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Immunophenotyping in Multiple Myeloma and Others Monoclonal Gammopathies

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1. Introduction

Clonal plasma cell disorders (PCD) including mostly monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (MM) are characterised by expansion of abnormal (clonal) plasma cells (PCs) producing monoclonal protein (M-protein, MIG). Although multiparametric flow cytometry (MFC) allows identification and characterisation of these neoplastic PCs, this approach is used in routine diagnostics of monoclonal gammopathies (MGs) complementarily, mostly in unusual cases [4-6]. The technological development of flow cytometry (FC) in connection with new findings reveal the need for MFC in clinical analysis of MGs. The main applications of immunophenotypisation in MGs are (1) differential diagnosis, (2) determining the risk of progression in MGUS and asymptomatic MM (aMM), (3) detection of minimal residual disease in treated patients with MM, and (4) analysis of prognostic and/or predictive markers. MFC is also very useful also for research analyses focused on different aspects of B and plasma cell (PC) pathophysiology in term of MG development as well as in looking for potential myeloma-initiating cells. MFC thus should be included as a routine assay in monoclonal gammopathy patients. Clinical significance, usefulness and examples of MFC analyses in MGs are reviewed in this chapter.

2. Flow Cytometry in MGs – Past, present and future

The basic principle of flow cytometry has not changed from the past, it is used for identification of cell subtypes according to their functional and structural properties. Flow cytometers are

usually equipped with 2-3 lasers allowing excitation of 6 or more standard fluorochromes and the term multiparametric and/or polychromatic flow cytometry is used for this approach [7]. The classical immunophenotypisation identifies cells based on their size and granularity/complexity as well as by the "visualization" of antigen-antibody binding. More than 360 antigens are currently known and commercial monoclonal antibodies conjugated with different fluorochromes are widely available.

Flow cytometry has been used in diagnostics of MM since 90th years of the 20th century. Mostly ploidy and proliferative characteristics were analysed, but also the combination of DNA analysis with cytoplasmic immunoglobulin detection was done [8-10]. Discovery of new monoclonal antibodies (MoAb) against PCs helped in the development of immunophenotypisation in MGs [11,12].

It is well known that MFC underestimate the number of PCs when compared to routine morphological evaluation. However, the sensitivity of MFC is similar to light microscopy, results obtained using both approaches correlate and the percentage of PCs provided by MFC is also an independent prognostic factor affecting the overall survival of patients [13]. MFC is precise in detecting even a small number of PCs and together with analysis of expression of selected markers, normal and abnormal PCs could be easily discriminated [4]. So MFC is helpful method for clinical analyses of MGs.

Development of flow cytometry, including powerful instruments with the possibility to analyze many fluorochromes, availability of new dyes and antibodies, together with accessible specific software for complex phenotype analysis, require reviewing of current settings in MG analyses. The shift towards polychromatic analyses should be associated with standardisation and validation of this method as it is necessary to be consistent in providing analyses and reporting results. Recently, the European Myeloma Network (EMN) started to use the Euroflow settings which led to the development of a uniform protocol for the analysis of biological material of MG cases [14].

3. Development and differentiation of B cells as PC precursors

B cells and PCs as their terminally differentiated stage play an essential role in humoral immune response. The antigen-dependent phase of B cell differentiation has been extensively studied for many years. Mature naive B cell (CD19⁺CD38[±]CD20⁺CD27⁺IgM⁺IgD⁺) pass from the circulation into lymph nodes. Recognition of antigen presented on a follicular dendritic cell together with a costimulatory signal from a specific T lymphocyte causes B cell activation [15,16]. The activated B cell either migrates to extrafollicular areas where it differentiates into a short term plasma cell or moves into a lymphoid follicle to establish a germinal centre (GC) [17,18]. Massive proliferation of B cell, somatic hypermutation of variable region of Ig chains, isotype switch and subsequent affinity maturation occur in GC [19,20,18,16]. The aim of these processes is to generate B cells able to bind the appropriate antigen with a high affinity. Part of these cells then differentiate into plasmablasts (CD19⁺CD38⁺⁺CD20⁻CD138⁺CD27⁺) migrating into the bone marrow where they mature into long-lived PCs (CD38⁺CD138⁺) producing high-

affinity antibodies. The second group differentiate into long-lived memory B cells (CD19⁺CD38⁺CD20⁺CD27⁺IgM^{-/+}IgD^{-/+}) [21-23]. Besides these GC derived memory B cells also exist memory B cells lacking their typical marker CD27 (CD19⁺CD38⁺CD20⁺CD27⁻IgM^{-/+}IgD⁻) [24], which likely arise independently from the germinal center reaction [25].

Different maturation stages of B cells give a rise to a variety of B cell lymphoproliferations including post-germinal centre (post-GC) neoplasms [26-28]. Knowledge of B and PC phenotype is thus important for determination of PCD diagnosis and its discrimination from other haematological malignancies (Fig 1).

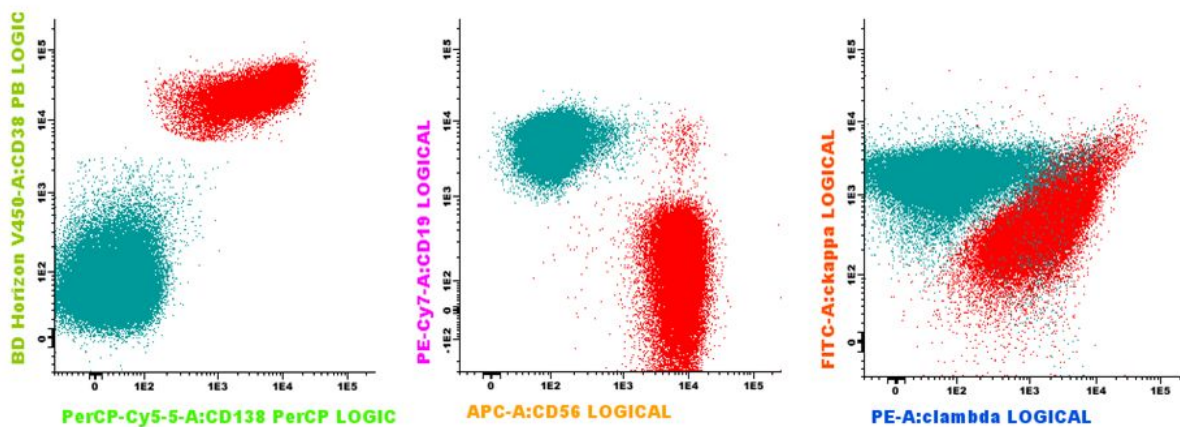


Figure 1. Coexistence of B-CLL and MM. Clone of B-CLL is represented by CD19⁺CD38⁻CD138⁻ B cells (turquoise dots) with cytoplasmic κ expression; clone of myeloma cells (red dots) are typical CD38⁺CD138⁺CD56⁺ PCs with cytoplasmic λ expression.

4. Identification and immunophenotype of PCs

Syndecan-1 (CD138) is a specific marker of PCs expressed on the surface of both, normal and malignant PCs from their early stages [29]. Expression of CD138 is usually missing and/or is not very intensive on circulating PCs and/or plasmablasts in peripheral blood as well as on immature PCs and/or lymphoplasmacytic cells in bone marrow. Another important marker is CD38, a non-specific marker, whose bright expression (brighter on normal than on abnormal PCs) was used to identify PCs for a long time period. Together with CD138 helps in precise identification of PCs. An important marker for pathological PCs identification is also CD45 which is usually missing on PCs. These surface antigens are still used in analyses, but adding of other antigens is necessary [30,31].

Mostly terminally differentiated clonal CD38⁺⁺CD138⁺CD45⁻ PCs are available in MM bone marrow. Relative number of PCs (determined by morphology and/or flow cytometry) corresponds to type of MG, although results could be distorted by dilution of aspirated bone marrow with peripheral blood. Lower amount of PCs is characteristic for MGUS, aMM and/or amyloidosis, on the other hand higher PC infiltration occurs in MM. There is no

possibility to determine PC “abnormality” in low-infiltrated cases without detailed phenotype study (Fig 2). There are also circulating pathological PCs in peripheral blood of some myeloma patients, which have usually the same phenotype as bone marrow PCs (mostly CD56⁺) [32].

Increased absolute ($>2 \times 10^9/l$) and/or relative ($>20\%$ of leukocytes) count of peripheral PCs serve as diagnostic criterion of plasma cell leukaemia (PCL). Primary PCL originates *de novo*, but secondary PCL occurs in patients with relapsed/refractory myeloma [33]. Primary PCL is a distinct clinic-pathological entity with different cytogenetic and molecular findings. The clinical course is aggressive with short remissions and survival duration [34].

Mixture of lymphoplasmacytic cell (LPC) subpopulations with different maturity status (from B cells CD19⁺CD20⁺CD38⁻CD138⁻ to PCs CD19⁺CD20⁺CD38⁺⁺⁺CD138⁺) is characteristic for Waldenström macroglobulinemia (WM), where abnormal LPCs multiply out of control and produce large amounts of IgM protein [35]. It is supposed that every MM is preceded mostly by non-IgM MGUS, however Waldenström macroglobulinemia and/or B-CLL probably arise from IgM MGUS or monoclonal B cell lymphocytosis (MBL) [36,37].

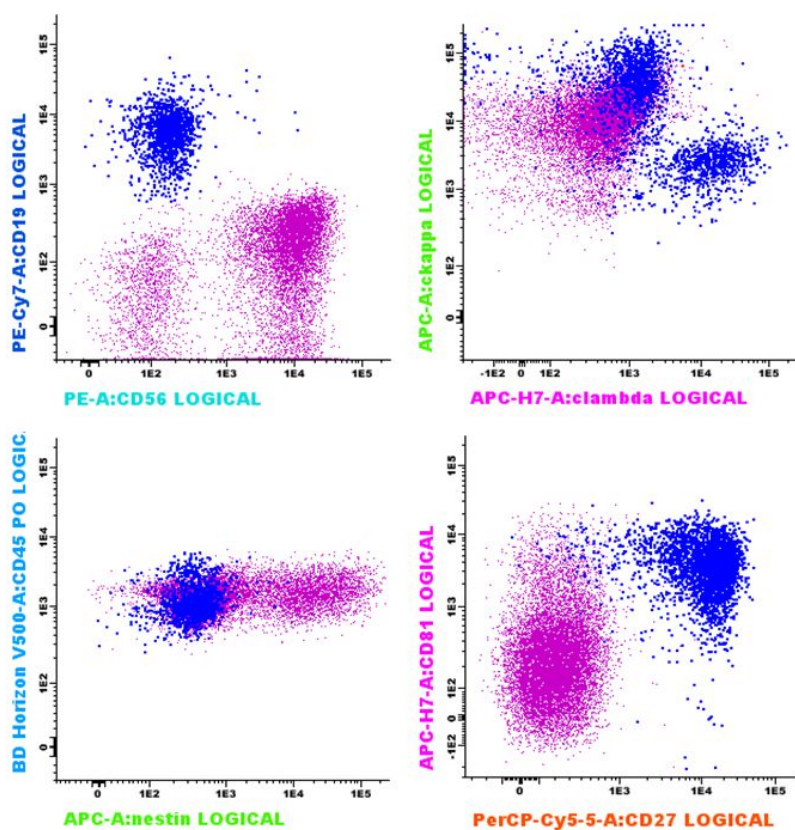


Figure 2. Mixture of polyclonal CD19⁺ (blue dots) and clonal CD56⁺ PCs (violet dots). Heterogeneous expression of CD56 and nestin, positivity for CD45, negativity for CD27 and CD81 was found in clonal CD38⁺CD138⁺ PCs.

Clinically important and necessary antigens allowing discrimination of abnormal from normal PCs are known and listed in Table 1 [4]. Similar antigens were used in Euroflow settings (Table

2) [38]. Detailed information about diagnostic and prognostic value of some interesting markers is mentioned in Table 3. Also other markers should be more and/or less expressed by PCs, mostly without clinical relevance.

Antigen	Normal expression	Abnormal expression	Patients with abnormal expression (%)	Requirement for diagnostics and monitoring
CD19	Positive (>70%)	negative	95%	necessary
CD56	Negative (<15%)	strongly positive	75%	necessary
CD117	Negative (0%)	positive	30%	recommended
CD20	Negative (0%)	positive	30%	recommended
CD28	Weak Positivity (<15%)	strongly positive	15-45%	recommended
CD27	Strong Positivity (100%)	weak/negative	40-50%	recommended

Table 1. List of surface antigens useful for detection of normal and abnormal CD38⁺CD138⁺ PCs in MGs [4].

Tube/ fluorochrom	Pacific Blue	Pacific Orange	FITC	PE	PerCP- Cy5.5	PE-Cy7	APC	APC-H7
1	CD45	CD138	CD38	CD28	CD27	CD19	CD117	CD81
2	CD45	CD138	CD38	CD28	CD56	β2m	clgk	clgλ

Table 2. EuroFlow PCD classification panel. Tube No.1 is useful for phenotype characterization of PCs and evaluation markers with potential prognostic significance, tube No.2 is used for detection and discrimination of normal PCs from aberrant and clonal PCs [38].

Cluster Designation	Normal distribution and functions	Expression in plasma cells of pre-malignant (MGUS) and malignant stage of myeloma	Diagnostic or prognostic significance	References
CD19	Expressed in all stages of B cells ranging from pro-B cells to PCs	MGUS – normal PCs express CD19 whereas malignant PCs do not MM – only negative or dim CD19 expression on PCs	Facilitate as an identification marker of malignant and physiological PCs in combination with CD56. Patients with $>5\%$ of normal PCs (CD19+CD56-) had better PFS and OS compared to patients with $\leq 5\%$ of normal PCs. Similarly, presence of $>5\%$ normal PCs or $<95\%$ of malignant PCs in MGUS and asymptomatic MM (AMM/ SMM) predicted better PFS compared to patients with $\leq 5\%$ normal PCs or $\geq 95\%$ of malignant PCs.	[39-41]
CD20	Expressed during maturation process of B cells and mostly absent on PCs	Only few patients express CD20 on their PCs ($<$ one third of patients)	Associated with poor prognosis	[41-43]
CD27	Helps in differentiation of B cells into PCs	MGUS - consistent expression on PCs MM- expression is heterogeneous and intensity of expression is lower compared to MGUS	Lack of CD27 expression associated with shorter PFS and OS	[44,45]
CD28	T cell activation	MGUS– only very few cases express CD28 MM– CD28 expressing PC represents aggressive phenotype and associates always with tumour expansion	Combination of CD28 and CD117 markers identified three groups of patients with different risk. Patients with CD28-CD117+ PCs (good risk group) had better PFS and OS compared to patients with CD28+CD117- PCs (poor risk) and patients with CD28-CD117- or CD28+CD117+ PCs (intermediate risk)	[46]
CD33	Myeloid and monocytic cells	A very few MM cases express CD33 on the surface of PCs	CD33 expression associated with poor OS and higher mortality rate	[47]

Cluster Designation	Normal distribution and functions	Expression in plasma cells of pre-malignant (MGUS) and malignant stage of myeloma	Diagnostic or prognostic significance	References
CD45	CD45 is a leukocyte common antigen and aids in activation and signaling processes of B and T cells	MGUS - heterogeneous distribution of CD45+ normal and CD45- abnormal PCs in bone marrow MM - mostly CD45 negative CD45 expression demonstrates proliferating compartment of normal, reactive and malignant PCs; immature PCs should be CD45+ as well	Patients with CD45 positive expression had better OS than patients with CD45 negative expression	[48-50]
CD56	NK and NKT cells	One of the most valuable markers to define the abnormal phenotype of PCs in PC proliferative disorders including myeloma. Loss of CD56 expression always associated with aggressive phenotype of myeloma cells. Lack of CD56 expression can be frequently found in patients with circulating PCs and extramedullary myeloma.	Possess substantial diagnostic value in PC disorders when combined with CD19 marker. Patients with CD56 negative expression on PCs found to have reduced OS compared to patients with CD56 positive expression. Also, CD56 negative myeloma cases strongly associated with adverse biological parameters.	[30,51-53]
CD81	Expressed on B cells including PCs and regulates CD19 expression	Less than 50% of MM cases express CD81 on PCs and expression is heterogeneous in most of the cases (ranging from 5%-92%)	Patients with CD81 expression on myeloma cells had inferior prognostic outcome (PFS and OS) compared to patients with CD81 negative expression	[54]
CD117	Progenitors of myeloid, erythroid and megakaryocytic lineage; mast cells	MGUS- 50% of cases express CD117 MM- only one third of myeloma cases express CD117	CD117 expression on PCs predicted better outcome in MM patients. Combination of CD117 and CD28 markers delineated MM patients with different risks; CD117 expression is associated with an altered maturation of the myeloid and lymphoid hematopoietic cell compartments and favorable disease features	[46,55-57]

Cluster Designation	Normal distribution and functions	Expression in plasma cells of pre-malignant (MGUS) and malignant stage of myeloma	Diagnostic or prognostic significance	References
CD138	Plasma cells	Both normal and malignant PCs from MGUS and MM cases express CD138 but the expression of CD38 marker is lower in malignant PCs	Universal marker of PCs and provides basis to quantify or to assess disease burden in PC proliferative disorders	[30]
CD200	Member of immunoglobulin superfamily and expressed on endothelial cells, neurons, B cells and a subset of T cells	MM - more than 70% of cases do express CD200	MM - more than 70% of cases do express CD200	Absence of CD200 expression on myeloma cells associated with better PFS [58, 59]
CD221 (insulin like growth factor-1 receptor)	Tyrosine kinase receptor family, expressed widely on all types of cells	MM - more than 70% and 85% of medullary and extramedullary cases express CD221 on the surface of PCs, respectively	Patients with CD221 expression had worse prognosis and CD221+ PCs were associated with adverse cytogenetic abnormalities	[55,60]
CD229	Signaling lymphocytic activation molecules (SLAM) family member	MM- consistent expression on PCs	Might represent an attractive diagnostic and therapeutic target for MM	[61]
nestin	Protein of class VI intermediate filaments, marker of multipotent proliferative precursors found in some embryonic and fetal tissues	MGUS - less than 30% express nestin; MM - more than 45% and 80% of medullary and extramedullary myeloma cases express nestin in the cytoplasm of PCs, respectively	Patients with nestin expression should have higher risk to develop extramedullary type of MM	[62]

Table 3. Myeloma cell specific antigens and their diagnostic and prognostic values. Abbreviations: PFS - progression free survival, OS - overall survival

5. Abnormality vs. clonality of PCs

The most useful antigens allowing basic orientation in context of PC normality are CD19 and CD56 which can allow relatively easy discrimination of immunophenotypically normal (CD19⁺CD56⁻) from immunophenotypically aberrant (CD19⁻CD56⁺) PCs [63-65]. As was verified by cytoplasmic analysis of immunoglobulin light chains kappa and lambda, this discrimination should be used just for orientation and does not have to correspond to a real number of polyclonal and clonal PCs, especially in unusual cases and/or time after treatment.

Thus polychromatic FC (minimum of 6 markers, but usually 8 markers) is required for sufficient PC analysis and combination of surface and intracellular antigens is necessary for identification and clonality assessment of PCs [66-68]. Only a limited number of cases requires more than 8 markers to detect a small clonal subpopulation of PCs on the prevailing background of polyclonal PCs. Use of marker with a known aberrant expression on analysed PCs (CD28, CD117 etc.) could help in precise identification of clonal PCs. Marker CD27 should be useful as loss of this antigen should reveal clonal PCs (Fig 3). Together with analysis of a sufficient number of PCs, the sensitivity of polychromatic FC should reach the sensitivity of the PCR approach [5,67].

6. Clinical application of flow cytometry in MGs

FC should be used not only for assessment of PCs in peripheral blood (PB) and/or bone marrow (BM), but in simultaneous analysis of 8 markers on a single cell could identify the type of PCs that has clinical and predictive value.

6.1. Differential diagnostics

Identification and enumeration of PCs is as important as discrimination between normal polyclonal PCs in reactive plasmocytosis and clonal PCs in plasma cell disorders (MGUS, MM, PCL, extramedullary plasmocytoma) [4]. It was found that BM of MGUS cases contained a mixture of polyclonal PCs with normal phenotype and clonal PCs with aberrant phenotype, on the other hand there is a majority of clonal PCs in MM [63,65]. Presence of more than 5% normal PCs in BM should be used as a cut-off value for differentiation between MGUS and MM [40]. Surprisingly there were found symptomatic MM patients with more than 5% normal PCs in BM, these should be signed as "MGUS-like MM" and have a low incidence of high-risk cytogenetic abnormalities with a longer progression-free survival and longer overall survival as well [39]. There are clonal non-myelomatous PCs present in Waldenström macroglobulinemia (WM) so careful PC analysis should be done in these patients especially when they have low number of PCs [35]. Discrimination of myelomatous from non-myelomatous PCs then should help in determination of other lymphoproliferations [28].

6.2. Determination of the progression risk in MGUS and MM

Conventional parameters related to the higher risk of progression of MGUS into MM are monoclonal Ig level (MIG) > 15 g/l and non-IgG isotype of MIG. Even so, a new parameter is serum free light chain (FLC) ratio. These parameters were used for risk stratification model [69]. Simultaneously evolving and non-evolving theory of MGUS type, based on evolutionary pattern of MIG (increasing vs. stable) was published [70]. Mentioned parameters are important in patient monitoring for decades, but FC approach based on pathological PCs enumeration is quicker with a better predictive value [40]. Finding $\geq 95\%$ pathological PCs (from all PCs) is an independent parameter with a predictive value, in term of risk of progression MGUS and/or a MM into symptomatic form. When compared FC results with a parameter describing

evolution of monoclonal component, the risk of progression was better described by immunophenotypisation [71]. Multiparametric FC is thus capable to distinguish patients which need more frequent monitoring and which need to start treatment earlier than usual. There is still not any marker allowing discrimination between benign MGUS and its malignant form at this moment.

6.3. Prognostic markers in MGs

Determination of immunophenotype should be used not only for discrimination of normal and pathological PCs, but it has also prognostic value. The loss of CD56 (neural cell adhesion molecule, NCAM) should be joined with extramedullary spread [72]. An association between the phenotype profile and cytogenetic abnormalities was found. Expression of CD19 and CD28 and/or absence of the CD117 on pathological PCs are joined with significantly shorter time without progression and overall survival in transplanted patients [46]. Expression of CD28 correlated with t(14;16) and del(17p), on the other hand no presence of CD117 was joined with t(4;14) and del(13q). The analysis combining both CD28 and CD117 was able to divide patients into 3 risk groups with different time without progression and overall survival. The correlation of CD117 expression with hyperdiploidy was found as well [73]. The expression of CD117 on PCs is associated with changes in production of haematopoietic stem cells from BM, lead to a decreasing number of neutrophils in PB and the presence of normal PCs in BM [57]. Recently, a rare MM case was described with PCs phenotype: CD19⁺CD56⁻CD20⁺CD22⁺CD28⁺CD33⁺CD117⁺HLA-DR⁺. Moreover, the cytogenetic analysis of this case revealed a hyperdiploid karyotype and no rearrangement of the IgH gene or deletion of 13q14 [74]. The very important genetic change in MM is loss of the gene for CD27 which is linked with clinically aggressive disease, but in about 50% of MM is expression of CD27 preserved and these patients have better prognosis [44]. Probably the best prognostic information until now serves a combination of two independent parameters: the presence of high-risk cytogenetics by FISH and persistent minimal residual disease evaluated by multiparameter flow cytometry at day +100 after autologous transplantation. These two parameters were able to identify patients in complete remission at risk of early progression [75]. The important thing is that these two methods are available in most hospitals taking care of patients with haematological malignancies.

6.4. Minimal residual disease analysis

It is known that conventional parameters (% PCs, MIG level) are not sensitive enough for analysis of treatment response in MM patients. As FC is applicable up to 80-90% of patients, this method is able to reach the sensitivity of allelic-specific oligonucleotide (ASO)-PCR (sensitivity 10^{-4} for FC vs. 10^{-5} for PCR) and is less time and monetary consuming as well. Hence FC looks as the optimal method for minimal residual disease (MRD) assessment after any treatment [76,77]. The advantage of FC in MRD analysis is the versatility of used markers allowing assessment of normal and abnormal PCs (CD19/CD56), removing the need to know the original phenotype of PCs before treatment. MRD negativity proved by FC (detection $<10^{-4}$ myeloma PC within all nucleated cells) was more informative than

positive immunofixation (IF) after autologous transplantation (regarding to time to progression and overall survival), so FC is sufficiently sensitive method and should be used for routine MRD analysis [78].

6.4.1 Better definition of complete remission

Using new treatment protocols led The International Myeloma Working Group (IMWG) to review treatment response criteria. There was included also FC analysis in the assessment of stringent complete response (sCR), more precisely the absence of phenotypically aberrant PCs in 3000 PC analysed by multiparametric FC (≥ 4 colours) [79]. Criteria of MRD level assessment are changing nowadays as newer more efficient treatment protocols are available and FC has technically developed. When used flow cytometry for confirmation of (s)CR, the new term „immunophenotype remission (iCR)” - a state without presence of any clonal PCs should be used [79, 80]. The evidence suggests that the 4-colour FC is not sufficiently sensitive for confirmation of iCR and standardized polychromatic flow cytometry is the best approach (Fig 3).

7. Conclusion

Flow cytometry analysis was performed only in a limited number of subjects with monoclonal gammopathies in the late 1990's and early 2000's. During the past decade and present, many analyses showed importance of MFC in differential diagnostics and monitoring (management) of plasma cell diseases. The MFC has developed significantly and with better understanding of PC pathophysiology is the mandatory diagnostic tool which should be included as a routine assay in monoclonal gammopathy patients.

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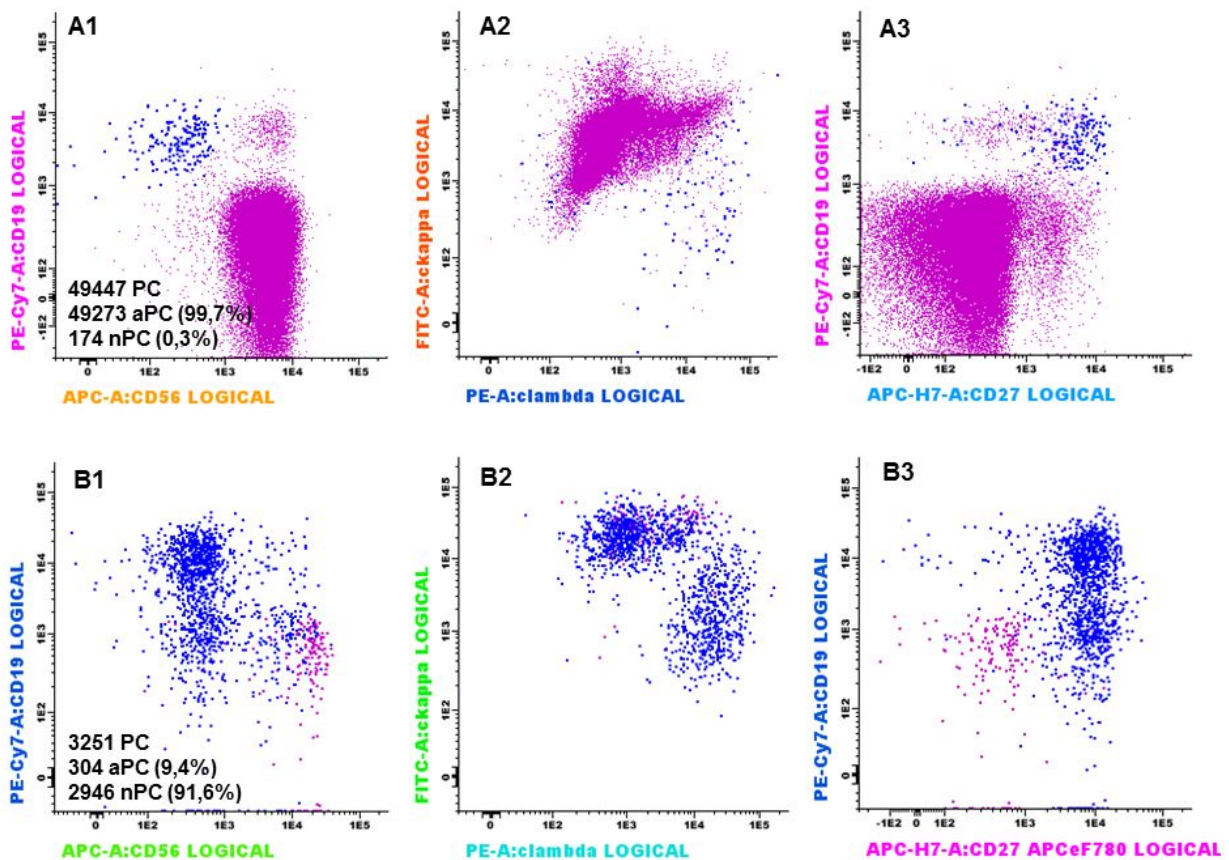


Figure 3. Polychromatic analysis of PC phenotype and clonality. A1-A3: Majority of clonal CD56⁺ckappa⁺CD27⁻ PCs (99,7% of aPCs according to clonality assessment) is visible in MM patient at the time of diagnosis (purple dots). B1-B3: Mixture of clonal (aPCs, purple dots) and polyclonal PCs (nPCs, blue dots) is visible at 3rd month after transplantation; assessment of % aPCs is possible only when CD27 is used as some nPCs are CD19⁻ and/or CD56⁺ as well.

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