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Multiple myeloma, immunotherapy and minimal residual disease

Minireview

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Multiple myeloma (MM) is an incurable heterogeneous hematological malignancy in which relapse is characterized by re-growth of residual tumor and immune suppression with a complex biology that affects many aspects of the disease and its response to treatment. The bone marrow microenvironment, including immune cells, plays a central role in MM pathogenesis, survival, and drug resistance. The advances in basic and translational research, introduction of novel agents, particularly combination therapies, improved indicators of quality of life and survival. Minimal residual disease (MRD) detection by multiparameter flow cytometry (MFC) has revolutionized monitoring of treatment response in MM. The importance of MFC methodology will be further strengthened by the ongoing international standardization efforts. Results of MRD testing provide unique and clinically important information and demonstrated the prognostic significance of MRD in patients, leading to regulate treatment intensity in many contemporary protocols. In this review, we will summarize the principal approaches in MM immunotherapy, focusing how new agents have potential in the treatment of MM and application of MRD detection by MFC as a surrogate endpoint would allow quicker evaluation of treatment outcomes and rapid identification of effective new therapies.

Key words: multiple myeloma, immunotherapy, monoclonal antibodies, minimal residual disease, multiparameter flow cytometry

Multiple myeloma (MM) is a malignant B-lymphoproliferative disease characterized by clonal proliferation of malignant plasma cells in the bone marrow (BM), lytic bone lesions, renal disease, and immunodeficiency associated with monoclonal immunoglobulin (Ig) in the blood and/or urine. It accounts for 1% of all cancers and more than 10% of all hematooncologic malignancies. In Europe, more than 40,000 new cases are diagnosed each year [1]. MM incidence is increasing with age and the median age at diagnosis is 65-70 years. First stage in the development of MM is the emergence of asymptomatic monoclonal gammopathy of undertermined significance (MGUS). In some of these patients, this progresses to smoldering MM and ultimately to symptomatic MM, with an annual risk of around 1% for patients with MGUS [2]. The etiology of MM is still unclear. MM pathogenesis is a multifactorial process. Progression to MM is associated with a series of complex genetic events in MM cells, as well as changes in the bone marrow microenvironment, including increased angiogenesis, suppression of the immune response, loss of immune surveillance, increased bone resorption, and the establishment of aberrant signaling-loops involving cytokines and growth factors associated with the clinical features of MM and its resistance to treatment [3]. MM is still incurable disease. If the disease is sensitive to treatment, remission of various lengths is usually reached. Relapse or disease progression is common, and response to therapy in advanced disease is always worse. In MM treatment, combination of high-dose chemotherapy is used. The high-dose chemotherapy with support of autologous stem cell transplantation (ASCT) significantly increases ratio of complete remission and average survival. This treatment possibility increases survival to more than 10 years for only

20% of MM patients [4]. Development in MM treatment in the first decade of this century has been unprecedented. The treatment strategy has been changed, and clinical protocols increase overall survival (OS) of more than 6-8 years with one third of these patients living more 10 years, while the intensity of treatment is lower and tolerance of one is higher [5]. This advancement is connected with novel highly efficient agents such as immunomodulatory drugs (thalidomide and lenalidomide) and proteasome inhibitors (bortezomib). However, despite clear treatment advances, virtually all myeloma patients will eventually relapse. Patients who relapse after bortezomib and either thalidomide or lenalidomide, the so called "double refractory" patients, have median OS of only 9 months [6]. This clearly demonstrates that there is a need for new treatment approaches that would be able to overcome a dismal course of the disease in these patients. To overcome this drug resistance, a number of therapeutic approaches have been developed in recent years [7]. New generation proteasome inhibitors, including carfilzomib, ixazomib, and marizomib, are active even in the setting of bortezomib-resistant MM. Pomalidomide, a third-generation immunomodulatory drug, has shown activity even in 17p (p53)-deleted MM. Monoclonal antibodies such as elotuzumab (anti-SLAMF7) and daratumumab (anti-CD38) show promising clinical efficacy, especially in combination with lenalidomide.

The development of novel anti-MM agents relies on an understanding of the biology of MM and the multiple factors involved in its pathogenesis and response to treatment. As well as genetic aberrations in essential growth- and tumor-suppressor genes, there is increasing evidence that interactions between tumor cells and their bone marrow microenvironment play a pivotal role in the development of drug resistance. In addition to their tumoricidal effects, immunomodulatory agents also act on the immune system, potentially helping to overcome MM-associated immunodeficiency and enhancing anti-MM immune activity [8].

In this review, we will summarize the major approaches in multiple myeloma immunotherapy, focusing how new agents have potential in the treatment of MM and application of MRD detection by MFC as a surrogate endpoint would allow quicker evaluation of treatment outcomes and rapid identification of effective new therapies.

Immunologic checkpoints inhibitors PD-1/PD-L1

The immune checkpoint molecule programmed cell death l (PD-1, CD279) is a type I transmembrane protein expressed on the surface of activated T cells and is upregulated on activated T lymphocytes and inhibits T-cell function by binding to its ligands PD-L1 (CD274) and PD-L2 (CD272). The association between PD-L1 on target cells and PD-1 on T and effectors cells acts as an immunologic checkpoint to suppress antitumor immunity [9]. PD-L1 is also expressed on many tumor cells, and clinical trials of m Abs directed against PD-1/PD-L1 are underway based on the hypothesis that blocking this inhibitory signal will break tolerance against tumor cells [10, 11].

MM cells express PD-L1, which is further upregulated in BM microenvironment [12]. PD-1 expression is upregulated on NK or T cells in MM patients [13]. The PD-1/PD-L1 interaction functions to prevent bystander tissue damage during inflammation, but it can also maintain an immunosuppressive tumor microenvironment that allows tumor cells to evade immune surveillance in MM. Growth of MM cells is inhibited in PD-1-deficient mice [14], and an anti-PD-1 antibody pidilizumab (CT-011) both enhances NK-cell cytotoxicity against MM cells and also enhances activated T-cell responses to vaccination with autologous dendritic/MM cell fusion [13]. Immune PD-L1 blockade has the capacity to inhibition of MM-tumor growth, and anti-PD1-L1 therapy, facilitates T cell-mediated anti-MM activity [15]. The checkpoint inhibitors PD-1/PD-L1 brings additional treatment options to patients with selected advanced cancers, include MM patients [16], alone or in combination with other immune-based therapies. Currently there are several clinical studies investigating the use of immune checkpoint inhibitors targeting PD-1, mAbs: pidilizumab, pembrolizumab and nivolumab in various combinations in MM. There is a growing interest in its cognate molecule, PD-L1 as this is the part of the signaling pathway that is harbored on the tumor itself and at least in theory has the additional potential for ADCC. This ligand was shown to be expressed on malignant plasma cells [17].

Immunomodulatory drugs (IMiDs)

Immunomodulatory drugs including thalidomide, lenalidomide, pomalidomide is a class of drugs that directly affect MM cells and bone marrow microenvironment leading to modulation of cytokines, inhibition of angiogenesis, and augmentation of immune effectors numbers and function (T-cell, NK cells, and NKT cells) from both the peripheral blood and BM of MM patients. Recently interaction anti-MM activity of IMiDs with ubiquitin ligase cereblon, triggering proteasomal degradation of Ikaros family zinc finger proteins was shown to be crucial for direct cytotoxic and immune related effects [18]. The immunomodulatory effects by IMiDs include activation of natural killer (NK) or NKT cells, stimulation of both CD4+ and CD8+ T cells, and lenalidomide and pomalidomide also inhibit regulatory T cells (Treg) proliferation [19]. IMiDs target not only MM cells, but also MM cell-immune cell interactions and cytokine signaling and diminished IL-2, IFN-γ, and IL-6 regulator suppressor of cytokine signaling (SOCS) 1 expression in immune cells from both peripheral blood and BM of MM patients and modulate SOCS1-mediated cytokine signaling in immune effectors cells, and may enhance immune response and efficacy of IMiDs in MM cells [20]. There is also evidence that IMiDs regulate humoral immune responses in MM. The positive co-stimulatory surface marker ICOS and its ligand, which are up-regulated by IMiDs [20], regulate T-cell-mediated

immune responses by controlling T-cell/B-cell interactions. These properties make IMiDs perfect companions to the clinical activities of monoclonal antibodies and immune based cellular therapies [21]. This is achieved by stimulating the production of cytokines, such IL-4 and IL-10, which play crucial roles in B-cell growth, maturation, and isotype switching [22]. Lenalidomide-based therapy was found to improve the humoral immune response in a significant proportion of responding patients [23]. Given the complexity of the immune response in vivo, the ultimate immunomodulatory effects of IMiDs in patients are probably considerably more complex and dynamic than current evidence indicates, and are also dependent on the individual´s immune status and cytokine profile in the BM [8, 24].

Myeloid-derived suppressor cells

PD-1 is also expressed on other cells as well such as on plasmocytoid dendritic cells and MDSC both of which play role in immunosuppressive state in MM. Myeloid-derived suppressor cells (MDSCs) are a heterogeneous, immature myeloid cell population with the ability suppress immune responses and which are expanded in the tumor environment [25]. MDSCs have been characterized in infections, inflammatory diseases, and solid tumors; however, their presence and role in immune-suppressive environment in hematologic malignancies remains unclear. In patients with MM, the number of MDSCs is significantly increased in both peripheral blood and BM [26]. Furthermore, MDSCs induced MM growth while suppressing of T-cell-mediated immune responses [27]. Recently, phosphodiesterase-5 (PDE5) inhibitors have been reported to reduce the function of MDSCs. The addition of the PDE5 inhibitor, tadalafil, in a patient with refractory MM reduced MDSCs function and generated a dramatic and durable anti-myeloma immune and clinical response. [28]. Inhibition of the tumor-promoting and immune-suppressive functions of MDSCs may represent a promising novel immune-based therapeutic strategy of tumor-directed therapies.

Targeting monoclonal antibodies (mAbs)

Monoclonal antibodies (mAbs) are currently the most investigated therapeutic compounds in oncology and represent new and interesting group of agents with a unique mechanism of action distinct from currently used drugs in the treatment of multiple myeloma. The toxicity seems to be minimal which is important for the incorporation of mAbs into combination with other more toxic drugs. Daratumumab and elotuzumab are the most promising molecules for several reasons. Target antigens (CD38, CS1) are highly expressed on the surface of most malignant plasma cells, while they are not present on other tissues or hematopoietic stem cells, so the expected side effects are not serious. Daratumumab is a fully human IgG1κ monoclonal antibody targeted against CD38, that is a 46kDa transmembrane glycoprotein with multiple proposed functions in cell adhesion, signaling and enzymatic activity and is expressed on a multiple hematopoietic cell types: thymocytes, subpopulations activated T a B lymphocytes, NK cells a dendritic cells [29]. Overexpression of CD38 is seen in a majority of lymphoid tumors, but on malignant plasma cells in MM this antigen is highly expressed in comparison to other types [30] making it an attractive target for antibody therapy. Daratumumab possess a broad spectrum of killing activities. ADCC (antibody-dependent cell-mediated cytotoxicity) is characterized by release of the content of cytotoxic granules or by the expression of cell death-inducing molecules. Effectors cells that mediate ADCC include NK-cells, monocytes, macrophages, neutrophils, eosinophils and dendritic cells. Others mechanisms of action are CDC (complement-dependent cytotoxicity) and ADCP (antibody-dependent cellular phagocytosis) by macrophages and direct induction of MM cell apoptosis [31]. It is important to mention that the mechanism of action of daratumumab (also elotuzumab) is strongly dependent on the function of host immune system and effectors cells. These monoclonal antibodies are likely to act synergistically with treatment modalities that stimulate host anti-myeloma immunity, so the combination approaches may be more effective than monotherapy [32]. For example, combination with immunomodulatory drugs (IMIDs) may improve clinical benefit as these agents enhance T- and NK-cell-mediated immune responses. For example combination with bortezomid or carfilzomib may also be effective [32, 33]. Daratumumab was brought to the clinic in phase I/II study, involving patients with relapsed/refractory MM who had received at least 2 prior lines of therapy. Daratumumab as a single agent yielded 36% overall response rate and in the responder group, 65% remained progression free in 12 months [34]. Elotuzumab is a humanized IgG1 monoclonal antibody targeted against the cell surface transmembrane glycoprotein CS1 (also as SLAMF7, CD2 subset-1, CD319). This surface antigen is highly expressed on MM cells and normal plasma cells, also at a lower level on NK-cells, NKT-.cells, and a subset of CD8 positive T cells. The function of CS1 on MM cells remains unclear. Elotuzumab exerts antimyeloma activity dominantly via NK-cell-mediated ADCC through both direct activation and engagement of NK-cells [35]. Recently at ASCO 2015 an interim analysis of phase III, randomized study of lenamidomide/dexamethasone with or without elotuzumab was reported. The study involving 646 relapsed MM patients. Progression free survival was 68% and 41% at 1 and 2 years (compared to 57% and 27% in controls) [36]. Combine daratumumab or elotuzumab with other mAbs that augment antitumor immune responses – e.g. immune check point inhibitors (anti-PD1 antibodies) or anti-KIR antibodies is research agenda for future. Currently many other mAbs targeting various antigens on the surface of myeloma cells or other molecules involved in their proliferation are under investigation, several of them have reached phase I/II of clinical trials and have showed some clinical efficacy warranting further testing. For example lirilumab is a second generation anti-

KIR (killer-cell Ig-like receptor) m Abs that was evaluated in a phase I trial in patients with solid tumors and hematological malignancies. There are several trials planned in combination with check point inhibitors or cytotoxic antibodies in myeloma patients as well [37]. Indatuximab ravtansine (BTO62) is an antibody- drug conjugate, comprising the anti CD138 (Syndecan 1) chimerized mAb and a cytotoxic agent maytansinoid DM4. It is designed to bind to CD138 on cancer cells, and then release DM4 after internalization to cause cell death. CD138 represent one of the most specific target antigens for identification of MM cells. The results of a phase I/II trial from 45 RRMM patients indicate that BTO62 is well tolerated overall response rate was 78% [38]. A new area of antibody research has recently focused on bispecific T cell engagers (BiTEs) that combine specificities of two antibodies by simultaneous binding to multiple epitopes, one which involves the engagement and activation of T cells via their CD3 molecules [39]. The first bispecific antibody generated specifically against myeloma was developed by combining single-chain variable fragments of mAb that binds normal and malignant plasma cells (Wue-1) and a mAb against CD3, forming BiTE product [40]. This led to desing and development of other BiTEs.

Targeting mAbs may have a broad range of activities that promote anti-tumor immunity and tumor cell death. Investigations into novel targets and mAbs may lead to better treatment strategies and rational combinations.

Chimeric antigen receptor (CAR) T cells

A new area of exploration in MM immunotherapy involves the in vivo generation of activated T cells specific to a particular antigen. Chimeric antigen receptor (CAR) T-cell based therapy, represent a huge leap in immune therapy. T cells, known as CAR T cells, are synthetically engineered via transduction of specific antibody into the T cell apparatus, thus promoting specific target binding and killing. Typically manufactured with the use of retroviral vectors, CARs function similarly to native T cells with activation through the zeta-chain of the CD3 complex [41]. Notable toxicity associated with CARs includes the cytokine release syndrome, which also correlates with treatment efficacy [42]. Anti-CD19 CAR T cells, constructed by fusing the single chain variable fragment (scFv) of a monoclonal antibody specific for a surface antigen with an intracellular signaling domain have shown activity in several CD19 related diseases as acute and chronic lymphocytic leukemia [43]. The MHC-independent tumor recognition, in vivo expansion and memory cell generation confers these cells a clear advantage over naked antibodies or adoptively transferred tumor-reactive T cells. CD19 CARs may in fact be effective in MM, targeting the "pre-myeloma" CD19 positive B cells. Currently, a successful example of CD19 targeted CAR – T cell approach with multiple pre-clinical and clinical trials utilizing in MM was published [44]. Again, another example of this strategy that employs an ontogenetically later target ie B-cell maturation antigen-directed lentiviral transduced CAR clinical trials, with restricted and consistent expression pattern of the target antigen are encouraging [45]. Additional attempts using other targets in MM have been less successful and have been attributed to the lack of expression of the target antigen on relevant clones and there are yet others not attempted due to low expression.

Multiple myeloma, minimal residual disease (MRD) and flow cytometry

Survivals of MM patients with relapse are still the main clinical problem. The source of these relapses is the persistence of minimal residual disease (MRD) that is defined as disease that is undetectable by standard diagnostic techniques (morphology). In MM, the majority of patients will inevitably relapse despite achievement of progressively higher complete remission (CR) rates. Novel treatment protocols with inclusion of monoclonal antibodies, immunomodulatory drugs, checkpoints inhibitors and others molecules might well be able to further increase remission rates and potentially also cure rates. Therefore, MRD diagnostics becomes essential to assess treatment effectiveness. MRD measurements should be inherently more informative than any myeloma cell features as they reflect the effect of several other variables that influence treatment response and outcome. Measurement of the response to the early phases of therapy using MRD-detection techniques provide a good indication of the susceptibility of MM cells to chemotherapy in each patient and represent the most accurate prognostic indicator that is currently available. MRD-detection methods have many potential applications in the clinical management of patients with MM including recognition of relapse before it is clinically evident and determination of the MM burden before autologous hematopoietic cells transplantation (HCT). A more recent application includes the use MRD as a parameter for measuring the efficacy of a new remission induction regimen in relation to that of a previous protocol; this can detect early whether the new regimen is significantly inferior to the previous one, thus prompting changes and reducing the number of patients exposed to suboptimal therapy. Consequently, an increasing number of treatment protocols use MRD as a tool for treatment stratification. MRD levels during remission induction therapy provide important prognostic information [46]. Over past decade, multiparameter flow cytometry (MFC) has emerged as the most attractive, well-suited, and sensitive approaches to detect MRD in the bone marrow of MM patients during and after therapy. Detection of MRD by MFC became the preferred method by several cooperative groups to adopt in myeloma clinical trials for several reasons. MFC is a apparent tool to study biological samples containing plasma cells because this worldwide-available technique allows: (a) simultaneous identification and characterization of single plasma cells based on multiple parameters, (b) evaluation of high cell numbers in a few hours, (c) quantitative assessment

of different cell populations and their corresponding antigen expression levels, and (d) combined detection of cell surface and intracellular antigens [47]. In recent years, the sensitivity of MFC has increased because of simultaneous assessment of more than 8 markers and evaluation of greater numbers of cells than what was previously feasible [48]. Single parameters cannot reliably distinguish clonal vs normal plasma cells, but highly sensitive MFC-based MRD with evaluation of at least 8 markers by color digital flow cytometer coupled to novel sample preparation procedures in a single tube can readily identify aberrant plasma cells phenotypes at MRD levels if sufficient cell numbers (more than $5x10^6$) are evaluated [47, 48]. Consensus exists that plasma cells identification markers (CD38, CD138 and CD45) plus discriminatory markers such as CD19, CD27, CD56, CD81, and CD117 should be simultaneously evaluated for accurate identification of bone marrow plasma cells and unequivocal distinction between clonal and normal plasma cells [48, 49]. It should be noted that normal plasma cells have a considerably heterogeneous immunophenotype according to the plasma cells maturation process, but this maturation pathway is highly conserved in all conditions from normal to regenerating and reactive BM samples. Because the aberrant phenotypes of clonal plasma cells are readily distinguishable from normal plasma cells, flow MRD is applicable in virtually every MM patients without requiring patients-specific diagnostic phenotypic profiles [48]. Additionally, discrimination between normal and myeloma plasma cells is still feasible in the event of phenotypic shifts from diagnostic to posttreatment MRD samples [50]. A potential limitation of MFC is that current strategies are designed to characterize the plasma cells compartment and could therefore miss potential MM cancer stem cells with more immature phenotypes, such as postgerminal center memory B cells [51]. Immunophenotypic complete response in MM has been shown to be one of the most relevant prognostic factors for patients undergoing autologous HCT, as well as in nontransplantat eligible patients treated with novel agents [52]. In addition, baseline MFC studies of BM may also contribute to prediction of outcome of MM patients after standard chemotherapy and high-dose therapy followed by autologous HCT [53]. Furthermore, circulating phenotypically aberrant/clonal plasma cells can be detected in approx. 80% of myeloma patients at presentation, and the level of circulating neoplastic plasma cells in newly diagnosed myeloma patients is a predictor of progression-free survival and overall survival (OS) [54]. OS is significantly reduced in MM patients undergoing autologous HCT, when FCM detects neoplastic myeloma cells in the stem cell grafts [55]. Therefore, assessment of peripheral blood samples obtained at different time points during the course of the disease may also be relevant for prognostication and clinical management in the near future, though it complementary role with BM MRD evaluation is yet to be demonstrated [56].

MRD monitoring variability between different clinical laboratories is a major challenge. Because of the prognostic value of MRD in MM, a key goal of the standardization effort is to eliminate or correct the relative differences between MRD negativity assessment and response rates across laboratories. Optimal use of clinical guidelines for disease diagnosis and patients management requires first standardization and then harmonization, to maximize compatibility, interoperability, safety, repeatability, and quality as well as achieve uniformity of results [57, 58]. Results that are neither standardized nor harmonized may lead to erroneous clinical, financial, regulatory, or technical decisions. The need for extensive expertise to analyze MFC data, together with lack of well-standardized and harmonized MFC-MRD methods [59], has been pointed out as the main drawback of MFC immunophenotyping [60]. In recent years, new multivariate computational tools and visualization plots have been developed and integrated into innovative software for multidimensional identification and classification of different cells coexisting in a sample. These tools further pave the way for automated detection and tracking of aberrant cell population deviate from normal phenotypic profiles [61]. Such innovative MFC-MRD strategies are currently being developed by the EuroFlow Consortium under the Black Swan Research Initiative promoted by the International Myeloma Foundation, and it is likely to become the method of choice for accurate, high-sensitive, and automated MFC-MRD monitoring in multiple myeloma.

Conclusions and considerations for the future

Multiple myeloma (MM) is a plasma-cell malignancy which remains incurable despite the recent emergence of multiple novel agents. Importantly, recent genetic and molecular analyses have revealed the complexity and heterogeneity of this disease, highlighting the need for therapeutic strategies to eliminate all clones. The bone marrow microenvironment plays a central role in MM pathogenesis. New classes of agents including monoclonal antibodies, immunomodulatory drugs, immunologic checkpoints inhibitors, CAR T cells etc. have shown remarkable efficacy. However, novel therapeutic approaches are still urgently needs to further improve patient outcomes. The introduction of MRD monitoring has transformed the way in which patients with MM are managed. MRD results can be applied to most patients with MM, and can be delivered in a timely fashion to satisfy the requirements for rapid changes in treatment timing and intensity. A further increase in cure rates for patients with MM will require accurate prediction of their relapse hazard. MRD assays now allow the objective and sensitive assessment of treatment response in virtually all patients. Despite this progress there are areas for continuing development. Methods to study MRD by MFC are constantly being refined by the introduction of new markers, which take advantage of the capacity of newer instruments to detect an increasingly higher number of fluorochromes. The recent technical innovations in routine FCM (3 lasers and≥8 colors) and the new developments in software for data analysis make this technology the most attractive for MRD diagnostics.

Immunophenotyping by mass cytometry, a new technology, provides the ability to measure>36 proteins at a rate of 1000 cells/second. Both MFC and mass cytometry have unique and powerful features as well as unique challenges and limitations. Over the next decade, these complementary technologies will play central roles in dissecting the complex interactions of cells [62]. An alternative approach to immunophenotypic analysis of MRD, based on high-speed cell imaging scanning technology, was also recently proposed [63]. The data indicate that this method has the potential to identify MRD with a very high sensitivity and ensure that the signals detected originate from viable cells. Precise MRD levels and optimal sampling time-points have to be defined for each treatment protocol before MRD-based risk stratification can be implemented. The implementation of MRD studies in treatment protocols requires a strong interaction between MRD specialists and clinical oncologists. Current goals should, thus, focus on integrating serial MRD assessment into cytogenetic and molecular results for optimized risk stratification and appropriately standardizing MRD technologies to allow reproducibility and multiinstitutional collaboration. MRD studies have multiple applications in the management of patients with MM.

Combination strategies are still keys for MM treatment, targeting not only MM cells, but also the tumor microenvironment, including host immunity [64].Many of these new strategies with more well-established treatments are under clinical development and have already started providing encouraging results.

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