Circulating clonotypic B-cells in multiple myeloma and monoclonal gammopathy of undetermined significance

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Circulating clonotypic B-cells in multiple myeloma and monoclonal gammopathy of undetermined significance

Running heads: Clonotypic B-cells in plasma cell neoplasms

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ABSTRACT

The B-cell compartment where the multiple myeloma stem cell resides, remains unclear. We investigated the potential presence of mature surface-membrane immunoglobulin-positive B lymphocytes clonally-related to the tumoral bone marrow plasma cells among different subsets of peripheral blood B-cells from 10 patients (7 multiple myeloma and 3 monoclonal gammopathies of undetermined significance). Presence of clonotypic immunoglobulin heavy chain gene rearrangements was determined in multiple highly-purified fractions of PB B-lymphocytes including surface membrane IgM+ CD27- naïve B-lymphocytes, plus surface membrane IgG+, IgA+ and IgM+ memory CD27+ B-cells, and normal circulating plasma cells, in addition to (mono)clonal plasma cells, by a highly-specific and sensitive allele-specific oligonucleotide polymerase chain reaction directed to the CDR3 sequence of the rearranged IGH gene of tumor plasma cells from individual patients. Our results showed systematic absence of clonotypic rearrangements in all different B-cell subsets analyzed, including, M-component isotype-matched memory B-lymphocytes, at frequencies <0.03 cells/µL (range: 0.0003-0.03 cells/µL); the only exception were the myeloma plasma cells detected and purified from the peripheral blood of 4/7 myeloma patients. These results indicate that circulating B-cells from patients with multiple myeloma and monoclonal gammopathies of undetermined significance are usually devoided of clonotypic B-cells while the presence of immunophenotypically aberrant myeloma plasma cells in peripheral blood of myeloma patients is a relatively frequent finding.

Keywords: myeloma, leukemic stem cell, monoclonal gammopathies
INTRODUCTION

A major challenge in the pathogenesis of both multiple myeloma (MM) and monoclonal gammopathy of undetermined significance (MGUS) has been the identification of the cell of origin of both neoplasias. Monoclonal plasma cells (M-PC) from MM usually show a low proliferative capacity as assessed by labelling indices of <0.5% \(^1\); this finding supports the notion that pre-PC compartments may contain the proliferative M-PC progenitor. In line with this hypothesis, circulating peripheral blood (PB) cells clonally identical to bone marrow (BM) M-PC have been recurrently detected in MM\(^2-7\). Apart from the presence of the malignant \(IGH\) gene rearrangement, these PB cells have also been found in some patients to carry karyotypic alterations -e.g. trisomy 11, del(17p)-, and oncogenic proteins (e.g. \(IGH\)-\(FGFR3\), \(IGH\)-\(MMSET\)) expressed by CD138\(^+\) M-PC\(^6-8\). Moreover, intraclonal heterogeneity as assessed through the analysis of the \(IGHV\) gene sequence\(^9\) or through the investigation of cytogenetic alterations\(^10\) and gene mutations evaluated by whole exome sequencing\(^11\), has been reported within the M-PC population, suggesting that the tumor PC compartment could be continuously repopulated by more than one stem cell.

Currently, there is a growing body of evidences showing the presence of circulating aberrant PC not only in MM but also in MGUS patients\(^12-16\) and recent studies have demonstrated the presence of cells sharing stem cell properties within the compartment of M-PC\(^17\). However, the question that remains to be answer is whether or not clonotypic cells are also identifiable in PB B-cell subsets, which would be the “earliest clonotypic cells”. Clonotypic cells were first identified in a sorted CD19\(^+\) fraction\(^2\) and the pattern of somatic hypermutation in the VH regions of the \(IGH\) gene suggested that the malignant cell had passed through the germinal center (GC)\(^18\). This was further supported through the detection of PB clonotypic memory B-cells in MM patients\(^2,6\) and the finding that \textit{in vitro} clonogenicity was reduced when memory B-cells were removed\(^19\). In contrast, screening for cells with a pre-PC phenotype in cell lines\(^4,19-21\), and analysis of the engraftment of PB B-cells from MM patients into
immunodeficient mice\textsuperscript{3,19,22,23} have provided inconsistent findings. In parallel, depletion of B-cells by rituximab therapy has shown no clear beneficial clinical effect in MM, except for a small fraction of patients with CD20\textsuperscript{+} PC\textsuperscript{24,25}, and evaluation of the clonal hierarchy in light chain-secreting myeloma did not detect clonotypic circulating cells, except for one patient showing PB infiltration by malignant PC\textsuperscript{26}. One explanation for these discrepant results is a simple technical pitfall: the B-cells analyzed could be contaminated in some cases by circulating PC. In fact, to the best of our knowledge, no highly-sensitive molecular analysis of different compartments of highly-purified circulating B-cells specifically devoided of contaminating circulating M-PC, has been performed so far, to confirm or rule out the presence of clonotypic B-cells in MM and MGUS.

Here, we investigated the presence of circulating B-cells which would be clonally related to the M-PC in patients with MM and MGUS, using an allele-specific oligonucleotide polymerase chain reaction (ASO-PCR) technique aimed at sensitive and specific detection of IGHVDJ monoclonal gene sequences unique for the tumor cells of individual patients. Such analysis was performed in multiple different highly-purified, maturation-associated subsets of PB B-cells (\textit{sm}IgM\textsuperscript{+}/CD27\textsuperscript{+} naïve, isotype non-switched \textit{sm}IgM\textsuperscript{+}, and isotype-switched \textit{sm}IgG\textsuperscript{+} and \textit{sm}IgA\textsuperscript{+} memory B-lymphocytes and PC) from a group of 7 MM and 3 MGUS patients. Our results indicate that if present, clonotypic B-lymphocytes would circulate in PB at extremely low levels within the different B-cell compartments analyzed, at the same time we confirmed that the majority of MM patients show detectable numbers of circulating M-PC.
METHODS

Patients and samples. A total of 10 patients (7 MM and 3 MGUS) diagnosed with monoclonal gammapathies (MG) were studied. In all cases, paired BM and PB samples were collected at diagnosis for further immunophenotypic characterization, isolation, and molecular analysis of M-PC and other B-cell subsets, after informed consent was given by each individual. The study was approved by the Ethics Committee of the Cancer Research Center of the University of Salamanca, (Salamanca, Spain) and it followed the Helsinki Declaration protocol. The most relevant clinical and biological characteristics of the patients studied are summarized in Supplementary Table 1.

Multiparameter flow cytometry immunophenotypic studies and cell purification. Multiparameter flow cytometry (MFC) immunophenotypic studies were performed as recently described (and described in the Online Supplementary Methods) in both BM and PB samples. Fluorescence-activated cell sorting was used to purify BM M-PC as well as PB B-cell subsets and N-PC; whenever present, circulating M-PC were also purified. The purity of the sorted M-PC and the multiple PB normal B-cell subsets was systematically ≥98%, and only those PB B-cell fractions which showed no contamination (<0.01%) by circulating PB M-PC were included in the study; all other fractions were not tested (Table 1).

Analysis of IGH gene rearrangements of BM M-PC and CDR3 sequencing. Identification of the malignant IGH gene rearrangement was performed on genomic DNA according to the BIOMED-2 protocol slightly modified to amplify the specific monoclonal complete VH-JH and incomplete DH-JH. PCR amplified products were identified by high-resolution capillary electrophoresis in an automated ABI PRISM 3130 Avant sequencer, using the GENEMAPPER 3.1 software (AB). Clonal PCR products
were purified, sequenced and identified as previously reported\textsuperscript{34}. Once the segments had been identified, the N-region was highlighted for ASO-primer design due to its high specificity for each individual rearrangement, as previously described\textsuperscript{35}.

A qualitative ASO-PCR with the CDR3 specific primer and its respective 6-FAM-labeled $IGH$ JH primer, was carried out on purified BM M-PC from each patient; purified PB B-cells from healthy donors were studied in parallel as negative controls. If a single (clonal) peak was detected in a sample, it was sequenced to confirm that the sets of primers were really specific. In order to assess the sensitivity of each patient-specific set of primers used in the ASO-PCR assay (e.g.: the lowest number of cells detected), ASO-PCR analyses were carried out in multiple tubes containing serial dilutions of $\geq 1$ previously purified BM M-PC from individual patients (see Online Supplementary Methods for a more detailed description).

**ASO-PCR analysis of purified PB subpopulations of B-cells and PC.** DNA was extracted from pre-defined numbers of highly-purified PB B-cell and PC subsets, and ASO-PCR conditions were as described above; all purified cells from each cell compartment from each patient were analyzed by ASO-PCR (7 to 10 ASO-PCR replicates/patient). Presence of clonotypic B-cells within a specific B-cell or PC compartment was defined when $\geq 2$ replicates were found to be positive. For every sample, DNA quality was evaluated by a control according to the BIOMED-2 protocols\textsuperscript{32}.
Results

Distribution of different subsets of B-lymphocytes and plasma cells in the PB of MM and MGUS patients. The following subsets of PB B-cells and PC were identified\(^{29,31}\): naïve (CD10\(^-\) CD19\(^+\) CD20\(^+\) CD27\(^-\) CD38\(^-\)) and memory (CD10\(^-\) CD19\(^+\) CD20\(^+\) CD27\(^+\) CD38\(^-\)) B-lymphocytes plus normal circulating PC (N-PC; CD10\(^-\) CD19\(^+\) CD20\(^-\) CD27\(^++\) CD38\(^++\)). PB memory B-lymphocytes were further subdivided into switched SmIgA\(^+\), SmIgG\(^+\), and non-switched SmIgM\(^+\) cells. In 4/7 MM patients (cases #3, #5, #6 and #7), PB circulating M-PC with restricted CyIg\(_\lambda\) or CyIg\(_\kappa\) expression were also identified as clearly different from N-PC based on differential expression of CD38, CD19, CD45, and light scatter\(^{26,29}\).

Overall, no statistically significant differences (p>0.05, Mann-Whitney U test) were observed in the distribution of circulating B-cells and PC in MM vs. MGUS patients (Figure 1). This included total B-cells (113±15 vs. 83±71 cells/µL), and the naïve (83±11 vs. 53±56 cells/µL), as well as total memory B-cells (30±10 vs. 22±16 cells/µL) and their SmIgM\(^+\) (9±4 vs. 9±6 cells/µL), SmIgG\(^+\) (14±2 vs. 4±2 cells/µL), SmIgA\(^+\) (12±1 vs. 6±4 cells/µL) subsets, and also N-PC (0.7±0.4 vs. 1.2±1.5 cells/µL) (Figure 1). In contrast, M-PC were found at frequencies >8.0 cells/µL in 4/7 MM patients (range: 0.08-29.8 cells/µL), but not in the three MGUS cases (although we have detected them in up to 21% of a larger series of MGUS cases\(^{16}\)) (p<0.05; Figure 1). Of note, all FACS-purified PB B-cell subsets (naïve and memory B-lymphocytes) and N-PC showed a normal protein expression profile, including a normal polyclonal Igk vs. Ig\(_\lambda\) light chain ratio, while the M-PC compartment systematically showed an aberrant immunophenotype associated with monoclonal light chain restriction (either CyIgk\(^+\) or CyIg\(_\lambda\)\(^+\)) profile.

Sensitivity of the molecular assays. The sensitivity assays performed aimed at both defining the minimum number of clonotypic M-PC required for a positive result and at
establishing the sensitivity of the ASO-PCR assay over a polyclonal background. Thus, the first set of experiments consistently showed the ability to systematically detect a (clonal) patient-specific IGH gene rearrangement peak whenever ≥ 5 clonotypic M-PC were present in the sample, for all ten patients analyzed; in 3/7 patients tested (cases #MM1, #MM2, #MGUS3) the sensitivity was even higher since patient-specific IGH CDR3 gene rearrangements were detected once a single M-PC was present in the sample. Regarding the ability to detect clonotypic M-PC over a background of polyclonal B-cells, sensitivity from dilution experiments was systematically <10⁻³, ranging between <10⁻³ to <10⁻⁵ (from 0.2% to 0.001% M-PC cells/total B-cells) (Supplementary Table 2), as illustrated in Figure 3 for one of the cases studied.

The sensitivity of the assay varied per patient for the different cell fractions from <0.08 to <0.0003 cells/µL of PB (i.e. <80 to <0.03 cells/mL), according to: the sensitivity and reproducibility of the assay; the number of purified PB B-cells and PC analyzed; and the distribution of the subpopulation (e.g. absolute count) in the PB of each individual patient at the moment the sample was collected (Table 1).

**Frequency of clonotypic cells in highly-purified PB subsets of circulating B-cells and PC.** The search for the presence of clonotypic cells was performed on highly-purified PB naïve B-lymphocytes, switched SmIgA⁺ and SmIgG⁺, as well as non-switched SmIgM⁺ memory B-cells and N-PC, present at variable numbers in the PB of each of the 10 MG patients (Table 1). In addition, circulating M-PC from 4 MM in which these cells were detected at frequencies of 0.79%, 0.09%, 0.03% and 0.17% of all PB nucleated cells (absolute M-PC count of 29.8, 0.7, 0.08, and 6.1 cells/µL, respectively) were also purified and analyzed. Overall, no cells carrying clonotypic IGH gene rearrangements were detected among the phenotypically normal B-cell subsets and N-PC from any of the MM (0/7) and MGUS (0/3) patients analyzed (Table 1). For Ig-switched memory B-cells sharing the same Ig heavy chain isotype as BM M-PC, a sensitivity of between <6 cells/mL and <24 cells/mL of PB (IgG⁺ MM) and of <9 cells/mL (IgA⁺ MM) of PB was
reached. As expected, analysis of PB M-PC confirmed the presence of clonotypic IGH
gene rearrangements identical to those identified in the corresponding (paired) BM M-PC, in all replicates of purified M-PC analyzed (Table 1 and Figure 2).
DISCUSSION

Since the pioneering experiments which used anti-idiotypic antibodies to track the oncogenic event in MM throughout the B-cell ontogeny\textsuperscript{36,37}, controversial results have been reported in the literature as regards the B-cell compartment where the potential myeloma stem cell (MSC) resides. For more than a decade now, it has been generally accepted that circulating CD19\textsuperscript{+} B-lymphocytes from MM patients could contain cells that carry clonotypic rearrangements identical to those of MM-PC\textsuperscript{2-7}. However, the frequency and nature of such clonally-related cells remains a matter of debate. While some groups suggest that the majority of circulating B-lymphocytes would belong to the malignant clone\textsuperscript{2,3}, others could not confirm these preliminary observations and have reported that, if present, circulating clonotypic B-cells would constitute a minor population of all PB leukocytes\textsuperscript{38,39}.

Important advances have been achieved in molecular as well as in cell purification techniques (e.g. improved exclusion of cell doublets and sorting efficiency, standard usage of ≥8-color experiments for better definition of the cell subpopulations of interest and exclusion of unwanted cells). Based on such technological advances we decided to revisit in the present study the detection of clonally related B-cells in the PB of patients with MG. For this purpose we used a highly-sensitive approach. First, an 8-color MFC technique was used for the identification of the distinct subsets of circulating PB B-cells and PC, in combination with multiparameter sorting of highly-purified, non-contaminated, cell fractions corresponding to such subsets of PB naïve and memory B-lymphocytes (including SmIgM\textsuperscript{+}, SmIgA\textsuperscript{+} and SmIgG\textsuperscript{+} memory B-cells), in addition to N-PC and, whenever present, also M-PC. In a second step, molecular detection of clonotypic B-cells was performed on DNA from the purified B-cell subsets and PC using a highly-sensitive ASO-PCR technique specifically designed for the detection of clonotypic B-cells of individual patients.
Our results showed systematic absence of clonotypic *IGH* gene rearrangements in all different B-cell subsets analyzed, including, M-component isotype-matched memory B-lymphocytes; the only exception were the M-PC detected and purified from the PB of 4 MM patients. In line with these observations, no altered patterns of protein expression were found in any of the purified fractions of naïve and memory B-lymphocytes and N-PC, all such cell subsets also showing a normal polyclonal Igκ vs. Igλ light chain ratio, except M-PC.

Upon considering the sensitivity of the assay used, the number of cells analyzed and the volume of blood investigated, it seems unlikely that circulating clonotypic B-lymphocytes are present in MM and MGUS patients at frequencies of between >6 and 30 cells/mL (<1 cell in between 33 and 166 µL of PB). Even more, when we specifically consider the absence of clonotypic B-cells among the compartment of memory B-cells (as the most susceptible of containing the myeloma stem cell) the frequency would go down to between <4.1 and < 37 cells/mL.

Overall, the sensitivity of the approach here used is far beyond that of most previously reported studies in which the presence of clonotypic B-lymphocytes has been reported. In this regard, the discrepancies observed between our and other studies cannot be due to patient selection since almost all our MM patients corresponded to symptomatic MM, and they had received no prior therapy for the disease. In turn, we confirm the presence of circulating M-PC at relatively high numbers in the PB of the majority of MM patients (range: 80 to 29,800 cells/mL), in line with previous observations by our and other groups. This frequency is lower than that reported in earlier studies for circulating clonotypic B-cells (range: 10,000 to 610,000 cells/mL). Noteworthy, in a substantial number of these previous studies, total PB B-cells were used to search for clonotypic *IGH* gene rearrangements and contamination by M-PC (e.g. due to doublet formation or altered time delay in sorting experiments) was not systematically excluded. In this regard, we have recently confirmed contamination of PB B-cells by M-PC through reanalysis of a frozen sample.
from a case which had been previously reported to carry clonotypic non-switched B-cells. Therefore, this could contribute to explain, at least in part, the discrepancies observed between our and other studies, even when with our approach we dramatically increased the sensitivity and specificity of analysis. In contrast, our results fully support recent findings by Kim et al. in SCID mice previously engrafted subcutaneously with fetal human bone, showing that only the MM-PC compartment was able to give rise to clonally-related, patient-specific MM cells in (two) serial transplants; although plasmablasts and B-cells were also able to engraft in those mice, their capacity was limited to the first xenotransplantation and these compartments could not give rise to any cell with the same patient-specific MM molecular signature. Alternatively, it might be possible that the tumor precursor would circulate only at the earlier stages of disease to fill/feed the BM niches, giving rise to tumor PC. However, this seems unlikely since also different PB B-cell compartments from MGUS patients here analyzed were found to be devoid of clonotypic cells with the same IGH gene rearrangement as BM M-PC.

Altogether, our results suggest that if PB clonal B-lymphocytes exist in MM and MGUS, in most patients these would be present at extremely low frequencies, and this would not explain the dissemination throughout the BM during active/progressive disease, as observed in many symptomatic MM patients. Conversely, since circulating M-PC were frequently detected at rather higher numbers, it could be hypothesized that the myeloma clonogenic cell could be part of the malignant PC compartment which is able to re-circulate and spread the disease. In line with this latter hypothesis recent results indicate that circulating PB M-PC from both MM and MGUS patients display a slightly more immature immunophenotype with lower expression of CD138, a phenotypic feature of M-PC that has been associated with an increased clonogenic potential and other myeloma stem cell features. Furthermore, presence of circulating PB M-PC is also frequently detected at the early phases of the disease including MGUS and smoldering MM patients. Further studies, in which the clonogenic
potential of PB M-PC is compared with that of BM tumor cells are required to validate this hypothesis.

In summary, here, based on a sensitive and well-controlled approach, we failed to demonstrate the presence of circulating clonotypic B-cells in the PB of MM and MGUS patients, outside the compartment of immunophenotypically aberrant clonal PC detected in a substantial fraction of MM patients. These findings suggest that tumor dissemination in MG could more likely be related to the PB compartment of circulating M-PC than to other populations of less differentiated naïve and memory B-lymphocytes.

AUTHORSHIP AND DISCLOSURES: LST performed the experiments, analyzed data and wrote the paper. MPA performed flow cytometry experiments, analyzed data and wrote the paper. AB, MAS, and MJ performed and analyzed molecular biology experiments. BP performed and analyzed flow cytometry experiments and collected data. PB and MLS performed sorting experiments. JA analyzed data and provided critical revision of the results. MG, JSM and RG provided critical revision of the results and wrote the paper. AO designed research, analyzed data and wrote the paper. The authors declare no conflicts of interest.

Supplementary information is available at Haematologica's website.
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lines and are not associated with a cancer stem cell phenotype. Haematologica 2012;97(7):1110-4.


**Table 1.** Frequency and absolute counts of clonotypic cells detected in different B-cell and PC subsets present in the PB of MGUS (n=3) and MM patients (n=7).

<table>
<thead>
<tr>
<th>Case ID / Isotype of the M-Component</th>
<th>Total B-lymphocytes</th>
<th>Naïve B-lymphocytes</th>
<th>Total memory B-lymphocytes</th>
<th>SmIgM+ memory B-cells</th>
<th>SmIgG+ memory B-cells</th>
<th>SmIgA+ memory B-cells</th>
<th>N-PC</th>
<th>M-PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGUS 1 IgG⁺</td>
<td>&lt;0.014 (&lt;0.06%)</td>
<td>&lt;0.004 (&lt;0.007%)</td>
<td>&lt;0.0105</td>
<td>&lt;0.008</td>
<td>&lt;0.015</td>
<td>&lt;0.015</td>
<td>&lt;0.0030 ND</td>
<td></td>
</tr>
<tr>
<td>MGUS 2 IgG⁺</td>
<td>&lt;0.019 (&lt;0.02%)</td>
<td>NT</td>
<td>&lt;0.0064</td>
<td>&lt;0.001</td>
<td>&lt;0.009</td>
<td>&lt;0.008</td>
<td>&lt;0.0058 ND</td>
<td></td>
</tr>
<tr>
<td>MGUS 3 IgG⁺</td>
<td>&lt;0.018 (&lt;0.02%)</td>
<td>NT</td>
<td>&lt;0.0041</td>
<td>&lt;0.002</td>
<td>&lt;0.011</td>
<td>&lt;0.005</td>
<td>&lt;0.00033 ND</td>
<td></td>
</tr>
<tr>
<td>MM 1 IgG⁺</td>
<td>&lt;0.02 (&lt;0.01%)</td>
<td>&lt;0.017 (&lt;0.1%)</td>
<td>&lt;0.0078</td>
<td>&lt;0.006</td>
<td>&lt;0.006</td>
<td>&lt;0.048</td>
<td>&lt;0.0031 ND</td>
<td></td>
</tr>
<tr>
<td>MM 2 IgA⁺</td>
<td>&lt;0.03 (&lt;0.03%)</td>
<td>&lt;0.037 (&lt;0.05%)</td>
<td>&lt;0.0178</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>MM 3 IgG⁺</td>
<td>&lt;0.015 (&lt;0.01%)</td>
<td>&lt;0.007 (&lt;0.1%)</td>
<td>&lt;0.037</td>
<td>NT</td>
<td>&lt;0.024</td>
<td>&lt;0.011</td>
<td>&lt;0.0042 29</td>
<td></td>
</tr>
<tr>
<td>MM 4 IgA⁺</td>
<td>&lt;0.11 (&lt;0.03%)</td>
<td>&lt;0.035 (&lt;0.3%)</td>
<td>&lt;0.026</td>
<td>NT</td>
<td>&lt;0.012</td>
<td>&lt;0.009</td>
<td>&lt;0.00029 ND</td>
<td></td>
</tr>
<tr>
<td>MM 5 IgG⁺</td>
<td>&lt;0.03 (&lt;0.03%)</td>
<td>&lt;0.077 (&lt;0.3%)</td>
<td>&lt;0.013</td>
<td>NT</td>
<td>NT</td>
<td>&lt;0.024</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>MM 6 IgA⁺</td>
<td>&lt;0.02 (&lt;0.02%)</td>
<td>&lt;0.025 (&lt;0.09%)</td>
<td>&lt;0.022</td>
<td>&lt;0.034</td>
<td>NT</td>
<td>NT</td>
<td>&lt;0.037 0.8</td>
<td></td>
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<tr>
<td>MM 7 IgG⁺</td>
<td>&lt;0.006 (&lt;0.01%)</td>
<td>&lt;0.005 (&lt;0.03%)</td>
<td>&lt;0.01</td>
<td>&lt;0.009</td>
<td>NT</td>
<td>NT</td>
<td>&lt;0.013 6.1</td>
<td></td>
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</tbody>
</table>

Results expressed as number of cells/μL and percentage of PB nucleated cells.
The sensitivity of the assay was calculated based on the numbers of cell purified for each cell subset and its distribution in PB.
ND: not detected; NT: not tested; PB: peripheral blood; MM: multiple myeloma; MGUS: monoclonal gammopathy of undetermined significance
LEGEND TO FIGURES

Figure 1: Distribution of B-lymphocytes and plasma cells and their subsets in the peripheral blood (PB) of patients with MGUS (n=3, white boxes) and MM (n=7; black boxes). N-PC: normal plasma cells; M-PC: (mono)clonal plasma cells. Boxes extend from the 25th to the 75th percentiles; the line in the middle and the vertical lines represent median values and both the 10th and 90th percentiles, respectively. The Mann–Whitney U test was used to estimate the statistical significance of differences observed. *p<0.05 vs MGUS.

Figure 2: Illustrating example of the results of molecular analysis of clonotypic B-cells and PC in purified B-cell and PC subsets from the PB of a MM patient (case #MM3). As shown, circulating (mono)clonal plasma cells (M-PC) are the only compartment of PB B-cells from this patient that showed a clonal CDR3 gene rearrangement signature similar to that of BM M-PC from the same patient (panel A). The quality of the DNA samples from all FACS-purified PB B-cell and PC subsets of each individual patient was analyzed in parallel for a control gene with PCR primers directed to human tromboxane synthase gene (TBXAS1) (panel B).

Figure 3: Illustrating example of a dilution experiment performed and analyzed by GeneScanning for one representative MM patient (case #MM6). To evaluate the sensitivity of the ASO-PCR assay for this patient, DNA from M-PC obtained from the BM was diluted into polyclonal DNA obtained from FACS-purified PB B-cells from a healthy donor. Numbers between brackets indicate the M-PC: normal B-cell dilution factor.
Figure 1.
Figure 2.

A

Naive B-lymphocytes

$s_{m}$IgG$^+$ memory B-cells

$s_{m}$IgA$^+$ memory B-cells

N-PC

M-PC

B

Naive B-lymphocytes

$s_{m}$IgG$^+$ memory B-cells

$s_{m}$IgA$^+$ memory B-cells

N-PC

Negative Control
Figure 3.

BM M-PC/Polyclonal B-cells
$(10^{-2})$

BM M-PC/Polyclonal B-cells
$(10^{-3})$

BM M-PC/Polyclonal B-cells
$(10^{-4})$

BM M-PC/Polyclonal B-cells
$(10^{-5})$

Polyclonal B-cells

Fluorescence Intensity

Base Pairs
SUPPLEMENTARY METHODS

Patients and samples
Patients with MM were diagnosed according to previously described criteria by the The International Myeloma Working Group (British Journal Hematology. 2003;121:749-57).

Multiparameter flow cytometry immunophenotypic studies and cell purification:
Erythrocyte-lysed whole PB and BM samples were stained using an 8-color stain-lyse-and-wash direct immunofluorescence technique with the following monoclonal antibody combinations - pacific blue (PacB), pacific orange (PacO), fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein/cyanin 5 (PerCPCy5), PECy7, allophycocyanin (APC), APC-hilite 7 (H7) – for PB samples: i) CD20, CD45, surface membrane (Sm)IgM, SmIgG, CD19, CD10, CD27, CD38; ii) CD20, CD45, SmIgM, SmIgA, CD19, CD10, CD27, CD38, and; iii) CD20, CD45, SmIgλ, SmIgκ, CD19, CD10, CD27, CD38 (for PB samples). For BM specimens, the CD45, CD138, CD38, CD56, β2-microglobulin, CD19, cytoplasmic Ig (CyIg)κ, CyIgλ antibody combination was used. Fluorescence measurements were performed in a FACSCanto II™ flow cytometer (Becton/Dickinson Biosciences [BD], San Jose, CA, USA) for a total of $\geq 2 \times 10^5$ cells showing CD19 and/or CD38 expression and low-to-intermediate sideward light scatter (SSC) values using a double-step data acquisition procedure described elsewhere and the FACSDiva software (version 6.1, BD). For data analysis the Infinicyt software (Cytognos SL, Salamanca, Spain) was used.

Fluorescence-activated cell sorting (FACS) based on the above antibody combinations was used (FACSAria II™, BD) to purify BM M-PC as well as PB smIgM+ naïve B-lymphocytes, non-switched smIgM+, switch smIgA+ and smIgG+ memory B-lymphocytes and N-PC; whenever present, circulating M-PC were also purified. The purity of the sorted M-PC and the multiple PB normal B-cell subsets was systematically
≥ 98%, and only those FACS-purified PB B-cell fractions (except M-PC), which showed no contamination (<0.01%) by circulating PB M-PC were included in the study; all other fractions were not tested (Table 1).

**Analysis of IGH gene rearrangements of BM M-PC and CDR3 sequencing.** Highly-purified BM M-PC were lysed and genomic DNA extracted and prepared for PCR using the REDExtract-N-Amp Blood PCR Kit™ (Sigma, St. Louis, MO, USA) following the recommendations of the manufacturer. Identification of the malignant IGH gene rearrangement was performed according to the BIOMED-2 protocol slightly modified, using 4.8µL of each individual M-PC lysate (final volume of 20 µl containing 10µL of PCR ReadyMix and 7.8 pmol of each primer) per PCR reaction. Complete VDJH and incomplete DJH rearrangements were amplified by different sets of family-specific primers (VH-FR2 and DH-JH) together with the JH consensus primer, in two different multiplex PCR reactions. Reverse primers were labelled with 6-carboxyfluorescein (6-FAM). PCR reactions were performed in a Verity™ 96-Well Thermal Cycler (Applied Biosystems [AB], Foster City, CA, USA) using the following conditions: incubation at 95°C for 3 min (pre-activation) followed by 40 cycles of sequential incubation at 95°C for 45s (denaturation), 60°C for 45s (annealing), and 72°C for 90s (extension), with a final extension step for 10 min at 72°C. The PCR amplified products were denatured, the FAM-labeled single-strand DNA fragments were size-separated by high-resolution capillary electrophoresis, and identified by GeneScanning in an automated ABI PRISM 3130 Avant sequencer, using the GENEMAPPER 3.1 software (AB). A clonal population was defined according to the recently criteria defined by the EuroClonality group (Langerak et al, Leukemia 2012). In all cases, at least two tubes were run in parallel for each PCR in order to avoid pseudoclonality.

Clonal PCR products were purified with ExoSap (USB Corp, Cleveland, OH, USA) and directly sequenced in both directions in an ABI 3130 DNA sequence
analyzer using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (AB). Germline VH, DH and JH gene segments from complete IGH V(D)JH gene rearrangements were identified through direct comparison with the IMGT database (Lefranc et al. Nucleic Acids Res. 2001; Giudicelli et al. Nucleic Acids Res. 2004) (http://imgt.cines.fr) using the DNAPlot software (MRC Center for Protein Engineering, Cambridge, UK). IGH DH and IGH JH germline gene segments from incomplete DH-JH gene rearrangements were identified using BLAST search in the IGH DH-JH germline locus sequence (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Once the segments had been identified, the N-region was highlighted for ASO-primer design due to its high specificity for each individual rearrangement, as previously described (Sarasquete et al. Haematologica 2005). If a single (clonal) peak was detected in a sample, it was sequenced as described above in order to confirm that the sequence was or not identical to the CDR3 signature of the reference sequence, indicating that the sets of primers were really specific. In order to assess the sensitivity of each patient-specific set of primers used in the ASO-PCR assay (e.g.: the lowest number of cells detected), ASO-PCR analyses were carried out in multiple tubes containing serial dilutions of ≥1 previously purified BM M-PC from individual patients. A signal was considered to be positive when a peak with the expected fragment size was detected clearly above the GeneScanning background signal in ≥ 2 replicates.

**Design of ASO-primers and sensitivity of the ASO-PCR technique.** ASO-primers complementary to the IGH VH-DH or IGH DH-JH junctional regions were designed using the Oligo 6.1 software (Molecular Biology Insights, Cascade, CO, USA). Design conditions avoided primer dimer formation with ΔG >-3.5 Kcal/M, GC-rich 3’ ends and Tm differences with IGHJ primers >2°C. Amplicon sizes were always <170 bp.

A qualitative fluorescent ASO-PCR with CDR3 specific primer and its respective 6-FAM-labeled IGH JH primer (intronic, allele-specific or consensus), was carried out
on purified BM M-PC from each patient; purified PB B-cells from healthy donors were studied in parallel as negative controls. ASO-PCR conditions were the same as described above. Each set of patient-specific *IGH* CDR3 primers were tested at different annealing temperatures in order to determine the maximum amplification efficiency for each pair of primers (Supplementary Table 2). In all cases, a single peak was present in the patient’s purified BM M-PC samples, while absent in purified normal PB B-cells. The single (clonal) *IGH* gene peak detected for M-PC from individual patients was sequenced as described above, and the amplified sequences systematically showed an identical CDR3 signature to those of the reference sequence, indicating that the sets of primers were highly specific.

In order to determine the sensitivity of each patient-specific set of primers used in the ASO-PCR assay (e.g.: the lowest number of cells detected), ASO-PCR analyses were performed in multiple tubes containing serial dilutions of ≥1 previously purified BM M-PC from individual patients. For this purpose, DNA from M-PC obtained from each patient was diluted into polyclonal DNA obtained from purified PB B-cells from healthy donors. Multiple replicates (range: 3 to 7) of each dilution were produced and analyzed in parallel with the ASO-PCR set of primers and technique described above. The sensitivity of the ASO-PCR assay for each pair of primers/patient was established as the highest dilution in which the clonal peak could be repeatedly detected (Supplementary Table 2). A signal was considered to be positive when a peak with the expected fragment size was detected clearly above the GeneScanning background signal in ≥2 replicates.

**ASO-PCR analysis of purified PB subpopulations of B-cells and PC.** DNA was extracted from pre-defined numbers of highly-purified PB B-cell and PC subsets, as described above, except for the volumes of the lysing solution that were lower; purified B-cells and PC were incubated with 5 µl of extraction solution (5 min, RT) to which 45 µL of neutralization solution was added. ASO-PCR conditions were as described above.
and all purified cells from each cell compartment from each patient were analyzed by ASO-PCR (7 to 10 ASO-PCR replicates/patient). Presence of clonotypic B-cells within a specific B-cell or PC compartment was defined when ≥2 replicates were found to be positive. For every sample, DNA quality was evaluated by a control PCR for the thromboxane synthase gene (TBXAS1 primers: forward 5’ GCCCGACATTCTGCAAGTCC 3’, Reverse 5’ GGTGTTGCGGGAAGGGTT 3’). The sensitivity of the assay was defined as the number of cells/µL of PB evaluated, based on the number of cells analysed per FACS-purified cell fraction and its absolute count in PB. Presence of clonotypic B-cells within a specific B-cell or PC compartment was defined when ≥ 2 replicates were found to be positive. For every sample, DNA quality was evaluated by a control according to the BIOMED-2 protocols.

**Statistical analyses** The Mann–Whitney U test was used to estimate the statistical significance of differences observed between distinct experimental groups (SPSS version 20, SPSS Inc., Chicago, IL, USA). P values ≤ 0.05 were considered to be associated with statistical significance.
### Supplementary table 1: Clinical and biological features of MM and MGUS patients at diagnosis

<table>
<thead>
<tr>
<th>Patient Group</th>
<th>MM (n=7)</th>
<th>MGUS (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical/Laboratory features</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>68±8</td>
<td>66±7</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male/Female</td>
<td>6/1</td>
<td>1/2</td>
</tr>
<tr>
<td>Disease at presentation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MGUS</td>
<td>NA</td>
<td>3/3</td>
</tr>
<tr>
<td>Symptomatic MM</td>
<td>6/7</td>
<td>NA</td>
</tr>
<tr>
<td>Smoldering MM</td>
<td>1/7</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Isotype of M-Component</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>4/7</td>
<td>3/3</td>
</tr>
<tr>
<td>IgA</td>
<td>3/7</td>
<td>0/3</td>
</tr>
<tr>
<td><strong>Clinical Stage (ISS)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>2/7</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>4/7</td>
<td>NA</td>
</tr>
<tr>
<td>III</td>
<td>1/7</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin ≤ 8.5 g/dL</td>
<td>1/7</td>
<td>0/3</td>
</tr>
<tr>
<td>β2-microglobulin&gt; 3.5 µg/L</td>
<td>5/7</td>
<td>0/3</td>
</tr>
<tr>
<td>Albumin≥ 3.5 g/dL</td>
<td>4/7</td>
<td>0/3</td>
</tr>
<tr>
<td>Creatinine≥ 2mg/dL</td>
<td>0/7</td>
<td>0/3</td>
</tr>
<tr>
<td>% S-phase BMPC &gt; 1.5%</td>
<td>0/7</td>
<td>0/3</td>
</tr>
<tr>
<td>% BM PC&gt;33%</td>
<td>4/7</td>
<td>0/3</td>
</tr>
<tr>
<td>% BM M-PC&gt;95% of all PC</td>
<td>7/7</td>
<td>0/3</td>
</tr>
</tbody>
</table>

BMPC: bone marrow plasma cells; NA: not applicable; M-PC: (mono)clonal PC. ISS: international staging system for multiple myeloma
<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Sensitivity of the assay</th>
<th>CDR3 Sequence</th>
<th>Primer</th>
<th>Tm (°C)</th>
<th>Length of the PCR product</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGUS 1</td>
<td>≤0.1 %</td>
<td>GTATTACGATCTTTGACTGGTTATTATCT TACACTAAATCAGTGAACGCTTTGACGAGG</td>
<td>GGTATTATCTAAATTCCCTAATGT GATGA</td>
<td>62</td>
<td>107 bp</td>
</tr>
<tr>
<td>MGUS 2</td>
<td>≤0.1 %</td>
<td>TACATCTATTACTGTGCAGCGAAGGAGGACGCTTTGACAAGGCGGAGGAGGACG</td>
<td>TCATCTATTACTGTGACGGAAAGAGAAG GCTTTGACCAGGACG</td>
<td>62</td>
<td>106 bp</td>
</tr>
<tr>
<td>MGUS 3</td>
<td>≤0.1%</td>
<td>AGGTGAGGGTCCTAAGCTGGATTCCTTGGGAGGACG</td>
<td>AGGTGAGGGTCCTAAGCTGGATTCCTTGGGAGGACG</td>
<td>69</td>
<td>108 bp</td>
</tr>
<tr>
<td>MM 1</td>
<td>ND</td>
<td>ATTACGTGTGAAGATCGATTGTGGTACGTC</td>
<td>ATTACGTGTGAAGATCGATTGTGGTACGTC</td>
<td>60</td>
<td>120 bp</td>
</tr>
<tr>
<td>MM 2</td>
<td>≤0.1%</td>
<td>AGGATATTTGTAATAAGAACCAGCTGGATTAACGCTTCCTTTTGGTGATAGTCGTC</td>
<td>AGGATATTTGTAATAAGAACCAGCTGGATTAACGCTTCCTTTTGGTGATAGTCGTC</td>
<td>68.5</td>
<td>125 bp</td>
</tr>
<tr>
<td>MM 3</td>
<td>≤0.1%</td>
<td>GCTAAGCGGAGCTACAACAGTGATACATGACTACTTTTCG</td>
<td>GCTAAGCGGAGCTACAACAGTGATACATGACTACTTTTCG</td>
<td>68</td>
<td>106 bp</td>
</tr>
<tr>
<td>MM 4</td>
<td>≤0.06%</td>
<td>TGGTATTATTTTGGGAGGAGCAGAAGTATG TGGTATTATTTTGGGAGGAGCAGAAGTATG TGGTATTATTTTGGGAGGAGCAGAAGTATG TGGTATTATTTTGGGAGGAGCAGAAGTATG</td>
<td>TGGTATTATTTTGGGAGGAGCAGAAGTATG TGGTATTATTTTGGGAGGAGCAGAAGTATG TGGTATTATTTTGGGAGGAGCAGAAGTATG</td>
<td>62</td>
<td>107 bp</td>
</tr>
<tr>
<td>MM 5</td>
<td>≤0.2%</td>
<td>TGGTATTATTTTGGGAGGAGCAGAAGTATG TGGTATTATTTTGGGAGGAGCAGAAGTATG TGGTATTATTTTGGGAGGAGCAGAAGTATG TGGTATTATTTTGGGAGGAGCAGAAGTATG</td>
<td>TGGTATTATTTTGGGAGGAGCAGAAGTATG TGGTATTATTTTGGGAGGAGCAGAAGTATG TGGTATTATTTTGGGAGGAGCAGAAGTATG</td>
<td>60</td>
<td>91 bp</td>
</tr>
<tr>
<td>MM 6</td>
<td>≤0.001%</td>
<td>TGGTATTATTTTGGGAGGAGCAGAAGTATG TGGTATTATTTTGGGAGGAGCAGAAGTATG TGGTATTATTTTGGGAGGAGCAGAAGTATG TGGTATTATTTTGGGAGGAGCAGAAGTATG</td>
<td>TGGTATTATTTTGGGAGGAGCAGAAGTATG TGGTATTATTTTGGGAGGAGCAGAAGTATG TGGTATTATTTTGGGAGGAGCAGAAGTATG</td>
<td>60</td>
<td>84 bp</td>
</tr>
</tbody>
</table>

The CDR3 sequences are shown in **bold.** Underlined nucleotides highlight the position of sense ASO primers. ND: Not Determined due to sample shortage.