Utility of flow cytometry studies in the management of patients with multiple myeloma

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Abstract

Purpose of review: Although the input of multiparameter flow cytometry (MFC) into the clinical management of multiple myeloma (MM) patients has faced some reluctance, continuously growing evidence supports the utility of MFC in this disease.

Recent findings: MFC immunophenotyping of bone marrow and peripheral blood plasma cells affords cost-effective assessment of clonality, and provides prognostic information on the risk of progression in smoldering MM, and the identification of active MM patients with dismal outcome (e.g.: high numbers of circulating tumor cells) or long-term survival despite sub-optimal responses through the characterization of MGUS-like phenotypes. Extensive data indicates that MRD monitoring can be used as biomarker to evaluate treatment efficacy and act as surrogate for survival. The time has come to address within clinical trials, the exact role of baseline risk factors and MRD monitoring for tailored therapy in MM, which implies systematic usage of highly sensitive cost-effective, readily available and standardized MRD techniques such as MFC.

Summary: Next-generation MFC should be considered mandatory in the routine evaluation of MM patients both at diagnosis and after therapy, and represents an attractive technique to integrate with high-throughput DNA and RNA-seq methods to help understanding the mechanisms behind dissemination and chemoresistance of MM.

Key words: flow cytometry; minimal residual disease; circulating tumor cells; survival

Introduction

Multiple myeloma (MM) is a plasma cell (PC) dyscrasia that accounts for 1% of all cancers and approximately 10% of all hematologic malignancies.(1) Almost all patients with MM evolve from an asymptomatic pre-malignant stage termed monoclonal gammopathy of undetermined significance (MGUS).(2) In some patients, an intermediate asymptomatic but more advanced premalignant stage referred to as smoldering MM (SMM) can be recognized clinically, which progresses to MM at a rate of approximately 10% per year over the first 5 years following diagnosis.(3)

Multiparameter flow cytometry (MFC) immunophenotyping is considered to be mandatory for the diagnosis and monitoring in a vast number of hematological malignancies.(4) Although MM should be no exception, the input of MFC into the clinical management of these patients has faced some reluctance.(5) According to the International Myeloma Working Group (IMWG), clonality of PCs should be preferably established by immunohistochemistry since MFC immunophenotyping may still not be widely available and standardized for general use.(6) Furthermore, MFC affords lower bone marrow (BM) PC frequencies versus reference morphological approaches, which are partly explained by the association of PC with lipid-enriched BM spicules.(7) That notwithstanding, MFC is particularly well-suited to study biological samples containing PCs because it allows: i) simultaneous identification and characterization of single PCs based on multiple parameters, ii) evaluation of high cell numbers in a few hours, iii) quantitative assessment of different cell populations and their corresponding antigen expression levels, iv) combined detection of cell surface and intracellular antigens.(8) Accordingly, the utility of MFC in the management of MM patients is here reviewed.

Immunophenotypic discrimination between normal vs clonal BM PCs and clinical outcomes

There is strong evidence that the phenotypic characteristics of clonal PCs differ from their normal counterpart in terms of antigenic expression(4,5), and phenotypically

aberrant PCs typically show amongst others: i) underexpression of CD19, CD27, CD38, and/or CD45; ii) overexpression of CD28, CD33 and CD56 and/or; iii) asynchronous expression of CD20, CD117 and/or surface immunoglobulins. From a clinical point of view, the distinction between normal and clonal PCs has shown to be of clinical utility in i) the differential diagnosis between MGUS and MM(9), ii) the evaluation of the risk of transformation of MGUS and smoldering MM into symptomatic MM (10)and, iii) the identification of a good prognosis subgroup of symptomatic MM patients.(11) Accordingly, the Spanish Myeloma Group (GEM/PETHEMA) has made an effort to strengthen MFC immunophenotyping using novel EuroFlow-based software analysis tools to develop an automated classification model focused on the analysis of the PC compartment and capable of identifying newly diagnosed symptomatic MM patients with a baseline MGUS-like profile (ie. coexistence of normal and clonal PCs). This small subset (8% of all cases) shows an unprecedented time to progression (TTP) of 59% at 10 years, and its prognosis is not dependent on the depth of response achieved [i.e.: complete remission (CR) vs no CR).(12) The prospective identification of this signature may contribute to discriminate a suboptimal response that requires additional treatment from a residual 'MGUS-like component' that may remain stable without further treatment. In addition, the model also contributed to the identification of smoldering MM patients at high risk of transformation to MM (median time to progression of 15 months).(12) Noteworthy, the reference data set and classification algorithm developed can be equally built or shared across different myeloma centers; this is particularly relevant due to the perception that MFC immunophenotyping is difficult to standardize, and those technological developments that could help standardizing MFC would be mostly welcomed.(13)

Quantification of circulating tumor plasma cells (CTCs)

The quantification of CTCs and its negative prognostic impact in PC disorders has been demonstrated along the entire spectrum of the disease, from MGUS to

smoldering MM, as well as in newly diagnosed and relapsed/refractory MM.(14-18) In particular, high levels of CTCs identify SMM patients with high risk of progression within the first years following diagnosis (71% at 2-years) (14,18), as well as symptomatic MM patients with standard-risk cytogenetics but dismal overall survival (OS) due to high CTCs numbers.(15) From a biologic point of view, we have recently shown that CTCs represent a unique subset of patient-paired BM clonal PCs with clonogenic potential and a quiescent phenotype, which may potentially be driven to circulate by circadian rhythms.(19) Thus, understanding the biologic features of CTCs may represent a unique model to understand and hopefully revert the extramedullary dissemination of MM.(20)

Minimal residual disease (MRD) monitoring

At present it is clear that in MM there is a direct correlation between depth of response, particularly CR, and prolonged progression-free survival (PFS) as well as OS.(21) With the introduction of highly-effective multidrug combinations almost 100% of overall response rates are observed, with over 50%-80% of patients reaching CR. Since a significant proportion of patients relapse despite achieving these “deep remissions”, it becomes clear that the definition of CR would benefit from an improvement that matches the unprecedented evolution observed in the MM treatment. Such improvement can only be accomplished by incorporating highly-sensitive techniques able to detect MRD at very low levels.

Several groups have demonstrated the utility of MFC in the detection of MRD.(22) In transplant-eligible MM patients, it has been shown that PFS of MRD-negative cases at least doubled that of MRD-positive CR patients.(23-29) Conversely, CR patients with persistent MRD had significantly inferior OS vs. MRD-negative cases. These results support the rationale for implementing MRD assessment to redefine and improve current CR criteria in MM. Since an optimal balance between treatment

efficacy and toxicity is of utmost importance in elderly MM, sensitive MRD monitoring might be particularly valuable in this patient population. However, the prognostic value of MRD assessment was not investigated outside of the (stem cell transplantation) SCT setting until the incorporation of novel agents into the treatment of patients who were not fit for SCT (24,30-33), increased CR rates and prolonged survival. Most recently, we have used 2nd generation MFC to monitor MRD in 162 transplant-ineligible MM patients enrolled in the PETHEMA/GEM2010MAS65 study. The transition from 1st to 2nd generation MFC resulted in increased sensitivity, and patients' MRD status was an independent prognostic factor for time-to progression and OS.(32) Accordingly (and similarly to transplant-candidates), MRD monitoring is one of the most relevant prognostic factors in elderly MM, irrespectively of patients' age and cytogenetic risk. This is particularly relevant since in MM it has been suggested that attaining CR could be critical only for patients with high-risk disease, while those with more indolent biology may not particularly benefit. However, after the PETHEMA/GEM reported that risk assessment by FISH and flow-MRD monitoring were of independent prognostic value(28), Rawstron et al. have reproduced and confirmed that the presence of MRD is a strong predictor of outcome in patients with both favorable and adverse cytogenetic profiles.(24) Further analyses by the PETHEMA/GEM have shown that combined cytogenetic evaluation of PCs at diagnosis plus MRD assessment after SCT resulted in a highly-effective approach to identify patients with unsustained CR and dismal outcomes (2-years median OS for cases with baseline high-risk cytogenetics plus persistent MRD).(27)

As noted above, the sensitivity of MFC has recently increased due to simultaneous assessment of >8 markers and evaluation of greater numbers of cells than what was previously feasible with analogical (4-color) instruments.(32) Thus, the availability of >8-color digital flow cytometers coupled to novel sample preparation procedures that allow fast and cost-effective routine evaluation of >5 million nucleated

cells, has boosted the sensitivity of modern MFC-based MRD monitoring into that achieved on molecular grounds (≤10^{-5}). It should be noted that current sensitivity of MFC is at least 1-log superior than that of previous MFC analyses (10^{-4}); therefore, ongoing MFC-based MRD monitoring should result in improved patient' risk stratification vs. 4- or 6-color analyses. Equally important, 8-color flow-MRD methods incorporate a sample quality check of BM cellularity via simultaneous detection of B-cell precursors, erythroblasts, myeloid precursors and/or mast cells. This information is critical to ensure sample quality and to identify hemodiluted BM aspirates that may lead to false-negative results.(8) The need for extensive expertise to analyze flow cytometric data, together with the lack of well-standardized flow-MRD methods have been pointed out as additional and perhaps the main limitations of conventional MFC immunophenotyping.(8) However, new software programs have been developed in recent years with improved multidimensional identification and classification of different cell clusters coexisting in a sample (e.g. through principal component analysis and canonical analysis). These tools together with the use of normal and tumor reference databases, allow for automated detection of normal vs. aberrant phenotypic profiles.(4) If such methods become now widely adopted, MFC would represent a method of choice for cost-effective yet highly-sensitive, standardized MRD monitoring. Accordingly, the IMWG has most recently developed novel response criteria that includes MRD monitoring and the flow-MRD negative criterion: absence of phenotypically aberrant clonal plasma cells by next-generation flow cytometry on bone marrow aspirates using the EuroFlow standard operation procedure for MRD detection in MM (or validated equivalent method) with a minimum sensitivity of 1 in 10^5 nucleated cells or higher.(34)

The importance of attaining an MRD-negative status was recently highlighted by Barlogie et al, which have shown that the vast majority of CR patients achieving long-term survival (10-years relapse-free), were also MRD-negative.(35) However, attaining

deep-remission is not a pre-requisite in order to achieve long-term disease control, at least in specific cases, and more accurate identification of such patients should also become a research priority. Accordingly, we have recently evaluated whether the BM immune profile of individual patients at the time of MRD assessment could also be predictive of outcome, and developed individual patient' immune signatures based on the unsupervised BM distribution of 13 immune cell populations identified with the 2nd generation MFC assay. This approach revealed the existence of 3 patient clusters that were segregated by progressively increasing numbers of erythroblasts and B-cell precursors, together with progressively decreasing numbers of mature naïve and memory B-cells, and were associated with significantly different OS.(32) These data showed for the first time that immune profiling in MM after therapy, in parallel to MRD monitoring, may contribute to identify a subset of patients that albeit being MRD-positive can still experience prolonged survival due to a unique immune signature specifically characterized by a more prominent regeneration of mature B-lymphocytes. In fact, a similar immune signature was previously found in both MRD negative and positive MM patients reaching long-term disease control.(36)

From a biologic point of view, it should be noted that MRD represents a very small fraction of all diagnostic tumor cells which are chemoresistant, potentially quiescent (not producing M-protein), and able to recapitulate the initial tumor burden at relapse. Thereby, a better understanding of the biologic signature of MRD vs. diagnostic cells could potentially contribute to gain insight in the mechanisms of chemoresistance at the MRD level, and the potential discovery of novel therapeutic targets. In this regard, we have recently reported for the first time the biologic features of MRD cells in MM.(37,38) Overall, our results revealed that the MRD subclone is enriched in cells over-expressing integrins (CD11a/CD11c/CD29/CD49d/CD49e), chemokine receptors (CXCR4) and adhesion molecules (CD44/CD54). Genetic profiling of MRD vs. diagnostic PCs showed either identical copy number alterations

(CNAs) or additional CNAs that emerged at the MRD stage. Accordingly, the MRD subclone showed significant downregulation of genes related to protein processing in endoplasmic reticulum, as well as novel deregulated genes such as ALCAM that is prognostically relevant in MM and may identify chemoresistant PCs *in vitro*. Altogether, these results suggested that therapy-induced clonal selection could be already present at the MRD stage, where chemoresistant PCs show a singular phenotypic signature that may result from the persistence of clones with different genetic and gene expression profiles.(37)

Other clinical applications of MFC immunophenotyping

As noted above, MFC is commonly used to monitor MRD in MM due to its widespread availability, fast turnaround, and the amount of information obtained upon enumeration of different cell populations and their corresponding antigen expression levels. Thus, MFC could potentially be used not only to monitor MRD, but also to offer additional prognostic information based on MM PC phenotypes. One of such markers is CD117, for which the favorable prognosis of CD117+ MM patients (39) has been hypothesized due to an altered homing of clonal PCs in the BM towards neutrophil precursor niches, thereby contributing to a greater maintenance of residual normal PCs.(40) Conversely, the adverse prognosis associated to CD28 expression initially attributed to a strong association with adverse cytogenetic alterations (39), has been more recently related to a pro-survival signaling provided through PC-dendritic cell interaction.(41,42) CD19 expression has been described in 5-10% of MM cases (39), and conferred inferior survival in a series of transplant-eligible patients treated prior to the incorporation of novel agents.(39) More recent studies from our group showed that the expression of CD81 in clonal PCs is an independent prognostic factor for patients with symptomatic MM and a marker for risk of progression in SMM.(43) In fact, we have most recently demonstrated in healthy individuals that the CD19-CD81

expression axis identifies three BM PC subsets with distinct age-prevalence, proliferation, replication-history, immunoglobulin-production, and phenotype, consistent with progressively increased differentiation from CD19+CD81+ into CD19-CD81+ and CD19-CD81- BMPCs.(44) Subsequently, we demonstrated that myeloma PCs fit into such a model of normal BMPC differentiation, and that patients with less-differentiated clones had dismal survival. PC differentiation is also related to therapy-induced selective pressure, through which less-differentiated PCs subclones become enriched from diagnosis into MRD stages in a subset of MM patients. Most interestingly, less-differentiated PCs maintain the expression of genes related to preceding B-cell stages, and show different mutation profiles as compared to fully-differentiated PC subclones within individual MM patients.(44) These observations have shed new light into PC plasticity and demonstrated that MM patients harboring less-differentiated PCs have dismal survival, which might be related to higher chemoresistant potential plus different molecular and genomic profiles.(44) In parallel, the breakthrough of immunotherapy in MM makes MFC an attractive technique to measure the expression of novel therapeutic targets such as the PD-1/PD-L1 axis.(45)

The assessment of PC ploidy and proliferation have been long shown to provide prognostic information in MM. However, it should be noted that while the detection of both non-hiperdiploid DNA content and ≥1% PCs in S-phase are of independent prognostic value for OS in newly diagnosed MM patients, treatment with bortezomib-based regimens might abrogate the inferior OS of patients with ≥1% PCs in S-phase.(46) Thus, the prognostic value of MFC-based DNA studies should be revisited.

On a different note, MM patients are living longer with increasingly effective therapies, but long-term complications including second primary malignancies (SPMs) are becoming new challenges in designing optimal patient care. It has been demonstrated in large studies that amongst others, risk is particularly high for SPMs such as myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML).(47) Importantly, such increased risk of MDS/AML has also been observed in MGUS

patients (48), suggesting that increased risk for MDS/AML may not only be treatment related but inheritably high in MGUS/MM. Thus, there is need to investigate for biomarkers that uncover cellular alterations predisposing for higher risk of MDS/AML in MM. Recently, we have shown that in a small proportion of MM and SMM patients, phenotypic alterations, detected by high-sensitivity MFC immunophenotyping, are already present in BM hematopoietic cell compartments at diagnosis.(49) Moreover, our results showed that immunophenotypic dysplastic features are intrinsically related to a genetically abnormal BM hematopoiesis (50), and did not support a protective nor a triggering effect between Len/Dex and MDS development.(49) Thus, given the clinical significance of MDS-CA and the multiple therapeutic options currently available in MM, the information provided by these biomarkers should be integrated in clinical trials, where the prospective identification of patients at higher risk of developing SPMs such as MDS/AML should become a goal.(50)

Conclusion

In the past, MFC immunophenotyping of PC has not been routinely applied in many laboratories for the diagnosis, classification and monitoring of patients with PC disorders. However, at present consensus exists about the clinical utility of MFC in at least three different areas (Table 1): i) the differential diagnosis and classification of PC disorders, ii) prognostic stratification of MGUS, smoldering MM or MM, and iii) MRD monitoring. Thus, MFC immunophenotypic studies should be considered mandatory in the routine evaluation of MM patients both at diagnosis and after therapy. In parallel, MFC has greatly contributed to a better understanding of MM pathogenesis and chemoresistance.

Key points

- MFC immunophenotyping of bone marrow and peripheral blood PCs affords cost-effective assessment of clonality, and provides prognostic information in MGUS and MM
- MFC is one of the methods of choice for cost-effective yet highly-sensitive, standardized MRD monitoring.
- MFC immunophenotypic studies should be considered mandatory in the routine evaluation of MM patients both at diagnosis and after therapy
- Next-generation MFC is an attractive technique to study rare MM subclones (eg.: CTCs and MRD) and help understanding the mechanisms behind disease dissemination and chemoresistance

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3. Conflicts of interest. None

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Table 1. Utility of flow cytometry studies in the management of patients with multiple myeloma (MM).

Disease stage	Application	References
Smoldering MM	Prediction of risk of progression based on the ratio of normal vs clonal PCs within the BM PC compartment	(10,12)
	Prediction of risk of progression based on the number of CTCs in peripheral blood	(14,18)
Active MM	Identification of MGUS-like patients based on the persistence of normal PCs	(12)
	Prognostic information based on the antigen profile of clonal PCs	(39,43,44)
	Assessment of potential therapeutic targets	(45)
	Detection of MDS-like phenotypic abnormalities in other hematopoietic cells	(49,50)
	MRD monitoring	(23-25,27-29,31-33)

PCs: plasma cells; BM: bone marrow; CTCs: circulating tumor cells; MGUS: monoclonal gammopathy of undetermined significance; MDS: myelodysplastic syndromes; MRD: minimal residual disease