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ORIGINAL ARTICLE

Enrichment of circulating myeloma cells by immunomagnetic beads combined with flow cytometry for monitoring minimal residual disease and relapse in patients with multiple myeloma

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Abstract

Difficulty in regularly analyzing marrow myeloma cells (MMCs) and low frequency of circulating myeloma cells (CMCs) in blood presents challenges for monitoring minimal residual disease (MRD) in multiple myeloma (MM). We have developed a set of method for enrichment of CMCs by immunomagetic beads (IMB) combined with flow cytometry (IMB-FCM) based on CD38-APC/CD138-APC antibodies in U266-spiked samples and in 122 patient samples. U266 cell capture efficiency of CD38/CD138-IMB-FCM (6.960, 2.574) was 6- and 2-fold higher than that of FCM (1.032), and the sensitivity of FCM and IMB-FCM was 0.01% and 0.001%, respectively. In MM cohort, the positive rate of CMCs by IMB-FCM increased from 60.5~70.0 to 85~87.2% in newly diagnosed/relapsed and partial remission (PR) patients compared with by FCM ($P < 0.05$). Two complete remission (CR) patients contain certain amounts of CMCs by IMB-FCM while no CMCs and MMCs were detectable by FCM. Patients exhibiting PR and CR upon therapy had much lower CMC and MMC counts than newly diagnosed/relapsed patients ($P < 0.005$). Based on MRD measurement in BM and PB samples, all FCM-negative BM samples were also paired with FCM/IMB-FCM-negative PB samples among newly diagnosed, relapsed, and PR patients, and FCM-positive BM samples were accompanied by IMB-FCM-positive results in 88% of corresponding PB samples. CMCs strongly associated with other clinical biomarkers of disease burden, including elevated MMCs, β2-MG, sCrea, and DS and ISS stages, and more serious anemia, bone destruction, and renal impairment ($P < 0.05$). Logistic regression analysis revealed that elevated β2-MG and moderate-to-more anemia were significant risk factors for the presence of CMCs ($P < 0.05$). As a noninvasive "liquid biopsy" of monitoring MRD, the potential of IMB-FCM for CMC detection may complement or minimize bone marrow aspiration in future treatment of MM patients.

Keywords Multiple myeloma · Circulating myeloma cells · Marrow myeloma cells · Immunomagnetic beads · Flow cytometry

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Introduction

Multiple myeloma (MM) is the second most common hematologic malignancy in the USA [1] and is characterized by proliferation of clonal plasma cells in the bone marrow [2], which results in hypercalcaemia, renal insufficiency, anemia, and osteolytic lesions [3]. The new therapeutic agents such as immunomodulatory drugs and proteasome inhibitors, as well as hematopoietic stem cell transplantation, has shown efficacy in bringing about better response rates and progression-free survival [2, 4]; however, the fact remains that the majority of patients experience multiple relapses which gradually causes refractory disease [5, 6].

The Revised International Staging System (R-ISS) based on serum albumin (Alb), β2-microglobulin (β2-MG), cytogenetic abnormalities, and lactic acid dehydrogenase (LDH) is a

widely applied procedure in prognosticating MM and thereby adopting the appropriate treatment [7]. Other clinical indicators such as hemoglobin (Hb), serum creatinine (sCrea), serum calcium (Ca), bone destruction, renal impairment, and DS stage can in some cases keep track of MM and reflect tumor burden; however, striking discrepancies in outcomes remain even in well-established groups [8]. There is evidence that the presence of minimal residual disease (MRD) is the source of recurrence for the disease, and bone marrow myeloma cell (MMC) examination is the best indicator to detect MRD [6] and the golden standard for evaluation of tumor burden in MM patients [3]. It does, however, require repetitive bone marrow puncture, an invasive procedure that causes patient pain and discomfort and a sample bias which provides limited molecular profile as the puncture cannot capture all subclones [9, 10]. Therefore, new biomarkers to better evaluate disease burden and recurrence are needed.

Several studies employing different methods have shown that circulating myeloma cells (CMCs) are detectable in peripheral blood of MM patients and demonstrated that it represents a capable indicator in detecting residual disease [11], determining prognosis [12, 13], and suggesting better therapeutic interventions [14, 15]. Therefore, it may be possible to use these circulating cells as a liquid biopsy to complement or partially replace bone marrow aspiration [9, 16]. However, it must be noted that CMC burden in peripheral blood is 100-fold lower than in bone marrow [11, 17]. Conventional CMC detection techniques primarily include slide-based immunofluorescence (IF) assay, polymerase chain reaction (PCR)–based methods, and flow cytometry (FCM). IF [18] is challenging for the ability of the morphologist to recognize the low frequency of CMCs with strong subjectivity and low sensitivity. In contrast, the PCR-based approach, such as next-generation sequencing (NGS), is currently the most sensitive (10^{-6}) [19], but the quantification of MRD by NGS is only approximate and is highly variable, as it requires detection of immunoglobulin (Ig) gene rearrangement in residual normal B cell that is obviously influenced by different treatment options. Additionally, highly frequent mutations of Ig gene further hinder its clinical application [8]. FCM for the detection of CMCs was most broadly applied with objectivity, high efficiency, and accurate quantification, since more than 90% of MM patients express plasma cell aberrant immunophenotype [20]. The only drawback is that the sensitivity of FCM is 10^{-4} [21] even through acquiring a large number of cells such as 150,000 events [18, 22], 100,0000 events [14].

CellSearch system, which is based on magnetic bead–labeled epithelial cell adhesion molecule (EpCAM) antibody, has received the FDA approval for the detection of circulating tumor cells in breast, colorectal, and prostate cancers [23]. One recent study has developed this platform to enumerate CMCs with more sensitivity and easier standardized protocols than FCM [16]. It has been reported that immunomagnetic beads (IMB) combined with other techniques such as PCR [24] and single-nucleotide polymorphism (SNP) microarray [25] can improve the applicability of MRD and diagnostic yield in clinical testing for patients with MM. Herein, we establish the CMC enumeration workflow, IMB-FCM system, for the first time through combining the specific enrichment of IMB with rapid quantification of FCM.

CD138 is the principal and specific surface marker for the detection of MM cells [26] but is absent in naive cells [27] and easily sheds during long specimen storage [28], while CD38 is strongly expressed and rarely downregulated on plasma cell membranes but also non-specifically expressed in other cell lines [26]. In our study, we enriched the CMCs using CD138-APC/CD38-APC together. The CD38-APC or CD138-APC antibodies were specifically bound to the CD38 or CD138 antigens on plasma membranes, respectively, then reacted with anti-APC labeling magnetic beads indirectly, and finally, the plasma cells were enriched and separated by the magnetic field.

Identification of isolated CMCs was performed by FCM via immunophenotyping with CD56-FITC/CD138-PE/CD45-PerCP/CD38-APC and CD38-FITC/CD19-PE/CD45-PerCP/CD138-APC, respectively. A cell displaying $CD38^+/CD138^+$ phenotype was classified as plasma cell and was further identified as cancerous cell with expression of $CD19^-$ and/or $CD56^+$ and/or $CD45^-$. Our IMB-FCM system with high sensitivity, specificity, and reproducibility based on peripheral blood could significantly facilitate the ability of CMCs to monitor residual disease and relapse in the future treatment of MM patients.

Methods

Cell culture and immunophenotyping

Multiple myeloma cell line U266 was purchased from Beijing Dingguo Biotech Co., Ltd., and cultivated in a mixture of 89% RPMI-1640 medium, 1% antibiotic/antimycotic (100 u/mL penicillin, 100 u/mL streptomycin, and 0.25 mg/mL amphotericin B), and 10% fetal bovine serum (HyClone, USA) at 37 °C, 5% CO_2 incubator for 2~3 days. The logarithmic phase cells were smeared with Wright-Giemsa within 24 h and observed under an optical microscope. Cells were washed three times with PBS, and the concentration was adjusted to 1~5 × 10^9/L. FCM was performed on the prepared cells stained with antibodies to CD38-FITC/CD138-PE/CD45-PerCP/CD38-APC (Becton Dickinson, USA) panel as well as CD38-FITC/CD56-PE/CD45-PerCP/CD138-APC/CD19-PE Cy7 (Becton Dickinson, USA) panel; the data was analyzed using FCS Express 4 Flow Research Edition.

U266 cells spiked in whole blood and detected by FCM and IMB-FCM

Healthy donor blood samples were collected from the First Affiliated Hospital of Zhengzhou University and anticoagulated by EDTA-K_2 with volume of 5 mL. $CD138^+$U266 cells were spiked into whole blood from the donors at 8 different levels of cells (10%, 5%, 1%, 0.5%, 0.1%, 0.01%, 0.001%, and 0.0001%). To verify reproducibility, every cell level was spiked into blood from a total of 5 samples. Then, the above 40 U266-spiked samples were washed three times with PBS, and the number of cells was adjusted to 4~10 × 10^9/L. According to whether anti-APC magnetic beads were used for enrichment, the U266-spiked experiment was divided into direct FCM group and IMB-FCM group; the latter was further divided into CD38 IMB-FCM and CD138 IMB-FCM group based on different APC-conjugated antibodies.

One hundred microliters of U266-spiked samples were incubated with CD56-FITC/CD138-PE/CD45-PerCP/CD38-APC, CD38-FITC/CD19-PE/CD45-PerCP/CD138-APC respectively at room temperature for 15 min, mixed with 2 mL red blood cell lysate, and then incubated for another 10 min. The mixture was centrifuged at 300×*g* for 5 min, and the supernatant was discarded. After washing with 2 mL PBS, cells were resuspended in 200 μL PBS for FCM and tested directly on the machine, while in the IMB-FCM group, 50 μL PBS was added into the cell pellet to prepare a single-cell suspension labeled with APC-conjugated antibody.

Ten to fifty microliters of anti-APC magnetic particles (Becton Dickinson, USA) for every 1 × 10^7 total cells was incubated with the cell suspension for 30 min at room temperature, then mixed with 500 μL PBS, and immediately placed the tube onto the EasySep™ Magnet (STEMCELL, Canada) for another 6~8 min. With the tube on the Magnet, the magnet and tube were inverted in one continuous motion to pour off the supernatant fraction in 2–3 s and then returned to upright position. The tube was removed from the magnet and mixed with 500 μL PBS, and then placed back in the magnet for another 2~4 min. The above steps were repeated once more for a total of 3 separations in the magnet. Finally, the positive fraction held by the magnetic field was resuspended in 200 μL PBS to proceed with FCM.

Patient sample preparation

One hundred twenty-two patients with MM were diagnosed in the First Affiliated Hospital of Zhengzhou University from July 2016 to January 2018, and 30 patients had anemia, but non-plasma cell-related disorder (control group) was selected, and risk indicators of MM such as Hb, Alb, β2-MG, sCrea, Ca, LDH, bone destruction, renal impairment, DS stage, and ISS stage were analyzed at the same time. According to the multiple myeloma diagnosis and treatment guidelines [3], 122 cases of MM patients were divided into newly diagnosed (ND) group (n = 39), relapse (RA) group (n = 20), partial remission (PR) group (n = 43), and complete remission (CR) group (n = 20). All study subjects submitted informed consent, and the study was approved by the Medical Research and Research Ethics Committee of the First Affiliated Hospital of Zhengzhou University. Two milliliters of bone marrow (BM) and peripheral blood (PB) was collected simultaneously and anticoagulated by EDTA-K_2. Sample processing was same as the above method.

Detection of CMCs and MMCs by FCM and IMB-FCM

Similar to the above U266 grouping method, this part was divided into BM FCM group (MMCs), PB FCM group (CMCs), and PB CD38 (CD138) IMB-FCM group (enriched CMCs) based on the specimen types and detection methods. Pre-treated PB and BM samples were stained with CD56-FITC/CD138-PE/CD45-PerCP/CD38-APC, CD38-FITC/CD19-PE/CD45-PerCP/CD138-APC to recognize plasma cells, and CD38-FITC/CD20-PE/CD45-PerCP/CD138-APC, CD38-FITC/CD33-PE/CD45-PerCP/CD117-APC and cKappa-FITC/cLambda-PE/CD19-PerCP/CD138-APC combination penals were added in BM group to identify plasma cell clonality. Fifty thousand events were collected using Becton Dickinson FACSCanto II instruments in the BM group, while 200,000 events were collected in the PB group. The IMB-FCM operation was as described above.

Statistical analysis

Statistical analysis was performed using the SPSS17.0 software. The data were presented as median (range). Two group correlation and regression were respectively analyzed by Spearman correlation and sample linear regression test. Chi-square test, Kruskal-Wallis H test, and logistic regression analysis were respectively used to compare rates, multiple sets of quantitative data, and multivariate data. P values < 0.05 were considered statistically significant. The Mann-Whitney U test was further conducted for multiple comparisons, and the test level was calibrated by Bonferroni method.

Results

Morphologic and immunophenotyping characteristics of U266 cell

U266 cells stained by Wright-Giemsa showed two types under oil microscopy; one type of cell was small and round, with little cytoplasm dyed blue, large and round nucleus, loose

chromatin; the expression of CD38, CD19, and CD45, and unexpression of CD138, CD56, and low SSC showed early B lymphocyte characteristics (Fig. 1a, b). Another type of cell was large, abundant cytoplasm with foam, nuclear deviation, binuclear or multinuclear; immunophenotype features were expression of CD138, CD38, CD56, CD45, weakened CD19 intensity, higher FSC and SSC as compared with the above cell, showing typical myeloma cell characteristics (Fig. 1b–d). Approximately 50% of U266 cells expressed CD138, and APC-conjugated CD138-positive cells increased to 80.22% when using magnetic beads to sort the cells. To verify APC fluorescence efficiency, PE-conjugated CD138 was applied to target the cells, and the similar percentage (81.67%) to APC indicated that anti-APC magnetic beads could increase the ratio of $CD138^{+}$U266 cells but did not affect APC fluorescence efficiency (Figs. 1c and 2).

Linearity, reproducibility, sensitivity, and specificity of myeloma cells capture

To determine the ability of the assays to capture plasma cells, we examined the capture efficiency upon blood samples with different concentration levels of $CD138^{+}$U266 cells (10%, 5%, 1%, 0.5%, 0.1%, 0.01%, 0.001%, and 0.0001% in blood, Fig. 3b). A linear fit to data with capture efficiencies for FCM, CD38 IMB-FCM, and CD138 IMB-FCM was 1.032, 6.960, and 2.574, and the correlation coefficients describe a clear direct relationship between the number of detected cells and nominal cell

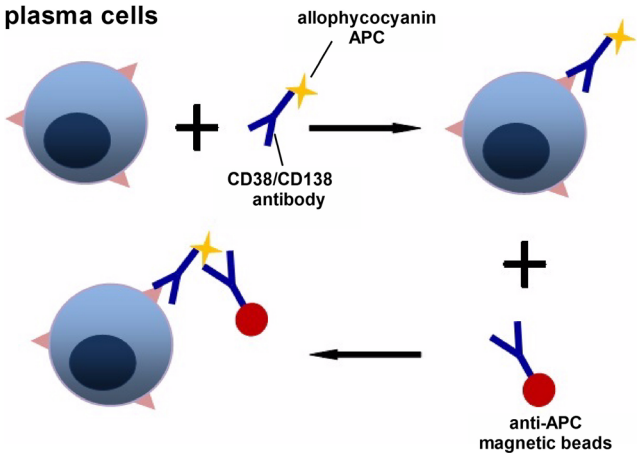

Fig. 2 APC-conjugated CD38/CD138 antibody and anti-APC magnetic beads indirectly label plasma cells

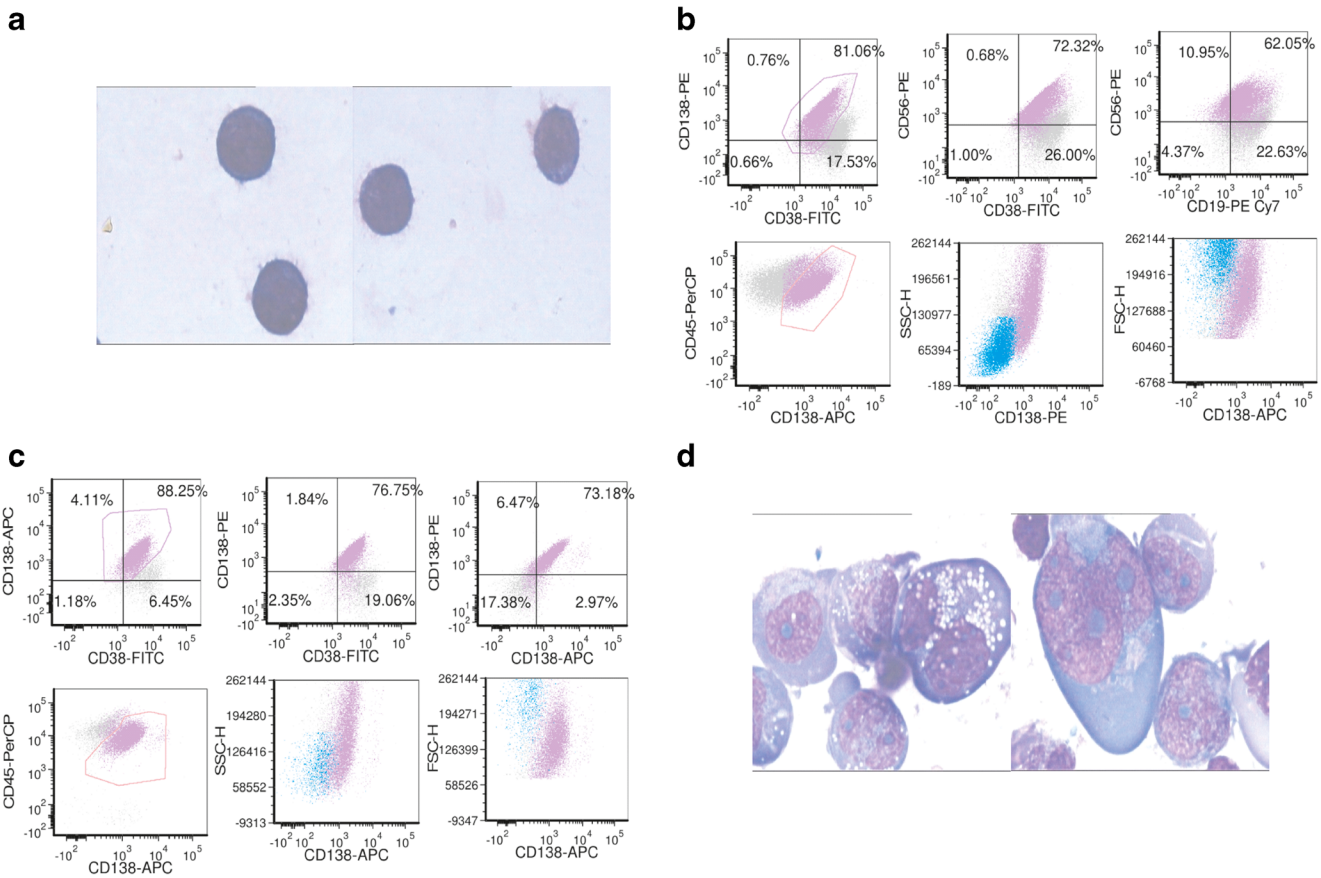

Fig. 1 Morphology and immunophenotype features of U266 cell. Lymphoblastic (**a**) and myeloma (**d**) characteristic U266 cells by image analysis (Wright-Giemsa, × 1000). Immunophenotyping features of U266 cell measured by FCM (**b**) and IMB-FCM (**c**). FCM, flow cytometry; IMB, immunomagetic beads

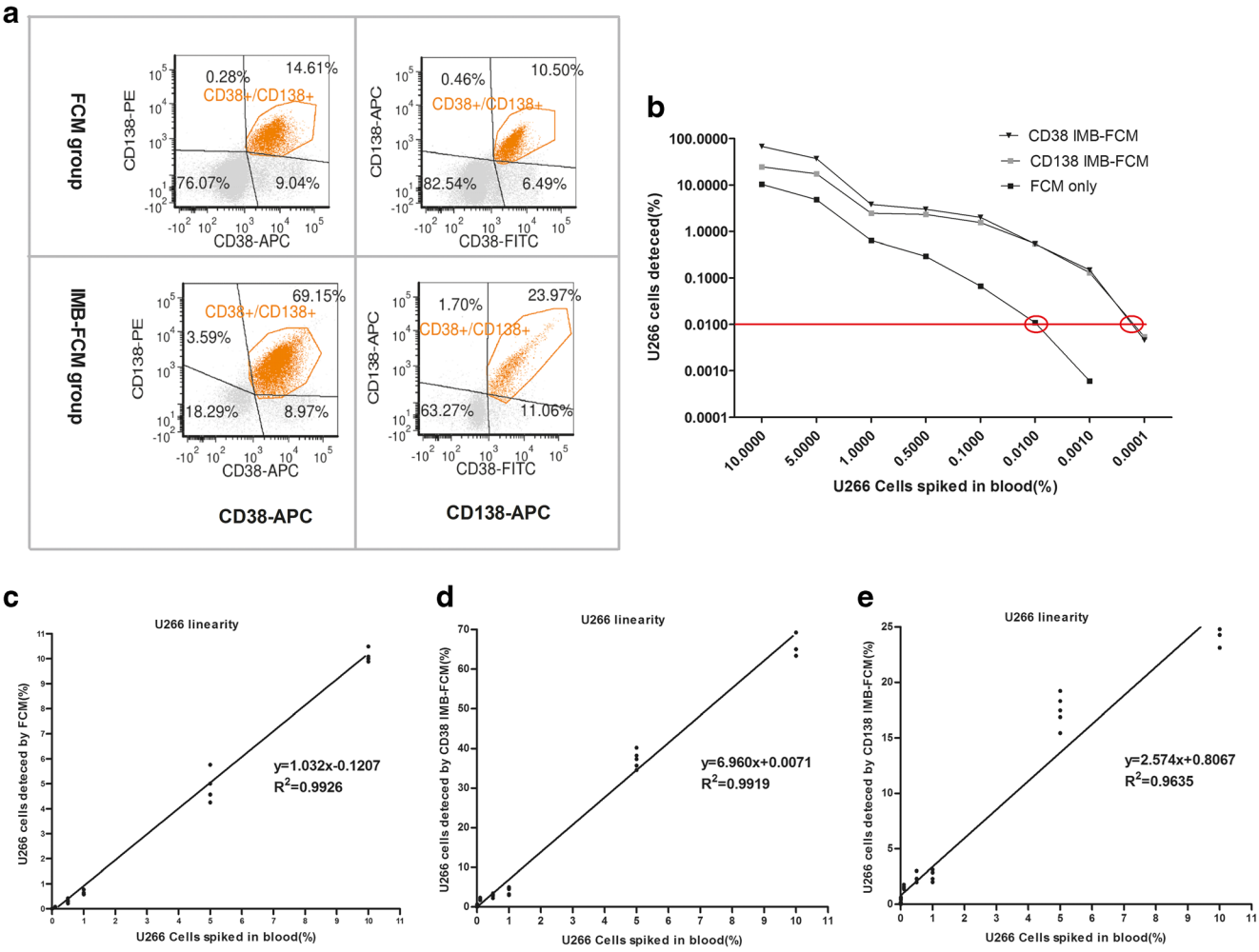

Fig. 3 Detection of U266 from healthy whole blood. **a** Flow cytometric analysis of U266 spiked in blood before and after IMB enrichment. **b** Changes of U266 cell concentration detected by FCM or IMB-FCM. Linearity of U266 cell capture using **c** FCM, **d** CD38 IMB-FCM, and **e** CD138 IMB-FCM. FCM, flow cytometry; IMB, immunomagetic beads

concentration (R^2 = 0.9926, 0.9919, and 0.9635, respectively, Fig. 3c~e). Herein, the detection efficiency of CD38/CD138-IMB-FCM was 6- and 2-fold higher than that of FCM, suggesting that the former was more sensitive. Additionally, detection of U266 using CD38 as a target antigen was better than CD138. Figure 3 b shows that the lower detection limit of myeloma cells by FCM and IMB-FCM was 0.01% and 0.001%, indicating that the sensitivity of FCM was increased by 10 times after magnetic bead enrichment. The curve demonstrated that when the tumor cell concentration was high (> 0.1%), the detection sensitivity of tumor cells based on CD38-APC magnetic sorting was higher than that of CD138-APC, and as the concentration decreased, the sensitivity of both decreased and tended to be consistent.

Association between treatment response and CMCs in MM patients detected by FCM and IMB-FCM

To explore the utility of the assay in MM, we collected 122 blood samples from patients to evaluate the transition of quantification of CMCs at various stages after treatment. CMCs were counted at new diagnosis (ND, n = 39), during partial remission (PR, n = 43) and complete remission (CR, n = 20), and at disease relapse (RA, n = 20). Simultaneously, CMCs were compared with bone marrow samples (MMCs) only measured by FCM.

When only utilizing FCM to detect MMCs and CMCs, the median values of MMCs were at ND, 7.5100×10^{-2} (range, 0.2660–91.6560); at RA, 6.0070×10^{-2} (range, 0.6000–56.9000); at PR, 1.0120×10^{-2} (range, 0.0400–5.6340); at CR, 0.0110×10^{-2} (range, 0.0010–0.6000); at control, 0.0021×10^{-2} (range, 0.0000–0.0080). The median values of CMCs were at ND, 3.0667×10^{-4} (range, 0.0000–373.0300); at RA, 2.0500×10^{-4} (range, 0.0000–365.0000); at PR, 1.0120×10^{-4} (range, 0.0333–3.0000); at CR, 0.1676×10^{-4} (range, 0.0000–0.8433); at control, 0.1000×10^{-4} (range, 0.0000–0.3000). Although MMCs and CMCs in CR patients and controls lacked the expression of CD19 or CD45, the cell number were extremely

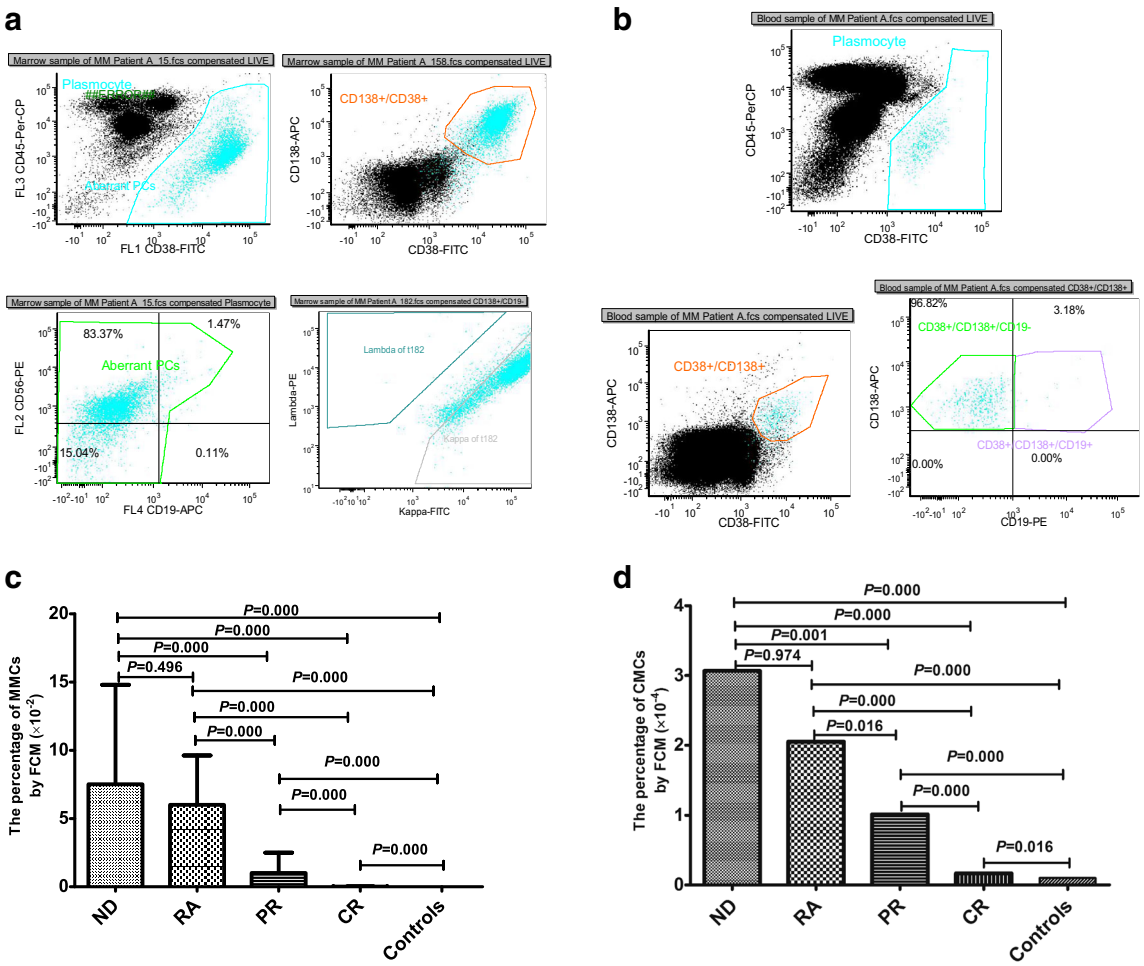

Fig. 4 Detection of myeloma cells by FCM in MM patient samples. **a** Flow cytometric analysis demonstrating $CD138^+/CD38^+/CD45^-/CD19^-/CD56^+$/intracellular kappa monoclonal MMCs. **b** Flow cytometric analysis demonstrating $CD138^+/CD38^+/CD45^-/CD19^-/CD56^+$ CMCs. **c** MMCs and **d** CMCs enumeration changes at various stages after treatment. FCM, flow cytometry; MMCs, marrow myeloma cells; CMCs, circulating myeloma cells; ND, new diagnosis; RA, relapse; PR, partial remission; CR, complete remission

low ($< 10^{-4}$) and did not show the light chain restrictive expression of cytoplasmic Ig, so no MMCs and CMCs were detected in both groups. Figure 4 c and d show that the proportions of CMCs and MMCs were gradually decreased at ND/RA, PR, and CR ($P < 0.005$), indicating that both of them were related to treatment efficacy. However, the number of MMCs and CMCs was not significantly different between ND and RA ($P = 0.496$, 0.974, respectively).

We also use the optimized IMB-FCM device to detected the 122-PB samples of MM patients, for the CD38 IMB-FCM group, the median values of CMCs were at ND, 0.1070×10^{-2} (range, 0.0001–13.9863); at RA, 0.1129×10^{-2} (range, 0.0001–9.090); at PR, 0.0421×10^{-2} (range, 0.0002–0.2000); at CR, 0.0005×10^{-2} (range, 0.0000–0.0167). Consistent with the above CMCs quantity fluctuations, patients exhibiting PR and CR as a result of therapy had fewer CMC counts than initial diagnosis; on the contrary, those patients who experienced relapse had increased CMC levels (Fig. 5c, d).

Seen from Fig. 5b, the number of CMCs in the CD138 IMB-FCM group was extremely close to that of the CD38 group ($P = 0.867$), and the median values of CMCs were as follows: at ND, 0.1097×10^{-2} (range, 0.0001–17.2721); at RA, 0.1570×10^{-2} (range, 0.0001–5.1608); at PR, 0.05210×10^{-2}(range, 0.0001–0.1900); at CR, 0.0012×10^{-2} (range, 0.0000–0.0821). The count of CMCs detected by IMB-FCM was significantly higher than that by direct FCM, but was still lower than the count of MMCs (Fig. 5b).

Correlation analysis between CMCs and MMCs

CMC analysis was compared with MMCs to verify whether CMCs could reflect tumor burden of bone marrow myeloma cells. The result revealed that, no matter whether CMC count was detected by IMB-FCM or FCM, it showed a significant

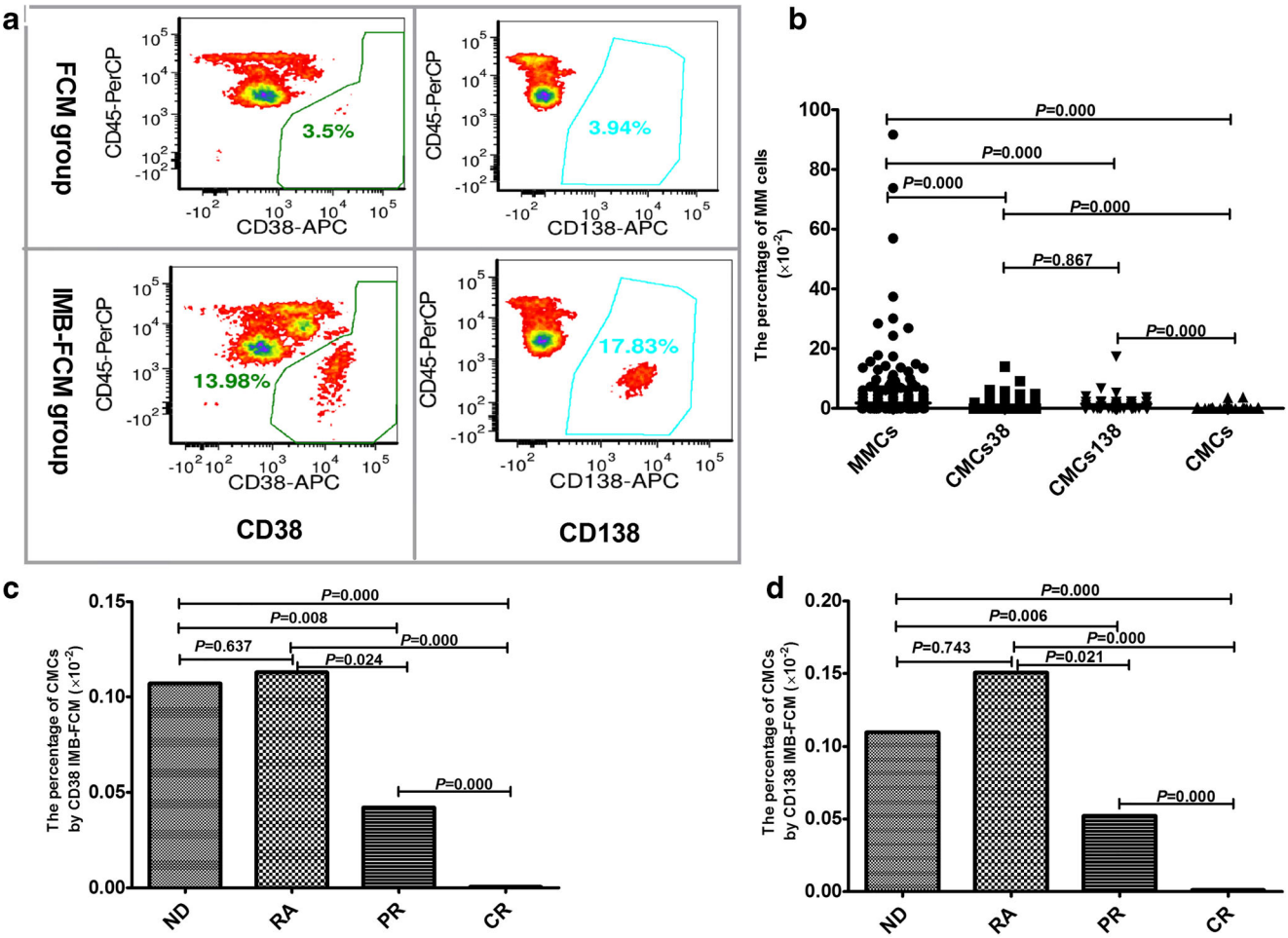

Fig. 5 Detection of CMCs by IMB-FCM in MM patient samples. **a** Flow cytometric analysis of CMCs before and after IMB enrichment. **b** Comparison analysis of myeloma cells according to different detection methods and sample type. **c** CD38 IMB-FCM and **d** CD138 IMB-FCM for CMCs enumeration changes at various stages after treatment. FCM, flow cytometry; IMB, immunomagetic beads; MMCs, marrow myeloma cells detected by FCM; CMCs38, circulating myeloma cells detected by CD38 IMB-FCM; CMCs138, circulating myeloma cells detected by CD138 IMB-FCM; CMCs, circulating myeloma cells detected by FCM; ND, new diagnosis; RA, relapse; PR, partial remission; CR, complete remission

association and strong correlation with MMCs as measured only by FCM ($r = 0.697, 0.732, P = 0.000$). Moreover, CMCs captured by IMB-FCM were also strongly correlated with those measured only by FCM ($r = 0.894$, $P = 0.000$).

MRD measurement in BM and PB

According to the sensitivity of FCM/IMB-FCM to detect U266 spikes and MRD, MM patients were divided into $CMCs^+$ and $CMCs^-$ groups. Seen from Table 1, CMC positive rate was not significantly different among ND, RA, and PR patients ($P > 0.05$), but CMC detection rate increased, especially at PR when captured by IMB-FCM ($P < 0.05$). It was interesting to note that there were 2 patients with CR captured CMCs by CD38 IMB-FCM and 1 patients captured CMCs by CD138 IMB-FCM, but when applied only FCM, no minimal residual cells were detectable in both PB and BM samples.

Although all FCM-negative BM samples were also paired with FCM/IMB-FCM-negative PB samples among ND, RA, and PR patients, FCM-positive BM samples were accompanied by IMB-FCM-positive results in 88% of corresponding PB samples (Table 1). Overall, the positive rate of MRD in PB samples detected by IMB-FCM was significantly higher than that by direct FCM, but was still lower than that in the corresponding BM samples.

Clinical characteristics of $CMCs^+$ MM patients

To ascertain the ability of CMCs as an indicator for keeping track of MM, CMC analysis was compared with other clinical measures of disease burden. The results indicated that CMC counts had significant association with elevated β2-MG, sCrea, and DS and ISS stages, and more serious anemia, bone destruction, and renal impairment (Table 2, $P < 0.05$), whereas CMC counts did not correlate closely with LDH, Ca,

Table 1 Correlation between MRD levels in BM and PB at different treatment response groups

Treatment response groups	MRD^+ in BM No. (%)	MRD^+ in PB No. (%)	$MRD38^+$ in PB No. (%)	$MRD138^+$ in PB No. (%)
ND (n = 39)	39 (100)	27 (69.2)	34 (87.2)	34 (87.2)
RA (n = 20)	20 (100)	14 (70)	17 (85)	17 (85)
PR (n = 43)	41 (95.3)	26 (60.5)	37 (86)	36 (83.7)
CR (n = 20)	0	0	2 (10)	1 (5)
Total (n = 122)	100 (82)	67 (54.9)	90 (73.8)	88 (72.1)

ND, new diagnosis; *RA*, relapse; *PR*, partial remission; *CR*, complete remission; *FCM*, flow cytometry; *IMB*, immunomagetic beads; *MRD^+ in BM*, minimal residual disease in bone marrow detected by FCM; *MRD^+ in PB*, minimal residual disease in peripheral blood (CMCs) detected by FCM; *$MRD38^+$ in PB*, minimal residual disease in peripheral blood (CMCs) detected by CD38 IMB-FCM; *$MRD138^+$ in PB*, minimal residual disease in peripheral blood (CMCs) detected by CD138 IMB-FCM

and Alb (Table 2, $P > 0.05$). Taking the presence or absence of CMCs as the dependent variable and the meaningful indicators of univariate analysis as independent variables, logistic regression analysis was performed. The results with 1.679 and 2.388 of regression coefficients, 5.362 and 10.886 of OR values, indicated that elevated β2-MG and moderate-to-more anemia were significant risk factors for the presence of CMCs (Table 3, $P < 0.05$)

Discussion

Bone marrow myeloma cell (MMC) examination is a gold standard for reflecting tumor burden and a prognostic factor for evaluating the therapeutic effect [3], but considering the invasive nature of bone marrow analysis, resorting to a "liquid biopsy" analysis of tumor cells in the circulation seems to be a better alternative [9, 16]. Therefore, determining the feasibility of CMCs to reflect tumor burden and the establishment of CMC detection device will facilitate the ability of CMCs to monitor and diagnose residual disease and relapse. Especially, our results have demonstrated that the potential of IMB-FCM for CMC detection has the advantage of being noninvasive in the attempt to monitor tumor burden and can serve as a complement to bone marrow aspiration or at least mitigate its effects on MM patients.

Studies have shown that CMCs detected by FCM could predict poorer survival outcomes [12, 13] and track early recurrence or disease progression [14, 15] of MM patients, but the sensitivity of FCM is only 10^{-4} [21], making it difficult to accurately determine the quantity of rare CMCs [16]. We established methods to enrich CMC-

Table 2 Clinical characteristics of 122 MM patients

Variables	$CMCs^+$ (n = 67) No. (%)	$CMCs^-$ (n = 55) No. (%)	P value
Median age, (years (range))	60 (40–82)	59 (41–81)	0.659
Sex, male	30 (44.8)	31 (56.4)	0.203
Hemoglobin < 85 g/L	30 (44.8)	3 (5.5)	0.000
Albumin < 35 g/L	22 (32.8)	18 (32.7)	0.990
Beta2-microglobulin > 3.5 mg/L	52 (77.6)	12 (21.8)	0.000
Creatinine > 177 μmm/L	21 (31.3)	6 (10.9)	0.007
Calcium > 2.65 mm/L	3 (4.5)	0 (0)	0.317
LDH > 245 U/L	16 (23.9)	10 (18.2)	0.444
Bone destruction	50 (74.6)	27 (49.1)	0.004
Renal inadequacy	31 (46.3)	11 (20.0)	0.002
DS stage			0.012
I–II	14 (20.9)	23 (41.8)	
III	53 (79.1)	32 (58.2)	
ISS stage			0.007
I–II	25 (37.3)	34 (61.8)	
III	42 (62.7)	21 (38.2)	

LDH, lactate dehydrogenase; *DS*, Durie-Salmon; *ISS*, International Staging System

Table 3 Multivariable logistic regression analysis of factors predicting presence of CMCs

Variables	OR	95% CI	*P* value
Hemoglobin < 85 g/L	5.362	1.252~22.976	0.024
Beta2-microglobulin > 3.5 mg/L	10.886	3.343~35.451	0.000
Creatinine > 177 μmm/L	0.498	0.099~2.509	0.398
Bone destruction	1.859	0.671~5.154	0.233
Renal inadequacy	1.255	0.329~4.785	0.739
DS III stage	2.047	0.699~5.995	0.192
ISS III stage	0.903	0.287~2.837	0.861

OR, odds ratio; *CI*, confidence interval; *DS*, Durie-Salmon; *ISS*, International Staging System

combined magnetic beads labeling anti-APC antibody with FCM, named IMB-FCM platform, which was more sensitive and with higher reproducibility than FCM alone. Herein, we developed two target antibody cocktails to enrich, recognize, and identify CMCs using CD56-FITC/CD138-PE/CD45-PerCP/CD38-APC panel and CD38-FITC/CD19-PE/CD45-PerCP/CD138-APC panel. CD38-APC and CD138-APC antibody played an important role in connecting magnetic beads and plasma cells; more importantly, U266 cell magnetic bead sorting results exhibited that APC fluorescence efficiency was not affected by upstream magnetic bead sorting operation and can be used for downstream FCM analysis without the competition with IMB for immune epitopes. Thence, for both kit configurations, cell population of $CD38^+/CD138^+$ phenotype was classified as plasma cells and plasma cells with expression of $CD56^+$ and/or $CD19^-$ and/or $CD45^-$ were further identified as malignant plasma cells.

The application of CD138/CD38 antigen combination to detect CMCs offers unique advantages over previous research measured by conventional tools. It is reported that downregulation of CD138 expression may occur in CMCs and MRD cells during chemotherapy and hypoxia [29]. Absence of CD138 in MM naive cells [27] and CD138 antigen shedding during long specimen storage [28] can lead to decreased CMC number. In contrast, CD38, another important marker, is strongly expressed [26] and is rarely downregulated in MM cells, and desirable for the purification and screening of MM cells, but is also target for modern monoclonal antibody therapies, such as daratumumab [16] and non-specifically expressed in other lines of cells [20]. To improve assay specificity and sensitivity in CMCs quantification, CD38-APC and CD138-APC are simultaneously used for enrichment of CMCs, followed by the addition of CD138-PE or CD38-FITC to their respective combination for downstream FCM analysis.

U266-spiked experiments displayed that the lower detection limit of myeloma cells by FCM and IMB-FCM was 0.01% and 0.001%. Obviously, the sensitivity of FCM for detection of tumor cells after enrichment with magnetic beads is increased by 10 times, but the detection limit is only reduced by one order of magnitude. It is reported that immunomagnetic beads combined with various detection techniques can effectively reduce detection limits by two orders of magnitude or more [30, 31]. Our inferior results may be related to the decrease in the efficiency of IMB sorting as the number of myeloma cells reduced [32] and the possible loss of targeted cells during IMB enrichment. Therefore, large sample size and standardized procedure are still needed to optimize enrichment conditions, combined with other advanced technologies such as NGS [19], next-generation flow (NGF) [33], and whole-genome/exome sequencing (WGS/WES) [34, 35] to further improve sensitivity of CMC detection at genetic, molecular, and protein levels. The linearity experiments showed that the detection efficiency of CD38/CD138-IMB-FCM for U266 was 6 and 2-fold higher than that of FCM; the better CD38 sorting efficiency than CD138 may be associated with the presence of population of $CD138^-/CD38^+$ cells in the U266 cell line. Overall, IMB-FCM has distinct advantages over FCM alone in detecting rare cells.

When using IMB-FCM to enrich and analyze CMCs in MM patients, the positive detection rate of CMCs increased from 60.5~70.0 to 85~87.2% in newly diagnosed/relapsed and PR patients as compared with only by FCM measurement. FCM-positive BM samples were accompanied by IMB-FCM-positive results in 88% of corresponding PB samples, indicating that the positive rate of MRD in PB samples detected by IMB-FCM was significantly higher than that by direct FCM. Especially, no CMCs and MMCs were detectable by FCM among 20 cases of CR patients, 2 patients of whom do contain measureable amounts of CMCs by IMB-FCM. This could be attributed to two factors: one is the problems associated with sampling bone marrow and the other is the fact that the relatively low frequency of CMCs in the circulation makes detection difficult without enrichment [16]. However, regardless of whether or not enrichment techniques were applied to CMCs, patients exhibiting partial and complete remission upon therapy had much lower CMC and MMC counts than initial diagnosis; meanwhile, patients who experienced relapse had increased CMC and MMC levels. Our results are consistent with Fujisawa et al.'s observations [36] that CMC count gradually decreased among patients with baseline/relapse, PR, and CR, and gradually increased among patients with CR, VGPR, and PR, indicating that the CMC count was related to disease progression and therapeutic effects. Our study displayed that CMC count detected by IMB-FCM or FCM showed a significant association and strong correlation with MMCs as measured only by FCM,

indicating that the number of myeloma cells in peripheral blood fluctuated synchronously with those in bone marrow, and CMCs in the circulation can reflect the tumor burden of MM patients. It has been reported that CMCs reduced as disease remits and increased as disease progresses, and evaluation of CMC kinetics, as a tumor response indicator, can provide important clinical information in the early identification and individualized treatment of aggressive disease [14]. However, CMC burden in peripheral blood is reported to be 100-fold lower than in bone marrow [11, 17], and in our study, CMC levels even with enrichment remain far lower than MMCs. This may be due to the low proliferation rate of CMCs, resulting from its independence from the bone marrow microenvironment and bone marrow mesenchymal cells [17]; on the other hand, harmful immune surveillance function, low concentration of promoting cell growth factors, and harsh growth environment in the circulation are reasons for the short life of CMCs [37].

The association between CMCs and MM prognostic indicators remains controversial. Some studies [38] showed that the presence of CMCs appeared to be largely independent of the levels of MMCs and β2-MG. However, in other studies [13, 18, 36], the levels of CMCs correlated more closely with decreased Hb and elevated MMCs, β2-MG, LDH, and DS and ISS stages. Our study supports the latter's relevant viewpoints [15, 18]. Patients with CMCs have higher β2-MG, sCrea, and DS and ISS stages, and more serious anemia, bone destruction, and renal impairment, indicating that CMCs strongly correlated with tumor burden and high-risk stage in MM patients. Logistic regression analysis showed that CMCs was more frequently detected in patients with elevated β2-MG and severe anemia, and more attention should be paid to follow up and thereby adopt appropriate treatment for such patients.

The specific pathophysiologic mechanism of CMCs in disease progression and prognosis is an open topic for discussion. CMCs have different cytogenetic abnormalities from MMCs [18]; downregulation of integrins (CD11a/CD11c/CD29/CD49d/CD49e), adhesion molecules (CD33/CD56/CD117/CD138), and activation molecules (CD28/ CD38/CD81) on the surface of CMCs showed a static phenotype [39]; the number of CMCs was positively correlated with the increase of neovascularization in the bone marrow [40]. WES of CMCs and corresponding MMCs indicated that CMCs acquired additional genetic mutations as CMCs migrate, invade, and spread into the extramedullary environment, gradually evolved into specific subclones from bone marrow plasma cell clones [34, 35]. Previous studies on the differences between CMCs and MMCs in surface antigens, cytogenetics, and gene mutations have achieved huge progress, but these studies only stayed at the initial or active stage of MM and did not monitor CMC kinetic distribution with changes in treatment efficacy. Moreover, during disease progression and treatment, molecular property of tumor cells is changed and drug-resistant subclones are formed [10]. Therefore, static analysis of molecular and genetic changes in CMCs and MMCs at a certain treatment point does not fully explain the role of CMCs in pathogenesis and disease progression. Future analyzes should adopt large-sample study and refine treatment response groups in MM to dynamically and deeply analyze the immunophenotype, genomic, and molecular characteristics of CMCs and MMCs for further revealing the mechanism of CMC metastasis into peripheral blood.

In conclusion, the sensitivity of IMB-FCM was 10-fold higher than FCM and was able to increase the positive detection rates of CMCs for monitoring MRD and relapse upon the peripheral blood of MM patients. Detection of CMCs may complement or minimize bone marrow aspiration in the future treatment of MM patients. Further multicentre studies designed with larger sample size to determine the role of these findings are warranted.

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Author contribution Baohong Yue conceived the idea and investigated the research study. Ningning Wang designed the study, performed research, analyzed and interpreted data, and wrote the manuscript. Shuai Liu guided and validated the statistical analysis. NahomTesfaluul, Jia Li, and Xiaojuan Gao contributed to writing and reviewing of the manuscript.

Compliance with ethical standards All study subjects submitted informed consent, and the study was approved by the Medical Research and Research Ethics Committee of the First Affiliated Hospital of Zhengzhou University.

Conflict of interest The authors declare that they have no conflict of interest.

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