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LETTER TO THE EDITOR

A single-tube multiparameter seven-colour flow cytometry strategy for the detection of malignant plasma cells in multiple myeloma

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The diagnosis and follow-up of multiple myeloma (MM) include the detection of malignant plasma cells (PCs) in the bone marrow (BM). Morphological observation is useful to establish the size of PC infiltration, yet does not allow to differentiate between malignant cells and the residual normal component of PCs. Multiparameter flow cytometry (MFC) is an additional valuable tool, very sensitive in differentiating normal and abnormal PCs.[1] The latter are identified by their restricted cytoplasmic immunoglobulin light chains together with aberrant patterns of surface antigen expression.[1] Indeed, normal polyclonal PCs retain the expression of CD19 and CD45, whereas lacking that of CD56 and CD28[refs 2,3]. Conversely, abnormal isotype-restricted PCs are most often negative for CD19 and CD45, yet frequently acquire surface CD56 and/or CD28 sometimes with such myeloid antigens as CD33 or CD117[refs 4,5]. Several MFC combinations have therefore been proposed in the literature to differentiate between abnormal and normal PCs in BM samples. The four-colour strategy of Rawstron *et al.*[6] proposed to combine a backbone of CD38, CD138 and CD45 associated with differentiating surface antigens or cytoplasmic light chains as clonality markers. Six-colour MFC panels later combined the CD38/CD138/CD45 backbone with CD19 and light chain restriction assessment.[7] This strategy limits the exploration of PC aberrancies to variations of the expression of CD19 and CD45, yet, among markers identified to be more specifically associated to malignant PCs, CD56 and CD28 appear to be of great value.[3] Indeed, we have observed that the combination of these two markers allows for the detection of abnormal PCs in 82% of MM cases (personal data). We devised a seven-colour/eight-antibody combination, adding antibodies to these two antigens to the combination recommended by Tute *et al.*,[7] and applied this strategy to 23 normal BM and 121 specimens from MM patients collected for minimal residual disease (MRD) detection. All patients provided informed consent according to the ethical rules of the institution, and BM sampling was a part of their medical management. BM collected on EDTA was processed for MFC after macrovolume red blood cell lysis (Versalyse, Beckman Coulter, Miami, FL, USA) and washes in phosphate-buffered saline (PBS). The cell suspension (4×10^6 cells/50 μl) was first stained for surface antigens, washed, and then permeabilized (Intrastain, Dako, Glostrup, Denmark) for intracytoplasmic immunoglobulin light chain detection. The seven-colour combination of monoclonal antibodies consisted in fluorescein isothiocyanate-conjugated anti-lambda (λ) light chains (BD-Biosciences, San Jose, CA, USA), PE-conjugated CD28 and CD56 (BD-Biosciences), PC5-conjugated CD138 (Beckman Coulter), PECy7-conjugated CD19 (Beckman Coulter), APC-conjugated anti-kappa (κ) light chains (BD-Biosciences), APCH7-conjugated CD45 (BD-Biosciences) and HorizonV450-conjugated CD38 (BD-Biosciences). After incubations for 10 min at room temperature and washes in PBS, the cells were resuspended in 1% paraformaldehyde-PBS and processed for MFC analysis on a FACSCanto II (BD-Biosciences) with FACSDiva v6.2 acquisition and analysis software (BD-Biosciences). In order to reach a sensitivity of 2.5×10^{-5} and the detection of at least 50 neoplastic cells (positive MRD), 2×10^6 events or more were acquired.

Two strategies of analysis were applied as shown in Figure 1 to compare the efficiency of Tute's six-colour approach[7] with that of our seven-colour approach. PC subset's clonality was considered for κ/λ ratios higher than 3 or lower than 0.66. Account was taken of the presence of residual normal PCs, using the normal κ/λ ratio observed in the CD19+/CD28−/CD56− quadrant. Statistical analyses (Kolmogorov–Smirnov and Mann–Whitney) were carried out using Medcalc software (Mariakirke, Belgium). PC subsets were expressed as a percentage of total $CD138^+/CD38^{++/-}$ PCs. The partition of κ- and λ-chains was assessed as percentages within each subset. Abnormal light-chain-restricted PCs were expressed as the percentage of total nucleated cells, taking into account all patterns of CD19/CD28/CD56 expression.

PCs were detected in all MM (median 0.113% of nucleated cells, range 0.003–13.7) and normal BM samples (median 0.29%, 0.037–1.2). In MM cases, this population was composed of a mixture of normal (median 0.089%, 0.003–1.45) and abnormal cells (median 0.163%, 0.000–13.7).

The display of $CD138^+/CD38^{++/-}$ cells according to their expression of CD19 and/or CD56/CD28 allowed to identify four subsets, respectively, CD19+CD56/CD28− (subset 1), CD19+CD56/$CD28^+$ (subset 2), CD19−CD56/CD28+ (subset 3) and CD19-CD56/CD28- (subset 4), both in normal and MM BM samples. Comparison of the proportions of each of these subsets among PCs between normal BM, MM samples with MRD and MM samples without MRD is shown in Figure 2a. The proportion of subset 1 was significantly higher in samples from patients without MRD (median 72,4%, 42.9–89.7) compared with both normal BM (median 54%, 35–94, $P<0.0001$) and samples with detectable MRD (median 54%, 0.2–89.6, $P=0.003$). Subset 2, in which CD19 is coexpressed with either or both CD28 and CD56, was the smallest, with a median of 7% (range 0–19.3) of PCs in normal individuals and 7% (range 1.3–34.9) and 8.2% (range 0.8–96.4) in MM patients without or with MRD, respectively. Subsets 3 and 4, both lacking CD19 expression, were in significantly different proportions in the three groups of patients tested. Subset 3, with the expression of CD28 and/or CD56, was the most represented in MM patients with MRD (median 18.5%, 0.7–98), significantly more than in controls (median 12%, 0.2–30.8, $P=0.01$) and even more than in MM patients without MRD (median 4.1%, 0.4–27, $P<10^{-4}$). It is noteworthy that the difference was also significant between the latter population and normal BM ($P=0.001$). Conversely, subset 4 was significantly lower in MM patients with MRD at a median of 7.75% (range 0–46.8) compared with 23% (range 1.7–37, $P<10^{-5}$) in normal BM and 14.1% (range 1.8–39.1, $P=0.0004$) in MM patients without MRD. Again, the two latter populations also differed significantly ($P=0.005$).

The expression of intracytoplasmic κ- and λ-chains characterized by definition the polyclonality of the four immunophenotypic subsets of PCs in normal BM samples and in those from MM patients without MRD. In MM patients with positive MRD,

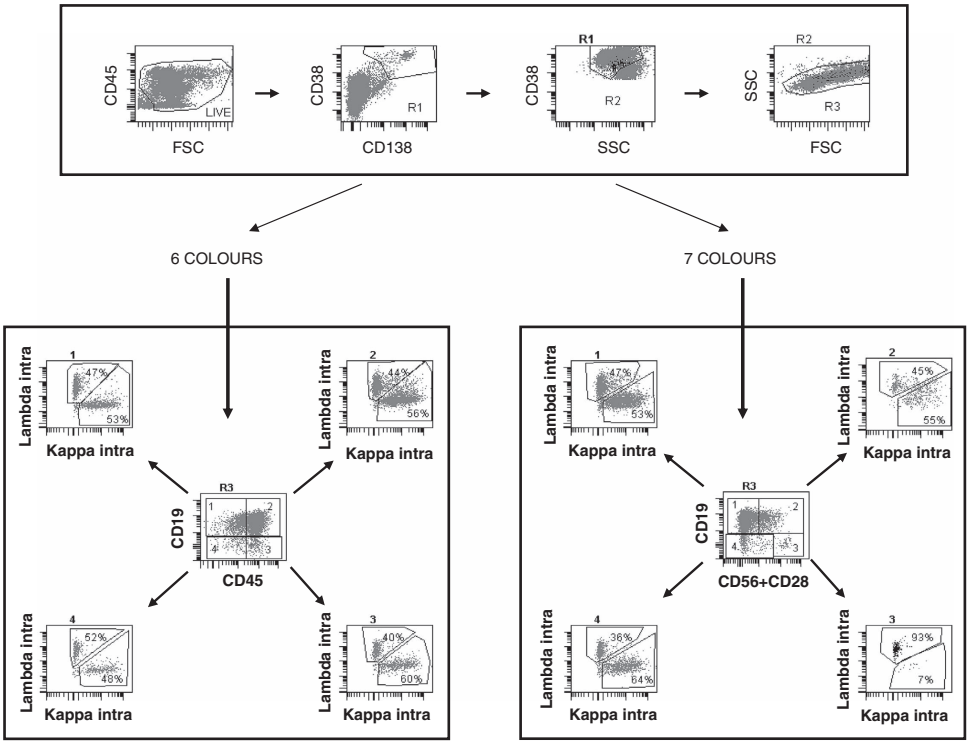

Figure 1. Analysis of the data from a single-tube seven-colour staining with a six-colour strategy (left) or a seven-colour strategy (right). After defining a nucleated cell gate on a FSC/SSC scattergram and excluding debris on an FSC/CD45 gate, PCs are first included in a broad R1 gate encompassing $CD138^+/CD38^{++}$ or $CD38^-$ cells. This population is refined on an SSC/CD38 scattergram conditioned on R1, thereby defining gate R2. Cells satisfying both R1 and R2 are then displayed on an FSC/SSC scattergram and included in an R3 gate. For six-colour analysis (left bottom panel), cells in R3 are displayed on a CD19/CD45 scattergram, allowing to define four populations as shown in the centre of the panel. For each of them, a κ/λ scattergram is established to discriminate normal polyclonal PCs and MM-restricted PCs. The right bottom panel shows that, in the seven-colour strategy, intracytoplasmic light chain restriction is examined among four different populations, delineated on the basis of the expression or not of CD19 combined to the mixture of CD28 and CD56 (either or both antigens expressed when positive). CD45, which can be abnormally expressed on the clonal population as shown in the six-colour strategy, is examined on a different plot. In this sample (same list mode), the six-colour strategy fails to identify light chain restriction (MRD $<0.24\times10^{-4}$), whereas the seven-colour strategy reveals that 93% of the cells in subset 3 ($CD19^-/CD56/CD28^+$) use lambda chains, thereby characterizing abnormal PCs (MRD 1.25×10^{-4}).

light chain restriction was never observed in subset 1 ($CD19^+$ $CD56/CD28^-$). Abnormal PCs were mostly present in subset 3 (47/50 samples) but were also found in subset 2 in four samples and in subset 4 in eight samples. Light chain restriction was concomitantly observed in subsets 2 and 3 in three cases and in subsets 3 and 4 in another three cases. Three samples displayed light chain restriction in subsets 2, 3 and 4. Abnormal MM cells were found only in subset 2 in one case and only in subset 4 in two other samples.

The 121 samples obtained from MM patients were tested in seven colours, and, as reported above, 50 were found to contain at least 2.5×10^{-5} abnormal PCs and therefore considered MRD-positive. All these samples were also interpreted using the six-colour strategy described above. Consistent data were found with both methods in all samples qualified as containing more than 2×10^{-4} abnormal PCs. There were four samples with more than 1% of abnormal PCs, 12 having between 10^{-3} and 10^{-2} and 17 having between 2×10^{-4} and 10^{-3}. However, of the 17 samples with less than 2×10^{-4} abnormal PCs, 7 were found to have undetectable MRD with the six-colour strategy (Figure 2b). In this specific subset of patients, the difference in abnormal PC counts was statistically significant between the seven-colour strategy (median 8.3×10^{-5}, range 2.6–14.7) and the six-colour strategy (median 3.7×10^{-5}, range 0–3.7, $P=0.0002$); $P<0.0001$ (Figure 2c).

Abnormal PCs display several immunophenotypic aberrancies delineating a MM-associated immunophenotype or MAIP by assimilation to the leukemia associated immunophenotypîc patterns of leukaemias. However, formal identification of clonal PCs ultimately relies on the characterization of light chain usage restriction. CD19 loss associated with an abnormal expression of CD28 and/or CD56 has been considered to be pathognomonic of abnormal PCs. However, we show here that the four combinations of CD19 and CD28 and/or CD56 expression can be observed also among normal non-restricted $CD138^+/CD38^{++/-}$ PCs. These data are consistent with those published in normal BM by Pecelunias *et al.*,[8] in which four subsets were delineated, based on the respective expression patterns of CD19 and CD56. By combining CD28 and CD56 detection with antibodies conjugated to the same fluorochrome, we increased the probability of detecting PCs with aberrant expression of these markers.

However, only clonotipy allowed to definitely assign PCs to the MM compartment in MRD samples from MM patients. Indeed, the partition of polyclonal subsets still differed significantly between the BM samples from normal controls and treated MM patients without detectable MRD.

Initially, four-colour immunophenotypic investigation of MRD in patients with MM has proven to be feasible and applicable on 90–95% of all cases, at a sensitivity of 10^{-4} (2, 3, 15). However, in the study by Tute *et al.*,[7] only 71% of tested samples allowed to establish the patient's MRD status. In our seven-colour assay, MRD could be assessed in all samples and was detected in three of the four subsets of MAIP. More importantly, by analysing the same listmodes with a six-colour or a seven-colour strategy, we could

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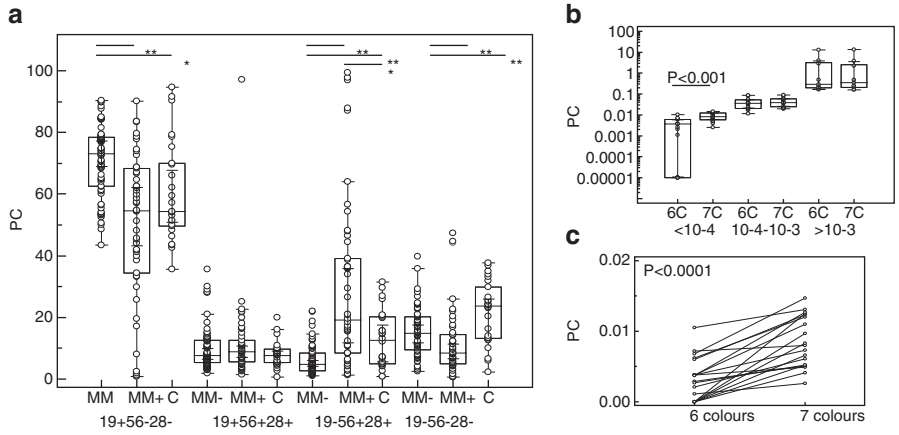

Figure 2. (**a**) Partition of plasma cells within the four subsets defined in seven-colour staining, based on the expression or not of CD19 and/or CD28/CD56 within samples with no MRD (MM−), positive MRD (MM+) or from normal controls (C). Statistically significant differences are indicated by asterisks: ** < 0.0001, * < 0.01. (**b**) Comparison of MRD results for the same listmodes interpreted with a six (6C)- or a seven (7C)-colour strategy. Data do not differ for samples with more than 1×10^{-4} cells, but the seven-colour strategy is more efficient to detect the lowest levels (** < 0.0001). (**c**) Paired comparison of the 17 samples with positive MRD below 10^{-4} in the seven-colour strategy analysed in six (left) or seven (right) colours.

directly demonstrate the increase in sensitivity of the seven-colour option. Finally, it should be stressed that this strategy does not depend on an initial definition of abnormal PCs at diagnosis as it allows for the detection of light chain restriction in one or more immunophenotypic subsets of PCs, thereby allowing for subclones assessment.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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