7, 8]and 1D09C3 (anti-HDR/MHC class II) [9, 10] that are also currently under clinical trials in MM. ActivMAb antibody discovery technology, for the direct selection of high-affinity, fully human antibodies that would otherwise be difficult to identify with other systems, was used to generate Azintrel® (mAb1339), a fully human anti-IL-6 mAb [11]. Azintrel, in addition to inhibit inflammation, showed significant anti-myeloma activities in preclinical studies [11]. On the other hand, targeted antibody payload (TAP) technology makes it possible to

use tumor-targeting antibodies to deliver a highly potent cell-killing agent specifically to cancer cells to kill these cells with minimal damage to healthy tissue [12]. Examples for such antibody-drug conjugates are anti-HER2 Trastuzumab-DM1 for breast cancer [13], anti-CD56-DM1 BB-10901 (IMGN901) [14], and anti-CD138-DM4 (nBT062) [15, 16] that are currently in MM clinical trials. Maytansinoid DM1 (or derivative DM4) is a highly potent anti-microtubule drug with potent inhibition of tumor cell division and growth. Another potent tubulin inhibitor monomethyl auristatin E (MMAE) was conjugated with anti-CD30 mAb (SGN-35) that showed even improved efficacy when combined with chemotherapeutic agents, suggesting possible advantages for the treatment of patients with relapsed or refractory Hodgkin lymphoma [17, 18]. In addition, anti-CD74 Ab-doxorubicin conjugate IMMU-110 [19] specifically binds to CD74 and induces cytotoxicity via intercalating DNA in CD74-positive B-cell cancers.

3.3 Mechanisms of Action of Therapeutic Monoclonal Antibodies

Antibodies of IgG, the most commonly used immunoglobulin form in cancer therapy, are unique proteins with dual functionality. Therapeutic mAbs use one or more following mechanisms (Fig. 3.2) to reduce tumor burden in patients. They can be categorized into direct and indirect actions. Three modes of action could be further subcategorized from the direct action (Fig. 3.2a) of mAb-based cancer therapy, including blocking the function of target signaling molecules or receptors, stimulating apoptosis signaling cascades, and targeting function to selectively target tumor cells and deliver toxins. The receptor functional blocking can occur by inhibiting ligand binding to inhibit cell cycle progression, DNA repair, or angiogenesis. It could also occur by increasing internalization of receptors or decreasing proteolytic cleavage of receptors. In the case of targeting function, mAbs could be conjugated with immunotoxins, i.e., anti-tubulin agents (DM1/DM4, auristatin), doxorubicin, radioisotopes, or other chemotherapeutic drugs, thus selectively targeting and killing tumor cells. Indirect action of mAb therapy is mediated by the immune system. The elimination of tumor cells using mAbs depends on Ig-mediated mechanisms, including antibody-dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC), to activate immune effector cells to lyse target tumor cells (Fig. 3.2b). These two mechanisms are believed to have the greatest impact, although there are conflicting views of which of these two pathways contributes the most to the response. ADCC involves the recognition of the Ab by immune cells that engage the Ab-marked cells and either through their direct action or through the recruitment of other cell types led to the tagged-cell's death. CDC (Fig. 3.2c) is a process where a cascade of different complement proteins becomes activated, usually when several IgGs are in close proximity to each other, either with one direct outcome being cell lysis or one indirect outcome being attracting other immune cells to this location for effector cell function.

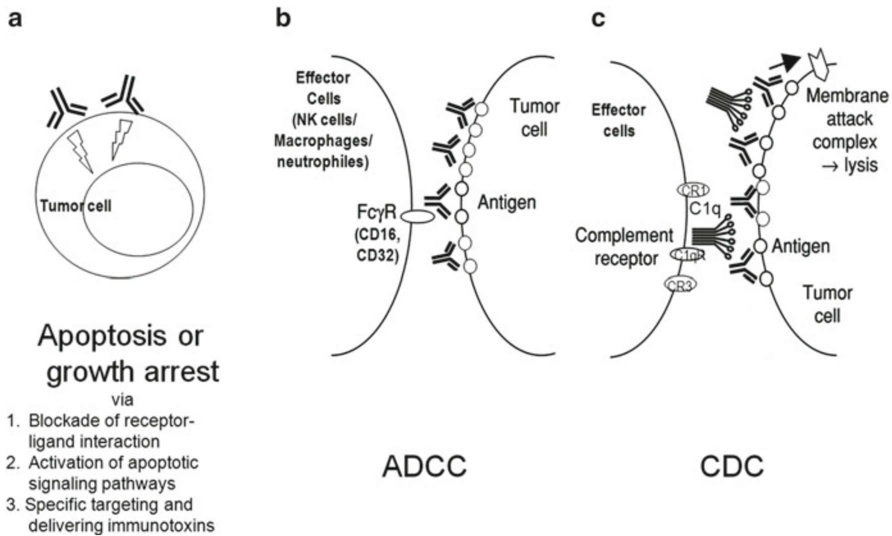

Fig. 3.2 *Mechanisms of actions associated with therapeutic monoclonal antibodies*. (**a**) Therapeutic antibodies could directly induce apoptosis or growth arrest upon binding to cell surface antigen on tumor cells. Rituximab and mapatumumab (anti-TRAILR1) could induce growth inhibition or apoptosis signaling to directly block tumor cell growth and survival. Such mechanism of action was employed by mAbs conjugated with toxins, i.e., maytansinoids (DM1, DM4) for BB-10901 (anti-CD56) and BT062 (anti-CD138), thus directly target and eliminate tumor cells. Most of the approved therapeutic mAbs belong to IgG1 subclass, which has a long half-life and trigger potent immune-effector functions. (**b**) Following the binding of mAbs to a specific target on a tumor cell, antibody-dependent cellular cytotoxicity (ADCC) is triggered by interactions between the Fc region of an antibody bound to a tumor cell and Fc receptors, particularly FcRI and FcRIII, on immune effector cells such as neutrophils, macrophages, and natural killer cells. MAb-coated tumor cells are phagocytosed by macrophages or undergo cytolysis by NK cells. (**c**) In the case of complement-dependent cytotoxicity (CDC), recruitment of C1q by IgG bound to the tumor cell surface is an obligatory first step. This triggers a proteolytic cascade that leads to generation of the effector molecule, C3b, and then to formation of a membrane attack complex that kills the target cell by disrupting its cell membrane

3.4 Antibodies Targeting Cell Surface Protein on MM Cells

Several mAbs directed against MM cell surface are being investigated as potential therapy in MM. Listed below are mAbs against receptor antigens that are currently under clinical development or investigation in MM.

3.4.1 Limited Clinical Benefit from Anti-CD20 mAb Rituximab in MM

MM is usually not considered as a disease suitable for anti-CD20 therapy due to weak CD20 expression in the majority of patients. For example, results from a

clinical phase II trial in relapsed MM showed that rituximab treatment yielded significant reductions in circulating B cells and serum IgM levels but had no beneficial clinical effect [20].

Moreover, rituximab was investigated for maintenance therapy in MM following autologous hematopoietic stem cell transplantation (SCT) [2]. Although the number of MM patients was too low to draw definitive conclusions, the use of rituximab in this setting was associated with an unexpectedly high rate of early relapse. The authors, therefore, hypothesized a possible role for rituximab in provoking a further decrease in the residual, normal B-cell activity within the context of the complex network of antitumor immune response. Taken together, the resistance of MM cells against rituximab could be due to the level of CD20 expression, dissociated action of CDC and ADCC, polymorphism in FGCR3 (CD16) receptor, and an inadequate dose schedule.

In contrast, other studies demonstrated that the $CD20^+$ phenotype is associated with patients with t(11,14)(q13;q32) and with shorter survival [21] and that occasional clinical responses have been achieved in selected patients with $CD20^+$ myelomatous plasma cells [22, 23].

Finally, new insights suggest that circulating $CD20^+$ clonotypic B cells act as precursors or “neoplastic stem cells” in MM patients representing the proliferative compartment of the disease able to play a role in determining relapse after effective treatments [24]. Thus, clinical trials using rituximab in MM may deserve further investigation.

3.4.2 Monoclonal Antibodies Targeting IL-6R to Overt IL-6/IL-6R Function

IL-6 is a major growth and survival factor in MM cells whose effects are mainly paracrine [25]. Various therapeutic agents which affect IL-6-mediated effects have been tested including IL-6-conjugated mAbs directed against IL-6R and IL-6 [26]. IL-6R antagonist SANT-7, in combination with Dex and all-trans retinoic acid (ATRA) or zoledronic acid, strongly inhibited growth and induced apoptosis in MM cells [27–29]. These studies suggest that overcoming IL-6-mediated cell resistance by SANT-7 potentiates the effect of glucocorticoids and bisphosphonates on MM cell growth and survival, providing a rationale for therapies using IL-6 antagonists in MM.

Tocilizumab (MRA, atlizumab, Roche Pharmaceuticals) is a humanized antihuman IL-6R mAb (rhPM-1, IgG1 class) designed by using genetic engineering technology and the first therapeutic mAb developed in Japan [30]. Tocilizumab specifically blocks IL-6 actions and ameliorates diseases associated with IL-6 overproduction [31]. For example, besides Castleman’s disease and rheumatoid arthritis (RA), tocilizumab has been shown to be effective in patients with juvenile idiopathic arthritis and Crohn’s disease [32, 33]. Tocilizumab treatment is generally well tolerated and safe. Moreover, blockade of IL-6R may prove effective in limiting

MM cell growth. Indeed, it is now evaluated in open-label Phase I (U.S.) and II (France) trials to assess its safety and efficacy as monotherapy in MM patients who are not candidates for or who have relapsed after stem cell transplantation (SCT).

In addition, NRI, another receptor inhibitor of IL-6 genetically engineered from tocilizumab, is under preclinical evaluation [34]. NRI consists of VH and VL of tocilizumab in a single-chain fragment format dimerized by fusing to the Fc portion of human immunoglobulin G1. The binding activity to IL-6R and the biological activity of the purified NRI were found to be similar to those of parental tocilizumab. Because NRI is encoded on a single gene, it is easily applicable to a gene delivery system using virus vehicles. An adenovirus vector encoding NRI was administered to mice intraperitoneally (i.p.) and monitored for the serum NRI level and growth reduction property on the xenografted IL-6-dependent MM cell line S6B45. These findings indicate that NRI is a promising agent applicable to the therapeutic gene delivery approach for IL-6-driven diseases.

3.4.3 Targeting CD40 by SGN-40 or HCD122

Novel monoclonal antibodies targeting CD40 activation in MM cells, SGN-40/dacetuzumab (Seattle Genetics, Genentech), and HCD122/lucatumumab (Novartis) have been investigated [35, 36]. In preclinical studies, SGN-40, a humanized IgG_1 partial agonistic mAb mediates cytotoxicity against CD40-expressing MM cell lines and patient MM cells via suppression of IL-6-induced proliferative and anti-apoptotic effects, as well as ADCC [35]. SGN-40 also induced significant antitumor activity in xenograft mouse models of human MM and lymphoma [37]. HCD122 (CHIR12.12) (Novartis), a novel, fully human, IgG_1 antagonistic mAb specifically blocked CD40L-induced adhesion, cytokine secretion, and survival of MM, as well as induced marked ADCC against $CD40^+$ MM cells [36]. In vivo anti-MM activity by HCD122 was demonstrated in a xenograft model of 12BM MM plasmacytoma in mice [38]. Early clinical trials have evaluated the pharmacokinetics, safety, and efficacy of dacetuzumab monotherapy in patients with relapsed/refractory MM and other B-cell tumors [39, 40]. Phase I data suggest both agents are well tolerated with no immunogenicity and show early evidence of single-agent clinical activity in relapsed and refractory MM and NHL [41, 42]. SGN-40 Phase Ib clinical trials in combination with lenalidomide and dexamathasone/or bortezomib are planned based on enhanced anti-MM activities when combining SGN-40 with lenalidomide [43].

3.4.4 Targeting CS1 by HuLuc63/elotuzumab in MM

Using subtractive hybridization of naïve B-cell cDNA from memory B/plasma cell cDNA, CS1 (CD2 subset-1, CRACC, SLAMF7, CD319), a novel member of

the signaling lymphocyte activating-molecule (SLAM)-related receptor family, was identified to be highly expressed in plasma cells [44, 45]. Specifically, CS1 mRNA and protein are expressed in CD138-purified primary tumor cells from the majority of MM patients (>97%), but neither in major body organs nor $CD34^+$ stem cells. To a low extent, its expression was also observed in NK cells, a subset of T-cells, activated monocytes, and activated dendritic cells. CS1 may contribute to MM pathogenesis by increasing MM-cell adhesion, clonogenic growth, and tumorigenicity via c-maf-mediated interactions with BMSCs [46]. A novel humanized ani-CS1 mAb HuLuc63 (elotuzumab) was selected for clinical development due to its potent tumor-killing activity in vivo and in vitro. Specifically, elotuzumab induced significant ADCC against MM cells even in the presence of BMSCs. Moreover, it triggered autologous ADCC against primary MM cells resistant to conventional or novel therapies including bortezomib and HSP90 inhibitor, and markedly enhanced HuLuc63-induced MM cell lysis when pretreated with conventional or novel anti-MM drugs [44, 47].

A phase I study of HuLuc63 was well tolerated in MM patients [48]. Preliminary PK data reveal that peak serum drug levels for the 0.5 mg/kg dosing cohort reached 10 mcg/mL, which was sufficient to achieve CS 1 saturation of at least 70% on the antigen rich NK cell subset. Drug levels dropped below 1 mg/mL by day 7, however, coinciding with a decrease in saturation. This indicates that the higher doses to be used in subsequent cohorts may achieve and surpass sustained concentrations in patients above this level. Enrollment is continuing to determine the MTD. Early results of clinical trials of HuLuc63 in combination with bortezomib or lenalidomide or dexamethasone were reported at the ASH meeting 2009 [49, 50], suggesting that elotuzumab may enhance the activity of bortezomib and lenalidomide in treating MM with acceptable toxicity. PK analysis suggests a serum half-life of 10–11 days at higher doses (10 and 20 mg/kg). Preliminary analysis of peripheral blood mononuclear cells and of the BM indicates that objective responses correlate well with complete saturation of CS1 sites by elotuzumab on BM plasma cells and NK cells. The combination of elotuzumab with lenalidomide and low-dose dexamethasone has a manageable adverse event profile, and compared to historical data for lenalidomide and high-dose dexamethasone, the preliminary efficacy data (PR of 92%) are very encouraging.

3.4.5 Targeting CD56 with Immunotoxin-Conjugated mAb

HuN901 conjugated with the maytansinoid $N^{2'}$-deacetyl-$N^{2'}$-(3-mercapto-1-oxopropyl)-maytansine (DM1), a potent antimicrotubular cytotoxic agent may provide targeted delivery of the drug to CD56-expressing tumors including MM. HuN901-DM1 has significant in vitro and in vivo anti-MM activity at doses that were well tolerated in a murine model [51].

The phase I clinical study of huN901-DM1 (BB-10901) (Lorvotuzumab Mertansine) in 23 MM patients determined the MTD as 140 mg/m^2/week dose and demonstrated an

overall favorable safety profile [14]. Exciting single-agent activity was observed in heavily pretreated MM patients. Continued investigation of this novel agent in MM patients in combination with lenalidomide and dexamethasone is underway.

3.4.6 Targeting CD38 in Multiple Myeloma

The CD38 molecule is expressed on cell surfaces in a majority of lymphoid tumors, notably MM [6, 52]. However, early studies using anti-CD38 mAb with or without an immunotoxin (ricin) have not led to useful clinical applications [4, 53].

Recently, a human anti-CD38 IgG_1 HuMax-CD38 (Daratumumab) was raised after immunizing transgenic mice (HuMax-Mouse®) possessing human, but not mouse, Ig genes. Preclinical studies indicated that HuMax-CD38 was effective in killing primary $CD38^+$ $CD138^+$ patient MM cells and a range of MM/lymphoid cell lines by both ADCC and CDC [54]. In SCID mouse animal models, using sensitive bioluminescence imaging, treatment with HuMax-CD38 inhibited $CD38^+$ tumor cell growth in both preventive and therapeutic settings. In addition, HuMax-CD38 inhibits the CD38 ADP-ribosyl cyclase activity in target cells, which may contribute to the effectiveness of HuMax-CD38 in killing both primary MM and plasma cell leukemia cells. Phase I clinical trial in MM is currently recruiting patients.

A chimeric version of SAR650984, another therapeutic humanized anti-CD38 antibody, was selected for its potent ADCC, CDC, and apoptotic activities in vitro and antitumor activity in vivo against CD38-expressing hematological tumors including MM [55, 56]. Thus, SAR650984 is a promising therapeutic antibody candidate for various hematological malignancies, especially in diseases, i.e., MM, where rituximab is inactive. Phase I clinical trial will be planned in year 2010.

Similarly, MOR202 (MorphoSysAG), a fully human anti-CD38 IgG_1 mAb produced by a human combinatorial antibody library (HuCAL) platform, also efficiently triggers ADCC against $CD38^+$ MM cell lines and patient MM cells in vitro as well as in vivo in a xenograft mouse model [6, 57]. One practical problem in applying anti-CD38 therapy is the wide expression on lymphoid, myeloid, and epithelial cells, especially following cell activation. However, mAbs specifically blocking CD38 might still provide a new approach for interfering with deleterious growth circuits, therefore increasing the susceptibility of MM and leukemic cells to conventional chemotherapy.

3.4.7 Targeting CD138 with BT062

The antitumor effect of murine/human chimeric CD138-specific monoclonal antibody nBT062 conjugated with highly cytotoxic maytansinoid derivatives against MM cells was investigated in vitro and in vivo [15]. These anti-CD138 immunoconjugates significantly inhibited MM tumor growth and prolonged host survival in both the

xenograft mouse models of human MM and SCID-hu mouse model. Preliminary data of phase I study for the treatment of MM demonstrated an acceptable toxicity profile and early evidence of clinical activity of BT062 in the clinics [16]. These encouraging results in combination with the observed pharmacokinetic properties support investigation of a more frequent dosing regimen for optimizing anti-MM responses.

3.4.8 Targeting HM1.24 on MM cells

HM1.24 (CD137) was originally identified as a cell-surface protein differentially overexpressed on MM cells [58] and later was found to be identical to bone stromal cell antigen 2 (BST-2). A role of HM1.24 in trafficking and signaling between the intracellular and cell surface of MM cells was suggested since it is one of the important activators of NF-kappaB pathway [59]. The humanized anti-HM1.24 mAb (IgG1/kappa, AHM, Chugai Pharmaceutical Co., Ltd.) is able to effectively induce ADCC against some human myeloma cells in the presence of human PBMCs as effectively as a chimeric anti-HM1.24 mAb [60]. Single intravenous injection of AHM significantly inhibited tumor growth in both orthotopic and ectopic human MM xenograft models [61]. Although limited, the only one phase I/II clinical study reported that a humanized anti-HM1.24 mAb did not cause any serious toxicity when administered to patients with relapsed or refractory MM (Powles R. Japanese MM forum proceedings Nov 3, 2003 [61]).

Most recently, we characterized XmAb®5592, a novel Fc-engineered and humanized anti-HM1.24 mAb, and studied mechanisms of its anti-MM activity [62]. XmAb®5592, with double amino acid substitution in Fc region of the wild type IgG1, has approximately 40-fold and 10-fold increases in affinity for Fc gamma receptor III (FcRIIIa) and (FcRIIa), respectively, expressed on effector cells including NK cells. It triggers 10–100-fold higher ADCC against these MM cell lines than a native/non Fc-engineered version (anti-HM1.24 IgG1) of the Ab. XmAb5592 also induced more potent anti-MM activity in murine subcutaneous xenograft murine models using RPMI 8226 cells. These results suggest that XmAb5592 is a promising next-generation immunotherapeutic for MM.

3.4.9 Targeting TRAIL Death Signaling Pathway

Two human agonistic mAbs directed against TRAILR1 (HGS-ETR1, TRM-1, mapatumumab) and TRAILR2 (HGS-ETR2) killed 68% and 45% of MM cell lines, respectively [63]. Only 18% of MM cell lines are resistant to either antibody. There is no correlation between TRAILR expression level and sensitivity to TRAILR1 or TRAILR2 triggering. Both the extrinsic (caspase 8, Bid) and the intrinsic (caspase 9) pathways are activated by anti-TRAIL mAbs. Mapatumumab is well tolerated in a phase I study in patients with advanced solid malignancies ($n=41$), and 12 patients

had stable disease for 1.9–29.4 months [64]. These studies encouraged clinical trials of anti-TRAILR1 mAb in MM. Based on enhanced cytotoxicity when combining mapatumumab with bortezomib in preclinical experiments [65], a randomized phase II study was recently started comparing TRM-1 plus bortezomib (Velcade®) versus bortezomib alone in patients with relapsed or refractory MM.

3.4.10 Targeting CD74 with Milatuzumab

CD74 is an integral membrane protein that functions as a MHC class II chaperone. Milatuzumab is a humanized anti-CD74 mAb constructed using the same human backbone as epratuzumab (anti-CD22), whose safety has been demonstrated in clinical trials of patients with B-cell malignancies and autoimmune disorders [66, 67]. MM cell lines express CD74 (~60% of samples), and milatuzumab caused growth inhibition and induction of apoptosis in CD74-expressing MM cell lines when cross-linked with an antihuman immunoglobulin G secondary antibody [68]. Moreover milatuzumab demonstrated promising therapeutic activity in a CAG-SCID mouse model of disseminated disease for MM when used alone or in combination with doxorubicin, dexamethasone, bortezomib, or lenalidomide [69, 70]. In a phase I trial, milatuzumab showed no severe adverse effects in patients with relapsed/refractory MM, and it stabilized the disease in some patients for up to 12 weeks [66]. Supporting the data in MM ongoing clinical trials testing, different treatment schedules of milatuzumab in chronic lymphocytic leukemia, non-Hodgkin's lymphoma, and MM indicate that milatuzumab shows no severe adverse effects in humans.

3.5 Antibodies Targeting MM cells in the Bone Marrow Microenvironment

MM cells are highly dependent on the BM microenvironment for growth and survival through interactions particularly with BM stromal cells (BMSCs) and osteoclasts, which secrete important MM growth factors and cytokines. Importantly, these factors/cytokines are further induced from BMSCs when MM cells adhere to BMSCs [25]. Thus, mAbs designed to block the binding of MM cell growth and survival factors to their cognate receptors have been under intensive development.

3.5.1 Blockage of IL-6 Binding to MM cells

Early work in developing mAb-based immunotherapies for MM has been focused on the blockade of IL-6 secretion from BM microenvironment because of its key

role in promoting MM cell growth and survival. Initial studies of mouse mAb to IL-6 (murine BE-4 and BE-8) demonstrated a transient tumor cytostasis and reduction in toxicities from IL-6 [71]. The potential of combination therapy, including BE-8 (250 mg), Dex (49 mg/day), and high-dose melphalan [220 mg/m [2] (HDM220)], followed by autologous SCT was demonstrated for the treatment of 16 patients with advanced MM. Overall, 13 of 16 patients (81.3%) exhibited a response, with a complete response (CR) seen in 6 patients (37.5%) without any toxic or allergic reactions. However, the incidence of thrombocytopenia and neutropenia increased. Subsequent clinical trials of BE-8 concluded that limitations of this regimen are, first, the amount of BE-8 that can be injected due to its short half-life (3–4 days) and, second, the continued production of IL-6 in vivo. Most recently, a high-affinity fully human version of BE-8, OP-R003-1 (or 1339, Azintrel®), was selected through ActivMAb antibody discovery technology. Indeed, it enhanced cytotoxicity induced by dexamethasone, as well as bortezomib, lenalidomide, and perifosine, in a synergistic fashion [11]. Importantly, Azintrel® also blocked bone turnover in SCID-hu mouse model of MM, providing an additional rationale for its use in MM.

Despite overcoming the safety concerns of human anti-mouse antibodies associated with murine anti-IL-6 mAb and a long half-life (17.8 days) in circulation, the chimeric mouse mAb to IL-6 CNTO 328 has been ineffective in producing a meaningful response in MM [72, 73]. Nevertheless, due to enhanced anti-MM activities of combined CNTO 328 and bortezomib/or dexamethasone in preclinical models, ongoing studies are investigating these regimens for their clinical value in treating MM. [74, 75] Specifically, results of a small safety analysis ($n=21$) done as a run-in to a larger ongoing Phase II trial showed promising preliminary efficacy of CNTO 328 in combination with bortezomib in relapsed/refractory MM. CNTO 328 is also being evaluated as part of a combination therapy for initial treatment of MM in a Phase II trial which compares the safety and effectiveness of CNTO 328 plus Velcade-melphalan-prednisone (VMP) with VMP alone.

3.5.2 Targeting MM-Induced Bone Lesion

3.5.2.1 Targeting RANK/RANKL/OPG Axis Using Denosumab for MM-Associated Bone Destruction

Receptor activator of nuclear factor-kappaB ligand (RANKL) is a cytokine member of the tumor necrosis factor family that is the principal mediator of osteoclastic bone resorption [76]. Osteoprotegerin (OPG), a natural soluble decoy receptor of RANKL, modulates the effect of RANKL and is able to prevent excessive bone resorption in the normal state. RANKL expression is elevated in patients with MM [77, 78]. Denosumab (AMG 162, Amgen Inc., Thousand Oaks, CA) is an investigational fully human mAb with high affinity and specificity for RANKL that mimics the natural bone-protecting actions of OPG [79]. A phase 1 clinical trial in patients with MM ($n=25$) or breast cancer with bone metastases ($n=29$) showed that,

following a single s.c. dose of denosumab (0.1, 0.3, 1.0, or 3.0 mg/kg), levels of urinary and serum N-telopeptide decreased within 1 day, and this decrease lasted through 84 days at the higher denosumab doses [80]. Mean half-lives of denosumab were 33.3 and 46.3 days for the two highest dosages. Larger trials are ongoing to investigate the effect of denosumab for the treatment of cancer-induced bone disease and other bone loss disorders [81].

3.5.2.2 Targeting the Wnt Inhibitor Dickkopf-1 (DKK-1)

Dickkopf-1 (DKK1), a soluble inhibitor of wingless (Wnt) signaling secreted by MM cells contributes to osteolytic bone disease by inhibiting the differentiation of osteoblasts. The effect of anti-DKK1 mAb on bone metabolism and tumor growth in a SCID-rab system has been evaluated [82]. The implants of control animals showed signs of MM-induced resorption, whereas mice treated with anti-DKK1 antibodies blunted resorption and improved the bone mineral density of the implants. Histologic examination revealed that myelomatous bones of anti-DKK1-treated mice had increased numbers of osteocalcin-expressing osteoblasts and reduced number of multinucleated TRAP-expressing osteoclasts. The bone anabolic effect of anti-DKK1 was associated with reduced MM burden ($P < .04$). Anti-DKK1 also significantly increased BMD of the implanted bone and murine femur in non-myelomatous SCID-rab mice, suggesting that DKK1 is physiologically an important regulator of bone remodeling in adults. Anti-DKK1 agents including BHQ880 (Novartis) may therefore represent the next generation of therapeutic options for the enhancement of bone repair in some malignant and degenerative bone diseases including MM [7, 8]. Although BHQ880 had no direct effect on MM cell growth, BHQ880 increased osteoblast differentiation, neutralized the negative effect of MM cells on osteoblastogenesis, and reduced IL-6 secretion. Furthermore, in a SCID-hu murine model of human MM, BHQ880 treatment led to a significant increase in osteoblast number, serum human osteocalcin level, and trabecular bone. Preliminary results from a phase I/II trial in MM where BHQ880 was given IV for 28 days showed patients to be well tolerated in combination with zoledronic acid.

3.5.2.3 Targeting the Activin Receptor Type IIA (ActRIIA)

ACE-011, a novel bone anabolic agent currently in a Phase 2 clinical trial in MM, is a protein therapeutic based on the activin receptor IIA. In numerous preclinical models of bone loss, ACE-011 has demonstrated beneficial effects on both trabecular and cortical bone [83, 84]. In addition, ACE-011 reversed osteoblast inhibition, ameliorated MM bone disease, and inhibited tumor growth in an in vivo humanized MM model [85]. ACE-011 increased bone mineral density, improved bone architecture, increased the mineral apposition and bone formation rates, and improved bone mechanical strength [86]. Results of the Phase 1 study in postmenopausal women demonstrated that a single dose of ACE-011 caused a rapid, sustained, dose-dependent increase in serum levels of bone-specific alkaline phosphatase (BSAP), a marker of

bone formation, while a marker of bone resorption, C-terminal type 1 collagen telopeptide (CTX), decreased. In MM, an ongoing multicenter Phase 2 trial is conducted in Russian patients, which are treated with melphalan, prednisone, and thalidomide and randomized to receive either monthly doses of ACE-011 or placebo for up to three months. Preliminary results show clinical significant increases in biomarkers of bone formation, improvement in skeletal metastases, decreases in bone pain, as well as antitumor activity [69]. These data indicate that ACE-011 is well tolerated and has significant hematologic activity in MM patients receiving myelosuppressive chemotherapy.

3.5.3 Targeting Angiogenesis by VEGF Inhibitor Bevacizumab (Avastin)

Vascular endothelial factor (VEGF) is important for the formation of new blood vessels and plays a key role not only in solid tumors but also in hematologic malignancies, including MM [87]. Bevacizumab targets and blocks VEGF and VEGF's binding to its receptor on the vascular endothelium [88]. Anti-VEGF Abs were active alone, and in combination with radiation in earlier preclinical studies [88, 89]. It is currently being studied clinically in many other solid and blood tumors including primary systemic amyloidosis and MM [90, 91]. NCI's Cancer Therapy Evaluation Program is sponsoring a phase II study of Bevacizumab plus Thalomid (Thalidomide, Celgene) in MM [91].

3.5.4 Targeting BAFF/ARPIL Growth and Survival Pathway by Atacicept (TACI-Ig) or BAFF Inhibitor

Recently, B-cell-activating factor of the tumor necrosis factor (TNF) family (BAFF; also known as B-lymphocyte stimulator, BLyS) and a proliferation-inducing ligand (APRIL) were identified as new survival factors for MM [92–94]. In addition to BMSCs, osteoclasts produce these factors to support MM cells in the BM microenvironment [94, 95]. Their cognate receptors are BAFF-R/BR3, transmembrane activator and calcium modulator (TACI), and B-cell maturation antigen (BCMA) with heterogeneous expression among patient MM cells. Specifically, RNA expression of BCMA and TACI is approximately >30-fold and >10-fold higher, respectively, than that of BR3 [94]. BR3 specifically bind BAFF but not APRIL and has very limited expression in mature B-cells plasma cells [96]. In fact, BCMA expression is only acquired in mature B cells accompanied by loss of BAFF-R expression [96]. These studies provide clinical rationale to target BAFF/APRIL survival pathway in MM.

Atacicept (TACI-Ig, ZymoGenetics; Serono) acts as a decoy receptor by binding to and neutralizing soluble BAFF and APRIL, and preventing these ligands from

binding to their cognate receptors on B-cell tumors, thereby enhancing cytotoxicity. An open-label, dose-escalation Phase I/II study enrolled 16 patients with refractory or relapsed MM ($n=12$) or active, progressive Waldenstrom's macroglobulinemia ($n=4$) [97]. Atacicept was well tolerated and showed clinical and biological activity consistent with its mechanism of action. TACI was expressed heterogeneously among patient MM cells, which may explain promising results for the treatment of TACI^{high} MM cells in a trial for atacicept [97, 98].

In addition, the in vivo antitumor activity of neutralizing anti-BAFF mAb in SCID-hu model of human MM provides the preclinical rationale for its evaluation in the treatment of MM [99]. Moreover, since all MM cell lines and patient MM cells express BCMA, BCMA might be a promising target for monoclonal antibody development against MM. Importantly, MM in remission post-allogenic transplant due to graft-versus-tumor response has donor derived anti-BCMA Abs that are tumor-lytic in vivo [100]. Indeed, BCMA antibodies show cytotoxic activity both as naked IgG and as drug conjugates, which warrant further evaluation as therapeutic candidates for plasma cell malignancies [101].

3.6 Other Potential Targets

Additional mAbs are directed against a variety of further MM cell targets including HLA-DR by 1D09C3 [102], HLA-class I by 2D7-DB [103], kininogen by C11C1 [104],and polyclonal rabbit anti-thymocyte globulin (rATG) [105].

Finally, since NK cells play a critical role in ADCC to lyse tumor target cells via therapeutic monoclonal antibodies and inhibitory-cell killer, immunoglobulin-like receptors (KIRs) negatively regulate natural killer (NK) cell-mediated killing of HLA class I-expressing tumors, mAbs targeting KIR might prevent their inhibitory signaling leading to enhanced ADCC. A novel fully human anti-KIR blocking mAb, 1-7F9 (or IPH 2101), antagonizes inhibitory KIR signaling, activates NK cells, and augments natural killer-mediated killing of tumor cells [106, 107]. Importantly, 1-7F9 enhances patient NK cell cytotoxicity against autologous MM tumor cells in vitro and appears safe in an ongoing phase I clinical trial [108]. A multicenter, open label Phase IIa clinical trial (trial IPH 2101–201, in France) has started to evaluate IPH 2101 as a single agent in patients with stable measurable MM after induction therapy. Another phase II clinical trial to assess the potential of lenalidomide combined with 1-7F9 will be initiated in patients with MM.

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Chapter 4
Defining Multiple Myeloma as a Target for DNA Vaccines

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4.1 Introduction

It is becoming increasingly clear that a combination of therapeutic strategies will offer the maximal potential for cure in multiple myeloma (MM). Remarkable advances in the combination use of new drug therapies alone have now reduced the burden of disease to a chronic state, at least in some patients, and this is a significant achievement in a disease that still persists in an incurable form in many cases. It is the efficacy of recent therapies in achieving remission in almost all cases across the spectrum of MM disease that now provides a window of opportunity to intervene with adjuvant therapies in the setting of minimal residual disease (MRD). For this, immunotherapy offers considerable scope and is being actively pursued in MM. Vaccination in particular provides multiple options to induce anti-tumour immunity, and our focus is on developing DNA vaccines as an intervention strategy. These vaccines allow a flexibility of design to deliver genes encoding antigen and immune modulators for transcription and translation in vivo and to then alert and engage the host immune system. Here, we discuss the development of DNA vaccination in harnessing effective anti-tumour immunity in MM. A prerequisite for this vaccination is knowledge of the nature of the tumour target in MM, importantly defining how tumour growth is fed or sustained and characterising specific tumour-associated antigens (TAAs) as targets. The biology of tumour cells as they re-emerge following therapeutic assault may differ, particularly with regard to antigen expression.

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N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 2: Translational and Clinical Research*,
DOI 10.1007/978-1-4614-5260-7_4, © Springer Science+Business Media New York 2013

Achieving MRD de-bulks disease and removes the anti-immune effects of tumour, but the effects of therapy can influence immune parameters significantly. These issues are addressed in relation to immunotherapy in MM and are under investigation to allow effective use of vaccines to prevent tumour escape.

4.2 Defining Multiple Myeloma as a Therapeutic Target

Understanding growth of the malignant cell population in MM has clear implications for therapy. The question that has persisted in this regard is the nature of the "feeder" cell that maintains growth, and its resolution becomes relevant for vaccination strategies against MM, as antigens associated with the feeder cell need to be defined. As discussed further in Sect. 4.3, antigenic molecules that play a vital role in sustaining tumour survival may serve as front-line targets for effective immunotherapy.

The low proliferative index of MM plasma cells led to early considerations that a less differentiated B cell may cycle to feed the tumour bulk. Identifying such cells and their functional relevance has remained controversial, but the advent of molecular probes based on tumour-derived immunoglobulin (Ig) variable (V) region genes has been instrumental in identifying clonally related progeny. V gene analysis in MM overall has revealed further important features of relevance to defining the cell of origin of disease and its clonal history. The question remains how the cell or origin relates to the "feeder" cell sustaining MM growth.

In defining clonal origins, maturation events that impinge on IgV genes in normal B-cell development are informative. V gene assembly occurs early in normal B-cell development, to generate signature CDR3 motifs that allow tracking of clonal progeny. B cells with functional surface Ig (sIg) molecules exit the bone marrow and mature to become sIgM + D + and, when encountering cognate antigen linked to T-cell help, form germinal centres (GCs) in secondary follicles of lymphoid tissue and initiate somatic hypermutation (SHM) of V genes to affinity mature fit for antigen [1]. Deletional class switch recombination (CSR) can also occur at this site, leading to deletion of unwanted heavy constant chain (C_H) genes at the IgH locus on chromosome 14q32. Post-GC B cells have two fates, circulating as memory B cells (which include sIgG/A in which the Cμ genes have been deleted) or home to the bone marrow (BM) [2].

Tumour-derived V genes in MM reveal extensive SHM, with a homogeneous pattern of intraclonal mutations, which is consistent with neoplastic transformation occurring at a stage when SHM has ceased (review, [3]). MM is typically isotype switched, and many cases reveal aberrant chromosomal translocations that map to switch site regions in 14q32 [4]. This indicates that isotype switch events are highly relevant to MM origins. Seminal observations identified tumour-derived Cμ transcripts in the BM in switched MM, suggesting an earlier progenitor (see review, [3]). There have been additional reports of circulating $CD19^+$ B-cell populations with V_H transcripts identical to $CD138^+$ malignant PCs [5, 6], suggesting "feeder"

cells, but both the frequency and malignant status of these earlier B cells have remained a consistent matter of debate.

More recent observations have reappraised hierarchical clonally related cells in MM. Rigolin et al. [7] described circulating endothelial cells (CECs) in MM which displayed tumour-associated 13q14 deletions and, in two cases, rearranged tumour V_H genes with the same nucleotide sequence as tumour cells. The authors suggest a number of mechanisms to explain these observations, including the possibility that endothelial progenitor cells (EPCs) and MM plasma cells could derive from the same multipotent hemangioblast precursor cell or that dedifferentiation of a precursor cell committed to the lymphoid lineage generates an aberrant cell with EPC characteristics, suggesting a cell with "stem cell" properties. However, we have argued that the complexity of SHM events in MM would make it unlikely that this degree of dedifferentiation could occur [8].

Nevertheless, in MM, the stem cell concept has gathered momentum. This argues for distinct characteristics in common with "cancer stem cells" (CSCs) that have emerged as a new paradigm in understanding malignancy. CSCs are perceived as a less differentiated minor component in a clonal hierarchy that possess the characteristic of asynchronous proliferation to generate a stem cell pool with a limitless self-renewal capacity and differentiation potential to feed the tumour bulk. Differentiated cells in this model lack the capacity for self-renewal. The first indication that such a cell might exist in cancer came from studies in acute myelogenous leukaemia (AML) (reviewed in [9]). Using a NOD/SCID xenotransplantation model, a CD34+ CD38-ve cell fraction representing 0.1–1% of the AML cell population was shown to contain the leukaemia-initiating cell or leukaemic stem cell (LSC).

Central to the current debate in MM are observations from the Matsui laboratory that a CD138-ve population in MM harbours "stem" cells [10]. These "myeloma stem cells" (MSCs), as proposed, are sIg^+ B cells. It has been shown that MM cells lacking CD138 (syndecan-1, which marks plasma cells) are more clonogenic in vitro and that circulating $CD19^+$ $CD27^+$ B cells from MM patients preferentially engraft NOD/SCID mice to give rise to $CD138^+$ human PCs in the BM [10, 11]. The hedgehog signalling pathway, which regulates progenitor fate in early development, was also shown to associate specifically with the CD138-ve MM "stem cell" population [12]. In a separate study of MGUS, elevated clonogenicity again specifically associated with the CD138-ve fraction in vitro [13]. Interestingly, SOX2, a gene critical for self-renewal in embryonal stem cells, was shown to be expressed in CD138 cells, the first molecular indicator of "stemness" [14]. Furthermore, early data on asymptomatic plasmaproliferative disease indicated that the presence of anti-SOX2 T cells very significantly reduced time to disease progression, revealing that immune control of the clonogenic fraction will be important [14]. Notably however, there was an early indicator from these studies that the "stem" cell fraction may actually alter as disease advances in MM and becomes a SOX2-expressing CD138+ cell [14]. Coculturing MM cell lines with dendritic cells led to the emergence of CD138-ve cells with enhanced clonogenicity in vitro [13] and induction of *BCL-6* expression, a gene which prevents terminal maturation and holds B cells at a stage of differentiation conducive to germinal centre events. This indicates a degree of

plasticity in MM-derived cells. However, in primary MM tumour cells, a CD138-ve fraction could be identified that had an enhanced potential for clonogenicity following coculture with DCs [13]. It is not clear from these experiments whether the CD138-ve fraction in primary tumour cells reflects emergence also from CD138$^+$ cells in situ, where comparable niche effects could impart a dedifferentiation signal, or at least lead to shedding of CD138.

However, in relation to a SOX2$^+$ CD138$^+$ “stem” cell in MM [14], supportive findings in the SCID-Hu model, in which human foetal bone has been implanted for homing of potential MM progenitor cells, reported that only fully differentiated CD38^{++} malignant (plasma) cells propagated growth [15]. Taken together, a plasma cell MSC (P-MSC) can clearly disseminate tumour growth. It has furthermore been argued that in CSC studies, the gross stromal species mismatch in xenotransplantation experiments may not adequately reflect the potential of all clonally related cells to engraft tumour in an immunodeficient background [16]. In a congenic tumour model, all clonal cells rather than a specific subpopulation engraft malignant cells, validating a stochastic or “clonal evolution” pathway of cancer growth [16]. P-MSCs would lend themselves as candidates for this pathway.

A putative sIg+MSC in MM must also fulfil additional requirement in relation to feeding the tumour bulk, that is, the acquisition of the repertoire of molecular lesions that are a feature of presenting malignant plasma cells, lesions that worsen as the tumour progresses and are seen again when tumour relapses. Very recent observations are revealing. In a study where some tumour-related B cells were identified as abnormal by elevated levels of transcripts for *CYCLIN D1* and *FGFR3*, a specific mutation in *K-RAS* was evaluated [17]. This mutation, which signifies the acquisition of critical oncogenic events that drive tumour progression, was not identifiable in the B-cell fraction but only in malignant plasma cells in MM patients. The likelihood that an sIg+MSC acquires such an identical mutation consistently as it periodically cycles to feed the tumour bulk appears unlikely, on the grounds of probability alone. Overall, defining whether a subpopulation regulates the clonogenic growth of MM remains crucial to therapy and for our aim of developing DNA vaccines against MM.

4.3 DNA Vaccines

Advances in genetic technology and our understanding of the immune system allow the rational construction and delivery of DNA vaccines capable of inducing powerful anti-tumour immune responses. The vaccine format is highly flexible and can encode not only tumour-derived antigens but also molecules desirable for coordinating and intensifying the immune response. This is crucial to overcome the immune tolerance/regulation and immunodeficiency likely to exist in many cancer patients.

DNA acts as a pathogen-associated molecular pattern (PAMP) and is a potent activator of innate immunity. This property is thought to contribute to the effectiveness of DNA vaccines at activating specific adaptive immune responses against

encoded antigen. The molecular mechanisms for sensing DNA are multilayered and appear to incorporate redundancy. Recent evidence suggests that high-mobility group box (HMGB) proteins serve as universal sentinels for the promiscuous sensing of nucleic acids within cells [18]. This promiscuous sensing precedes, and may be necessary for, the more discriminative sensing that is afforded by pattern recognition receptors (PRR).

Several PRR families recognise microbial nucleic acids, including the Toll-like receptors (TLRs), the retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and the NOD-like receptors (NLRs). Tissue-specific PRR expression, nucleic acid form (e.g., DNA vs RNA) and intracellular location (e.g., endosomes, cytosol) will influence receptor binding and trigger common pathways of pro-inflammatory cytokine secretion (e.g., type I interferons, IL-1β) leading to a cascade of activation, proliferation and differentiation of immune cell subsets. Although TLR9 was the first PRR found to interact with DNA [19], several cytosolic DNA sensors have recently been identified, including DAI (DNA-dependent activator of interferon regulatory factors) [20], AIM2 (absent in melanoma 2) [21] and RNA polymerase III [22].

Clearly plasmid DNA engages multiple pathways to activate innate immunity, imparting DNA vaccines with adjuvant properties—an important first step for immune targeting of weak tumour antigens. However, key to the induction and maintenance of effective and durable adaptive immunity is the activation of $CD4^+$ helper T cells (T_H) [23]. The programming of $CD8^+$ T cells for secondary expansion is influenced by T_H cells, and in their absence the "helpless" $CD8^+$ T cells undergo activation-induced cell death on secondary stimulation [24]. T-cell help is also necessary for the induction of effective high-affinity antibody responses [25]. It is unlikely that autologous tumour antigens are able to promote significant T_H responses, due to mechanisms of tolerance and/or regulation. We have overcome this by developing DNA vaccines that encode weakly immunogenic tumour antigen sequences linked to microbe-derived sequences, thereby engaging T-cell help from a functional and intact antimicrobial repertoire to help immune responses against the tumour antigen [26]. Although strategies to remove regulatory $CD4^+$ T cells are being developed, activation of $CD4^+$ helper T cells remains key to the induction of effective and durable anti-tumour immune responses.

We have developed several DNA fusion vaccine constructs (Fig. 4.1) that, depending on the target antigen, are designed to provide help to stimulate antigen-specific antibody, $CD4^+$ or $CD8^+$ T cell responses for tumour destruction [26]. The microbial sequences that we have selected are derived from tetanus toxin (TT) [27, 28] or a plant viral coat protein [29]. A similar approach has also been developed by others [30, 31]; although using different nonself antigens as vaccine fusion partners, all are potentially capable of stimulating the desired T-cell help. It is likely that this mechanism of T_H stimulation may also be implicit in the induction of immunity to DNA vaccines encoding xenogeneic antigens with MHC class II-binding sequence differences: that is, human antigens in preclinical murine models and vice versa in human clinical trials [32, 33].

To test our DNA fusion vaccine approach, we initially used the idiotypic (Id) antigen of B-cell malignancies as a tumour target. This antigen represents an ideal

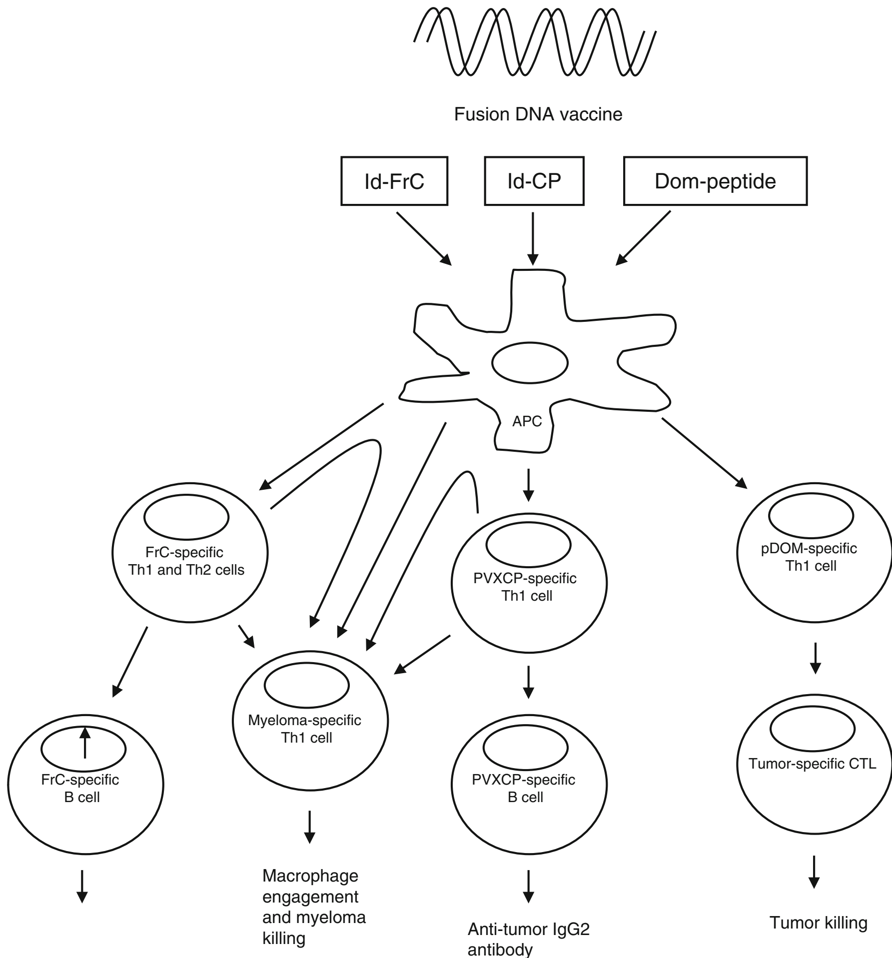

Fig. 4.1 Three fusion gene DNA vaccine designs tailored to engage multiple arms of the immune response

target for antibody attack since it is expressed on the B-cell surface and is tumour specific. DNA vaccines encoding Id antigen alone proved ineffective at stimulating an anti-Id antibody response, likely due to the poor provision of CD4+ T-cell help afforded by this self-antigen. However, fusion of a microbial sequence (fragment C (FrC)) derived from TT was able to engage a non-deleted CD4+ T-cell repertoire and promote anti-Id antibody responses which led to protective antibody-mediated immunity in several models of B-cell malignancies [28, 34]. CD4+ T-cell help was also crucial for maintenance of the anti-Id antibody response and had to be included for booster vaccinations [35]. In fact, removal of CD4+ T-cell help or exposure of vaccine-induced memory B cells to Id antigen in the absence of T-cell help led to irreversible silencing of the anti-Id antibody response [35].

We have also tested an alternative fusion sequence, the potato virus X (PVX) coat protein (CP) [29]. It is unlikely that pre-existing immunity to this antigen would be expected in humans, removing an additional complication which could be introduced by pre-existing antibody to TT. An Id-CP DNA fusion vaccine induced IFNγ-secreting CD4+ protective responses against murine lymphoma as well as anti-Id antibody of the IgG 2a and 2b isotypes with no IgG1 [29]. This contrasted with the Id-FrC DNA vaccine which induced a potent anti-Id humoral response of mixed IgG1 and IgG2 isotypes. Clearly, the immune outcome induced to the tumour antigen was influenced by the nature of the DNA vaccine fusion partner.

The Id-CP DNA vaccine design was also tested in a myeloma model. Vaccination with the Id-CP DNA vaccine induced significant protection against myeloma challenge [29]. Since the tumour cells do not carry Id on the surface, the role of anti-Id antibody in protection was not significant. Instead, protection was mediated by CD4+ T cells [29].

An alternative Id-specific DNA vaccine strategy for attacking myeloma has been developed by others [36]. This approach targets bivalent Id antigen to antigen-presenting cells using an scFv antibody fragment against MHC class II. It leads to the induction of Id-specific T- and B-cell responses and protective immunity in murine models of myeloma and lymphoma.

Id-specific CD4+ T cells have been shown to play an essential role in eradication of myeloma in murine models [37]. Since myeloma cells are MHC class II-negative, these primed T cells are unable to kill the tumour cells directly. Instead, through release of IFNγ, they engage macrophages at the tumour site to engulf and destroy the myeloma cells [38]. The effector CD4+ T cells are of classical Th1 phenotype, and adoptive transfer protects mice against myeloma. Secretion of Id antigen by the tumour cells may result in differing outcomes: Id antigen secretion in the tumour vicinity appears important for attracting CD4+ cells to the tumour site [39], but excessive levels of Id immunoglobulin may play a deleterious role in anti-Id immune induction ([40]; Sect. 4.5). In fact, the induction of tolerance by secreted protein in myeloma patients has made the application of Id vaccines very challenging. However, vaccinating patients when serum Id immunoglobulin is low may provide an ideal setting for successful anti-Id vaccination.

The activation of tumour-specific CD8+ cytolytic T lymphocytes (CTL) is a clear objective for tumour immunotherapy, given their ability to seek out and destroy tumour cells. This is especially desirable when targeting intracellular antigens that are not present at the cell surface for recognition by antibody. The induction of effective CD8+ T-cell responses and the establishment of immunological memory also require T-cell help [41]. The key for our DNA fusion vaccines is to focus the immune response on tumour-derived CD8+ T-cell peptides rather than immunodominant peptides derived from the FrC fusion partner. For this we have used a shorter sequence (DOM) derived from FrC, retaining T-cell help but reducing the potential to produce competing MHC class I-binding peptides [27]. Positioning of the tumour-derived target peptide at the C-terminus of DOM provides an additional advantage, possibly by simplifying the requirements for correct peptide processing and presentation on MHC class I [42]. This p.DOM-peptide DNA fusion vaccine can

break tolerance and induce high levels of tumour-specific CD8+ T cells in numerous preclinical models [26]. The approach also appears to be superior to peptide vaccination [43]. The p.DOM-peptide vaccine is being tested in a clinical trial in patients with prostate cancer, with vaccine-specific immune responses, including tumour-specific CD8+ T cells, detected in the majority of patients [44, 45].

Despite their success in murine models, clinical testing of DNA vaccines in humans initially proved disappointing, with a general failure to induce robust immune responses. A key factor was injection volume, which greatly influences plasmid uptake and immune induction [46]. To overcome this translational barrier, physical methods to improve DNA vaccine delivery are being developed [44, 47–49]. Electroporation has emerged as a very effective strategy to induce robust immune responses to DNA vaccines, overcoming the issue of low injection volume ([46]; Low et al. 2009a). The technique involves the immediate application of electrical stimulation at the site of DNA vaccine injection [50, 51]. This improves cell transfection efficiency, resulting in increased antigen expression (10–100-fold). It is also accompanied by local tissue damage, inflammation and an influx of immune cell subsets [52, 53]. This will likely increase cross-presentation of DNA vaccine-encoded antigen resulting in improved immunity [54]. Significantly, electroporation increases DNA vaccine-specific immune responses in large animals and primates [55], including human subjects [44], providing a safe and effective delivery system for clinical testing of DNA fusion vaccines.

4.4 Antigen Targets for DNA Vaccines in Multiple Myeloma

Prime candidates for vaccination targets are antigens that are patient specific or are essential for survival of tumour cells as the latter are unlikely to be downmodulated under therapeutic pressure. The patient-specific antigen that comes to the fore in MM is the idiotype and has been extensively investigated as a target for immunotherapy. It raises little concern in relation to unwanted side effects that may result when antigen is more widely shared by normal cells. A molecular characterisation of V genes encoding the idiotype-containing domains served to inform clonal origins (Sect. 4.2) and revealed that in typical MM, there are no on-going events that modify this antigen.

As with other tumours, there has been considerable interest in mapping the full repertoire of available tumour-associated antigens (TAAs) in asymptomatic disease and MM. Antigen discovery in MM has relied on a number of approaches. Many of the early TAAs described in MM were examined to parallel known patterns of expression in other tumours, such as the cancer testis antigens (CTAs) (Table 4.1), a family of mostly intracellular antigens. The classification of CTAs has been refined more recently based on a comprehensive screen of patterns of expression in testis and somatic tissues [56]. This classification has established genes as testis-restricted (39 genes), testis/brain-restricted (14) and a testis-selective (85) group, the latter showing a wider pattern of expression in somatic tissues. The testis-restrictive (T-R)

antigens are expressed only in normal testis and cancer cells and are effectively tumour specific as testis lacks MHC class I expression as an immune-privileged site and will avoid vaccine-generated CTLs. A number have been described in MGUS and MM and expression analysis augmented by assays for presence and persistence of immune responses to these antigens (Table 4.1). While initial findings suggested a restriction of CTA expression to late, advanced stages of MM [57], more recent observations indicate a diverse pattern of expression, importantly also at the MGUS stage and at disease presentation (Table 4.1), with CT7 (MAGE-C1) noteworthy. By using an in silico-based strategy, we identified PASD1 as a new CTA in MM, confirming expression at the transcript and protein level [58]. PASD1 falls in the category of a testis/brain-restricted (T/B-R) CTA [56]. A more systematic comparison of genes at the pangenomic scale of expressed genes in normal testis and MM has shown that 98% of MM patients express at least one CTA, 86% at least two and 70% at least three CTA genes [59]. By using a probe set of 10 CTAs, this study also showed that three genes or more can provide immunotherapeutic targets to avoid tumour escape in ~70% of MM. This combinatorial approach may also be necessary in view of the intraclonal nature of CTA expression in cancer cells, with some T-R and T/B-R antigens found to be expressed in some but not all clonal cells by transcript or IHC analyses. However, some CTAs may be more predominant, and this requires further analysis. Recent observations indicate that CTAs are important therapeutic targets, as a role for MAGE-A and MAGE-C proteins has emerged in supporting tumour survival by complexing with KAP1, a scaffolding protein that corepresses p53 expression to suppress apoptosis [60].

Gene expression profiling at the whole genome level has generated a vast data set that has as yet not been fully mined to identify the full repertoire of MM-associated antigens. Many may fall in the category of antigens over-expressed in MM in relation to normal tissues, but may nevertheless serve as useful targets. Profiling has also yielded antigens with a more restricted pattern of expression. Dickkopf-1 (DKK1) is an example [61]. As a secreted protein, it functions as an inhibitor of the Wnt/β-catenin signalling cascade. DKK1 mRNA has not been detected in most normal human tissues, except for prostrate, testis, placenta and uterus, but appears ubiquitous in MM primary cells [61]. It clearly is recognised by immune surveillance, as DKK1-specific $CD8^+$ T cells can be identified at low frequencies in MM patients and able to lyse cell lines and primary tumour cells in an HLA-restricted manner [61]. Transcription factors that associate with the maturation status of plasma cells, such as X box-binding protein 1 (XBP-1) and positive regulatory domain-binding factor 1 (PRD-BF1), are also being considered as appropriate targets for MM [62].

The immunogenicity of TAAs has been exploited more recently in MGUS and MM using a serum antibody detection array (SADA) to profile serum reactivity against 83 antigens [14]. Strikingly, both antibody and cellular responses were identified in MGUS to SOX2, a protein central to control of pluripotency in embryonic stem cells, and anti-SOX2 T cells mitigated against malignant progression in asymptomatic plasmaproliferative disease. These observations of immune response to “stemness” genes are highly notable, suggesting that immunisation by vaccination against,

Table 4.1 Tumour-associated antigens in multiple myeloma: expression and immune responses

Targeted antigen	Method of analysis	Treatment	Results	Reference
1. *Cancer testis antigens*				
1.1. *Testis restrictive*				
CT7 (MAGE-C1)	Expression analysis of BMMCs from MGUS and MM patients (RT-PCR, IHC)	P	1/6 MGUS, 2/3 stage I/II MM and 6/7 stage III MM specimens expressed CT7 transcript 2/15 MGUS, 3/4 stage I/II MM and 27/33 stage III MM specimens expressed CT7 protein CT7 protein expression increased with advanced stage of disease	Jungbluth et al. [125]
	Ex vivo analysis of BM lymphocytes from MM patients (ELISA,ELISPOT)	P	CT7-specific cellular immune responses in 2/4 stage III MM patients	Lendvai et al. [126]
MAGE-A1	Characterisation of $CD4^+$ and $CD8^+$T-cell immune responses to MAGE-A1/A2/A3 in MGUS and MM patients (cytokine secretion assay)	P/T	19/53 MM and 2/25 MGUS patients displayed a MAGE-A1/A2/A3 specific $CD8^+$-T-cell responses 1/32 MM and 4/30 MGUS patients showed a MAGE-A1/A2/A3-specific $CD4^+$ T-cell responses	Goodyear et al. [86]
	Ex vivo analysis of BM lymphocytes from MM patients (ELISA)	P	1/24 patients sera showed a humoral immune response (stage III MM)	Lendvai et al. [126]
NY-ESO-1	Expression analysis of BMMCs from MGUS and MM patients (RT-PCR, IHC)	P	0/5 MGUS, 1/3 stage I/II MM and 1/5 stage III MM specimens expressed NY-ESO-1 message 0/15 MGUS, 1/4 stage I/II MM and 7/33 stage III MM specimens expressed NY-ESO-1 protein	Jungbluth et al. [125]
	Expression analysis of NY-ESO-1 in BMMCs from 55 patients with MM (RT-PCR) Analysis of 66 patients with MM for Ab response against NY-ESO-1	P/T	7% MM patients expressed NY-ESO-1 Strong Ab responses against NY-ESO-1 were found in 4/66 MM patients preferentially following ASCT	Atanackovic et al. [69]
	In vitro study of the effects of DCs loaded with tumour cells previously coated with anti-tumour mAbs	N/A	Tumour cell-loaded DCs induced a strong $CD8^+$ T-cell response that was specific for the CTAs expressed in the tumour, one of which was NY-ESO-1	Dhodapkar et al. [127]

	Analysis of MM patients for NY-ESO-1 expression and NY-ESO-1 immune responses (ELISA, flow cytometry, IHC)	P and P/T	Detection of spontaneous NY-ESO-1-specific Abs in 33% of patients with NY-ESO-1^{+} MM Detection of spontaneous NY-ESO-1-specific T cells in patients with NY-ESO-1^{+} MM, able to kill primary MM cells when expanded	van Rhee et al. [88]
	In vitro study of the effects of DCs transduced with NY-ESO-1 fused to a protein transduction domain (PTD) (tetramer analysis and ELISPOT)	N/A	Superior generation of HLA-A2.1 CD8^{+} T lymphocytes specific for NY-ESO-1 compared with NY-ESO-1 control protein (44 vs 2%) The NY-ESO-1 specific T lymphocytes generated secreted IFN-γ	Batchu et al. [128]
SPAN-Xb	Ex vivo analysis of PBMCs of 13 healthy donors and 5 patients with MM (stages II to III) (IFN-γ ELISPOT)	P/T	Peptide-specific T-cell precursors could be detected and expanded in 9/13 tested normal donors and 3/5 tested patients	Frank et al. [129]
SSX1	Screening of a panel of MGUS and MM patients BMMCs for SSX1 expression (RT-PCR)	P	4/45 MGUS patients and 48/114 MM patients showed SSX1 expression	Taylor et al. [130]
	Ex vivo analysis of BM lymphocytes from MM patients (ELISA)	P	1/24 patients sera showed a humoral immune response (stage III MM)	Lendvai et al. [126]
SSX2	Screening of a panel of MGUS and MM patients BMMCs for SSX2 expression (RT-PCR)	P	0/45 MGUS patients and 26/114 MM patients showed SSX2 expression Of SSX1, 2, 4 and 5, SSX2 had the strongest association with reduced survival	Taylor et al. [130]
	Expression analysis of SSX2 in BMMCs from 55 patients with MM (RT-PCR) Analysis of 66 patients with MM for Ab response against SSX2	P/T	16% MM patients expressed SSX2 IgG Abs against SSX2 were found in 3/66 MM patients (all 3 following ASCT)	Atanackovic et al. [69]

(continued)

Table 4.1 (continued)

Targeted antigen	Method of analysis	Treatment	Results	Reference
1.2. *Testis/brain restrictive*				
PASD1	Evaluation of PASD1 expression in MM cell lines and in primary MM (RT-PCR, Q-PCR, IHC)	P and P/T	PASD1 expression was observed in 5/11 MM cell lines (RT-PCR) and in 14/16 primary MM (Q-PCR), both at presentation and post-treatment Protein expression was confirmed in 2/4 primary MM (IHC)	Sahota et al. [58]
1.3. *Testis/selective*				
MAGE-A3	Expression analysis of BMMCs from MGUS and MM patients (RT-PCR, IHC)	P	4/6 MGUS, 1/3 stage I/II MM and 2/7 stage III MM specimens expressed MAGE-A3 message 6/15 MGUS and 2/4 stage I/II MM specimens expressed MAGE – A family protein 23/33 stage III MM specimens expressed MAGE-A3 protein	Jungbluth et al. [125]
	Expression analysis of MAGE-A3 in BMMCs from 55 patients with MM (RT-PCR) Analysis of 66 patients with MM for Ab response against MAGE-A3	P/T	55% MM patients expressed MAGE-A3 IgG Abs against MAGE-A3 were found in 4/66 MM patients following ASCT	Atanackovic et al. [69]
Sp17	Ex vivo generation of Sp17-specific HLA class I-restricted CTLs from PBMCs of 4 MM patients (CTL assays)	P/T	Generated CTLs were able to lyse autologous tumour cells that expressed Sp17	Chiriva-Internati et al. [131]
SSX4	Screening of a panel of MGUS and MM patients BMMCs for SSX4 expression (RT-PCR)	P	9/45 MGUS patients and 43/114 MM patients showed SSX4 expression	Taylor et al. [130]
SSX5	Screening of a panel of MGUS and MM patients BMMCs for SSX5 expression (RT-PCR)	P	0/45 MGUS patients and 40/114 MM patients showed SSX5 expression	Taylor et al. [130]

2. *Others*				
DKK1	Expression analysis of BM from 10 MM patients and MM cell lines for DKK1 expression (RT-PCR and WB) Ex vivo generation of peptide-specific T-cell lines from MM patients.	?	DKK1 mRNA was detected in 8/8 myeloma cell lines and 10/10 primary myeloma cells DKK1 protein was detected in 7/10 myeloma cell lines and 10/10 primary myeloma cells DKK1-specific T-cell lines efficiently lysed DKK1$^+$ MM cell lines U266 and IM-9 and primary myeloma cells from patients	Qian et al. [61]
Heat shock proteins	In vitro study of the effects of Hsp90 inhibition in MM cell lines and MM primary cells	N/A	siRNA-mediated knockdown of Hsp90 or treatment with Hsp90 inhibitor 17-DMAG induced apoptosis in MM cell lines The induced apoptosis was not attenuated in the presence of cells from BMM Hsp90 inhibition induced apoptosis in MM primary cells cultured with cells from BMM	Chatterjee et al. [132]
	Evaluation of the anti-tumour activities of KW-2478, a nonansamycin Hsp90 inhibitor in MM cells both in vitro and in vivo	N/A	Exposure of MM cells to KW-2478 resulted in growth inhibition and apoptosis in vitro, and showed a significant suppression of tumour growth in vivo	Nakashima et al. [133]
	Generation of specific CTL lines after repeatedly stimulating T cells with autologous, HLA-A*0201$^+$ DCs pulsed with gp96 from U266 cell line or primary myeloma cells	P	The generated T cells were able to lyse gp96-pulsed DCs, U266 and other MM cell lines, as well asHLA-A*0201$^+$ primary myeloma cells The response was mainly MHC class I/HLA-A*0201 restricted	Qian et al. [134]
HM1.24	Expression analysis of 20 MM cell lines and of CD138$^+$ MCs from 7 ND, 7 MGUS and 65 MM patients (microarray analysis) In vitro study of the effects of HM1-24-peptide-loaded DCs derived from HLA-A2$^+$ ND PBMCs (ELISPOT, tetramer analysis and ^{51}Cr-release assay)	P	HM1.24 gene was expressed at comparable levels in 65 MM patients, 7 MGUS patients and 7 ND HM1.24 gene median expression level was higher in MM cell lines as compared to ND, MM and MGUS patients 1/8 nano-peptides tested showed the most frequent activation of CD8$^+$ T cells in 8/11 ND HM1.24-specific CD8$^+$ T cells lysed HLA-A2$^+$ MM cell lines	Hundemer et al. [135]

(continued)

Table 4.1 (continued)

Targeted antigen	Method of analysis	Treatment	Results	Reference
mHag	Analysis of an HLA class II-restricted CD4+ cytotoxic T-cell line (CTL) and cytotoxic T-cell clone (CTC), 3AB11, isolated from the blood of an MM patient with clinical GvM following allogeneic SCT (ELISA and ^{51}Cr-release assay)	P/T	The antigen 3AB11 was expressed by patient-derived EBV-transformed B-cell lines (EBVp), the patient-derived myeloma plasma cell line UM9 and monocytes CTC 3AB11 was strongly activated by the HLA-DP*0401+ EBVp, mildly by monocytes and UM9 and poorly by patient-derived PHA blasts	Holloway et al. [136]
Melan-A/MART1	Evaluation of the anti-tumour activity of PBMCs from HLA-A2+ healthy donors and HLA-A2+ MM patients stimulated and expanded in vitro with Melan-A analogue peptide-loaded autologous DCs (IFN-γ ELISPOT, ^{51}Cr-release assay, tetramer analysis)	?	Melan-A analogue-specific T cells showed IFN-γ secretion and lysed specifically human myeloma-derived cell lines Melan-A analogue-specific T cells from MM patients specifically lysed autologous MM cells	Christensen et al. [137]
MUC-1/DF3	Induction of MM-specific CTLs by transfection of MHC class I-matched DCs with total RNA from LP-1 and U266 myeloma cell lines	N/A	Induction of CTLs that lyse the LP-1 and U266 myeloma cells in an antigen-specific and MHC class I-restricted manner showed a MUC-1 specificity of the CTLs induced with U266-derived RNA	Milazzo et al. [138]
	Ex vivo generation of MUC-1-specific human T-cell lines from normal volunteers and pancreatic cancer patients using agonist epitopes	N/A	T-cell lines generated with one of the agonist epitopes were more efficient than those using the native epitope in the lysis of HLA-A2 human tumour cells expressing MUC-1	Tsang et al. [139]
	Generation of mAbs (DCM209) specifically binding the MUC1 α/β junction by cDNA/protein immunisations	N/A	DMC209 specifically binds the MUC1 α/β junction expressed by MUC-1 positive malignant plasma cells of MM	Rubinstein et al. [140]
	Screening of 12 MM patients for T-cell responses toward MUC-1 after ASCT	P/T	5/12 patients showed a MUC-1-specific T-cell response. Only 1 out of those 5 relapsed in contrast to 4/7 without MUC-1-specific CTL response	Kapp et al. [141]

OFA-iLR	Evaluation of the anti-tumour activity of OFA-iLR1 and iLR2 peptides-specific CTLs generated by in vitro priming with peptides-pulsed monocyte-derived DCs (ELISPOT and ^{51}Cr-release assay)	P/T	6/12 MM patients showed a reactivity against iLR1 and iLR2 OFA-iLR peptide-specific CTLs efficiently lysed tumour cells endogenously expressing OFA-iLR	Siegel et al. [142]
PRDI-BF1 and XBP-1	Ex vivo analysis of PRDI-BF1 and XBP-1 specific A2.1-restricted CTL generated in HLA-A*0201 (A2.1) transgenic mice (^{51}Cr-release assay)	N/A	A2.1^{+} MM cell lines expressing PRDI-BF1 and XBP-1 were selectively killed by PRDI-BF1- and XBP-1-specific CTLs	Lotz et al. [62]
RHAMM	RHAMM-R3 peptide vaccination (4 *s.c.* injections at a biweekly interval) of 9 patients (3 MM, 5 MDS and 1 AML)	P and P/T	Positive immunological responses detected in 4/9 patients (2 MDS and 2 MM): Increase of CD8^{+}RHAMM-R3tetramer^{+}/CD45RA^{+}/CCR7^{-}/CD27^{-}/CD28^{-} effector T cells and of R3-specific CD8^{+} T cells 3 patients (2 MDS and 1 MM) showed clinical effects	Greiner et al. [143]
SOX2	Analysis of ND, MM, MGUS and AMM patients (BMMCs and PBMCs) for SOX2 expression and SOX2-specific immune responses (flow cytometry, ELISA, Luminex)	?	Intranuclear expression of SOX2 marked the CD138^{-} compartment in MGUS and a proportion of CD138^{+} cells in MM patients Anti-SOX2 IgG Abs were detected in12/52 MGUS, 1/92 ND but in none of AMM and MM patients tested SOX2-specific T cells were detected in fresh PBMCs from 11/16 MGUS, 2/21 AMM but in none of MM patients, and ND SOX2 stimulated marrow MNCs from MGUS but not MM patients inhibited clonogenic growth in 3/3 MGUS patients	Spisek et al. [14]
WT-1	Evaluation of WT1 expression in BMMCs from MM patients (qRT-PCR) Examination of the effects of induced WT1-specific CTLs from MM patients	?	Expression level of WT1 mRNA was significantly lower than acute leukaemia cells Myeloma cells were lysed efficiently by WT1-specific CTLs in a HLA-restricted manner	Azuma et al. [144]

Abbreviations: *Ab* antibody, *mAb* monoclonal antibody, *AML* acute myeloid leukemia, *AMM* asymptomatic multiple myeloma, *BM* bone marrow, *BMM* bone marrow microenvironment, *BMMCs* bone marrow mononuclear cells, *CTA* cancer testis antigen, *IHC* immunohistochemistry, *mHag* minor histocompatibility antigens, *MC* mononuclear cells, *MDS* myelodysplastic syndrome, *MGUS* monoclonal gammopathy of undetermined significance, *MM* multiple myeloma, *N/A* not applicable, *ND* normal donors, *OFA-iLR* oncofetal antigen-immature laminin receptor protein, *P* presentation, *PRDI-BF1* positive regulatory domain I-binding factor 1, *P/T* post-treatment, *RHAMM* receptor for hyaluronan-mediated motility, *SCT* stem cell transplantation, *WB* western blot, *XBP-1* X box-binding protein 1, *?* not known

e.g. SOX2, in the asymptomatic phase of disease may prevent disease escalation. DNA vaccines, in particular, lend themselves for use as prophylactic vaccines to generate antigen-specific cellular responses that associate with preventing malignant transformation. A more direct approach employed serological analysis of recombinant cDNA expression library (SEREX) to also identify unique immune responses to ten antigens in MGUS, of which specific T-cell responses to OFD1 (oral-facial-digital type I syndrome) were observed in MGUS but not MM patients [63]. It would also be of interest to examine if these T-cell immune responses in MGUS are also relevant to preventing malignant escalation of disease. OFD1 was also found as a target for T-cell responses post-transplant [63].

Antigen expression at disease presentation will also need to be fully re-evaluated post-therapy if it is to serve as a target for vaccine delivery in the MRD phase in MM. Focusing on CTAs, we had previously shown that PASD1 is retained after therapy, and a limited number of other CTAs have also now been reported, such as MAGE-C1 [58, 64]. To assess this more systematically, we have recently analysed the expression of a probe set of 66 CTA genes for presence calls in gene expression profiles obtained from large cohorts of presentation and post-therapy MM [65, 65a]. The data revealed retention of >80% of CTA genes in MM cells despite therapy, but with distinct patterns of expression. These CTAs are under current investigation as targets for DNA vaccines in our laboratory.

In allogeneic stem cell transplantation (ASCT), durable remissions can result from donor-derived T cells being able to attack tumour cells in the graft-versus-myeloma (GvM) effect. While some of the TAAs targeted by GvM-inducing T cells are known, at present, these are limited, and new targets need to be identified. In particular, those antigens that are either exclusively restricted to MM cells or have a narrow pattern of expression limited to hematopoietic cells present an opportunity to allow educated donor lymphocyte infusion (EDLI) by donor vaccination. These are likely to lessen GvH disease. However, antigens with a broader pattern of expression may still be targets for GvM activity as expression on normal tissues may not be recognised or may not occur under steady state conditions [66]. Of known T-cell targets following HLA-identical ASCT, relevant hematopoiesis-restricted minor histocompatibility antigens (mHags) are HA-1, HA-2, BCL2A1 and the B-lymphocyte-restricted mHag HB-1 [66]. In MM, interesting evidence for additional targets is beginning to emerge. An HLA-B7-restricted CTL clone recognising the angiogenic endothelial cell growth factor-1 *(ECGF1)* gene product was recently identified following DLI post-allo [67] as well as T-cell recognition of the mHag encoded by the ATP-dependent interferon-responsive *(ADIR)* gene [68]. Post-allo T-cell responses specific for NY-ESO-1 and other CTAs (in a panel of ten antigens) have been identified [69, 70]. This indicates that new CTAs are likely to be additional CD8^{+} T-cell targets in MM following ASCT. For DNA vaccines, there is considerable scope for generating antigen-specific responses for EDLI, given that a healthy donor is vaccinated with an intact immune system.

While Table 4.1 is not an exhaustive listing of TAAs defined in MGUS and MM over the last 10 years, it serves to highlight some of the approaches taken in evaluating

important antigens. Surface antigens open the additional arm of intervention by both passive antibody therapy and vaccination, and many such TAAs have been described in these tumours (reviewed by [71]). It is also anticipated that whole genome mutation analysis in MM may yield up specific mutations that could generate novel antigen epitopes that could be exploited as vaccine targets.

4.5 Immune Status in MM: Potential for Vaccination

4.5.1 Immune Capacity

DNA vaccines have clear potential to generate both humoral and cellular immune responses, as has been amply demonstrated in a variety of preclinical models and against an array of antigens (Sect. 4.3). The challenge in MM is to define to what extent immunity can be induced therapeutically and how effective the level of immunity is likely to be in this tumour setting.

As discussed above (Sect. 4.4), donor T cells infused with allogeneic stem cells attack leukaemia-associated polymorphic antigens in MM, indicating that tumour cells are susceptible to T-cell-mediated lysis: importantly, this indicates that polymorphic antigens are processed and presented at a suitable level and that this critical machinery is sufficiently intact in MM cells. As MM cells express MHC class I molecules, direct vaccination should include induction of $CD8^+$ CTLs, targeting antigenic peptides presented via MHC class I molecules. Vaccination to harness $CD4^+$ T cells will also be important, as these have been shown to mediate protection against myeloma by Id vaccination, able to attack myeloma cells by an indirect pathway (Sect. 4.2). These $CD4^+$ T cells secrete IFN-γ and engage macrophages to fight against myeloma. The role of $CD4^+$ cells as helper T cells is equally important for vaccination, if not more so in priming $CD8^+$ CTLs and antibody responses.

4.5.2 Immune Status at Disease Presentation

4.5.2.1 DCs

Antigen delivery by DNA vaccine will need to harness dendritic cells (DCs), either by cross-presentation following release from muscle depots that have been transfected, and serve as long-term source of antigen, or by direct transfection [72]. These activated APC will then play a central role in recruiting host immunity.

In MM however, DC dysfunction had been recognised early, posing a potential problem for vaccine delivery, but more recent investigations have shown that this can be rescued (reviewed by [73, 74]). Enumeration of CMRF4 + CD14-veCD19-ve DC numbers in PB in MM appeared normal, but these DCs exhibited stimulatory

defects as a reduced ability to upregulate CD80 expression following CD40 ligand + IL-2 stimuli, more so during progressive disease [75]. These effects were likely mediated by tumour-derived TGF-β1 and IL-10 and could be abrogated using neutralising antibodies to the two growth factors. High levels of β2 microglobulin, which correlate with tumour burden, negatively modulate monocyte-derived DC function by reducing co-stimulatory molecules and IL-12 production, with IL-12 and IFN-γ rescuing CD80 expression on DCs [73, 74]. Another aspect delineated by loading MM DCs directly with tumour lysates mitigated activation, but the dysfunction could be overcome by neutralising VEGF [76]. Comparable and additional effects were observed in the 5TGM1 murine model of MM, revealing that tumour cells directly compromise differentiation and function of bone marrow-derived DCs, but that this could be partially overcome by neutralising antibodies to IL-6, IL-10 and TGF-β and inhibition of the p38 MAPK signalling pathway [77]. Extending their observations in MM patients, these authors also found monocyte-derived DCs displaying functional defects, showing weak activation of alloreactive T cells, but treatment with anti-IL-6 neutralising antibody and p38 signal transduction inhibition again restored function [78]. The ability to overcome functional limitations, however, including use of in vitro manipulations, has led to harvesting CMRF44+ DCs from PB at clinical grade for therapy in MM [79], and a host of patient-derived DC vaccine strategies have been utilised in vaccination studies to deliver idiotype (Table 4.2), often revealing at least measurable responses to vaccination [74].

More recently, unexpected observations have revealed an additional complication associated with a role for DCs in MM. Remarkably, plasmacytoid DCs interactions directly augment MM cell growth [80]. DCs have also been shown to support the clonogenic growth of CD138-ve MM cells preferentially, with implications for the MSC as discussed ([13]; Sect. 4.1). The modulation of CD138 on MM plasma cells, by stromal cells and possibly by DCs, may also be pertinent to survival in the BM niche [81]. In a two-way interaction, MM cells can impart a strong dedifferentiating stimulus on DCs in their vicinity, leading to osteoclast (OC) formation from DCs [82]. These OCs may also be relevant as APCs, as native OCs have been reported as effective antigen-presenting cells in MM to stimulate both $CD4^+$ and $CD8^+$ T-cell responses, although their overall contribution to anti-tumour responses is as yet not defined [83]. With regard to DNA vaccination, however, uptake of antigen occurs from muscle sites and DCs home to local lymph nodes; this should minimise such bidirectional potential interactions with MM cells as these peripheral DCs are distant to the primary tumour site.

4.5.2.2 T-Cell Function

Both the circulating and BM compartments are accessible to evaluating T-cell role and function in monoclonal gammopathies (MGs). Circulating T cells represent a dynamic state of flux, with cells homing to organ sites and re-entering the periphery, and enumeration is likely to vary as a result. T-cell flux in the BM is less well defined and may differ.

Table 4.2 Antigens targeted by vaccines in multiple myeloma

Antigen	Type of therapy	Number	Results	Reference
Id	Immunised with *i.v.* autologous Id- or Id-KLH pulsed DCs + *s.c.* boosts of Id-KLH	26 patients post-autologous PBSCT	4/26 with Id-specific proliferative immune responses	Liso et al. [145]
Id	Immunised with $CD34^+$ stem cell-derived DCs pulsed with Id peptides, autologous M protein and GM-CSF	11 patients with advanced MM	3/10 Id-specific humoral immune responses 4/10 Id-specific T-cell responses	Titzer et al. [146]
Id	Immunised *s.c.* with Id-pulsed DCs	5 patients post-autologous PBSCT	4/5 Id-specific T-cell responses 5/5 Id-specific B-cell responses	Yi et al. [147]
Id	Immunised with autologous M protein with IL-12 alone or with GM-CSF	10 patients with stage I IgG MM – 6 evaluable by PCR for MRD	Reduction in blood tumour mass in 4/6 patients 3 of them showed an Id-specific T-cell response	Rasmussen et al. [148]
Id	Immunised with autologous Id-pulsed DC followed by Id-KLH + GM-CSF booster immunisation	12 MM patients post-autologous PBSCT	Id-specific immune response in 2/12 patients of which one showed CTL responses 2/12 patients in PR 25 and 29 months after the start of the treatment	Reichardt et al. [149]
mHag	Treatment of $HA\text{-}1^+$ or $HA\text{-}2^+$ patients after ASCT with DLI from $HA\text{-}1^-$ and/or $HA\text{-}2^-$ donors	3 patients (2 CML and 1 MM) after allogeneic SCT	HA-1 and HA-2-specific $CD8^+$ T cells emerged in the blood of the recipients 5–7 weeks after DLI (tetramer analysis) CR and restoration of 100% donor chimerism in 3/3 patients Cloned tetramer positive T cells inhibited the growth of leukaemic precursors cells in vitro (^{51}Cr-release assay)	Marijt et al. [150]
Id	Immunised with autologous Id-KLH with GM-CSF	15 patients post-autologous PBSCT	Immune responses that lasted almost 2 years including humoral responses Positive Id-specific DTH skin tests in 85% of tested patients	Coscia et al. [151]

(continued)

Table 4.2 (continued)

Antigen	Type of therapy	Number	Results	Reference
Id	Donors and recipients immunised with either Id mixed with GM-CSF or Id-KLH mixed prior to BMT. Recipients received booster Id immunisation post-BMT	5 IgG or IgA MM patients with at least a partial response	2/5 died within 30 days of BMT from transplant-related complications Id- and KLH-specific cellular and/or humoral immune responses induced in donors and 3 remaining recipients (persisted for 18 months) Conversion from PR to CR following BMT 2/3 disease-free 7 and 8 years after BMT 1/3 died of renal failure after 5.5 years while in CR	Neelapu et al. [152]
Sp17	Immunised with Sp17-pulsed DCs (phase I study)	One $Sp17^+$ MM patient after ASCT	Sp17-specific B-cell immunity (IgG) was induced (T-cell immunity not determined)	Dadabayev et al. [153]
Id	Immunised with a combination of Id-pulsed allogeneic DCs and Id-KLH	4 MM patients post-reduced-intensity conditioning ASCT	Strong anti-KLH but not anti-Id antibody response Secretion of Th1 cytokines 2 TR and 1 SD after stopping vaccination 3 patients ultimately progressed	Bendandi et al. [154]
Id	Immunised with the autologous Id protein together with GM-CSF and/or IL-12	18 patients with stage I–II MM	Id-specific T cells developed in 78% of IL-12/GM-CSF group and in 22% of IL-12 group No clinical response during the first 32 weeks of follow-up	Abdalla et al. [155]
Id	Immunised *i.d.* with autologous M protein combined with IL-12 ($n=15$) or with IL-12 and GM-CSF ($n=13$)	28 patients with IgG MM stages I–II	Id-specific immune response in 5/15 in IL-12 group and 11/13 in GM-CSF/IL-12 group Gradual increase of the immune response in 3/16 responders whereas 11/16 showed initial response which then disappeared Immune nonresponse associated with an increase of Tregs Median time to progression for immune responders was 108 weeks compared to 26 for nonresponders	Hansson et al. [96]

Id	Immunised with increasing doses of Id-loaded DCs x3 *s.c.* and ×2 *i.v.* at 2 weeks interval	15 patients who had previously received double ASCT	8/15 developed an Id-specific T-cell response, 8/15 increased IFN-γ-secreting T cells and 4/15 showed an Id-positive DTH test 7/15 patients had an SD after a median follow-up of 26 months, 1/15 achieved durable PR and 7 progressed	Abdalla et al. [156], Curti et al. [157]
Id	Immunised with the autologous Id protein together with GM-CSF and/or IL-12	10 patients with stage I–III IgG MM	Id-specific response detected in PBMCs of 5 patients and in BMMCs of 4 patients (3 patients with responses in PBMCs and BMMCs) Decrease with time of the frequency and magnitude of the responses	Abdalla et al. [156]
MAGE-A3	Syngeneic PBSCT after immunisation of the twin donor with MAGE-A3 protein formulated in AS02B	One patient with MAGE-A3-positive MM	MAGE-A3-specific antibody, cytotoxic T lymphocyte and T-helper responses in both twins Remains in remission 2.5 years after second transplant	Szmania et al. [158]
WT1	Immunised with weekly *i.d.* injections of peptide with Montanide ISA51 adjuvant for 12 weeks	One patient with chemotherapy-resistant MM	Decrease of the proportion of myeloma cells in the BM from 85 to 25% Decrease of M protein in the urine from 3.6 to 0.6 g/day	Tsuboi et al. [159]
Id	Immunised with Id-loaded DCs ×4 over 16 weeks following ASCT	27 patients: 11 relapsed off treatment, 10 in plateau and 6 primary refractory to initial chemotherapy	6/27 CR, 2/27 PR, 19/27 with SD 2-year increase of OS	Lacy et al. [160]

(continued)

Table 4.2 (continued)

Antigen	Type of therapy	Number	Results	Reference
RHAMM-R3 peptide	Immunised 4x at a biweekly interval with *s.c.* injections of peptide with IFA	9 patients (3 MM, 5 MDS and 1 AML)	Positive immunological responses in 4/9 patients (2 MDS and 2 MM) Increase of $CD8^{+}$RHAMM-R3tetramer^{+}/$CD45RA^{+}$/$CCR7^{-}$/$CD27^{-}$/$CD28^{-}$ effector T cells and of R3-specific $CD8^{+}$ T cells 3 patients (2 MDS and 1 MM) showed clinical effects	Greiner et al. [143]

Abbreviations: *AML* acute myeloid leukemia, *ASCT* autologous stem cell transplant, *BMMC* bone marrow mononuclear cells, *BMT* bone marrow transplant, *CML* chronic myeloid leukemia, *CR* complete response, *DCs* dendritic cells, *DLI* donor lymphocyte infusion, *DTH* delayed type hypersensitivity, *i.d.* intradermal, *Id* idiotype, *i.v.* intravenous, *IFA* incomplete Freund adjuvant, *KLH* keyhole limpet haemocyanin, *MDS* myelodysplastic syndrome, *MGUS* monoclonal gammopathy of undetermined significance, *mHag* minor histocompatibility antigens, *MM* multiple myeloma, *MRD* minimal residual disease, *OS* overall survival, *PBMC* peripheral blood mononuclear cells, *PBSCT* peripheral blood stem cell transplant, *PR* partial remission, *s.c.* subcutaneous, *SCT* stem cell transplantation, *SD* stable disease

Inverted ratio of CD4+ to CD8+ circulating T cells in MG, coupled with a state of activation and clonal expansions in both CD4+ and CD8+ T cells as defined by TCR usage, was reported early [73, 84]. A frequent observation has been that CD4+ and CD8+ T cells are CD28-ve [84, 85], with CD8+ CD57+ CD28-ve/perforin+ cells indicative of a cytotoxic phenotype [85]. Clonal expansions appear to correlate with low tumour mass and stable disease and remain stable over time until advanced disease. In the BM compartment, whole population studies appear limited, but no inversion of CD4+/CD8+ T cells has been observed [85a]. In fact, both the CD4+ CD8− and CD4−CD8+ T-cell subsets are significantly elevated in this environment in MGUS and MM, with a high proportion of CD28-veCD4+ and CD28-veCD8+ indicative of a memory and/or effector T-cell phenotype, and increased IFNγ production suggesting a role in immunosurveillance. These T cells also display significant expansions of one or more TCR-Vβ families in both CD4 and CD8 T cells, suggesting recruitment of cytotoxic T cells in BM at an early MGUS stage that persists to MM.

The question remains whether expanded numbers or clones of T cells actually reflect an active role in controlling tumour growth. At present, this is difficult to establish in the absence of advances in imaging in MG patients that can show direct killing of tumour cells *in vivo* by tagged T cells. A further and significant obstacle to T-cell activation and effector function is tolerance exerted by tumour cells, potentially ranging from central tolerance that deletes T cells from the available repertoire to peripheral tolerising effects where TAA is seen in the absence of co-stimulation, resulting in anergy. The question of a T-cell repertoire tolerised to tumour-derived antigens is perhaps best exemplified by investigations of the tumour-derived idiotype in MM. A large amount of secreted idiotype protein is problematic and can lead to deletion of CD4+ Id-specific T cells. This has been demonstrated by the Id (λ2315) specific TCR transgenic model, where myeloma protein exceeding 50 μg/ml led to deletion of thymocyte and circulating Id-specific T cells [85b], a cautionary issue for use of anti-idiotype DNA vaccines (discussed in Sect. 4.3). Nevertheless, as pointed by Bogen's group [85b], endogenous Id-specific T-cell responses have been detected in MM patients, and it is conceivable that a suitable Id-based vaccination protocol could harness dormant low-avidity T cells that have escaped tolerance to directly or indirectly mediate anti-tumour effects. Certainly, many of the Id-based vaccine trials (Table 4.2) show that the tolerising effects are not complete, as anti-idiotype T-cell responses are frequently observed and reveal a responsive repertoire.

Many observations have revealed an *ex vivo* cytotoxic potential of T cells in both the circulation and tumour beds against other important MM-associated antigens, as discussed in Sect. 4.4 (Table 4.1), indicating that tolerance against these specific antigens is also either absent or incomplete. As an example, T-cell immune responses to CTAs are informative. These were assessed using a cytokine secretion assay, and 19/53 MM and 2/25 MGUS patients exhibited a MAGE-A1/A2/A3-specific CD8+-T-cell response, and 1/32 MM and 4/30 MGUS patients showed a MAGE-A1/A2/A3-specific CD4+ T-cell response [86] (Table 4.1). CD8+ CTLs are dependent on CD4+ T cells, and in this study, the latter appeared to be more frequent in MGUS

than MM, and as suggested by the authors, efforts to sustain antigen-specific $CD4^+$ T-cell responses in asymptomatic disease may play an important role in preventing disease progression [86]. These $CD4^+$ T cells were cytolytic against target cells that processed and presented MAGE-A3 antigen. $CD8^+$ CTA-specific T cells displayed a $CD45RA^+$ $CCR7^{-ve}$ resting memory phenotype, suggesting absence of recent antigenic stimulation. A previous study from this group had measured the mean $CD8^+$ T-cell frequency of anti-CTA response as 0.02% that correlated with disease burden [87]. An increase in $CD8^+$ anti-CTA T-cell numbers in advancing MM disease indicated that other localised factors ameliorate anti-tumour control. There are also conflicting reports as to the extent to which T cells may be functionally impaired *in vivo* in MM [73].

Ex vivo data again shows that $CD8^+$ CTLs directed at CTAs, e.g. NY-ESO-1, are capable of lysing primary MM tumour cells [88], importantly revealing that antigen presentation via class I is at a sufficient level to mediate tumour lysis. For NY-ESO-1, pMHC levels have been specifically quantified in MM using a high-affinity TCR tag [89]. This type of analysis is important as a comparable tag to examine the HLA-A2-restricted hTERT(540–548) peptide showed that tumour cells do not display this epitope at an appropriate density, revealing that natural processing is inadequate to allow lysis by CTLs [90].

An important area to address in relation to active vaccination to induce anti-tumour effector T cells is the role of regulatory T cells (Tregs) in modulating therapeutic intervention. A number of Treg subtypes have now been described, the "naturally occurring" $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs (nTregs), generated in the thymus, and inducible forms that depend on localised immune responses, converting $CD4^+$ T cells to type I T-regulatory (Tr1) secreting IL-10 and Th3 regulatory cells producing TGF-β [91]. In addition, $CD8^+$ Tregs and double-negative Treg (DN Treg) cells have also been described. $FoxP3^+$ T cells can lose FoxP3 and suppressive activity, revealing a dynamic interconversion in specific cytokine environments [91a]. Although initially appreciated in suppressing reactivity to self-antigens, Tregs are now known to have potentially profound effects in the cancer setting. It has been shown that depleting $CD25^+$ Tregs can accentuate recognition of tumour antigens and augment vaccination [92, 93]. Treg interconversion and homeostasis as a product of tumour-mediated regulation of immune responses will clearly impact on vaccination strategies.

In MM, both a decrease in $FoxP3^+$ Treg populations [93a] and an increase [94, 95] have been observed, and more recently, a marked decrease in DN Tregs is reported in the circulation in MM, expanding the spectrum of defined Tregs in MGs [95]. The specificity and role of these Tregs in MGUS and MM are as yet, largely undefined, although a dysfunctional association has been described [93a]. $CD4^+$ Tregs can dampen responses to NY-ESO-1 (reviewed in [91]), impacting on vaccination directed against CTAs, suggesting concurrent depleting strategies for Tregs. Idiotype vaccination in MM can also lead to the amplification of $CD4^+$ $CD25^+$ Tregs, but this also associates with specific growth factor and cytokine use [96], further indicating a requirement to balance strategies to induce effective anti-tumour immunity. Interestingly, the use of immunomodulatory drugs (IMIDS) appears to inhibit

Treg expansion and FOXP3 expression on Treg, but without affecting survival and apoptosis [96a]; (Sect. 4.6).

4.5.3 *Immune Evasion and Immunosuppression*

Combining vaccination with strategies to overcome or block immune evasion and immunosuppression mediated by MM cells will need to be considered in relation to effective immunotherapy. As in other cancers, MM cells orchestrate a number of responses aimed at reducing anti-tumour immunity, either directly or indirectly by modulating stromal cells and the niche. The complexity of these events has been reviewed elsewhere [73, 97], and here, some key aspects are outlined. Immunosuppressive agents, including TGF-β, IL-10, IL-6, VEGF, β2-microglobulin and MUC-1 exert anti-maturation effects in DC function, influence Treg generation or lead to inadequate T- and B-cell responses. Cyclooxygenase-2 overexpression by MM cells is also implicated in suppressing macrophage- or T-cell-mediated tumour lysis. The role of indoleamine 2,3-dioxygenase (IDO) in MM as a direct immunosuppressive mechanism is less well clear, as our observations reveal that tumour cells express low levels of IDO; however, an indirect role for mesenchymal stem cells stimulated by IFN-γ that results in high levels of IDO leading to local tryptophan depletion appears relevant [97a].

4.6 Clinical Setting for DNA Vaccine Use in MM

A key issue is defining when vaccination is most likely to be optimal clinically, which raises questions of what effect therapy will have on immune capacity, on antigen modulation in tumour cells and on tolerance, immune evasion, and protective niche effects.

In advanced MM, increased numbers of $CD8^+$ T cells specific for CTAs can be observed, but seem to be of limited value in controlling tumour growth [86]. This indicates that a high disease load associates with a severe immunoparesis, which would make this situation less attractive for active immunotherapy directed at tumour cells. Furthermore, release of immunosuppressive cytokines, dendritic cell dysfunction, disruption of normal hematopoiesis and paraprotein load may all contribute to immunodeficiency in advanced myeloma. This indicates a role for vaccination at a stage in patients where induction therapy has achieved a pronounced response (very good partial remission = VGPR or better), with minimal numbers of myeloma cells persisting as a target.

Currently, induction regimens including new agents achieve a complete remission (CR) rate of > 20%, which increases to > 40% after additional autologous transplantation [98, 99]. Lenalidomide consolidation and/or maintenance therapy after autologous transplantation has the potential to increase the CR rate further to > 50%

and the rate of VGPR or better to nearly 90% [100]. Thus, the vast majority of myeloma patients fit a therapeutic approach, which includes autologous transplantation, and would be expected to reach a clinical status where active immunotherapy is feasible. In elderly patients, CR rates of 30% can be achieved with multidrug combination regimen including new agents, and VGPR or better in more than 40% [101, 102]. In this subset, the pronounced tumour reduction would provide a window of opportunity for vaccination.

Minimal residual disease (MRD) has been assessed by ASO-PCR after autologous and allogeneic transplantation in myeloma. In these early studies, PCR negativity was demonstrated in approx. 50% of patients in CR after allogeneic transplantation, while PCR negativity was rarely seen after autologous transplantation [103, 104]. PCR negativity seemed to be associated with a low rate of relapse and a favourable clinical course. Evidence for the clinical significance of MRD detection also comes from a Spanish trial [105], where an MRD negative status after autologous transplantation was associated with an improved outcome. In this trial, an MRD negative status was achieved in 42% of patients, with MRD here defined by flow cytometry, which is less sensitive than ASO-PCR techniques. Recently it was shown that consolidation therapy with bortezomib, thalidomide and dexamethasone (VTD) after autologous transplantation can further decrease the tumour burden by approx. 4 log and increase the rate of molecular remissions from 3 to 18% by ASO-PCR [106]. The latter figure probably adequately reflects the percentage of patients in which a PCR-grade molecular remission can be achieved by maximal therapy (excluding allogeneic transplantation) in the younger patient subset. Even in patients with a molecular remission, a consolidating vaccine strategy could be very useful, as it is known that these patients can experience relapse after a prolonged disease-free interval.

The question arises if a certain in vivo modulation of target achievable by drug therapy can be coupled with vaccine use. Downstream effector mechanisms induced by a CTL attack include caspase activation and mitochondrial outer membrane permeabilisation. High levels of expression of XIAP or dysfunctional mitochondrial apoptotic pathways – often observed in myeloma cells – can eventually induce resistance to a CTL attack. In a model using T cells engineered to express an immunoreceptor with binding specificity for high-molecular-weight melanoma-associated antigen, it was shown that bortezomib can sensitise melanoma cells for an antigen-specific T-cell attack by enhancing the mitochondrial apoptotic response [107]. Similar observations were made by other authors [108], although one group reported conflicting data, with bortezomib altering proteasomal processing and presentation of tumour antigens to render tumour cells less susceptible to a CTL attack [109].

Bortezomib mediates direct effects more widely on immune cells. Clinical observation of an increased frequency of viral infections during bortezomib treatment, especially herpes virus reactivations, necessitates antiviral prophylaxis during treatment with the proteasome inhibitor. The mechanisms of increased susceptibility to

viral infections during bortezomib treatment were further investigated in a mouse model [110], with data indicating an interference of this drug with priming of naïve T cells due to altered antigen processing.

Bortezomib can downmodulate DC activation [111]. In contrast, bortezomib treatment of MM cells, when compared with other therapeutic regimens (irradiation, chemotherapy), potentiated cross-presentation of antigens from dying tumour cells as a result of elevated surface hsp90, which acted as a "danger" signal to increase IFNγ producing T-cell responses [112], although the possibility that treatment disrupted DC-MM interactions could not be excluded. Interestingly, bortezomib in combination with IFNγ can reveal cryptic epitopes from a tumour antigen [113]. Bortezomib therapy has been shown to have a marked effect on diminishing $CD3^+/CD4^+$ T-cell counts in 41 of 53 patients (77%), from a median of 494/ml (range, 130–2,187/ml) to 274/ml (range, 41–1,404/ml), with minimal $CD4^+$ T-cell numbers reached at a median of 6 weeks (range, 2–22 weeks) posttreatment [114]. Importantly however, these nadir values recovered in 19 of 28 patients (68%) with a median time of 6 weeks, indicating that the bortezomib-mediated decrease is transient. In relation to the treatment arms in the study, no difference was apparent in nadir levels of $CD3^+/CD4^+/CD8^+$ or CD4:CD8 ratio between bortezomib alone, bortezomib/dexamethasone or bortezomib/chemotherapy [114]. These observations reveal a transient immunodeficiency, with timing of recovery of $CD4^+$ T cells important to any consideration of vaccine intervention.

IMIDs thalidomide and lenalidomide can augment the proliferative and cytokine response when T cells are stimulated through the T-cell receptor complex [115, 116]. Furthermore, for lenalidomide, an inhibitory effect on T-regulatory cells has been described, associated with decreased FOXP3 expression [117]. The IMIDs thus have the potential to enhance tumour-specific immunity, and there is a strong rationale for linking immunotherapy to treatment with either thalidomide or lenalidomide. The maintenance phase after successful induction therapy would thus appear as a favourable setting for vaccination, possibly coupled with IMIDs.

An alternative setting for active prophylactic immunotherapy would be the early-stage disease, MGUS or smouldering myeloma. Seminal observations have recently revealed that MGUS almost invariably precedes MM, based on retrospective analysis of M protein at a disease-free stage in long-term follow-up of large population cohorts of normal elderly individuals [118]. In these early stages, immune dysfunction is less prevalent. Also, antigen presentation of MGUS plasma cells may be more efficient than of myeloma plasma cells. A study showed that—in relation to MGUS—myeloma plasma cells are characterised by a lower expression of proteins linked to antigen presentation and that MGUS plasma cells are more efficiently lysed by cytotoxic T cells [119]. The authors concluded that plasma cells may elude immunosurveillance in the progression from MGUS to myeloma. As lenalidomide has been used successfully also in smouldering myeloma [120], a combination of lenalidomide and active immunotherapy could be tested in this early-disease phase.

4.7 Clinical Trials with DNA Vaccines

DNA vaccines have demonstrated a potentially broad usage against tumours based on preclinical models, with vaccine design shown to overcome tolerance even in a stringent setting, suggesting efficacy to counter effects of tumour (Sect. 4.3). The fusion of bacterial "alert" genes allows recruitment of CD4$^+$ T-cell help to aid CD8$^+$ T-cell activation and antibody response. In the 5 T murine models of MM, the DNA fusion gene vaccine design targeted tumour idiotype and demonstrated potency in tumour challenge experiments, as described in Sect. 4.3. These data allowed ethical and regulatory approval for a DNA vaccine trial against patient-specific idiotype in MM in our institution, an on-going phase I/II trial (Clinical lead, Prof. C. Ottensmeier, Cancer Sciences Unit).

To assess the efficacy of the pDOM-epitope vaccine design to generate antigen-specific CD8$^+$ CTLs, a preclinical strategy has been to test efficacy in the HLA-A2 transgenic murine model (HHD). Although restricted to clinical evaluation in A2 patients, it nevertheless allowed the first goal to be assessed, whether specific immune responses could be detected in a therapeutic setting, coupled with a safety evaluation. The first use of a pDOM-epitope DNA vaccine in a clinical phase I/II trial was to target prostate-specific membrane antigen (PSMA) in prostate cancer, coupled with electroporation (EP) as prime/boost to augment antigen presentation [45]. Most patients revealed anti-DOM antibody responses, improved by EP, as well as CD4$^+$ T-cell responses. Notably, CD8$^+$ T cells were detected against PSMA in ~60% of cases, at a higher level following EP [45]. This has demonstrated safety and immunogenicity. A second pDOM-epitope study in patients with carcinoembryonic antigen (CEA) expressing cancers targeted a CEA-derived HLA-A2-restricted epitope, with specific T-cell responses detected to CEA and DOM protein [121].

In relation to MM, we have been evaluating the pDOM-epitope DNA vaccine design for clinical application. For this, we selected PASD1 as target antigen in the HHD model [65]. We examined DNA vaccines to target full-length PASD1 and 2 HLA-A2 restricted epitopes and used human MM cell lines transfected with HHD MHC molecules as natural targets [122]. These studies revealed that a DNA vaccine encoding full-length PASD1 generated CTLs that lysed MM tumour cells effectively, revealing dominance of 1 epitope over the other examined. This indicates that parental MM cells processed and presented PASD1-derived epitopes via class I at a sufficient density to permit killing. These data support a clinical intervention with DNA vaccines against PASD1 in HLA-A2$^+$ MM, and a trial is currently under planning. The anti-PASD1 DNA vaccines are generic, with potential for use not only in MM but also in other histologically distinct tumours known to express this CTA.

Following phase I/II trials with DNA vaccines, the challenge now is to examine whether clinical benefit will result. For this, an extensive trial of a DNA vaccine to target WT1 in chronic myeloid leukaemia has been approved, with control arms, and is currently recruiting in our institution (lead: Prof. Christian Ottensmeier). The primary end point of this randomised trial is control of disease. Clinical efficacy of

vaccination in a therapeutic setting is now clearly feasible, as recently demonstrated by the dendritic cell-based vaccine (Provenge) to yield a significant survival advantage in prostate cancer after phase III trials, and leading to FDA approval [123, 124].

More recently, attention is focusing on use of vaccination in a prophylactic setting in MM, at a stage of asymptomatic disease to prevent progression to MM. Remarkably, specific T-cell responses against the embryonal antigen SOX2 in asymptomatic plasma cell tumours correlate strongly with a reduced risk of progression to MM [14]. A number of CTAs are also expressed in the asymptomatic stage of disease, including MGUS, and these, together with SOX2, could be potential prophylactic targets. The expectation would be that DNA vaccines will function optimally in a prophylactic setting, as a relatively intact immune system is armed in the absence of more severe tumour-associated tolerising effects and immunosuppression in advanced disease.

4.8 Concluding Remarks

Several strands of investigation are improving the prospects for successful vaccination in MM. Notably new drug therapies in combination with transplantation are achieving almost universal remission in this tumour, which can be durable. There is considerable interest in how disease can re-emerge in this setting, and the MSC has recently emerged as a potential "feeder" cell. The balance of evidence, however, suggests that the "feeder" cell is most likely to be a malignant plasma cell, or P-MSC, and that therapy should target this cell. This stage of residual disease is also generally considered optimal for vaccination to induce immunity against tumour cells that have persisted. The immune status following therapy, although under assessment in MM, suggests challenges as immunomodulation is evident. A fuller evaluation of immune dysfunction effects will be important to inform vaccination; to enable counteractive measures, many of which have already been reported. Many relevant TAAs certainly persist post-therapy, and these can be targeted. Specific antigens are also being defined for EDLI in allo-transplantation protocols in a parallel option for vaccination. There is also the recent perception that prophylactic vaccination may now be eminently feasible. DNA vaccines provide a highly versatile strategy for vaccination in MM, with possibilities for altering design to engage specific immune pathways, and demonstrated potential for overcoming tolerance. They become especially relevant as a prophylactic tool in asymptomatic disease in MM. Current DNA vaccine clinical trials are progressing with optimism, with the aim of reaching phase III where progression-free survival can be assessed, and this will be the goal in MM. There is light at the end of the vaccination tunnel.

Acknowledgement This work was funded by Leukaemia and Lymphoma Research (UK) and an EU FP6 Grant EU FP6 Grant LSHC-CT-2006-037602 (MSCNET).

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Chapter 5
Harnessing Allogeneic Immunity for Anti-myeloma Response

Roberto Bellucci and Edwin P. Alyea

5.1 Introduction

Multiple myeloma (MM) is a clonal B-cell neoplasm of terminally differentiated B cells. Conventional chemotherapy such as melphalan and prednisone or combination therapy of vincristine, doxorubicin, and dexamethasone (VAD), which have been used for few decades, resulted in a median overall survival of only 2–3 years [1–3]. These results lead many groups to explore high-dose chemotherapy followed by autologous bone marrow transplant (auto-SCT). This treatment approach resulted in a better survival rate with a 5-year probability of event-free survival of 28% compared to 10% of the standard chemotherapy treatments [4–6]. In the last decade, the new understanding of the pathology of this disease and new treatments such as thalidomide, bortezomib, and lenalidomide with or without auto-SCT significantly increased the survival of many myeloma patients [7–11]. However, despite all these new developments in the past decade, MM remains still an incurable disease.

Allogeneic stem cell transplantation (allo-SCT) is the only potential curative treatment for MM; although part of the benefits of allo-SCT are provided by the eradication of myeloma cells after administration of high-dose chemotherapy and radiation, several studies have shown that donor immune cells are also involved in the curative effects of allo-SCT. This has been termed graft-versus-myeloma effect (GVM) [12–14]. The clearest evidence for GVM is provided by the example of donor lymphocyte infusions (DLI), where some patients can achieve complete responses (CR) after DLI in the absence of any other therapy [15–17]. Unfortunately,

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N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 2: Translational and Clinical Research*,
DOI 10.1007/978-1-4614-5260-7_5, © Springer Science+Business Media New York 2013

these potential curative effects are associated with a significant transplant-related mortality (TRM) mostly related to graft-versus-host disease (GVHD) and posttransplant relapses which limit the overall success of this treatment [12, 18]. In the past decade, in the attempt to reduce TRM while maintaining the GVM benefits, several groups have explored a reduced-intensity conditioning strategy followed by allo-SCT alone or in combination with auto-SCT. New generation drugs have been also tested in combination with auto-SCT or allo-SCT. These new approaches are opening the door for new strategies aimed at reducing toxicity and enhancing the allogeneic immune response.

In this chapter we will review the role of the allogeneic transplantation in patient with multiple myeloma and how current clinical and laboratory strategies are being developed to enhance the effectiveness of allogeneic immune responses in eliminating myeloma cells in vivo.

5.2 Myeloablative Conditioning and Allo-SCT

Myeloablative conditioning and allo-SCT in MM have shown a potential curative effect for some patients. Over the last 2 decades, several large studies have been performed [12–15, 18, 19]. The largest study was performed by the European Bone Marrow Transplant Registry (EBMTR). The EBMTR study involved more than 40 centers with 162 patients treated. The majority of patients received high-dose chemotherapy consisting of cyclophosphamide or melphalan with total body irradiation (TBI). GVHD prophylaxis consisted in cyclosporine and methotrexate in 48% patients. T-cell depletion, alone or in combination with other agents, was used in 33% of patients. The TRM in this study was 45% mostly due to infection, interstitial pneumonitis, and GVHD. This study reported a CR rate of 43% and an overall actuarial survival rate of 32% and 28% at 4 and 7 years, respectively [13, 18]. Other large single-center studies reported similar results. Investigators at the Fred Hutchinson Cancer Center treated 80 patients with 60 patients receiving marrow from HLA identical sibling donor and 20 from mismatched or unrelated donors. Fifty-seven patients were treated with cyclophosphamide and busulfan and 23 with cyclophosphamide and TBI, while GVHD prophylaxis consisted in cyclosporine plus methotrexate or prednisone. This study reported a CR rate of 36% and a progression-free survival rate of 20% at 4.5 years, but similar to the EBMTR study, the TRM was 49% [12]. In an attempt to reduce toxicity related to GVHD, investigators at Dana-Farber Cancer Institute treated 66 patients with myeloablative conditioning and T-cell-depleted bone marrow transplant with the T-cell depletion used as the only form of GVHD prophylaxis. Although T-cell depletion was very effective in lowering the GVHD rate (17% grade II or greater), the non-relapse mortality was 24% due to other complication such as increased incidence of infection. The study reported a 22% CR rate and 60% PR with an overall and progression-free survival of 39 and 23%, respectively, at 4 years after transplantation. [15, 20].

Overall these studies demonstrated similar results with a benefit for some patients (20–30%) who remained disease-free at 7 years after transplant with an apparent

plateau in the relapse-free survival curves. Unfortunately this treatment was also characterized by an excessively high toxicity with a TRM of 40–50% mostly due to GVHD, infections, and regimen-related toxicities. Interestingly, a retrospective analysis performed by the EBMT comparing 334 patients who received myeloablative allo-SCT between 1983 and 1993 with 356 patients who received allo-SCT between 1994 and 1998 showed a significant reduction in the overall TRM in the latter period with a TRM of 30% compared to 46% of the patients treated between 1983 and 1994 [21]. Although this marked reduction, a TRM of 30% is still high, and new strategies to reduce toxicity while improving the GVM effects are needed.

5.3 Autologous Versus Allogeneic Immunity in Myeloma

The effectiveness of allogeneic immunity against myeloma has been investigated in several studies that compared the overall outcome of autologous and allogeneic transplant. A retrospective analysis of the EBMT compared 189 patients who underwent allo-SCT with a similar cohort who received auto-SCT. The two groups received similar myeloablative therapy before transplant; the only significant differences were the median age (43 allo-group vs. 49 in the auto-group) and the follow-up (46 months in the allo-group vs. 30 months in the auto-group) [22]. The analysis showed that while patients in the two groups had a similar CR rate (48% in the allo-group vs. 40% in the auto-group), the TRM was significantly higher in the allo-group, resulting in a higher median survival in the autologous transplant group. There was a lower relapse rate in the allo-group compared with the auto-group at 4 years (50% vs. 70%) and a tendency for a better long-term survival in the allo-group. The high TRM reported in patients who received allo-SCT leads several groups to explore T-cell depletion of the allo-graft. In a single-center study performed at Dana-Farber Cancer Institute, investigators compared the outcome of 166 patients, who received autologous transplant, with 66 patients who received T-cell-depleted allo-SCT. The TRM was lower than the EMBT study (24% vs. 41%, respectively) but still significantly higher than the patients who received autologous transplant (24% vs. 16%), resulting in a similar overall and progression-free survival in the 2 groups. Similar to the EMBT analysis, the relapse rate was significantly higher in the auto-group compared with allo-group (66% vs. 46%, respectively) at 5 years and the prospect of a long-term outcome were better in the allo-group [20]. Additional evidence of the effectiveness of allogeneic immunity against myeloma was also highlighted by two other studies conducted by Martinelli and Corradini. In these studies, investigators monitored minimal residual disease in patients who received autologous and allogeneic stem cell transplant using a highly sensitive PCR analysis. These studies demonstrated that molecular complete responses, which were achieved in larger proportion in patients who underwent allo-SCT, were also associated with prolonged relapse-free survival [14, 19, 23]. In a follow-up study, Corradini et al. analyzed a larger cohort of patients (n = 48) who received allo-SCT. Sixteen (33%) achieved durable PCR negative molecular remission, while 13 (27%) remained PCR positive and 19 (40%) showed a mixed pattern.

The study confirmed that the risk of relapse at 5 years was 0% for patients who achieved molecular remission, while 100% and 33% of patients who remained PCR positive or had mixed positive and negative results relapsed [24]. Finally, a prospective analysis of the US intergroup trial (S9321), which compared high-dose therapy and auto-SCT with standard dose therapy, also included an arm where patients could be treated with an allo-SCT. The TRM in the allo-group was even higher than other previous studies (53%) probably due to the high TBI dose used in this trial. However, the comparison between the autologous and allo-SCT outcomes, showed that the progression-free survival at 7 years was higher in the allo-SCT group compared to the auto-group (22% vs. 15%), and while the risk of relapse still continued in the auto-SCT group, patients treated with allo-SCT reached a plateau with a follow-up at 10 years [25].

Overall all these studies support the evidence of the potential curative GVM effect in the allo-SCT setting; however, the potential benefits are limited by the extremely high toxicity associated with this treatment. In the past decade, several groups have been exploring reduced-intensity conditioning transplantation with the aim to reduce treatment toxicities while maintaining the allogeneic anti-myeloma activity.

5.4 Reduced-Intensity Conditioning (RIC) Allo-SCT

Many studies have demonstrated that non-myeloablative regimens could facilitate engraftment of hematopoietic cells with a marked reduction in toxicity and early mortality when compared to ablative conditionings [26–29]. Given these encouraging results and the clear benefit that the GVM effect plays in the allo-SCT, different groups have been exploring RIC and allo-SCT in MM. Badros et al. demonstrated the safety and efficacy of this approach with 25 out of 31 patients achieving complete donor hematopoiesis at 30 days after transplant and an excellent tumor response in 61% of patients treated. However, 18 patients still developed acute and chronic GVHD [30, 31]. Further follow-up studies, using different RIC, confirmed the lower incidence of TRM compared with the ablative regimens and an achievement of CR in approximately 50% of the patients treated. Acute and chronic still impacted in approximately 30% and 50% of the patients with some association of chronic GVHD and better responses [32–34]. T-cell depletion showed an improved incidence of GVHD but was also associated with poor responses, suggesting that an excessive immunosuppressive approach combined with a reduced-intensity regimen would affect immune responses even when a DLI was used to restore the GVM effect [35]. Follow-up analysis of these trials demonstrated that low tumor burden, chemosensitive disease, and no prior auto-SCT were the prognostic factors associated with long-term durable responses. Heavily pretreated patients and patients with progressive disease did not benefit from this approach [36–38].

Considering prognostic factors such as low tumor burden, several groups also investigated the combination of auto-SCT and reduced-intensity allo-SCT with the aim to reduce tumor burden with a myeloablative auto-SCT followed by an induction

Table 5.1 Tandem auto-SCT versus auto-/allo-SCT

No of patients (auto/auto vs. auto/allo)	CR (%)	EFS (Mo)	OS (Mo)	References
166 vs. 46	32.5 vs. 32.6	35 vs. 31.7	47.2 vs. 352	Garban et al. [43]
85 vs. 25	11 vs. 40	26 vs. 19.6	58 vs. NR	Rosinol et al. [45]
80 vs. 82	26 vs. 55	36 vs. 29	54 vs. 80	Bruno et al. [46]
436 vs. 189	24 vs. 24	46 vs. 43 (%)	80 vs. 77 (%)	Pasquini et al. [48]

CR complete remission, *EFS* event-free survival, *OS* overall survival, *NR* not reached

of allogeneic GVM with the allo-SCT [39–42]. Recent updates from the 2 largest studies from Italy and Seattle showed encouraging results with a CR rate of 53 and 62%, respectively [39, 42]. The median follow-up at 5 years showed an event-free survival of 37 and 36 months, respectively, with a median overall survival not reached in both studies. Long-term disease control and GVHD, which impacted in 38% and 42% (acute) and 74% (chronic), respectively, still remain key issues, and a longer follow-up will be needed to evaluate the efficacy of this auto/allo combination approach.

5.4.1 Comparative Studies of Tandem Auto-SCT Versus Auto-/Allo-SCT

Studies comparing the outcomes of tandem auto-SCT with auto-SCT followed by RIC allo-SCT have been also performed with differential results probably due to the differences in the conditioning regimens and inclusion criteria (Table 5.1). In a French study conducted by Garban and colleagues, authors compared 166 patients who underwent tandem auto-SCT (IFM99-04 trial) with 46 patients who received auto-SCT followed by allo-SCT (IFM99-03 trial). There was no difference in overall survival and event-free survival in the 2 groups with a trend of better overall survival in patients who received double auto when authors compared only patients who completed the entire treatment [43]. There were some concerns regarding the high dose of ATG used in this study that could have affected the GVM effect in the allo-group [44]. The Spanish PETHEMA study also compared 85 patients who received tandem auto-SCT with 25 patients treated with auto-/allo-SCT. Although authors observed a trend for a better progress-free survival in the allo-group, there were no differences in terms of overall survival and event-free survival [45]. In the Italian study, published by Bruno and colleagues, 82 patients who received auto/allo were compared with 80 patients who underwent double auto. Patients treated with auto-/allo-SCT showed a better CR rate (55% vs. 26%), progression-free survival (36 vs. 29 months) and overall survival (80 vs. 54 months) with a TRM of only 11% [46]. Issues regarding this study included the total number of patients who completed the entire treatment and the poor outcome in the double auto-group [47]. Finally, a recent study from the US Blood and Marrow Transplant Clinical Trial Network (BMT

CTN) reported the results of a multicenter phase III trial where the outcome of 625 MM patients treated with tandem auto-SCT or auto-/allo-SCT was compared at 3 years follow-up. In this study, 436 patients were assigned to the tandem auto-SCT using 200 mg/m2 melphalan conditioning, while 189 patients received auto-SCT with 200 mg/m2 melphalan followed by allo-SCT with 2 Gy TBI. The GVHD prophylaxis was cyclosporine and mycophenolate mofetil. Complete and near-complete response rate at the study entry were equal in both groups (24%). Three years follow-up showed no differences between the 2 groups with a PFS of 46% and 43% and OS of 80% and 77% in the tandem auto-SCT and auto-/allo-SCT, respectively. Although further follow-up will be necessary to make final conclusions, even in this study, the tendency was that the potential benefits of GVM activity to reduce disease progression or relapse, in the group of patients who received auto-/allo-SCT, were balanced by the increased TRM compared with the tandem auto-SCT [48].

5.5 Treatment of Relapse After Allo-SCT

5.5.1 Donor Lymphocyte Infusion (DLI)

The effectiveness of GVM is demonstrated most convincingly by the clinical responses achieved after infusion of donor lymphocytes in patients with relapsed myeloma after allo-SCT. The clinical observation that DLI, in the absence of any other therapy or radiation, is able to induce CR demonstrates that immune effector cells derived from the donor are responsible for generating potent allogeneic antitumor immune responses. Early studies by Tricot and Verdonck described individual cases of relapsed myeloma patients after T-cell-depleted bone marrow transplant that achieved CR after the infusion of DLI, in both studies patients developed GVHD after DLI [49, 50]. The 2 largest studies were conducted by Salama and Lokhorst with 25 and 54 patients treated, respectively. Salama and colleagues reported a CR rate of 28% with all responding patients developing GVHD. Although the dose of DLI infused to patients varied significantly, responses were primarily noted in patients who received the higher DLI doses [51]. In the study conducted by Lokhorst et al., 19 out of 54 patients (35%) showed a PR and 9 out 54 (17%) achieved a CR. Interestingly, acute and chronic GVHD, developed in 57% and 47% of patients, respectively, were the strongest predictors for response [52]. These and other smaller clinical studies have demonstrated the efficacy of DLI in relapsed MM patients after allo-SCT with a response rate of approximately 50% and a CR rate ranging between 20 and 30% [17, 51, 53–55]. In contrast to other hematological malignancies such as chronic myeloid leukemia (CML) [56–58], long-lasting CRs were only achieved by less than 20% of patients. In a study conducted at Dana-Farber Cancer Institute, investigators used DLI as a prophylaxis to enhance the GVM effect after T-cell-depleted bone marrow transplant [15]. Twenty-four patients were enrolled in this study, but only 14 patients were able to receive DLI at 6 months after transplant. Eleven of these 14 patients had still evidence of disease after transplant, but prophylactic DLI induced a significant GVM response in 10 of

them with 6 patients achieving CR and 4 PR. Although this strong induction of responses after prophylactic DLI, one limitation was caused by the toxicity after myeloablative conditioning with only 14 out of 24 patients being able to receive DLI. The efficacy of different doses of DLI was also explored in MM patients who received RIC followed by allo-SCT. Ayuk and colleagues studied 21 patients treated in 5 different centers with RIC regimens based of melphalan and fludarabine followed by allo-SCT from related or unrelated donors. DLIs were infused only if no signs of GVHD were present, and patients were in progression or relapse after transplant or with more than 10% plasma cells in the bone marrow. Initial doses of DLI were 1x106 and 5x106 CD3+ cells/kg for related and unrelated transplantation, respectively, and if no responses and GVHD were noted, a second dose of 5x106 and 1x107 was infused after 3 months. A further log increase in the DLI doses was recommended if no response or GVHD was noted after the second dose. The incidence of acute and chronic GVHD was low, and responses were only observed after the first (29%) and the second DLIs (22%) with no further responses after the third and the fourth DLI suggesting that remissions after RIC allo-SCT could be achieved with relatively low numbers of infused T cells [53].

5.5.2 *Target Therapy*

Although allo-SCT has shown a high rate of responses, its potential curative effect for patients with MM still remain limited with many patients only achieving a PR, while in other cases, even after achieving a CR, patients experience early relapses. For these reasons many investigators have attempted to develop strategies aimed to direct the allogeneic immune response toward GVM effects without increasing GVHD.

5.5.2.1 Immunoglobulin Idiotype (id)

Given their restricted tumor expression, id could be an ideal target for targeted immunotherapies. Several groups have explored their role as a myeloma-specific antigen, Massaia and colleagues showed that specific T-cell responses could be generated after high-dose chemotherapy or auto-SCT [59]. In another study, donor immunization with patient-specific id showed specific T-cell responses in MM patients after allo-SCT [60]. Follow-up of this study showed prolonged disease-free survival in 2 out of 5 patients for 5 and 7 years after allo-SCT. Also in this case, T cells against patient-specific id were present in all patients for several months after transplant [61].

5.5.2.2 Cancer Testis Antigens (Mage, NY-ESO-1, Muc-1)

Other potential targets of immune responses are cancer testis (CT) antigens. This class of antigens is a group of proteins expressed in the human germ line tissue. Since their restricted expression, they have not been exposed to the immune system

and therefore more immunogenic than broadly expressed antigens. Early in 1999, Van Baren and colleagues showed that several CT antigens in the MAGE family were highly expressed in myeloma cells. Interestingly, expression of at least one of the MAGE genes was restricted to all samples from patients with stage III myeloma, while none of the MGUS or stage I and II myelomas were positive [62]. These evidences were also confirmed from another study by Jungbluth et al., where authors showed that messenger RNA for CT7 and MAGE-A family members could be detected in 87% and 100% of stage III myeloma, and their expression correlated with elevated plasma cell proliferation [63]. Later in 2007, Atanackovic et al. studied whether these promising antigens could also be target of GVM activity. Authors analyzed the expression of 11 CT antigens in bone marrow samples from 55 MM patients and 32 healthy donors. As shown in previous studies, these antigens were frequently expressed in MM and, importantly, strong antibody responses against MAGEA3, SSX2, and NY-ESO-1 were found predominantly in patients who received allo-SCT. The antibody response against NY-ESO-1 also correlated with NY-ESO-1-specific CD4+ and CD8+ T-cell responses in 1 of these patients. Importantly, these allogeneic immune responses were not present in pre-transplant and donor samples analyzed, suggesting that immune responses against some of these CT antigens are associated with the GVM response and they could be ideal targets for antigen-specific immunotherapies associated with stem cell transplant [64]. This strategy has been shown to be feasible by a report where clinical investigators transplanted stem cells from a donor previously immunized with MAGE-A3 protein to her identical twin diagnosed with MM and followed by further immunization in the patient. Strong MAGE-A3 antibody and T-cell responses could be detected in both donor and recipient with CTLs specific for a previously unknown MAGE-A3 epitope lasting for more than a year after the last immunization [65]. Another potential tumor-associated antigen is MUC-1, several groups found this antigen to be highly expressed on malignant plasma cells, and they were able to isolate MUC-1-specific T-cell lines from the bone marrow of myeloma patients [66, 67]. Vaccination studies targeting these antigens have been described for other diseases such as breast and ovarian cancer [68, 69], and recently, Kapp et al. described immune responses against MUC-1 in patients with acute myeloid leukemia (AML), MM, and acute lymphoid leukemia (ALL) after allo-SCT. Patients with T-cell responses after stem cell transplant showed a significantly better clinical outcome, suggesting that T-cell responses against MUC-1 or other tumor-associated antigens can significantly contribute to GVL/GVM effects after transplant [70].

5.5.2.3 B-cell Targets

Although T cells play a central role in anti tumor immunity, several studies in animal models have shown coordinated B- and T-cell responses in tumor rejection [71, 72]. These evidences have been also confirmed in human studies where T-cell responses against NY-ESO-1 or other defined antigens also induced concurrent strong humoral responses against the same antigens [73, 74]. Polyclonal B cells and

T cells have been also found in histological examination of vaccination sites of patients who responded to tumor vaccines [75, 76]. In our laboratory, we extensively studied the immune reconstitution of MM patients who received T-cell-depleted allo-SCT followed by CD4+ prophylactic DLI, and we showed that after DLI, patients had an enhanced and significantly faster immune reconstitution when compared to patients who received the same T-cell-depleted bone marrow transplant but without DLI [77]. One of the clearest differences, in these patients who received DLI, was the significant increase in the number of polyclonal CD20+ B cells. The increase was evident at 3 months after DLI (9 months after allo-SCT) and persisted for more than 1 year after DLI. The GVM responses, induced by prophylactic infusions of CD4+ DLI [15], led us to investigate whether the expansion of B cells at the time of CR after DLI reflected a strong antibody response directed against MM-associated antigens. Serum, at 2 different time points after DLI from 4 MM patients who had a CR after DLI, was used to screen a cDNA expression library derived from CD138+ bone marrow cells from a patient with MM. As shown in Table 5.2, using this molecular approach, we identified 13 gene products that were specifically reacting with patient serum post-DLI but not with the pre-transplant or pre-DLI serum from any of these patients. Importantly, none of these antigens were recognized by serum from 20 healthy donors, 5 and 20 patients with acute and chronic GVHD, respectively, or patients who did not respond to DLI. Some of these antigens, such as BCMA, PDC-E2, ROCK1, and Homer 3, were reactive with serum from several MM DLI responders and were also found to be highly expressed in primary MM cells as well as MM cell lines [78]. Most of these identified proteins have not been previously described as potential targets of humoral responses. However, antibodies specific for some of these proteins, as for example, ROCK1, have been also found in patients with breast cancer, renal cancer, and fibrosarcoma, suggesting that some of these antigens, targeted by high-titer antibodies after DLI, can also be immunogenic in other tumors [79, 80]. In most of the studies, antibody responses have been found to target intracellular proteins, and the mechanisms whereby these antibodies contribute to tumor immunity are still debated. Several evidences have shown that these antibodies can facilitate presentation of the antigens to dendritic cells increasing T-cell responses to peptides presented by the major histocompatibility complex molecules [81]. Interestingly, one of the 13 antigens identified in our studies was a transmembrane protein highly expressed on B cells as well as MM cells (BCMA) [82–84]. These antibodies, found in 2 MM patients who achieved CR after DLI, were directed against the extracellular domain of the protein, and we showed that they were able to mediate complement-mediated lysis and antibody-dependent cellular cytotoxicity (ADCC) of BCMA-positive cell lines as well as primary myeloma tumor cells. These antibodies were presumably derived from donor B cells since these patients converted to full donor chimerism after DLI [77], suggesting that antibody responses after allo-SCT can also contribute directly to the elimination of myeloma cells in vivo [85].

Another protein, known to be highly immunogenic in autoimmunity and that we found to induce high-titer antibody response in MM, was dihydrolipoamide acetyltransferase (PDC-E2). PDC-E2 is the E2 component of the multienzyme pyruvate dehydrogenase complex (PDC) [86, 87], and in addition to its role in cell

Table 5.2 Identified target antigens tested with different sera from healthy donors, patients post-BMT and post-DLI

Gene products	Normal donors	Acute GVHD	Chronic GVHD	TCD BMT	Myeloma DLI responders	Myeloma DLI nonresponders	CML DLI responders
PDC-E2	0/20	0/5	0/20	0/10	2/9	0/5	1/5
KIAA0053	0/20	0/5	1/20	0/10	1/9	0/5	0/5
BCMA	0/20	0/5	0/20	0/10	2/9	0/5	0/5
FLJ10330	0/20	0/5	0/20	0/10	2/9	0/5	0/5
ROCK-1	0/20	0/5	0/20	0/10	4/9	0/5	0/5
Similar to hepatoma-derived growth factor	0/20	0/5	0/20	0/10	1/9	0/5	0/5
Homer-3	0/20	0/5	1/20	0/10	2/9	0/5	0/5
Bax-interacting factor 1 (Bif-1)	0/20	0/5	0/20	0/10	1/9	0/5	0/5
Heterog. nuclear ribonucleoprotein D-like	0/20	0/5	0/20	0/10	1/9	0/5	0/5
SON DNA	0/20	0/5	0/20	0/10	1/9	0/5	0/5
FLJ10534	0/20	0/5	0/20	0/10	1/9	0/5	0/5
SFRS	0/20	0/5	0/20	0/10	1/9	0/5	0/5
AT-rich sequence binding protein	1/20	0/5	1/20	0/10	1/9	0/5	0/5

Table 5.3 Combination of allo-SCT with new generation drugs

Drug	No of patients	CR (%)	GVHD (%)	References
Thalidomide	18	22	11	Kroger et al. [94]
Lenalidomide	13	23	38	Minnema et al. [99]
	24	8	12.5	Lioznov et al. [100]
Bortezomib	18	30	22	Kroger et al. [104]
	37	13.5	5.4	El-Cheikh et al. [103]

CR complete remission

metabolism, it represents the main target of antimitochondrial autoantibodies present in up to 95% of patients with primary biliary cirrhosis (PBC), an autoimmune disease of the liver [88]. Our studies showed that distinct patient populations such as MM and CML who received allo-SCT and DLI developed strong antibody responses to PDC-E2 after DLI. Interestingly, the anti-PDC-E2 antibodies, developed in patients after DLI, were only targeting epitopes located in the catalytic domain of PDC-E2 rather than the commonly targeted inner lipoyl domain of PDC-E2 described for PBC patients [89, 90]. MM patients did not have any sign of PBC and considering the described overexpression of PDC-E2 within these tumor cells and the temporal association of antibody production with DLI, this immune response appears to be more associated with tumor rejection rather than the development of autoimmune disease [91].

5.5.3 Potential Combinations with New Immunomodulatory Drugs

Several new generation drugs such as thalidomide, lenalidomide, and bortezomib have shown potential immunomodulatory effects on T and NK cells [92, 93]. These evidences have prompt several groups to investigate their combinations with allo-SCT (Table 5.3).

5.5.3.1 Thalidomide

Kroger and colleagues have used low doses (100 mg) of thalidomide to enhance the GVM effect of DLI after allo-SCT in 18 MM patients with progressive disease. The study showed an overall survival of 67% with 22% of patients achieving CR. Importantly, no grade II/IV of acute GVHD was reported, and only 11% of patients reported de novo chronic GVHD, suggesting that the combination of low doses of thalidomide and DLI can induce a strong GVM effect with very low incidence of GVHD [94]. Thalidomide has been also used at higher doses (median 200 mg range 50–600) as salvage therapy in 31 MM patients in disease progression after allo-SCT. Although the treatment was discontinued in 6 patients (19%) for high toxicity, 29%

of the patients achieved a PR or very good PR with only 5 patients developing mild GVHD after thalidomide treatment, showing that thalidomide can be potentially effective also in these patients who fail allo-SCT [95].

5.5.3.2 Lenalidomide

Lenalidomide is another immunomodulatory drug [96] that has been successfully used in newly diagnosed as well as relapsed and refractory MM patients, and several studies have investigated its use in combination with allo-SCT. In a recent study Minnema et al. showed the efficacy of lenalidomide alone or in combination with dexamethasone in patients with MM who failed allo-SCT. Twenty-three percent of the treated patients achieved CR. Five out of 13 patients treated only with lenalidomide still developed GVHD, but none of the patients where dexamethasone was added to lenalidomide showed GVHD. Interestingly, in 7 out of 8 patients who received lenalidomide, there was a significant expansion of CD4+ Foxp3+ T regulatory cells (T-regs). T-regs have been found to be associated with less GVHD in allo-SCT [97, 98]; however, probably due to the small number of patients, authors did not find any correlation between T-reg numbers, GVHD, and clinical responses [99]. Recently, another study reported the treatment with lenalidomide of 24 heavily pretreated patients who relapsed after allo-SCT. Authors reported a response rate of 66% with 8% of patients achieving CR, and also in this study, the treatment with lenalidomide was associated with an increased number of T-reg cells (CD4+, CD25+, CD127 lo) and activated NK cells (NKp44+) as well as T cells (HLA-DR+), confirming the immunomodulatory effects of lenalidomide [100].

5.5.3.3 Bortezomib

Several preclinical studies have also shown the ability of the proteasome inhibitor bortezomib to prevent GVHD while maintaining the GVT effect, [101, 102] and recently, bortezomib has been used as salvage therapy in 37 patients with MM who relapsed after RIC allo-SCT. Authors reported an objective response of 73% with an estimated overall survival at 18 months of 65%, which was significantly higher in patients achieving an objective response [103]. In another study, clinical investigators studied the effects of bortezomib in MM patients after RIC allo-SCT. Eighteen patients without progressive disease were treated at a median time of 8 months after allo-SCT with 2 cycles of bortezomib (1.3 mg/m2) to enhance or maintain remission status. All patients were evaluated for toxicity, CD3+ cells, GVHD, and responses. While 14 patients (78%) completed the 2 cycles of bortezomib, 4 patients discontinued the therapy for neurotoxicity or gastrointestinal toxicity. Fifty percent of patients had thrombocytopenia, 17% leucopenia, and 17% neuropathy, while the median number of circulating CD3+ cells significantly decreased from 550 μl to 438 μl resulting in herpes zoster infection in 3 patients. Three patients showed a mild increase of existing acute and chronic GVHD of the skin, while 1 patient developed de novo skin grade I acute GVHD. Although CR, PR, and minor response were achieved by 30%, 50%, and 20% of patients, respectively, some level of toxicity in

terms of aggravation of GVHD, neurotoxicity, and infection complications suggests that further studies should be performed to better evaluate the balance between toxicity and efficacy [104]. These concerns were also raised from a recent study where, in a retrospective analysis, 30 patients who relapsed from allo-SCT were evaluated for the anti-myeloma effect of bortezomib in combination with DLI. According to the analysis, the combined treatment did not result in durable remissions [105].

Overall these novel agents show great promises, and their immune-modulating effect can be a strong weapon to enhance the GVM effect after transplantation especially if they can control the level of GVHD. Further studies and longer follow-up will be needed to evaluate the balance between efficacy and toxicity.

5.6 Conclusion Remarks

The sensitivity of MM to high-dose chemotherapy combined with an allogeneic immune response against residual MM cells has shown great potential in prolonging disease-free survival in some patients with MM. The direct evidence for a GVM effect is provided by the ability of DLI to induce significant responses in 30–50% of MM patients who have relapsed after allo-SCT. However the high rate of toxicity and TRM associated with myeloablative transplant has significantly limited this approach. In the past 10 years, clinical investigators have explored RIC to reduce the high toxicities related with the myeloablative regimens. Although this approach has shown to reduce TRM, the relapse rate is high. While the RIC regimen remain the most suitable approach for MM, data analysis of large cohort of patients has shown the importance of prognostic factors in predicting the outcome, and in several cases, a more aggressive approach can overcome the predicted poor prognosis. In this respect, a myeloablative conditioning followed by allo-SCT could still be the most effective treatment for patients with high risk factors. Future studies and clinical trials on allo-SCT should point the aim at improving the GVM effect while reducing the TRM related to the toxicity of the approach. The new immunomodulatory drugs show great promises on harnessing the allogeneic immune system toward the GVM effect, and although still early, further studies will determine whether these new agents could be a valid support of a more effective allo-SCT. Finally, new strategies that will better explore NK cell therapy, adoptive T-cell therapy, and vaccines to enhance the allogeneic immunity could increase the number of MM patients that will benefit from this approach.

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Chapter 6
Dendritic Cells and Peptide-Based Vaccine In Multiple Myeloma

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6.1 Introduction

Since Steinman and Cohn [1]'s initial report on dendritic cells (DC) with a distinctive stellate morphology, DC have been extensively studied by many other investigators for their major role as antigen-presenting cells (APC) to stimulate T lymphocytes and induce the disease-specific cytotoxic T lymphocytes (CTL). As major regulators of the adaptive immune response, DC have been known as the most potent APC for initiating cellular immune responses through the stimulation of naive T cells and to mediate antitumor responses in both preclinical studies and clinical trials [2–4]. The unique ability of DC to induce and sustain primary immune responses makes them prime candidates in vaccination protocols as a cancer therapy [5–8]. Therefore, translating the accumulating knowledge on DC subsets and their unique functional specializations into designs for novel vaccines is emerging as a key topic in the field of immunotherapy. More than 200 clinical trials have been performed using DC as cellular adjuvants in cancer [9]. The first US Food and Drug Administration approval in history for a therapeutic cancer vaccine was sipuleucel-T (Provenge; Dendreon, Inc.) that is an autologous DC-based vaccine loaded with a prostatic acid phosphatase (PAP)-GM-CSF fusion protein for treatment of men with advanced castrate-resistant prostate cancer. These ongoing studies have been accompanied by the development of a wide range of therapies using DC in other types of

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N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 2: Translational and Clinical Research*,
DOI 10.1007/978-1-4614-5260-7_6, © Springer Science+Business Media New York 2013

cancer, and we will specifically focus on the development of current DC therapies to treat multiple myeloma.

Multiple myeloma (MM) is a B-cell malignancy characterized by the clonal proliferation of malignant plasma cells in the bone marrow and the development of osteolytic bone lesions. Despite recent advances in treatment using new drugs, the disease still remains incurable; thus, novel approaches are required to improve therapeutic outcome [10–12]. In the post-allograft relapse setting, in which myeloma patients are chemotherapy refractory, long-lasting disease remission has been achieved after donor lymphocyte infusion (DLI) [13, 14]. Based on the success of allogeneic transplantation as well as graft-versus-myeloma responses following DLI, other types of immunotherapeutic approaches are being evaluated to treat the disease. The current focus has been on augmenting and directing autologous anti-MM immune responses as allogeneic immune manipulations put patients at risk of developing graft-versus-host disease with associated significant morbidity and mortality [15, 16]. It has been reported that the efficient generation of mature DC from peripheral blood $CD14^+$ monocytes in the majority of myeloma patients by culturing them with GM-CSF and IL-4 followed by TNF-α and/or other DC maturation factors can be utilized for immunotherapeutic purposes [17–22]. A number of approaches have been investigated including use of patient-specific idiotype, MM cell lysates, or MM cell-dendritic cell fusions.

6.2 Idiotype-Based DC Vaccine

Among the antigens identified on myeloma cells as potential targets, idiotype protein (Id) which is the immunoglobulin produced by myeloma cells has been investigated extensively [23–28]. The idiotypic determinants of the immunoglobulin are generated by rearrangement between the variable (V), diversity (D), and joining (J) regions in the heavy chain and between the V and J regions in the light chain. During maturation, a B cell may accumulate further diversity by somatic hypermutation [29, 30]. Tumor-specific Id secreted by MM cells can be easily detected in the blood of patients at concentrations which correlate to disease status [31]. Thus, the Id protein provides a clear tumor-specific antigen for B-cell tumors including MM and serves as a target antigen in various immunotherapeutic strategies. Several investigators have demonstrated that MM patients' T cells stimulated in vitro with Id-pulsed DC can kill autologous tumor cells in a MHC-restricted fashion and induce Th1-specific cytokines in vitro, thus demonstrating that MM cells process and present idiotypic peptides in the context of their MHC molecules and thereby can serve as targets of Id-specific T-cell-mediated antitumor responses [32–35].

Past and current clinical immunotherpies for MM patients have mainly been performed using Id as the antigen to boost patients' immune responses (Table 6.1).

Table 6.1 Idiotype-based clinical trials in myeloma

Vaccine	Patients(#)	Cellular responses	Clinical responses	Comments	Reference
Id alone Repeated vaccination	5 I–III stage	Increased T cell res 3/5 ELISPOT	Insufficient CR IFNg/IL4—2–5-fold up Anti-Id abs up	Show feasibility	Bergenbrant et al.
Id+GM-CSF 6 shots	5	ELISPOTs IFNg/IL2 3–5 times up No IL4, proliferation 1/5 CD4/CD8 responded Class I restricted(46–100%) Class II restricted(5–37%) NO DTH responses	No clear CR	Paraprotein levels unchanged	Osterborg et al.
Id+KLH+GM-CSF (IL-2)	12 HD chemo autoSCT	Very little T-cell responses (2/11) No anti-Id abs w/ remission 85% had DTH	Residual tumor burden was not reduced after 36 m	Paraprotein levels not changed	Massaia et al. Coscia et al.
Id+IL12 ± GM-CSF 7 shots	6 stage I	5/6 up w T-cell responses	4/6 down tumor	No change in paraprotein	Rasmussen et al.
Id+GM-CSF 4 shots hepB Vac	3 autoSCT	T-cell responses up (1/3)	No alteration in	No abs to HepB	Bertinetti et al.
Tumor cell+ GMCSF K562 8 shots after paraprotein autoSCT	16	T/B-cell responses up antitumor abs up	1/16 CR/PR	3/16 rise in 4/10 had DTH 3/16 increased in	Borrello et al.
Id + KLH + GMCSF 52 in 3coharts	18 BMT1/2+3shots 21 BMT1/2+6shots 13 BMT1+3shots+ BMT2+3shots	Prolif/cytokines up 14/18 7/19 7/11	6/18 improved 12/21 improved 8/13 improved	6/13 had DTH 5/10 had DTH 1/5 had DTH	Munshi et al.

In a pilot study by Lim and coworkers [36], six patients with IgG MM were vaccinated with intravenous infusions of DC derived from peripheral blood mononuclear cells (PBMC) pulsed with autologous Id protein. Although both a B-cell and a T-cell immune response were found, tumor-specific responses were only minor. In order to boost the Id-specific response, Reichardt and colleagues [37] conjugated myeloma-specific Id with keyhole limpet hemocyanin (KLH) and used the fusion protein to pulse autologous DC in vitro. They reported on 12 patients who had undergone autologous peripheral stem cell transplantation followed by a series of monthly immunizations of two intravenous infusions of Id-pulsed autologous DC and by booster immunizations with subcutaneous Id-KLH. This strategy was well tolerated as patients had only minor side effects. Furthermore, 2 of 12 patients developed Id-specific cellular proliferation, while 1 of 3 patients developed an Id-specific CTL response. In other studies, DC pulsed with Id-KLH have elicited potentially useful immunologic responses such as Id-specific T-cell proliferation detected from 15% [38] to as many as 83% of the patients [39] in clinical trials. In the latter study, the response was associated with production of IFN-γ in 2 out of 6 patients and an increase in CTL precursor frequency in these patients. In a study from Cull et al. [40], two patients with advanced refractory MM were vaccinated with Id-pulsed DC combined with GM-CSF. An anti-Id T-cell proliferative response was detected in both patients, which was also associated with IFN-γ production by the T cells. Titzer et al. [41] treated 11 patients with advanced MM with Id-pulsed, $CD34^{+}$ stem cell-derived DC and GM-CSF. Three of ten vaccinated patients showed an increased anti-Id antibody titer, and four of the ten patients had Id-specific T-cell responses.

Overall, meaningful immunologic responses and antitumor effects have been reported in lymphoma patients using different formulations of Id vaccine [42, 43]. However, the Id vaccination in B-cell cancers other than lymphoma is less advanced, and the vigorous Id-specific immune responses reported in lymphoma have not been detected yet in MM although DC-based Id vaccination can elicit Id-specific T-cell responses in patients with MM. This may be explained by the following aspects: (1) Id protein can induce humoral immunity; however, in contrast to lymphoma, myeloma cells do not express the IgG Id on the cell surface, and hence, the contribution of anti-Id antibodies to any vaccine-induced clinical response in myeloma is unclear [44]. (2) Early stage I myeloma patients with competent immune systems upon receiving DC-based Id vaccination displayed specific T-cell responses, and 89% of these patients demonstrated specific T-cell-mediated cytokine release after Id stimulation [26, 27]. In contrast, immune system suppression such as a functional defect in peripheral blood DC was observed in advanced myeloma patients when treated with Id-DC therapy [45]. In advanced myeloma, T-cell responses may be shifted to a type 2 inflammatory cellular response, and the functional activity of these T cells is a matter of debate [46, 47]. (3) Route of administration should be considered to help overcome the limitation of Id-pulsed DC vaccination. Most Id-pulsed DC vaccination trials have been administered intravenously [36, 37, 40, 41, 48]. However, several investigators report that

intravenous injection of DC led to accumulation of the cells in the lung, liver, and spleen during the first 24–48 h [49, 50], whereas DC injected subcutaneously migrated to the T-cell regions of draining lymph nodes and induced a strong protective immune response or a Th1-specific response [51]. In addition, Curti et al. [52] reported in a phase I/II clinical trial comparing subcutaneous and intravenous delivery of DC pulsed with Id that a more robust T-cell response was observed after subcutaneous DC injections along with increased Id-specific T-cell proliferation up to 1 year after vaccination in the myeloma patients. (4) Quality of DC should be explored in the clinical setting. Although monocyte-derived immature DC are both efficient in uptaking and processing antigens, the administration of these immature DC showed a limitation in triggering T-cell responses due to a lower expression of costimulatory and MHC molecules on their cell surfaces. In addition, monocyte-derived immature DC are not stable and may differentiate back to macrophages when IL-4 and GM-CSF are withdrawn [53]. In a study of functional differences between mature and immature DC, Yi et al. [54] concluded that mature DC derived from peripheral blood monocytes would better serve as APC than immature DC. Their clinical study using subcutaneous DC vaccination of Id-pulsed mature DC in MM patients with stable partial remissions following high-dose chemotherapy showed promising results, whereby Id-specific T-cell responses were observed in 80% of these myeloma patients. In a recent study, Yi et al. [25, 28] showed that intranodal administration of Id-pulsed CD40 ligand-matured DC induced Id-specific T-cell and B-cell responses in patients. (5) Several studies suggest that Id vaccination may have a therapeutic effect in the setting of autologous or allogeneic transplantation. Lacy et al. [55] showed that idiotype-pulsed DC following autologous stem cell transplantation for MM might be associated with prolonged survival. They demonstrated that 96% of the patients in the vaccine trial had achieved an objective response following autologous transplantation and suggested that Id vaccines are attractive as a consolidation therapy after autologous transplantation for MM. Exploitation of the potential antitumor effect of stem cell grafts in the allogeneic setting relies on strategies for enhancing graft-versus-tumor effects without aggravating graft-versus-host disease. In a study by Kwak et al. [56], donor-Id-specific T-cell immunity was detected at the time of allografting of Id-immune marrow. In another study, Li et al. [32, 35] showed release of high levels of Th1-type cytokines in an MHC-restricted fashion in response to stimulation with recipients' myeloma cells in two donors immunized with Id proteins obtained from their transplant recipients. These results set the stage for an ongoing phase I/II clinical trial at the National Cancer Institute of donor immunization prior to allogeneic stem cell transplantation followed by a nonmyeloablative conditioning regimen for MM. In the same clinical setting, to avoid any potential complications associated with immunization of healthy donors with tumor-derived products, in vitro priming of donor T cells using Id-pulsed DC may provide an alternative to in vivo donor immunization and allow the transfer of highly enriched populations of Id-specific T cells from donor to recipient [57] (Table 6.2).

Table 6.2 Clinical trials using DCs pulsed with myeloma patient idiotype

Vaccine	Patients	Clinical outcome	Reference
Id + KLH+ DCs 7 shots 2-iv Id + DC 5-Id + KLH	12 autoSCT hdose Chemo	Stable	Reichardt et al.
Id + DCs 3 shots	6	Progressed	Lim et al.
Id+ 4 shots GMCSF+ DCs	2 Adv refrat	1 progressed 1 stable	Cull et al.
2-Id + Dcs 7 shots 5-Id + KLH	26 hdose chemo autoSCT	17 live/stable	Liso et al.
1-Id + DC 4 shots 3-Id + GMCSF	11 III stage	Progressed	Titzer et al.
Id + DCs 3 shots IL2/5d	5 hdose chemo stable PR	4 stable 1 relapsed	Yi et al.
2-Id + DCs 7 shots 5-Id + KLH+ GMCSF	12 hdose chemo autoSCT at remission	10 progressed 2-PR	Reichardt et al.
alloDCs + Id Id + KLH+ GMCSF 4-7shots	4 RIC alloSCT	3 progressed	Bendandi et al. .
Id + DCs+ KLH 4 shots	9	All idiotype abs 5/9 CTL responses 3 progressed 4 stable	Yi et al.
Id + DCs 5 shots	9 stage I	5/9 anti-Idiotype abs 8/9 cytokine responses 3/9 dropped slightly	Rolliq et al.

6.3 DNA-Based DC Vaccine

Although proven effective in experimental models and in clinical trials, the traditional Id vaccine approach based on the culture of heterohybridomas is complicated in view of its clinical application by the need for large amounts of custom-made and individually tailored proteins that must be prepared and certified for each case within an appropriate time scale. The DNA vaccination technique provides ease of

vaccine generation and the specific protein production by host cells following immunization. The first requirement to make Id DNA vaccines is the identification of Id-encoding variable region genes (V_H and V_L) from tumor biopsies or blood. To construct Id DNA vaccines, the Id-encoding regions are isolated from malignant B cells using PCR-based techniques and formatted into a refined tumor-specific single-chain immunoglobulin (sFv) that retains the conformation of the native immunoglobulin. The weakly immunogenic, self-sFv is genetically fused to carriers, thus avoiding the need for purified Id protein, carriers, and adjuvants [58, 59]. For Id DNA vaccines, scFv alone was unable to reproducibly induce anti-Id antibody responses, even in the presence of the "immune stimulatory sequence" in the plasmid DNA backbone [60]. To improve the potency of Id DNA vaccines, investigators have constructed DNA fusion vaccines with scFv genetically linked to FrC, which is the nontoxic C fragment of tetanus toxin as an adjuvant to deliver a "danger signal" to the immune system [61, 62]. All of the fusion constructs were able to induce an antibody response against FrC in mice, and more importantly the linkage to FrC dramatically improved antibody responses against the patients' tumor IgM [63]. King and colleagues [64] further investigated a fusion DNA vaccine for induction of anti-Id responses and protection against challenge in syngenic mouse models, a surface Ig-positive lymphoma (A31) and a surface Ig-negative myeloma (5 T33). Their study showed that fusion of FrC enhanced anti-Id antibody responses, and the immunized mice were protected against tumor challenge in both cases. Lauritzsen and colleagues [65] have demonstrated that $CD4^+$ T cells are capable of protecting mice against challenge with a surface Ig-negative myeloma using anti-Id $CD4^+$ transgenic mice. The ability of scFv–FrC DNA fusion vaccines to induce an FrC-specific Th response suggests that the antitumor immunity observed by the fusion of FrC in the 5 T33 myeloma model may operate through the Th cooperation pathway [64].

A variety of different approaches have been explored using DNA fusion vaccines incorporating various immune-enhancing molecules or tumor-associated antigens (TAA) that can be used to promote immunity against attached tumor antigens. Different designs of these molecules can be used to circumvent tolerance and activate specific pathways of attack. Several investigators have developed a DNA vaccine approach using mediators of innate immunity such as proinflammatory chemokines or cytokines [66–68] and defensins [69] as genetic carriers, which deliver Id or a potential TAA to DC in vivo [70]. In two different mouse B-cell tumor models, this strategy converted Id into a potent immunogen with generation of both humoral and cellular antitumor immunity [69, 71]. Testing in pilot clinical trials showed insignificant toxicity, opening the way for the assessment of efficacy. Trudel et al. [72] in a phase I study evaluated the feasibility and safety of vaccinating MM patients after high-dose chemotherapy with adenovector-engineered, IL-2-expressing autologous plasma cells. These vaccines were well tolerated and induced a local inflammatory response consisting predominantly of $CD8^+$ T cells. However, no specific antitumor immunity or clinical responses were noted, and this indicates that further studies are needed to examine this clinical approach for treatment of patients.

6.4 Cell-Based DC Vaccine

A major drawback of an antigen-specific vaccine approach is that immune responses will be restricted to the single TAA with the subsequent risk of relapsing when tumors no longer express the antigens against which they were vaccinated, a phenomenon known as "antigen escape variants." An alternative to overcome this potential limitation is represented by whole tumor cell immunization (polyvalent vaccination), which may present to the host immune system a whole array of both known and as yet unidentified tumor antigens. This approach relies on the ability of the individuals' immune system to induce stronger immunity against tumor-selective antigens than against normal tissue antigens present on the tumor cells' surface.

Critical to this type of vaccine development is the ability to modify the tumor cell with genes encoding immunologically relevant molecules that produce a sustained, local release of its product, leading to a local inflammation at the vaccine site without systemic toxicity. Because of advances over the past decade in gene-transfer techniques, various tumor cells have been genetically modified to either secrete cytokines (e.g., IL-2, GM-CSF) or to express components of the cell membrane such as adhesion molecules or costimulatory molecules [73–77] that can enhance T-cell responsiveness. The means of active specific immunization using autologous tumor cells has been tested in trials for MM following their uptake and processing by DC in vivo. Trudel et al. [72] evaluated eight MM patients after vaccination with IL-2 expressing adenovirus engineered autologous plasma cells. Two months after high-dose therapy, six patients received from one to five injections of $3.5–9.0 \times 10^7$ of the engineered plasma cells. A phase I assessment found that the vaccine was effective in seven of eight patients with MM. Injection with tumor cells induced a local inflammatory response, and the clinical response, manifested as a decrease in serum paraprotein, was not observed in the one patient who had measurable disease at the time of vaccination. However, the limitation of this type of vaccine is that development of using cytokine-producing autologous tumor cells is hindered by the time needed for labor-intensive preparation of the vaccine and by the variability in the cytokine production of each patient's vaccine formulation. To overcome such drawbacks, investigators have developed an allogeneic bystander cell line (called K562) that secretes large and stable amounts of GM-CSF [78]. This cell line can be grown easily in suspension and has no detectable expression of HLA class I or class II molecules, and thus minimizes the likelihood of antibystander allogeneic responses with multiple vaccinations. This strategy of a universal bystander vaccine obviates the need for gene modification for each individual tumor source and ensures uniform cytokine production, thereby eliminating intrapatient and interpatient variability. In addition, GM-CSF produced at the vaccine site promotes the recruitment and activation of the host's APC, which efficiently uptake, process, and present tumor antigens to antigen-specific T cell, leading to strong antitumor responses.

In another effort to stimulate a broader antitumor immunologic response, investigators have explored the use of tumor lysate as a source of multiple antigens for vaccination. Wen et al. [79] demonstrated that patient-derived DC loaded with autologous tumor lysate induced antitumor immunity after repetitive stimulation in vitro. The T cells recognized and lysed autologous myeloma protein-pulsed DC and killed autologous primary myeloma cells. Another study also demonstrated the potent cytotoxic activities of CTL lines generated by DC pulsed with myeloma lysate against autologous target cells and showed the importance in the optimization of concentration of myeloma lysates utilized in pulsing of the DC [80]. Their results suggested that the DC pulsed with purified and optimized myeloma lysates could generate potent myeloma-specific CTL.

DC vaccines can also be made by fusing with myeloma cells. Several investigators have shown some efficacy using this vaccine approach in MM. Zhang et al. [81] showed that a DC-based tumor vaccine created by the formation of hybrid-engineered J558 tumor cells after fusion with DC induced an efficient tumor-specific CTL cytotoxicity against wild-type tumor cells in vitro and an efficient antitumor immunity in vivo. In other studies, investigators demonstrated that engineered J558 myeloma cells secreting IL-4, IL-12, or CD40 ligand, respectively, helped eradicate the established tumors [82–84]. They demonstrated that immunization of mice with the engineered fusion hybrid elicited stronger J558 tumor-specific CTL responses in vitro as well as more potent protective immunity against J558 tumor challenge in vivo than immunization with the conventional fusion hybrid DC/J558 created from the fusion of DC and unmanipulated J558 tumor cells alone. In addition, Grossman et al., [85] performed a DC fusion study using either primary myeloma cells from patients or a myeloma cell line (U266) and demonstrated that fusions with mature DC, as compared to immature DC, induced higher levels of T-cell proliferation and activation, as assessed by IFN-γ production and higher CTL activity against the myeloma cells. Tumor cell fusion has been known to induce maturation and the development of an activated DC phenotype necessary for their effectiveness as cancer vaccines [86]. Based on these results, a clinical trial was designed to evaluate the efficacy of vaccination of myeloma patients using fusion cells with myeloma cells and autologous mature DC. Rosenblatt et al. [87] have completed a phase 1 study in which patients with MM underwent serial vaccination with the DC/MM fusion product in conjunction with GM-CSF. Their study of vaccination was well tolerated, without evidence of toxicity and resulted in the expansion of circulating $CD4^{+}$ and $CD8^{+}$ T lymphocytes reactive with autologous myeloma cells in 11 of 15 evaluable patients. The vaccination with DC/MM fusions resulted in antitumor immune responses and disease stabilization in a majority of patients. In a separate report, they demonstrated that increased PD-1 expression was observed on T cells of patients with active myeloma compared with a control population of normal volunteers. However, it was returned to levels seen in normal controls, and anti-PD1 antibody enhances activated T-cell responses after DC/tumor fusion stimulation; thus, they suggested the potential enhanced vaccine efficacy in combination with the anti-PD1 antibody [88].

6.5 Peptide-Based Vaccines in Multiple Myeloma

6.5.1 Introduction

Active-specific immunotherapy has the distinct advantage of inducing highly effective T lymphocytes with antitumor activities [89, 90]. Long-term stabilization of disease with good quality of life has been demonstrated as a characteristic of cancer immunotherapy. To avoid a patient-specific immunotherapy requires individualized patient-specific products, which are labor intensive and costly, peptide vaccines can be used as an attractive therapeutic option for a broader applicability, low toxicity, and easy production [91]. Although there is MHC restriction in this therapeutic approach, use of cocktails of immunogenic peptides to different HLA molecules would broaden the induction of CTL specific to tumor cells of multiple MHC classifications. Based on the recent progress on the discovery of tumor-associated antigens (TAA), epitopes have been identified from multiple potential antigens and evaluated for the development of vaccines by eliciting the antigen-specific $CD8^+$ T-cell responses against MM cells. Strategies for further improvement in the efficacy of therapy, including combined use of chemotherapy drugs and molecular target-based drugs, are being proposed. Peptide vaccination in an "adjuvant setting" should be considered a promising treatment to protect against progression or relapse of malignancies in cases with minimal residual disease. The following are the types of TAA utilized and progress made for the development of peptide-based vaccines in MM.

6.5.2 Receptor for Hyaluronic acid Mediated Motility

Receptor for hyaluronic acid mediated motility (RHAMM) is an immunogenic antigen that is strongly expressed in several hematological malignancies including MM and induces humoral and cellular immune responses [92–94]. Schmitt et al. [95] and Greiner et al. [96] have investigated both immunological and therapeutic clinical responses to a RHAMM-R3 peptide vaccine in patients with MM. In their phase I trial, the RHAMM-R3 peptide (ILSLELMKL) was administered four times (300 μg or 1 mg/vaccination) subcutaneously at a biweekly interval to $HLA\text{-}A2^+$ MM patients who were in partial remission or near complete remission after high-dose chemotherapy with melphalan and autologous stem cell transplantation and had detectable free light chains in serum and/or urine and expression of RHAMM-mRNA in bone marrow or peripheral blood. Immune monitoring during or after vaccination for positive immune responses was performed on patient cells using the following criteria: (1) ELISpot analyses as an increase (>50%) in $IFN\text{-}\gamma^+$ and $granzyme^+$ spots, (2) tetramer analyses as an increase (>50%) in $HLA\text{-}A2/R3\text{-}tetramer^+/CD8^+$ T lymphocytes and with an increase (> 25%) in $RHAMM\text{-}R3\text{-}tetramer^+/CD8^+$ T lymphocytes, and (3) $CD8^+$ T-cell responsiveness demonstrated by a response 2/3

or 1/2 of the monitoring assays (tetramer staining, IFN-γ, and granzyme B ELISpot). Those patients having a positive immunological response showed an increase of $CD8^+$ $tetramer^+/CD45RA^+/CCR7^-/CD27^-/CD28^-$ effector T cells and an increase of RHAMM-R3-specific $CD8^+$ T cells. In addition, high-dose RHAMM-R3 peptide vaccination induced positive clinical effects. Two of four patients with MM showed a reduction of free light-chain serum levels.

6.5.3 Wilms' Tumor Gene

Wilms' tumor gene (WT1), which possesses oncogenic functions, is expressed in various kinds of malignancies. A series of investigations indicated that WT1 is a highly immunogenic antigen in patients with MM [97–99]. CTL epitopes were identified from WT1 specific to HLA-A2 and HLA-A-24, and evaluated in clinical trials. Vaccination of cancer patients with the WT1 CTL peptides induced immunological responses, which were assessed by ex vivo immunomonitoring, such as the tetramer assay, and in vivo immunomonitoring, such as the peptide-specific delayed type hypersensitivity reaction. The induced immunological responses then led to clinical responses as reduction of M-protein [100, 101]. The vaccination with a single WT1 peptide elicited an immunological response strong enough to induce a clinical response, suggesting that the WT1 peptide vaccine has therapeutic potential. The number of reports of the successful treatment of cancer patients with WT1 vaccination is increasing.

6.5.4 Dickkopf-1 (DKK1)

The DKK1 protein, a secreted protein and Wnt signaling pathway inhibitor, is produced by myeloma cells and overexpressed in myeloma microenvironment of patients with extensive bone disease [102, 103]. In addition to its direct inhibitory effect of DKK1 on osteoblasts, DKK1 disrupts the Wnt3a-regulated osteoprotegerin and receptor activator of NF-kappaB ligand (RANKL) expression in osteoblasts, and thus, it indirectly enhances osteoclast function in MM [104–107]. It is highly expressed by the tumor cells of almost all myeloma patients, and therefore, it has been suggested as an ideal target for immunotherapy in MM. However, DKK1 mRNA is detected in some normal tissues such as testis, prostate, placenta, and uterus, in addition to myeloma cells; thus, DKK1 resembles cancer–testis antigens because the most commonly used cancer–testis antigens NY-ESO-1 and MAGE are also found in the uterus, placenta, ovary, and even brain, in addition to tumors and testis [108, 109]. Qian et al. [110] identified an HLA-A2-specific peptide derived from DKK1 that was capable of inducing DKK1-specific T-cell lines and clones from $HLA\text{-}A2^+$ normal donors and MM patients. These CTL showed

peptide-specific and MM-specific responses in vitro and showed the therapeutic efficacy in vivo against established tumor cells in a HLA-A2 transgenic mouse model. These data show that DKK1 is a novel target for the management of myeloma patients with lytic bone disease.

6.5.5 Telomerase

Telomerase plays a critical role in cellular immortality and tumorigenesis. Its activity is normally not detectable in most somatic cells, while it is reactivated in the vast majority of cancer cells resulting in a tight correlation between telomerase activity and malignant potential of tumor cells [111–113]. Thus, inhibition of telomerase has been considered as a promising anticancer approach. Telomerase includes three major components: the telomerase reverse transcriptase (TERT) protein subunit that catalyzes the enzymatic reaction of DNA synthesis, the telomerase RNA (TR) component that serves as a template for TERT, and a protein termed dyskerin which binds to hTR. These three components are known to be essential for telomerase activity and telomere lengthening [114, 115]. Telomerase activity in a cell is associated with the expression of hTERT-related peptides on its surface and is present in more than 85% of human tumors [116, 117]. Recently, a multipeptide vaccine derived from the human telomerase reverse transcriptase (hTERT I540 (ILAKFLHWL), hTERT D988Y (YLQVNSLQTV), hTERT D988Y (YLQVNSLQTV)) and the antiapoptotic protein surviving (Sur1M2 peptide (LMLGEFLKL)) have been evaluated in a phase 1/2 two-arm trial [118, 119]. A total of 54 patients with myeloma received autografts followed by ex vivo anti-CD3/anti-CD28 costimulated autologous T cells at day 2 after transplantation. Study patients positive for HLA-A2 ($n=28$) also received pneumococcal conjugate vaccine immunizations before and after transplantation and the multipeptide vaccine. A subset of patients vaccinated (36%) developed immune responses to the tumor antigen vaccine by tetramer assays, but this cohort did not exhibit better median event-free survival (EFS). Adoptive transfer of tumor antigen vaccine-primed and costimulated T cells leads to augmented and accelerated cellular and humoral immune reconstitution, including antitumor immunity, after autologous stem cell transplantation for myeloma.

6.5.6 Cancer Testis Antigen

Cancer testis antigen (CTA) has been extensively studied in MM by many investigators. It exhibits physiological expression within germ cells and is frequently expressed in malignant tissue. Interestingly, immunological tolerance to CTA does not appear to be established, and the expression of CTA within malignant cells can therefore lead to induction of cellular and humoral immunity [120]. Antigen expression is detected most commonly in MM patients with advanced disease [121, 122],

but is also found in a significant proportion of patients with MGUS [123]. Recently, van Duin et al. [124] evaluated CTA expression in newly diagnosed MM patients ($n=320$) and in relapse cases ($n=264$) using Affymetrix GeneChips. They reported that relapse MM reveals extensive CTA expression and confirmed that the antigens are as useful prognostic markers in newly diagnosed MM patients and in relapse MM patients. The mechanisms that underlie this expression are unclear but are at least partially related to demethylation of gene promoter sequences [125]. DNA microarray analysis of gene expression of >95% pure myeloma cells from more than 300 patients showed that the genes of MAGE-3 and NY-ESO-1 were expressed in the tumor cells from patients with relapsed disease or abnormal cytogenetics [126]. The HLA-A1-restricted or HLA-A2-restricted MAGE-3- or NY-ESO-1-specific peptide have been identified and the tumor-specific CTL generated by the peptide were demonstrated against myeloma cells [127, 128]. In addition, MUC-1, HM1.24, and survivin are expressed on MM cells and have been shown to induce T-cell reactivity against the antigen in patients with MM [129–132]. Antigen-specific peptides have been identified from these potential target proteins [133–136] and have shown immunogenicity both in vitro and in vivo against myeloma cells. In a phase 1/2 two-arm trial, a combination of survivin and hTERT peptides was evaluated for their efficacy [118, 119]. The investigators showed that adoptive transfer of tumor antigen vaccine-primed and costimulated T cells leads to augmented and accelerated antitumor immunity after autologous stem cell transplantation for MM. In another study, MUC-1 and hTERT peptides were evaluated in vitro for their immunogenicity [137]. Following repeated stimulation of T lymphocytes with DC loaded with hTERT- and MUC1-derived nonapeptides, the resulting CTLs were identified by their high IFN-γ production. Next, these activated CTL were separated immunomagnetically, expanded in vitro, *and* tested for their cytolytic activity against a myeloma cell line. There were no statistically significant differences in the cytotoxic activities between the different antigen-specific CTL and their specific antigens expressed on MM cells. Christensen et al. [138] explored the possibility in vitro of using Melan-A peptide (aa26-35, EAAGIGILTV) with the hypothesis that Melan-A and Melan-A analog (ELAGIGILTV, aa26-35*A27L) peptide-specific T cells can be expanded reliably for immunotherapeutic application. They showed the ability of Melan-A analog (ELAGIGILTV, Melan-A (aa26-35*A27L))-specific T cells to recognize the HM1.24 (aa22-30: LLLGIGILV) peptide within the HM1.24 antigen presented by normal and malignant plasma cells. In addition, they found that Melan-A analog-specific T cells from HLA-A2$^+$ healthy donors and HLA-A2$^+$ MM patients secrete IFN-γ in response to HM1.24 (aa22-30) peptide-pulsed T2 cells. These peptide-specific CTL also lysed HLA-A2$^+$ HM1.24$^+$ U266 and XG-1 human MM-derived cell lines as well as the IM-9 B-lymphoblastoid cell line, and demonstrate that Melan-A analog-specific T cells cross-react with the HM1.24 peptide. Anderson et al. [139] discovered peptides derived from MAGE-C1 (CT-7), which is the most commonly expressed CTA found in MM. The CT-7-specific CTL recognizing two peptides targeted both MM cells as well as CT-7 gene-transduced tumor cells. They demonstrated that these epitopes are promising targets for developing an immunotherapy against myeloma or other CT-7$^+$ malignancies. In another study, Goodyear et al. [140] identified CTA-specific immune responses in patients with MM and

reported that recognition of HLA-B*0702-specific MAGE-A1 (289–298) peptide was the most dominant response seen with the their peptide panel. $CD8^+$ T-cell clones specific for the MAGE-A1 (289–298) peptide were isolated from three MM patients and demonstrated cytotoxic activity against MM cell lines. Interestingly, three clones from a HLA-B*0702-negative patient recognized the MAGE-A1 (289–298) peptide on a lymphoblastoid cell line expressing HLA-Cw7. The T-cell receptor gene usage was determined in five clones and showed conserved features in both α and the β chain genes indicating correlation between T-cell receptor usage and peptide specificity of CTA-specific T-cell clones. Clinical applicability of the peptides derived from the cancer–testis antigens is under evaluation.

6.5.7 XBP1

Besides CTA, other MM-associated antigens have been identified and evaluated as potential immunogenic epitopes for development of a vaccine therapy to treat MM. XBP1 is a basic leucine zipper-containing transcription factor, which is required for the terminal differentiation of B lymphocytes to plasma cells. To date, XBP1 is the only transcription factor found to be essential for plasma cell differentiation and immunoglobulin secretion. The expression of XBP1 is uniformly found in primary MM cells and cell lines, selectively induced by exposure to IL-6, and has been implicated in the proliferation of malignant plasma cells [141–144]. A splice variant of XBP1 has known to have a crucial role in normal plasma cell differentiation [145], and XBP1 splicing has been recognized to occur in terminal B-cell differentiation and correlates with plasma cell differentiation. Based on these observations, Bae et al. [146] proposed the XBP1 as a unique therapeutic target antigen and identified two heteroclitic peptides, YISPWILAV and YLFPQLISV, with improved HLA-A2-binding and stability from their respective native peptides, $XBP1_{184–192}$ (NISPWILAV) and XBP1 $SP_{367–375}$ (ELFPQLISV). CTL generated by stimulation of $CD3^+$ T cells with each HLA-A2-specific heteroclitic peptide showed an increased percentage of $CD8^+$ (cytotoxic) and $CD69^+$/$CD45RO^+$ (activated memory) T cells and a lower percentage of $CD4^+$ (helper) and $CD45RA^+$/$CCR7^+$ (naïve) T cells, which were distinct from the control unstimulated T cells. The CTLs showed functional activities and demonstrated MM-specific and HLA-A2-restricted proliferation, IFN-γ secretion, and/or cytotoxic activity in response to MM cell lines and primary MM cells. These data demonstrate the distinct immunogenic characteristics of unique heteroclitic XBP1 peptides, which induce MM-specific CTL.

6.5.8 CD138, CS1

Furthermore, Bae et al. ([147], 2012) introduced immunogenic peptides specific to CD138 and CS1 antigens, which offer additional targets to develop an

immunotherapy targeting MM. The CD138, also known as syndecan-1, is a transmembrane heparan sulfate-bearing proteoglycan expressed by most MM cells. It has cytoplasmic domain which is linked to cytoskeletal elements to potentiate anchorage of the cells and stabilize cell morphology, while their extracellular domain has up to three heparan sulfate chains that bind to numerous soluble and insoluble molecules. These associations include interactions with heparan-binding molecules on adjacent cells to mediate cell–cell adhesion, binding to molecules to mediate cell adhesion to the extracellular matrix, as well as binding to growth factors and cytokines; thus, CD138 is known to be critical for the growth of tumor cells [148, 149]. In patients with MM, shed syndecan-1 accumulates in the bone marrow, and soluble syndecan-1 is known to facilitate MM tumor progression, angiogenesis, and metastasis in vivo. Therefore, preventing or reducing high levels of syndecan-1 in the serum, an indicator of poor prognosis in MM [150–152], would have a direct clinical benefit by targeting CD138 on malignant plasma cells. A novel immunogenic HLA-A2-specific peptide, $CD138_{260\text{-}268}$ (GLVGLIFAV), identified by Bae et al. [147] induces antigen-specific CTL, and the CD138 peptide-specific CTL displayed a unique immunological phenotype, and HLA-A2-restricted responses and functional activities against both primary MM cells and MM cell lines expressing CD138 antigen. Additionally, CS1 (CD2 subset 1, CRACC, SLAMF7, CD319) has been utilized as a target antigen to potentially develop immunotherapy against MM. CS1 is a member of the signaling lymphocyte activating-molecule-related receptor family, which is highly expressed on MM cells and is absent in the vast majority of acute leukemia, B-cell lymphoma, and Hodgkin lymphomas [153]. In addition, CS1 antigen is not expressed by normal tissues or stem cells, but is expressed at low levels on NK cells and a subset of T lymphocytes compared with malignant plasma cells [153]. CS1 expression was observed on MM cells from all patients, including MM with high-risk and low-risk molecular profiles and those with and without cytogenetic abnormalities, suggesting that this antigen is not restricted to any particular MM subgroup [154]. Equally important for the development of immunotherapy, CS1 expression is maintained on patients' MM cells even after relapse of disease. Based on these findings, Bae et al. [155] identified a novel immunogenic HLA-A2-specific epitope, $CS1_{239–247}$ peptide (SLFVLGLFL), which is derived from the CS1 antigen and has the ability to evoke MM-specific CTL. The CS1 peptide-specific CTL demonstrated HLA-A2-restricted antitumor cytotoxicity and degranulation against $HLA\text{-}A2^{+}$ primary MM cells and MM cell lines. In addition, the specific CTL demonstrated cell proliferation and IFN-γ secretion in response to antigen restimulation, which is also HLA-A2 restricted and the antigen specific. They also observed distinct immunologic activities specific to MM cells within the CD8 effector memory ($CD45RO^{-}CCR7^{-}/CD3^{+}CD8^{+}$) T-cell subset, and proposed an immunotherapeutic approach using the $CS1_{239–247}$ peptide to effectively target MM cells and improve treatment outcome in patients with MM. These results highlight their potential application for immunotherapy to treat the patients with MM or its premalignant condition. Clinical applicability of the peptides derived from the antigens is under evaluation.

6.6 Future Directions

Active cancer immunotherapy has been proven to be an effective approach to induce T-cell immune responses and overcome a number of issues by passive cancer immunotherapy including the requirement for repeated dosing and its high cost, the development of resistance through loss of immunodominant epitopes and undesired immunogenicity of humanized or chimerized antibodies. Dendritic cell-based or peptide-based treatments have been proposed as promising candidates for development of active cancer immunotherapy by generation of TAA-specific CTL. Clinical trials with dendritic cell-based or peptide-based therapy in patients with MM show that the vaccinations were well tolerated and induced clinical benefit in the patients. However, the effectiveness of active cancer immunotherapy to induce the specific immune response and clinical benefit depends on several factors. Besides element of antigens, it is becoming critical to optimize various conditions of the immune system to generate a clinically effective antitumor response. Generally, vaccine alone is not sufficient to evoke a potent immune response. Future challenge for successful immunotherapy is to skew the immune response towards a Th1 and to increase the antigen-induced T cells that bear high-avidity T-cell receptor to the specific TAA by using optimal adjuvant. Adjuvant should be important to enhance the immune response through a wide range of mechanisms including a depot action causing slow release of antigen to local inflammation causing enhanced recruitment of antigen-presenting cells to the injection site and facilitation of cross priming and mimic a danger signal. Furthermore, administration of optimal cytokine would be supportive, not only for the activation and expansion of tumor-associated T cells, but also for potential induction of the migration of vaccine-induced circulating T cells to the tumor site. Importantly, it would be highly potential to reverse the tolerance to tumor by blocking the CTLA-4 or by depleting regulatory T cells. Additionally, type of antigen-presenting cell and its activation status in the subjects vaccinated should be considered for successful therapeutic outcomes by cancer vaccines. Clinical responses of active cancer immunotherapy have been shown as promising in patients with minimal residual disease; thus, the combination of tumor debulking treatment and vaccination has been considered as a potential strategy to lead a successful therapeutic outcome in patients. Lastly, the complexity of the immune network and of the interactions between the tumor and the immune system makes the task to optimize the regimen including vaccine dose and route and schedule of immunization.

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Part II
New Agents

Chapter 7
Novel Proteasome Inhibitors

Robert Z. Orlowski

7.1 Introduction

The initial regulatory approval of the first-in-class proteasome inhibitor bortezomib for relapsed/refractory multiple myeloma based on data from phase I [1] and II [2] trials showing antitumor activity validated the proteasome as a rational target for cancer therapy. This was followed later by additional approvals, both as a single agent [3] and with liposomal doxorubicin [4], for relapsed disease, and with melphalan and prednisone for previously untreated symptomatic patients with myeloma [5]. Proteasome inhibitors exert their anti-myeloma effects through a number of molecular mechanisms, given the role of the proteasome in turnover of the majority of cellular proteins [6]. Among the more prominent include stabilization of proapoptotic B cell CLL/lymphoma (Bcl)-2 homology 3 (BH3) proteins and cleavage of antiapoptotic Bcl-2 and myeloid cell leukemia sequence (Mcl)-1, accumulation of cyclin-dependent kinase inhibitors resulting in cell cycle arrest, induction of stress-response pathways such as c-Jun-N-terminal kinase (JNK) and the unfolded protein response (UPR), and inhibition of nuclear factor kappa B (NF-κB) signaling, as detailed in several recent reviews [7–9]. The successful translation of bortezomib from the bench to the bedside spurred interest in the development of novel inhibitors that might have attractive properties which could be different from this first-generation agent. Broadly speaking, these agents can be divided into those that, like CEP-18770 and MLN9708, bind the proteasome reversibly and those like carfilzomib

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N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 2: Translational and Clinical Research*,
DOI 10.1007/978-1-4614-5260-7_7, © Springer Science+Business Media New York 2013

and marizomib that bind catalytic subunits in an irreversible manner. Also, inhibitors such as bortezomib and carfilzomib seem to bind relatively indiscriminately to all isoforms of the proteasome. In contrast, some agents in development are able to bind more specifically to the so-called immunoproteasome, which is expressed to a large extent in both normal and malignant hematopoietic tissues, but not in other organs. Finally, intriguing data are emerging about the potential utility of dual proteasome inhibition with combinations of more than one inhibitor, providing students of the field and investigators with a broad range of studies to be completed before the full utility of the "zomib" class of drugs in multiple myeloma is defined.

7.2 Irreversible Proteasome Inhibitors

The first-in-class proteasome inhibitor bortezomib is a slow-binding and reversible agent [10, 11], which allows recovery of cellular proteasome activity through a number of mechanisms, including new proteasome synthesis, drug metabolism, and release of its intended target. While boronic acid peptides have the benefits of enhanced potency and specificity compared to the traditional peptide aldehydes used as laboratory probes of proteasome function, other chemistries can provide similar properties. Among these are several that bind the proteasome and form irreversible bonds, which have the theoretical advantage of providing a longer-lasting target inhibition and possibly therefore greater therapeutic efficacy. Examples of these include peptide epoxyketones such as carfilzomib, previously known as PR-171, and also lactacystin and related agents, such as marizomib, previously known as NPI-0052. Both of these drugs have been validated in preclinical studies and are now undergoing clinical trials, and other irreversible inhibitors are showing promise as well.

7.2.1 Carfilzomib

7.2.1.1 Biological Basis

Carfilzomib is a peptide epoxyketone related to epoxomicin which, like marizomib, was originally isolated from a bacterium and is currently being developed by Onyx Pharmaceuticals (Emeryville, CA). In the case of epoxomicin, the bacteria of origin was the actinomycete strain No. Q996-17 [12]. Later, epoxomicin was synthesized chemically and shown to have potent anti-inflammatory and antiproliferative effects due to its ability to induce proteasome inhibition [13–15]. Studies in models of multiple myeloma showed that low doses of this agent specifically bound the β5 constitutive proteasome and, to some extent, also the $\beta5_i$ immunoproteasome

Table 7.1 Comparison of the ability of bortezomib, marizomib, and carfilzomib to inhibit the proteolytic activities of the 20S proteasome[a]

Proteasome activity	Bortezomib	Marizomib	Carfilzomib
Trypsin-like	4,200 nM	28 nM	3,600 nM
Caspase-like	74 nM	430 nM	2,400 nM
Chymotrypsin-like	7 nM	3.5 nM	6 nM

[a]Data represent the concentration needed to reduce the indicated constitutive proteasome activity by 50% and are derived from reference [17]. Please note that, due to the use of different assays and conditions, data from Tables 7.1, 7.4, and 7.5 are not strictly comparable

subunit and inhibited their chymotrypsin-like (ChT-L) activities [16]. However, at higher concentrations of carfilzomib, binding and inhibition were also seen of the trypsin-like (T-L) activity and also the post-glutamyl peptide hydrolyzing (PGPH) or caspase-like (C-L) activity. Carfilzomib induced accumulation of ubiquitin-protein conjugates and proteasome substrates such as Bax and induced apoptosis through dual activation of caspases-8 and −9, along with the downstream effector caspase-3. This was accompanied by mitochondrial membrane depolarization with release of cytochrome c and second mitochondria-derived activator of caspases (Smac), as well as activation of JNK. Notably, carfilzomib activated caspases and programmed cell death to a greater extent than was the case for bortezomib in both myeloma cell lines and primary samples. Furthermore, carfilzomib overcame drug resistance to both conventional agents and also to bortezomib in these model systems, providing a strong rationale for its translation into the clinic.

Biochemical characterization of carfilzomib supported these in cellulo studies and showed that this agent inhibited the ChT-L activity with comparable potency to that of bortezomib but was a weaker inhibitor of the PGPH function, while both were poor inhibitors of the T-L activity (Table 7.1) [17, 18]. These studies showed that carfilzomib was able to reduce tumor cell viability with equal to greater potency than bortezomib in experiments with continuous exposure to either drug. Interestingly, when both were given as a pulse followed by a washout, to somewhat mimic what might be expected based on in vivo pharmacokinetics, carfilzomib proved superior in myeloma models, as well as cell lines representing other hematologic malignancies and solid tumors. Systemic administration of radioactive drug in animal models induced proteasome inhibition in virtually all tissues tested with the exception of the brain, and drug accumulation was seen in the adrenals, bone marrow, intestine, liver, lung, and urine. Whereas bortezomib could not be dosed on consecutive days in animal models [10, 11], carfilzomib was tolerated either on a schedule of 5 days daily or on a schedule of two consecutive days in each week [17]. The latter regimen showed enhanced antitumor efficacy in murine xenograft models of HT-29 colorectal adenocarcinoma and HS-Sultan lymphoma cells compared to bortezomib. Notably, correlative studies revealed that carfilzomib provided greater tumor tissue proteasome inhibition in these xenografts, possibly accounting for the greater activity.

Carfilzomib is active not just alone but also seems able to induce chemosensitization and overcome drug resistance. In combination with dexamethasone, for example, carfilzomib showed strongly synergistic anti-myeloma activity [16]. Inhibition of antiapoptotic Bcl-2 family members including Bcl-2, Bcl-x_L, Bcl-w, and Mcl-1 appears also to be a rational strategy with carfilzomib. Using either ABT-737 [19] or AT-101 [20], the activity of carfilzomib was potentiated against models of mantle cell lymphoma, diffuse large B cell lymphoma, and chronic lymphocytic leukemia. Suppression of histone deacetylases with agents such as vorinostat also has been shown to be synergistic with carfilzomib in diffuse large B cell lymphomas, including both germinal-center B cell-like and activated B cell-like models [21]. Finally, an additional attractive approach may be to first induce cell cycle arrest through the use of a cyclin-dependent kinase (CDK)-4/6 inhibitor, such as PD 0332991, which sensitizes cells to later cytotoxic agents including carfilzomib [22], due at least in part to loss of interferon regulatory factor 4 [23].

7.2.1.2 Clinical Development

Carfilzomib as a Single Agent

Preclinical studies with carfilzomib validated consecutive-day dosing with this agent for either 2 or 5 days as being tolerable, and these schedules were therefore translated into the clinic into two phase I trials for patients with hematologic malignancies. The first study, PX-171-001, administered carfilzomib as an intravenous push on days one through five, followed by 9 days off in every 14-day cycle, at doses ranging from 1.2 to 20 mg/m^2 [24]. Adverse events seen in at least 20% of the 29 patients treated included fatigue, nausea, diarrhea, cough, dyspnea, hypoesthesia, pyrexia, headache, peripheral edema, constipation, exertional dyspnea, and paresthesias. These rarely reached grade 3 or 4 severity, with only dyspnea and thrombocytopenia being seen in more than one patient. Dose-limiting toxicities (DLTs) in the 20 mg/m^2 cohort included one episode of grade 3 febrile neutropenia requiring hospitalization and one of grade 4 thrombocytopenia. Pharmacodynamic studies showed a carfilzomib dose-dependent inhibition of the 20S proteasome in peripheral blood mononuclear cells (PBMCs) and in whole blood. This inhibition exceeded 75% after single doses of at least 15 mg/m^2 and reached levels above 90% after the fifth consecutive dose, though these generally returned to baseline in PBMCs during the 9-day rest period. Evidence of antitumor activity was seen in one patient with mantle cell lymphoma who achieved an unconfirmed complete remission (CR), one patient with Waldenström macroglobulinemia who experienced a minor response (MR), and two patients with multiple myeloma, including one MR and one PR. Notably, the latter was in a patient with previously bortezomib-refractory disease, corroborating in part the preclinical data [16]. Importantly, peripheral neuropathy was not seen at the grade 3 or 4 level in any patients, possibly due to the greater specificity of carfilzomib for the proteasome over other targets compared to bortezomib [25].

A different schedule, with twice-weekly dosing for 3 weeks out of 4, which led to drug administration at doses ranging from 1.2 to 27 mg/m^2 on days 1, 2, 8, 9, 15, and 16 of every 28-day cycle, was evaluated in the second phase I study of carfilzomib, PX-171-002 (ClinicalTrials.gov Identifier NCT00150462)[26]. Dose-limiting toxicities occurred at 27 mg/m^2 and included an episode of hypoxia and also grade 4 thrombocytopenia. In addition, though this did not reach criteria for a DLT, reversible elevations in the serum creatinine were seen in three of five myeloma patients treated at 27 mg/m^2, which in at least some patients seemed to be associated with a rapid decline in monoclonal protein levels and possible tumor lysis. The minimal effective dose was defined as 15 mg/m^2, and among 16 patients who received dosing at this level or higher, five responses were seen, including four PRs and one MR in myeloma patients, while another two had stable disease. Some of these responses also were in previously bortezomib-refractory disease, and response durability ranged from 134 to 392 days. Since the aforementioned episodes of renal insufficiency tended to not recur with drug rechallenge, this study was later amended to allow for a lower initial dose level at 20 mg/m^2 to be given during cycle 1 and a higher dose of 27 mg/m^2 to be given during subsequent cycles. This has been reported to be well tolerated and to show evidence suggesting the possibility of an enhanced antitumor activity, though these data have not yet appeared in a peer-reviewed format.

Successful completion of the phase I studies of carfilzomib was followed by two phase II studies specifically targeting patients with multiple myeloma. The first of these, PX-171-003 (NCT00511238), enrolled patients with relapsed and refractory disease utilizing the day 1, 2, 8, 9, 15, and 16 schedule, which has been the regimen taken forward in most of the phase II and phase I combination studies. Patients also later received tumor lysis prophylaxis in the form of allopurinol and intravenous hydration, as well as a low, 4-mg dose of dexamethasone during cycle 1 only to prevent a possible cytokine release syndrome [27]. These data were updated after 46 patients had been enrolled, at which time common adverse events were anemia, diarrhea, fatigue, increased creatinine, nausea, thrombocytopenia, and upper respiratory infection. Among evaluable patients, five had achieved at least a PR, with another five having an MR, for a clinical benefit ratio of 26%, including 10 out of 39 subjects, some of whom were bortezomib refractory. Median TTP was 6.2 months, while the median DOR for patients with at least an MR was 7.4 months. Another 16 patients had achieved stable disease or better for at least 6 weeks, further supporting the activity of this agent. More recently, updated data from this study have been provided in a press release [28], which reported that the final overall response rate in this trial was 24%, while the duration of response (DOR) was 7.4 months in patients with a median of five prior lines of therapy. These data could in the future form the basis for a filing with the Food and Drug Administration for accelerated approval of carfilzomib in patients who have previously received an immunomodulatory agent and bortezomib and were refractory to their last line of therapy.

The second phase II study of carfilzomib, PX-171-004 (NCT00530816), targeted patients with relapsed disease who were earlier in their course with multiple

myeloma and had received between one and three prior lines of therapy. Cohorts were enrolled for treatment who were bortezomib naïve, bortezomib-exposed but bortezomib sensitive, and bortezomib-exposed and bortezomib refractory. In a cohort of thirty-five bortezomib-treated patients, the only grade 3 or 4 adverse events seen in at least 10% of subjects were neutropenia and anemia, and only one grade 3 neuropathy was recorded [29]. An overall response rate of 18% was seen, including patients with at least a PR, showing some evidence of clinical cross-resistance between carfilzomib and bortezomib. However, 70% of patients achieved at least stable disease, and median DOR and time to progression (TTP) were a respectable 10.6 and 5.3 months, respectively. In a larger cohort of 54 patients who were bortezomib naïve, grade 3 or 4 adverse events seen in at least 10% of subjects were fatigue, pneumonia, and thrombocytopenia [30]. Among patients who received dosing with 20 mg/m^2 of carfilzomib, the overall response rate was 46%, while 53% of those who received 27 mg/m^2 starting in cycle 2 achieved at least a PR. Median DOR and TTP values were 8.4 and 7.5 months, respectively, with the latter being superior to the TTP seen with bortezomib in bortezomib-naïve patients with relapsed disease, which was 6.2 months [3].

Carfilzomib-Based Combination Regimens

The synergistic interaction between carfilzomib and dexamethasone [16], as well as the remarkable activity of the regimen of bortezomib with lenalidomide and dexamethasone in both the relapsed/refractory [31] and up-front settings [32], prompted an evaluation of carfilzomib with lenalidomide and dexamethasone. This recently completed study, PX-171-006 (NCT00603447), used the standard carfilzomib schedule along with lenalidomide at 25 mg on days one through 21 of every 28-day cycle and once weekly dexamethasone at 40 mg [33]. Common adverse events seen in at least 25% of patients included fatigue (in 45%), diarrhea (37%), neutropenia (30%), and anemia (25%), while common grade 3/4 adverse events seen in at least 5% were neutropenia (23%), thrombocytopenia (18%), and anemia (12%). As had been the case in previous studies of carfilzomib, peripheral neuropathy was not reported at the grade 3 or 4 level. An overall response rate of 66% was seen among 80 patients, including 27.5% with at least a very good PR and 6.3% with either a CR or sCR. In the cohort that received the doses selected for further study, the overall response rate was 75%, with response rates being aided by the use of carfilzomib at 20 mg/m^2 in cycle 1, followed by dosing at 27 mg/m^2 in cycle 2 and later. Responses were robust in all patient subgroups (Table 7.2), including patients with prior exposure to bortezomib, lenalidomide, or both. These encouraging findings have formed the basis for a phase III randomized study, which will compare lenalidomide with low-dose dexamethasone to carfilzomib with lenalidomide and low-dose dexamethasone in the relapsed setting (Table 7.3). If positive, as is to be hoped, this trial would provide the confirmatory data needed to support approval of carfilzomib and its use in an earlier, less refractory patient population.

Table 7.2 Response rates to the regimen of carfilzomib, lenalidomide, and dexamethasone in subgroups of patients with relapsed and relapsed/refractory multiple myeloma[a]

	Total *n*	CR/sCR n (%)	≥VGPR *n* (%)	ORR (≥PR) *n* (%)
Prior lines of therapy				
1	17	0 (0)	8 (47.1)	13 (76.5)
2	63	5 (7.9)	19 (30.2)	40 (63.5)
Types of prior therapies				
Bor	59	2 (3.4)	16 (27.1)	34 (57.6)
Len	54	2 (3.7)	15 (27.8)	32 (59.3)
Thal	34	3 (8.8)	12 (35.3)	27 (79.4)
Len or Thal	69	5 (7.2)	21 (30.4)	44 (63.8)
Len and Thal	19	0 (0)	6 (31.6)	15 (78.9)
Bor and Len	44	1 (2.3)	10 (22.7)	23 (52.3)
Bor and Thal	22	1 (4.5)	5 (22.7)	15 (68.2)
Bor, Len, and Thal	13	0 (0)	3 (23.1)	9 (69.2)
Cytogenetics				
Normal/favorable[b]	40	2 (5.0)	15 (37.5)	28 (70.0)
Poor prognosis[c]	31	3 (9.7)	9 (29.0)	17 (54.8)
Unknown	9	0 (0)	3 (33.3)	8 (88.9)
ISS stage				
Stage I	34	2 (5.9)	13 (38.2)	25 (73.5)
Stage II	31	2 (6.5)	10 (32.3)	21 (67.7)
Stage III	9	0 (0)	2 (22.2)	4 (44.4)

[a]*Abbreviations*: *Bor* bortezomib, *CR* complete remission, *ISS* International Staging System, *Len* lenalidomide, *ORR* overall response rate, *PR* partial remission, *sCR* stringent CR, *Thal* thalidomide, *VGPR* very good PR

[b]Normal/favorable cytogenetics included patients with t(11;14) or normal cytogenetics by metaphase analysis

[c]Poor prognosis cytogenetics included patients with t(4;14), t(14;16), del17p, del13q, gain 1q21, or other abnormalities by metaphase analysis

7.2.2 ONX 0912

7.2.2.1 Biological Basis

Preclinical studies with carfilzomib to determine if it was orally bioavailable unfortunately revealed that this drug did not induce inhibition of blood or target tissue proteasomes after oral administration [34, 35]. Additional screening and rational design efforts looking for smaller peptides that might be better absorbed through the gastrointestinal tract led to the identification of PR-047, which is now known as ONX 0912 (Onyx Pharmaceuticals), as a potential drug candidate. This N-capped tripeptide epoxyketone contains leucine in the P1 position, which is the residue that forms a bond with the N-terminal threonine active site of proteolytically active proteasome subunits, and methoxylated serine residues in the P2 and P3 positions, which are the next residues towards the N-terminus of the peptide, providing greater aqueous solubility. Like carfilzomib, ONX 0912 exhibited strong specificity for the

Table 7.3 Ongoing studies of carfilzomib in patients with multiple myeloma[a]

Study title	Carfilzomib dosing
A Study of Carfilzomib maintenance therapy in subjects previously enrolled in Carfilzomib treatment protocols	IV push on days 1, 2, 15, and 16 of a 28-day cycle
Multicenter, open-label, single-arm, phase 1b/2 study of the safety and efficacy of combination treatment w/ Carfilzomib, Lenalidomide, and Dexamethasone in subjects w/ newly diagnosed, previously untreated multiple myeloma requiring systemic chemotherapy	IV infusion on days 1, 2, 8, 9, 15, and 16 of a 28-day cycle for cycles 1–8 (induction) and on days 1, 2, 15, and 16 of a 28-day cycle for cycles 9+ (maintenance)
Phase 2 Study of the safety and pharmacokinetics of Carfilzomib in subjects with relapsed and refractory multiple myeloma and varying degrees of renal function	IV on days 1, 2, 8, 9, 15, and 16 of a 28-day cycle
Compassionate use study of Carfilzomib for patients with relapsing or resistant multiple myeloma	Carfilzomib (20 mg/m^2) IV push to be given at maximum rate of 10 ml/min on day 1 and day 2 of cycle 1 only Carfilzomib (27 mg/m^2) IV bolus to be given at maximum rate of 10 ml/min on days 8, 9, 15, and 16 of cycle 1, then through cycle 2 and beyond if initial dosing with 20 mg/m^2 tolerated For patients who tolerated 27 mg/m^2 through cycle 2 days 1 and 2, carfilzomib dose may be escalated to 36 mg/m^2 on days 8, 9, 15 and 16 of cycle 2
Phase 1b multicenter dose escalation Study of Carfilzomib with lenalidomide and dexamethasone for safety and activity in relapsed multiple myeloma	First 12 cycles, IV infusion twice weekly for 3 weeks of a 28-day cycle. Remaining 6 cycles, twice weekly during weeks 1 and 3 of a 28-day cycle
An open-label, single-arm, Phase 2 study of Carfilzomib in patients with relapsed and refractory multiple myeloma	IV push twice weekly for three weeks followed by 12 days of rest
A Phase I/II Trial of Cyclophosphamide, Carfilzomib, Thalidomide, and Dexamethasone in patients with newly diagnosed active multiple myeloma	Patients receive carfilzomib IV on days 1, 2, 8, 9, 15, and 16
A randomized, multicenter, Phase 3 study comparing Carfilzomib, Lenalidomide, and Dexamethasone vs Lenalidomide and Dexamethasone in subjects with relapsed multiple myeloma	IV on days 1, 2, 8, 9, 15, and 16 of a 28-day cycle
Phase 1b/2, multicenter open-label study of the safety and activity of Carfilzomib in subjects with relapsed solid tumors and in multiple myeloma	IV on days 1, 2, 8, 9, 15, and 16 of a 28-day cycle
An open-label, single-arm, Phase 2 study of Carfilzomib in patients with relapsed multiple myeloma	IV on days 1, 2, 8, 9, 15, and 16 of a 28-day cycle

[a]Data are from a search of http://www.clinicaltrials.gov performed on October 10, 2010, using the terms “carfilzomib” and “multiple myeloma”

chymotrypsin-like proteasome activity, with an IC_{50} for the β5 subunit of 36 nM, and for the low molecular mass polypeptide (LMP)-7 immunoproteasome subunit of 82 nM [35]. Following oral dosing in rodents and dogs, proteasome inhibition in excess of 80% could be achieved in virtually all tissues examined, except for the brain, with an onset in 15 min, which was comparable to that of intravenous carfilzomib. Doses needed to achieve this level of inhibition were up to tenfold below the maximum tolerated dose and were tolerated on a daily for 5 days in a row schedule [34, 36]. Murine studies with ONX 0912 in BNX mice in vivo utilizing RL cell- and CT-26 cell-based models of human non-Hodgkin lymphoma and colorectal carcinoma, respectively, showed significant antitumor activity using oral dosing on days 1 and 2 of each week [35]. With respect to plasma cell dyscrasias, activity was also seen in an in vivo model of human multiple myeloma based on MM1.S cells [34]. Waldenström macroglobulinemia may be another attractive target, since ONX 0912 induced cytotoxicity in primary Waldenström and IgM-secreting lymphoma cell lines through proteasome inhibition, suppression of NF-κB, activation of c-Jun-N-terminal kinase, and induction of the unfolded protein response [37].

Combination regimens based on ONX 0912 have been investigated preclinically as well, and as was the case for carfilzomib, combinations with inhibitors of CDK-4/6 appear to be attractive. By using the CDK-4/6 inhibitor PD 0332991 to induce a G1 arrest, myeloma cell lines and primary cells were sensitized to ONX 0912 in a synergistic manner even in the presence of protective stromal cells. This combination was active through mitochondrial membrane depolarization and activation of caspase-9, as well as induction of proapoptotic BH3-only proteins such as Bcl-2-interacting mediator of cell death (Bim), which presumably negated the effects of antiapoptotic proteins such as Bcl-2 [22, 38]. ONX 0912 could also have promise in combination with bortezomib [37], since this regimen showed synergistic cell killing of Waldenström cells.

7.2.3 Marizomib

Salinosporamide A, later renamed NPI-0052, and more recently marizomib, was initially isolated as a metabolite of Salinispora tropica strain CNB-440, a seawater-requiring marine actinomycete [39]. This agent, which is being developed by Nereus Pharmaceuticals (San Diego, CA), is structurally related to omuralide and lactacystin [40] and can now be chemically synthesized quite efficiently through a number of approaches [41], making it accessible for large-scale preclinical and clinical studies.

7.2.3.1 Biological Basis

Initial studies of marizomib in models of multiple myeloma showed that it was able to suppress all three major proteolytic activities of the proteasome [42]. In comparison

Table 7.4 Comparison of the ability of bortezomib and marizomib to inhibit the proteolytic activities of the 20S proteasome[a]

Proteasome activity	Bortezomib	Marizomib
Trypsin-like	67 nM	2 nM
Caspase-like	10 nM	34 nM
Chymotrypsin-like	7.9 nM	3.5 nM

[a]Data represent the concentration needed to reduce the indicated proteasome activity by 50% and are derived from reference [42]. Please note that, due to the use of different assays and conditions, data from Tables 7.1, 7.4, and 7.5 are not strictly comparable

with bortezomib, it was a more potent inhibitor of the ChT-L activity in erythrocyte proteasomes and a much more potent inhibitor of the T-L activity, though a weaker inhibitor of the PGPH activity (Table 7.4). Notably, using maximally tolerated doses of both agents in an in vivo model, marizomib provided a longer duration of ChT-L activity inhibition than bortezomib and suppressed the T-L activity, whereas bortezomib actually stimulated it, though, in agreement with the in vitro data, it was a weaker inhibitor of the PGPH function. Later studies have shown that marizomib may also be able to provide a longer duration of ChT-L activity suppression in tumor tissues as compared to some other organs such as peripheral blood [43]. Functional assays showed that marizomib blocked activation of NF-κB more potently than was the case for bortezomib [42]. Indeed, other studies have documented that marizomib is not only superior to bortezomib in this regard but also compared to other proteasome inhibitors such as MG-132, ALLN, and lactacystin [44]. Also, in primary samples, it induced programmed cell death with DNA fragmentation to a greater extent, was able to overcome both adhesion- and cytokine-mediated drug resistance, and retained activity in samples from bortezomib-refractory patients [42]. Using a human plasmacytoma xenograft model, marizomib was shown to be able to significantly delay tumor growth and to prolong survival. Apoptotic induction was associated with activation of caspases-8, -9, and -3, but studies with dominant negative constructs showed a greater reliance for cell death on the caspase-8-dependent arm than was the case for bortezomib. Other mechanisms that appeared to contribute to cell death included mitochondrial release of cytochrome c and Smac, cleavage of poly-(ADP-ribose) polymerase, and activation of Bax. Finally, marizomib may have other benefits for patients with multiple myeloma, based on preclinical studies that documented its ability to inhibit tumor necrosis factor-mediated receptor activator of NF-κB ligand (RANKL)-induced osteoclastogenesis [44].

As is the case for other proteasome inhibitors, marizomib may prove most active in combination with other agents. Intriguingly, synergistic anti-myeloma activity has been seen in dexamethasone-sensitive MM1.S cells and in dexamethasone-resistant MM1.R cells, when bortezomib and marizomib were combined [42]. These latter findings were later confirmed in other multiple myeloma cell lines and in primary patient samples [45], and this regimen was found to suppress myeloma cell migration and measures of angiogenesis. Combination proteasome inhibitor therapy was more active against in vivo models of multiple myeloma and was effective

through enhanced activation of caspases and JNK, as well as increased suppression of NF-κB. Dual targeting strategies of this type have also shown promise in preclinical studies in models of Waldenström macroglobulinemia [46]. Another combination of interest for myeloma may be that of marizomib and the immunomodulatory agent lenalidomide, which have been shown to interact synergistically through induction of caspases-8, -9, -3, and -12, cleavage of poly-(ADP-ribose) polymerase, and activation of Bim [47]. These findings were also borne out in studies of in vivo models, where low-dose combinations of marizomib and lenalidomide significantly inhibited tumor growth and also prolonged survival. Finally, regimens of marizomib with inhibitors of CDK-4/6 are also intriguing, based on studies showing that G1 arrest markedly sensitized primary myeloma cells to proteasome inhibitors, including bortezomib and NPI-0052 [48].

7.2.3.2 Clinical Development

Marizomib is currently being studied predominantly in the phase I setting, with trials focusing on patients with solid tumors, as well as with hematologic malignancies, including multiple myeloma. The first-in-man study dosed marizomib once weekly for 3 weeks out of every 4-week cycle (NCT00396864) and did not initially report any dose-limiting toxicities. However, one serious adverse event was seen in the form of an episode of methicillin-resistant Staphylococcus aureus sepsis and postinfectious glomerulonephritis with renal failure, which did recover after antibiotic treatment [49]. Interestingly, preclinical studies have shown that the renal medulla and cortex are areas in which there is substantial accumulation of marizomib [43], and the previously noted episodes of renal insufficiency with carfilzomib do suggest caution in the use of irreversible inhibitors in patients with renal compromise. Later updates did not reveal any further renal adverse events, and toxicities included nausea, vomiting, diarrhea, fatigue, muscle stiffness, dizziness, headache, insomnia, hypotension, hypomagnesemia, anemia, and febrile neutropenia, with minimal thrombocytopenia or neuropathy [50, 51]. Proteasome inhibition of up to 100% was seen in peripheral whole blood, with a return to baseline within 1 week of each dose. Responses were not seen in patients with solid tumors, though stable disease was noted in patients with cervical carcinoma, as well as others with colorectal, hepatocellular, adenoid cystic, melanoma, granulosa cell, and ovarian tumors [51]. A second study using the same dosing schedule (NCT00629473)[52] has reported two DLTs, including one of dizziness and an unsteady gait, while another was described as “transient hallucinations” with “visual imprints when (the patient’s) eyes (were) closed [53].” Stable disease was seen as the best outcome in this study as well, including in patients with mantle cell and follicular non-Hodgkin lymphoma, Hodgkin lymphoma, sarcoma, prostatic adenocarcinoma, and melanoma. Later, this study was amended to include a bortezomib-like twice-weekly dosing, which produced common adverse events including fatigue, dysgeusia, reversible infusion site pain, lymphopenia, headaches, dizziness and/or unsteady gait, and changes in cognition [54]. A clinical benefit, including either stable disease

or evidence of regression, was then noted in a larger array of patients, including some with myeloma and cutaneous marginal zone lymphoma.

One study has focused exclusively on patients with relapsed and/or refractory multiple myeloma and also using the weekly for three out of every 4-week schedule (NCT00461045) [55]. Dose-limiting toxicities included fatigue and mental status changes with loss of balance, and other patients required dose reductions due to nausea and vomiting, as well as vertigo and confusion with word-finding difficulties. These toxicities have since been ameliorated with the addition of prophylactic anti-emetics and with meclizine. An unconfirmed partial response was seen in one patient with IgA myeloma who was bortezomib-exposed and bortezomib sensitive, along with one minor response, and several patients had stable disease, including two who were previously bortezomib refractory.

7.3 Reversible Proteasome Inhibitors

While irreversible inhibitors have the theoretical advantage of binding and inhibiting the proteasome for an extended period of time, preclinical and clinical studies have shown that their duration of inhibition is only modestly longer than what would be expected for bortezomib [17, 18, 24, 42–44]. These findings suggest that new proteasome synthesis and/or assembly remains the predominant mechanism for recovery of proteolytic function in cells challenged with proteasome inhibitors. In the absence of a clear advantage for irreversible agents, therefore, reversible inhibitors with properties distinct from bortezomib, such as CEP-18770 and MLN9708, are moving forward in development for multiple myeloma and other malignant and even nonmalignant diseases.

7.3.1 CEP-18770

7.3.1.1 Biological Basis

CEP-18770 is being developed by Cephalon, Inc. (Frazer, PA) and is a dipeptide boronic acid which, like bortezomib, contains leucine in the P1 position. Unlike bortezomib, which has a phenylalanine in the P2 position, CEP-18770 contains threonine in this location instead [56–58], possibly reducing its hydrophobicity. Preclinical studies in myeloma models demonstrated the ability of CEP-18770 to inhibit the chymotrypsin-like proteasome activity with comparable potency to that of bortezomib. Slightly weaker inhibition was seen of the caspase-like activity by CEP-18770 than with bortezomib, while neither agent impacted on the trypsin-like function. Consistent with its ability to target the proteasome, CEP-18770 inhibited tumor necrosis factor-mediated activation of NF-κB by stabilizing IκB. It induced

apoptosis mediated by caspases-3, -7, and -9 in cell line and primary myeloma models and reduced endothelial cell survival, proliferation, and tubular morphogenesis. Also, this agent was shown to suppress macrophage colony-stimulating factor/ receptor activator for NF-κB ligand-mediated osteoclastogenesis. Notably, CEP-18770 showed enhanced antitumor activity and increased levels of tumor proteasome inhibition compared to bortezomib in an in vivo myeloma model when both agents were administered intravenously. This occurred in conjunction with reduced cytotoxic effects on bone marrow stromal cells and a lesser impact on colony formation by bone marrow progenitor cells of both myeloid and erythroid lineages [57], suggesting the possibility that it may have a superior therapeutic index. More recent studies have shown that CEP-18770 could be combined with melphalan or bortezomib to induce synergistic anti-myeloma activity in vitro, that it could overcome either melphalan- or bortezomib-resistant tumors in vivo, and that it was effective with oral dosing [59].

7.3.1.2 Clinical Development

These encouraging preclinical findings have supported the translation of CEP-18770 to the clinic. One phase I study that administered CEP-18770 as an intravenous infusion on days 1, 4, 8, and 11 of every 21-day cycle to patients with solid tumors and non-Hodgkin lymphoma was completed in Europe (NCT00572637), but results of this trial have not yet been reported. A second, international phase I/II study to evaluate the safety and efficacy of CEP-18770 given intravenously on days 1, 8, and 15 of every 28-day cycle is currently underway in patients with relapsed and refractory multiple myeloma (NCT01023880). Finally, a combination study of CEP-18770 with lenalidomide and dexamethasone is being planned as well. All of these will be following the pharmacokinetics of CEP-18770 using a novel, high-pressure liquid chromatography/mass spectrometry-based technique to determine plasma drug levels [60].

7.3.2 MLN9708

7.3.2.1 Biological Basis

The first proteasome inhibitor to have reached the clinic in an oral formulation is MLN9708, which is being developed by Millennium: The Takeda Oncology Company (Cambridge, MA). This dipeptide has leucine in the P1 position and glycine in the P2 position and is a prodrug with a protected cyclic boron that is hydrolyzed to the active boronic acid, MLN2238, upon exposure to aqueous solutions or plasma [61]. MLN2238 preferentially bound to the β5 constitutive proteasome subunit with comparable potency to that of bortezomib (IC_{50} 3.4 nM/L for the former

Table 7.5 Comparison of the ability of bortezomib and MLN2238 to inhibit the proteolytic subunits of the 20S proteasome[a]

Proteasome activity	Bortezomib	MLN2238
Trypsin-like	1,200 nM	3,500 nM
Caspase-like	24 nM	31 nM
Chymotrypsin-like	2.4 nM	3.4 nM

[a]Data represent the concentration needed to reduce the indicated proteasome activity by 50% and are derived from reference [61]. Please note that, due to the use of different assays and conditions, data from Tables 7.1, 7.4, and 7.5 are not strictly comparable

versus 2.4 for the latter), and the two showed similar abilities to inhibit activation of NF-κB in cell-based assays. Substantially weaker binding was seen to the β1 and β2 subunits in a pattern that was also similar to bortezomib, but the binding affinity seemed even weaker than was the case for its predecessor (Table 7.5). A major difference was seen in the proteasome dissociation half-life, which was 110 min for bortezomib, but only 18 min for MLN2238, suggesting the possibility of a more rapid recovery of proteasome function, which was confirmed in washout studies in cell culture models in vitro. While this may at first seem to be a disadvantageous feature, it could in fact be a strength compared to bortezomib, if MLN2238 could more rapidly dissociate from its binding sites on proteasomes in the blood and redistribute into tumor tissues bindings. Consistent with this possibility, MLN2238 showed a greater blood volume of distribution than bortezomib at steady state in in vivo studies utilizing maximum tolerated doses of each agent [61].

In xenograft models of human lymphoma and prostate cancer, MLN2238 showed comparable peak blood proteasome inhibition levels to that of bortezomib but a shorter area under the effect versus time curve. In contrast, in tumor tissue itself, treatment with MLN2238 induced a greater and more sustained level of proteasome inhibition, as well as of downstream pharmacodynamic markers, including accumulation of growth arrest DNA damage 34. Three models were evaluated for antitumor activity, including one of prostate cancer using CWR22 cells and both subcutaneous and disseminated models of lymphoma using WSU-DLCL2 and OCI-Ly7-Luc cells, respectively. Whereas bortezomib showed modest activity against the CWR22 model system, MLN2238 induced a significantly greater growth delay, and comparable findings were obtained in both lymphoma models [61]. Subsequent studies have shown the ability of MLN2238 to retain activity in a lymphoma model that was resistant to bortezomib therapy [62].

One in vivo plasma cell dyscrasia model, the double transgenic F1 hybrid iMyc^{Ca}/Bcl-x_L mouse, which develops plasma cell malignances with a short onset, has also been studied to determine the activity of MLN2238. Pharmacodynamic studies showed that 83–84% proteasome inhibition was achieved in both the blood and marrow compartments [63]. Treatment with MLN9708, as well as with bortezomib, produced a reduction in tumor burden and a significant prolongation in the median tumor-free survival [63–65].

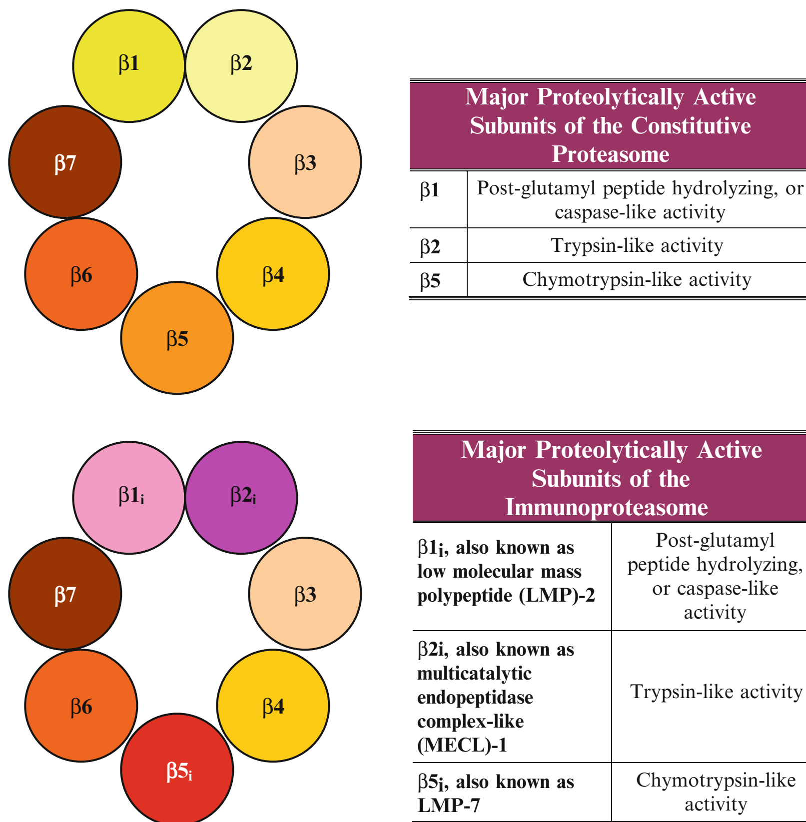
7.3.2.2 Clinical Development

Clinical trials of MLN9708 are currently underway utilizing both intravenous as well as oral dosing, and one report has been presented of a study in patients with non-hematologic malignancies [66]. In this trial (NCT00830869), patients received MLN9708 intravenously on the standard bortezomib schedule of days 1, 4, 8, and 11 of every 21-day cycle. Common adverse events have included fatigue, nausea, and pyrexia, grade 3/4 adverse events included anemia and thrombocytopenia, and dose-limiting toxicities included rash, reversible thrombocytopenia, and reversible renal failure. The available pharmacokinetic data suggested that MLN9708 showed a dose-proportional systemic exposure and a half-life of about 7 days after dosing on day 11. Moreover, reversible blood target inhibition was seen as predicted from the preclinical studies, with substantial return of proteasome function to normal within 2–4 h of dosing. A second trial evaluating MLN9708 using intravenous dosing on days 1, 8, and 15 of every 28-day cycle is targeting patients with Hodgkin and non-Hodgkin lymphoma (NCT00893464).

MLN9708 is also being studied in patients with relapsed and refractory multiple myeloma. One of these is a phase I trial of oral MLN9708, which is being administered on days 1, 8, and 15 of an every 28-day cycle (NCT00963820). The second is a phase I/II study using the standard bortezomib schedule of days 1, 4, 8, and 11 given every 21 days (NCT00932698). Data from these trials will hopefully be available for presentation at the 2010 meeting of the American Society of Hematology.

7.4 Immunoproteasome Inhibitors

The proteasome variant expressed in most somatic tissues is known as the constitutive proteasome, and its 20S catalytic core contains at least three subunits with proteolytic activity, known as β1, β2, and β5 (Fig. 7.1). In the presence of cytokines such as tumor necrosis factor α or γ-interferon, however, production of three alternate subunits, known as low molecular mass polypeptide (LMP)-2, multicatalytic endopeptidase complex subunit (MECL)-1, and LMP-7, or $\beta1_i$, $\beta2_i$, and $\beta5_i$, is stimulated. These subunits may be preferentially incorporated into new proteasomes under these conditions to replace β1, β2, and β5, respectively, producing a variant known as the immunoproteasome [7–9, 67]. This has been so named due to data supporting a role for its ability to generate more hydrophobic, antigenic peptides that can be presented in the context of major histocompatibility class I molecules [67]. Interestingly, cells of hematopoietic origin normally express the immunoproteasome, which may be present in conjunction with the constitutive proteasome in myeloma, while in some cases it is the predominant isoform found in primary plasma cells [18, 68]. Given the restricted, tissue-specific expression of the immunoproteasome, it may represent a target for myeloma therapy whose inhibition could provide an enhanced therapeutic index due to the paucity of expression in neural and gastrointestinal tissues. Immunoproteasome-specific inhibitors could therefore



Major Proteolytically Active Subunits of the Constitutive Proteasome	
β1	Post-glutamyl peptide hydrolyzing, or caspase-like activity
β2	Trypsin-like activity
β5	Chymotrypsin-like activity

Major Proteolytically Active Subunits of the Immunoproteasome	
β1i, also known as low molecular mass polypeptide (LMP)-2	Post-glutamyl peptide hydrolyzing, or caspase-like activity
β2i, also known as multicatalytic endopeptidase complex-like (MECL)-1	Trypsin-like activity
β5i, also known as LMP-7	Chymotrypsin-like activity

Fig. 7.1 *Proteasome variants that have been validated as targets for multiple myeloma*. The constitutive proteasome is present in most tissues in the body and consists of a barrel-shaped structure with four rings surrounding a central pore. Each of the inner two rings contains seven unique β subunits, three of which encode the major proteolytic activities of the proteasome. A cross section through one of these rings of the constitutive proteasome is shown in the *top panel*, whereas the *bottom panel* shows a comparable cross section through the immunoproteasome

target the proteasome specifically in hematologic malignancies, unlike agents such as bortezomib and carfilzomib, which do not discriminate between the constitutive and immunoproteasomes.

7.4.1 *Peptide Aldehyde Inhibitors*

The first immunoproteasome-specific inhibitor (IPSI) developed and tested against models of multiple myeloma was IPSI-001, a dipeptide aldehyde with norleucine in the P1 position and leucine in the P2 position [68]. Screening efforts using purified

constitutive and immunoproteasome preparations suggested that amino acid residues with greater hydrophobic character in the P1 position, such as norleucine or phenylalanine, provided a measure of immunoproteasome specificity. Consistent with this hypothesis, IPSI-001 showed a more than 100-fold increased potency to inhibit the chymotrypsin-like and branched chain amino acid preferring activities of the immunoproteasome over the constitutive proteasome. This agent bound specifically to the $\beta 1_i$ subunit both in vitro and in cellulo, which was to some extent unexpected, since $\beta 5_i$ contains the chymotrypsin-like activity, suggesting that binding to $\beta 1_i$ caused an allosteric shift that precluded substrate entry into the $\beta 5_i$ binding site. IPSI-001 induced accumulation of ubiquitin-protein conjugates, including ubiquitinated IκBα, proteasome substrates such as p21, and activated Bax as well as c-Jun-N-terminal kinase. These effects in part contributed to stimulation of programmed cell death through both caspase-8- and caspase-9-mediated pathways, resulting in dual downstream activation of the effector caspase-3. Notably, these effects were preferentially seen in immunoproteasome-expressing model systems, while those expressing the constitutive proteasome were relatively spared. Importantly, IPSI-001 was able to induce cell death in patient-derived plasma cells and in primary cells from patients with other hematologic malignancies. Also, IPSI-001 overcame drug-resistant phenotypes and was even active in primary samples from patient with clinically bortezomib-refractory disease. Further studies of IPSI-001 and other related peptide aldehydes with specificity for the immunoproteasome will, however, remain restricted to the preclinical arena, since these agents do not have sufficient potency and in vivo stability to warrant clinical application.

7.4.2 Ketoepoxide Inhibitors

Immunoproteasome-specific inhibitors with enhanced potency have been developed based on the ketoepoxide pharmacophore, which may prove to be more clinically relevant. The first of these was PR-957 (Onyx Pharmaceuticals) which, like IPSI-001, was shown to target the chymotrypsin-like proteasome activity [69]. However, unlike IPSI-001, which bound to $\beta 1_i$, PR-957 bound specifically to LMP-7, or $\beta 5_i$, demonstrating the ability of a directly binding agent to inhibit the chymotrypsin-like activity of the proteasome. While this drug has not been tested against multiple myeloma, it did show the ability to block inflammatory cytokine production from mononuclear cells and attenuated progression of experimental arthritis in animal models. Of potential interest to the myeloma field was its ability to reduce production of interleukin-6, which plays a role in myeloma pathobiology [70–72], as well as in resistance to drugs such as bortezomib [73] and dexamethasone [74].

A second ketoepoxide immunoproteasome-specific inhibitor that has been studied in models of multiple myeloma is PR-924 [75]. This agent was also found to be LMP-7 selective and exerted antiproliferative and proapoptotic effects with drug concentrations in the micromolar range. PR-924 was able to overcome resistance to standard chemotherapeutics such as dexamethasone, doxorubicin, and melphalan

and also subverted resistance due to cell-mediated adhesion to stroma, as well as resistance due to cytokines such as interleukin-6. At the molecular level, PR-924 activated caspases-8, -9, and -3, reduced levels of antiapoptotic Bcl-2, induced cleavage of (BH3-interacting domain death agonist) Bid to tBid, and caused loss of the normal trans-mitochondrial membrane potential with migration of cytochrome c into the cytoplasm. Finally, PR-924 was active against myeloma in both a severe combined immunodeficiency-hu model and a human plasmacytoma xenograft model.

While the data with IPSI-001 and PR-924 provide a strong rationale for translation of immunoproteasome-specific agents to the clinic to fight multiple myeloma, it should be noted that one study has suggested that inhibiting the immunoproteasome alone is not sufficient to induce cytotoxicity. Using a different specific ketoepoxide compound, these investigators found that, in MM1.S myeloma cells, inhibition of either the constitutive proteasome alone or the immunoproteasome alone did not reduce cell viability [18]. Only when these agents were combined, or when carfilzomib was used, which inhibits both proteasome variants, was there substantial cytotoxic activity. Moreover, these findings were paralleled by the effects of these agents on intracellular accumulation of ubiquitin-protein conjugates, which were marginal with either specific inhibitor alone but substantial with the combination or carfilzomib. Further preclinical studies seem therefore to be in order to validate the potential of immunoproteasome inhibitors before their translation into the clinic.

7.5 Conclusions

Second-generation, novel proteasome inhibitors are making significant progress both preclinically and clinically along the drug development path leading to regulatory approvals. Among irreversible inhibitors, carfilzomib and marizomib, which may bind the proteasome more exclusively than other proteases or with broader specificity compared to bortezomib, respectively, have already reached the clinic. Carfilzomib has shown activity in relapsed and relapsed/refractory myeloma, and though there is evidence for cross-resistance in patients with prior bortezomib therapy, it appears to have a more favorable toxicity profile, especially in regard to peripheral neuropathy. A combination regimen with lenalidomide and dexamethasone has also shown encouraging tolerability and activity, and regimens using higher doses of carfilzomib starting with the second cycle may enhance the efficacy of this agent further. Marizomib has also shown activity against multiple myeloma in a smaller number of studies, and, while like carfilzomib it does not appear to confer a significant risk of peripheral neuropathy, other neurologic effects have been noted. Interestingly, unlike bortezomib, which can be safely given in patients with renal failure without dose adjustments [76–78], early phase studies of both carfilzomib and marizomib have documented rare episodes of treatment-emergent renal insufficiency. This suggests that formal studies of these agents in patients with renal impairment will be needed, and indeed one such study with carfilzomib is already underway (NCT00721734).

Reversible inhibitors are being developed as well, including novel boronic acids that can be delivered either intravenously or orally, and which may have superior pharmacokinetics to bortezomib. The latter agents, if ultimately approved, could also more easily be used in settings such as maintenance after either standard-dose induction therapy or after high-dose therapy with autologous stem cell transplantation. More targeted agents that suppress only the immunoproteasome may have a role to play, though there is disagreement in the literature as to whether immunoproteasome inhibition is by itself sufficient to induce programmed cell death in models of multiple myeloma. Finally, and perhaps most intriguingly, combination regimens with more than one proteasome inhibitor have shown enhanced preclinical activity. If similar synergy were seen clinically, these agents could possibly be used at lower doses to achieve the same or even a superior antitumor efficacy, with the potential for a much reduced toxicity profile. Taken together, these findings strongly argue that proteasome inhibitors will not only remain part of our arsenal against multiple myeloma but will probably play an ever increasing role in our armamentarium against this disease.

Abbreviations

Bax	Bcl-2-associated X protein
Bcl-2	B cell CLL/lymphoma-2
BH3	Bcl-2 homology 3
Bid	BH3-interacting domain death agonist
BIM	Bcl-2-interacting mediator of cell death
Bor	Bortezomib
CDK	Cyclin-dependent kinase
ChT-L	Chymotrypsin-like
C-L	Caspase-like
CR	Complete remission
DLT	Dose-limiting toxicity
DOR	Duration of response
IκB	Inhibitor of nuclear factor kappa B
IPSI	Immunoproteasome-specific inhibitor
ISS	International Staging System
JNK	c-Jun-N-terminal kinase
Len	Lenalidomide
LMP	Low molecular mass polypeptide
Mcl-1	Myeloid cell leukemia sequence 1
MECL	Multicatalytic endopeptidase complex-like
MR	Minor response
MTD	Maximum tolerated dose
NF-κB	Nuclear factor kappa B
ORR	Overall response rate

PBMCs	Peripheral blood mononuclear cells
PGPH	Post-glutamyl peptide hydrolyzing also referred to as the caspase-like (C-L) activity
PR	Partial remission
RANKL	Tumor necrosis factor-mediated receptor activator of NF-κB ligand
sCR	Stringent CR
Smac	Second mitochondria-derived activator of caspases
T-L	Trypsin-like
Thal	Thalidomide
TTP	Time to progression
UPR	Unfolded protein response
VGPR	Very good PR

Acknowledgements The author would like to acknowledge research support from the National Cancer Institute (P50-CA-142509, P01-CA-124787, R01-CA-102278, R01-CA-134786), as well as the Multiple Myeloma Research Foundation and The Leukemia & Lymphoma Society.

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Chapter 8
Newer IMiDs

Abhishek Singla and Shaji Kumar

8.1 Introduction

Immunomodulatory drugs (IMiDs) are a series of compounds that were developed by using the first-generation IMiD thalidomide as the lead compound. Thalidomide, initially introduced as a sedative and used for morning sickness, was withdrawn from the market in the early sixties after it was found to be a teratogen. However, it was later found to be beneficial in the treatment of erythema nodosum leprosum, oral ulcers, graft vs. host disease, and wasting associated with the human immunodeficiency syndrome. Its anti-angiogenic properties were recognized in the early nineties and led to the evaluation of thalidomide as an anti-angiogenic agent in the treatment of several cancers. Following initial trials in relapsed and newly diagnosed multiple myeloma (MM), where it was used alone or in combination with dexamethasone and other anti myeloma agents, it became part of standard therapy for the treatment of MM. The thalidomide structural backbone was used as a template to design and synthesize compounds with increased immunological and anticancer properties but lacking the toxicity associated with the parent compound. In the mid-1990s, a series of amino-phthaloyl-substituted thalidomide analogues were generated, and these were found to be up to 50,000 times more potent at inhibiting TNF- than the parent compound in vitro [1]. Further preclinical testing of these compounds led to the identification of second-generation IMiDs namely lenalidomide (Revlimid, CC-5013) and pomalidomide (Actimid, CC-4047) for study in

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N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 2: Translational and Clinical Research*,
DOI 10.1007/978-1-4614-5260-7_8, © Springer Science+Business Media New York 2013

clinical trials for patients with myeloma. The introduction of these IMiDs and other novel therapeutic agents such as bortezomib has favorably affected the survival of patients with myeloma in the last decade [2].

8.2 Lenalidomide (Revlimid)

8.2.1 Preclinical Studies

The chemical name of lenalidomide is 3-(4-amino-1-oxo 1,3-dihydro-2H-isoindol-2-yl) piperidine-2,6-dione and the empirical formula is $C_{13}H_{13}N_3O_3$. The mechanism of action of lenalidomide involves direct cytotoxicity as well as indirect effects by modulating different components of the immune system such as altering cytokine production, inhibiting angiogenesis, regulating T cell co-stimulation, and augmenting the NK cell cytotoxicity.

8.2.1.1 Alteration of Cytokines

Lenalidomide inhibits the production of pro-inflammatory cytokines TNF-α, IL-1, IL-6, and IL-12 and elevate the production of anti-inflammatory cytokine IL-10 from human PBMCs [3]. Reduction in IL-6 and TNF-α levels can partially explain the action of lenalidomide in multiple myeloma. IL-6 inhibits the apoptosis of malignant myeloma cells and helps in their proliferation [4]. Lenalidomide downregulates the production of IL-6 directly and also inhibits multiple myeloma—bone marrow stromal cell (BMSC) interaction [5, 6], which augments the apoptosis of myeloma cells [7]. The precise mechanism of TNF-α downregulation by lenalidomide is not known; however, like thalidomide, it possibly increases the degradation of TNF-α mRNA [8]. The downregulation of TNF-α secretion is up to 50,000 times more when compared to thalidomide [1].

8.2.1.2 T Cell Activation

Besides stimulation of T cell receptor (TCR), a secondary interaction of B7 molecule on APC and CD28 on the T cell surface provides a co-stimulatory signal that augments the T cell response followed by a cascade of cytokine and cellular responses [9]. Lenalidomide and other IMiDs directly induce tyrosine phosphorylation of CD28 on T cells leading to activation of downstream targets such as PI3K, GRB-2-OS, and NF-κb. There is an increase in Th1 type cytokine response resulting in increased genetic expression of IL-2 and IFN-γ which subsequently increases T cell and natural killer (NK) cell-mediated lysis of myeloma cells [3, 10]. IMiDs have been shown to stimulate both cytotoxic $CD8^+$ as well as helper $CD4^+$ cells [11].

Their effects on T helper cells can also potentially mediate Th1 type antitumor immunity in response to tumor cell vaccination in animal models [10].

8.2.1.3 Augmentation of NK Cell Function

Natural killer (NK) cells are an important component of innate immunity against cancer cells and kill the cell with antibody-dependent cell-mediated cytotoxicity (ADCC) and natural cytotoxicity. Modulation of NK cell function is also believed to contribute to the antitumor activity of lenalidomide in MM. Treatment with thalidomide is accompanied by increased NK cell numbers as well as IL-2 levels, and the mechanism is probably indirect. Hayashi et al. in their study of IMiDs in MM cell lines have demonstrated that culturing PBMC with IMiDs leads to 1.2–1.3-fold increase in the percentage of CD56 cells [12]. IMiDs enhanced ADCC when 51 Cr-labeled MM cells that express CD40 were incubated with rhuCD40 and then subsequently treated with PBMC cells incubated in the presence of IMiDs for 5 days. The increase in NK cell function may be related to the increase in IL-2 production by the T cells as the presence of a monoclonal Ab against IL-2 R blocked the NK cell cytotoxicity. IMiDs also were shown not to directly activate the NK cells, as evidenced by lack of phosphorylation of signaling molecules (ERK/p38MAPK/Akt/PKC) in NK cells [12]. Lenalidomide-enhanced Fc-γ receptor signaling may also play a role in increasing the potency of NK cells.

8.2.1.4 Anti-angiogenic Activity

Thalidomide and IMiDs has been shown to have antiangiogenic properties that are independent of their immunomodulatory effects [13, 14]. Tumor associated endothelial cells are more dependent on the VEGF receptor signaling for growth and survival compared to normal endothelial cells [15]. Early studies showed that thalidomide had anti angiogenic activity in a rabbit model of corneal neovascularization that was induced as a response to bFGF [13]. Thalidomide and the newer IMiDs have also been shown to significantly decrease the expression of angiogenic factors VEGF and IL-6 in multiple myeloma [16]. The overall superiority of newer IMiDs over thalidomide regarding antiangiogenic effect is controversial [14, 17], but the data suggests that thalidomide is a potent inhibitor of endothelial cell migration whereas lenalidomide and pomalidomide are more potent inhibitors of other aspects of the angiogenic process, such as inhibition of endothelial cell attachment, migration, and differentiation [14]. Apart from alteration in the levels of VEGF, lenalidomide partially inhibits Akt phosphorylation after VEGF stimulation in endothelial cells and also has inhibitory effects on phosphorylation of Gab1, a protein upstream of Akt 1 [18, 19]. These observations demonstrate that IMiDs may affect angiogenesis by multiple mechanisms.

8.2.1.5 Direct Antitumor Activity

Lenalidomide treatment has also shown anti proliferative activity against MM cells in the absence of immune effector cells [20]. Malignant plasma cells derived from refractory cases of myeloma were shown to be susceptible to IMiD-induced growth arrest. Lenalidomide upregulates cyclin-dependent kinase (CDK) inhibitor, p21 waf-1, a key cell cycle regulator that modulates the activity of CDKs. Similarly reductions in CDK2 activity have been demonstrated in myeloma-derived cell lines, U266 and LP-1 [21]. In contrast, the normal B cells obtained from healthy donors were immune from growth inhibition and did not show any upregulation of p21 expression after 3 days of lenalidomide treatment. In other studies, thalidomide and its analogues have also been shown to induce apoptosis in MM cell lines [22]. Effects on apoptosis in MM cells is secondary to increased potentiation of TNF-related apoptosis inducing ligand (TRAIL), inhibition of apoptosis protein-2, increased sensitivity to Fas-mediated cell death, upregulation of caspase-8 activation, downregulation of caspase-8 inhibitors (FLIP, cIAP2), downregulation of NF-κb activity, and inhibition of prosurvival effects of IGF-1 [23].

8.2.1.6 Effects on Multiple Myeloma Microenvironment

In multiple myeloma, osteoclasts lead to bone resorption and secrete survival factors for MM cells. The interaction between MM cells and BMSC in turn leads to increased production of IL-6 and other growth factors for MM cells and osteoclasts [24]. Lenalidomide alters the myeloma microenvironment by directly decreasing the formation of tartrate-resistant acid phosphatase (TRAP) positive cells which form osteoclasts [5]. Additionally, it decreases αVβ3-integrin levels, an adhesion molecule needed for osteoclast activation, and downregulates cathepsin K, a major cysteine protease expressed in osteoclasts, pertinent for matrix degradation in the resorption process [5]. It downregulates the important mediators of osteoclastogenesis such as transcription factor PU.1 and MAP kinase pERK and reduces the levels of bone remodeling factor-receptor activator of NF-κb ligand. IMiDs are also known to decrease the cell surface adhesion molecules such as ICAM-1, VCAM-1, and E-selectin and inhibit the adhesion of MM cells to BMSC [6]. Thus, lenalidomide interferes with the synergism among the osteoclasts, MM cells, and BMSC and decreases osteoclastogenesis by acting at various levels.

8.2.2 Safety

Though teratogenicity of lenalidomide in humans is not proven, its structural similarity to thalidomide and induction of malformations in the offspring of female monkeys has raised concerns [25]. Caution should be taken in women with childbearing potential and in sexually active male patients.

The most common grade 3 or higher adverse events reported in MM-009/010 patients treated with Len/Dex was neutropenia found in more than one-third followed by thromboembolic events (16%), thrombocytopenia (13%), anemia (11%), and pneumonia (9%) [26]. An expanded access program (MM-016) over 1,400 similar patients showed that at least one grade 3 or 4 adverse event was reported in 70% of patients, most common being myelosuppression (45%), fatigue (10%), and pneumonia (7%) [27]. Toxicity effects noted in various studies involving lenalidomide in MM are listed in Table 8.1.

Previously untreated patients are at a lower risk for myelosuppression (12–21%) than patients with refractory or relapsing myeloma (38–69%). Neutropenia is much more common than thrombocytopenia and anemia but is generally predictable and associated with low rate of febrile neutropenia (3%) [39]. Particular vigilance needs to be kept especially during the initial cycles as the risk of myelosuppression appears to be highest during this phase [40]. Myelosuppression can usually be managed with growth factor support and/or lenalidomide dose reductions but may require discontinuation of treatment in a few (less than 4%) [27].

The risk of venous thromboembolism (VTE) is low when lenalidomide is given as monotherapy but increases significantly when it is used in combination with dexamethasone, particularly at high dose as well as with concomitant administration of erythropoietic agents [36, 41, 42]. The risk also appears to increase in combinations with cytotoxic chemotherapy, particularly anthracyclines [42]. The incidence of VTE in patients treated with Len-Dex without thromboprophylaxis in MM-009/010 was 16% [26]. However most recent studies have shown that prophylaxis with low-molecular-weight heparin (LMWH) or low-dose aspirin effectively reduces the risk of VTE to less than 5%, which is comparable to the background risk in patients with MM [42–46]. Like myelosuppression, risk of venous thromboembolism (VTE) also appears to be highest during the initial cycles [40]. In a pooled analysis, 60% thrombotic events occurred between the third and sixth cycle of treatment [47]. Uncommonly arterial thrombosis such as in coronary arteries leading to myocardial infarction can also occur [47]. In patients who develop VTE, it is reasonable to briefly discontinue lenalidomide and resume the treatment when full anticoagulation has been established [48]. Low-dose aspirin (81–100 mg) provides sufficient thromboprophylaxis for patients with standard risk of VTE during Len/Dex therapy, while LMWH for at least the first four cycles should be considered for patients with a higher risk of VTE, especially immobilized patients and those with a history of VTE [46, 49].

Fatigue is very frequently encountered and is a common reason for treatment discontinuation in elderly patients with MM. Common causes of fatigue, such as anemia, hypothyroidism, infection, and depression should be ruled out [49]. Infections are common and combination with dexamethasone therapy increases the risk. Routine antibiotic prophylaxis should be considered for the first 3 months of therapy and is particularly recommended for patients with aggressive disease, history of infectious complications, or neutropenia.

More than a fifth of patients suffer from neurological complications such as dizziness (20%), headache (21%), and/or insomnia (32%). Unlike bortezomib and thalidomide,

Table 8.1 Toxicity profile of lenalidomide

Regimen	Study	Phase	Dose	Hematological complications (grade ≥ 3)	VTE	Prophylaxis	Non-hematological complications (grade ≥ 3)	Other common complications
Relapsed/refractory								
R ± D	Richardson et al. [28]	II	Len 30 mg OD vs. 15 mg BID on days 1–21; 28-day cycles [Dex 40 mg on days 1–4 and 15–18 for suboptimum response]	Neutropenia (61%vs. 69%); leukopenia (37% vs. 34%); thrombocytopenia (31% vs. 43%); anemia (16% vs. 14%)	1.5% vs. 5.7% (on adding dex)	None	Fatigue (7% vs. 9%)	Peripheral neuropathy (10% vs. 23%), constipation (25% vs. 31%), diarrhea
R	Richardson et al. [29]	II	Len 30 mg on days 1–21; 28-days cycles	Neutropenia (60%); thrombocytopenia (39%); anemia (20%)	5%; DVT (4%), PE (1%)	Some prophylaxis in 45%	Pneumonia (12%), fatigue (12%)	Peripheral neuropathy (3%), renal failure (3%)
RD vs. D	Weber et al. [30]	III	Len 25 mg vs. placebo 25 mg on days 1–21; dex 40 mg on days 1–4, 9–12, and 17–20; 28-day cycles	52.5% vs.13.7%; Neutropenia (41·2% vs.4.5%); thrombocytopenia (14·7% vs. 6.9%); anemia (13·0% vs. 5.1%)	15·7% vs. 3.4%	None	Infections (21.5% vs. 12%), pneumonia (11·4% vs. 7.4%), fatigue (6·2% vs. 6.3%)	Fatigue, insomnia, diarrhea, constipation, muscle cramps, infection
RD vs. D	Dimopoulos et al. [31]	III	Len 25 mg vs. placebo 25 mg on days 1–21; dex 40 mg on days 1–4, 9–12, and 17–20; 28-day cycles	Neutropenia (29.5% vs. 2.3%); thrombocytopenia (11.4% vs. 5.7%); anemia (8.6% vs. 6.9%)	11.4% vs. 4.6%	None	Infections (11.3% vs. 6.2%), muscle weakness (7.4% vs. 4.6%); fatigue (6.8% vs. 3.4%); asthenia (6.2% vs. 5.7%); dyspnea (2.9% vs. 1.7%)	Muscle cramps, constipation, nausea, tremor, dizziness

DVd- R	Baz et al. [86]	I/II	Liposomal doxorubicin 40 mg/m2 and vincristine 2 mg on day 1; dex 40 mg on days 1–4; len on days 1–21; 28-day cycles	Neutropenia (32%); thrombocytopenia (13%); leukopenia (8%); anemia (3%)	9%; DVT (6%), PE (3%)	Aspirin 81 mg	Infections (13%), peripheral neuropathy (5%), muscular cramps (3%), acute renal failure (5%), gastrointestinal bleeding (5%), dyspnea (2%)	Asthenia, rash
RAD	Knop et al. [87]	I/II	Len 25 mg on days 1–21; adriamycin 9 mg/m2 on days 1–4; dex 40 mg on days 1–4 and 17–20; six 28-day cycles	Neutropenia (48%), thrombocytopenia (38%), leukopenia (36.5%), anemia (16.5%)	4.5%	Aspirin 100 mg or LMWH	Infections (10.5%), pain (1.5%)	Fatigue, nausea, vomiting, diarrhea
RCD	Morgan et al. [88]	II	Len 25 mg days 1–21; cyclophosphamide 500 mg days 1, 8, 15, and 21; dex 40 mg days 1–4 and 12–15; 28-day cycles	Neutropenia 38%	14%		Infections (29%)	
RCd	Schey et al. [89]	I/II	Cyclophosphamide 300–700 mg on days 1 and 8; len 25 mg on days 1–21 and dex 20 mg days 1–4 and 8–11; 28-d cycles. MTD: 600 mg cyclophosphamide	26%	6%	Aspirin 75 mg	Infections (3%), cardiac arrhythmias (6%)	Peripheral neuropathy (6%), cramps, somnolence, constipation/diarrhea, musculo-skeletal aches and pains

(continued)

Table 8.1 (continued)

Regimen	Study	Phase	Dose	Hematological complications (grade ≥ 3)	VTE	Prophylaxis	Non-hematological complications (grade ≥ 3)	Other common complications
CPR	Reece et al. [90]	I/II	Cyclophosphamide 150–300 mg/m^2 on days 1, 8, and 15; len 15–25 mg on days 1–21 and prednisone 100 mg q 2 days; 28-day cycle	Neutropenia (29%); thrombocytopenia (22%)	6%	Asprin 81 mg	Abdominal pain/ bacteremia, hypokalemia, fatigue, sick sinus syndrome, cardiac amyloidosis, perforated diverticulum	
RV ± D	Richardson et al. [29]	I	Len 5–15 mg on days 1–14; bortz 1.0 or 1.3 mg/m2 on days 1, 4, 8, and 11; 21-day cycles. Dex 20 mg or 40 mg added for progressive disease. MTD: len 15 mg plus bortz 1.0 mg/m2.	Neutropenia (63%); thrombocytopenia (45%); anemia (18%); leukopenia (18%)	3% (on LMWH)	39% received some thromboprophylaxis	Fatigue	Diarrhea (39%), pruritus (29%), cramps(26%), and nausea (26%)
RVD	Richardson et al. [32, 33]	II	Bortz 1.0 mg/m2 on days 1, 4, 8, 11; len 15 mg on d 1–14; dex 40/20 mg/day (cycles 1–4); and 20/10 mg/day (cycles 5–8) on days 1, 2, 4, 5, 8, 9, 11, 12; eight 21-day cycles. Maintenance therapy : btz (d 1, 8); len (d 1–14); dex (d 1, 2, 8, 9)	Neutropenia (30%); thrombocytopenia (22%); lymphopenia (11%); leucopenia (9%)	2% (grade 2)	Aspirin 81 or 325 mg	Hyperglycemia(9%), hyponatremia(8%), hypophosphatemia (8%)	Sensory neuropathy (64%), fatigue (48%), neutropenia (42%), diarrhea (39%), muscle pain (39%), hyperglycemia (36%);

RD vs. VRD	Dimopoulos et al. [34]	II	RD: Len 25 mg on days 1–21; dex 40 mg on days 1–4 and 15–18 (cycle1–4)/days 1–4 thereafter; 28-day cycle vs. VRD: Bortz 1 mg/m2 on days 1, 4, 8, and 11; len 15 mg on days 2–14 and dex 40 mg on days 1–4; eight 21-day cycles	Neutropenia (28% vs. 26%); anemia (12% vs. 20%); thrombocytopenia (10% vs. 14%)	2% vs. 4%	Aspirin 100 mg	Infections (16% in both), fatigue (16% vs. 22%); elevation of serum creatinine (4% vs. 8%)	
Newly diagnosed								
RD	Lacy et al. [91]	II	Len 25 mg on days 1–21 plus dex 40 mg on days 1–4, 9–12, 17–20; 28-day cycles	Neutropenia (12%); leukopenia (9%); lymphopenia (6%); anemia (6%)	3%	Aspirin 81 mg or 324 mg	Fatigue (21%), muscle weakness (6%), anxiety (6%), pneumonitis (6%), rash (6%)	Neuropathy, constipation, depression, confusion, dizziness, dyspepsia

(continued)

Table 8.1 (continued)

Regimen	Study	Phase	Dose	Hematological complications (grade ≥ 3)	VTE	Prophylaxis	Non-hematological complications (grade ≥ 3)	Other common complications
RD vs. D	Zonder et al. [35]	III	Len 25 mg vs. placebo 25 mg on days 1–28; 40 mg dex on days 1–4, 9–12, and 17–20; three 35-day cycles. Maintenance: Len 25 mg vs. placebo 25 mg on days 1–21; 40 mg dex on days 1–4 and 15–18; 28-day cycles	Neutropenia (21% vs. 5%)	23.5% vs. 5%	Aspirin 325 mg in the second phase	Infections	
RD vs. Rd	Rajkumar et al. [36]	III	Len 25 mg on days 1–21 plus dex 40 mg on days 1–4, 9–12, and 17–20; 28-day cycles (high dose) vs. len given on the same schedule with dex 40 mg on days 1, 8, 15, and 22; 28-day cycles (low dose)	Neutropenia (12% vs. 20); anemia (8% vs. 7); thrombocytopenia (6% vs. 5%)	26 vs. 12%	Aspirin (coumadin or aspirin in expansion phase)	Infections/pneumonia (16% vs. 9%); fatigue (15% vs. 9%); hyperglycemia, cardiac ischemia, atrial fibrillation or flutter, neuropathy	

RD vs. TD	Gay et al. [37]	retrospective	Len 25 mg on days 1 to 21 vs. thal 100–400 mg/day; 28-day cycles. Dex either at high dose (40 mg orally on days 1–4, 9–12, and 17–20) or at low dose (40 mg orally on days 1, 8, 15, and 22); 28-day cycles	Neutropenia (14.6% vs. 0.6%)	9.2% vs. 15.3%		Peripheral neuropathy (0.9% vs. 10.4%), infections (13.1% vs. 8.2%), fatigue (10.1% vs. 7.1%), dermatologic toxicity (9.7% vs. 6.6%, and cardiovascular events (4.4% vs. 5.5%)
MPR	Palumbo et al. [38]	I/II	Melphalan 0.18–0.25 mg/kg on days 1–4; prednisone 2-mg/kg dose on days 1–4; len 5–10 mg on days 1–21; nine 28-day cycles. Maintenance: len alone. MTD: 0.18-mg/kg melphalan and 10-mg len	Neutropenia (67.9%); thrombocytopenia (32.1%); anemia (17.0%)	5.70%	Aspirin 100 mg	Infections (9.4%), fatigue 3.8%, rash 3.8%, vasculitis 3.8%, mood alterations 1.9%, diarrhea 1.9%

(continued)

Table 8.1 (continued)

Regimen	Study	Phase	Dose	Hematological complications (grade ≥ 3)	VTE	Prophylaxis	Non-hematological complications (grade ≥ 3)	Other common complications
BiRD	Niesvizky et al. [92]	II	Clarithromycin 500 mg BID on days 1–28; len 25 mg on days 1–21; dex 40 mg on days 1, 8, 15, 22; 28-day cycles	Neutropenia (19.4%); thrombocytopenia (22.2%); anemia (13.8%),	12.50%	Aspirin 81 mg	Infections (4%), myopathy (11.1%), rash (5.6%), diverticular abscess (5.6%), hypocalcemia (4.2%), neuromood (4.2%), tremor (4.2%)	
RCd	Kumar et al. [2]	II	Len 25 mg on days 1–21; dex 40 mg on days 1, 8, 15, and 22; cyclophosphamide 300 mg/m2 (300 mg in 20 patients) on days 1, 8, and 15; 28-day cycle	Neutropenia (60%)	13%	Aspirin 325 mg	Fatigue	
VRD	Richardson et al. [32, 33]	I/II	Bortz 1.0 or 1.3 mg/m2 on days 1, 4, 8, 11; len 15–25 mg on days 1–14; dex 40 or 20 mg on days 1, 2, 4, 5, 8, 9, 11, 12; eight 21-day cycles. Phase II: bortz 1.3 mg/m2, len 25 mg, dex 20 mg	Lymphopenia (14%); neutropenia (9%); thrombocytopenia (6%).	6%	Aspirin 81 or 325 mg	Infections (2%), sensory neuropathy (2%), fatigue (3%), hypokalemia (5%), and hypophosphatemia (5%)	Sensory neuropathy, fatigue, electrolyte imbalance

VDCR	Kumar et al. [93]	I	Cyclophosphamide 100–500 mg/m2 on days 1 and 8; bortz 1.3 mg/m2 on days 1, 4, 8, and 11; dex 40 mg on days 1, 8, and 15; len15 mg on days 1–14; eight 21-day cycles. Maintenance: bortz 1.3 mg/m2 on days 1, 8, 15, and 22); Four 42-day cycles	Neutropenia (24%); thrombocytopenia (12%); anemia (12%)	0%	Aspirin 325 mg	Peripheral neuropathy (4%), fatigue (8%), back pain (12%)	Constipation, nausea, diarrhea, vomiting, dizziness, insomnia, peripheral neuropathy

neuropathy is rarely seen with lenalidomide alone, thus making it an optimal therapeutic choice in patients with high risk or existing neuropathy [50]. Musculoskeletal problems like arthralgia, backache, and cramp are common but rarely severe.

A variety of rashes (morbilliform, acneiform, urticarial, etc.) have been described in approximately 30% of myeloma patients treated with lenalidomide with or without dexamethasone. Severe rashes requiring permanent discontinuation of lenalidomide therapy are rare [51]. Peripheral edema, dyspnea, constipation, diarrhea, and nausea are other common toxicities of this drug. Lastly, case report of lenalidomide-induced Coomb's positive autoimmune hemolytic anemia are also there [52].

8.2.3 Lenalidomide for Relapsed MM

Two large, multicenter, randomized, placebo-controlled phase III pivotal trials MM-009 ($n=353$) conducted in North America and MM-010 ($n=351$) conducted in Europe, Australia, and Israel, which collectively included 704 patients, assessed the efficacy and safety of lenalidomide plus dexamethasone vs. dexamethasone alone in patients with relapsed/refractory multiple myeloma (RRMM) [26, 30, 31]. Patients were randomized to receive either oral lenalidomide 25 mg per day or placebo for three weeks along with 40 mg oral dexamethasone for four days starting 1, 9, and 17 day of each 28-day cycle (for 4 cycles) until disease progression. After four cycles, dexamethasone (40 mg/day) was limited to days 1–4 only. The results of both studies were similar, and the pooled analysis showed that treatment with lenalidomide plus dexamethasone significantly improved overall response (OR: 60.6 vs. 21.9%, $P<0.001$), complete response rate (CR: 15.0 vs. 2.0%, $P<0.001$), time to progression (TTP: median of 13.4 vs. 4.6 months, $P<0.001$), and duration of response (DOR: median of 15.8 months vs. 7 months, $P<0.001$) compared with dexamethasone-placebo. Even at a median follow-up of 48 months for surviving patients, a significant benefit in overall survival (median of 38.0 vs. 31.6 months, $P=0.045$) was retained [26]. Thus the data confirmed the significant response and survival benefit with lenalidomide and dexamethasone, and this led to approval of lenalidomide in combination with dexamethasone for the treatment of MM in patients who have received at least one earlier therapy by the US FDA in June 2006 followed by European Medicines Agency in June 2007.

Sub-analysis of MM-09 and MM-10 by Harousseau et al. revealed that half of the patients who initially had a partial response achieved a complete or very good partial response with further treatment [53]. The probability of achieving a complete or very good partial response with continued lenalidomide treatment decreased with delayed achievement of a partial response (by cycle 4 vs. later); however, it still remained clinically significant. The quality of response also showed a positive prognostic impact with an extended follow-up of 48 months, as patients who achieved a CR/VGPR as their best response had significantly longer median response duration, time-to-progression, and overall survival than in those with a

partial response (24.0 vs. 8.3 months, $P<0.001$; 27.7 vs. 12.0 months, $P<0.001$; not reached vs. 44.2 months, $P=0.021$, respectively), and this was regardless of when the CR/VGPR was achieved [53]. Another sub-analysis of the same studies determined that continued lenalidomide treatment until disease progression after achievement of ≥PR is associated with a significant survival advantage when controlling for patient characteristics [54].

A Dutch study showed that treatment with len-dex is highly effective and feasible in heavily pretreated multiple myeloma patients by analyzing the clinical data of more than 100 patients who had been on a median of 3 previous lines of therapy, including thalidomide in most [45]. With a median of 7 cycles of treatment, an overall response rate of 69%, including complete response in 6%, was achieved, and this was not influenced by previous thalidomide and/or bortezomib treatment. Using the recommended prophylaxis, incidence of venous thrombotic events was low (5%), but grade ≥3 myelosuppression occurred in more than a third (37%) [45].

Chromosomal aberrations such as del (17p), t(4;14), t(14;16), and t(14;20) have been associated with poor outcome in MM. The combination of lenalidomide and dexamethasone induces durable responses among relapsed t(4;14) disease but appears to be ineffective in patients with del(17p) [55]. Also, it is postulated that lenalidomide may overcome the eventual negative impact of del(13q) on OS by reducing the relapse rate.

Although comparisons across different trials must be interpreted with caution, it appears that the response rate and the depth of response reported for lenalidomide plus dexamethasone is more favorable than that reported in phase III trials of other active treatment regimens, such as the proteasome inhibitor bortezomib in combination with pegylated liposomal doxorubicin (VPLD) [56].

Recently an expert panel published consensus statement on use of lenalidomide in RRMM [49]. Len-dex is considered to be most effective when used at first relapse and can be administered regardless of the type of previous therapy and age. The optimal starting dose of lenalidomide is 25 mg once daily orally on days 1–21 of each 28-day cycle but has to be modified according to renal function and the presence of cytopenias. The use of low-dose dexamethasone in combination with lenalidomide can result in better tolerability with no loss of efficacy compared with the standard regimen. The recommended dose of dexamethasone in combination with lenalidomide is 40 mg but has to be modified according to age in patients (20 mg in >75 years). Len-dex at best-tolerated dose may continue in responding patients until evidence of disease progression [49].

Toxicities from dexamethasone can sometimes be dose limiting, and this led to evaluation of the efficacy and safety of lenalidomide monotherapy in patients with RRMM by Richardson et al. [29]. This phase II study enrolled more than 200 patients of which two-third had received 3 or more prior anti-MM treatment regimens including prior autologous stem cell transplants in 45%. Lenalidomide alone for three weeks in monthly cycles induced a partial response or better in more than one-fourth of patients. Myelosuppression was reported in more than half of patients but was manageable with dose reduction [29]. Lenalidomide monotherapy was thus shown to be active in RRMM with acceptable toxicities.

Another multicenter, open-label, randomized phase II study evaluated two dose regimens of lenalidomide (30 mg once-daily or 15 mg twice-daily) in over 100 patients with RRMM [28]. Analysis showed a similar response rate (complete, partial, or minor was 25%) in the two groups, but increased grade 3/4 myelo-suppression was noted in patients receiving 15 mg twice daily (41% vs. 13%, $P=0.03$). Though lenalidomide monotherapy was effective, addition of dexamethasone in patients in whom lenalidomide either failed to achieve a response (after 2 cycles) or who subsequently progressed did induce a response in 29% and SD in 21% [28].

Combinations of lenalidomide with other chemotherapeutic agents have also been studied (8.2). Due to lack of overlapping toxicity, lenalidomide has been tried concomitantly with bortezomib, and the clinical evaluations showed that RVD regimen (lenalidomide, bortezomib, and dexamethasone) is well tolerated and shows promising activity with durable responses in patients with RRMM, including in patients who were prior treated with lenalidomide, bortezomib, and/or thalidomide. Two different phase II studies evaluated more than 60 patients in each and after a median of 8 cycles have reported a high ORR (84–86%) and a good depth of response (more than 20% complete response [CR]/near-complete response [nCR]) even in patients with high-risk cytogenetic profiles [57, 58]. A recent prospective study also found that the RVD regimen was able to overcome the negative impact of certain abnormalities [e.g., del(13q), t(4;14)] to a greater extent than lenalidomide plus dexamethasone alone but failed to improve outcomes for patients with del(17p) [34].

Besides bortezomib, combinations with doxorubicin or cyclophosphamide have also shown to be safe and effective options. Combinations of lenalidomide plus dexamethasone in with novel agents such as panobinostat, bevacizumab, SGN-40, perifosine, vorinostat, dasatinib, NPI-0002, everolimus, and carfilzomib are currently being investigated in phase I and II trials. A summary of the important trials in this setting with lenalidomide are given in Table 8.2.

8.2.4 Lenalidomide for Newly Diagnosed Myeloma

Lenalidomide has shown high efficacy in newly diagnosed MM patients (Table 8.3). The Southwest Oncology Group conducted a randomized trial comparing lenalidomide (Len) plus dexamethasone (Dex) to dex (about 100 patients in each group) in newly diagnosed myeloma [35]. Three 35-day induction cycles followed by monthly maintenance induced superior response rates in len-dex group (1-year OS of 78% vs. 52%, $P=0.002$; ORR of 78% vs. 48%, $P<0.001$, and VGPR of 63% vs. 16%, $P<0.001$). However in initial part of this study, there was a very high incidence of thromboembolic events in len-dex group (75%). Adding aspirin prophylaxis significantly reduced this risk, but it still continued to be more than the dex group [35, 59].

A case–control retrospective study by Mayo clinic involving more than 400 newly diagnosed patients revealed len-dex to be well-tolerated and more effective than thal-dex as initial therapy for newly diagnosed myeloma [37]. The incidence of

one grade 3/4 adverse event was similar (57.5% vs. 54.6%, $P=0.568$) in the two groups, but the main grade 3/4 toxicities of len-dex were hematologic while that in thal-dex were venous thromboembolism and peripheral neuropathy [37].

In an open-label randomized controlled trial by Eastern Cooperative Oncology Group (ECOG), 445 patients with untreated symptomatic myeloma were randomly treated with lenalidomide 25 mg for three weeks along with either high-dose dexamethasone (40 mg on days 1–4, 9–12, and 17–20) or low-dose (40 mg weekly) in monthly cycles [36]. Within four cycles, 79% of patients receiving high-dose therapy and 68% of patients on low-dose therapy had complete or partial response (odds ratio 1·75, 80% CI 1·30–2·32; $P=0.008$). At the second interim analysis at 1 year, overall survival was 96% (95% CI 94–99) in the low-dose dexamethasone group compared with 87% (82–92) in the high-dose group ($P=0.0002$). Even though patients on high dose of dex showed a better response rate, they experienced much more toxicity and mortality (12 of 222 on high dose and one of 220 on low-dose) compared to those on the low-dose regimen [36].

Role of lenalidomide as a monotherapy in this group is still unknown. However, recently, a retrospective study observed an overall response rate (≥partial remission) to be 47% at a median follow-up of 7 months (range 1–26) to lenalidomide alone [60]. Though the study was limited by the small size ($n=17$), it reassures that lenalidomide alone has the potential to induce significant clinical response in newly diagnosed patients as well.

Combinations with various chemotherapeutic agents in front-line myeloma have also been evaluated (Table 8.3). The combination of melphalan-prednisone-lenalidomide (MPR) has shown promising results in elderly newly diagnosed myeloma patients [38, 61]. Combinations with bortezomib, clarithromycin, or cyclophosphamide have shown overall response rates of more than 80% with acceptable toxicity. A recent phase I/II study using lenalidomide-bortezomib-dexamethasone has shown a partial response of 100% [32].

8.2.5 Maintenance

Being orally available, IMiDs have a distinct advantage over intravenous drugs such as bortezomib as maintenance therapy. Thalidomide has been proven to improve OS as well as time to progress in three separate phase III studies in post transplant patients [62–65]. Despite these findings, concerns about cumulative toxicity have limited the use of thalidomide for maintenance.

Recently maintenance therapy with oral lenalidomide in multiple myeloma patients who had undergone stem cell transplantation has shown a significant reduction in the risk for relapse in two separate phase III trials—one conducted in the USA and the other in France. The American study reported result of 460 randomized patients which showed that after 17.5 months of follow-up, only 20% of patients in the lenalidomide group had experienced an event (progression or death), compared with 41% of those in the placebo group. Estimated hazard ratio was 0.40, thus

Table 8.2 Lenalidomide in RRMM

Regimen	Study	Phase	Dose	Median no. of prior therapies (range)	Evaluable patients (n)	≥MR	≥PR	CR + nCR	PFS/TTP/EFS	OS
R ± D	Richardson et al. [28]	II	Len 30 mg OD vs. 15 mg BID on days 1–21; 28-day cycles [Dex 40 mg on days 1–4 and 15–18 for suboptimum response]	4 (1–13)	67 vs. 35	24% vs. 29%	18% vs. 14%	6% vs. 0%	PFS: 8 vs. 4 months	28 months vs. 27 months
R	Richardson et al. [29]	II	Len 30 mg on days 1–21; 28-days cycles	(1–3+)	222	44%	26%	2%	PFS: 4.9 months; TTP: 5.2 months	23.2 months; 1-year OS 67%
RD vs. D	Weber et al. [30]	III	Len 25 mg vs. placebo 25 mg on days 1–21; dex 40 mg on days 1–4, 9–12, and 17–20; 28-day cycles	(1–2+)	177 vs. 176		61% vs. 20%	14% vs. 0·6%	TTP: 11·1 months vs. 4·7 months	29·6 months vs. 20.2 months
RD vs. D	Dimopoulos et al. [31]	III	Len 25 mg vs. placebo 25 mg on days 1–21; dex 40 mg on days 1–4, 9–12, and 17–20; 28-day cycles	(1–2+)	176 vs. 175		60% vs. 24%	15.9 vs. 3.4%	TTP: 11·1 months vs. 4·7 months	Not reached vs. 20.6 months
DVd-R	Baz et al. [86]	I/II	Liposomal doxorubicin 40 mg/m2 and vincristine 2 mg on day 1; dex 40 mg on days 1–4; len on days 1–21; 28-day cycles	3 (1–7)	52		75%	29%	PFS : 12 months	Not reached
RAD	Knop et al. [87]	I/II	Len 25 mg on days 1–21; adriamycin 9 mg/m2 on days 1–4; dex 40 mg on days 1–4 and 17–20; six 28-day cycles	(1–2+)	69	73%	69%	15%	PFS: 40 weeks; TTP: 45 weeks	1-year OS: 88%

RCD	Morgan et al. [88]	II	Len 25 mg days 1–21; cyclophosphamide 500 mg days 1, 8, 15, and 21; dex 40 mg days 1–4 and 12–15; 28-day cycles	4 (1–8)	20	75%	65%	5%		
RCd	Schey et al. [89]	I/II	Cyclophosphamide 300–700 mg on days 1 and 8; len 25 mg on days 1–21 and dex 20 mg days 1–4 and 8–11; 28-d cycles. MTD: 600 mg cyclophosphamide	3 (1–6)	31		81%	29%	Median PFS: not reached; 2-year PFS: 56%	Median OS: not reached; 80% OS at 30 months
CPR	Reece et al. [90]	I/II	Cyclophosphamide 150–300 mg/m2 on days 1, 8, and 15; len 15–25 mg on days 1–21 and prednisone 100 mg q 2 days; 28-day cycle	2 (1–5)	32	94%		16%	1-year PFS: 78%	1-year OS: 93%
RV ± D	Richardson et al. [29]	I	Len 5–15 mg on days 1–14; bortz 1.0 or 1.3 mg/m2 on days 1, 4, 8, and 11; 21-day cycles. Dex 20 mg or 40 mg added for progressive disease. MTD: len 15 mg plus bortz 1.0 mg/m2	5 (1–14)	36	61%	39%	8%	PFS : 6.9 months; TTP : 7.7 months	37 months; 2-year OS: 50%

(continued)

Table 8.2 (continued)

Regimen	Study	Phase	Dose	Median no. of prior therapies (range)	Evaluable patients (n)	≥MR	≥PR	CR + nCR	PFS/TTP/EFS	OS
RVD	Richardson et al. [32, 33]	II	Bortz 1.0 mg/m2 on days 1, 4, 8, 11; len 15 mg on d 1–14; dex 40/20 mg/day (cycles 1–4) and 20/10 mg/day (cycles 5–8) on days 1, 2, 4, 5, 8, 9, 11, 12; eight 21-day cycles. Maintenance therapy: btz (d 1, 8); len (d 1–14); dex (d 1, 2, 8, 9)	2	64	78%	64%	25%	PFS: 9.5 months; TTP: 9.5 months	26 months
RD vs. VRD	Dimopoulos et al. [34]	II	RD: Len 25 mg on days 1–21; dex 40 mg on days 1–4 and 15–18 (cycle1–4) / days 1–4 thereafter; 28–day cycle vs. VRD: Bortz 1 mg/m2 on days 1, 4, 8, and 11; len 15 mg on days 2–14 and dex 40 mg on days 1–4; eight 21-day cycles	2 (1–6) vs. 2 (1–8)	50 vs. 49		63% (similar for RD and VRD)	9%	PFS : 8 months, (9 vs. 7 months)	16 months (no difference in RD vs. VRD)

Table 8.3 Lenalidomide in newly diagnosed

Regimen	Study	Phase	Dose	Evaluable patients (n)	≥PR	CR + nCR	PFS/TTP/EFS	OS
RD	Lacy et al. [91]	II	Len 25 mg on days 1–21 plus dex 40 mg on days 1–4, 9–12, 17–20; 28-day cycles	34	91%	18%	2-year TTP: 71%	3-year OS: 88%
RD vs. D	Zonder et al. [35]	III	Len 25 mg vs. placebo 25 mg on days 1–28; 40 mg dex on days 1–4, 9–12, and 17–20; three 35-day cycles. Maintenance: Len 25 mg vs. placebo 25 mg on days 1–21; 40 mg dex on days 1–4 and 15–18; 28-day cycles	97 vs. 95	78% vs. 48%		1-year PFS: 78% vs. 52%	1-year OS: 94% vs. 88%
RD vs. Rd	Rajkumar et al. [36]	III	Len 25 mg on days 1–21 plus dex 40 mg on days 1–4, 9–12, and 17–20; 28-day cycles (high dose) vs. len given on the same schedule with dex 40 mg on days 1, 8, 15, and 22; 28-day cycles (low dose)	214 vs. 208	81% vs. 70%	18% vs. 14%	PFS: 19·1 vs. 25·3 months; TTP: 22·3 vs. 26·1 months	Median OS: not reached. 1-year OS: 87% vs. 96%
RD vs. TD	Gay et al. [37]	Retrospe-ctive	Len 25 mg on days 1 to 21 vs. thal 100–400 mg/day; 28-day cycles. Dex either at high dose (40 mg orally on days 1–4, 9–12, and 17–20) or at low dose (40 mg orally on days 1, 8, 15, and 22); 28-day cycles	228 vs. 183	80% vs. 61%	13.6% vs. 3.3%	PFS: 26.7 vs. 17.1 months; TTP: 27.4 vs. 17.2 months	Median OS not reached vs. 57.2 months

(continued)

Table 8.3 (continued)

Regimen	Study	Phase	Dose	Evaluable patients (n)	≥PR	CR + nCR	PFS/TTP/EFS	OS
MPR	Palumbo et al. [38]	I/II	Melphalan 0.18–0.25 mg/kg on days 1–4; prednisone 2-mg/kg dose on days 1–4; len 5–10 mg on days 1–21; nine 28-day cycles. Maintenance: len alone. MTD: 0.18 mg/kg melphalan and 10 mg len	53	81%	24%	1-year EFS 92%	1-year OS 100%
BiRD	Niesvizky et al. [92]	II	Clarithromycin 500 mg BID on days 1–28; len 25 mg on days 1–21; dex 40 mg on days 1, 8, 15, 22; 28-day cycles	72	90%	39%	EFS: not reached	
RCd	Kumar et al. [2]	II	Len 25 mg on days 1–21; dex 40 mg on days 1, 8, 15, and 22; cyclophosph-amide 300 mg/m2 (300 mg in 20 patients) on days 1, 8, and 15; 28-day cycle	53	83%	2%		2-year OS: 87%
VRD	Richardson et al. [32, 33]	I/II	Bortz 1.0 or 1.3 mg/m2 on days 1, 4, 8, 11; len 15–25 mg on days 1–14; dex 40 or 20 mg on days 1, 2, 4, 5, 8, 9, 11, 12; eight 21-day cycles. Phase II: bortz 1.3 mg/m2, len 25 mg, dex 20 mg	66	100%	39%	18-month PFS: 75%	OS: not reached; 18-month OS: 97%
VDCR	Kumar et al. [93]	I	Cyclophosphamide 100–500 mg/m2 on days 1 and 8; bortz 1.3 mg/m2 on days 1, 4, 8, and 11; dex 40 mg on days 1, 8, and 15; len15 mg on days 1–14; eight 21-day cycles. Maintenance: bortz 1.3 mg/m2 on days 1, 8, 15, and 22); Four 42-day cycles	25	96%	40%	Could not be assessed	Could not be assessed

a 60% reduction in the risk of disease progression with lenalidomide. The estimated median TTP was 42.3 months in lenalidomide group vs. 21.8 months for the placebo arm [66].

The other set of results come from an interim analysis of a French study involving 614 patients which revealed that lenalidomide maintenance halved the risk for relapse. The 3-year progression-free survival was 68% with maintenance lenalidomide, compared with 35% with placebo (hazard ratio, 0.46; $P < 10^{-6}$), reducing the rate of relapse by 54% [67].

The two studies were similar, but the French study used a consolidation phase of therapy before moving on to maintenance with lenalidomide. Both trials showed a significant improvement in time to disease progression, although no significant data available is yet for overall survival. However, since the time to progression of disease is dramatically increased, lenalidomide maintenance therapy could become the new standard of care for these patients.

8.2.6 Early Stage Disease (Smoldering Myeloma)

Smoldering MM (SMM) is a MM precursor defined by an M-protein of ≥3 g/dL and/or ≥10% bone marrow plasma cells with no evidence of end-organ damage (hypercalcemia, renal insufficiency, anemia, or bone lesions [CRAB]) (Criteria for the classification of monoclonal gammopathies, multiple myeloma and related disorders: a report of the International Myeloma Working group 2003 [68]). SMM is differentiated from MGUS based on the size of the M protein and the level of bone marrow involvement. The natural history of SMM varies greatly, and the overall risk of progression is approximately 10% per year for the first 5 years, 3% per year for the next 5 years, and 1% per year for the last 10 years with the cumulative probability of progression being 73% at 15 years [69].

Standard management of smoldering myeloma at present consists of monitoring the patient every 3–6 months until the disease has progressed to a point at which intervention is warranted. Three phase II studies showed that thalidomide could prolong the TTP; however, proven benefit in prospective randomized trials is required before approval [70–72]. The activity of lenalidomide and its acceptable safety profile has prompted evaluation of its efficacy in preventing or delaying progression of high-risk smoldering myeloma to symptomatic myeloma. A multicenter, phase III study compared the efficacy of induction therapy with lenalidomide (25 mg daily for 21 days every 28-day cycle, for 9 cycles) plus dexamethasone (20 mg on days 1–4 and days 12–15 every 28 days, for 9 cycles) and maintenance therapy with lenalidomide (10 mg/day for 21 days every 2 months) with that of therapeutic abstention in patients with high-risk smoldering myeloma [73]. After a median of four cycles, the overall response rate in the lenalidomide arm was 81% ($n = 47$), which increased to 91% after nine cycles. After a median follow-up of 14 months, the median TTP was not reached in the lenalidomide group (n = 47) and was 19.3 months in the abstention arm ($n = 47$). OS at 2 years was 100% for lenalidomide-treated patients and 96% for those abstaining from treatment [73].

8.3 Pomalidomide

Pomalidomide (CC-4047) is yet another derivative of thalidomide with similar mechanism of action and is considered to be most potent of the IMiDs [21, 74]. Preclinical studies showed that it significantly increases serum IL-2 receptor and IL-12 levels serum within a month, which correlated with the percentage decrease in paraprotein [75]. A decrease in $CD8^{+}/CD45RA^{+}$ cells and $CD4^{+}/CD45RA^{+}$ during the first month of study was also accompanied by a corresponding increase in $CD8^{+}/CD45RO^{+}$ cells and $CD4^{+}/CD45RO^{+}$, which suggests a switch from naive cells to activated effector T cells [75]. This drug also potently blocks osteoclasts differentiation and thus, might also have a role in preventing or treating myeloma bone disease [76]. Pomalidomide also affects inflammation via transcriptional inhibition of cyclooxygenase-2 (COX-2) production, which is associated with increased prostaglandins in human lipopolysaccharide (LPS)-stimulated monocytes [77].

Like thalidomide, pomalidomide may have the potential for severe birth defects, and caution in reproductive age group is advised. Myelosuppression is the major and dose-limiting toxicity noted in all clinical trials. Grade 3/4 neutropenia has been seen in about 30–60% of patients and is more common than thrombocytopenia or anemia (Table 8.4). Thromboembolic complications occurred with a frequency similar to that reported with other IMiDs. Neuropathy is infrequent, but worsening of neuropathy has been reported by previously heavily pretreated patients. Noninfectious acute lung injury is a rare but serious drug complication. Fortunately it responds well to the use of corticosteroids. Other common side effects include orthostatic hypotension, skin rash, and constipation.

Low-dose pomalidomide is effective in the treatment of anemia associated with JAK2V617F-positive myelofibrosis [83]. Among patients with multiple myeloma, pomalidomide has been tried in only relapsed cases. Initial phase I trials established pomalidomide as well tolerated in maximum tolerated dose (MTD) of 2 mg QD or 5 mg on alternate days and demonstrated a potent immune-activating effect of this agent in myeloma [75, 78]. These studies using pomalidomide predominantly as monotherapy have shown excellent long-term responses with an overall response rate of 52% [84].

The first phase II trial conducted by Lacy and colleagues presented data on a cohort of 60 relapsed patients who were administered 2 mg of oral pomalidomide daily along with weekly 40 mg oral dexamethasone [79]. About two-third patients achieved confirmed response including complete response in 5%. Responses were shown even among 40% of patients who were lenalidomide and 60% of patients who were bortezomib-refractory. Also, 74% of patients with high-risk cytogenetic or molecular markers (hypodiploidy or karyotypic deletion of chromosome 13, FISH showing presence of translocations t(4;14) or t(14;16) or deletion 17p, or plasma cell labeling index ≥3%) showed a response. This observation carries great importance since lenalidomide and its combinations have so far being unsuccessful in improving the outcomes of patients with deletion 17p [34]. Pomalidomide was well tolerated with primary issue being grade 3/4 hematologic toxicity in about a

Table 8.4 Pomalidomide in RRMM

Regimen	Study	Phase	Dose	Median no. of prior therapies (range)	Evaluable patients (*n*)	≥PR	PFS	OS	Hematological complications (grade ≥ 3)	Non- hematological complications (grade ≥ 3)
Pom	Schey et al. [75]	I	Pom 1–10 mg on days 1–28; 28-day cycles. MTD 2 mg	3 (1–6)	24	54%	9 months	21 months	Neutropenia (58%); thrombocytopenia (12.5%)	DVT (16.67%)
Pom	Streetly et al. [78]	I	Pom 1–10 mg on alt day; 28-day cycles. MTD 5 mg alt day	4 (1–7)	20	50%	10.5 months	36 months	Neutropenia (45%)	
Pom + Dex	Lacy et al.[79]	II	Pom 2 mg on days 1–28; dex 40 mg, days 1, 8, 15, and 22; 28-day cycles	2 (1–3)[a]	60	63%	11.6 months	94% at 6 months	Neutropenia (32%); anemia (5%); thrombocytopenia (3%)	Fatigue (17%), pneumonia (8%), DVT (2%), diarrhea, constipation, hyperglycemia, and neuropathy
Pom + Dex	Lacy et al.[80, 81]	II	Pom 2 mg on days 1–28; dex 40 mg on days 1, 8, 15, and 22; 28-day cycles	4 (1–7)[a]	34	32%	4.8 months	14 months	Neutropenia (29%); anemia (12%); thrombocytopenia (9%)	Fatigue (9%), pneumonitis, edema, pneumonia, folliculitis, and hyperglycemia

(continued)

Table 8.4 (continued)

Regimen	Study	Phase	Dose	Median no. of prior therapies (range)	Evaluable patients (*n*)	≥PR	PFS	OS	Hematological complications (grade ≥ 3)	Non- hematological complications (grade ≥ 3)
Ongoing trials										
Pom + Dex	Lacy et al.[80, 81]	II	Pom 2 mg (Cohort A) vs. 4 mg (Cohort B) on days 1–28; dex 40 mg daily on days 1, 8, 15, and 22; 28-day cycles	6 (1–8+)[b]	35 vs. 35	26% vs. 26%	6.4 months vs. 3.3 months	78% vs. 69% at 6 months	49% vs. 66%; neutropenia (37% vs. 55%); thrombocy-topenia (11% vs. 13%); anemia (9% vs. 16%)	Fatigue, neuropathy, pneumonia, hyperglycemia, hypercalcemia, atrial fibrillation, renal failure, thrombosis (9% vs. 6%)
Pom + Dex	Leleu et al. [82]	II	Pom 4 mg on days 1–21 (arm A) vs. days 1–28 (arm B); dex 40 mg on days 1, 8, 15, and 22; 28-day cycles	4 (1–8)[b]	43 vs. 41	42% vs. 39%	6.5 months vs. 9 months	88% at 4 months vs. 85% at 5 months	Neutropenia (34% vs. 33.5); thrombocy-topenia (18% vs. 21); anemia (11% vs.14)	Asthenia, bronchitis, cramps, diarrhea, generalized pain, chest pain, dyspnea, fever

Pom ± Dex	Richardson et al. [32, 33]	I	Pom 2–5 mg on days 1–21; 28-day cycle; dex 40 mg/week after 4 cycles if lack of response or progressive disease. MTD 4 mg	6 (2–17)[b]	38	25%	4 months	17 months	Neutropenia (52.6%); anemia (21.0%); thrombocytopenia (15.8%)	Fatigue (18.4%), peripheral neuropathy (13.2%), VTE (10.53%)
Pom ± Dex	Richardson et al. [32, 33]	II	Pom 4 on days 1–21; 28-day cycle; dex 40 mg/week vs. pom 4 mg on days 1–21; 28-day cycle	5 (2–13)[b]	120	25%	NA	NA	Neutropenia (42%); thrombocytopenia (22%); anemia (20%)	Infections (31%), fatigue (12%), renal failure (7%), cardiac disorders (4%), and DVT (1%)

[a]Includes patients refractory to lenalidomide

[b]Includes patients refractory to lenalidomide and bortezomib

third [79]. To better define its efficacy in lenalidomide refractory disease, Lacy et al. also treated a cohort of 34 of these patients with the same regime of pom-dex, and the overall response (PR or better) was near 50% [80].

Dual refractory myeloma (refractory to both bortezomib and lenalidomide) is a great challenge in current scenario, and ongoing studies have established pomalidomide to be effective in this group of patients with an overall response of 25% or more [33, 81, 82]. Also, it is being postulated that its effectivity goes beyond marrow pathology as it has been also shown effective in treatment of extramedullary disease with a response rate of ~30% including the extramedullary component [85].

The optimal dose of pomalidomide is still unclear. While earlier studies advocated 2 mg daily as the maximum tolerated dose, Richardson and colleagues in a recent phase I/II dose escalation study proved 4 mg pomalidomide daily to be well tolerated as well [33]. In the study by Lacy et al., eight patients with suboptimal response were escalated from 2 to 4 mg daily, and one patient improved from stable disease to PR [80]. However, in an ongoing trial by Lacy et al. starting with higher pomalidomide dose (4 mg) has not shown any superiority of response over starting with 2 mg dose and is associated with higher risk of myelosuppression [81].

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Chapter 9
Novel Agents in Multiple Myeloma

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9.1 Introduction

Multiple myeloma (MM) represents a paradigm in drug development with an improved understanding of the biology and derived clinical trials translating into six new US Food and Drug Administration (FDA)-approved treatments over the past 10 years. The proteasome inhibitor, bortezomib, and the immunomodulatory drugs, thalidomide and lenalidomide, have been the cornerstone of the improvement in outcomes during the last decade [1, 2]. However, almost all patients with MM relapse and the outcome of patients who progress after therapy with the immunomodulatory drugs and bortezomib remain dismal [3]. Novel biologically based therapeutic approaches that target not only the MM cell but also the interaction with other cells and cytokines in the bone-marrow milieu have the potential to overcome resistance to conventional agents and improve patient outcomes in MM, with next generation targets now emerging [4, 5]. Here we will review novel targets in MM used either alone or in combination strategies.

9.2 Drug Combinations of Novel Agents in Myeloma

The introduction of thalidomide, lenalidomide and bortezomib has led to important changes in the management of patients with MM. Bortezomib received accelerated FDA approval for the treatment of patients with relapsed and refractory multiple

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N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 2: Translational and Clinical Research*,
DOI 10.1007/978-1-4614-5260-7_9, © Springer Science+Business Media New York, 2013

myeloma in 2003 [6]. Subsequently, bortezomib also received full approval for the treatment of patients with relapsed multiple myeloma and as initial therapy on the basis of favorable results from phase III trials [7, 8]. The immunomodulatory drugs thalidomide, lenalidomide and pomalidomide target myeloma cells in the bone-marrow microenvironment. Specifically, these agents trigger caspase-8-mediated apoptosis, decrease binding of tumour cells to bone-marrow stromal cells, inhibit secretion of cytokines from the bone marrow (through both constitutive secretion as well as secretion induced by the binding of myeloma cells), inhibit angiogenesis and stimulate immunity against myeloma cells mediated by autologous natural killer cells, T cells or both [9, 10].

In the upfront setting, thalidomide with dexamethasone (thal/dex) and bortezomib (Velcade) in combination with melphalan and prednisone (MPV) increased the overall response rate (RR) and significantly prolonged time to progression (TTP) and are FDA-approved for this indication, [8, 11] with overall RRs for thal/dex of 64% and 71% with MPV. In the relapsed setting, bortezomib alone [6, 7] and the combinations of lenalidomide/dexamethasone (len/dex) [12, 13] and bortezomib and liposomal doxorubicin (Vel/Doxil) have all been approved [14]. Importantly, results of a phase III randomized trial suggest that lower doses of dex (40 mg weekly for 4 weeks) in combination with len provide a survival advantage mainly due to the decreased toxicity associated with lower doses of dex [15].

In order to improve upon current outcomes, optimal combinations of bortezomib, thal and len have been evaluated in phase II/III clinical trials, with the combination of lenalidomide–bortezomib–dexamethasone (RVD) showing particularly promising activity [16]. Preclinical data indicate synergistic cytotoxicity results from combining lenalidomide (which induces caspase-8-mediated apoptosis) with bortezomib (which induces predominantly caspase-9-mediated apoptosis) in in vitro models of myeloma (Fig. 9.1). Lenalidomide and bortezomib achieved 61% responses in patients with relapsed and refractory multiple myeloma and who were refractory to each agent alone [17]. In the setting of newly diagnosed disease, RVD produced an unprecedented overall RR of 100%, with 74% of patients achieving at least a very good partial response and 52% of patients showing complete or near-complete responses [16].

9.3 Next Generation Novel Agents in Clinical Development

9.3.1 Monoclonal Antibodies

9.3.1.1 CS1-, CD38- and CD138-Targeting Antibodies

One of the major ongoing efforts is to identify MM cell-surface antigens and design-specific antibodies with cytotoxic properties. CS-1, CD38 and CD138 are multifunctional glycoproteins widely and highly expressed on MM cell surface. Elotuzumab (HuLuc63) is a CS1-targeting monoclonal antibody which triggers ADCC-mediated cell death *in vitro* and effectively reduces tumour growth in an in vivo MM model [18].

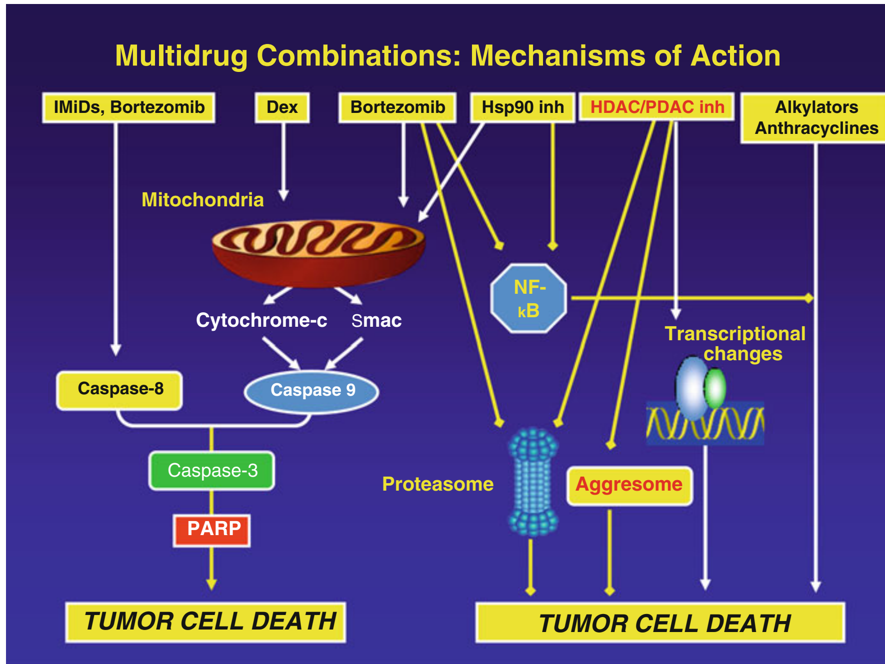

Fig. 9.1 Rationale for combination therapies in multiple myeloma (adapted from Richardson et al. Br J Haematol 154(6):755–762)

In relapsed and refractory MM patients, elotuzumab has a manageable toxicity profile, and stable disease was observed on a low-dose schedule with monotherapy [19]. Preliminary data indicate exciting results with the combination of elotuzumab with lenalidomide and dexamethasone [20], with efficacy evaluable patients, 22/26 (85%) achieving a confirmed or an unconfirmed response, including 31% VGPR/CR and the remaining 4/26 (15%) stable disease in one study.

In vitro, antibodies against CD38 induce antibody-dependent cell cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) against MM cells. There are ongoing clinical trials to further evaluate the CD38 antibodies with early results showing promise [21, 22].

Similarly, the maytansanoid toxin conjugated to an anti-CD138 monoclonal antibody has shown promising results in vitro, and xenograft models of human MM in mice have provided the framework for a clinical trial of this immunotoxin [23].

9.3.1.2 IL-6-Targeting Antibodies

Interleukin-6 (IL6) is an inflammatory cytokine that is both an autocrine and paracrine survival factor for malignant plasma cells. IL-6 is secreted by myeloma cells which also stimulate its production in the tumour niche by both bone-marrow

Table 9.1 Promising novel agents in clinical trials in multiple myeloma

Drug	Category	Comments
Pomalidomide	Immunomodulatory drug	Ongoing phase III trial [NCT01311687]
Carfilzomib	Proteasome inhibitors	Ongoing phase III trial [NCT01080391]
NPI-0052 MLN 9708 ONX 0912		Orally bioavailable proteasome inhibitors currently in phase I, II, III trials
Elotuzumab	Anti CS-1 antibody	Ongoing phase III trials [NCT01239797; NCT01335399]
ACY-1215	Histone deacetylase inhibitors	Phase I [NCT01323751]
Panobinostat		Phase III [NCT01023308]
Romidepsin		Phase I-II trials
Perifosine	Phosphatidylinositol 3-kinase/ Akt pathway inhibitor	Ongoing phase III trial [NCT01002248]

stromal cells (BMSC) and osteoclasts (OC). In addition, IL-6 stimulates osteoclastogenesis [24]. CNTO328 is a novel human–mouse chimeric monoclonal antibody against IL6 currently undergoing clinical evaluation. CNTO328 enhances bortezomib-induced cytotoxicity on MM cells increasing the activation of the pro-apoptotic caspases 8, 9 and 3 [25], with stable disease and partial responses observed in MM patients treated with single-agent CNTO328, which in turn has led to combination studies of bortezomib-based therapy and CNTO328.

9.3.1.3 BAFF-Targeting Antibody

B-cell activating factor (BAFF) is a potent osteoclast (OC)-derived MM growth factor, and its inhibition reduces tumour burden as well as OCs and lytic lesions in in vivo models of myeloma bone disease [26]. Clinical trials of BAFF-neutralizing antibody in combination with bortezomib are currently ongoing to confirm the effects on bone lesions and tumour burden [NCT00689507].

9.3.1.4 Pomalidomide

CC-4047 (Pomalidomide) is a potent immunomodulatory analog (IMiDs), derived using the thalidomide backbone [27]. As mentioned above, IMiDs have multiple mechanisms of action beyond immunomodulation alone. Phase I clinical studies of pomalidomide in combination with low-dose dexamethasone showed activity in relapsed patients with MM who were resistant to other agents, including thalidomide, lenalidomide and bortezomib [28]. Pom/dex was found to be highly active and well tolerated including responses among patients who were lenalidomide and bortezomib refractory [29, 30]. No grade 3 neuropathy was seen, and thromboembolic events have been rare. Pom therefore appears to be a very promising agent in

the therapy of MM and provides an alternative to patients who have received lenalidomide-, thalidomide- and bortezomib-based treatments (Table 9.1).

9.4 Modulators of Protein Homeostasis

Bortezomib, the first in class boronate peptide proteasome inhibitor, reversibly inhibits chymotrypsin-like activity of the 20S proteasome. Peripheral neuropathy, thrombocytopenia and gastrointestinal symptoms, although manageable, are important side effects. More potent inhibitors of chymotryptic activity, including carfilzomib and MLN 9708, have been noted to overcome bortezomib resistance in preclinical and early clinical trials. Carfilzomib, an irreversible proteasome inhibitor in the epoxyketone-category-induced partial response in approximately 23% of heavily pretreated relapsed and refractory MM patients, and importantly, the overall RR was noted to be 57% in a subset of bortezomib-naïve patients [31]. The toxicity profile was manageable, consisting mainly of myelosuppression and markedly reduced rates of neuropathy. Phase III clinical trials comparing carfilzomib–lenalidomide–dexamethasone with lenalidomide–dexamethasone in patients with relapsed multiple myeloma are now ongoing [32]. MLN 9708 is an oral proteasome inhibitor in the boronate peptide category [33] that has shown encouraging results in early phases I–II clinical trials both as a single agent and in combination with lenalidomide and dexamethasone [NCT00963820, NCT01383928]. ONX 0912, an oral epoxyketone proteasome inhibitor, is also now undergoing evaluation as a single agent in hematologic malignancies [NCT01416428] [34].

A broader and more potent proteasome inhibitor, NPI-0052 or marizomib, targets chymotryptic, tryptic and caspase-like activities and overcomes bortezomib resistance in preclinical studies and with early clinical trials confirming consistent activity in bortezomib-refractory patients [35–37]. Importantly, in preliminary results from a phase I study in patients with relapsed and refractory MM, NPI-0052 has not appeared to induce significant peripheral neuropathy or myelosuppression and was generally well tolerated and demonstrated unique safety profiles compared to bortezomib in spite of up to 100% proteasome inhibition [37].

Inhibitors of de-ubiquitinating enzymes located upstream of the proteasome, such as the USP-7 inhibitor P5091, have shown activity against multiple myeloma [38].

PR-924, an inhibitor of the LMP-7 immunoproteasome subunit, inhibits myeloma cells *in vitro* and *in vivo*. Owing to the selective expression of immunoproteasome subunits in malignant, but not in normal, haematological cells, inhibitors of the immunoproteasome should also have a favourable therapeutic index, and studies of these are awaited with interest [39].

In a similar context, NEDD8-activating-enzyme inhibitor MLN4924 targets the neddylation pathway upstream of the 20S proteasome, with downstream molecular sequelae which generates significant preclinical anti-myeloma activity that is distinct from that of established 20S proteasome inhibitors [40].

9.5 Histone Deacetylase Inhibitors

Histone deacetylase (HDAC) inhibitors are novel antineoplastic agents that correct the transcriptional deregulation of genes involved in the induction of apoptosis and cell-cycle arrest. They have multiple mechanisms of action, including mediating tumour cell death via caspase-dependent and non-caspase-dependent apoptosis as well as autophagy. They also block the aggresome complex which represents a protein–scavenger system that mediates protein degradation in the event of either proteasome overload or inhibition. Intriguingly, the high protein turnover characteristic of plasma cells and MM cells requires aggresome formation, and so the synergistic activity seen in combination with the proteasome inhibitor, bortezomib, is particularly promising. Specifically, HDAC inhibitors suppress proteasome activity, decrease expression of proteasome subunits and critically inhibit the aggresome. For example, inhibition of this pathway via tubacin, a specific HDAC6 inhibitor, synergizes with proteasome inhibition achieved with bortezomib. The HDAC6 inhibitors also have the potential of reduced toxicity, and the HDAC6-specific inhibitor, ACY 1215, is currently being studied in a phase I clinical trial. HDAC inhibitors have been shown to be effective anticancer agents in both in vitro and in vivo studies [41, 42].

Other HDACi which have been developed in the clinical setting include SAHA (suberoylanilide hydroxamic acid, vorinostat), LBH589 (panobinostat) and romidepsin with both vorinostat and romidepsin FDA-approved in cutaneous lymphomas. The multitude of effects of these compounds are complex, with the transcriptional signature of SAHA, for example, revealing downregulation of IGF-1R/AKT and IL6R/STAT3-signalling pathways, as well as DNA synthesis and repair enzymes [43].

The effects and toxicities of HDACi differ according to the specific compound, the formulation and schedule of administration. Intravenous doses of SAHA cause myelosuppression and thrombocytopenia, while with the oral formulation, fatigue, diarrhoea and dehydration are more common. Adverse effects of oral LBH589 consist of thrombocytopenia and neutropenia. HDACi have now been assessed also in combination strategies with novel anti-MM agents, including bortezomib and len with considerable promise shown with both panobinostat and bortezomib, vorinostat and lenalidomide and romidepsin and bortezomib [44–48].

The combination of HDACi and bortezomib in vivo not only effectively reduced tumour burden but also improved osteolytic lesions in a mouse model of bone disease [49].

9.6 HSP90 Inhibitors

Heat-shock protein 90 (HSP90) is a chaperone protein that regulates protein folding and translocation into the different cellular compartments. Studies demonstrate that bortezomib treatment of MM cells in vitro induces death signalling, downregulates survival signalling and upregulates both ubiquitin/proteasome and stress response

gene transcripts. In vitro studies show that Hsp90 inhibitor 17AAG can block the Hsp90 stress response induced by bortezomib and thereby increase MM cell apoptosis. These studies therefore provided the framework for a clinical trial coupling of these agents in MM with favourable tolerability and encouraging responses seen in relapsed and refractory patients [50, 51]. As a result of production difficulties with 17 AAG, studies of this compound are no longer going forward. However, other HSP 90s are now under study.

9.7 PI3K/Akt Inhibitors

Cytokine-induced activation of Akt has been reported to induce growth and survival advantage to MM cells and mediate dex-resistance in MM cells in the context of the BM microenvironment [52]. Agents targeting PI3K/Akt network directly, in particular the pleiotropic Akt inhibitor perifosine, the PKC inhibitor enzastaurin and the mTOR inhibitors RAD001 and CCI-779, have been examined in MM preclinical models.

The novel oral Akt inhibitor perifosine (Keryx Biopharmaceuticals) triggers cytotoxicity against MM cells, both in vitro and in vivo [53, 54]. Molecular studies revealed that perifosine-induced inhibition of Akt phosphorylation and its downstream molecules (GSK)-3β and FKHRL1 was associated with c-jun NH2-terminal kinase activation. Perifosine treatment also triggered the formation of the death-inducing signalling complex as well as the recruitment of TRAIL-R1/DR4 and TRAIL-R2/DR5, resulting in potent apoptosis [55].

Preclinical data has also been reported on bortezomib-induced activation of Akt as a putative mechanism of resistance, which in turn has been completely blocked by perifosine, while bortezomib successfully abrogated perifosine-induced ERK phosphorylation [54]. This blockade of both Akt and ERK signalling cascades by perifosine and bortezomib enhances JNK phosphorylation, caspase/PARP cleavage and apoptosis. Results of a phases I–II trial with the combination of perifosine and bortezomib showed durable responses, even in the setting of bortezomib refractoriness. In 73 evaluable patients, an overall response rate (ORR; defined as minimal response or better) of 41% was demonstrated with this combination, including an ORR of 65% in patients who relapsed following bortezomib treatment and 32% in bortezomib-refractory patients. Median PFS was 6.4 months, with an encouraging median overall survival of 25 months (and 22.5 months in bortezomib-refractory patients) [56]. A phase III clinical trial of bortezomib versus bortezomib with perifosine in patients with relapsed multiple myeloma is ongoing [NCT01002248].

9.8 mTOR Inhibitors

PI3K/Akt/mTOR kinase cascade plays a critical role in cell proliferation, survival and development of drug resistance in MM [57]. Rapamycin is a universal inhibitor of mTORC1-dependent S6K1 phosphorylation [58, 59]. Rapamycin-induced

cytotoxicity is predominantly triggered as a consequence of autophagy (programmed cell death type II) via excessive cell digestion. Therefore, activated Akt can be a key upstream inhibitor of two cell death-inducing events: autophagy via mTOR activation and apoptosis via phosphorylation of BAD and inhibition of the catalytic subunit of caspase-9. In vitro and in vivo preclinical studies have demonstrated anti-MM activity of rapamycin and its analogs (CCI-779 and RAD001) [59, 60].

However, resistance to rapamycin results from a strong positive feedback loop from mTOR/S6K1 to Akt with consequent Akt activation [61, 62]. This effect in some cancer types is due to rapamycin activity only on mTORC1 complex, whereas mTORC2, the one responsible for Akt activation, remains unaffected. Promising data reported on combined targeting of mitogen-activated protein kinase (MAPK) and PI3K/mTOR pathways by rapamycin with len [59] have been translated to clinical trials. In the phase I study of RAD001 with lenalidomide, stable disease or better was observed in 68% of patients (13/19–90%, CI: 30–76%) with grade 3/4 adverse events (5%) included thrombocytopenia (11%) and neutropenia (22%) [63]. In the phase 2 study of the combination of temsirolimus with bortezomib in heavily pretreated, advanced MM patients , the proportion of patients with a partial response or better was robust at 33% (14 of 43; 90% CI 21–47) [64].

There are ongoing and planned trials with dual inhibitors of mTORC1/2-INK 128 and AZD 8055 [65, 66] and the composite mTORC1/2 and PI3-kinase inhibitors NVP-BEZ235 [67].

9.9 Cyclin-Dependent Kinase Inhibitors

Dysregulated and/or increased expression of cyclin D1, D2 or D3 occurs as an early, unifying event in MM pathogenesis, predisposing MM cells to proliferative stimuli, and is frequently seen in relapsed patients with poor prognosis [68]. Specific inhibition of Cdk4/6 by PD 0332991, an orally bioavailable small-molecule Cdk inhibitor, has demonstrated only growth arrest in MM cells [69], suggesting that selective cyclin-dependent kinase (CDK) inhibition may not be sufficient in inducing MM cell death. Rather, effective MM cytotoxicity may be best achieved when multiple CDKs are inhibited concurrently, as demonstrated in preclinical studies with multi-targeted CDK inhibitors such as AT7519 [70]. Additionally, they target CDK complexes that phosphorylate RNA pol II resulting in inhibition of RNA pol II phosphorylation and transcriptional inhibition and also modulate expression/activity of multiple signalling pathways critical for MM cell proliferation and survival in the context of the bone-marrow microenvironment. AT7519, independent of its potent inhibitory effects on CDKs, effectively induces the dephosphorylation of glycogen synthase kinase (GSK)-3β [71], another important target in MM therapy. AT7519 is being evaluated in combination with bortezomib and dexamethasone in patients with relapsed and/or refractory MM [NCT01183949].

9.10 Aurora Kinase Inhibitors

The aurora kinases regulate cell-cycle transit from G2 through to cytokinesis. Myeloma is characterized by genetic instability and disruption of cell-cycle checkpoints which renders myeloma cells suspectible to induction of apoptotic death in mitosis. Aurora kinase inhibitors have been shown to inhibit the growth of MM cell lines and primary myeloma samples at nanomolar concentrations with minimal effect on proliferating lympocytes and hematopoietic cells [72–75]. Phase I/II studies of MLN8237, an aurora kinase inhibitor, are now ongoing in multiple myeloma [NCT01034553].

9.11 Telomerase Inhibitors (GRN163L)

Telomerase is a reverse transcriptase that protects chromosome endings and therefore expands cell lifespan. It is expressed at high levels in cancer cells, including MM, while almost no expression detected in normal somatic cells. Targeting telomerase via a novel inhibitor, GRN163L, results in MM cell death *in vitro*. In vivo studies demonstrated that GRN163L impaired tumour growth and enhanced animal survival [76]. There is a completed phase I study of the telomerase inhibitor GRN163L alone and in combination activity with bortezomib and dexamethasone in patients with relapsed or refractory MM and an ongoing phase II study of GRN 163 L (Imetelstat) currently under way [NCT00594126, NCT00718601].

9.12 Farnesyltransferase Inhibitors

Mutations of Ras are commonly encountered and are associated with disease progression and decreased survival [77]. Because Ras and other proteins require farnesylation, a lipid posttranslational modification, for malignant transformation activity, farnesyltransferase inhibitors (FTIs) were studied as potential anticancer drugs. In a phase II trial of patients with advanced MM, disease stabilization was achieved in 64% of patients treated with FTI5777 (Zarnestra) [78]. Preclinical evaluation of the combination of the specific FTI, tipifarnib, and bortezomib revealed synergistic anti-MM activity. This combination has been shown to enhance the ER-stress-induced apoptosis and overcome the CAM-DR phenotype, therefore delineating a treatment strategy that specifically targets microenvironment-mediated drug resistance [79]. Based upon these observations, a phase I trial combining escalating doses of tipifarnib (100–400 mg/BID) with bortezomib (1.0 mg/m^2) in patients with relapsed MM was initiated, and encouraging preliminary data reported stabilization of disease or better seen among 7/16 patients with 2 of the 7 achieving an MR; no serious drug-related toxicities were noted, including the absence of cardiac events or DVT [80]. Future studies are anticipated with interest.

9.13 Conclusions and Future Directions

The availability of several classes of agents targeting biologically relevant pathways and proteins in MM remains remarkably exciting and productive. Patients with MM now have increasing therapeutic options with agents active alone and in combination. Future studies will focus on biologic risk stratification and optimizing drug combinations relevant to specific patient profiles, including adverse cytogenetics and extramedullary disease. Given that several of these agents have different toxicity profiles, the future holds promise in terms of novel drug combinations with improved efficacy and tolerability with rational combination strategies derived from both preclinical models and clinical experience, providing real hope for further improving patient outcome [81, 82].

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Chapter 10
Early Combination Studies in Multiple Myeloma

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10.1 Introduction

Multiple myeloma (MM) is the second most common haematological malignancy. It accounts for 20,580 new cancer cases in the USA in 2009, including 11,680 cases in men, 8,900 cases in women and 10,580 deaths overall [1]. Although the disease remains still incurable, outcomes have improved substantially over recent years, thanks to the use of high-dose therapy and the availability of novel agent-based therapies [2, 3].

Prolongation of both progression-free survival (PFS) and overall survival (OS) remains the main and ultimate goal, but newer and more effective therapies enabled to achieve a complete response (CR) in a larger proportion of patients.

The proteasome inhibitor bortezomib and the immunomodulatory agents thalidomide and lenalidomide are basic components of first-line therapy. Different induction therapies combining novel agents have been introduced for the treatment in both transplant and non-transplant settings. Physicians should choose the best treatment strategy by taking into account patients' baseline comorbidities and the possible regimen-associated toxicities, in particular peripheral neuropathy, thrombotic risk, changes in renal function and bone disease.

Despite recent advances, patients with MM eventually relapse. Efforts to prolong PFS and at least ensure long-term survival with a good quality of life are needed. Several studies have recently focused on the role of achieving a CR. In the transplant setting, CR was found to be closely related to overall survival. Conversely, CR was not associated with a survival advantage in elderly patients, mainly due to the small proportion of subjects achieving a CR. With the introduction of novel agents, a greater number of elderly patients were able to obtain a CR, but only rarely was

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N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 2: Translational and Clinical Research*,
DOI 10.1007/978-1-4614-5260-7_10, © Springer Science+Business Media New York 2013

this associated with an enhanced survival. The achievement of a durable CR remains a crucial treatment goal, but it should carefully be balanced with an acceptable toxicity. Longer follow-up is still required to assess the impact of this increased CR on long-term survival [4–6].

10.2 Diagnosis and Treatment Strategy

MM is characterized by malignant plasma cell infiltration in the bone marrow and is associated with an increased level of monoclonal protein in the blood and/or urine. Besides the monoclonal protein, the presence of an abnormal serum-free light-chain ratio is a further sign of MM. Identifying symptomatic MM is the very first step to start treatment. Patients with symptomatic MM should be treated immediately, while asymptomatic patients do not benefit from early intervention.

Symptomatic disease is defined by evidence of end-organ damage caused by plasma cells proliferation according to the CRAB criteria: C, hypercalcemia (>11.5 mg/dL); R, renal failure (serum creatinine >1.73 mmol/L); A, anaemia (haemoglobin <10 g/dL or >2 g/dL below the lower limit of normal); and B, bone disease (lytic lesions, severe osteopenia or pathologic fractures) [7]. Afterwards, physicians should recognize organ damage and its correlation with MM and finally choose the most appropriate treatment approach [8].

A preliminary distinction within MM patient population is needed. The choice of treatment is based on both scientific evidence and patient's characteristics, in particular age. Young patients are subjects younger than 65 years, usually fit enough and without severe comorbidities, who are able to undergo intensive treatments or repetitive therapies. This group of patients is commonly considered eligible for autologous stem cell transplantation (ASCT). On the contrary, elderly patients are older than 65 years or have serious comorbidities. These patients are usually not considered ASCT candidates, and a gentler approach is necessary. However, physiological age and chronological age do not always correspond, and in some countries, like the USA, a greater emphasis is placed on the former rather than the latter. The incidence of MM varies between the two groups: the median age at diagnosis is 70 years, with 36% of patients younger than 65 years, 27% aged 65 to 74 years and 37% older than 75 years [9].

Other factors may determine whether a patient is eligible for ASCT or not, such as performance status, impaired renal failure and comorbidities. Patients with normal cardiac function (normal electrocardiogram [EKG] and echocardiography or multiple-gated acquisition (MUGA) evaluation and New York Heart Association [NYHA] class I/II), normal pulmonary function (normal chest X-ray, normal spirometry and normal diffusion capacity), normal liver function and normal renal function are good candidates for ASCT. Reduced dose-intensity transplantation (melphalan 100 mg/m^2, Mel100) may be a valuable option for patients with a good performance status and a physiological age ranging between 65 and 75 years [10].

The major adverse events associated with novel agents include venous thromboembolism (thalidomide and lenalidomide), myelosuppression (lenalidomide and bortezomib), gastrointestinal discomfort and peripheral neuropathy (thalidomide and bortezomib). These toxicities are easily manageable by using appropriate supportive care, dose reduction and eventually drug interruption. During treatment, a constant monitoring is needed to enable physicians to intervene promptly.

The National Cancer Institute Toxicity Criteria (NCI-CTC) are used to grade adverse events. At the occurrence of any serious adverse event, namely grade 4 or higher haematological or grade 3 or higher non-haematological toxicities, treatment should be immediately withheld. It can be restarted once the event resolves completely or turns into a grade 1, and appropriate dose reductions are necessary [11].

Prognostic factors play a controversial role in determining the best treatment approach for MM. According to the International Staging System (ISS), symptomatic patients may be classified in three different risk groups: stage I (serum β2-microglobulin < 3.5 mg/L and serum albumin ≥ 35 g/L) is associated with a median survival of 62 months, stage II (serum β2-microglobulin > 3.5 mg/L and serum albumin < 35 g/L, or serum β2-microglobulin 3.5–5.5 mg/L) is associated with median survival of 44 months and stage III (β2-microglobulin ≥ 5.5 mg/L) is associated with a median survival of 29 months [12]. Serum-free light-chain incorporated into the ISS may improve the risk stratification [12, 13]. Chromosomal abnormalities can be detected by using cytogenetics and fluorescent in situ hybridization (FISH). In particular, patients with isolated deletion 13 (del13) on FISH analysis do not have a worse outcome, unless this abnormality is associated with 17p deletion (del17) or t(4;14). By FISH, t(4;14) and t(14:16) are associated with poorer outcome, t(11:14) does not have negative impact, and hyperdiploid is associated with more favourable outcome. Although new drugs, such as bortezomib and/or lenalidomide, may overcome poor prognosis, no specific therapy is routinely recommended for patients with chromosome abnormalities. Risk stratification on the basis of cytogenetics or FISH warrants confirmation from further studies with large numbers of patients [14].

This chapter will provide an overview of the latest combinations including novel agents used for the treatment of both young and elderly patients with newly diagnosed MM.

10.3 Therapeutic Options for Young Patients with Newly Diagnosed MM

10.3.1 The Traditional Approach: Vincristine plus Adriamycin and Thalidomide (VAD)

Since its introduction in the 1980s, VAD combination became one of the most commonly used treatments for young patients with MM eligible for ASCT. Patient deemed as candidates for transplant would receive VAD for 4–6 cycles and then proceed to

collection of stem cells and to transplantation. VAD was then adopted as the standard induction regimen for MM in major randomized studies, leading to a partial response (PR) rate ranging from 52 to 63%, with 3 to 13% of CR rate [13].

In recent years, the treatment of myeloma has undergone substantial changes. The use of novel agents, such as the first in-class proteasome inhibitor bortezomib and the immunomodulatory drugs (IMIDs) thalidomide and lenalidomide, in combination with established antimyeloma agents such as dexamethasone, adriamycin and cyclophosphamide, provided physicians with various new and more effective combinations that have replaced VAD regimen. Here follows a description of the main induction treatments for myeloma patients eligible for ASCT.

10.3.2 The Latest Combinations Including Novel Agents

10.3.2.1 Thalidomide-Based Therapies

The use of thalidomide in combination with adriamycin and dexamethasone (TAD) has been investigated in the prospective phase III HOVON-50/GMMG-HDR study. This trial explored the role of TAD in comparison with VAD as induction treatment [15]. One thousand two hundred and forty patients aged 34 to 65 years were enrolled in this study. A first interim analysis was performed on 402 patients, 201 per each treatment group. The at least PR rate after the 3 planned courses of TAD was significantly higher compared with the response after 3 courses of VAD (72% vs. 54%, $P<0.001$). The corresponding figures for the very good PR (VGPR) were 33% vs. 15% ($P<0.001$), with 4% of CR in the TAD group as compared to 2% in the VAD group. Despite the better quality of response induced by TAD, these results should be balanced against the greater proportion of venous thromboembolism (VTE) associated with the use of thalidomide: induction with TAD caused 8% of VTE, while the incidence of VTE in the VAD group was 4% only ($P=0.08$). No other significant difference in terms of serious adverse events was detected between the two groups. It is not yet known whether the higher responses achieved with TAD translate into prolonged event-free survival (EFS) and overall survival (OS). The benefit in favour of TAD remained after ASCT when considering the VGPR rate but not for the CR rate. This also translated into a superior PFS for TAD compared to VAD (33 months vs. 25 months, $P<0.001$), but OS was similar (59 months vs. 62 months) [13].

The British group explored the role of thalidomide in combination with cyclophosphamide and dexamethasone (CTD), compared to cyclophosphamide plus VAD (CVAD) as induction therapy before ASCT [16]. A total of 1,800 patients were enrolled in this large study. Preliminary results were in favour of CTD, which led to better responses than CVAD: at least PR rate was 96% after induction with CTD vs. 83% after CVAD and CR rates were 20% vs. 12%, respectively. Higher responses with CTD were also confirmed after ASCT, thus confirming its superiority over CVAD. A longer follow-up of patients entered into this large study will assess whether these increased and enhanced responses will translate into improved PFS and OS.

10.3.2.2 Bortezomib-Based Therapies

The association bortezomib-dexamethasone (VD) is a valuable induction option before ASCT. The IFM phase III study compared the combination VD with the standard VAD [17]. Patients were randomized to 4 arms: 119 patients received induction with VD followed by consolidation with dexamethasone, cyclophosphamide, etoposide and platinum (DCEP); 121 patients received VD without subsequent consolidation; 121 patients received VAD followed by DCEP and 121 received VAD without subsequent consolidation. After 4 cycles, VD induction resulted in higher response rates than VAD: in the intention to treat analysis, VD resulted in significantly higher CR plus near CR as compared to VAD (21% vs. 8%, $P=0.0023$) and at least VGPR of 47% vs. 19%, respectively ($P<0.0001$). The advantage obtained with VD was also maintained after ASCT, but the subsequent consolidation DCEP did not increase response rates. The incidence of adverse events was similar in the two groups (38% vs. 41%, respectively); serious adverse events were less frequent with VD than with VAD (25% vs. 31%) and caused death in less than 1% of patients who received induction with VD and in 3% of those who received induction treatment with VAD. Despite its higher efficacy, VD was associated with a higher incidence of all grade neuropathy than VAD (35% vs. 23%).

The role of bortezomib induction has been also explored in a recent phase III study conducted by the HOVON group [18]. In this study the combination of bortezomib–adriamycin–dexamethasone (PAD) was compared with VAD regimen. At least PR achieved with PAD was 78% and was significantly higher than 54% achieved after induction with VAD ($P<0.001$). At least VGPR was 42% after induction with PAD and 14% after VAD ($P<0.001$), with few CR (7% vs. 2%, $P<0.001$), which increased after transplantation (21% vs. 9%, $P<0.001$). Despite better responses with PAD, induction with VAD proved to be less toxic: in particular, grade 2 to 4 peripheral neuropathy occurred in 40% of patients in the PAD group and in 18% of patients who received induction with VAD; similarly, deep vein thrombosis occurred in 4% and 3% of patients (P<0.001), respectively.

An open, prospective, multicenter, uncontrolled phase II study conducted in Germany further investigated the role of bortezomib-containing induction regimens in combination with cyclophosphamide and dexamethasone (VCD) [19]. In this study, 200 patients aged up to 60 years with untreated myeloma were enrolled to receive 3 induction cycles with VCD. At least PR rate was 84%, with a CR rate of 12%. Eighty-four patients (24%) experienced a serious adverse event, which was due to bortezomib in 16% of patients, cyclophosphamide in 14% of patients and dexamethasone in 9% of patients. The mortality rate was 1% only. Fifty-three percent of the patients experienced grade 3 to 4 adverse events: grade 3 to 4 infections were reported in 2%, and grade 3 paraesthesia occurred in 2% of patients. These results confirm that VCD is a highly effective induction option for patients younger than 60 years. The benefits of VCD are further supported by another smaller study, where 33 patients were included [20]. By intention to treat, at least PR rate was 88%, with 22% of patients achieving VGPR and 39% of CR/near CR rate. Grade 3 and 4 toxicities included neutropenia (13%), thrombocytopenia (25%), hyperglycemia

(13%), thrombosis (7%) and peripheral neuropathy (7%). Grade 1 to 3 peripheral neuropathy was the main toxicity associated with this regimen; no grade 4 neuropathy was reported.

10.3.2.3 Lenalidomide-Based Combinations

Different studies have been designed to evaluate the feasibility and efficacy of lenalidomide-containing regimens as induction therapy in untreated patients with MM. The randomized ECOG trial compared lenalidomide and high-dose dexamethasone (RD; with dexamethasone given at 40 mg on days 1–4, 9–12, and 17–20 of a 28-day cycle) vs. lenalidomide plus low-dose dexamethasone (Rd; with dexamethasone 40 mg on days 1, 8, 15, and 22 of a 28-day cycle) showing a better short-term OS and lower toxicity with Rd [21].

A case-match study proved that the combination lenalidomide–dexamethasone–clarithromycin (BiRD) is superior to Rd [22]. Seventy-two newly diagnosed patients with myeloma received BirD regimen. In both groups patients were allowed to discontinue treatment to pursue transplant. CR was significantly higher with BiRd compared to Rd (46% vs. 14%, respectively, $P<0.001$); similarly, VGPR or better was higher with BiRd (74% vs. 33%, $P<0.001$). Median time to progression (48.3 vs. 27.5 months, $P=0.071$) was higher with BirD, and there was a trend towards better OS no statistically significant (3-year OS –90% vs. 73%, HR 0.48; 95% CI 0.17–1.37; $P=0.170$). Main grade 3–4 toxicities with BiRd were haematological, in particular thrombocytopenia (24% vs. 8%, $P=0.012$), whereas neutropenia was similar between the 2 groups (19% vs. 17%, $P=0.665$). Infections (17% vs. 10%, $P=0.218$) and dermatological toxicity (12% vs. 4%, $P=0.129$) were higher in patients who received Rd. The rate of venous thromboembolism was similar in the two groups (10% vs. 12%, respectively, in Rd and BiRd patients, $P=0.596$). This analysis shows that there may be a significant additive value when clarithromycin is added to Rd as induction treatment; however, these results still need to be confirmed in future prospective, randomized phase III studies.

Kumar and colleagues confirmed the additive positive effect of cyclophosphamide in combination with Rd (RCd) as initial therapy for newly diagnosed MM patients [23]. In this phase II dose finding pilot study of 53 patients, the best response was CR 2%, VGPR 38% and PR 43%. Grade 4 haematological toxicity was detected in 15% of patients, whereas 11% of patients experienced a severe non-haematological adverse event attributed to the drug (thrombosis, confusion, depression and sepsis). Myelosuppression was a significant toxicity and was lower with decreased dose of cyclophosphamide without any apparent loss of responses.

10.3.2.4 Bortezomib and IMID-Based Combinations

Several studies have been designed to assess the activity of bortezomib associated with either thalidomide or lenalidomide. A phase III study by Cavo and colleagues

investigated the efficacy and safety of bortezomib–thalidomide–dexamethasone (VTD) vs. TD as induction and consolidation therapies in a randomized trial of 474 patients [24]. The response rate was significantly higher with VTD induction therapy compared to TD: CR 19% vs. 5% and at least VGPR 62% vs. 31% ($P<0.001$). However, no difference in OS was seen between the two treatment groups, and longer follow-up is required. Grade 3 peripheral neuropathy was reported more frequently with VTD induction therapy than with TD (10% vs. 2%, respectively; $P<0.001$). The once-weekly administration of bortezomib and a reduced dose of thalidomide in VTD as consolidation therapy resulted in a dramatic decrease in the frequency of grade 3 peripheral neuropathy (2%).

Richardson and colleagues performed a phase I/II study to evaluate the role of bortezomib–lenalidomide–dexamethasone (VRD) in front-line treatment [25]. Sixty-six patients received 8 three-week cycles of the study combination. VRD showed to be highly effective, reporting a response rate of 100%, including 74% of at least VGPR. After a median follow-up of 21 months, estimated 18-month PFS and OS for the combination treatment were 75% and 97%, respectively. VRD demonstrated favourable tolerability as well: grade 3 to 4 haematologic toxicities included lymphopenia (14%), neutropenia (9%) and thrombocytopenia (6%). Thrombosis was rare (6% overall) and no treatment-related mortality was seen.

A most powerful combination of bortezomib, lenalidomide, cyclophosphamide and dexamethasone (VRCD) was studied in 25 patients to define the dose [26]. The maximum tolerated dose was not reached, so the recommended phase II 2 cyclophosphamide dose in VDCR is 500 mg/m^2, which was the highest dose tested. The overall response rate was 96%, including 20% stringent CR, 40% CR/near CR and 68% at least VGPR. This regimen showed to be effective and well tolerated.

Efficacy and safety profile of regimens discussed above are summarized in Tables 10.1 and 10.2.

10.4 Therapeutic Options for Elderly Patients with Newly Diagnosed MM

10.4.1 The Old Standard: Melphalan and Prednisone (MP)

Newly diagnosed elderly patients with MM, as well as younger patients ineligible for ASCT, have traditionally been treated with the oral combination MP for more than 40 years. A meta-analysis including 27 randomized studies, including MP and other chemotherapy-containing regimens, showed that higher response rates were reported with chemotherapy compared with MP (60% vs. 53%, $P<0.0001$), and MP was better tolerated; no significant difference in terms of survival was detected ($P=0.6$) [27].

Similar results were seen in a randomized trial comparing MP with melphalan plus dexamethasone (MD), high-dose dexamethasone (HD) and HD plus interferon-α. Response rates and PFS were superior in patients receiving melphalan-containing

Table 10.1 Efficacy of regimens used as front-line treatment in young patients with multiple myeloma

Regimen		*N*	Pre-transplant		Post-transplant		PFS/ EFS/TTP	OS	References
			At least PR	CR + VGPR	At least PR	CR + VGPR			
Thalidomide-based									
TAD vs. VAD	*TAD* Thal: 200–400 mg po days 1–28 Doxo: 9 mg/m^2 IV days 1–4 Dexa: 40 mg po days 1–4, 9–12, 17–20 *VAD* Vcr: 0.4 mg IV days 1–4 Doxo: 9 mg/m^2 IV days 1–4 Dexa: 40 mg po days 1–4, 9–12, 17–20	402	72% vs. 54%	33% vs. 15%	76% vs. 79%	49% vs. 32%	PFS 33 vs. 25 months	59 vs. 62 months	Lokhorst et al. [15]
CTD vs. CVAD	*CTD* CTX: 500 mg days 1,8,15 Thal:100 mg/day Dexa: 40 mg days 1–4,12–15 *CVAD* CTX: 500 mg days 1–8,15 Vcr: 0,4 mg IV days 1–4 Doxo: 9 mg/m^2 IV days 1–4 Dexa: 40 mg po days 1–4, 9–12	254	96% vs. 83%	20% vs. 12% (CR)	99% vs. 96%	58% vs. 41% (CR)	NA	NA	Morgan et al. [16]

Bortezomib-based									
VD vs. VAD	*VD* Vel: 1,3 mg/m² days 1,4,8,11 Dexa: 40 mg po days 1–4 9–12 *VAD* Vcr:0,4 mg IV days 1–4 Doxo: 9 mg/m² IV days 1–4 Dexa: 40 mg po days 1–4 9–12, 17–20	480	NA	47% vs. 19%	NA	72% vs. 51%	NA	NA	Harousseau et al. [17]
PAD vs. VAD	*PAD* Vel: 1.3 mg/m² IV days 1, 4, 8, 11 Doxo: 9 mg/m² IV days 1–4 Dexa: 40 mg po days 1–4, 9–12, 17–20 *VAD* Vcr: 0.4 mg IV days 1–4 Doxo: 9 mg/m² IV days 1–4 Dexa: 40 mg po days 1–4, 9–12, 17–20	827	78% vs. 54%	42% vs. 14%	88% vs. 75%	62% vs. 36%	PFS 35 vs. 28 months	NA	Sonneveld et al. [18]
VCD	Vel: 1,3 mg/m² days 1,4,8,11 CTX: 900 mg IV days 1 Dexa: 40 mg po days 1–2, 4–5, 8–9, 11–12	200	84%	12% (CR)	NA	NA	NA	NA	Knop et al. [19]
VCD	Vel: 1.3 mg/m² IV days 1, 4, 8, 11, CTX: 300 mg/m² po days 1, 8, 15, 22 Dexa: 40 mg po days 1–4, 9–12, 17–20	33	88%	61%	NA	NA	NA	NA	Reeder et al. [20]

(continued)

Table 10.1 (continued)

Regimen		*N*	Pre-transplant		Post-transplant		PFS/ EFS/TTP	OS	References
			At least PR	CR + VGPR	At least PR	CR + VGPR			
Lenalidomide-based									
RD vs. Rd	*RD* Len: 25 mg days 1–21 Dexa: 40 mg days 1–4, 9–12, 17–20 *Rd* Len: 25 mg days 1–21 Dexa: 40 mg days 1, 8, 15, 22	445	81% vs. 70%	50% vs. 40%	NA	NA	NA	1-year.OS 87% vs. 96% 2-year OS 75% vs. 87%	Rajkumar et al. [21]
BiRD vs. Rd	*BiRD* Len: 25 mg po days 3–21(cycle1) and days 1–21 of subsequent cycles Dexa: 40 mg po days 1, 2, 3, 8, 15 and 22 (cycle 1) and days 1, 8, 15, 22 of subsequent cycles Cl: 500 mg po twice daily *Rd* Len: 25 mg po days 1–21 Dexa: 40 mg po days 1, 8, 15, 22	144	NA	74% vs. 33%	NA	NA	TTP 48 vs. 27.5 months	3-year OS 90% vs. 73%	Gay et al. [22]
RCd	Len: 15 mg days 1–21 CTX: 300 mg or 300 mg/m^2 days 1, 8, 15 Dexa: 40 mg 1,8,15, 22	53	83%	40%	NA	NA	NA	NA	Kumar et al. [23]

Bortezomib- and IMID-based									
VTD vs. TD	*VTD* Vel:1.3 mg/m² IV days 1, 4, 8, 11 Thal: 200 mg/d po days 1–63 Dexa: 320 mg/cycle *TD* Thal: 200 mg/d po days 1–63 Dexa: 320 mg/cycle	474	NA	62% vs. 31%	NA	80% vs. 65%	2-year PFS 85% vs. 75%	OS no differences	Cavo et al. [24]
VRD	Vel: 1.0–1.3 mg/m² IV days 1, 4, 8, 11 Len: 15–25 mg po days 1–14 Dexa: 40 or 20 mg po days 1, 2, 4, 5, 8, 9, 11, 12	66	100%	74%	NA	NA	18-month PFS 75%	18-month OS 97%	Richardson et al. [25]
VRCD	Vel:1.3 mg/m² IV days 1, 4, 8, 11 CTX: 500 mg/m² IV days 1 and 8 (MTD) Dexa:40 mg po days 1, 8, 15 Len: 15 mg po days 1–14	25	96%	68%	NA	NA	NA	NA	Kumar et al. [26]

N indicates number of patients, *CR* complete remission, *PR* partial response, *VGPR* very good partial response, *PFS* progression-free survival, *EFS* event-free survival, *TTP* time to progression, *OS* overall survival, *M* melphalan, *P* prednisone, *T* thalidomide, *Vel* bortezomib, *Len* lenalidomide, *CTX* cyclophosphamide, *Dexa* dexamethasone, *Doxo* doxorubicin, *Vcr* vincristine, *Cl* clarithromycin. *TAD* thalidomide–Adriamycin–dexamethasone, *VAD* vincristine–adriamycin–dexamethasone, *CTD* cyclophosphamide–thalidomide–dexamethasone, *CVAD* cyclophosphamide–bortezomib–thalidomide–dexamethasone, *VD* bortezomib–dexamethasone, *VCD* bortezomib–cyclophosphamide–dexamethasone, *PAD* bortezomib–doxorubicin–dexamethasone, *RD* lenalidomide plus high-dose dexamethasone, *Rd* lenalidomide plus low-dose dexamethasone, *BiRD* clarithromycin–lenalidomide–dexamethasone, *RCd* lenalidomide–cyclophosphamide plus low-dose dexamethasone, *VTD* bortezomib–thalidomide–dexamethasone, *TD* thalidomide–dexamethasone, *VRD* bortezomib–lenalidomide–dexamethasone, *VRCD* bortezomib–lenalidomide–cyclophosphamide–dexamethasone, *NA* not available

Table 10.2 Safety (grade 3–4 adverse events) of regimens used as front-line treatment in young patients with multiple myeloma

Regimen	*N*	Neutropenia	Thrombocytopenia	Infection	Peripheral neuropathy	VTE	References
Thalidomide-based							
TAD	201	NA	NA	NA	12% (neurology)	8%	Lokhorst et al. [15]
TD	238	NA	NA	NA	2%	NA	Cavo et al. [24]
Bortezomib-based							
VD	240	NA	NA	NA	Grade 1-4, 35%	NA	Harousseau et al. [17]
PAD	413	3%	10%	26%	24%	4%	Sonneveld et al. [18]
VCD	200	NA	NA	2%	2%	NA	Knop et al. [19]
VCD	33	13%	25%	NA	7%	7%	Reeder et al. [20]
Lenalidomide-based							
RD	223	12%	6%	16%	2%	26%	Rajkumar et al. [21]
Rd	222	20%	5%	9%	2%	12%	Rajkumar et al. [21]
Rd	72	17%	8%	17%	NA	10%	Gay et al. [22]
BiRD	72	19%	24%	10%	NA	12%	Gay et al. [22]
Bortezomib- and IMID-based							
VTD	236	NA	NA	NA	9.7%	NA	Cavo et al. [24]
VRD	66	9%	6%	NA	NA %	Grade 1-4, 6%	Richardson et al. [25]
VRCD	25	Grade 3, 20% Grade 4, 4%	Grade 4, 12%	NA	48%	0	Kumar et al. [26]

N indicates number of patients, *TAD* thalidomide-adriamycin-dexamethasone, *TD* thalidomide-dexamethasone, *VD* bortezomib-dexamethasone, *PAD* bortezomib-adriamycin-dexamethasone, *VCD* bortezomib-cyclophosphamide-dexamethasone, *RD* lenalidomide-high-dose dexamethasone, *Rd* lenalidomide-low-dose dexamethasone, *BiRD* lenalidomide-dexamethasone-clarithromycin, *VTD* bortezomib-thalidomide-dexamethasone, *VRD* bortezomib-lenalidomide-dexamethasone, *VRCD* bortezomib-lenalidomide-cyclophosphamide-dexamethasone, *NA* not available

regimen, such as MP or MD, but this did not translate into an improved survival. Moreover, dexamethasone-containing regimens proved to be more toxic than MP, thus negatively affecting outcome [28].

In another randomized study comparing MP with TD, a higher response rate and longer PFS were reported with TD. However, patients receiving MP had a significantly longer survival, probably due to the better tolerability of MP compared to TD: extra-haematological toxicities, mainly related to high-dose dexamethasone, were superior in patients treated with TD, thus leading to a higher treatment-discontinuation rate. During the first year of therapy, non-disease-related deaths in the TD group were doubled compared to MP, with infections being the primary cause of death, especially in patients older than 72 years with poor performance status [29].

These findings suggest the benefit of incorporating an alkylating agent in the induction regimens of elderly MM patients and provided the rationale to explore the role of novel agents in combination with the standard MP.

10.4.2 New Treatments Containing Novel Agents

10.4.2.1 Thalidomide-Based Therapies

The role of thalidomide plus MP (MPT) has been extensively explored. Five randomized studies compared the combination MPT with the standard MP: PR rate was 42–76% with MPT and 28–48% with MP, and at least VGPR rate was 15–47% with MPT and 6–8% with MP; longer PFS (14–28 months) was reported in the MPT arms [10, 30–35]. In the two French studies, the PFS advantage observed with MPT also translates into a significant OS improvement (45–52 vs. 28–32 months) [10, 34], but this trend was not confirmed in the three other trials [30–33, 35]. In the Nordic study (NMSG), these results were also affected by the use of higher doses of melphalan (0.25 mg/kg) and thalidomide (200 mg every day) in a patient population older than 75 years and with approximately one-third patients having poor performance status (World Health Organization [WHO] performance status of 3 or 4 in 30% of patients) [31].

A recent meta-analysis pooled the existing data related to the efficacy of MP vs. MPT [36]. A total of 1,682 patients were included, 868 in the MP arm and 814 in the MPT arm. Median PFS was 15 (14, 17) months in the MP arm and 20 (19, 22) months in the MPT arm. Median OS was 33 (95% CI 30.4–36.5) months in the MP arm and 39 (35.6–39.0) months in the MPT arm. Overall hazard ratio of MPT compared to MP was 0.67 (0.55–0.80) for PFS when a random effects model was used and 0.82 (0.66–1.02) for OS. These results confirmed the role of MPT as one of the new standards of care for newly diagnosed elderly patients.

The main toxicities associated with MPT were grade 3–4 neutropenia, ranging from 16 to 48% and mainly linked to melphalan administration; peripheral neuropathy, reported in 6–20% of patients, particularly related to thalidomide; and venous thromboembolism (VTE) that varies from 3% to 12% [10, 30–34].

Another alkylating agent, cyclophosphamide, has been assessed in combination with thalidomide and dexamethasone (CTD). The Medical Research Council (MRC) Myeloma IX trial analysed and compared the combination CTD with the standard MP in 900 patients. Patients treated with CTD had higher responses than MP (at least PR was 83% vs. 46% and CR was 21% vs. 4%, respectively), but this did not translate into a longer survival. CTD showed to be a valuable option for elderly patients and also proved to be well tolerated, despite a slight increase of VTE [37].

An Italian study also reported positive results with thalidomide in association with pegylated liposomal doxorubicin and dexamethasone (ThaDD), followed by maintenance with thalidomide, in 62 patients transplant ineligible [38]. ThaDD resulted in 92% of at least PR, including 59% patients with at least VGPR and 24% of CR. After a median follow-up of 36 months, median TTP and PFS were 31 and 39 months, respectively, and five-year OS was 49%. Treatment was well tolerated; grade 3 or higher infections were reported in 14% of patients, thromboembolism, peripheral neuropathy in 10% and neutropenia in 8% of patients after 6 courses of therapy.

10.4.2.2 Lenalidomide-Based Therapies

A phase III randomized trial showed the superiority of RD vs. high-dose dexamethasone alone. Results with RD are promising: CR rate was 22% and was higher than with dexamethasone alone. A significant improvement in 1-year PFS (77% vs. 55%, $P=0.002$), without difference in OS, was observed with RD. As expected, RD also proved to be more toxic with grade 3–4 neutropenia 14% vs. 3% ($P=0.001$) [39].

In newly diagnosed MM, Rd showed to improve TTP, PFS and OS as compared to RD. In particular, the 1-year OS was 96% vs. 87% ($P<0.001$) and the 2-year OS was 87% vs. 75% ($P<0.001$). Responses were in favour of the high-dose dexamethasone regimen: CR rate was 5% vs. 4% ($P=0.04$), at least PR was 81% vs. 70% ($P=0.009$) with RD and Rd, respectively. However, RD administration was associated with a higher proportion of early deaths and adverse events, particularly thromboembolic events. Because of the safety advantages associated with Rd, patients crossed over to low-dose dexamethasone treatment, thus resulting in the premature interruption of the protocol. As a consequence of the crossover, 3-year OS rates are similar in the two treatment groups. A landmark analysis at 4 months was performed to assess the impact of the two different approaches: 3-year OS for patients who continued on primary therapy with RD beyond 4 months was 79%, whereas in patients who stopped treatment after 4 months, it was only 55% [21]. Considering its good tolerability and efficacy, Rd continued until progression can be considered a valuable option for patients older than 65 years.

The ECOG phase III study analysed the role of RD vs. Rd in a subset of 147 patients older than 70 years. PR was 75% with RD and 74% with Rd, including an at least VGPR of 42% and 48%, respectively. Median PFS was 16 months with RD and 22 months with Rd ($P=0.11$). Survival was significantly superior in the Rd

group, and 3-year OS was 73% compared to 61% with RD ($P = 0.03$). Toxicities were again higher with RD (grade 3–4 non-haematologic toxicities with RD 78% and Rd 59%) and included 30% of VTE and 20% of infections, while the corresponding figures for Rd were 20% and 10%. This study further supported the positive role of Rd also in very elderly patients, and future comparison with standard regimen such as VMP is needed [40].

A phase I/II dose escalating study explored the combination of MP in combination with lenalidomide (MPR). At the maximum tolerated dose (lenalidomide 10 mg/daily for 21 days and melphalan 0.18 mg/kg for 4 days every 4–6 weeks, plus prednisone 2 mg/kg days1–4), PR rate or better was 81%, including 48% of at least VGPR and 24% of patients with immunofixation-negative CR [41]. The 2-year EFS and OS rates for all patients were 80% and 91%, respectively [42]. These data provided the basis for the European Myeloma Network phase III study, comparing MP with MPR, with or without lenalidomide maintenance [43]. Responses were significantly higher with MPR followed by lenalidomide maintenance (MPR-R) compared to MP: at least PR rate was 77% vs. 50%, with 23% vs. 9% VGPR and 10% vs. 3% CR, respectively ($P < 0.001$). Similarly, the median PFS was higher in patients who received MPR-R than in those who received MP (31 months vs. 14 months). No differences were detected in the median OS (45 months in the MPR-R group vs. not reached in the MP group; $P=0.81$). The main grade 3 toxicities associated with both regimens were neutropenia (67% of patients treated with MPR-R vs. 29% with MP), thrombocytopenia (35% vs. 12%), infections (9% vs. 7%) and fatigue (5% vs. 3%). No grade 3–4 peripheral neuropathy was reported in the two groups. These data suggest that MPR-R may be considered a new and valuable option for myeloma patients in the non-transplant setting.

10.4.2.3 Bortezomib-Based Therapies

The VISTA trial explored the role of the combination bortezomib, melphalan and prednisone (VMP) compared to standard MP. This is the largest MP-based phase III study so far conducted, and a total of 682 patients were evaluated. VMP proved to be superior to the traditional MP for all efficacy endpoints: CR rate was 30% vs. 4% ($P < 0.001$), median TTP was 24 months vs. 16.6 months ($P < 0.001$) and the 3-year OS was 72% vs. 59% ($P = 0.0032$) [44]. Haematologic toxicities were similar in the two groups, with grade 4 thrombocytopenia (17% in the VMP group vs. 14% in the MP group) and grade 4 neutropenia (10% with VMP vs. 15% with MP) being the most serious toxicities. Peripheral neuropathy (13% with VMP vs. 0% with MP), gastrointestinal adverse events (20% vs. 5%) and fatigue (8% vs. <1%) were higher in patients given VMP than in those given MP. Grade 4 peripheral neuropathy was less common (<1% of VMP patients). The positive results achieved with VMP made it a new standard of care for myeloma patients who are not eligible for ASCT. A recent update of the VISTA trial further confirmed the benefits of the VMP regimen on survival. The 3-year OS from diagnosis was 69% with VMP as compared to 54% with MP. The median survival from start of subsequent therapy was longer with VMP than with MP (30 vs. 22 months; HR 0.815, $P = 0.219$) [45].

10.4.2.4 Bortezomib- and Thalidomide-Based Therapies

The new standard VMP has been compared to the combination of bortezomib, thalidomide and prednisone (VTP) as induction therapy in a randomized trial. Response rates were similar between the two groups: at least PR was 79% in both groups, with a CR rate of 22% vs. 27% (*P* nonsignificant [NS]), respectively, in the VMP regimen and VTP regimen. After a median follow-up of 22 months, there were no significant differences between the two arms in terms of 2-year TTP (VMP 75% vs. VTP 70%), PFS (VMP 71% vs. VTP 61%) and OS (VMP 81% vs. VTP 84%). Despite similar responses, VTP was more toxic than VMP: grade 3–4 cardiac toxicity rate was 8.5% vs. 0% ($P<0.001$), thromboembolic events were 4% vs. <1% ($P=$NS) and peripheral neuropathy was 9% vs. 5% ($P=$NS) with VTP and VMP, respectively. Thus, a higher proportion of patients in the VTP group discontinued treatment (17% vs. 8%, $P=0.003$). However, patients receiving VMP had a higher rate of neutropenia (37% vs. 21%, $P=0.003$), thrombocytopenia (22% vs. 12%, $P=0.03$) and infections (7% vs. <1%, $P=0.01$). These results lend further support to good tolerability of VMP, thus confirming its role as new standard of care for elderly myeloma patients [46].

Another recent, US community-based, randomized, phase IIIb study investigated the safety and efficacy of three bortezomib-based regimens (bortezomib–dexamethasone [VD], bortezomib–thalidomide–dexamethasone [VTD] and VMP) in previously untreated MM patients ineligible for high-dose therapy and ASCT. At least PR rate was 60%, 70% and 52% in the VD, VTD and VMP arms, respectively; at least VGPR 15%, 23% and 24%, respectively, including CR/near CR rates of 13%, 18% and 15%. VD was better tolerated, with a lower incidence of grade 3–4 AEs (58% compared to 71% seen in both the VTD and VMP arms). The incidence of serious AEs was 39% with VD, 50% with VTD and 36% with VMP. Discontinuation due to AEs was 10% in VD, 18% in VTD and 16% in VMP arm. VTD thus showed to be rather toxic. Consistently, any grade peripheral neuropathy occurred in 29% of patients in the VD group, 48% in the VTD group and 30% in the VMP group, and the rates of serious thromboembolic events was 6% with VD, 8% with VTD and 3% with VMP [47].

A recent phase III trial compared the combination of bortezomib, melphalan, prednisone and thalidomide followed by maintenance with VT (VMPT-VT) and VMP without maintenance. Responses were in favour of the four-drug regimen: at least PR rate was 89% vs. 81% ($P=0.01$), VGPR rate was 59% vs. 50% ($P=0.03$) and CR rate was 38% vs. 24% ($P=0.0008$), respectively. The improvement in response rate translated into prolonged survival: after a median follow-up of 17.8 months, the 2-year PFS was significantly longer in the VMPT-VT group (70% vs. 58%, HR=0.62, 95% CI 0.44–0.88, $P=0.008$). No differences in OS were detected between the two arms. Grade 3–4 neutropenia (37% vs. 28%, $P=0.02$) and cardiac complications (10% vs. 5%, $P=0.04$) were more common among VMPT-VT patients. The incidence of other grade 3–4 AEs was similar in the two groups: thrombocytopenia (21% vs. 19%), peripheral neuropathy (5% vs. 8%), infections (12% vs. 9%) and gastrointestinal complications (6% vs. 8%) with VMPT-VT and

VMP, respectively [48]. In both arms, bortezomib was initially administered twice weekly and was subsequently reduced to a once-weekly schedule to reduce toxicity, particularly peripheral neuropathy. After the amendment, the incidence of grade 3–4 peripheral neuropathy considerably decreased in both VMPT-VT (from 18% to 4%, $P=0.0002$) and VMP arms (from 13% to 2%, $P=0.0003$, respectively), without negatively affecting efficacy and PFS [49]. This is the first trial demonstrating the superiority of a 4-drug combination followed by maintenance over the latest standard of care VMP. This study also showed the effectiveness and good tolerability of the once-weekly schedule of bortezomib.

The efficacy of the treatments described above has been summarized in Table 10.3. The most frequent grade 3–4 AEs associated with these treatments have been summarized in Table 10.4. Table 10.5 shows the main treatment-related toxicities associated with the use of novel agents and provide some basic management information.

10.5 Role of Transplant in Elderly Patients

Patients older than 65 years, as well as those with significant comorbidities, are generally considered ineligible for standard melphalan 200 mg/m^2 followed by ASCT. A randomized trial exploring the efficacy of high-dose chemotherapy and transplant in patients with newly diagnosed MM showed a significantly higher 5-year OS in patients younger than 65 years undergoing ASCT compared to elderly patients (68% vs. 50%, respectively; $P=0.008$) [50]. Two randomized studies compared intermediate-dose melphalan (melphalan 100 mg/m^2, Mel100) and reduced-intensity ASCT with standard MP. The first study included patients aged 65 to 70 years and showed an improvement in EFS and OS with reduced-intensity ASCT compared with MP [51]. The second study included patients aged 65–75 years and compared reduced-intensity ASCT with MP and MPT. In this trial, PFS and OS were higher with MPT than with MP or Mel100, and no differences between MP and Mel100 were noted [10]. A recent phase II trial evaluated the efficacy of novel agents incorporated in both pre-transplant induction (PAD) and post-transplant consolidation and maintenance with lenalidomide, in patients aged 65–75 years, who received reduced-intensity ASCT: the CR rate was 13% after induction with bortezomib, 43% after Mel100 and 73% after consolidation-maintenance with lenalidomide. These data show that a sequential approach, including bortezomib as induction, followed by reduced-intensity ASCT and lenalidomide as consolidation-maintenance progressively improves responses, by taking advantage of a subsequent exposure to different drugs. Grade 3–4 toxicities during PAD induction included thrombocytopenia (17%), neutropenia (10%), peripheral neuropathy (16%) and pneumonia (10%). Lenalidomide therapy was well tolerated, with no cumulative or persistent neutropenia (grade 3–4 reported in 16%) and/or thrombocytopenia (6%); pneumonia (5%) and cutaneous rash (4%) were the more frequent extra-haematologic AEs [52].

Table 10.3 Efficacy of regimens used as a front-line treatment in elderly patients with multiple myeloma

Regimen		*N*	CR	≥ PR	PFS/EFS/TTP	OS	References
Thalidomide-based							
MPT	M: 4 mg/m² d 1–7 P: 40 mg/m² d 1–7 for six 4-week cycles T: 100 mg/day until PD	129	16%	76%	50% at 22 mo	50% at 45 mo	Palumbo et al. [32, 33]
MPT	M: 0.25 mg/kg d 1–4 P: 2 mg/kg d 1–4 T: 400 mg/day for 12 6-week cycles	125	13%	76%	50% at 28 mo	50% at 52 mo	Facon et al. [10]
MPT	M: 0.25 mg/kg d 1–4 P: 2 mg/kg d 1–4 T: 100 mg/day for 12 6-week cycles	113	7%	62%	50% at 24 mo	50% at 45 mo	Hulin et al. [34]
MPT	M: 0.25 mg/kg d 1–4 P: 100 mg d 1–4 T: 200–400 mg/day in a 6-week cycle until plateau T: 200 mg/day until disease progression	182	6%	42%	50% at 20 mo	50% at 29 mo	Waage et al. [31][a]
MPT	M: 0.25 mg/kg P: 1 mg/days 1–5 T: 200 mg/day for eight 4-week cycles, followed by T: 50 mg/day until disease progression	165	2%	66%	50% at 14 mo	50% at 37 mo	Wijermans et al. [35][a]
CTD	C: 500 mg d 1, 8, 15 T: 100–200 mg/day D: 40 mg d 1–4, 12–15 in a 3-week cycles	450	21%	83%	NA	NA	Morgan et al. [37]
ThaDD	Dox: 40 mg/m² d 1 D: 40 mg d 1–4, 9–12 T: 100 mg/d continuously	62	24%	92%	NA	66% at 36 mo	Offidani et al. [38]
Lenalidomide-based							
RD	R: 25 mg d 1–21 D: 40 mg d 1, 8, 15, 22 in a 4-week cycle	223	5%	81%	NA	96% at 12 mo	Rajkumar et al. [21]
Rd	R: 25 mg d 1–21 d: 40 mg d 1, 8, 15, 22 in a 4-week cycles	222	4%	70%	NA	87% at 12 mo	Rajkumar et al. [21]

MPR	M: 0.18 mg/kg d 1–4 P: 2 mg/kg d 1–4 for nine 4-week cycles R: 10 mg d 1–21 until relapse or progressive disease	152	10%	77%	50% at 31 mo	50% at 45 mo	Palumbo et al. [43]
Bortezomib-based							
VMP	M: 9 mg/m² d 1–4 P: 60 mg/m² d 1–4 V: 1.3 mg/m² d 1, 4, 8, 11, 22, 25, 29, 32 for the first four 6-week cycles; d 1, 8, 15, 22 for the subsequent five 6-week cycles	344	30%	71%	50% at 24 mo	72% at 36 mo	San Miguel et al. [44]
VMP	M: 9 mg/m² d 1–4 P: 60 mg/m² d 1–4 V: 1.3 mg/m² d 1, 8, 15, 22	257	24%	81%	70% at 36 mo	87% at 36 mo	Palumbo et al. [48]
VMP	M: 9 mg/m² d 1–4 P: 60 mg/m² d 1–4 V: 1.3 mg/m² twice weekly (d 1, 4, 8, 11; 22, 25, 29 and 32) for one 6-week cycle, followed by once weekly (d 1, 8, 15 and 22) for five 5-week cycles	344	22%	79%	72% at 24 mo	81% at 24 mo	Mateos et al. [46]
Bortezomib- and thalidomide-based							
VTP	T: 100 mg/day P: 60 mg/m² d 1–4 V: 1.3 mg/m² twice weekly (d 1, 4, 8, 11; 22, 25, 29 and 32) for one 6-week cycle, followed by once weekly (d 1, 8, 15 and 22) for five 5-week cycles	130	27%	79%	61% at 24 mo	84% at 24 mo	Mateos et al. [46]

(continued)

Table 10.3 (continued)

Regimen		*N*	CR	≥ PR	PFS/EFS/TTP	OS	References
VMPT	M: 9 mg/m^2 d 1–4 P: 60 mg/m^2 d 1–4 V: 1.3 mg/m^2 d 1, 8, 15, 22 T: 50 mg d 1–42 for nine 5-week cycles followed by Bor: 1.3 mg/m^2 every 15 days and T: 50 mg/day as maintenance	254	38%	89%	60% at 36 mo	88% at 36 mo	Palumbo et al. [48]

N indicates number of patients, *CR* complete remission, *PR* partial response, *PFS* progression-free survival, *EFS* event-free survival, *TTP* time to progression, *OS* overall survival, *M* melphalan, *P* prednisone, *T* thalidomide, *V* bortezomib, *R* lenalidomide, *C* cyclophosphamide, *D* high-dose dexamethasone, *d* low-dose dexamethasone, *Dox* doxorubicin, *MPT* melphalan-prednisone-thalidomide, *VMP* bortezomib-melphalan-prednisone, *VTP* bortezomib-thalidomide-prednisone, *VMPT* bortezomib-melphalan-prednisone-thalidomide, *CTD* cyclophosphamide-thalidomide-dexamethasone, *ThaDD* thalidomide-doxorubicin-dexamethasone, *MPR* melphalan-prednisone-lenalidomide, *NA* not available, *PD* progressive disease

[a]Updated information was presented at the meeting (American Society of Clinical Oncology, European Haematology Association and American Society of Hematology congress)

Table 10.4 Safety (grade 3–4 adverse events) of regimens used as front-line treatment in elderly patients with multiple myeloma

Regimen	*N*	Neutropenia	Thrombocytopenia	Infection	Peripheral neuropathy	VTE	References
Thalidomide-based							
MPT	129	16%	3%	10%	8%	9%	Palumbo et al. [32, 33]
MPT	125	48%	14%	13%	6%	12%	Facon et al. [10]
MPT	113	23%[a]	NA	NA	Grade 2-4, 20%	6%	Hulin et al. [34]
MPT	165	NA	NA	14%	9%	3%	Wijermans et al. [35][a]
CTD	450	NA	NA	NA	NA	NA	Morgan et al. [37]
ThaDD	62	8%	0%	14%	10%	10%	Offidani et al. [38]
Lenalidomide-based							
MPR[b]	152	67%	35%	9%	0%	1%	Palumbo et al. [43]
Bortezomib-based							
VMP	344	40%	37%	10%	13%	3%	S Miguel et al. [44]
VMP	257	28%	20%	9%	8%	2%	Palumbo et al. [48]
VMP	344	40%	38%	NA	13%	NA	Mateos et al. [45]
Bortezomib- and Thalidomide-based							
VTP	130	21%	12%	<1%	9%	4%	Mateos et al. [46]
VMPT	254	38%	22%	13%	12%	5%	Palumbo et al. [48]

N indicates number of patients, *MPT* melphalan-prednisone-thalidomide, *VMP* bortezomib-melphalan-prednisone, *VTP* bortezomib-thalidomide-prednisone, *VMPT* bortezomib-melphalan-prednisone-thalidomide, *CTD* cyclophosphamide-thalidomide-dexamethasone, *MPR* melphalan-prednisone-lenalidomide, *NA* not available

[a]Updated information was presented at the meeting (American Society of Clinical Oncology, European Haematology Association and American Society of Hematology congress)

[b]Grade 3 only

Table 10.5 Management of adverse events in multiple myeloma patients treated with novel agents

Adverse event	Antimyeloma agents involved	Management	Dose modification
Neutropenia	Lenalidomide, bortezomib and combinations	G-CSF until neutrophil recovery in case of uncomplicated grade 4 AE or grade 2–3 AEs complicated by fever or infection	25–50% drug reduction
Thrombocy topenia	Bortezomib and combinations, lenalidomide and combinations	Platelet transfusion in case of grade 4 AE	25–50% drug reduction
Anaemia	Bortezomib and combinations, lenalidomide and combinations	Erythropoietin or darbepoietin in case of haemoglobin level $\leq$ 10 g/dL	25–50% drug reduction
Infection	All the agents	Trimethoprim-co-trimoxazole for *Pneumocystis carinii* prophylaxis during high-dose dexamethasone. Acyclovir or valacyclovir for HVZ prophylaxis during bortezomib-based therapy	25–50% drug reduction
Neurotoxicity	Bortezomib and combinations, thalidomide and combinations	Neurological assessment before and during treatment. Prompt dose reduction of the suspected drug is recommended	Bortezomib: 25–50% reduction for grade 1 with pain or grade 2 peripheral neuropathy; dose interruption until peripheral neuropathy resolves to grade 1 or better with restart at 50% dose reduction for grade 2 with pain or grade 3 peripheral neuropathy; treatment discontinuation for grade 4 peripheral neuropathy. Thalidomide: 50% reduction for grade 2 neuropathy; discontinuation for grade 3; resume thalidomide at a decreased dose if neuropathy improves to grade 1
Cutaneous toxicity	Thalidomide and combinations, lenalidomide and combinations	Steroids and antihistamines	Interruption in case of grade 3–4 AE 50% reduction in case of grade 2 AE
Gastrointestinal toxicity	All the agents	Appropriate diet, laxatives, exercise, hydration, antidiarrheic drugs	Interruption in case of grade 3–4 AEs 50% reduction in case of grade 2 AEs

Thrombosis	Thalidomide and combinations, lenalidomide and combinations	Aspirin 100–325 mg if no or one individual/myeloma thrombotic risk factor is present. LMWH or full-dose warfarin if two or more individual/myeloma risk factors are present and in all patients with thalidomide-related risk factors	Drug temporary interruption and full anticoagulation, then resume treatment
Renal toxicity	Lenalidomide	Correct precipitant factors (dehydration, hypercalcemia, hyperuricemia, urinary infections and concomitant use of nephrotoxic drugs)	Reduce dose according to creatinine clearance: If 30–60 mL/min: 10 mg/day; If <30 mL/min without dialysis needing: 15 mg every other day; If <30 mL/min with dialysis required: 5 mg/day after dialysis on dialysis day
Bone pain	None	Start with simple non-opioid analgesics. If no benefit is detected continue with weak opioids (e.g. codeine 8 mg/paracetamol 500 mg as co-codamol tablets; usual dosage is 2 tablets 6 hourly). In case of no relief, use strong (natural) opioids (for instance, morphine 5–10 mg orally, given 4 hourly in case of severe pain) or synthetic opioids. Local radiotherapy is also effective for pain relief of bone disease	NA
Bone disease	None	Vertebroplasty (percutaneous injection of polymethacrylate or equivalent material into the vertebral body). The use of balloon kyphoplasty improves vertebral height. Long-term biphosphonate treatment helps prevent bone disease. Other options are intravenous pamidronate, intravenous zoledronic acid as well as oral clodronate (used, e.g. in the UK)	NA

G-CSF granulocyte colony-stimulating factor, *HVZ* herpes-varicella-zoster, *LMWH* low-molecular-weight heparin, *NA* not available, *AE* adverse event

Data from these trials support the use of reduced-intensity ASCT for both elderly and younger patients with pre-existing comorbidities, for whom full-dose chemotherapy and ASCT would be too toxic. However, further validation in randomized trials is needed.

10.6 Conclusion

The availability of new targeted therapies in combination with conventional chemotherapy or low-dose dexamethasone has substantially changed the treatment of MM. The treatment should be initiated only in symptomatic MM patients and should be tailored on the basis of patients' characteristics, comorbidities and expected toxicity profile associated with each regimen.

Full-dose melphalan followed by ASCT is the treatment of choice in patients younger than 65 years, and induction therapy including new drugs seems the most suitable preparatory regimen before transplant. The incorporation of new drugs as induction followed by ASCT appears to lead to VGPR rates slightly superior to those achieved with conventional chemotherapy with new drugs. Randomized trials are needed to directly compare the current best chemotherapeutic approach with the best ASCT strategies and to determine the best induction, consolidation and maintenance therapy.

In elderly patients, the combination of an alkylating drug with a novel agent should be considered as standard approach. Randomized phase III studies have shown that MPT, MPV and MPR proved to be more effective than the traditional treatment with MP; hence, they can now be regarded as new standards of care for patients ineligible for ASCT. The four-drug combination VMPT-VT recently showed to be more effective than VMP, thus it can be considered a new valuable option for elderly patients with MM. Preliminary results on Rd are also encouraging, but they still need to be further validated in comparative studies with confirmed regimen MPT, MPV and MPR.

The wide variety of treatment options now available will support the choice of a more personalized therapy, by balancing efficacy and toxicity of each drug. Patients with renal impairment can be treated with both thalidomide- and bortezomib-based therapies. Lenalidomide should be preferred in patients with pre-existing neuropathy, and appropriate dose reduction is needed in case of renal insufficiency. Patients with risk factors for thrombosis can be safely treated with bortezomib, and IMIDs can be administered with appropriate antithrombotic prophylaxis.

These novel agents and combinations alter the natural history of MM and improve both the quality of life and outcome, with a subsequent great advantage for the patient.

Disclosure Statement Antonio Palumbo has received honoraria from Celgene, Janssen-Cilag, Bristol-Myers Squibb, Millennium, Merck and Onyx, and served on the advisory board for Celgene and Janssen-Cilag. Alessandra Larocca has received honoraria from Celgene and Janssen-Cilag.

Acknowledgment Our thanks to Giorgio Schirripa, Barbara Lupo and Mariella Genuardi for help in writing the manuscript.

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Chapter 11
The Effect of Novel Anti-myeloma Agents on Bone Metabolism

Evangelos Terpos

11.1 Introduction

Multiple myeloma (MM) is a relatively common hematological malignancy characterized by the accumulation of abnormal plasma cells in the bone marrow. Myeloma patients often develop osteolytic bone lesions that result in debilitating skeletal complications such as pathologic fractures, severe bone pain, and hypercalcemia. At diagnosis, two thirds of patients have lytic lesions, as determined by skeletal survey, and approximately 60% have bone pain [1]. In addition to the bone disease associated with MM itself, glucocorticoids such as dexamethasone and prednisone, which are a mainstay of MM treatment, are well known to be associated with loss of bone tissue [2]. The development of lytic bone lesions is related to an uncoupled bone remodeling: the increased osteoclast-mediated bone resorption is accompanied by a reduction in new bone formation [3–5]. Lytic lesions rarely heal, even in patients in complete remission. Bone disease in MM is often assessed by plain radiographs that show radiolucent lesions without calcification, known as "punched-out" lesions. Magnetic resonance imaging (MRI) and computed tomography (CT) are more sensitive than conventional radiography for detecting lytic lesions, with MRI offering complementary information and positron emission tomography (PET)/CT scanning being a useful additional diagnostic tool for MM bone disease [6]. Although radiographs are useful in diagnosing lytic lesions, they do not provide information about ongoing bone remodeling. Therefore, biochemical markers of bone metabolism have been used in an attempt to assess the rate of bone turnover in patients with MM and to monitor MM bone disease. Bone resorption is mainly assessed by the measurement of serum or urinary degradation products of

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N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 2: Translational and Clinical Research*,
DOI 10.1007/978-1-4614-5260-7_11, © Springer Science+Business Media New York 2013

bone collagen, namely, N- and C-terminal cross-linking telopeptide of collagen type I (NTX and CTX or ICTP, respectively), and the serum levels of tartrate-resistant acid phosphatase type-5b (TRAcP-5b), a molecule which is produced primarily by activated osteoclasts. On the other hand, bone formation is assessed by the serum measurement of molecules that are produced by osteoblasts, such as the bone-specific alkaline phosphatase (bALP) and osteocalcin (OC) [7].

Over the last decade, novel agents have been used in the management of MM. Immunomodulatory agents (IMiDs), such as thalidomide and lenalidomide and proteasome inhibitor, bortezomib, have shown significant anti-myeloma activity in both newly diagnosed and relapsed/refractory MM [8–10]. Besides their potent efficacy against myeloma cells, these agents modify the interactions between malignant plasma cell and bone marrow microenvironment and alter abnormal bone metabolism in MM.

11.2 The Abnormal Coupling of Bone Remodeling in Myeloma

The pathogenesis of bone disease in MM occurs via the disruption of the finely tuned balance between the bone-forming activity of osteoblasts and the bone-resorptive activity of osteoclasts, through which bone health is normally maintained. In MM bone disease, the activity of osteoclasts is substantially increased through multiple interactions between MM cells and the bone marrow microenvironment, resulting in increased bone resorption, while osteoblast differentiation and activity is impaired, thus decreasing bone formation [3–5]. The increased osteoclast activity seen in MM is primarily mediated through disturbances in the receptor activator of nuclear factor kappaB ligand (RANKL)/RANK/osteoprotegerin (OPG) axis, with RANKL expression being enhanced and OPG being suppressed [4, 11]. The RANKL/OPG ratio is important in regulating the level of osteoclast activity. RANKL signaling promotes the formation of osteoclasts from their precursors [12], as well as osteoclast survival and the expression of osteoclast-specific genes such as the lytic enzyme TRAcP, stimulating bone resorption [13]. OPG is a decoy receptor for RANKL that is produced by osteoblasts and interrupts osteoclastogenic signaling [14]. Notably, RANKL expression is enhanced by glucocorticoids. In addition, macrophage inflammatory protein-1α (MIP-1α) stimulates osteoclast formation [15] and is associated with bone destruction [16, 17] and poor prognosis [18] in MM. Cytokine signaling from MM cell–bone marrow microenvironment interactions stimulate osteoclast activity, which produces other cytokines and growth factors that subsequently stimulate MM cell adhesion and growth, creating a “vicious cycle” of disease development and bone destruction [5, 19, 20].

Bone formation through osteoblast activity is inhibited in MM due to inhibition of the osteoblast transcription factors Runx2/Cbfa1 and osterix and the Wnt/β-catenin signaling pathway by myeloma cells [21–23]. Dickkopf-1 (Dkk-1) is a *Wnt* pathway antagonist expressed predominantly in the bones of adults and is upregulated in

myeloma patients with osteolytic lesions [23–25]. Previous studies indicated that overexpression of Dkk-1 by myeloma cells may disrupt the normal balance between osteoblasts and osteoclasts by blocking osteoblast differentiation and thus promote bone resorption [22]. Furthermore, Dkk-1 may indirectly increase osteoclastogenesis via a Dkk1-mediated increase in RANKL/OPG ratio [26, 27]. The inhibition of Dkk-1 removes suppression of bone formation and prevents the development of osteolytic bone disease in MM murine model [28]. Sclerostin is another Wnt inhibitor which reduces osteoblast function and is increased in MM patients with lytic bone disease [29].

These data support the complex interactions between myeloma and stromal cells and the multifactorial pathogenesis of osteolytic disease in MM.

11.3 Management of Myeloma Bone Disease

Bisphosphonates (BPs) are the current standard of care for the management of myeloma patients. Oral clodronate, intravenous pamidronate, and intravenous zoledronic acid have been licensed for use in myeloma patients with osteolytic disease or diffuse osteoporosis (only the intravenous drugs have been approved by FDA). Although BPs can reduce skeletal-related events (SREs) and bone pain, improve performance status, and conserve patient quality of life, patients need to be monitored for signs of renal impairment and osteonecrosis of the jaw. Bisphosphonates are also effective in preventing steroid-induced osteoporosis. However, the optimal duration of BP administration and the best BP has to be determined [30].

Denosumab is a fully human immunoglobulin (Ig)G2 monoclonal antibody that binds to RANKL with high affinity and specificity, thereby inhibiting osteoclastogenesis. A number of recent studies demonstrated that denosumab improved SREs among patients with bone metastases from solid tumors or MM. More specifically, denosumab was non-inferior to zoledronic acid in delaying time to first SRE in 1,776 patients with advanced solid tumors or MM [31]. However, further studies are needed in the MM setting before the approval of this very interesting agent in myeloma bone disease.

Novel drugs that target molecules that are implicated in the biology of myeloma bone disease, such as Dkk-1 or IL-6, are studied in phase 2 trials, and the results of these studies are eagerly anticipated [20].

11.4 Immunomodulatory Drugs and Myeloma Bone Disease

11.4.1 Preclinical Studies

Thalidomide and other IMiDs, such as lenalidomide and pomalidomide, are very effective for the management of patients with MM [8, 32, 33]. The first evidence of

the effect of IMiDs on bone metabolism came from the work of Anderson et al. [34] who found that thalidomide and mainly pomalidomide almost completely abrogated RANKL-induced osteoclast formation by downregulation of the expression of PU.1, which is a major transcriptional factor for osteoclastogenesis. Pomalidomide inhibited the formation of mature multinucleated osteoclasts, while it induced the production of an overgrowing population of small cells that lacked the features and activity of osteoclasts. Pomalidomide acted especially at the early stages of the osteoclast differentiation. In that study, significant inhibition of osteoclast formation was observed at concentrations of 1 μM of pomalidomide, which is similar or even lower than that achieved in vivo after the therapeutic administration of this agent. Thalidomide exhibited similar but less potent effects than pomalidomide, suggesting that thalidomide is less potent than pomalidomide, at least in the inhibition of osteoclast formation [34].

Lenalidomide also inhibited osteoclast formation through similar mechanisms, such as the downregulation of PU.1 gene expression. Lenalidomide decreased $alpha_V beta_3$-integrin, TRAcP-positive cells, and bone resorption on dentin disks in a dose-dependent manner. However, lenalidomide did not alter the counts of mature osteoclasts, but strongly inhibited B-cell activation factor (BAFF) and a proliferation-inducing ligand (APRIL) that are major MM growth and survival factors and are produced mainly by osteoclasts [35].

In another in vitro study, all IMiDs (thalidomide, lenalidomide, and pomalidomide) at a dose of 10 microM showed an anti-osteoclast effect without cytotoxicity to osteoblast differentiation, at which dose myeloma cells underwent apoptosis [36]. These studies suggest that IMiDs through the inhibition of the proliferation of myeloma cells and the reduction of osteoclast formation seem to break the vicious cycle between myeloma cells and osteoclasts, leading to further reduction of tumor burden and bone resorption. However, despite the inhibitory effect on osteoclast, no effect on osteoblast activity and bone formation was observed with IMiDs in the preclinical setting. All these interactions are depicted in Fig. 11.1.

11.4.2 Clinical Studies

Two clinical, phase II, trials have studied the effect of thalidomide on bone metabolism of patients with MM (Table 11.1). In the first study, Terpos et al. [38] showed that thalidomide in combination with dexamethasone reduced bone resorption in 35 patients with relapsed/refractory myeloma. Thalidomide was administered at a dose of 200 mg/daily, while dexamethasone was given at a dose of 40 mg/daily for 4 days every 15 days until maximal response and then at 40 mg/daily for 4 days monthly for a median period of 10 months. Patients who responded to treatment or had stable disease continued on treatment until disease progression. All patients have been on zoledronic acid since diagnosis and continued to receive zoledronic acid at a dose of 4 mg every 28 days while on study. According to EBMT criteria, the objective response (OR) rate was 58%, while 31% of the patients achieved either minor

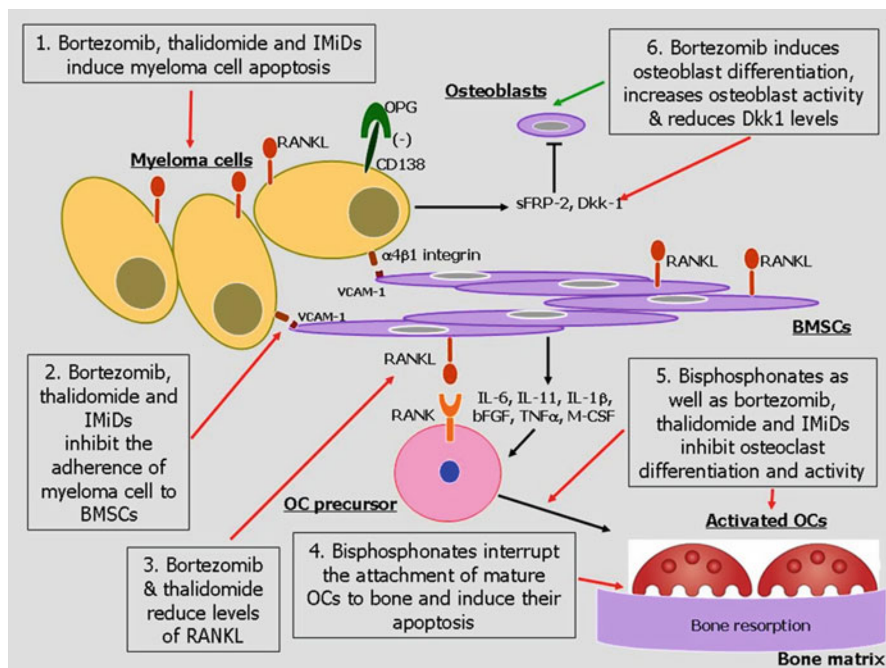

Fig. 11.1 The effect of thalidomide, IMiDs, and bortezomib on myeloma cells and their interactions with bone marrow stromal cell (BMSCs), osteoblasts, and osteoclasts

response (8%) or stable disease (23%). The combination of this intermediate dose of thalidomide with dexamethasone produced a significant reduction of both studied serum markers of bone resorption, i.e., CTX and TRAcP-5b, at the 3rd month post-initiation of treatment, which continued at the 6th month of the study. The combined treatment also reduced circulating soluble RANKL (sRANKL) levels and sRANKL/OPG ratio at 6 months post treatment initiation. Furthermore, there was a strong correlation between changes of sRANKL/OPG ratio and changes of c-5b and CTX, suggesting that the reduction of bone resorption by thalidomide is, at least partially, due to the reduction of RANKL levels [38]. This result is also supported by the in vitro finding that thalidomide can abrogate RANKL-induced osteoclast formation [34]. Despite the reduction of bone resorption, intermediate doses of thalidomide and dexamethasone showed no effect on bone formation, as assessed by serum levels of bALP and OC. This regimen produced no healing of the observed lytic lesions after radiographic evaluation of responders at six months post treatment, though only one of four patients who progressed while on treatment presented with new lytic lesions at the time of progression [38].

In the second study, Tosi et al. [37] showed that thalidomide can reduce bone resorption in newly diagnosed MM patients too. In this study, 40 patients received the combination of thalidomide (100 mg/d for 2 weeks and then 200 mg/d), dexamethasone (40 mg/day on days 1–4, 9–12, 17–20/28 days on odd cycles and on days

Table 11.1 Clinical studies for the effect of novel anti-myeloma agents on bone metabolism

Regimen	MM study population	No. of patients	Results	Subpopulation analysis
Thalidomide (+Dexa)				
Tosi et al. [37][a]	Newly diagnosed	40	↓ Bone resorption markers (CTX & NTX)	In responders
			↓ Bone formation markers (bALP & OC)	In all patients
Terpos et al. [38][a]	Relapsed/refractory	35	↓ Bone resorption markers (CTX & TRAcP-5b)	In all patients
			↓ Osteoclast stimulators (sRANKL, sRANKL/OPG ratio)	In all patients
			↔ Bone formation markers (bALP & OC)	In all patients
Lenalidomide				
Breitkreutz et al. [35]	Relapsed/refractory	20	↓ Osteoclast numbers	ND
			↓ Osteoclast differentiation	
			↓ Bone resorption	
Bortezomib-based regimens				
Zangari et al. [39]				
Analysis of UARK 2001-37 (VT±D)	Relapsed/refractory	24	↑ Total ALP	In responders
Analysis of APEX (V vs. D)	Relapsed/refractory	V: 217 D: 205	↑ Total ALP only in bortezomib group	In all patients
Heider et al [40][a]—V±D	Relapsed/refractory	58	↑ Bone formation markers (bALP & OC)	In all patients
Terpos et al [41][a]—V±D	Relapsed/refractory	34	↓ Bone resorption markers (CTX & TRAcP-5b)	In all patients
			↓ Osteoclast stimulators (sRANKL, sRANKL/OPG ratio)	In all patients
			↑ Bone formation markers (bALP & OC)	In all patients
			↓ Osteoblast inhibitors (Dkk-1)	In responders[c]
Giuliani et al [42][a]—V	Relapsed/refractory	21	↓ Bone resorption markers (CTX)	In all patients[d]
			↑ Osteoblast numbers	In responders
Ozaki et al. [43]—VD	Relapsed/refractory	14	In two patients, a "dramatic improvement" in osteolytic lesions was observed (detected via multidetector CT scans)	In responders
			↑ Total ALP and OC	In all patients
Terpos et al. [44, 45][a] (VMDT)	Relapsed/refractory	62	↓ Bone resorption markers (CTX & TRAcP-5b)	In all patients In all patients
			↓ Osteoclast stimulators (sRANKL, sRANKL/OPG, MIP-1α)	In all patients
			↔ Bone formation markers (bALP & OC)	In all patients

			↓ Osteoblast inhibitors (Dkk-1)	In all patients
Terpos et al. [45]—VTD post ASCT	Newly diagnosed (post-ASCT consolidation)	32	↓ Dkk-1	In all patients
			↓sRANKL/OPG ratio	In all patients
			↓ Bone resorption markers (CTX & TRAcP-5b)	In all patients
Zangari et al. [46]—V	Relapsed/refractory	18	↑ Bone volume in 6/7 patients	In all patients
			↑ OC	
Delforge et al. [47][b]	Newly diagnosed not eligible for ASCT	VMP: 344	Progression of bone disease: VMP 3% vs. MP 11%	
(analysis of VISTA study: VMP vs. MP)		MP: 338	SREs: VMP 4% vs. MP 5%	
			↑ bALP in VMP vs. MP (50% vs. 30%)	In all patients
Terpos et al. [45][a]	Relapsed/refractory	91	↑ Bone formaton markers (bALP & OC) only in VRD arm	In all patients
(VRD vs. RD)			↓ Dkk-1 and sRANKL/OPG ratio only in VRD arm	In all patients
Lund et al. [48]—VD	Relapsed/refractory	20	↑ Bone formation markers (bALP & PINP)	In responders
			↓ NTX	In all patients
			↓ Dkk-1	In responders
Terpos et al [49][a]—V ± D	Relapsed/refractory	27	Significant increase in BMD after 8 cycles of therapy in the lumbar spine (assessed via DXA); 4 patients showed at least a 10% increase in lumbar spine BMD (all had responded to therepy)	
			↑ Bone formation markers (bALP & OC)	In all patients
			↓ NTX	In all patients
Terpos et al. [29][a]—V ± D	Relapsed/refractory	25	↓ Sclerostin	In all patients

[a]Concomitant bisphosphonates administration in the majority of patients
[b]Use of bisphosphonates: VMP 73% of patients, MP 82%
[c]bALP was increased only in responders while OC was elevated in all patients
[d]This reduction was not reached statistical significance

1–4 on even cycles), and zoledronic acid (4 mg/28 days) for 4 months. The OR rate was 77.5%. A significant reduction in both studied markers of bone resorption, i.e., urinary NTX and serum CTX, was observed, but only in responders. This reduction was accompanied by a reduction in bone pain in 60% of the patients. However, markers of bone formation (bALP and OC) were also reduced in all patients (responders and refractory), suggesting that the combined regimen may have a negative effect on the already exhausted osteoblasts of newly diagnosed patients, possibly due to the concomitant use of dexamethasone [37].

In a small series of myeloma patients ($n=20$) treated with lenalidomide, serum RANKL and serum RANKL/OPG ratio were significantly reduced, whereas OPG was increased after 2 cycles of lenalidomide administration [35]. In a recent study contacted by the Greek Myeloma Study Group, the effect of lenalidomide and dexamethasone (RD) combination on one metabolism was evaluated in 106 consecutive patients with relapsed/refractory MM. Lenalidomide was given at the standard dose of 25 mg, p.o., daily (or adjusted to creatinine clearance) on days 1–21 of a 28-day cycle, and dexamethasone was given at a dose of 40 mg on days 1–4 and 15–18 for the first four cycles and only on days 1–4 thereafter. All patients were under zoledronic acid both pre- and during treatment period. The objective response was 55% (CR 12%, VGPR 11%, PR 32%). The administration of RD produced a reduction in CTX serum levels in responders compared to nonresponders who showed an increase in both CTX and TRAcP-5b after 3 cycles of treatment compared to baseline. There were no changes in markers of bone formation even in responders with RD [50]. In that study, a reduction of Dkk-1 was also observed after 3 cycles of treatment only in responders. Similar results were reported in another study with lenalidomide but not with thalidomide-based regimens in responding patients [51]. However, lenalidomide was unable to modify osterix transcription in osteoblasts of myeloma patients. Osterix is a key transcription factor required for osteoblast differentiation, and this may explain the lack of influence of lenalidomide on markers of bone formation [52].

In conclusion, IMiDs reduce bone resorption either directly through the inhibition of osteoclast formation or indirectly through the reduction of tumor burden, and therefore, they have a beneficial effect on altered bone remodeling in MM. However, these agents seem to have minor effects on osteoblast function and bone formation.

11.5 Proteasome Inhibition and Myeloma Bone Disease

11.5.1 Preclinical Studies on the Effect of Bortezomib on Bone Metabolism

Bortezomib is a first-in-class proteasome inhibitor with known activity against myeloma cells [53] and has proven activity in both newly diagnosed and relapsed/

refractory myeloma patients [54, 55]. Bortezomib affects osteoclast differentiation and function in a dose-dependent manner, thus reducing subsequent bone resorption. Bortezomib seems to act in both early and late phase of osteoclast differentiation through the inhibition of p38 mitogen-activated protein kinase (MAPK) pathways (early phase), activator protein-1 (AP-1), and nuclear factor-kappaB (NF-κB) signaling (late phase) [56, 57]. The concentrations of bortezomib used in these studies were typically less than that required to induce tumor cell apoptosis. Bortezomib also inhibited the secretion of BAFF and APRIL by osteoclasts [35]. In another study a 50% decrease in the number of osteoclasts was observed in bortezomib-treated 5T2MM mice vs. control mice [58]. This is supported by an in vitro study of human bone marrow cultures, which demonstrated significant inhibition of osteoclast formation following treatment with bortezomib vs. controls [59, 60]. However, two in vivo studies which determined osteoclast numbers/activity by TRAcP staining showed no significant differences between bortezomib-treated and control mice [61, 62]. The molecular mechanisms underlying the effect of bortezomib on osteoclasts have not been fully clarified. Proteasome inhibitors are known to affect NF-κB signaling [63], which is activated by RANK/RANKL/TRAF6 signaling and is important in osteoclast differentiation and survival [64, 65]. The presence of proteasome inhibitors is thought to prevent degradation of IκB (which is bound to and inhibits NF-κB), thus NF-κB activation is prevented [66]. Indeed, decreased bone resorption through proteasome inhibition has been shown to correlate with the extent of NF-κB binding [67]. Bortezomib has also been shown to downregulate TRAF6, both at protein and mRNA levels [68], which is a key signaling mediator between RANK and NF-κB. NF-κB is also involved in IL-6 regulation [69], which is a major factor for growth and survival of MM cells—as MM cells act to promote bone resorption, disruption of the MM cell–bone marrow microenvironment interaction could, therefore, also result in reduction of osteoclast activity [70]. RANKL signaling has also been shown to stimulate Jak1 ubiquitination and degradation, thereby promoting osteoclastogenesis [71]; proteasome inhibition would therefore be expected to stabilize or upregulate Jak1 by preventing its degradation and thus inhibiting osteoclast differentiation and activity.

The major interest for the effect of bortezomib on bone metabolism in MM has been created by the observation that bortezomib stimulates osteoblast function. The stimulation of new bone formation was firstly reported by Oyajobi et al. [72]. Ubiquitin–proteasome pathway was known to regulate osteoblast differentiation [73]. Proteasome inhibitors have been shown to stimulate bone formation in vitro and can regulate expression of bone morphogenic protein (BMP)-2 by preventing processing of Gli3—the truncated form of which stimulates BMP-2 expression [74]. BMP-2 can increase levels of the transcription factor Runx2/Cbfa1, which promotes the formation and differentiation of osteoblasts, and levels of which can be increased by bortezomib [42, 75]. Runx2 and Smad1 are also regulated by proteasomal degradation (with degradation leading to systemic bone loss); this degradation is triggered by Smurf1-mediated ubiquitination [76]; thus, proteasome inhibition in this pathway suggests another possible mechanism by which bortezomib might prevent the downregulation of factors associated

with osteoblastogenesis. The ubiquitin–proteasome system also plays a role in transforming growth factor-β (TGF-β)-mediated degradation of $P57^{KIP2}$; degradation of $P57^{KIP2}$ leads to inhibition of osteoblast differentiation [77]; thus, inhibition of the proteasome would be expected to prevent this.

Bortezomib was shown to induce osteoblast differentiation and increase the size of osteoblastic colony-forming units [42, 62]. Giuliani et al. [42] reported that bortezomib significantly increased the transcription factor Runx2/Cbfa1 activity in human osteoblast progenitors and mature osteoblasts, without affecting nuclear and cytoplasmatic active beta-catenin levels. The stimulatory effect of bortezomib on Runx2/Cbfa1 activity was observed at low concentrations of the drug (2 nM), whereas higher doses did not show any effect. This behavior was not related to a toxic effect of bortezomib as the authors found that bortezomib did not induce apoptosis or inhibit proliferation of both osteoblast progenitors and mature osteoblasts at concentration ranging between 2 and 5 nM [42].

Several in vivo studies have also shown that bortezomib is associated with an increase in bone formation. Pennisi et al. [56] reported a significant increase in bone mineral density (BMD) in SCID-rab mice engrafted with MM cells treated with bortezomib; increases in bone volume, trabecular thickness, and bone formation were also observed in bortezomib-treated mice, both in myelomatous and nonmyelomatous bones. Similarly, a decrease in osteolytic lesions and increases in trabecular number and bone volume have been reported following bortezomib treatment of 5T2MM mice [58].

Separating the direct effect of bortezomib on osteoblast differentiation and indirect effects via the inhibition of tumor growth in vivo is difficult (Fig. 11.1). However, it does highlight the potential interaction between myeloma growth and osteoblasts, which seems to be crucial for myeloma cell survival [78].

11.5.2 Clinical Studies on the Effect of Bortezomib on Myeloma Bone Disease

An increasing number of studies are reporting the effects of bortezomib on bone formation in the clinical setting, confirming preclinical observations. The first indications that bortezomib may have a positive effect on bone formation came from Zangari et al. *[*39*,* 79*]* who observed a significant increase in serum ALP levels in patients who responded to treatment with bortezomib. Similarly Shimazaki et al. [80] showed in a patient with refractory MM, who was treated with the combination of bortezomib and dexamethasone, that response to treatment was accompanied by increases in both serum total ALP and bALP. This association of ALP with response to therapy echoed an analysis of the phase III APEX study of single-agent bortezomib vs. high-dose dexamethasone in patients with relapsed MM, which showed that a 25% increase in ALP after 6 weeks' treatment with bortezomib was a strong indicator of both quality of response and time to progression [81].

Osteoblast stimulation by bortezomib was also reported by Heider et al. [40], who measured changes in bALP and OC, in patients who received bortezomib ± dexamethasone (*n* = 25) and in a control group of patients who received Adriamycin/dexamethasone, melphalan/prednisone, or thalidomide-containing therapy (*n* = 58). Significant increases in bALP and OC following bortezomib treatment were observed in both responders and nonresponders, irrespective of whether dexamethasone was included in the treatment regimen. Conversely, in the control group of patients who did not receive bortezomib, no increase in osteoblast markers was seen, suggesting that the effect on osteoblasts is unique to the proteasome inhibitor. These results are in accordance with those by Giuliani et al. [42] who found significant increases in the number of osteoblasts/mm^2 of bone tissue and Runx2-/Cbfa1-positive osteoblasts in the trephine biopsies of responding patients to bortezomib, but not in those who did not respond. Furthermore, Terpos et al. [41] investigated the effect of bortezomib on bone turnover in patients (*n* = 34) with relapsed MM (88). Patients received bortezomib 1.3 mg/m^2 on days 1, 4, 8, and 11 of a 3-week cycle for four cycles. Responders could continue bortezomib for four more cycles and nonresponders could receive dexamethasone in addition to bortezomib. After four cycles of treatment, the OR rate was 66% (8% CR, 58% PR). Bortezomib administration resulted in a significant reduction in Dkk-1 and RANKL levels, with concomitant reduction in osteoclast function and bone resorption, as assessed by TRACP-5b and CTX serum levels, respectively. The reduction in osteoclast function and bone resorption occurred irrespective of response to therapy. In addition, bortezomib significantly increased levels of bALP and OC (Table 11.1). CR or vgPR patients had greater elevations of bALP levels. Interestingly, 75% of nonresponders had an increase in bALP levels following four cycles of bortezomib treatment. In 27 of these patients BMD data were available; 10% or greater increases in lumbar spine BMD were seen in four patients who responded to the regimen as their second-line therapy. Among all 27 patients lumbar spine BMD increased significantly from baseline after eight cycles of therapy, but no change was seen in femoral neck BMD [49]. Similarly, in a prospective phase II study of single-agent bortezomib in patients with relapsed/refractory MM, bortezomib was associated with a significant increase from baseline in bone volume/total volume (as assessed via comparative histomorphometric microCT analysis after three 3-week cycles of treatment) in six out of seven patients [46]. Uptake of tetracycline as part of bone deposition was also observed after bortezomib treatment in bone samples [82]. This study also indicated that response to bortezomib was associated with increases in serum parathyroid hormone concentrations in responding patients but not in nonresponders [46]. Results from two additional studies also indicate that bortezomib treatment results in the development of new bone tissue. In a study of bortezomib plus dexamethasone in 14 relapsed/refractory MM patients, two patients exhibited dramatic improvements in bone lesions upon CT scanning after 3 months and 1 year of therapy, respectively. Both patients also exhibited marked increases in their levels of bALP and OC [43].

Direct evidence for the impact on bone health-related clinical aspects of the addition of bortezomib to treatment is provided by the findings of the phase III VISTA

trial of bortezomib plus melphalan–prednisone (VMP) vs. melphalan–prednisone (MP) alone, in which 344 and 338 previously untreated MM patients who were ineligible for high-dose therapy received up to 54 weeks' therapy with VMP or MP, respectively [83]. Patients were administered bisphosphonates if they had evidence of lytic destruction of bone or osteopenia, per American Society of Clinical Oncology (ASCO) guidelines [84]. An analysis of concomitant bisphosphonate use and bone disease-related MM progression events demonstrated lower rates of both among patients in the VMP arm, suggesting a positive effect of the addition of bortezomib [47]. VMP therapy was also associated with a 50% increase in median maximum ALP compared with a 30% increase in the MP arm. This change was most evident in patients with CR, in whom a median maximum increase in ALP of 69% was recorded during treatment with VMP [47]. However, when bortezomib was combined with other anti-myeloma agents, such as melphalan, dexamethasone, and intermittent thalidomide (VMDT regimen), in relapsed/refractory patients, the reduction of Dkk-1, sRANKL, sRANKL/OPG ratio, MIP-1α, and CTX was not accompanied by an increase in bALP and OC. This observation may suggest that bortezomib in combination with other anti-myeloma agents may lose its beneficial effect on osteoblasts in patients who have received several lines of previous therapies [44]. Indeed, Heider et al. found a lower increase in bALP in patients with relapsed/refractory MM who received the combination of bortezomib with dexamethasone compared with patients who received bortezomib alone [40].

Bortezomib has also been shown in a number of clinical studies to reduce levels of Dkk-1. Terpos et al. [85] demonstrated that administration of bortezomib in combination with lenalidomide–dexamethasone in patients with relapsed/refractory MM resulted in a significant decrease in Dkk-1 levels—an effect that was not observed in the lenalidomide–dexamethasone only arm. In a study by Heider et al. [51], bortezomib treatment was associated with a significant decrease in Dkk-1 levels after 3 months' therapy in 29 patients with MM. However, a similar effect was also observed in patients treated with either HDCT+ASCT, Adriamycin+dexamethasone, or lenalidomide. Furthermore, similar to that observed for changes in ALP, across all groups evaluated, a significant decrease in Dkk-1 was only recorded in patients who achieved at least a partial response, including those receiving thalidomide [51].

In general, markers of bone resorption and osteoclast regulators were decreased following treatment with bortezomib. Three studies reported that the bone resorption markers CTX and TRAcP-5b were significantly reduced following treatment with bortezomib-based therapy [41, 44, 45]. Furthermore, concentrations of the osteoclast regulators sRANKL, OPG, MIP-1α, and osteopontin were also shown to be reduced following treatment with bortezomib [41, 44, 45]. At this point, it is crucial to mention that different effective anti-myeloma regimens in combination with bisphosphonates also reduce bone resorption through the reduction of tumor burden and the inhibition of osteoclast function [86–88]. However, among current therapies that are widely used for the treatment of MM, the bone anabolic effect of bortezomib indicated by the findings of the above studies appears to be unique [20]. It remains to be determined whether this effect of bortezomib on bone is independent of its direct anti-MM activity, although some bone marker studies indicate that changes are seen in both responding and nonresponding patients, as described above.

11.6 Effect of Other Novel Anti-myeloma Agents on Bone Metabolism

Several novel agents with anti-myeloma activity have also an impact on bone metabolism in MM. Histone deacetylase (HDAC) inhibitors have been recently used in the treatment of myeloma patients with promising results, mainly in combination with proteasome inhibitors [89]. HDAC inhibitors, such as trichostatin A, sodium butyrate, KD5170, and FR901228, can block osteoclastogenesis [90–92]. PXD 101 was also shown to inhibit osteoclast formation synergistically with bortezomib [60]. JNJ-26481585 is a novel "second-generation" pyrimidyl-hydroxamic acid-based HDAC inhibitor. In a recent report, treatment of myeloma mice with JNJ-264815 significantly reduced the development of bone disease [93].

SDX-101 is a structural analog of etodolac that is already used in clinical trials for the treatment of B-cell chronic lymphocytic leukemia. SDX-308 is another analog of etodolac which has been used in MM. Compared with SDX-101, a 10-fold lower concentration of SDX-308 induced potent (60%–80%) inhibition of osteoclast formation, and a 10- to 100-fold lower concentration inhibited multiple myeloma cell proliferation. Bone resorption was completely inhibited by SDX-308, as determined in dentin-based bone resorption assays. SDX-308 also decreased constitutive and RANKL-stimulated NF-κB activation and osteoclast formation in an osteoclast cellular model. SDX-308 effectively suppressed TNF-α-induced IKK-γ and IκB-α phosphorylation and degradation and subsequent NF-κB activation in human multiple myeloma cells. These results indicate that SDX-308 effectively inhibits multiple myeloma cell proliferation and osteoclast activity, potentially by controlling NF-kappaB activation signaling [59].

AZD6244 is another anti-myeloma agent, which blocks the extracellular signal-regulated kinase 1/2 (ERK1/2) MAP kinase pathway and is very active in myeloma cell lines. AZD6244 blocked osteoclast differentiation and formation in a dose-dependent manner, evidenced by decreased alphaVbeta3-integrin expression and TRAP (+) cells. Functional dentin disk cultures showed inhibition of osteoclast-induced bone resorption by AZD6244. Major MM growth and survival factors produced by osteoclasts including BAFF and APRIL, as well as MIP-1α, were also significantly inhibited by AZD6244. In addition to ERK inhibition, NFATc1 (nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1) and c-fos were both downregulated, suggesting that AZD6244 targets a later stage of osteoclast differentiation. These results indicate that AZD6244 inhibits OCL differentiation, formation, and bone resorption, thereby abrogating paracrine MM cell survival in the bone marrow microenvironment [94].

An interesting agent which seems to also restore osteoblast function is SB431542, an inhibitor of TGF-β type I receptor kinase. This agent antagonized the inhibitory effects of conditioned media from MM cell lines (RPMI8226 and U266) and bone marrow plasma from patients with MM, enhancing the BMP-induced mineralized nodule formation. In addition, the induction of osteoblast maturation caused by SB431542 downregulated the production of IL-6 and upregulated the production of OPG leading to osteoclast inhibition. Therefore, SB431542, through the blockade of

TGF-β actions, releases osteoblasts from the differentiation arrest in MM bone disease while concomitantly suppresses osteoclastogenesis to ameliorate bone destruction and at the same time suppressing MM expansion by disrupting the MM niche [95].

11.7 Conclusions

Novel anti-myeloma agents, such as IMiDs, bortezomib, and more recent ones, alter abnormal bone metabolism in myeloma patients. Most of them reduce bone resorption either directly through the inhibition of osteoclast formation or indirectly through the modification of interactions between malignant plasma cells and osteoclasts. Regarding restoration of osteoblast function, based on available evidence, we may suppose that bortezomib is able to directly stimulate osteoblast differentiation. However, to date, evidence of the effect of bortezomib on clinical endpoints specific to bone, such as SREs and BMD, is limited. It is therefore important to design prospective trials that investigate endpoints related to bone formation; the results of which will be eagerly anticipated. It would be also of great interest to see the results of studies using combination regimens including novel agents with or without the presence of bisphosphonates or other targeted bone therapies (denosumab, anti-RANKL agent; BHQ880, anti-Dkk-1 agent). In this period of skepticism about the prolonged use of bisphosphonates due to side effects, the administration of agents, such as bortezomib, that alter bone metabolism by both reducing bone resorption and enhancing bone formation may alter our way of management of myeloma bone disease in the near future. However, more data with clinical endpoints are needed before making specific recommendations[96].

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N.C. Munshi and K.C. Anderson (eds.), *Advances in Biology and Therapy of Multiple Myeloma: Volume 2: Translational and Clinical Research*,
DOI 10.1007/978-1-4614-5260-7, © Springer Science+Business Media New York 2013

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