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Immunophenotyping in multiple myeloma and related plasma cell disorders

Shaji Kumar, M.D, Teresa Kimlinger, B.A, and William Morice, M.D, Ph.D.

Divisions of Hematology and Hematopathology, Mayo Clinic, Rochester, MN 55905

SUMMARY

Plasma cell disorders form a spectrum ranging from the asymptomatic presence of small monoclonal populations of plasma cells to conditions like plasma cell leukemia and multiple myeloma, in which the bone marrow can be replaced by the accumulation of neoplastic plasma cells. Immunophenotyping has become an invaluable tool in the management of hematological malignancies and is increasingly finding a role in the diagnosis and monitoring of plasma cell disorders. Multiparameter flow cytometry has evolved considerably during the past decade with an increasing ability to screen large numbers of events and to detect multiple antigens at the same time. This, along with a better understanding of the phenotypic heterogeneity of the clonal plasma cells in different disorders, has made immunophenotyping an indispensable tool in the diagnosis, prognostic classification and management of plasma cell disorders. This book chapter addresses the approaches taken to evaluate monoclonal plasma cell disorders, and the different markers and techniques that are important for the study of these diseases.

INTRODUCTION

Clonal plasma cell disorders make up a wide spectrum of disorders, ranging from incidental findings in asymptomatic individuals to life threatening conditions, and can present with a myriad of clinical manifestations.^{1–4} Currently, we still depend primarily on morphological features and limited immunophenotypic studies for identification of the clonal plasma cells as well as the clinical manifestations for diagnosis and classification of plasma cell disorders. However, in the past decade there has been increasing appreciation of the genetic and phenotypic heterogeneities that underlie the plasma cell dyscrasias and the changes that parallel disease evolution. Increased use of multi-parameter flow cytometry and a broadening array of available reagents for surface and intracellular staining of specific antigens have enhanced our understanding of various aspects of these diseases. These improvements have led to a better insight into the disease biology and have enhanced our diagnostic and prognostic abilities. Moreover, the ability of flow cytometry to rapidly analyze a large number of cells provides the level of sensitivity of detection required for assessing disease response to treatment and for demonstrating minimal residual disease. This is a major advantage when compared to conventional methods of morphological assessment and immunohistochemistry. While flow cytometric methods have become part of the

Address correspondence to: Shaji Kumar, M.D, Division of Hematology, Mayo Clinic, 200 First St SW, Rochester, MN 55905. Phone 507-284-2017, Fax: 507-266-4972, kumar.shaji@mayo.edu.

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standard diagnostic approach in other hematological malignancies, such as the acute and chronic leukemias, consensus in regards to their routine use in plasma cell disorders is lacking. Much of this stems from the contradictory results seen in early studies and the lack of universally acceptable plasma cell specific markers. Some of the controversies in earlier studies most likely ensued from the wide spectrum of disease stages included in the studies, variability in the reagents used, technical differences both in terms of acquisition and gating strategies and inconsistencies in the appreciation of the high inherent autofluorescence of plasma cells.^{5,6} Despite these challenges, high-sensitivity flow cytometry is evolving into an integral part of the laboratory evaluation and management of plasma cell disorders and can play an important role in the (i) diagnosis and classification, (ii) prognostic stratification, (iii) monitoring of response to therapy and minimal residual disease, (iv) understanding of the biology of disease progression, (v) studying of the role of tumor microenvironment in plasma cell disorders and (vi) identification of potential therapeutic targets on the surface of malignant plasma cells.^{7,8}

Classification of clonal plasma cell disorders

Monoclonal gammopathies form a large spectrum of disorders ranging from the asymptomatic presence of small clonal plasma cell populations to the near replacement of bone marrow with malignant plasma cells.¹⁻⁴ The common denominator for these disorders is the presence of a monoclonal protein, which can be in the form of intact immunoglobulin, immunoglobulin fragments, or free immunoglobulin light chains, in either the serum or urine. This is typically accompanied by the accumulation of clonal plasma cells primarily in the bone marrow or as localized soft tissue deposits or plasmacytomas, with few circulating plasma cells, except in more advanced stages of the disease. A classification of clonal plasma cell and related disorders are given in Table 1.

Plasma cells are terminally differentiated, non-dividing, effector cells of the B-cell lineage.⁹⁻¹² They are the primary mediators of humoral immunity, secreting antigen specific immunoglobulins. As a consequence, abnormalities of these cells are responsible for a variety of autoimmune diseases in addition to plasma cell neoplasms. The development and function of plasma cells is tightly regulated. The precise etiological events that alter the normal development of B-cells into mature plasma cells, to date, are not well understood and are most likely multifactorial. The progenitor B cell undergoes an initial development process in the marrow that is antigen independent, gaining the ability to produce an intact IgM immunoglobulin; followed by migration to the spleen. A subsequent antigen dependent development phase is typically triggered by exposure to an antigen, which results in the formation of memory B cells and long lived plasma cells.⁹⁻¹² Plasma cells typically secrete an intact immunoglobulin that is made up of two identical light chains and two heavy chains. There are five major classes of heavy chains, designated mu, delta, gamma, alpha, and epsilon corresponding to the major classes of immunoglobulins; IgM, IgD, IgG, IgA, and IgE respectively. In each of the immunoglobulin molecules the heavy chains are bound to one of the two light chains (either Kappa or Lambda), but not to both.

Monoclonal gammopathy of undetermined significance (MGUS) represents the majority of patients with a diagnosis of monoclonal gammopathy and is found in approximately 1 to 2 percent of adults in studies from Sweden, the United States, France, and Japan.^{3,13-15} (Figure 1) In fact, its prevalence increases with age and nearly 6% of individuals over 70 years have MGUS in large epidemiological studies. It is characterized by a relatively small burden of clonal plasma cells (<10%) in the bone marrow, secretion of small amounts of monoclonal protein (< 3.0 gm/dl in the serum) with small amounts detectable in the urine, and lack of any symptoms related to the clonal process. A small fraction of these patients (typically 1% every year) will undergo disease progression into a symptomatic stage, which

usually presents as multiple myeloma characterized commonly by anemia, bone destruction, renal insufficiency or hypercalcemia. In contrast, patients with amyloidosis, which is much less common than myeloma, present with widespread organ deposition of light chain-derived amyloid fibrils. These patients typically have a lower burden of clonal plasma cells compared to myeloma, as is also the case in other related conditions such as light chain deposition disease or POEMS syndrome. Waldenström's macroglobulinemia is another related disorder, where the clonal population is made up of lymphoplasmacytoid cells with morphological characteristics of plasma cells and B-lymphocytes. Immunophenotypic evaluation of the malignant plasma cells is an invaluable tool in the diagnosis and classification of these disorders, their prognostic assessment, the serial monitoring of disease burden and its reduction as a reflection of response to ongoing therapy.

Sample preparation and analysis approaches

Sample preparation

Flow cytometric evaluation of plasma cells in whole blood/or bone marrow aspirates begins with either isotonic lysis of the erythrocytes or density gradient separation methods to isolate the mononuclear cells. Both methods involve the use of commercially available reagents, the former (isotonic lysis) is advantageous in that it involves minimal labor or reagent costs, however it does little for plasma cell enrichment as all mononuclear cells, including granulocytes, are isolated and stained. Density gradient cell isolation is typically performed using Ficoll Hypaque and provides for much greater plasma cell enrichment. This methodology also has drawbacks, however, as it is more time and labor intensive. Furthermore, Ficoll gradient separation accelerates the loss of antigens such as CD38 and CD138 from the plasma cell surface and, therefore, the cells must be quickly stained and analyzed when isolated by this method.

Plasma cell identification

Identification of the plasma cells has typically been based on the demonstration of high CD38 and CD138 expression, in conjunction with the scatter properties of the cell population.^{16,17} While CD38 expression is also seen in other cell populations, especially activated T cells, the bright expression by plasma cells sets it apart with reasonable specificity to suggest its routine use for identification of plasma cells.^{5,18} It is important to bear in mind, however, that in many abnormal plasma cell populations the surface density of CD38 is slightly decreased and, therefore, the abnormal plasma cells may have a staining intensity similar to that of normal B-cell precursors (hematogones) or activated T-cells. Therefore coincident analysis of CD138 or syndecan, another antigen characteristically expressed by plasma cells, greatly enhances the sensitivity of flow cytometry. Similar to CD38, however, heterogeneity of CD138 expression can be seen between plasma cell populations.^{17,19} Moreover, CD138 can be shed from the cell surface as a result of heparanase activity. The expression of CD45 and CD19 allows further refinement of the plasma cell identification process. In addition to CD38 and CD138, normal plasma cells are generally positive for these antigens whereas abnormal plasma cells characteristically lack CD19 and variably express CD45. In plasma cell dyscrasias, one typically finds a mix of CD45 positive and negative plasma cells and the proportions appear to change with disease evolution. Using 6-color flow cytometry, we were able to detect a distinct CD45 positive plasma cell fraction in the majority of bone marrow and nearly half of the peripheral blood specimens from patients with plasma cell disorders.²⁰ The clonal nature of these cells was confirmed by light chain restriction. Interestingly, the bone marrows contained less CD45 positive than CD45 negative plasma cells, while the proportions were generally equal in the peripheral bloods. Given the higher than previously reported presence of CD45 positive cells plasma cells, additional studies were done which ruled out antibody variations or the

adherence of T-cells as a potential explanation for the findings. Figure 1 shows the typical gating strategy for identification of plasma cells.

Use of controls

Although plasma cells can exhibit autofluorescence, immunoglobulin isotype controls are usually not required for accurate plasma cell analysis by flow cytometry. In all studies, it is useful to run positive and negative control cases to confirm the composition of the antibody cocktail used and to verify the instrument compensation settings. Also, for accurate analysis, cellular viability should be assessed with a vital exclusion dye, such as 7-AAD. In addition, DDM (doublet discrimination) may be important to use to control for cell aggregates in certain situations.

Sample acquisition and event numbers

The advent of advanced flow cytometry instrumentation and analysis software has allowed for the rapid collection of very high numbers of cellular events (>500,000). This is often required in the analysis of blood and marrow specimens potentially involved by a plasma cell neoplasm as the variation between assays can be considerable when the number of neoplastic plasma cells evaluated falls below 100. The European myeloma network recommends that at least 100 neoplastic plasma cell events be acquired for accurate enumeration. This will often require acquisition of a million or more total events in assays designed for minimal residual disease assessment to provide the adequate sensitivity.

Role of flow cytometry in the diagnosis and classification of plasma cell diseases

The diagnosis of plasma cell disorders requires confirmation of the clonal nature of the plasma cells, typically against the background of increased numbers of non-malignant plasma cells. While this is most commonly performed on a bone marrow aspirate specimen, there is increasing appreciation of circulating plasma cells in these disorders due to the increased sensitivity of flow cytometry techniques and their ability to screen large number of events in a rapid manner. Given that polyclonal expansion of plasma cells can be seen in several inflammatory and infectious conditions, the ability to demonstrate clonality of a plasma cell population is critical to making the diagnosis. The pattern of surface antigens on the clonal plasma cells can help classifying the disorders and is an important adjunct to other laboratory studies and clinical features.

The quantitation of plasma cells is routinely performed using a slide-based readout of the aspirate or biopsy sections, based on morphology with or without immunohistochemistry with antibodies to plasma cell associated antigens, such as CD138 or MUM-1. The proportion of plasma cells in the aspirate can also be assessed using flow cytometry, but results have consistently shown significantly lower plasma cell numbers from flow cytometry than from slide-based morphological evaluation.^{21–24} The reasons for this discrepancy are not entirely clear. Various mechanisms have been suggested, including the draw sequence (the aspirate used for flow studies coming after the aspirate for the slide-based studies and resultant hemodilution), adherence of plasma cells to bone spicules and hence loss during cell isolation, clustering of plasma cells, loss of surface expression of CD138 and CD38 as a result of cell processing prior to flow cytometry, or shedding of CD138 related to cellular viability among others.^{21–24} While the quantity of plasma cells may be underestimated by flow cytometry compared to morphological assessment, the increased sensitivity of multi-color flow cytometry allows the detection of very small numbers of plasma cells, which may be missed by morphological or immunohistochemical evaluation.^{20,25} Ideally, a minimum of 1×10^5 nucleated events should be screened to

adequately characterize plasma cells. Use of a two-step process, involving a live gate for the acquisition of plasma cell events, allows for increased sensitivity of the assay.^{16,26} The consensus recommendation from the European Myeloma Network implies the use of the first bone marrow aspirate for flow cytometry and the evaluation of at least 100 neoplastic plasma cells. More recently, we have developed a lyse, no-wash method, that involves minimal processing of the aspirate sample, which in combination with enumeration beads allows for the quantitation of absolute numbers of plasma cells/ μL . Approaches to distinguish abnormal from normal polyclonal plasma cells are discussed later.

The clonality of plasma cells can be easily confirmed based on the sole presence of either kappa or lambda cytoplasmic immunoglobulin light chains in all cells. This test for light chain restriction requires the use of antibodies to kappa or lambda light chains in fixed and permeabilized cells. Plasma cell populations can be selected based on surface antigen expression, prior to determining clonality. Figure 2 explains the determination of plasma cell clonality based on kappa or lambda light chain expression. In some settings, proof of light chain restriction can be difficult; for instance, when very few abnormal plasma cells are present, when the abnormal plasma cells contain very low levels of cytoplasmic light chains, or when the disorder is bi-clonal expressing both kappa and lambda light chains. Moreover, permeabilization of plasma cells can affect their light scatter properties and, in some instances, may impair the ability to assess the expression of cell surface antigens.

Differential expression of a wide variety of surface antigens helps distinguishing normal from malignant plasma cells and permits the recognition of plasma cells from different stages of disease evolution as well as from different types of plasma cell disorders.^{7,8} The surface antigens that have been studied in plasma cell disorders are described in Table 2. Flow cytometry allows the simultaneous assessment of multiple markers on small populations of plasma cells as well as determination of clonality.

Normal versus abnormal plasma cell

Non-neoplastic polyclonal plasma cells are normal components of a bone marrow aspirate and thus need to be distinguished from neoplastic, clonal plasma cells. While the majority of plasma cells in myeloma (> 95%) belong to the neoplastic population, the proportion of normal plasma cells is typically higher in MGUS and smoldering myeloma (SMM).²⁷ Several surface antigens have been suggested to characterize individual plasma cells as malignant or normal, but no single marker can make the distinction with any degree of specificity. This distinction is based on the differential expression of a panel of antigens (Table 3). Overall, compared to normal plasma cells, abnormal plasma cells tend to be low in the expression of CD19, and CD27, have weaker expression of CD38 and CD45, increased expression of CD28, CD33 and CD56 and variable expression in a small proportion of cells of CD20 and CD117.⁸

Plasma cell leukemia

Plasma cell leukemia (PCL) is characterized by circulating neoplastic plasma cells and may present de novo (primary PCL) or at the time of relapse of a previously diagnosed multiple myeloma (secondary PCL). The antigens commonly used for plasma cell identification, such as CD38 and CD138, are similarly expressed PCL and multiple myeloma.²⁸ However, PCL cells are more likely to express CD20 and usually tend to be negative for CD56, CD117 and HLA-DR.²⁹ Others have shown that CD28 and CD27 expression can be useful in distinguishing primary PCL from secondary PCL.^{30,31}

Waldenström's macroglobulinemia

Waldenström's macroglobulinemia is typically associated with lymphoplasmacytoid cells secreting an IgM monoclonal protein. The lymphoplasmacytic cells are typically CD19 and CD38 positive and variably express CD138. In a study of 35 lymphoplasmacytic lymphoma cases,³² both immunohistochemistry and flow cytometry were useful in identifying the cells with lymphoid and plasmacytic features. In 19 cases, immunohistochemistry revealed a pattern in which plasma cell infiltrates were physically separate from the lymphoid infiltrates. Surface immunoglobulin light-chain restricted, mature B-cells were identified by flow cytometry in 96% of the cases and in approximately half of these the cells expressed CD5 and/or CD23. Using highly sensitive 6-color flow cytometry, clonal plasma cells were seen in over 90% of patients; and in over half of these cases, the distinctive bright CD38 and CD138 co-expression was identical to that seen in true plasma cell neoplasms, such as multiple myeloma. In contrast to typical plasma cell neoplasms, however, the plasma cells in lymphoplasmacytic lymphoma were almost always CD19 and CD45 positive.

Amyloidosis

There is limited information available on characteristic features of plasma cells in light chain (AL) amyloidosis.^{33–35} In a small study of 36 cases with well documented AL amyloidosis, expression of CD20, CD79a, CD56, cyclin D1 and EMA was noted in 42%, 86%, 50%, 53% and 83% of cases, respectively.³⁴ Expression of CD56 and/or cyclin D1 was seen in 79% of cases. In 9/10 patients, small lymphoid-like plasma cells were positive for CD20 and all the 10 cases positive for cyclin D1. On the other hand, among cases without small lymphoid-like morphology, CD20 and cyclin D1 expression was seen in only a quarter of patients. More recently, expression of the low-affinity IgG Fc receptor, CD32B, was found to be elevated in plasma cells from patients with amyloidosis.³⁵ Gene expression profiling demonstrated significantly higher expression of CD32B compared to all other Fc receptor family members. Reverse-transcriptase polymerase chain reaction (RT-PCR) using enriched CD138 positive plasma cells showed uniform expression of the stable cell surface CD32B1 isoform at diagnosis and relapse, and flow cytometry showed intense CD32B cell surface staining on 99% of CD138 positive plasma cells at diagnosis and relapse.³⁵

Assessment of plasma cell proliferation, apoptotic rates and DNA ploidy status

The proliferative rate of malignant cells is a powerful prognostic factor in most malignancies, and this is also true for plasma cell disorders. Various methodologies have been proposed for determining proliferation, including immunohistochemistry for markers such as Ki67, labeling of the nucleus in proliferating cells with 5-bromo-2'-deoxyuridine (BrdU) or thymidine, or examination of the cells in S-phase using simple DNA stains.

Initial methods of labeling index determination in myeloma utilized the incorporation of radioactive thymidine by replicating DNA^{36,37}. The percentage of thymidine-labeled cells was then determined by autoradiography. Plasma cells in the S-phase of the cell cycle actively incorporate BrdU. The availability of antibodies to BrdU led to the development of a slide-based immunofluorescence method for identifying dividing plasma cells that are labeled with BrdU³⁶. This method allows for the morphological confirmation of plasma cells among all labeled cells, thereby improving specificity. By staining concurrently for cytoplasmic kappa or lambda immunoglobulin light chains, the percentage of dividing clonal plasma cells can be counted.

More recently, there has been increasing interest in using flow cytometry for the determination of proliferative rates. This approach involves cell fixation followed by

staining with a dye, which stoichiometrically binds to DNA, such as propidium iodide. Sophisticated software is available to determine the fraction of cells currently in S-phase of the cell cycle. In addition to being less cumbersome than slide-based techniques, flow cytometric measurements also allow for the evaluation of a much greater number of cells and the simultaneous determination of the plasma cell DNA ploidy, valuable prognostic information in patients with myeloma. Figure 3 provides an illustrative example for identifying plasma cells in S-phase in the bone marrow from a patient with multiple myeloma. There are many benefits to assessing the bone marrow plasma cell labeling index in the diagnosis and management of multiple myeloma^{38–41}. It discerns patients with stable disease not requiring treatment, including MGUS and SMM, from patients with active myeloma requiring treatment for their disease^{37,42}. It provides valuable prognostic information in patients with SMM, where a high labeling index suggests high risk of progressing to active myeloma in the short term. In patients with active multiple myeloma, a high labeling index at diagnosis portends a shorter overall survival^{43,44}.

In addition to the proliferative rate of plasma cells, the proportion of cells undergoing apoptosis also appears to have prognostic significance in myeloma and related disorders.⁴⁵ Cells in apoptosis are typically assessed using Annexin V and propidium iodide staining to determine the loss of membrane integrity. Phosphatidylserine, which in non-apoptotic cells is inside the membrane, will be exposed on the outer surface of apoptotic cells, where it can be labeled with Annexin V. The addition of propidium iodide allows for staining of DNA, which will further discriminate stages of apoptosis.

Prognostic value of immunophenotypic studies

It is clear from the discussion so far that the phenotypic features of plasma cells are quite heterogeneous, depending on the disease stage, diagnosis, a patient's other biologic characteristics, and the type of therapies employed. Multiple studies have demonstrated the prognostic value of specific patterns of antigen expression by neoplastic plasma cells. Furthermore, the expression of specific antigens has been shown to correlate with genetic abnormalities commonly seen in plasma cell disorders. While these associations are not specific enough to propose flow cytometry in lieu of conventional genetic analyses, such as metaphase cytogenetics or fluorescence in-situ hybridization (FISH), such relations explain the prognostic value of antigen expression profiles.

Significance of normal plasma cells

It has been shown that the proportion of normal plasma cells in patients with plasma cell disorders is a powerful prognostic factor in all stages of disease as well as following therapy.^{26,27} Patients with MGUS and SMM, who have >5% phenotypically normal plasma cells, have a better outcome with lower rates of disease progression to symptomatic myeloma.^{26,46} In a series of 594 newly diagnosed multiple myeloma patients, uniformly treated on clinical trials, those with more than 5% normal plasma cells in the marrow (14% of patients) had favorable baseline clinical characteristics and a significantly lower frequency of high-risk cytogenetic abnormalities.⁴⁷ These patients had a better response to therapy and longer progression-free and overall survival compared to the rest.

Circulating Plasma Cells

Clonal plasma cells and B-lymphocytes can be detected in the peripheral blood of patients with plasma cell disorders by immunofluorescence microscopy and flow cytometry.^{48,49} The number of circulating plasma cells has been found to be a measure of disease activity in myeloma³⁸ and an important, independent prognostic factor for survival in myeloma,

SMM^{50,51} and primary amyloidosis⁵². Among patients with MGUS, presence of circulating plasma cells predicted for higher risk of progression to symptomatic myeloma.⁵³

CD45

Expression of CD45 on plasma cells in myeloma has been shown to be of prognostic significance. In normal hematopoiesis, CD45 is predominantly expressed in the early stages of plasma cell development and decreasing in expression with maturation; clonal plasma cells in myeloma show a heterogeneous expression pattern.^{54,55} Studies suggest the presence of two populations of myeloma cells in the marrow; early plasma cells with CD45 expression and late plasma cells with dim or no expression.^{54–56} Important biological differences have been demonstrated between these two populations, including their proliferative rates, IL-6 responsiveness and dependence, chromosomal abnormalities and angiogenic capability. In plasma cell disorders, the ratio of CD45-positive to CD45-negative plasma cells appears to be altered in relation to the disease stage. Moreau et al in a study of 95 patients with myeloma undergoing high dose therapy demonstrated a shorter survival for patients lacking CD45 expression on the plasma cells⁵⁷. The early stages of plasma cell proliferative diseases, namely MGUS and SMM, are characterized by a nearly equal distribution of CD45 positive and negative plasma cells, whereas in newly diagnosed and relapsed myeloma the CD45 positive population dominates. The CD45-positive fraction appears to be more responsive to IL-6 stimulation^{58,59} and contains the myeloma cells with higher proliferative activity⁶⁰. The interaction between CD45, which is a protein tyrosine phosphatase, and the src kinases (lyn, hck, lyk) may mediate the effect of CD45 on IL-6 signaling⁵⁹.

CD56

Expression of CD56 (NCAM-Neural cell adhesion molecule) is commonly used to identify abnormal plasma cells, since normal plasma cells are typically CD56 negative.^{61,62} However, heterogeneity exists among clonal plasma cells with respect to CD56 expression, and this may have prognostic implications.⁶³ CD56 expression has been associated with poor outcome in myeloma patients treated with conventional therapies while no such effect was seen in another group of patients undergoing autologous stem cell transplantation.^{64,65} In one study, CD56 negative patients had higher beta-2 microglobulin levels, a higher incidence of extramedullary disease, Bence Jones protein, renal insufficiency, and thrombocytopenia and were more likely to have a plasmablastic morphology compared to CD56 positive patients.⁶⁴ CD56 expression may correlate with the ability of cells to migrate out of the bone marrow and modulation of its expression is a potential mechanism of controlling plasma cell homing to extramedullary sites.^{66,67} In one study, the presence of circulating plasma cells was inversely correlated with CD56 expression.⁶⁸ Another study suggested that lack of CD56 expression is associated with lambda light chain expression.⁶⁹

CD20

CD20 is typically not expressed by normal plasma cells. However, several studies have suggested the presence of a small proportion of neoplastic plasma cells that express CD20 on their surface. In one study in patients with multiple myeloma, CD20 expression was associated with small mature plasma cell morphology and with t(11;14).⁷⁰ Although the presence of t(11;14) is associated with better outcome in patients with myeloma, the expression of CD20 does not appear to have any prognostic impact. Given the success of anti-CD20 directed monoclonal antibody therapy in lymphoma, trials have been carried out with Rituximab in myeloma, without any clear efficacy.

CD27

Expression of CD27 is uniformly seen in normal plasma cells as well as plasma cells in MGUS and its loss has been correlated with disease progression in patients with MGUS.⁷¹ Progressive loss of CD27 has also been observed with disease progression in myeloma, and lack of CD27 expression at diagnosis was associated with shorter overall survival.⁷² Gene expression data have demonstrated that the multiple myeloma group with the highest prevalence of poor prognostic factors had the lowest CD27 RNA levels.^{31,71}

CD28

CD28 is a major costimulatory molecule on T-cells and aberrantly expressed on neoplastic plasma cells in a fairly consistent manner.⁷³ Its expression level appears to increase with disease progression, with one study showing CD28 expression on plasma cells from 19% of MGUS and 41% of myeloma patients, and in 100% of human myeloma cell lines.⁷⁴ Among patients with myeloma, CD28 expression seems to increase with disease evolution in that CD28 positive plasma cells were detected in 26% of myeloma patients at diagnosis, in 59% at medullary relapse, in 93% at extramedullary relapse, and in 100% of patients presenting with secondary plasma cell leukemia. In individual patients, there was increasing expression of CD28 as the disease relapsed. CD28 in multiple myeloma can up-regulate the transcription of the interleukin-8 gene, a chemokine that promotes angiogenesis, thus potentially contributing to disease progression.⁷⁵ Direct activation of CD28 on myeloma cells by anti-CD28 monoclonal antibody leads to the suppression of plasma cell proliferation and protects the malignant cells from dexamethasone-induced cell death.⁷⁶

CD33

CD33 is a myeloid antigen that can be aberrantly expressed by plasma cells.^{16,62,77} In one study, CD33 positive patients had higher beta-2 microglobulin and lactate dehydrogenase levels and a higher incidence of anemia and thrombocytopenia than did CD33 negative patients.⁷⁸ The overall survival was significantly shorter in the CD33 positive group, especially due to early mortality from disease. Serial evaluation of CD33 expression showed that the amount of CD33 antigen on the surface of plasma cells significantly increased after therapy in individual patients, possibly suggesting its expression as a marker of drug resistance.

CD44

CD44 is an adhesion molecule that is encoded by a single gene but can be expressed as CD44 standard (CD44s) or variant forms (CD44v). While no significant differences have been observed in the expression of CD44s, the CD44v9 and v10 containing isoforms were differentially expressed on bone marrow plasma cells from normal individuals (predominantly CD44v9+v10+) versus myeloma patients with stable (predominantly CD44v9-v10+) or progressive (predominantly CD44v9+v10-) disease.⁷⁹ Some of the CD44 isoforms play a key role in myeloma cell migration, homing and adhesion to stromal cells.^{80,81} Expression of CD44v6 has been associated with disease progression, with very low expression found in patients with MGUS and Stage I myeloma, and with the presence of chromosome 13 deletions, a well-known risk factor in myeloma.⁸²

CD52

CD52, a pan-lymphoid antigen, is expressed by a proportion of plasma cells in various plasma cell disorders, including MGUS, myeloma and amyloidosis.^{17,83,84} In one study, 67%, 52%, and 35% of patients with MGUS, myeloma and amyloidosis, respectively, demonstrated CD52 positive plasma cells.⁸³ The CD52 expression was predominantly confined to the clonal CD38 and CD45 positive plasma cell fraction with a median of 68%,

88%, and 82% of positive cells in MGUS, myeloma, and amyloidosis, respectively, compared with 18%, 6%, and 9% of CD52 positive but CD45 negative plasma cells, respectively. However, use of CD52 as a therapeutic target in myeloma using monoclonal antibodies has not been successful.

CD117

CD117 (c-kit) is a hematopoietic growth factor receptor with tyrosine kinase activity that is aberrantly expressed by a proportion of neoplastic plasma cells.^{85,86} In one study, plasma cells from normal bone marrow samples did not show any reactivity for CD117, compared with 83 – 99% of CD117 positive plasma cells found in the marrows of one-third of myeloma patients. Expression of CD117 has been associated with a good prognosis in myeloma.^{86,87} Recent studies have suggested that CD117 is a valuable marker to distinguish abnormal, neoplastic from normal plasma cells.¹⁶

CD200

CD200 is a membrane glycoprotein that mediates suppression of T-cell-mediated immune responses. Patients with CD200 negative myeloma cells had an improved event-free survival compared with patients with CD200 positive myeloma cells, following high-dose therapy and stem cell transplantation. In a Cox proportional-hazard model, the prognostic value of CD200 expression for event free survival was independent of International Staging System (ISS) stage and beta-2 microglobulin levels.⁸⁸

Response assessment and monitoring of minimal residual disease (MRD)

Improvements in therapy are very dependent on the sensitivity of tests used for the detection of residual disease. MRD determination allows us to estimate the real impact of new drugs and treatment approaches. Chronic myelogenous leukemia (CML) offers the best example for this concept. When hematological response was considered as the endpoint for successful therapy, high response rates, albeit poorly sustained, were seen with interferon and hydroxyurea. With the advent of PCR and FISH testing for the BCR/ABL fusion, the detection of MRD in CML improved dramatically and provided an explanation for high relapse rates in these patients.

In the setting of multiple myeloma, we know that MRD is an important predictor of outcome in patients undergoing aggressive therapy^{26,89–93}. Rawstron et al used a sensitive flow cytometry assay that quantitated normal and neoplastic plasma cells in the bone marrow of 35 patients undergoing autologous stem cell transplantation (ASCT). These investigators identified a high-risk group of patients (66%) in whom at 3 months after transplantation the flow cytometric assay confirmed the presence of neoplastic plasma cells, whereas in the low-risk group, only normal phenotype plasma cells were present at that time-point. Low-risk patients experienced significantly longer progression-free and overall survival than the high-risk group. In this study, flow cytometry demonstrated increased sensitivity for MRD when compared with the detection of paraprotein by immunofixation, which is the conventional method for the monitoring of residual disease. Among patients with detectable malignant plasma cells by flow cytometry, approximately half became immunofixation negative; however, these patients had an outcome identical to patients who remained immunofixation positive⁹⁰. Bakkus et al examined the utility of detecting MRD using a quantitative allele-specific oligonucleotide PCR (ASO-qPCR) assay at 3–6 months post-transplant in 67 patients. Using specific cutoffs for the quantitative PCR results, the authors identified patients with residual disease and a short time to relapse⁹¹.

Lipinski et al retrospectively analyzed the tumor load in blood and bone marrow samples of 13 patients at the time of remission after ASCT and during progression using ASO-qPCR⁹².

Progression was detected earlier by this method compared to monoclonal protein estimation, demonstrating the increased sensitivity of the PCR technique. Galimberti et al examined the prognostic value of MRD in 20 patients after a tandem transplant, consisting of an autologous peripheral blood stem cell transplantation (PBSCT) followed by a non-myeloablative allogeneic peripheral blood stem cell infusion (NMT)⁹³. MRD levels were determined by PCR for immunoglobulin heavy chain rearrangements. After PBSCT only 3 patients (15%) became PCR-negative, versus 12 (60%) patients after NMT. Eradication of MRD favorably impacted on overall survival with 76% of MRD-negative patients still alive 20 months post NMT versus 34% of persistently PCR-positive cases. Although MRD evaluation by ASO-PCR is slightly more sensitive and specific than flow cytometry, it is applicable in a lower proportion of myeloma patients and is more time-consuming, while providing similar prognostic information.⁹⁴ In a recent study of 295 newly diagnosed myeloma patients who received uniform treatment including stem cell transplantation, a prospective analysis by multiparameter flow cytometry showed MRD to be one of the most important predictors of outcome.⁹⁵ MRD negativity on day 100 after ASCT translated into significantly improved progression-free and overall survival.

PRACTICE POINTS

1. Plasma cells can be separated from other white cells based on strong CD38 and CD138 expression.
2. The CD38/CD138 antigen combination can also be used for the gating of abnormal plasma cells by flow cytometry; once identified, the antigen profile of gated plasma cells allows for the distinction between normal and abnormal plasma cells.
3. The most important findings that characterize malignant versus normal plasma cells are: absent or low expression of CD27, CD19 and/or CD45, and increased expression of CD28, CD33, CD117 and/or CD56.
4. In all instances, monoclonality for intracytoplasmic immunoglobulin light chains is the ultimate proof for the presence of an abnormal plasma cell population.
5. Some subtypes of plasma cell dyscrasias show specific immunophenotypic characteristics, such as positivity for CD19 in Waldenstrom's macroglobulinemia, or expression of CD20 in plasma cell leukemia.
6. Flow cytometric MRD assessment in plasma cell disorders is easy, rapid, more sensitive and better correlated with outcome than conventional immunofixation of paraprotein.

RESEARCH AGENDA

1. Large phase III trials for the various subtypes of plasma cell disorders, which employ immunophenotyping prior to treatment, are needed in order to confirm correlations of specific antigen expression profiles with outcome.
2. To date, only few examples exist for associations of specific antigen expression with cytogenetic aberrations in plasma cell disorders, e.g., CD44v6 with chromosome 13 deletion; given the prognostic significance of cytogenetic abnormalities in plasma cell disorders, further such surrogate markers for genetic lesions should be identified.

3. Considering that we are in a period of new drug developments for the treatment of plasma cell disorders, flow cytometric measurements of MRD levels will be essential for the evaluation of drug efficacy and patient response.

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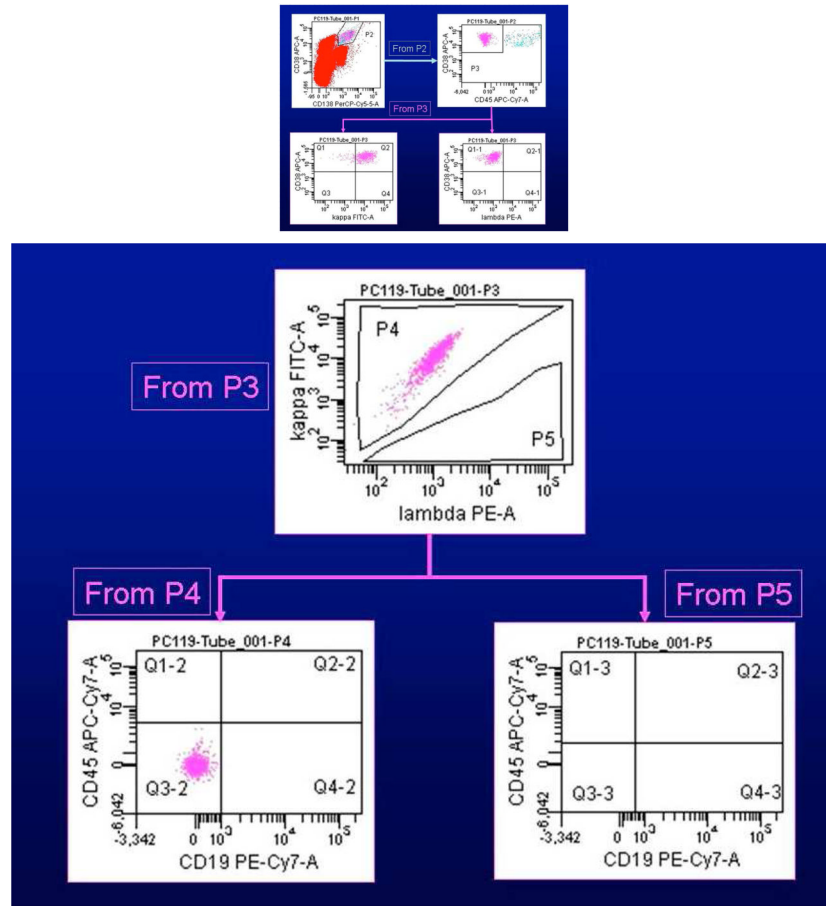
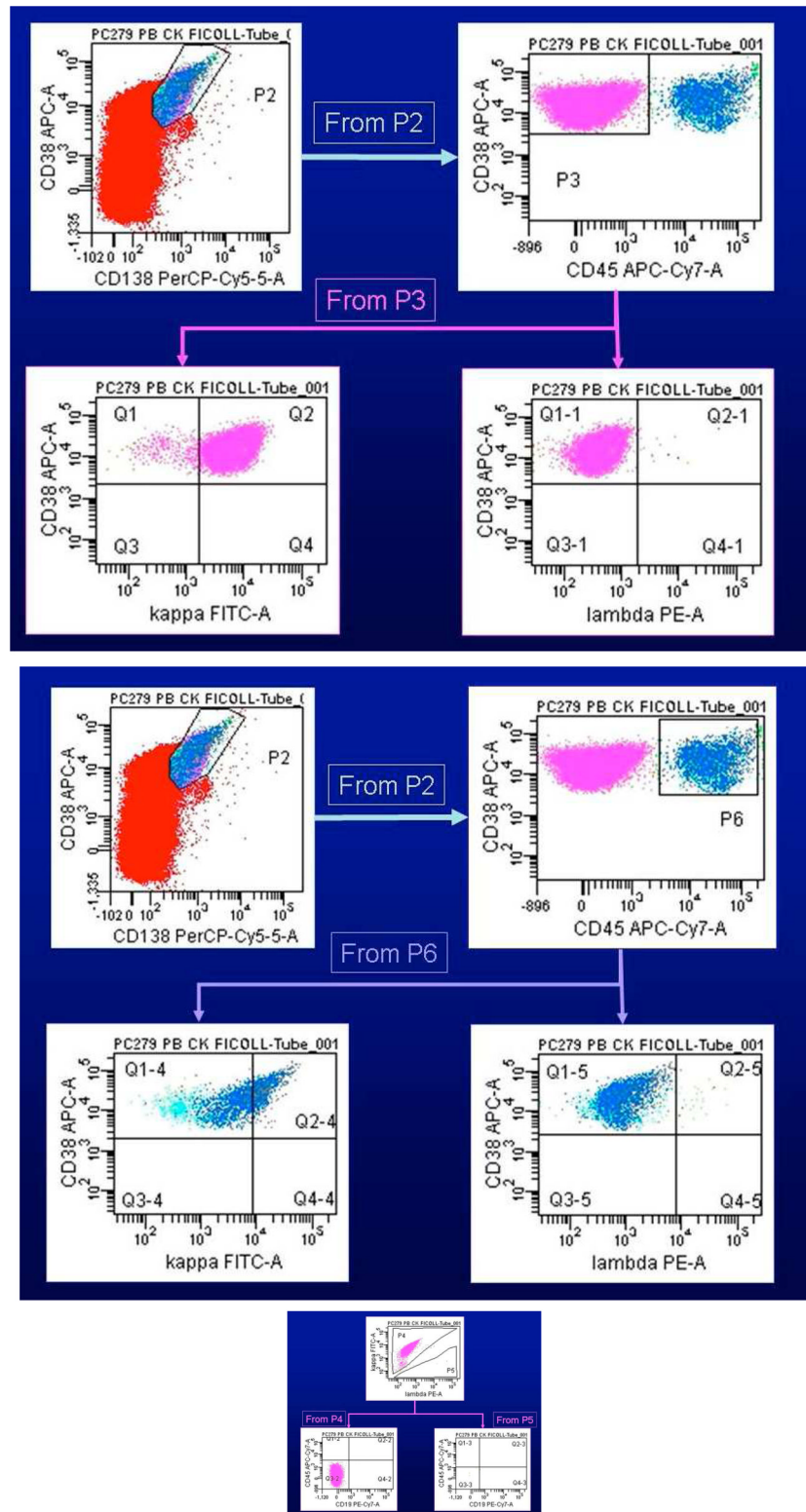


Figure 1.

A) Gating on CD38 and CD138 positive plasma cells (upper left dotplot, gate P2) reveals them to be CD45 negative (upper right dotplot). Selective gating on this CD45 negative plasma cell population (gate P3) reveals them to be monoclonal for cytoplasmic kappa immunoglobulin light chains (lower left dotplot) and to lack expression of lambda cytoplasmic immunoglobulin light chains (lower right dotplot). B) Selective analysis of the kappa light chain restricted plasma cells (gate P4) reveals them to lack expression of CD19 as well as CD45 (lower left dotplot). No lambda light chain positive plasma cells are present in gate P5 as the plasma cells are kappa light chain restricted (lower right dotplot).



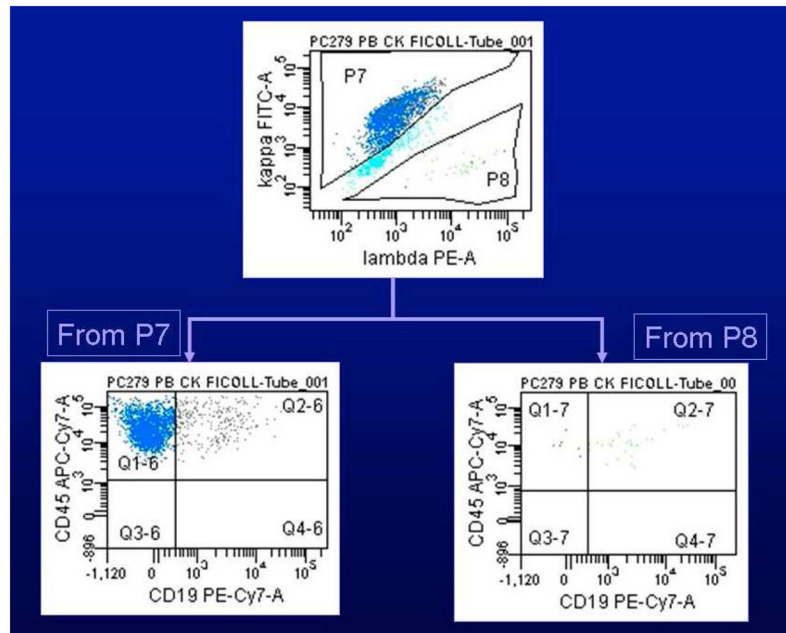


Figure 2.

Gating on CD38 and CD138 positive plasma cells reveals both CD45 negative (Figure 2A, gate P3) and CD45 positive (Figure 2B, gate P6) subsets, both of which are kappa cytoplasmic immunoglobulin light chain restricted (lower panels). Selective analysis of the light chain restricted plasma cells reveals both the CD45 negative and CD45 positive subsets to lack expression of CD19 (Figures 2C and 2D respectively).

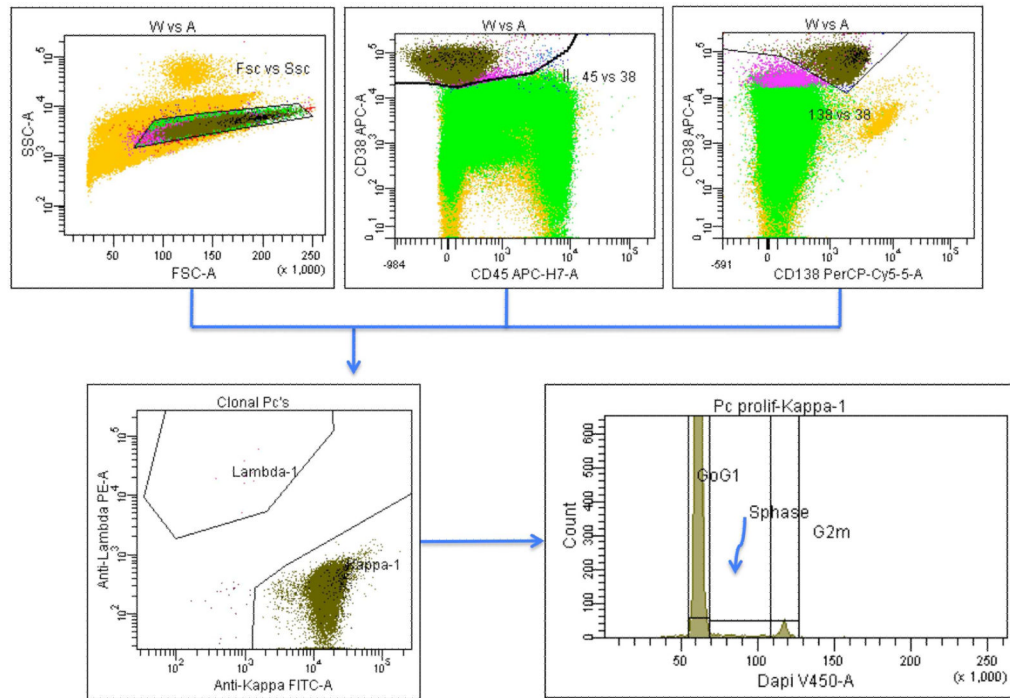


Figure 3. The plasma cell population is identified in 3 separate gating steps using forward scatter (FSC)/side scatter (SSC), the CD45/CD38, and the CD38/CD138 gate. Subsequently, clonality of the plasma cell population is identified by using antibodies to kappa and lambda. The left lower histogram shows binding of diamino-2-phenylindole (DAPI) to the DNA in kappa monoclonal plasma cells. Based on nuclear content, a significant fraction of plasma cells is in S-phase, reflective of proliferative activity.

Table 1

Differential diagnosis of monoclonal gammopathies

Diagnosis	Description
<i>Monoclonal gammopathy of undetermined significance (MGUS)</i>	<ul style="list-style-type: none"> Serum monoclonal protein < 3.0 g/dL, bone marrow plasma cells < 10% with minimal or no monoclonal urinary protein and no evidence of end organ damage Always precedes symptomatic myeloma, usually progresses at a rate of 1% per year, which is constant
<i>Smoldering multiple myeloma (SMM)</i>	<ul style="list-style-type: none"> Serum monoclonal protein \geq 3.0 g/dL, or bone marrow plasma cells \geq 10%, asymptomatic with no evidence of end organ damage This intermediate stage may not be recognized in all patients, progression rate is 10% per year during the first five years after diagnosis
<i>Symptomatic multiple myeloma</i>	<ul style="list-style-type: none"> Plasma cell proliferation in the presence of end organ damage; typical symptoms/findings include hypercalcemia, renal insufficiency, anemia and bone lesions (CRAB features)
<i>Solitary Plasmacytoma</i>	<ul style="list-style-type: none"> Presence of a plasmacytoma (homogenous plasma cell collection) in the absence of multiple osteolytic lesions, no evidence of monoclonal plasma cells in bone marrow, or other features of myeloma. Half of the patients will eventually develop myeloma, especially patients with persistent monoclonal protein after treatment
<i>Primary Systemic Amyloidosis</i>	<ul style="list-style-type: none"> Tissue deposition of light chain derived amyloid fibrils (beta pleated sheets), apple green birefringence on polarizing microscopy May present as cardiomyopathy, nephrotic syndrome, malabsorption, hepatic failure, peripheral neuropathy or other symptoms based on organ involvement Typically, lytic lesions and hypercalcemia not seen, associated with low level of marrow plasmacytosis
<i>POEMS syndrome (polyneuropathy, organomegaly, endocrinopathy, monoclonal protein, skin changes)</i>	<ul style="list-style-type: none"> <u>Major criteria:</u> polyneuropathy, presence of monoclonal plasma cells <u>Minor criteria:</u> sclerotic bone lesions, Castleman's disease, organomegaly (spleen, liver, or lymph nodes), Volume overload (peripheral edema, pleural effusion, ascites), endocrinopathy (adrenal, thyroid, pituitary, gonadal, parathyroid, pancreatic), skin changes (hyperpigmentation, hypertrichosis, hemangiomas, white nails), papilledema Two major criteria plus at least one minor criterion needed for diagnosis
<i>Waldenström's Macroglobulinemia</i>	<ul style="list-style-type: none"> IgM monoclonal gammopathy \geq 10% bone marrow infiltration by lymphoplasmacytic cells, often accompanied by lymphadenopathy and or hepatosplenomegaly Typical immunophenotype (surface IgM+, CD5+/-, CD10-, CD19+, CD20+, CD22+, CD23-)

Table 2

Surface markers in the assessment of plasma cell disorders

<i>Lymphoid markers</i>
CD10
CD19
CD20
CD22
CD27
CD28
CD52
<i>Myeloid markers</i>
CD33
CD117
<i>Other hematopoietic cell markers</i>
CD38
CD45
<i>Adhesion molecules</i>
CD11
CD44
CD49d
CD49e
CD56
CD138
<i>Other aberrantly expressed markers</i>
CD32
CD146
CD200
CD221
CD307

Table 3

Comparison of immunophenotypic features between normal and abnormal plasma cells

	Normal Plasma Cell	Abnormal Plasma Cell	Comments
CD19	Positive (>70%)	Negative (95%)	Present on all B-cells and most normal plasma cells. Clonal plasma cells have dim or no expression.
CD20 ⁷⁰	Negative	Positive (30%)	Typically seen during the maturation process of B-cells and absent from plasma cells. CD20 expression is seen on a subset of clonal plasma cells.
CD27 ^{32,71,72}	Positive (100%)	Negative/weak (15–45%)	This molecule is involved in the differentiation of mature B cells into plasma cells. Weakly or not expressed in myeloma.
CD28 ^{73,74}	Negative (<15%)	Positive (45%)	CD28 is involved in T-cell activation. Expression on plasma cells in myeloma correlates with aggressive disease.
CD33 ^{77,78}	Negative	Positive in a small subset of patients	This myeloid-lineage affiliated antigen may be present aberrantly in a subset of myeloma patients.
CD56 ^{61,96}	Negative (<15%)	Positive (75%)	The neural-cell adhesion molecule, typically present on NK-cells. Almost invariably present on neoplastic plasma cells, but expression may be low or lost in cells in circulation and in extramedullary disease.
CD81	Positive (100%)	Negative/weak	Member of CD19/CD21/Leu-13 signal transduction complex. Functions of this and other members of the tetraspan family are as yet poorly understood.
CD117 ^{28,97}	Negative	Positive	Typically seen on progenitors of myeloid and megakaryocytic lineage. Adapted from: Paiva et al, Clinical Cytometry, 12 Feb 2010

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