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Immunophenotypic Profile of Multiple Myeloma: A Tertiary Care Centre Experience

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Abstract

Background Immunophenotyping and enumeration of plasma cells (PCs) by flow cytometry are deemed to be prognostically significant. However, PCs enumeration by flow cytometry is challenging owing to discrepancy with morphology and PCs loss during sample processing. Enumeration and differentiation of abnormal plasma cells (APCs) and normal plasma cells (NPCs) is difficult because abnormal antigen expression can be seen in subsets of NPCs. This is particularly true when a limited panel of antibodies are relied upon.

Aims and purpose To study the immunophenotypic profile of newly diagnosed multiple myeloma (MM) cases by flow cytometry and evaluate the sensitivities and specificities of individual antigens and combinations.

Methods We studied immunophenotype of PCs in newly diagnosed MM cases ($n = 48$) and control cases ($n = 10$) by a 6-color, 3-tube flow cytometry panel. The sensitivities and specificities of antigens in MM were evaluated and compared with control cases.

Results Majority of MM cases ($n = 43$) had $< 3\%$ NPCs. CD19 was the most sensitive (100%) and CD81 was the most specific marker (100%) for differentiating APCs from NPCs. CD38 MFI came out as a useful marker for APCs identification. In combination, CD19 and CD81 had a higher sensitivity and specificity to detect APCs.

Conclusion NPCs may show aberrant antigen expression. A combination of multiple markers including CD81 and CD38 MFI should be used for accurate APC detection.

Keywords
- multiple myeloma
- immunophenotype
- flow cytometry
- CD38MFI

Introduction

Plasma cell dyscrasias (PCDs) results from clonal expansion of PCs that secrete single homogenous monoclonal immunoglobulin called an M protein.[1] The diagnostic criteria included the presence of $> 10\%$ clonal bone marrow PCs on morphology or an extramedullary plasmacytoma along with one or more myeloma defining events defined by the International Myeloma Working Group (IMWG).[2] Multiparametric flow cytometry (FCM) has been the forerunner in diagnosing and monitoring in many hematological neoplasms mostly because of the high sensitivity, specificity along with the ability to provide results within a few hours.[3] The importance of FCM in diagnosing, monitoring, and prognosticating PCDs, especially MM, have progressively increased in recent years. FCM is useful in differentiating PCDs from reactive plasmacytosis or other B cell neoplasms with extensive plasmacytic differentiation.[3] FCM has been

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useful in differentiating APCs from NPCs based on the different surface antigen expressions as well as the clonality analysis.[4] APCs characteristically show under-expression of CD19, CD45, CD27, CD81, CD38, and overexpression of CD56, CD117, CD28, CD33, and CD200.[3,4] NPCs predominantly express CD19, CD45, CD27, and CD81 and are negative for CD56, but a varying minor population amongst NPCs can have altered expression of surface antigens.[3,4] The enumeration of APCs and NPCs is also useful in differentiating MM from monoclonal gammopathy of unknown significance (MGUS) and smoldering MM (SMM) based on the percentage of NPCs in a total PC compartment in the bone marrow.[5] The most useful application of enumerating NPCs is in SMM, where FCM can differentiate between an MGUS-like profile from an MM-like phenotypic profile.[6] Newly diagnosed MM cases (NDMM) who have > 5% NPCs from all bone marrow PCs have a lower bone marrow PC burden, higher hemoglobin level, longer progression-free survival (PFS), and longer overall survival (OS) compared with patients who had less than 5% NPCs.[7] However, different FCM panels incorporating different antigens for plasma cells have been used.[8] Scarce studies have been published evaluating the sensitivities and specificities of individual and combination of antigen-differentiating APCs from NPCs.[9] Literature on objective evaluation of CD38 and CD138 in differentiating APCs and NPCs is also rare.[10] Enumeration of PCs by FCM has been particularly difficult owing to discrepancy with bone marrow (BM) morphology, hemodilution, and cell loss secondary to sample processing.[9,11–13] FCM was found to have greater sensitivity and limit of detection compared with morphology supplemented with IHC despite being limited to more hemodilute second aspirate.[13] Despite discrepancies in PCs enumeration, there has been significant correlation between FCM and BM PCs numbers.[14–16] Complete absence of CD19 and/or CD45 along with dim expression of CD38 and aberrant expression of CD56 has been used to identify APCs in most MM cases; however, precise enumeration of APCs has been challenging, especially in the evaluation of minimal residual disease (MRD).[9] Therefore, a combination of highly sensitive and specific markers differentiating APCs and NPCs is necessary.

We evaluated sensitivities and specificities of CD19, CD45, CD81, CD27, CD56, and CD117 to differentiate APCs from NPCs. Objective evaluation of CD38 and CD138 in terms of median fluorescence intensity (MFI) was done in APCs and NPCs. PCs enumeration by FCM was correlated with BM morphology. Correlation of total PC percentage and NPCs percentage with baseline characteristics of newly diagnosed MM cases was also evaluated.

Patients and Methods

BM aspirate samples of 48 newly diagnosed MM cases were evaluated for diagnostic FCM. The diagnosis of MM was done in these cases according to IMWG criteria.[2] Ten nonmyeloma cases with reactive bone marrows (solid tumor/Hodgkin's lymphoma staging marrow/immune thrombocytopenic purpura [ITP]/chronic kidney disease/ATTR amyloidosis) were recruited for controls.

A 3-tube, 6-color multiparameter FCM panel was used for plasma cell immunophenotyping (►**Table 1**).

BM aspirate samples were received in EDTA anticoagulant and processed within 12 hours. An ammonium chloride-based bulk lysis/pre-lysis protocol (RBC lysis buffer, BioLegend, San Diego, CA) was used for all samples. Antibodies against surface antigens and intracytoplasmic light chain antigens were stained according to previously described protocols.[17] For cytoplasmic light chain staining permeabilizing solution (BD Perm/wash, BD biosciences, San Jose, CA) was used after surface staining. In all washing steps aspiration of supernatant was done in the place of simple decantation of tube to minimize cell loss. A minimum of 0.5 million events were acquired on BD FACSCanto II 3-laser flow cytometer (BD Biosciences, San Jose, CA) immediately after sample processing in all cases.

A sequential gating strategy was used for immunophenotyping of PCs. BD FACSDiva v6.0 software (BD biosciences, San Jose, CA) was used in all the cases for analysis.

Gating Strategy

a. A CD38 versus time dot plot was used to assess the quality of data acquisition.
b. FSC-H versus FSC-A dot plot to exclude doublets.
c. FSC-A versus SSC-A to exclude debris.
d. A broad gating (PC gate) on CD38 versus CD138 dot plot to include all CD38- and CD138-positive events.

Table 1 FCM Panel for plasma cell immunophenotyping

Tube	FITC	PERCPCy5.5	PE	PECy7	APC	APCH7
1	CD81	CD45	CD138	CD19	CD56	CD38
COMP./CLONE	BL/JS81	BL/30-F11	BD/MI15	BL/HIB19	BD/B159	BD/HB7
2	CD27	CD45	CD138	CD19	CD117	CD38
COMP./CLONE	BD/M-T271	BL/30-F11	BD/MI15	BL/HIB19	BD/104D2	BD/HB7
3	cLAMBDA	CD45	CD138	CD19	cKAPPA	CD38
COMP./CLONE	BL/MHL38	BL/30-F11	BD/MI15	BL/HIB19	BL/MHK49	BD/HB7

Abbreviations: COMP, company; BL, BioLegend.
San Diego, CA, BD-BD biosciences, San Jose, CA

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e. A refined PC gate (CD38 vs. CD45 dot plot) on nondebris cells to include only CD38+ bright events.
f. A CD19 versus CD45 plot was used to characterize refined PCs with expression of CD19 and CD45.
g. Further characterization of each subset of PCs was done with CD56, CD81, CD117, and CD27.
h. Each subset on a CD19/CD45 dot plot was assessed for cytoplasmic kappa or lambda restriction.
i. Mast cells, hematogones, and NK cells were also evaluated to asses sample dilution.

NPCs were defined based on polyclonal cytoplasmic kappa and lambda light chain expression. APCs were based on antigen expression profile and monoclonality on cytoplasmic light chain staining. An aberrant antigen expression profile was assigned when at least two surface antigen expression were abnormal. Antigen expression intensity were characterized as negative (N), dim (D), partial positive (PP), subpopulation positive (SPP), subpopulation negative (SPN), or moderate/strong positive (P).

Bone marrow aspirate smear and peripheral smears were stained with Jenner–Giemsa stain and evaluated for PCs. The baseline characteristics including serum protein electrophoresis (SPEP), immunofixation electrophoresis (IFE), free light chain assay (sFLC), and radiological features were retrieved from patients' medical records.

Results

We studied 48 MM cases with a male predominance (males = 33, females = 15). The mean age in our cohort was 58.4 years (range, 40–79 years). Staging of the disease in MM patients was done according to the published guidelines as the international staging system (ISS) ($n = 48$)/revised international staging system (RISS) ($n = 41$).[1] Most patients in our cohort were in ISS-3 (31 cases, 65%) or RISS-2 (26 cases, 63%). The demographics, baseline characteristics including staging are shown in ►**Table 2**.

Table 2 Characteristics of MM patients

Age in years ($n = 48$) mean/median/range	58.4/60.5/40–79
Sex ($n = 48$)	Male(M) = 33, Female(F) = 15 M:F = 2.2:1
Lytic Lesions on radiology ($n = 48$)	68.75% (33/48)
Hb (g/dL) ($n = 48$) mean, median, range	8.3/8.2/4–15.1
TLC ($\times 10^9$/L) ($n = 48$) mean/median/range	7.0/6.0/1.39–17.6
Platelets ($\times 10^9$/L) ($n = 48$) mean/median/range	158.29/144.0/20.0–503
Calcium (mg/dL) ($n = 48$) Mean/median/range	9.34/9.1/7.9–13.8
Creatinine (mg/dL) ($n = 48$) Mean/median/range	2.01/1.4/0.5–7.2
M Band (g/dL) ($n = 44$) Mean/median/range	3.58/3.8/0.3–8.63
No M bands	4 of 48 cases
Albumin (g/dL) ($n = 48$)	3.37/3.45/1.5–5.6
LDH (U/L) ($n = 48$) Normal range (120–240) Mean/median/range	241/184/56–940
sFLC ratio ($n = 48$) Normal sFLC ratio (0.26–1.65) Mean/median/range	67.8/6.25/0.001–657.3
Beta2M (mg/L) ($n = 48$) Mean/median/range	8.02/6.25/3.04–20
Type of immunoglobulin ($n = 48$)	IgG Kappa- 20 IgG Lambda-09 IgA Kappa-07 IgA lambda-06 Kappa light chain-01 Lambda light chain-04 Not available-01
FISH (fluorescent in situ hybridization) abnormalities ($n = 41$)	Del 13q-17 Del17p-05 $t^{4,14}$-04 $t^{11,14}$-03 No abnormalities-17 Other anomalies (trisomy17, hyperdiploidy, del6p, monosomy 16)-08
International staging system (ISS) ($n = 48$)	ISS1 = 2, 4% ISS2 = 15, 31% ISS-3 = 31, 65%
Revised International staging system (RISS) ($n = 41$)	RISS1 = 2, 5% RISS2 = 26, 63% RISS3 = 13, 32%

Comparison between BM Morphology and FCM PC

There was a significant reduction in PCs in FCM processing in comparison to morphology (BMPCs). Only two cases had FCMPC% more than BMPCs and both the cases had marrow fibrosis of grade 1–2 (WHO scoring system grade 0–3). A mean of 59.77% reduction was seen in PCs enumeration by FCM in comparison to BM. However, a moderate correlation was present between PCs in both the methods ($R^2 = 0.458$, $p = 0.001$).

Enumeration of NPCs

NPCs in BM ranged from 0–35% (median-0.41%, mean-1.67%). Five patients (10.41%) had NPC > 3% and only 3 (6.25%) patients had NPC > 5%.

Patterns of Antigen Expression in MM Cases

None of the 48 cases showed a moderate to strong positivity for CD19. CD45 was moderate to strong positive in seven cases (14.6%). CD56 positivity (D/PP/P) was seen in 38 cases (79.1%) cases. CD81 and CD27 were abnormal (N/D/PP) in 44 (93.6%) and 42 (89.3%) cases, respectively. CD117 expression was abnormal in 15 (31.9%) cases only (►**Table 3**).

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Table 3 Patterns of antigen expression of APCs in MM cases

	CD19 ($n=48$)	CD45 ($n=48$)	CD56 ($n=48$)	CD81 ($n=47$)	CD27 ($n=47$)	CD117 ($n=47$)
N	45 (93.7%)	31 (64.6%)	10 (20.8%)	39 (82.9%)	31 (66%)	32 (68.0%)
D	3 (6.3%)	4 (8.3%)	3 (6.3%)	2 (4.3%)	6 (12.8%)	6 (12.8%)
PP	0 (0%)	6 (12.5%)	4 (8.3%)	3 (6.4%)	5 (10.6%)	0
P (Moderate/strong positive)	0 (0%)	7 (14.6%)	31 (64.6%)	3 (6.4%)	5 (10.6%)	9 (19.2%)

Table 4 Distribution of immunophenotypic abnormalities in MM cases

	CD19 ($n=48$)	CD45 ($n=48$)	CD81 ($n=47$)	CD27 ($n=47$)	CD56 ($n=48$)	CD117 ($n=47$)
Number of cases with abnormalities	48	41	44	42	38	15
%	100	85.4	93.6	89.3	79.1	31.9

Distribution of Immunophenotypic Abnormalities in MM Cases

CD19 showed abnormalities in 100% of cases followed by CD81 (93.6%) and CD27 (89.3%) cases. In all, 14.6% of cases ($n=7$) showed a moderate to strong positivity for CD45. CD56 showed a dim, partial or strong positivity in 79.1% of cases ($n=38$). CD117 had the lowest frequency of abnormalities with positivity in only 31.9% of cases ($n=15$). The distribution of antigen expressions is shown in ►**Table 4**.

Patterns of Abnormal Antigen Expression in NPCs in Control Cases

Eight out of 10 cases of controls run for immunophenotyping of NPCs showed antigen expression abnormalities in the form of an SPN or SPP (►**Table 5**). CD56 SPP was most frequent with six cases showing expression in a mean of 11.7% of cases (range, 1.9–26.4%). No case showed an abnormal expression in CD81 or CD117. All control cases were polyclonal for kappa and lambda light chains.

Sensitivity and Specificity of Individual Antigens/Immunophenotypic Markers

When compared with the immunophenotype of NPCs in control cases, CD19 was abnormal in 100% of MM cases showing a sensitivity of 100%. However, four control cases showed abnormal CD19 expression in NPCs, reducing the CD19 specificity to 60%. However, CD81 showed a sensitivity of 93.62% and specificity of 100% as no control cases showed anomalous CD81 expression. Though CD117 was 100% specific, it was expressed in a fewer number of MM cases (sensitivity-31.91%), reducing its utility as a single marker defining APCs. Both CD56 and CD27 had a lower sensitivity and specificity (79.17%/40% and 89.36%/70%).

Sensitivity and Specificity of Combination of Markers

We evaluated the combination of the commonly used antigens in MM immunophenotyping. A combination of CD19 and CD81 showed the highest sensitivity of 93.62% and specificity of 100% when compared with a similar combination in NPCs in controls. A combination of CD19/CD45/CD56 showed only 68.75% sensitivity. However, no combination of markers showed 100% sensitivity and specificity simultaneously, emphasizing the importance of simultaneous use of multiple surface markers for reliable identification of APCs.

Comparison of CD38 Median Fluorescence Intensity (MFI) in APCs and NPCs

CD38 MFI was evaluated in APCs and NPCs (only MM cases where NPCs were $>0\%$ were included, $n=42$) in diagnostic

Table 5 Patterns of abnormal antigen expression in NPCs in control cases ($n=10$)

Total cases	CD19 (SPN)	CD45 (SPN)	CD56 (SPP)	CD81 (SPN)	CD27 (SPN)	CD117 (SPN)
10	04 (40%)	03 (30%)	06 (60%)	00(0.0%)	03(30%)	00(0.0%)
Mean/range of abnormalities	17.25%/ 6.1–29.6	11.70% /3.1–19.7	11.70% /1.9–26.4	–	15.33% /10.9–17.7	–

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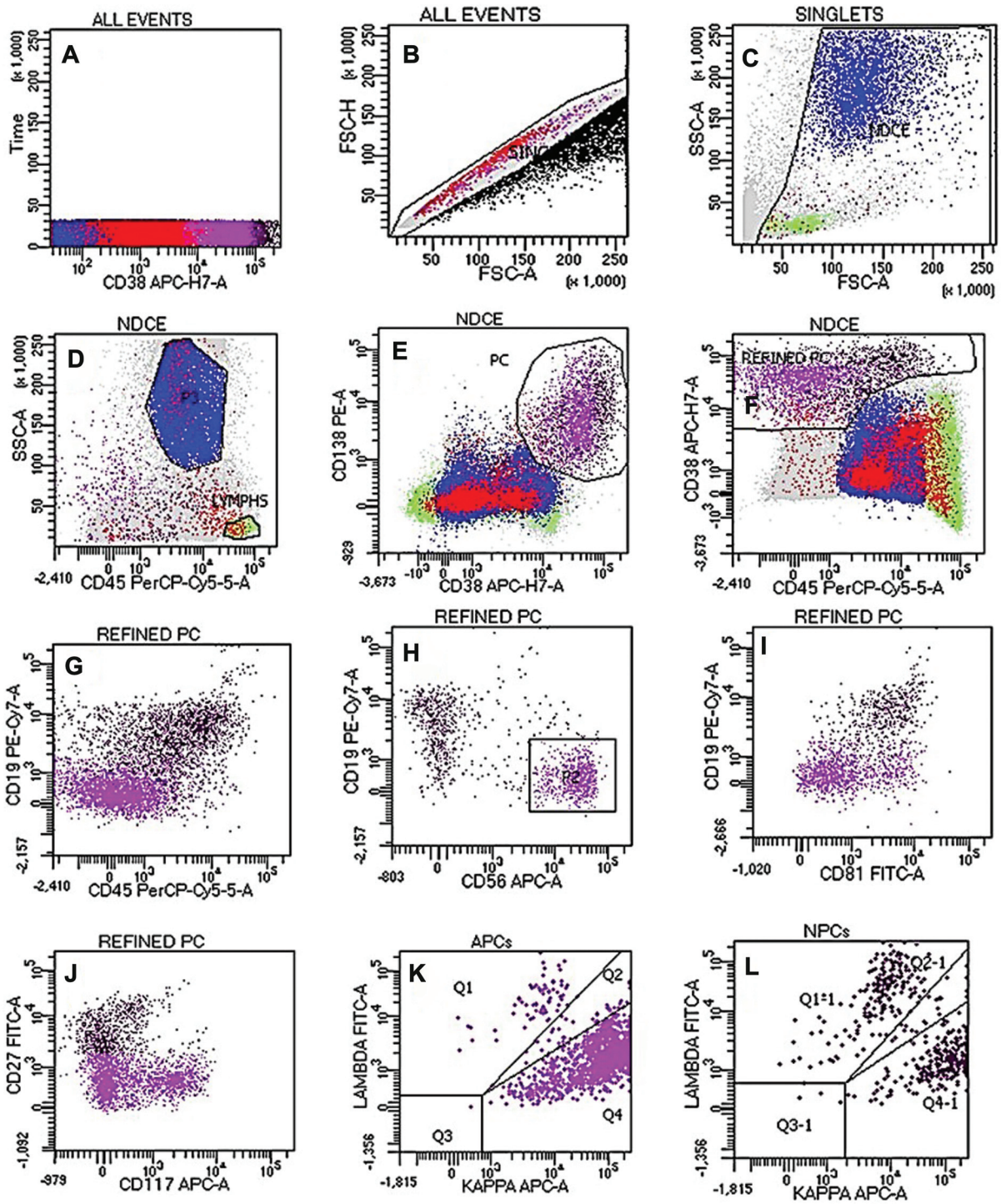

Fig.1 MM immunophenotyping gating strategy. (**A**)Time vs. CD38 gate. (**B**) Singlet gate (FSC-A vs. FSC-H). (**C**) Non-debris cells (NDCE) gate (FSC-A vs. SSC-A). (**D**) CD45 vs. SSC plot. (**E**) PC gate (CD38 vs. CS138) (**F**). Refined PC gate on NDCE (CD38 vs. CD45). (**G**) CD19 vs. CD45 plot on refined PC. (**H**) CD19 + CD56-(purple) NPCs and CD19-CD56+ (pink) APCs (**I**) CD19 + CD81+ NPCs and CD19-CD81-APCs. (**J**) CD27 + CD117-NPCs and CD27-CD117 + APCs (**K**) Kappa-restricted APCs. (**I**) Polyclonal NPCs.

immunophenotyping. Median CD38 MFI was 18204.5 (range 1029–109052) and 40895 (range, 3943–144856) in APCs and NPCs, respectively. CD38 had a significant low MFI or was dimmer in expression in APCs in comparison to NPCs ($p < 0.0001\%$), making it a useful marker for APCs and NPCs discrimination. However, no significant difference was established in CD138 MFI in APCs versus NPCs ($p = 0.361$).

Discussion

FCM PC enumeration can accurately discriminate between MGUS and MM.[6,18] The number of residual polyclonal PCs (NPCs) is a useful discriminating marker between MGUS and MM at diagnosis.[6,19] MGUS usually has more than 5% NPCs within total bone marrow PCs.[18] The proportion of NPCs in

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patients with PCDs is a powerful prognostic factor in all stages of the disease as well as during follow-up post-therapy.[6,19] MGUS and SMM with abnormal to total PC ratio more than 95% have a higher risk of progression to symptomatic MM with time to progression at 5 years of 25% versus 5% and 64% versus 8%, respectively.[6] MM patients with more than 5% NPCs in BM have a lower frequency of immunoparesis (42% vs. 83%, $p = 0.003$) and a greater response to autologous stem cell transplantation (ASCT) (complete remission [CR] rate after ASCT of 64% vs. 33%, $p \leq 0.001$).[20] These findings suggest that FCM is very useful in defining subgroups of patients with better prognoses, irrespective of achievement of CR or not. Because the exact enumeration of APCs and NPCs have prognostic significance, instituting a multiparametric FCM panel is important to differentiate APCs from NPCs. A classical immunophenotype of APCs is described as CD138 positive, dim CD38 (compared with NPCs), CD19 negative, CD45 dim to negative and aberrant CD56/CD117/CD20.[8] CD200 and CD33 are expressed on APCs in a smaller subset of myeloma cases.[8,9] CD28 is usually negative in NPCs but is overexpressed in APCs.[8,9] The antigen expression in plasma cell leukemias (PCLs) vary from only MM cases. PCLs are more likely to express CD20 and tend to be negative for CD56 and CD117.[8] Subpopulations of NPCs are known to show aberrancies in CD19, CD45, and CD56 expressions.[9,19] Tembhare et al, in their patient cohort, found small sub-populations of NPCs showing abnormalities in CD19, CD45, or CD56 expression in 68% of cases.[9] These normal variations in NPCs may pose significant difficulty in APC enumeration and NPCs versus APCs differentiation.[21] Classically, CD38 and CD138 along with scatter properties and CD45 are being used for PC identification by FCM. However, with increasing use of anti-CD38 targeted therapies, such as daratumumab, additional PC gating marker should be used for monitoring of MM cases post therapy. Signaling lymphocyte activation molecules (SLAM) family proteins such as CD229 and CD319 have been increasingly used in combination with CD138 for PC identification post anti-CD38 therapy minimal residual disease analysis.[22]

In lines with previous studies, our study confirmed that there is a significant PCs loss when enumerated by FCM but a significant correlation exists between enumerations by the two methodologies. The significant loss can be attributed to either sample dilution, patchy nature of the disease or cell loss in processing. We found CD19 to be 100% sensitive and CD81 to be 100% specific to identify APCs. Combination of CD19/CD45/CD56 though gives a 100% specificity, it is less sensitive in identifying APCs owing to presence of similar aberrancies in subset of NPCs. Next, 80% of control cases evaluated for immunophenotype of NPCs in our cohort showed abnormal antigenic expression. None of the control cases showed abnormality in CD81 in NPCs. Incorporating CD81 into the panel increased the sensitivity to 93.62% for accurate discriminations of APCs from NPCs. Individually, specificities of CD19 and CD56 (60% and 40%) were much lower than that of CD81 (100%). CD27 showed a decent sensitivity of 89.36%, however, a low specificity (70%). CD38 MFI was significantly lower in APCs in the majority of cases in comparison to NPCs. CD38 MFI can be used a useful discriminator between APCs versus NPCs wherever possible. The majority of our cases had $< 5\%$ NPCs by FCM, similar to previous published literature.

Conclusion

FCM at diagnosis is highly sensitive for differentiating APCs from NPCs. Along with CD19, CD45, CD56, CD117, and CD27, CD81 increases the specificity for APC and NPC identification and enumeration. A combination of multiple markers increases the accuracy of APC identification. CD38 MFI calculation in APC and NPC subset could help as an adjunct to different surface markers in APC enumeration. Though there is discrepancy in morphology and FCM PC enumeration, FCM should be routinely incorporated in MM cases at diagnosis owing to its prognostic significance at diagnosis. However, due to limitations in study sample, larger studies are required to confirm the findings.

Ethical Standards

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Ethical Approval

The study is approved by institute ethics committee with reference number IECPG-293/29.05.2019.

Informed Consent

Informed consent was obtained from all individual participants included in the study.

Publication Consent

Consent for publication was obtained for every individual person's data included in the study.

Data Availability

The datasets generated and analyzed during this study are available from the corresponding author on reasonable request.

Authors' Contributions

A.R. processed the samples, analyzed the flow cytometry data, and wrote the manuscript. S.T. conceptualized and designed the study, analyzed the data, and wrote the manuscript. J.D. reviewed the morphology and analyzed the data. T.S. and M.M. reviewed the data and provided the clinical information.

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None.

Conflict of Interest

None declared.

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