

Flow Cytometric Immunophenotypic Analysis of 306 Cases of Multiple Myeloma

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Abstract

Bone marrow aspirates from 306 patients with multiple myeloma were analyzed by flow cytometric immunophenotyping. The plasma cells (PCs) were identified by their characteristic light scatter distribution and reactivity patterns to CD138, CD38, and CD45. Monoclonality was confirmed by immunoglobulin light chain analysis. The immunophenotypic profile of the PCs was determined with a panel of antibodies. Moderate to bright expression of CD56, CD117, CD20, CD45, and CD52 was detected in 71.7%, 17.8%, 9.3%, 8.8%, and 5.2% of cases, respectively. These antigens were expressed by a distinct subpopulation of the PCs in 6.3%, 2.2%, 3.7%, 2.9%, and 2.6% of additional cases. CD19 was negative in more than 99% of cases. The combination of CD38 and CD138 was superior to CD38 alone for identifying CD45+ myeloma and separating CD20+ myeloma from B-cell lymphoma. PC immunophenotyping might be useful for detecting minimal residual disease in cases with aberrant antigen expression and for selection of therapeutic agents that have specific membrane targets.

Recent advances in flow cytometry (FC) have permitted more specific and sensitive evaluations of plasma cell (PC) populations. Neoplastic PCs traditionally have been identified by their CD38+++CD45-/dim staining pattern on FC histograms.^{1,2} With the use of multiparameter FC and the introduction of the monoclonal antibody CD138 (syndecan-1), it is clear that CD38+++CD45-/dim gating alone might fail to identify myeloma composed largely or partly of CD45+ PCs.^{3,4} CD38 is a nonspecific marker that can be detected on hematopoietic stem cells and T and B cells. Neoplastic PCs typically express CD38 at a lower intensity than normal PCs and might be indistinguishable from contaminating T or B cells.^{5,6} Thus, PC immunophenotyping is best determined by multiparameter FC using at least a 3-color assay that includes CD138 (syndecan-1) in the analysis. Syndecan-1 is a transmembrane heparan sulfate proteoglycan that typically is expressed by PCs and not by T or B cells.⁷ Therefore, syndecan-1 is considered the most specific marker for PCs. The commercially available monoclonal antibody B-B4 recognizes an epitope of the syndecan-1 molecule and can be used in FC or immunohistochemical analysis.⁸ CD138 is reported to be detectable in 60% to 100% of myeloma cases and in 70% to 100% of neoplastic cells in each case.^{7,9,10} Causes for the wide variation in detection sensitivity are unclear and might be related to technical or biologic factors.^{8,11}

Aberrant immunophenotypes are observed in a majority (87%) of myeloma cases at diagnosis including overexpression of CD56 (62%-75%) and aberrant expression of CD117 (28%) and CD20 (10%).^{3,5,12,13} While expression of CD56 distinguishes malignant from benign PCs, myeloma without CD56 expression might be associated with more aggressive disease and extramedullary dissemination.¹⁴⁻¹⁶ The purposes

of the present study were to examine antigens on myeloma cells that are potential targets of immunotherapy and to determine the incidence of aberrant markers that potentially could be used in the detection of minimal residual disease in a large series of myeloma cases. Bone marrow samples from 306 patients with myeloma were analyzed with a panel of monoclonal antibodies to determine the frequency and intensity of antigen expression. Three-color multiparameter FC with a 2-step acquisition procedure using a CD138, CD38, and CD45 live gate was used. CD117, CD52, CD20, and CD19 were evaluated to identify patients who might benefit from therapy with imatinib mesylate, alemtuzumab, rituximab, and anti-CD19 immunotoxin, respectively. Differences in antigen expression of myeloma cells between previously treated and untreated patients also were evaluated.

Materials and Methods

Patient Selection

The study patients were selected from consecutive patients with myeloma referred to the Myeloma Institute for Research and Therapy, University of Arkansas for Medical Sciences, Little Rock, between January 2001 and March 2002. The diagnosis of myeloma was established in each patient according to published criteria and staged according to the Salmon and Durie criteria.¹⁷ Cases in which the patient had a monoclonal IgM protein shown on serum protein electrophoresis or immunofixation or those with too few PCs in the aspirate owing to a dry tap were excluded. PC monoclonality was first confirmed by cytoplasmic immunoglobulin light chain analysis vs DNA ploidy.¹⁸ Only rare contaminating normal PCs (<1%) were found. Corresponding clinical information was obtained from the Myeloma Institute for Research and Therapy patient database.

FC Immunophenotyping

Whole bone marrow collected in EDTA anticoagulant was washed in phosphate-buffered saline and resuspended in phosphate-buffered saline with 2% fetal calf serum. Cells (1.25×10^4 in 50 μ L) were incubated with each of the following monoclonal antibodies for 20 minutes at room temperature in the dark: CD138 phycoerythrin (PE), CD138 phycoerythrin–cyanin 5.1 (PC5), and CD38 PC5 (Immunotech, Marseille, France); CD138 fluorescein isothiocyanate (FITC) and CD52 FITC (Serotec, Raleigh, NC); CD138 peridinin chlorophyll protein (PerCP), CD45 FITC, CD20 FITC, CD38 PE, CD117 PE, CD56 PE, and CD19 FITC (Becton Dickinson Immunocytometry Systems, San Jose, CA); immunoglobulin κ and λ light chains (DAKO, Carpinteria, CA).

The overall sensitivity of the assay was determined to be approximately 1/10,000 cells. All antibodies were titered specifically using PCs to obtain antibody concentrations for optimum peak fluorescence and appropriate compensation for 3-color analysis. After lysing with ammonium chloride, the cells were washed and fixed with 1% methanol-free formaldehyde. In 20 consecutive cases, FACS Lyse (Becton Dickinson) and ammonium chloride were tested in parallel for comparison. PCs were first identified by live gating using CD138, CD38, and CD45 and then analyzed for additional antigen expression using CD138 in combination with the other antibodies. The number of events acquired ranged from 10×10^3 to 100×10^3 to yield a minimum of 100 PCs in the analyzed region. Analyses were performed on a FACScan (Becton Dickinson, Mountain View, CA) using Cell-Quest software (Becton Dickinson).

Data Analysis

An antigen was considered positive when detected on more than 20% of the PCs relative to the isotype control. Intensity of expression was compared with the appropriate control and graded as follows: 1+, weakly positive population that overlaps the control; 2+, positive population that is either distinct from or at least 1 log brighter than the control; 3+, positive population that is at least 2 logs brighter than the control. In cases with a wide range of expression intensity, the mean fluorescent intensity for the population was recorded. If a distinct subpopulation of PCs equal to or greater than 20% of the gated population was identified at 2+ or greater intensity, this also was recorded and considered partial expression. The signal for the expression of CD117 antigen was weaker than other antigens, and the measuring scale for CD117 was adjusted to reflect this feature.

Results

Clinical Findings

The study population consisted of 123 women and 183 men with a median age of 61 years (range, 32–81 years). The Salmon–Durie stage was I in 24 cases, II in 46 cases, and III in 236 cases. Of 306 patients, 244 (79.7%) had received previous treatment. The chemotherapy regimens included VAD (vincristine, doxorubicin [Adriamycin], and dexamethasone), MP (melphalan and prednisone), or glucocorticosteroids only.

Immunophenotypic Findings

Analyses were performed on gated cells containing more than 99% monoclonal PCs as assessed by dual analysis of cytoplasmic immunoglobulin light chain expression and

DNA ploidy. Intensity of antibody fluorescence was moderate to strong against some antigens but weak to moderate in others (Table 1). Some antigens were expressed by the large majority of PCs, while others showed a different pattern of expression in a distinct subpopulation.

CD138 and CD38 primarily showed the first pattern of antigen expression, with strong staining clearly separating the majority of PCs from the other cellular components (Image 1). Despite strong staining in the majority of PCs, a fraction of less intensely staining PCs was identified in occasional cases that gradually merged with other hematopoietic elements. This was observed less frequently for CD138 than for CD38. The 3-color combination of CD38, CD138, and CD45 facilitated identification of PCs with moderate CD38 expression following adjustment of the gate to include all CD138+ cells, regardless of CD45 expression (Image 1). When CD138 PC5 became available and was substituted for CD138 FITC or CD138 PerCP later in the study, its brighter signal conferred a better signal-to-noise ratio and permitted better separation of the PC population from other cell populations. Only rare cases had weaker CD138+ cells using this fluorochrome.

In comparison with ammonium chloride, FACS Lyse enhanced the signal-to-noise ratio for CD138 antigen but resulted in a loss of CD138+ cells in 8 of 20 samples. FACS Lyse yielded better cell recovery with regard to CD138 staining in 1 of 20 samples. Differences between the 2 methods were not discernible in the remaining 11 samples, which all had a large number of PCs (Image 2).

CD45 and CD20 were expressed in a small subset of myeloma cases; both were found frequently in distinct subpopulations of cells. CD45 was expressed in 54 (17.6%) of 306 cases, of which 9 (17%) of 54 showed partial expressions and 27 (50%) demonstrated moderate to strong expression. CD20 was expressed in 47 (15.6%) of 301 cases, of which 11 (23%) of 47 showed partial expression and 28 (60%) demonstrated moderate to strong expression (Image 3). CD45+ neoplastic PCs differed from normal PCs by the absence of CD19 coexpression and the presence of cytoplasmic light chain restriction. Coexpression of CD19 and CD20 was not found in any cases, thereby excluding the

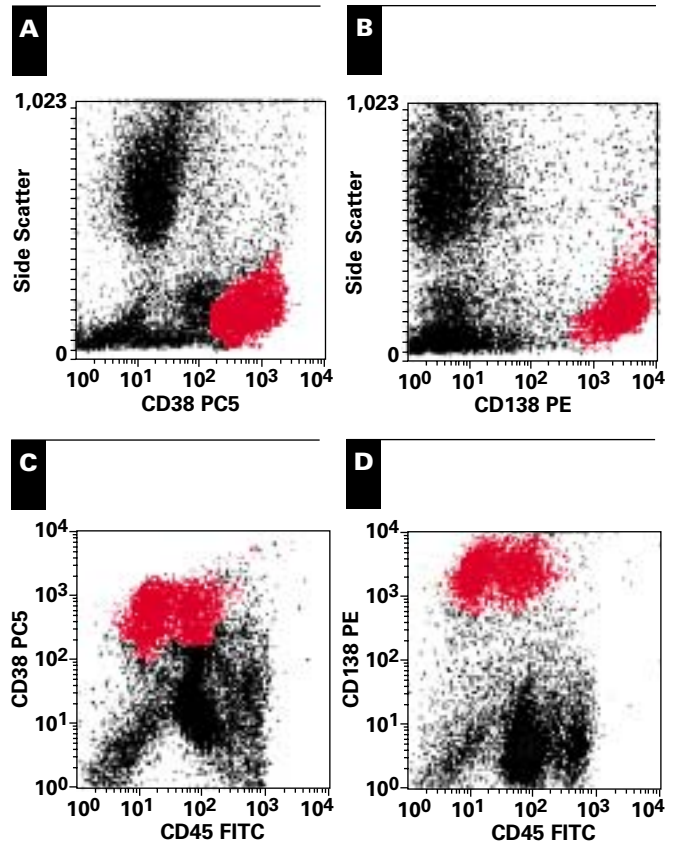


Image 1 A and B, CD38 PC5 and CD138 PE identify plasma cell (PC) populations (red) that strongly express these antigens. C and D, When combined with CD45, CD138 is better than CD38 for separating PCs (red) from other cell populations. CD138 also permits better delineation of 2 distinct subpopulations of PCs; one population is CD45–/dim, and the other is moderately positive for CD45. FITC, fluorescein isothiocyanate; PC5, phycoerythrin–cyanin 5.1; PE, phycoerythrin.

possibility of contaminating B cells in the analysis. Coexpression of CD45 and CD20 was found in only 5 (1.7%) of 301 cases. Neoplastic PCs expressing CD45 or CD20 showed a spectrum of morphologic features ranging from small mature Marschalko type to more immature forms.

Table 1 Overall Frequency and Intensity of Antigen Expression by Myeloma Cells

Intensity*	CD138	CD38	CD56	CD117	CD45	CD20	CD52
3+	306	305	89	26	20	24	8
2+	0	1	2	13	7	4	8
1+	0	0	1	33	18	8	18
Partial†	0	0	8	5	9	11	8
Frequency (%)	306/306 (100.0)	306/306 (100.0)	100/127 (78.7)	77/219 (35.2)	54/306 (17.6)	47/301 (15.6)	42/306 (13.7)

* 1+, weakly positive; 2+, moderate positive; 3+, strongly positive (see the “Materials and Methods” section).
 † Distinct subpopulation of plasma cells (≥20%) with at least 2+ staining intensity.

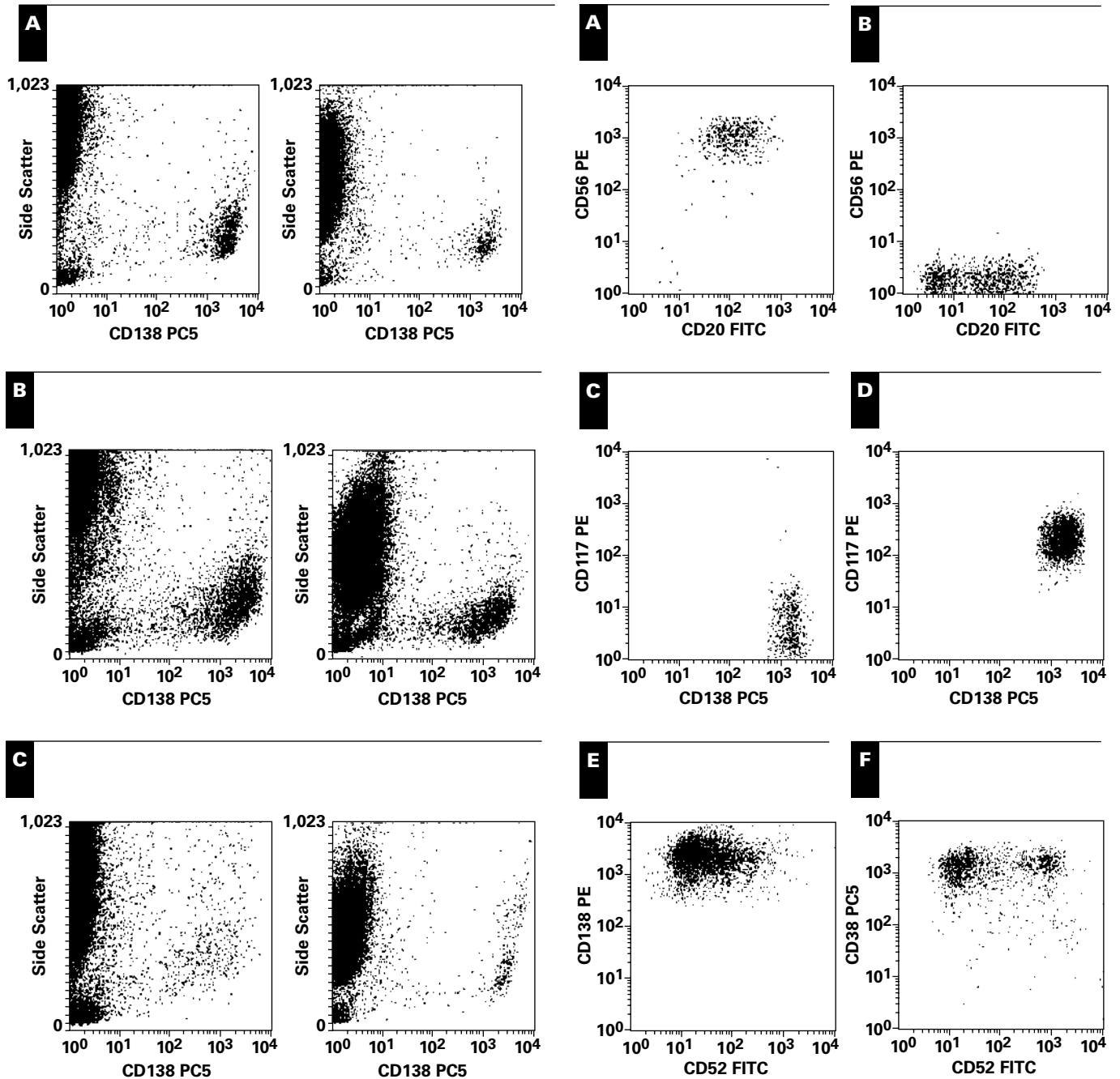


Image 2 Comparison of 2 RBC lysis methods on CD138 staining in 3 cases. The left panels represent samples processed with ammonium chloride (NH₄Cl), and the right panels represent samples processed with FACS Lyse (Becton Dickinson, San Jose, CA). **A** (Case 1), Loss of CD138+ cells is seen with FACS Lyse vs NH₄Cl. **B** (Case 2), A greater number of bone marrow plasma cells (PCs) show no significant difference in CD138+ cells between the 2 lysis methods. **C** (Case 3) FACS Lyse enhances the signal-to-noise ratio among the small number of CD138+ PCs. PC5, phycoerythrin–cyanin 5.1.

Image 3 Plasma cells (PCs) are first identified by live gating using CD45, CD38, and CD138. Examples of additional antigen expression are shown for 6 myelomas. **A**, Moderate (2+) CD20 and strong (3+) CD56 expression. **B**, CD56 is negative and CD20 is positive in a distinct subpopulation of PCs, consistent with partial expression. **C**, The strongly CD138+ PCs show weak (1+) staining for CD117. **D**, Bright (3+) CD117 expression. **E**, Weak CD52 in strongly CD138+ cells. **F**, A subpopulation of strong CD52 expression in strongly CD38+ cells. FITC, fluorescein isothiocyanate; PC5, phycoerythrin–cyanin 5.1; PE, phycoerythrin.

Table 2
Comparison of Myeloma Cell Immunophenotype Between Previously Treated and Untreated Patients*

	CD138		CD38		CD56		CD117		CD45		CD20		CD52	
	T	U	T	U	T	U	T	U	T	U	T	U	T	U
3+	244	62	243	62	68	21	20	6	18	2	18	6	7	1
2+	0	0	1	0	1	1	9	4	5	2	4	0	6	2
1+	0	0	0	0	1	0	25	8	16	2	7	1	11	7
Partial	0	0	0	0	8	0	3	2	7	2	10	1	7	1
Frequency (%)	244/244 (100.0)	62/62 (100.0)	244/244 (100.0)	62/62 (100.0)	78/95 (82)	22/32 (69)	57/174 (32.8)	20/45 (44)	46/244 (18.9)	8/62 (13)	39/239 (16.3)	8/62 (12.9)	31/244 (12.7)	11/62 (18)

T, treated; U, untreated.

* 1+, weakly positive; 2+, moderate positive; 3+, strongly positive (see the "Materials and Methods" section). *P* values, generated from comparing the overall frequency of each marker using the χ^2 test, were as follows: CD138, 1.0; CD38, 1.0; CD56, 0.2; CD117, 0.2; CD45, 1.0; CD20, 1.0; CD52, 1.0.

Both CD117 and CD52 were expressed at weak to moderate intensity in most positive cases (Image 3). CD117 was expressed by 77 (35.2%) of 219 cases, of which 5 (6%) of 77 showed partial expression and 33 (43%) showed weak expression. CD52 was primarily weakly expressed on PCs compared with the bright expression found on T or B lymphocytes. Although 42 (13.7%) of 306 cases expressed this marker, only 16 (38%) of the 42 positive cases showed moderate to strong expression in the entire PC population. The remaining 26 (62%) displayed weak or partial expression. In 9 (33%) of 27 myeloma cases with moderate to strong CD45 expression, moderate to strong CD52 expression also was detected, compared with 7 (2.6%) of 270 cases in the CD45–dim group ($P < .001$; χ^2).

CD56 was expressed in the majority of cases (100/127 [78.7%]) with primarily moderate to bright expression (91/100 [91%]) (Image 3). CD19 was expressed moderately in a subset of PCs in only 1 case and was negative in the remaining 127 cases assessed.

The immunophenotype of myeloma cells was not significantly different in untreated vs treated patients. **Table 2** shows the comparative immunophenotypes. An insufficient number of cases had repeated evaluations to determine whether the immunophenotype remains stable in an individual patient over time.

Discussion

Myeloma cells commonly are identified by their characteristic CD38+++CD45–/dim staining pattern.^{1,2} The panel of antibodies recommended by the international consensus group for PC immunophenotyping consists of, in the order of frequency, cytoplasmic immunoglobulin light chain, CD38, CD45, CD56, CD19, CD20, immunoglobulin heavy chain, and CD138. Of these, CD138 was considered essential by 5 and useful by 9 of 12 panelists, whereas CD38 and immunoglobulin light chain were considered

essential by all 12 panelists.¹⁹ We evaluated these antibodies in a large series of cases of myeloma. In addition, we tested for antigens on neoplastic PCs that might be important for targeted therapy.

Although CD38+++CD45–/dim gating identifies many cases of myeloma, this method has several potential pitfalls. Neoplastic PCs with heterogeneous CD38 expression might be difficult to separate from activated lymphocytes and hematopoietic stem cells that also express CD38. Although myeloma cells most commonly show dim to negative CD45 reactivity, 11.8% (36/306) of the cases in our series had moderate to strong CD45 expression in at least a subpopulation of the PCs.³ Inclusion of all PC subpopulations is essential for complete immunophenotypic profiling and functional analysis of subpopulations of interest. This also has therapeutic implications as monoclonal antibodies become more widely used in hematologic diseases.²⁰

We found CD138 to be a sensitive marker for identification of PCs in bone marrow samples. CD138 gating identified a much more homogeneous population of myeloma cells than did CD38 gating, particularly when CD138 was labeled with PC5 as compared with FITC, PE, or PerCP (weaker signals, data not shown). CD138 permits identification of PCs with weak or moderate CD38 reactivity, as well as the moderate to bright CD45-expressing cells, that may be undetectable by CD38+++CD45–/dim gating alone. The expression intensity of CD138 in cells from patients previously treated with chemotherapy was not significantly different from that in their untreated counterparts, making it feasible to use CD138 for analysis of follow-up samples.

The wide range of detection sensitivity (60%-100%) for CD138 reported in the earlier literature likely represents technical problems. We observed loss of CD138 in samples stored in a cold environment (refrigerator) or when sample processing was delayed at the initial stage of setting up the assay. It is clear that for evaluation of CD138 on PCs, the method and reagents need to be optimized in the individual laboratory. We found that identical samples analyzed with

different RBC lysis methods or incubation periods yielded variable results. Samples that showed the most significant loss of CD138 were those that had been separated with Ficoll-Hypaque or lysed with overnight incubation before testing. Processing with FACS Lyse also was accompanied by loss of CD138+ cells in some cases, but the signal-to-noise ratio was enhanced with this reagent. In contrast with what has been reported by others,⁴ the RBC lysis reagent ammonium chloride did not alter detection sensitivity in our laboratory. We, therefore, use ammonium chloride when evaluating CD138 on PCs because it leaves the greatest number of PCs intact for analysis. FACS Lyse is reserved for only the rare cases that require enhanced CD138 signal owing to background noise. Because our study focused primarily on bone marrow specimens, it is possible that the expression pattern of CD138 differs for PCs in the peripheral blood and extramedullary sites.

Approximately 13% of cases in the present series expressed moderate to bright CD20. These patients are good candidates for rituximab (anti-CD20).²⁰ However, CD20+ myeloma needs to be distinguished from B-cell non-Hodgkin lymphoma with plasmacytoid differentiation. Entities such as chronic lymphocytic lymphoma with plasmacytoid features, lymphoplasmacytic lymphoma, and diffuse large B-cell lymphoma with plasmacytoid differentiation often are in the differential diagnosis. We found CD19 to be an especially useful antigen to separate these processes. CD19 was expressed in fewer than 1% of myeloma cases in this study and was not expressed in any of the CD20+ myeloma cases, in contrast with its nearly universal expression by B-cell lymphomas and leukemias. Immunophenotyping with a complete panel of markers including CD138, CD19, and both surface and cytoplasmic light chain immunoglobulin is recommended to avoid misdiagnoses in questionable cases.

A substantial number of patients with myeloma had a distinct subpopulation of PCs that expressed an antigen not otherwise identified on the remainder of the tumor cells. This phenomenon was common for CD45 and CD20, whereby partial expression with at least 2+ staining intensity was observed in 17% (9/54) and 23% (11/47) of cases, respectively. CD45 expression did not correlate with CD20 reactivity but instead correlated strongly with CD52 expression in our study group. CD52 positivity was significantly more common in cases of CD45+ myelomas than CD45–/dim myelomas ($P < .001$; χ^2). We found that only 13.7% of myeloma cases expressed CD52, unlike the 52% of cases reported by Kumar and colleagues.²¹ In addition, the majority of our positive cases (26/42 [62%]) exhibited weak or partial CD52 expression. The disparity between the 2 studies might result from different gating strategies for identifying PCs. Our analysis used CD138, CD38, and

CD45 gating, whereas Kumar and colleagues²¹ used only CD38 and CD45 gating. As previously discussed, inclusion of contaminating lymphocytes, particularly T cells that normally are CD52+, is problematic when CD138 is not examined. Our criterion for defining CD52 positivity also was rigorous. We required the presence of CD52 on at least 20% of the PCs; if present in only a discrete subset, the subset had to show at least moderate (2+) expression intensity. Whether subsets of myeloma cells expressing CD45, CD20, or CD52 represent functionally or biologically distinct fractions of tumor requires further study. However, these findings suggest that alemtuzumab (anti-CD52) is unlikely to target a majority of myeloma cells for therapeutic purposes.

The other antigen of interest for potential targeted therapy is CD117. The overall frequency of CD117 expression among myelomas in this study was 35.2%. Approximately half of the CD117+ cases showed only weak or partial expression isolated to a subset of the cells. The remaining cases (39/219 [17.8%]) are potential candidates for therapy with imatinib mesylate.

When we compared the immunophenotypes of myeloma cells in untreated and previously treated patients, no significant differences were found, suggesting preservation of aberrant antigen expression. Ideally, identification of a unique myeloma immunophenotype for each patient would permit optimized detection of minimal residual disease, similar to the approach used for acute leukemia. To evaluate whether the myeloma immunophenotype changes in individual patients over time requires further study.

The combination of CD138, CD38, and CD45 is a more effective approach for identifying PCs than CD38 and CD45 gating alone in FC immunophenotyping. Analysis of CD138 requires optimization of reagent and processing methods for PC detection. The complementary effects of CD138 and CD38 permit inclusion of PCs with total or partial expression of antigens that are biologically or therapeutically relevant. While aberrant antigen expression is helpful for the diagnosis of myeloma, it also might be helpful for the detection of minimal residual disease, given the similar incidence of marker expression in treated vs untreated patients. Finally, this study shows that targeted therapy with rituximab or imatinib mesylate is warranted for the substantial subset of patients with CD20+ or CD117+ myelomas. The usefulness of alemtuzumab for the rare CD52+ cases is less clear.

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