

Immunophenotyping of Plasma Cells in Multiple Myeloma

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Summary

Multiparametric immunophenotyping of multiple myeloma (MM) and other plasma cell (PC) dyscrasias represents an attractive approach not only for research purposes but also in clinical practice. Based on well-established antigenic patterns, discrimination between myelomatous and normal PCs can be easily achieved in various types of samples, and this can be particularly valuable for the differential diagnosis between MGUS and MM and for monitoring residual disease in the latter. In addition, immunophenotyping may be an alternative and more reproducible method than morphology for evaluating PC infiltration, as well as for specifically analyzing DNA content and the cell-cycle distribution of different subsets of PCs. Despite the widespread use, standardization of methods and protocols still remains a challenge. In this chapter, we describe in detail the protocols and precise instructions for specimen collection, sample preparation, together with the methods for staining PCs and flow cytometry, data acquisition, and data analysis, including the more recent developments in the field. We highlight the most frequent limitations, and provide troubleshooting and practical recommendations that could help to solve them. The goal of this chapter is to emphasize the relevance of methodological issues in order to obtain reproducible and high-quality results regarding the phenotypic analysis of PCs.

Key Words

Multiple myeloma; plasma cell; flow cytometry; immunophenotyping; antigen.

1. Introduction

During the last decade, immunophenotyping of multiple myeloma (MM) has been frequently debated. The complexity of assaying plasma cells (PCs) is owing, to a large extent, to their low representation in bone marrow aspirates, despite obvious morphological involvement in biopsy specimens. Additional

From: *Methods in Molecular Medicine*, Vol. 113: *Multiple Myeloma: Methods and Protocols*
Edited by: R. D. Brown and P. J. Ho © Humana Press Inc., Totowa, NJ

difficulties owing to the maturation-related loss of most of the B-lineage-associated markers, together with the intrinsic antigen heterogeneity of PCs (1–3), have made it difficult to specifically characterize this cell population for many years. Moreover, a relatively large number of studies have been reported in which single or double antigen stainings were used, PCs were not specifically identified, and the total number of PCs evaluated was frequently relatively small; this would not allow appropriate assessment of weakly expressed antigens or antigens present in only a small proportion of all PCs. Altogether these and other factors may contribute to explaining some of the overt controversies found in the literature regarding the exact phenotype of myelomatous PCs and its clinical significance.

In recent years, efforts have been made to solve these methodological problems, to standardize the immunophenotyping protocols, and to gain further insight into the phenotype of normal and myelomatous PCs. The value of multi-parameter immunophenotyping by flow cytometry for distinguishing between normal/polyclonal and tumoral/clonal PCs is now well recognized, even when PCs are present in the sample at very low frequencies. Such distinction can be of clinical interest for the differential diagnosis between distinct PC dyscrasias (4,5), for monitoring of residual disease after treatment (6–8) and for investigation of the presence of contaminating PCs in peripheral blood-derived products for an autologous transplant (9,10). Consequently, immunophenotyping has progressively been incorporated into routine practice in many clinical laboratories, while also continuing to be a useful technique in basic research in MM. Accordingly, in parallel with the methodological advances, an increasingly large amount of knowledge has accumulated in recent years about the biology of MM, to which immunophenotyping has actively contributed by providing not only technical support but also intrinsically relevant information.

2. Materials

The materials described outline the type of specimens and samples and basic requirements for specimen collection, and the equipment and supplies as well as the reagents necessary to conduct immunophenotypic studies of PCs.

1. Specimens and samples: Specimens from patients with MM containing PCs that are susceptible to being studied by immunophenotypic approaches may include the following (*see* **Notes 1–4**):
 - a. Bone marrow.
 - b. Peripheral blood.
 - c. Liquors or malignant effusions (e.g., ascitic or pleural effusions).
 - d. Other solid tissues (e.g., plasmacytomas, tonsillar tissues, lymph nodes).
 - e. Immortalized PCs.

2. Multiparameter flow cytometer with 488- and 653-nm double-excitation lasers and filters for the detection of at least green, orange, red, and deep-red fluorescence.
3. Fluorescence-activated cell sorter or magnetic cell-sorting equipment (optional).
4. Computer workstation equipped with calibration, quality control, and appropriate software for data acquisition, transfer, and analysis.
5. Additional computer support (PCs, CDs, ZIP drive).
6. Benchtop centrifuge at 540g adapted for 5-mL tubes and/or plates.
7. Refrigerator (at $\approx 4^{\circ}\text{C}$), for storage of reagent.
8. Polystyrene tubes (12×75 mm) or 96-well culture plates.
9. Set of precision-adjustable calibrated micropipets capable of dispensing in the range of 5–10 μL and in volumes of 100–1000 μL .
10. Appropriate pipet tips.
11. Vortex mixer.
12. Tube mixer/roller (optional).
13. Monoclonal antibodies (MAbs) directly conjugated to fluorochromes optimized for detection of cell surface and intracellular epitopes resistant to conditions of fixation and permeabilization. Staining reagents may be used as a multicolor cocktail.
14. Filtered (0.40- μm pore filter) phosphate-buffered saline (PBS) with 0.1–0.2% sodium azide and 0.1–2% protein solution (e.g., bovine serum albumin), pH 7.6, as cell wash buffer.
15. Red cell lysing solution (*see Note 5*).
16. Fixation and permeabilization solution for staining of intracellular antigens (*see Note 6*).
17. Reagents and cells for instrument setup (*see Note 7*).

3. Methods

Technical procedures used for the immunophenotypic analysis of human PCs typically may include up to five sequential steps:

1. Preparation of specimen and sample.
2. Staining of cells.
3. Acquisition of data.
4. Analysis of data.
5. Interpretation of the results.

3.1. Preparation of Specimen

The most important requirement of a specimen for multiparameter flow cytometry analysis is that it contain a single cell suspension that allows specific immunophenotypic characterization of individual cells and facilitates interaction of the antibody with the antigen. Since in the bone marrow and other solid tissue specimens cell aggregates exist, adequate mechanical disaggregation procedures should be used. Accordingly, these specimens should be passed several

times through a 25-gage needle using a syringe either directly (bone marrow and fine-needle aspirates) or after the tissue has been placed in a Petri dish containing 1 to 2 mL of PBS and cut into small (1-mm^3) pieces (solid tissues); alternatively, semiautomated instruments, which are commercially available (BD MedimachineTM, BDB Biosciences), can be used to disaggregate solid tissues mechanically. Just prior to starting staining procedures, a nucleated blood cell count should be performed and the cell concentration adjusted to a final count of 10^7 nucleated cells/mL. For that purpose, and if it is necessary, the sample should be diluted in filtered PBS and gently mixed.

3.2. Staining of Cells and Preparation of Samples

In this section, we discuss (1) the requisites to choose the most appropriate combinations of reagents and (2) techniques used to identify the antigens expressed, either at the cell surface or cytoplasmic/nuclear level, in PCs.

3.2.1. Panels of MAb Reagents

3.2.1.1. COMBINATIONS OF MABS

For the immunophenotypic analysis of PCs, two different groups of MABs should be combined for simultaneous assessment of their expression. First, MABs aimed at the specific identification of PCs or their subsets should be chosen from a relatively restricted number of reagents. Second, these should be further combined with a set of one or more reagents selected for characterization of the PCs and/or their subsets of interest. Despite the increasingly large number of antigens known to be expressed by normal and pathological PCs, CD38 and CD138 are the most efficient ones for their specific identification. Both antigens are considered to be highly sensitive and specific for the identification of PCs. However, note that PCs that express low levels of CD138 can be frequently found, and this antigen can also be expressed on nonhematopoietic cells; in turn, CD38 is an antigen widely expressed on both hematopoietic and nonhematopoietic cells that shows uniquely high amounts on normal PCs but that can be expressed at lower levels in myelomatous PCs. Consequently, some researchers (5,11–13) have proposed the combined assessment of both markers for a more efficient identification of PCs. In addition to CD38 and CD138, other surface and cytoplasmic antigens, such as CD13, CD19, CD20, CD28, CD33, CD40, CD52, CD56, CD45, CD86, and CD117, as well as surface and cytoplasmic immunoglobulin (Ig) κ and λ light chains, have been shown to be of variable utility for the specific identification within the PC compartment of normal/polyclonal vs pathological/clonal PCs in patients with monoclonal gammopathies. If enumeration of PCs or their subsets is pursued, the use of CD45 or a DNA dye can be of great help in creating a common denominator of all leukocytes or nucleated cells present in the sample, respectively.

Accordingly, overall consensus exists on the use of the CD38/CD56/CD19/CD45 or CD38/CD56/CD19/CD45/CD138 four- or five-color combinations of MAb reagents for the specific identification and enumeration of normal and pathological PCs from a sample. In addition to these markers, an increasingly large number of surface and intracytoplasmic molecules have been found to be of relevance in MM. According to their functional role, these may be classified as (1) coreceptors for signal transduction that modulate PC response to various stimuli, (2) crucial molecules in the cell-cell and cell-matrix interactions, (3) molecules involved in the differentiation and maturation of PCs, as well as (4) proteins associated with apoptosis and cell survival. **Table 1** summarizes the most relevant molecules that have been described on myelomatous PCs. Many high-quality and validated MAb reagents that specifically bind to unique epitopes of these molecules are currently available (*14*).

3.2.1.2. FLUOROCHROMES

Primary MAbs can be labeled with different fluorescent molecules or fluorochrome tandems from which fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), PE/cyanin-5 (PE/Cy5), peridin/chlorophyll protein (PerCP)/Cy5, PE/Cy7, APC/Cy7, and PE/Texas red (TR) are the most frequently used. In recent years, new fluorochromes have been incorporated into fluorochrome-conjugated MAbs, including pacific blue and the Alexa[®] 647 and 488 dyes; such an increase in the number of available fluorochrome conjugates has been pushed by the development of new flow cytometry instruments that allow the simultaneous detection of more than 10 different fluorescent signals. However, note that once conjugated to an MAb, each fluorochrome behaves differently regarding fluorescence intensity, requirements for fluorescence compensation, or fluorescence-resonance energy transfer properties, among others. As a result, fluorochrome conjugates should be carefully selected for each MAb reagent to be included in a multicolor combination. As an example for the four-color combination of MAbs just listed, the following combinations of fluorochrome-conjugated reagents would be recommended: CD38 FITC/CD56 PE/CD19 PerCPCy5 or PE/TR/CD45 APC or PE/Cy5 depending on the characteristics of the flow cytometer. Additionally, differences in the reactivity obtained for different MAb clones and in the quality of the reagents available from different manufacturers might also be observed.

3.2.1.3. CONTROLS

Parallel measurement of a negative control specifically stained for PCs is required, because PCs show variable levels of intrinsic autofluorescence that differ from those of other nucleated cells. Negative controls are necessary to establish a cutoff for positivity based on the levels of natural

Table 1

Summary of Most Relevant Molecules Described in PCs

Molecules involved in differentiation/maturation process of PCs

PCs represent the last stage of B-cell development. Maturation of B-lymphocytes toward PCs is a multistep process in which a coordinated acquisition/loss of surface and intracellular antigens occurs. During malignant transformation of PCs, the expression of these antigens may be altered.

CD19	pan-B-cell marker that functions as a general rheostat for defining signaling thresholds critical for expansion of B-cells. Specific loss in malignant/clonal PCs (~98% of myeloma cases) correlates with altered PAX-5, a gene that encodes for the transcriptional positive regulatory factor BSAP (B-cell-specific activating protein), necessary for CD19 expression.
CD20	B-cell-specific antigen that is expressed from intermediate stages of B-cell maturation in BM and is downregulated in normal PCs. In ~20% of MM patients and up to 50% of "PC leukemia" cases, partial or total expression of CD20 is seen, suggesting the potential utility of CD20-directed serotherapy (Rituximab®).
CD38	A transmembrane glycoprotein with enzymatic activity that is widely expressed on both hematopoietic and nonhematopoietic cells. It has a discontinuous and variable pattern of expression in B-cells with PCs showing uniquely high amounts of CD38. CD38 is downregulated in PCs from ~80% of all myeloma cases. It is one of the most useful markers for the specific identification of PCs.
CD45 (leukocyte common antigen)	Protein tyrosine phosphatase required for activation of lymphocytes and development. Its expression decreases during PC differentiation. In most MM cases, PCs lack CD45 expression. Brighter CD45 expression has been correlated with a higher proliferative capacity and a high degree of immaturity of PCs.
CD52 (CDw52)	Characterized as a human leukocyte differentiation antigen. It is present on the surface of malignant PCs from MGUS and MM patients. It is a potential target for specific immunotherapies (e.g., Alemtuzumab®, Campath-1H) for MM and other hematological disorders.

Cytokine/chemokine receptors

Cytokines are soluble proteins produced and secreted by different hematopoietic cells including PCs (IL-6, IGF-1, TNF- α , VEGF). IL-6 is a key growth and survival factor for PCs (induces PC proliferation and inhibits apoptosis) as well as a major morbidity

Table 1 (continued)

factor (bone destruction, hypercalcemia, renal failure) in MM. Chemokines are a superfamily of cytokines that play a critical role in selective cell trafficking and homing acting through specific receptors on the cell surface.

• **IL-6 receptor**

CD126 subunit (IL-6R α , gp 80)

α -chain of the IL-6R that specifically binds IL-6. High levels of soluble IL-6R have been associated with poor prognosis in MM.

CD130 subunit (IL-6R β , gp 130)

Common β -chain of the IL-6R that is responsible for signal transduction. IL-6 triggers proliferation via the Ras/Raf/MEK/MAPK cascade and promotes PC survival through phosphorylation of STAT3 and upregulation of antiapoptotic molecules (Mcl-1, Bcl-xL, and bcl-2). IL-6 interaction protects against dexamethasone through PI3K/AKT signaling.

• **SCF receptor**

CD117 (c-kit ligand)

Class III family tyrosine kinase strongly expressed by mast cells, stem cells and myeloid precursors. It is also present in PCs from $\approx 1/3$ of myeloma patients. SCF induces via CD117 an increased in PC proliferation. It is a potential target for novel drugs (Imatinib mesylate, STI571, Gleevec[®]) for treatment of myeloma.

• **Chemokine SDF-1 receptor**

CXCR4

A chemokine receptor for SDF-1 that induces chemotaxis of PC toward BM. In addition, it modulates the adhesion activity of VLA-4 ($\alpha 4\beta 1$ integrin).

• **RANTES and MIP- α ligand**

CCR1

BM in MM produces high levels of RANTES and/or MIP- α . Both chemoattractants bind to the CCR1 receptor on PCs, inducing migration and homing.

Cell adhesion

Adhesion molecules bind to their ligands on BMSCs, or on extracellular matrix proteins and proteoglycans, allowing individual cells to form and stabilize close contacts relevant for the maintenance of a higher order of tissue specialization and facilitate transfer of information. Adhesive interactions play a crucial role in the pathogenesis of the growth and survival of MM.

(continued)

Table 1 (continued)

• **Integrin family**

β 1-integrins Ligation of VLA-4 (CD49d/CD29; α 4/ β 1 integrin) via vascular cellular adhesion molecule-1 present on BMSCs upregulates IL-6 secretion by BMSCs, induces proliferation, and blocks apoptosis of myeloma PCs. Altogether, it decreases secretion of osteoprotegerin and increases expression of RANKL, promoting osteolysis.

VLA-5 (CD49e) binds to fibronectin. Expression of CD49e identifies “mature” PCs, whereas negativity for CD49e correlates with more “immature” PCs with a higher proliferation rate and resistance to chemotherapy.

β 2-integrins LFA-1 (CD11a-CD18) interacts with ICAM-1 on BMSCs; expression on myeloma PCs has been correlated with a higher tumor cell growth.

LFA-3 (CD58) mediates adhesion to T-cells via CD2, stimulating them to produce those cytokines necessary for MM growth. CD58 is not expressed by normal PCs but is commonly positive on malignant PCs.

• **Ig superfamily**

CD56 (NCAM) Typically, CD56 is weakly expressed on normal/polyclonal PCs and strongly positive on the surface of myelomatous/clonal PCs (\approx 60% of myeloma cases) and stromal cells. PC leukemia usually lacks on CD56, suggesting disease progression. It is a useful marker for follow-up of minimal residual disease.

• **Cell-surface proteoglycans**

CD138 (BB4; syndecan-1)

CD138 is a heparan sulfate proteoglycan that promotes cell-surface adhesion to type I collagen, fibronectin, and thrombospondin. In BM, CD138 is expressed exclusively by PCs. This molecule is rapidly lost in apoptotic cells and shed from the surface of PCs into the serum in advanced phases of the disease (e.g., PC leukemia). It is commonly used for the identification and purification of myeloma PCs from clinical samples.

Other markers of interest

• **Costimulatory molecules**

CD28 CD28, a T-cell-restricted antigen, is also present in both normal and myelomatous PCs from >20% of the cases. The activation

Table 1 (continued)

	and function of CD28 in myelomatous PCs are not well known, but its overexpression correlates with disease progression and relapse, suggesting an important contribution of CD28 signaling to myeloma cell survival.
CD40	Normal and malignant PCs retain CD40 antigen at varying levels of expression. CD40 signaling inhibits apoptosis via nuclear factor- κ B-mediated pathways, suggesting the possible use of proteasome inhibitors (PS341, Velcade®) and CD40 antagonists for the treatment of myeloma.
CD80 (B7-1) CD86 (B7-2)	CD80 is expressed by antigen-presenting cells (macrophages, activated B-cells, and dendritic cells) whereas CD86 is present on immature unstimulated antigen-presenting cells. On PCs, CD86 is variably expressed and CD80 is almost constantly absent. Studies have reported that a high expression of CD86 on malignant PCs may confer a poor prognosis in myeloma patients.
• Myelomonocytic-associated antigens	
CD13 CD33	Both antigens are expressed by normal and malignant PCs. Their biological function and clinical significance in a PC that belongs to a different cell lineage remain unclear. CD13 and CD33 are useful markers for follow-up investigations of minimal residual disease in about 15% of all MM patients.

^aBM = bone marrow; MGUS = monoclonal gammopathy of undetermined significance; IL-6 = interleukin-6; IGF-1 = insulin-like growth factor-1; TNF- α = tumor necrosis factor- α ; VEGF = vascular endothelial growth factor; MEK = MAPK/ERK (extracellular signal-regulated)/kinase; MAPK = mitogen-activated protein kinase; PI3K = phosphatidylinositol 3-kinase; AKT = also known as protein kinase B; SCF = stem cell factor; SDF-1 = stromal cell-derived factor-1; MIP-1 α = macrophage inflammatory protein- α ; BMSCs = bone marrow stromal cells; NCAM = neural cell adhesion molecule; RANKL = receptor activator of nuclear factor- κ B ligand.

autofluorescence and/or nonspecific staining, and they are typically a sample aliquot treated in a way identical to that of the test sample. At present, it is well established that isotype-matched fluorochrome-conjugated mouse Igs are not the most appropriate control for specific fluorescence, especially when a large panel of MAbs of different isotypes is used. Accordingly, most appropriate negative controls include the specific measurement of basal autofluorescence levels of PCs (**Fig. 1A**) together with the confirmation that the expected reactivity is observed for the markers analyzed in the normal cells also present in the sample, including those cellular compartments

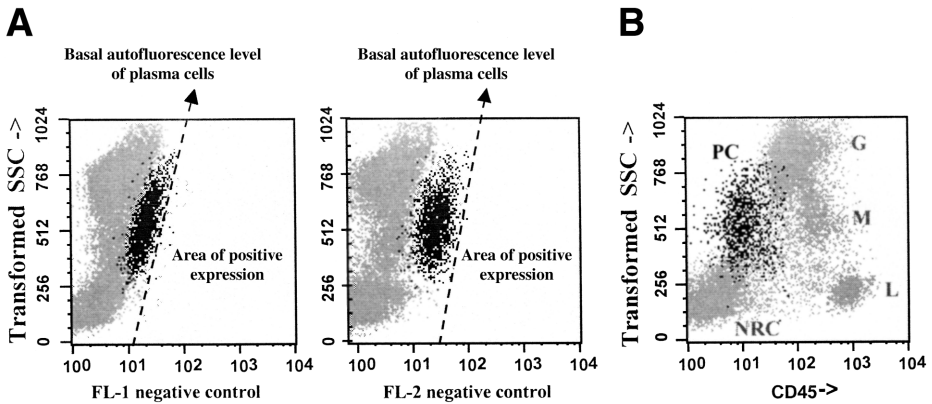


Fig. 1. (A) Level of natural autofluorescence of PCs (in black) and/or nonspecific green (FL-1; left dot plot) and orange (FL-2; middle dot plot) fluorescence. Negative controls are necessary to establish a cutoff for positivity. (B) SSC vs CD45 dot plot showing position of PCs (PC) referred to other normal cell populations present in sample. CD45 expression on PCs could be considered as negative because it is comparable with CD45-negative nucleated red cells (NRC). CD45 expression on positive cells—lymphocytes (L), monocytes (M), and granulocytes (G)—can be used as an internal positive control for this marker.

expressing high amounts of FcIg receptors. The use of a relatively large panel of MAbs also provides adequate positive controls. As an example, CD45 should be strongly expressed on lymphocytes, whereas neutrophils show low positivity for CD45, and nucleated red cells lack reactivity for this antigen (Fig. 1B).

3.2.2. Antigen Staining

3.2.2.1. PREVIOUS CONSIDERATIONS

Immunophenotyping staining assays on blood-containing samples (e.g., peripheral blood or bone marrow) are based on three steps: (1) lysing of non-nucleated red cells, (2) staining, and (3) washing with a fixation of nucleated cells. Erythrocyte contamination of leukocyte preparations is particularly vexing for flow cytometry procedures; consequently, samples must be depleted from erythrocytes prior to analysis. As stated in **Note 5**, there are several types of red cell lysing solutions that can be used either prior to or, more frequently, immediately after the staining of whole blood or bone marrow samples with the appropriate panel of MAbs. Usually, it is preferable first to stain and then to lyse and wash because lysing solutions may start to damage cells or even

facilitate an increase in intracellular specific or nonspecific staining, especially if they contain fixatives. Depletion of erythrocytes through density gradient centrifugation (e.g., Ficoll-Hypaque) procedures for the purification of mononuclear cells to be used for further staining and analysis of PCs is currently not recommended owing to the high degree of manipulation that the sample is submitted to and the occurrence of an uncontrolled variable degree of cell loss.

3.2.2.2. DIRECT IMMUNOFLUORESCENCE TECHNIQUES

In this section, the exact steps used for the staining of surface antigens alone or in combination with intracytoplasmic markers on PCs are described. Such procedures can be simplified in nonnucleated red cell-free samples (e.g., cell lines, isolated/purified PCs) as pointed out in **Note 8**.

3.2.2.2.1. Protocol I: Staining for Surface Antigens

1. Label the tubes according to the MAb combinations (see the combinations listed in **Subheading 3.2.1.1**).
2. Add the appropriate amounts of each of the selected MAbs (typically between 0.1 and 1.5 μg of antibody in a volume of 5–10 μL) to a final incubation volume of $\leq 250 \mu\text{L}$.
3. In each of the tubes, place 100 μL of the PBS-diluted bone marrow sample containing 10^6 nucleated cells; gently mix the tubes for 10 s.
4. Incubate for 15 min at room temperature in the dark.
5. Add 2 mL/tube of lysing solution and gently mix.
6. Incubate for 10 min at room temperature in the dark.
7. Centrifuge the nonnucleated red cell-lysed sample for 5 min at 500g.
8. Discard the supernatant with a Pasteur pipet, and resuspend the cell pellet.
9. Add 2 mL/tube of filtered PBS.
10. Centrifuge for 5 min at 500g.
11. Discard the supernatant with a Pasteur pipet, resuspend the cell pellet, and add 0.5 mL/tube of filtered PBS containing 1% paraformaldehyde.
12. Read in a flow cytometer or store at 4°C for a maximum of 24 h. Preferentially, samples should be acquired just after being processed, although they can be stored at 4°C overnight.

3.2.2.2.2. Protocol II: Staining for Surface Ig κ and λ Light Chains (or Ig Heavy Chains) and Other Membrane Antigens

For the surface detection of Ig κ and λ light chains (κ and λ) or Ig heavy chains, it is recommended that the soluble Ig molecules potentially present in the sample in high amounts (e.g., peripheral blood and bone marrow) be eliminated. Soluble Igs specifically bind to the anti-Ig MAb reagents, and this decreases the intensity of staining for the Ig present at the surface of PCs toward undetectable levels. Accordingly, the following protocol should be followed:

1. Add 2 mL of filtered PBS to a tube.
2. Place 100 μ L of the PBS-diluted bone marrow sample containing 10^6 nucleated cells in that tube, and vortex the sample for a few seconds.
3. Centrifuge for 5 min at 500g.
4. Discard the supernatant with a Pasteur pipet and resuspend the cell pellet; add 2 mL/tube of filtered PBS and gently mix for a few seconds.
5. Centrifuge for 5 min at 500g.
6. Discard the supernatant with a Pasteur pipet and resuspend the cell pellet.
7. Label the tubes according to the MAb combinations containing anti-Ig reagents in combination with other PC markers.
8. Proceed with **steps 2–12** of protocol I (*see Subheading 3.2.2.2.1.*).

3.2.2.2.3. Protocol III: Staining for Intracellular and Surface Antigens

When an antigenic target is sequestered or is present inside a cell, in the cytoplasmic or another intracellular compartment, its appropriate staining requires permeabilization of the cellular membranes to allow the antibody to enter the cell structures where the target protein is localized. To avoid extensive cell damage and cell loss, fixation procedures should be applied prior to permeabilization of the cellular membranes. The technique described next combines staining for intracellular antigens with detection of antigens expressed at the cell surface, which is useful for the identification of the target PCs (e.g., double staining with CD38 and CD138 on the cell surface to identify the PC and cytoplasmatic κ and λ Ig light chains to assess clonality).

1. Label the tubes according to the MAb combinations to be applied.
2. Add the appropriate amounts of each of the MAbs directed against the surface antigens.
3. To each tube add 100 μ L of sample containing approx 10^6 nucleated cells; gently mix the tubes for a few seconds.
4. Incubate for 15 min at room temperature in the dark.
5. Add 2 mL/tube of filtered PBS.
6. Centrifuge for 5 min at 500g.
7. Discard the supernatant with a Pasteur pipet, and resuspend the cell pellet in a volume of <50 μ L.
8. Add 100 μ L/tube of a fixative reagent as detailed in **Note 6**; gently mix.
9. Incubate for 15 min at room temperature in the dark.
10. Add 2 mL/tube of filtered PBS.
11. Centrifuge for 5 min at 500g.
12. Discard the supernatant with a Pasteur pipet, and resuspend the cell pellet in a volume of <50 μ L.
13. Add 100 μ L/tube of a permeabilizing solution (*see Note 6*) and the appropriate amounts of those MAbs directed against the intracellular antigens to be detected; gently mix.
14. Incubate for 15 min at room temperature in the dark.

15. Add 2 mL/tube of filtered PBS.
16. Centrifuge for 5 min at 500g.
17. Discard the supernatant with a Pasteur pipet and resuspend the cell pellet.
18. Add 0.5 mL/tube of filtered PBS.
19. Read in a flow cytometer or store at 4°C for a maximum of 24 h. Preferentially, samples should be acquired just after being stained, although they can be stored overnight at 4°C.

3.3. Acquisition of Data on PC Phenotypes

Prior to acquisition of the data on the distribution of PCs in the sample and their phenotypes in a flow cytometer, instrument settings should be placed in optimal conditions for measurement.

3.3.1. Instrument Setup

Appropriate instrument settings should be preferentially established according to the protocol proposed by the European Working Group in Clinical Cell Analysis (EWGCCA) (15); alternatively, the instrument setup protocol specifically recommended by the manufacturer may be used, either automatically, or manually if instrument setup software is not available. Typically, a threshold is set in forward scatter (FSC) at a relatively low channel value. A four-decade logarithmic amplification with a minimum 256-channel resolution is required for appropriate detection of immunofluorescence signals in conventional benchtop flow cytometers. Linear amplification of the forward and 90° light scatter amplifiers with the voltage of detectors set to provide the best discrimination between debris/platelets and different populations of nucleated cells is preferentially used. Although in most currently available benchtop flow cytometers, fluorescence compensation is done electronically after the voltages of the photomultiplier tubes have been set and prior to data acquisition, it can also be performed during or after data acquisition by software. In such cases, appropriate uncompensated files containing a set of single-antigen-stained samples with all fluorochromes/fluorochrome tandems used should be acquired with identical instrument settings to those used to measure the test sample (16). Once established, instrument settings may be optimized by running unstained or stained PCs as described in the EWGCCA protocol (15).

3.3.2. Acquisition of Data

All commercially available flow cytometer instruments are equipped with a proprietary software for data acquisition. Two-dimensional dot plots should be generated in which light scatter parameters (FSC and side scatter [SSC]) are correlated between them and with each fluorescence emission. Additional bivariate plots of all possible combinations of fluorescence emission should be

created. After appropriately labeling the files to be stored, the data acquisition protocol should be established. Because of the limited capabilities of most software programs regarding the maximum number of events that they may handle for one file and the relatively low frequency of PCs in many samples, in these latter samples, it is recommended that data acquisition be performed in two consecutive steps for each combination of MABs.

In the first step, fluorescence emissions and light scatter signals from 20 to 30×10^3 nucleated cells corresponding to the whole sample cellularity are acquired and stored in a list mode file without any selection of regions or gates apart from the threshold set in FSC. Acquisition of data on the normal cellular components of the sample together with those of the myelomatous PCs is necessary because it provides ideal internal negative and positive controls and will allow subsequent enumeration of the percentage of myelomatous PCs present in the sample (**Fig. 2**). After storing the first data file, broad electronic regions are set in an FSC vs SSC dot plot (R1 in **Fig. 2**) to exclude cell debris and in a CD38 vs CD138 dot plot (R2 in **Fig. 2**); in R1 all cells present in the sample are included; R1 plus R2 is enriched in PCs and includes all PCs. An alternative region to R1 (R3 in **Fig. 2**) may be drawn on FSC vs SSC to better define the population of PCs, especially when PCs show abnormally low CD38 and CD138 expression.

If information on <10,000 PCs of interest was acquired in the first step, as occurs for studies of minimal residual disease (MRD), a second acquisition step should be performed by activating an electronic live gate (R4 = R2 plus R3), with information specifically stored for the PCs present in a total of up to 10^6 nucleated cells (*see Note 9*).

3.4. Analysis of Data

Analysis of flow cytometry data stored in list mode files (FSC format) is performed using dedicated software programs. During analysis of data, the following steps are typically performed for the study of each of the populations of cells of interest: (1) identification, (2) enumeration, and (3) phenotypic characterization of the populations of PCs of interest; information on the intensity and homogeneity/heterogeneity of both antigen expression and light scatter features of each population of PCs in comparison with the pattern of expression observed for the same antigens in the other cells present in the sample should be recorded. If a combination of CD19, CD38, CD56, and CD45 has been employed to stain the sample, the following protocol can be used for data analysis:

1. Use the FSC/SSC dot plot to exclude dead cells and debris.
2. In an SSC/CD38 bivariate dot plot, gate on CD38 strong-positive cells.

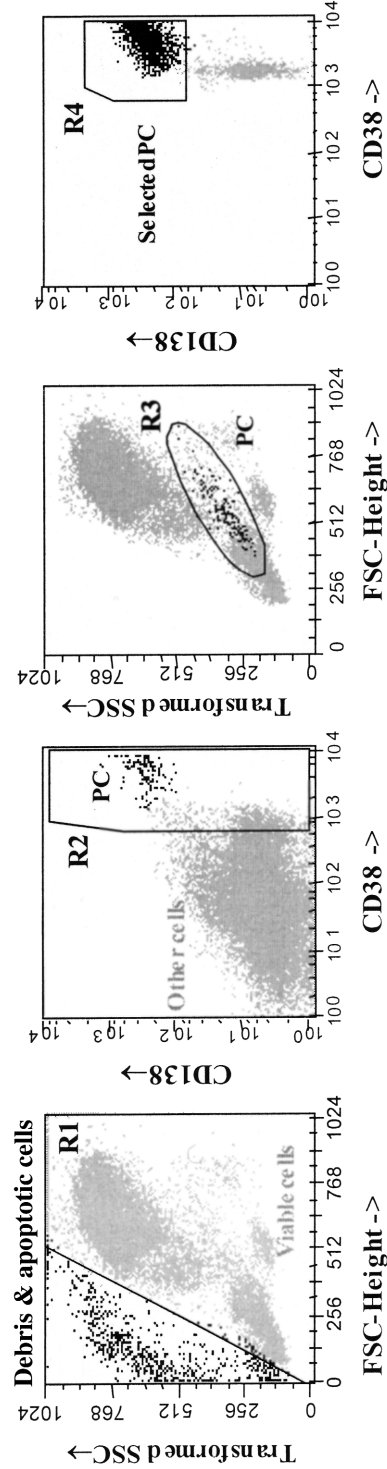


Fig. 2. Representative bivariate dot plots showing appropriate gate regions for correct acquisition of PCs. Based on the light scatter properties, it is possible to exclude debris and apoptotic cells (R1) while including PCs in a well-defined area of size (FSC) and internal complexity (SSC) (R3). The coexpression of CD38 and CD138 is used to specifically identify the PCs and distinguish them from other cells in the same sample (R2); such an analytical strategy can be further used to specifically acquire information on a higher number of PCs in a second-step acquisition through an electronic live gate as shown in the dot plot on the right (R4).

3. Redefine the gated CD38 strong-positive PCs in an FSC/SSC bivariate dot plot as a homogeneous population of cells in SCC with a relatively heterogeneous FSC distribution.
4. Report the percentage of PCs as defined in **step 3**.
5. Report on the mean and coefficient of variation (CV) of PCs for CD38, FL2 (orange), FL3 (red), and FL4 (deep-red) associated-fluorescence emissions.
6. Report on the immunophenotypic characteristics of the CD38 strong-positive PCs:
 - a. Define the presence of normal PCs as low SSC/low FSC/CD38^{high} and CD56⁻ and most expressing CD19 and CD45.
 - b. Define the phenotypic characteristics of myelomatous PCs and report on its percentage from the total CD38^{high}CD138⁺ PCs and both the mean and CV of the fluorescence emissions associated with the CD38, CD19, CD56, and CD45 markers.
7. Report on the percentage of events being the PC population of interest from the total number of events acquired, after excluding those corresponding to debris/platelets.

3.5. Interpretation of Results

Typically in MM, most of the myelomatous PCs accumulate in the bone marrow. In the marrow of patients with MM, normal PCs are either undetectable or outnumbered by the myelomatous PC compartment. Compared with normal PCs, myelomatous PCs frequently show decreased expression of CD38, strong expression of CD56, and negativity for both CD19 and CD45 (**Fig. 3**). In addition, other markers that are negative (CD117, CD20) or only dimly expressed in a small proportion of all normal PCs (CD28 and CD33) might also be strongly expressed by myelomatous PCs in up to 25% of all cases. In other monoclonal gammopathies such as monoclonal gammopathies of undetermined significance (MGUS), clonal PCs predominantly infiltrate the bone marrow as well, and they display a similar phenotype to that of MM; however, these clonal PCs from MGUS coexist in the bone marrow with the normal PCs at frequencies constantly lower than 97% of all PCs (**4**). In contrast to MGUS and MM, in primary PC leukemias and primary plasmacytomas, pathological PCs infiltrate other tissues with variable levels of bone marrow involvement. Interestingly, in the two latter conditions, PCs more frequently display a CD56⁽⁻⁾ and CD45⁽⁺⁾ phenotype (**13,17**). Actually, in other more immature disease conditions in which clonal PCs are also detected (e.g., Waldenström's macroglobulinemia [**18**]), clonal PCs show an aberrant, but clearly different, immunophenotype: CD56⁽⁻⁾, CD19⁽⁺⁾, CD20⁽⁺⁾, CD22^(-/ +), CD45⁽⁺⁾, and sIg⁺ (typically with κ light chain restriction).

Once clearly identified, further characterization of antigen expression on clonal/myelomatous PCs should be performed. For each antigen analyzed, the following may be of utility: (1) the presence or absence of antigenic expression,

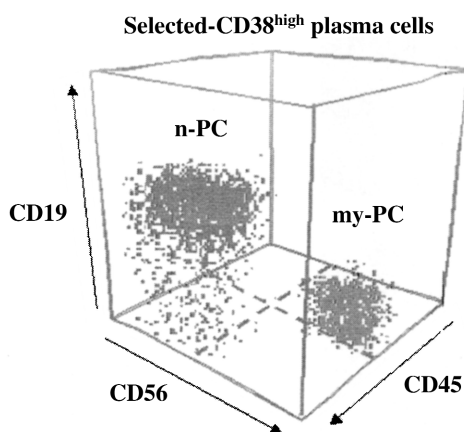


Fig. 3. Representative trivariate dot plot showing antigenic differences between myelomatous PCs (my-PC) and normal PCs (n-PC) among all CD38^{high} cells selected from a bone marrow sample. The combination of the four markers shown—CD19, CD45, CD56, and CD38—for selective gating of PCs represents the best combination of markers for a clear distinction between my-PC and n-PC in most patients with monoclonal gammopathies.

(2) the median amount of antigen expressed per cell within a given population of PCs as expressed by the median fluorescence intensity or other equivalent standardized units (molecules equivalent of soluble fluorochrome [MESF]; antibody binding capacity [ABC]), and (3) the pattern of antigen expression (homogeneous vs heterogeneous) as expressed by the CV for the reactivity observed for a particular antigen specifically on the population of PCs under analysis.

4. Notes

1. Bone marrow is the specimen most frequently used for immunophenotypic studies in myeloma because it usually contains the highest proportion of clonal PCs. However, there are specific clinical situations in which the investigation of other specimens, such as peripheral blood infiltrated with malignant PCs in advanced phases of the disease (in the so-called plasma cell leukemia) as well as solid tissues (e.g., plasmacytomas) or malignant effusions involved in extramedullary disease, are required. From all these different types of biological specimens, cells of interest can be directly studied in a sample aliquot or submitted to subsequent procedures to be further used with the patient's informed consent in basic research or in routine clinical practice. Among others these may include the following:
 - a. Isolation/purification of PCs: Highly enriched PC fractions are typically obtained using magnetic activated cell sorting (MACS) procedures after specifically labeling PCs with PC-specific antibodies (e.g., CD138) coupled to

magnetic particles of different sizes; or single-cell-based fluorescence activated cell sorting (FACS), which currently allows simultaneous isolation of up to four different populations of PCs that might coexist in a sample. An increasingly high number of alternative cell-sorting procedures are currently available and have been reviewed in detail elsewhere (19).

- b. **Immortalization:** The generation of cell lines derived from human myelomatous PCs through different procedures, including Epstein-Barr virus infection, can be done. These procedures have been detailed elsewhere (20).
2. **Specimen collection procedures:** Bone marrow and peripheral blood specimens are obtained by conventional aspiration or venipuncture procedures, respectively; once obtained, they should be placed in a sterile tube containing a sufficient amount of anticoagulant. Ideally, 1 to 2 mL is required for immunophenotyping, but it depends on the assay to be performed. Pleural effusions or ascitic fluid are obtained by conventional thoracic and abdominal puncture procedures. Cellular specimens derived from solid tissues may be collected by either fine-needle aspiration or surgical procedures; once obtained, these specimens should be immediately placed in a sterile isotonic saline solution.
3. **Anticoagulant used in collection:** EDTA is the preferred anticoagulant for immunophenotypic studies for all types of specimens because it preserves cell morphology and phenotype the best. However, in cases in which the specimen will also be used for additional functional studies (e.g., in vitro cell culture experiments), sodium heparin can be alternatively employed.
4. **Storage conditions and specimen integrity:** Freshly obtained peripheral blood, bone marrow, and other body fluid specimens should be preferentially stored at stable room temperature (18–22°C) for no longer than 24 h. Longer storage periods should be avoided in order to prevent significant changes in the surface expression of specific antigens and/or selective loss of cells (e.g., under inadequate conditions PCs may lose CD138 expression and rapidly enter apoptosis) (21,22). However, if required, storage for longer periods should be performed at lower temperatures (≈ 2 –8°C). In the case of samples aged longer than recommended or any specimens showing evidence of freezing, hemolysis, or clotting, cell viability should be assessed prior to phenotypic studies. The criteria for rejection of sample based on suboptimal conditions that may preclude successful analysis may vary, depending on the specific goals of the immunophenotypic studies. Stabilizing agents and solutions such as TransFix™ and Cytocheck (Cytochecks Laboratory) might allow prolonged storage of samples, but at present they have not been specifically and extensively tested for the preservation of PCs.
5. **Red cell lysing solutions that contain a fixative or one fixative free can be used.** The most frequently used solutions including those commercially available include 1X ammonium chloride, Optilyse and Versalyse (both from Immunotech, Marseille, France), FACSlysing (Becton/Dickinson, San José, CA), Quicklysis (Cytognos, Salamanca, Spain), Whole Blood Lysing Solution (Caltag, San Francisco, CA), and Uti-Lyse™ (DAKOCytomation, Glostrup, Denmark).

6. The following commercially available reagent kits containing both permeabilization and fixation reagents can be applied as reference reagents: Fix & Perm (Caltag), Intraprep (Immunotech), Intrastain (DAKOCytomation).
7. For instrument setup follow the protocol proposed by EWGCCA guidelines (15).
8. In the case of samples corresponding to single cell suspensions that do not contain nonnucleated red cells, the lysing steps can be detected from protocols I (steps 5–8) and II (steps 5–8 of protocol I in step 8 of protocol II).
9. In any case, for their phenotypic characterization, a minimum of 10^3 PCs should be acquired using a single- or double-step acquisition. For studies of MRD, at least 100 events corresponding to pathological PCs should be collected to allow their unequivocal identification and accurate enumeration (CV of <10%).

Acknowledgment

This work was supported in part by a grant from the Instituto de Salud Carlos III for the “Spanish Myeloma Network” (G03-136).

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Multiple Myeloma

Methods and Protocols

Brown, R.D.; Ho, P.J. (Eds.)

2005, XIV, 310 p. 70 illus., 1 illus. in color., Hardcover

ISBN: 978-1-58829-392-3

A product of Humana Press