Differentiation stage of myeloma plasma cells: biological and clinical significance

Running title: Differentiation stage of normal and myeloma plasma cells

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Abstract: The notion that plasma cells (PCs) are terminally-differentiated has prevented intensive research in multiple myeloma (MM) about their phenotypic plasticity and differentiation. Here, we demonstrated in healthy individuals (n=20) that the CD19-CD81 expression axis identifies three bone marrow (BM)PC subsets with distinct age-prevalence, proliferation, replication-history, immunoglobulin-production, and phenotype, consistent with progressively increased differentiation from CD19+CD81+ into CD19-CD81+ and CD19-CD81- BMPCs. Afterwards, we demonstrated in 225 newly-diagnosed MM patients that, comparing to normal BMPC counterparts, 59% had fully-differentiated (CD19-CD81-) clones, 38% intermediate-differentiated (CD19-CD81+), and 3% less-differentiated (CD19+CD81+) clones. The latter patients had dismal outcome, and PC differentiation emerged as an independent prognostic marker for progression-free (HR:1.7;P=.005) and overall survival (HR:2.1;P=.006). Longitudinal comparison of diagnostic vs. minimal-residual-disease samples (n=40) unraveled that in 20% of patients, less-differentiated PCs subclones become enriched after therapy-induced pressure. We also revealed that CD81 expression is epigenetically regulated, that less-differentiated clonal PCs retain high expression of genes related to preceding B-cell stages (e.g.:PAX5), and show distinct mutation profile vs. fully-differentiated PC clones within individual patients. Together, we shed new light into PC plasticity and demonstrated that MM patients harboring less-differentiated PCs have dismal survival, which might be related to higher chemoresistant potential plus different molecular and genomic profiles.
Introduction

Multiparameter flow cytometry (MFC) is currently considered a sensitive co-adjuvant test in the diagnostic screening of patients with multiple myeloma (MM) to demonstrate bone marrow (BM) clonality. (1) Tumor plasma cells (PCs) from virtually all MM patients show phenotypic aberrancies that allow for clear distinction between these and normal PCs (2); furthermore, the expression levels of some antigens are significantly associated with differences in outcome. (3-7) One example is CD19, whose expression has been found in 5-10% of MM cases and correlated with inferior survival (3); however, the biological explanation behind such correlation remains unknown. Recently, we showed that the expression of CD81 in clonal PCs is also an independent prognostic factor in MM (4), but similarly to CD19, there is no knowledge on the biologic significance of CD81 expression in the surface of clonal PCs.

Normal PC differentiation is characterized by the acquisition of secretory capacity, cell-cycle exit and changes in both surface phenotype and gene expression. (8) Accordingly, CD19 which is a co-receptor of the B-cell receptor and is solely regulated by PAX5, becomes lost in a subset of normal BMPCs after PAX5 downregulation during B-cell into PC differentiation. (9,10) After the initial observation that CD19 expression was decreased in mature PCs generated in vitro (11), most recent analyses suggested that CD19^CD38^{hi}CD138^{+} PCs share similarities with murine long-lived PCs and could represent their human counterpart. (12,13) Since CD19 expression requires CD81 (14), a tetraspanin widely expressed at all stages of the B-cell lineage (4,15), it could be hypothesized that both markers might contribute to identify unique PC subsets during the transition from less- into more-differentiated BMPCs. In such cases, further investigations in MM would be warranted to unravel whether clonal PCs follow a similar pattern of normal PC differentiation according to CD19-CD81 expression levels, and to determine the clinical sequelae of myeloma PCs’ differentiation stage.

Here, we started by showing that the combined expression of CD19 and CD81 identified three unique BMPC subsets in healthy individuals with distinct functional and phenotypic features, consistent with progressively increased differentiation from CD19^{+}CD81^{+} into CD19^{+}CD81^{+} and CD19^{+}CD81^{+} normal BMPCs. Subsequently, we demonstrated that myeloma PCs fit into such a model of normal BMPC differentiation, and that patients with less-differentiated clones had dismal survival. PC differentiation is also related to therapy-induced selective pressure, through which less-differentiated PCs subclones become enriched from diagnosis into minimal residual disease (MRD) stages in a subset of MM patients. Most interestingly, less-differentiated PCs maintain
the expression of genes related to preceding B-cell stages, and show different mutation profiles as compared to fully-differentiated PC subclones within individual MM patients.

Methods

Patients, controls and samples. A total of 225 elderly, transplant-ineligible patients with newly-diagnosed symptomatic MM staged according to the International Myeloma Working Group criteria (16) were prospectively studied after inclusion in the PETHEMA/GEM2010MAS65 trial (NCT01237249). In all cases, BM aspirates were collected at diagnosis and in 40 out of the 225 patients, also after induction therapy for preplanned MRD monitoring. BM aspirates were additionally taken from 20 healthy individuals (median age: 46; range: 19-64 years) to study the functional and phenotypic characteristics of normal PCs. All samples were collected after informed consent was given by each individual, according to the local ethical committees and the Helsinki Declaration.

Multidimensional Flow Cytometry (MFC) immunophenotyping. Approximately 200μL of EDTA-anticoagulated BM aspirated samples from newly-diagnosed MM patients were immunophenotyped using two different 8-color combinations of monoclonal antibodies (MoAb) and a direct immunofluorescence stain-and-then-lyse technique - [Pacific Blue (PacB)/Pacific Orange (PacO)/fluorescein isothiocyanate (FITC)/phycoerythrin (PE)/peridinin chlorophyll protein-cyanin 5.5 (PerCP-Cy5.5)/PE-cyanin 7 (PE-Cy7)/allophycocyanin (APC)/APC7]; i) CD45/CD138/CD38/CD56/β2microglobulin/CD27/CD19/cyKappa/cyLambda; ii) CD45/CD138/CD38/CD28/CD27/CD19/CD117/CD81 – following the EuroFlow guidelines (17) to identify clonal PCs, and characterize their pattern of expression for CD19 and CD81. Patients with no reactivity for CD19 and <10% CD81+ clonal PCs were classified as CD19-CD81-, whereas those cases with <50% CD19+ clonal PCs but CD81 expression (≥10%) were classified as CD19+CD81+; all remaining patients showing ≥50% CD19+ clonal PCs were classified as CD19+CD81+ (all of them were positive for CD81). After induction therapy, a single 8-color MoAb combination (PacB/PacO/FITC/PE/PerCP-Cy5.5/PE-Cy7/APC/APC7) was used to monitor MRD, and whenever persistent MRD was detected, the percentage of CD19+ and/or CD81+ clonal PCs was determined to compare, at the individual-patient-level, with that found at diagnosis. The same 8-color MoAb combination was used to characterize the BMPC compartment of the 20 healthy individuals. In 5 out of the former 20 cases, an additional 8-color MoAb combination (BV421/BV510/FITC/PE/PerCP-Cy5.5/PE-
with CD138/CD27/cyIgM+cyclgA/cyclgA+cyclgG/CD38/CD19/cyKappa/CD81 was stained to quantify the cytoplasmic (cy) immunoglobulin (Ig) heavy chain distribution in different PC subsets according to CD19-CD81 expression. Data acquisition was performed for approximately 10^6 leukocytes/tube in a FACSCantoll flow cytometer (Becton Dickinson– BD – San Jose, CA) using the FACSDiva 6.1 software (BD). Data analysis was performed using the Infinicyt software (Cytognos SL, Salamanca, Spain).

**Quantitation of replication history.** B lymphocyte precursors, transitional, naive and memory B cells, CD19^+ and CD19^- PCs were FACS-sorted (FACSAria II, BD; purity ≥97%) from BM samples of healthy individuals (n=5), according to their respective phenotypic characteristics as described elsewhere.(18-20) The replication history of B lymphocytes and PCs was determined using the κ–deleting recombination excision circle (KREC) assay, which is based on the quantification of coding joints and signal joints of an Ig-deleting rearrangement (intron RSS-Kde) by real-time quantitative PCR (RQPCR).(21) Primers and probes were designed to specifically amplify the intronRSS–Kde rearrangements (coding joint) and the corresponding signal joint using TaqMan-based RQ-PCR from DNA isolated from FACS-sorted cell subsets.(21) The RQ-PCR mixture of 25μl contained TaqMan Universal MasterMix (Applied Biosystems), 900nM of each primer, 100nM of each FAM-TAMRA–labeled probe, 50ng of DNA, and 0.4ng BSA, and was run on the ABI PRISM 7700 sequence detection system (Applied Biosystems).(21)

**Cell cycle analyses.** The proliferation index of different normal PC subsets according to CD19-CD81 expression was analyzed in BM samples from 5 healthy individuals using 5-color staining for nuclear DNA and four cell surface antigens (CD19-PacB/CD45-PacO/CD38-FITC/CD81-PE as described elsewhere.(22)

**Single-cell multidimensional phenotyping.** BM aspirates from healthy individuals (n=10) were immunophenotyped using four different 8-color combinations of MoAb: [PacB, PacO, FITC, PE, PerCP-Cy5.5, PE-Cy7, APC, alexafluor 700 (AF700)]: i) CD29, CD45, CD11a, β7, CD79b, CD49d, CD19, CD38; ii) CD11c, CD45, CD41a, CD49e, CD33, CD117, CD19, CD38; iii) CD20, CD45, CD81, CD54, CD138, CD56, CD19, CD38, and; iv) HLA-DR, CD45, CD44, CXCR4, CD27, CD28, CD19, CD38. The expression of all 23 phenotypic markers was analyzed at the single PC level and compared between the CD19^+CD81^+, CD19^-CD81^+ and CD19^-CD81^- subsets, using the merge and calculation functions of the Infinicyt software as described elsewhere (23-25),
Fluorescence-in-situ-hybridization (FISH) and deep-targeted sequencing. FISH was performed at diagnosis on immunomagnetic-enriched PCs from 169 out of the 225 cases with available phenotypic data. DNA from two PC clones FACS-purified according to their differentiation status from 4 newly-diagnosed MM patients was analyzed including the corresponding germline samples. DNA was extracted from cells using AllPrep DNA/RNA Micro Kit, Qiagen. Targeted gene sequencing was performed using 20 ng of input DNA and applying the MM Mutation Panel Version 2.0 (M3P 2.0). Targeted panel consists of 1271 amplicons from 77 genes commonly mutated in MM. Enriched templates were sequenced using semiconductor technology (Ion Proton, Life Technologies) and analysed with Ion Reporter Software v4.4 (Life Technologies). A median of 1700x depth coverage was obtained. Mutation calls were considered positive when called by ≥5% variant reads and has a minimum depth coverage of 10 reads.

Gene Expression Profiling (GEP). A total of 71 newly-diagnosed MM patients screened at the University of Arkansas for Medical Sciences and with simultaneously available information on CD19 and CD81 immunophenotypic patterns of expression and GEP were included in this analysis. An aliquot of BM aspirate was collected to isolate CD138⁺ PCs with immunomagnetic bead selection (autoMACS; Miltenyi Biotec), as described elsewhere.(26) Purity of PC was monitored by flow cytometry and was ≥85%. Total RNA was used to measure GEP with Affymetrix U133 Plus 2.0 microarrays. Differentially expressed genes between classes were identified using the Significant Analysis of Microarrays (SAM) algorithm. Analyses were performed using BRB-ArrayTools (version 4.4.1) developed by Dr. Richard Simon and the BRB-ArrayTools Development Team, available at http://linus.nci.nih.gov/BRB-ArrayTools.html.

DNA methylation studies. We used the EZ DNA Methylation Kit (Zymo Research) for bisulfite conversion of 500 ng genomic DNA. Bisulfite-converted DNA was hybridized onto the HumanMethylation 450K BeadChip kit (Illumina). Data from the 450k Human Methylation Array were analyzed as described previously.(27)

Statistical analysis. Correlation studies between PC subset distribution and age were performed using the Pearson test. The Wilcoxon signed rank test was used to evaluate the statistical significance of the percentage of each PC subset in the distinct phases of the cell cycle, as well as for the replication history of each PC subset. Conversely, the Friedman test was used to compare the distribution according to the heavy-chain Ig
isotype across the different PC subsets. The Mann-Whitney U and the Kruskal-Wallis tests were used to estimate the statistical significance of differences observed between two or more groups, respectively. Survival was analyzed by the Kaplan-Meier method, and differences between curves were tested for statistical significance with the two-sided log-rank test. Progression-free survival (PFS) was defined as the time from diagnosis to disease progression or death from any cause, and overall survival (OS) as time from diagnosis to death from any cause. A multivariate Cox proportional hazard model was developed to explore the independent value of significant variables on the univariate analysis, and variables were retained in the model for levels of significance $P < .05$. The SPSS software (version 20.0; IBM, USA) was used for all statistical tests.

Results

**Combined expression of CD19 and CD81 identifies three unique normal BM PC subsets.** We first determined the distribution of the CD19$^+$CD81$^+$, CD19$^-$CD81$^+$ and CD19$^-$CD81$^-$ subsets within total BM PCs from healthy individuals; overall, the CD19$^+$CD81$^+$ subset accounted for the majority of PCs (median of 79% within the BMPC compartment), followed by the CD19$^-$CD81$^+$ and CD19$^-$CD81$^-$ subsets (14% and 5%, respectively). However, when we compared the distribution of each subset within the BMPC compartment across different age decades, we noted that while CD19$^-$CD81$^+$ and CD19$^-$CD81$^-$ PCs were almost absent among healthy individuals aged 10-20, their frequency progressively increased from younger to older individuals (Figure 1A). Accordingly, there was a significant ($P \leq .006$) correlation between age and the distribution of the CD19$^+$CD81$^+$, CD19$^-$CD81$^+$ and CD19$^-$CD81$^-$ subsets (Figure 1A), suggesting that CD19$^+$CD81$^+$ normal BMPCs appear earlier in the development of antibody responses, whereas CD19$^-$CD81$^+$ and CD19$^-$CD81$^-$ PCs accumulate in the BM later in life. Since during PC differentiation acquisition of secretory capacity is accompanied by progressive cell cycling exit, we subsequently explored the distribution of the CD19$^+$CD81$^+$, CD19$^-$CD81$^+$ and CD19$^-$CD81$^-$ subsets within G$_0$/G$_1$ and S-phase/G$_2$M normal BMPCs. As expected, the majority of BM PCs were in G$_0$/G$_1$ (data not shown), but while the relative distribution of all three CD19$^+$CD81$^+$, CD19$^-$CD81$^+$ and CD19$^-$CD81$^-$ subsets in G$_0$/G$_1$ was inside the normal ranges described above, there were virtually no CD19$^-$CD81$^-$ PCs in S-phase/G2M (Figure 1B; $P=.03$). Thus, CD19$^-$CD81$^-$ normal PCs were not only enriched in the BM of elderly healthy individuals, but also showed virtually no proliferation, suggesting that among CD19$^-$ PCs, those lacking CD81 could be more differentiated than CD19$^-$CD81$^+$ BMPCs. Additional analysis was performed to assess the replication history of CD19$^+$CD81$^+$ and total CD19$^-$ BMPCs, since it was not possible to purify sufficient cells numbers for the KREC assay from
CD19^+CD81^- and CD19^-CD81- BMPCs separately (Figure 1C); that notwithstanding, we confirmed that PCs have a superior median number of cell cycles compared to B lymphocytes \((P=.04)\), but also showed that within the BMPC compartment, the median number of cell cycles in CD19^- PCs was slightly superior to that of CD19^+ PCs \((P=.08)\). Additionally, there was a trend \((P=.07)\) for an altered distribution of Ig heavy-chain isotypes between PC subsets according to their CD19-CD81 expression, with progressively decreasing frequencies of IgA^+ PCs counterbalanced with increasing numbers of IgG^+ PCs along the respective CD19^-CD81^+, CD19^-CD81^- and CD19^-CD81^- BMPC subsets (Figure 1D). Further phenotypic differences were observed after single-cell analysis of 21 markers within the CD19-CD81 phenotypic pathway, with decreasing MFI of CD27, CD38, CD44 and CD54 combined with progressively increased expression of CD28 and CD56 being observed along the CD19^-CD81^+, CD19^-CD81^+ and CD19^-CD81^- BMPC subsets (Figure 1E). Overall, our results indicate that the combined CD19-CD81 pattern of expression identifies three BMPC subsets with singular functional and phenotypic characteristics, consistent with an accumulation of long-lived, less active and fully-differentiated PCs from the CD19^-CD81^- and CD19^-CD81^- into the CD19^-CD81^- BMPC subsets.

**Clinical sequelae of the differentiation stage of myeloma PC clones.** After demonstrating the existence of three well-defined normal BMPC subsets with distinct differentiation, we sought to determine how myeloma PC clones fit in such a model of normal BMPC differentiation. Upon specific analysis of the CD19-CD81 pattern of expression in clonal PCs from 225 newly-diagnosed MM patients, we found that more than half \((132/225; 59\%)\) had clonal PCs that phenotypically matched the fully-differentiated normal PC counterpart \(\text{i.e.: }\) CD19^-CD81^-; conversely, 86 out of the 225 patients \((38\%)\) displayed intermediate-differentiated myeloma PCs \(\text{i.e.: }\) CD19^-CD81^+, whereas only 7 cases \((3\%)\) showed clonal PCs for which the normal counterpart would correspond to the less-differentiated BMPC subset \(\text{i.e.: }\) CD19^-CD81^+. Interestingly, patients with less- and intermediately-differentiated clonal PCs had a different phenotypic profile vs cases with a fully-differentiated PC phenotype (Table 1), with significantly less frequent CD28^+ and CD117^+ expression; conversely, CD45 positivity was more frequent among patients with less-differentiated PC clones (Table 1). Furthermore, we noted a trend \((P=.07)\) for higher frequencies of cytogenetic abnormalities \(\text{i.e.: }\) t(IGH), +1q, del(13q), and/or del(17p) from patients with less- into intermediate- and fully-differentiated PCs (Table 2); in fact, cases with less-differentiated clones only showed +1q, and no IGH translocations nor del(13q) nor del(17p). Patients with less- and intermediately-differentiated clonal PCs achieved
lower MRD-negative rates as compared to cases with a more mature PC phenotype (25% and 20% vs. 40%; \( P = .03 \)). Upon investigating if the differentiation stage of myeloma PC clones influenced patients' prognosis, we noted that PFS and OS of cases in less- and intermediate-differentiation stages was significantly inferior as compared to patients with fully-differentiated CD19-CD81- myeloma PC clones (Figures 2A and 2B). The treatment arm had no impact in patients' outcomes according to PC differentiation (data not shown). Multivariate analysis of prognostic factors for survival including the differentiation stage of clonal PCs plus patients' age, ISS and FISH cytogenetics showed that the best combination of independent predictive parameters for PFS and OS were PC differentiation and FISH cytogenetics (Table 3). Accordingly, the differentiation stage of clonal PCs emerged again as a relevant prognostic factor for PFS and OS when the analysis was restricted to cytogenetically-defined standard-risk cases (Figures 2C and 2D), suggesting that the presence of less-differentiated myeloma PC clones identifies a subgroup of patients with more aggressive disease despite standard-risk cytogenetic profiles.

**Less-differentiated PC clones may become predominant at the MRD stage.** Since the differentiation stage of clonal PCs at baseline was intrinsically related to patients' response to therapy and survival, we subsequently evaluated the in vivo chemoresistant profile of different myeloma PC clones according to their differentiation stage, by performing a longitudinal comparison of the CD19-CD81 pattern of expression in clonal PCs at diagnosis (baseline) vs. after treatment during MRD monitoring (the chemoresistant subclone) in 40 MM patients. Overall, we found that while the expression of CD19 remained mostly stable between baseline and MRD (Figure 3A), there was a significant increase in the percentage of CD81+ chemoresistant clonal PCs after therapy (mean of 31% vs. 21% at baseline, \( P = .04 \)). Accordingly, 30/40 (75%) patients displayed the same differentiation stage during baseline and MRD monitoring [16 corresponding to the fully-differentiated PC subset (i.e.: CD19-CD81-) and 14 to the intermediate stage (i.e.: CD19-CD81+)], whereas 10/40 (25%) patients showed clonal selection of PCs with altered differentiation upon therapy-induced selective pressure (Figure 3B). Namely, 8 cases with fully-differentiated phenotypes at diagnosis showed intermediate stage chemoresistant clonal PCs after therapy; conversely, the remaining 2 patients transitioned from a CD19-CD81+ into a CD19-CD81- phenotype (Figure 3B). These results demonstrate that in approximately one-fourth of MM patients there might be clonal selection upon therapy of PC subsets with a distinct differentiation stage to that observed in the
majority of myeloma PCs at diagnosis; such clonal dynamics usually favoring less-differentiated PC subclones.

**Mutation profiles of intrACLonal heterogeneity according to PC differentiation.**
Upon observing that in selected patients less-differentiated PC subclones became predominant under therapeutic pressure, we decided to investigate whether less- and fully-differentiated PC subclones could eventually display different genomic profiles. In order to address this hypothesis, we investigated the presence of mutations in PC subclones sorted according to their differentiation stage within individual patients (n=6), by using a comprehensive panel covering 77 genes. While one case had no detectable mutations among those tested in any of the FACS-purified CD19+CD81⁺ and CD19⁻CD81⁻ PC subsets (#1; Figure 4A), the five remaining patients had detectable mutations and their pattern differed within PC subclones sorted according to their differentiation stage. Namely, in case#2 CD19⁺CD81⁺ myeloma PCs displayed mutations in **SP140** that were not present among more differentiated CD19⁻CD81⁻ PCs. Similarly, patient#3 had a mutation in **EGFR** among less-differentiated tumor cells while absent in intermediate- and fully-differentiated clones. Patient#4 showed a mutation in **DIS3** that was simultaneously present in CD19⁺CD81⁺ and CD19⁻CD81⁻ myeloma PCs; however, intermediate-differentiated cells had an additional mutation in **IKZF3**. Cases #5 and #6 showed the highest differences between the mutation profiles, with mutually exclusive mutations among intermediate- vs fully-differentiated myeloma in both cases. Overall, these results suggest that tumor heterogeneity dissected according to PC differentiation on phenotypic grounds, may uncover the presence of subclones with different mutation profiles.

**GEP of MM patients according to the differentiation stage of myeloma PC clones.**
After demonstrating that myeloma PCs followed the same model of differentiation as observed in BMPCs from healthy individuals, and that such a model had a clear implication in patients’ survival, we decided to investigate if the differentiation stage of myeloma PC clones would underlie different mRNA expression. Our results showed that newly-diagnosed MM patients with less-differentiated clonal PCs (i.e.: CD19⁺CD81⁺; n=8) displayed 39 deregulated genes as compared to cases with intermediate-differentiation (i.e.: CD19⁺CD81⁺; n=33) (Supplementary Excel File 1). **CD19** mRNA expression was consistent with that observed on phenotypic grounds and was down-regulated among CD19⁺CD81⁺ patients; most-interestingly, down-regulation of other B-cell related genes such as **CD79A**, **MS4A1** (CD20) and **PAX5** was also observed. **PTPRCAP**, which stabilizes the expression of CD45, the pre-B lymphocyte 3
protein coding gene VPREB3, TNFSF8 and CCND1 were also found to be down-regulated among CD19⁺CD81⁺ patients. Although no significantly deregulated genes were observed upon comparing patients with CD19⁺CD81⁺ vs. CD19⁺CD81⁻ (n=28) phenotypes, gene set enrichment analysis (GSEA) showed that patients with intermediate-differentiated CD19⁺CD81⁺ PCs had significantly up-regulation of cell cycle, nucleotide excision repair, and DNA replication pathways as compared to those with fully-differentiated CD19⁺CD81⁻ PCs, which is consistent with the higher proliferative potential of the former PC subset. Conversely, patients with fully-differentiated PCs showed down-regulation of pathways related to protein processing in ER, amongst others (Supplementary Excel File 2). The comparison between patients with less- vs. fully-differentiated (i.e.: CD19⁺CD81⁺ vs. CD19⁺CD81⁻) PCs showed up-regulation of FCRLB, MS4A1 (CD20), CTGF, BEND5 and CD81 in less-differentiated clones (Supplementary Excel File 1). Overall, these results confirm a correlation between the phenotype and the GEP of PCs, but also that phenotypically less-differentiated CD19⁺CD81⁺ myeloma clones retain higher expression of genes associated with preceding B-cell stages.

Discussion

In other hematological malignancies such as acute myeloid leukemia, it is current practice to classify blasts according to their differentiation stage, and the concept of cellular plasticity with more immature clones being typically enriched at the MRD and relapse stages has been recognized.(28) In MM, it has recently been suggested that progenitor organization exists within clonal PCs that recapitulates maturation stages between B-cells and PCs, and may contribute to in vitro chemoresistance.(29) However, there is no accurate knowledge on the myeloma PC differentiation pathway, nor how these correlate with patients’ clinical behavior; in fact, information on the correct identification of less- vs. fully-differentiated normal BMPCs is yet very limited.(12,13) Here, we showed the existence of three well-defined maturation stages in both normal and clonal BMPCs identified through the CD19-CD81 expression axis, and that MM patients harboring less-differentiated PCs have dismal survival. We also showed that the level of PC differentiation in MM could be related, at least in part, to different chemoresistant potential together with different molecular and genomic profiles.

The variable half-life of different serum antibodies (e.g.: in response to measles and mumps vs. influenza viruses’) (30) is consistent with specific survival patterns among unique PC subsets, with long-lived PCs being responsible for maintaining such antibody titers for a life-span of several years or decades.(31) Two recent studies have
characterized CD19+ normal PCs and concluded that these are specifically enriched in the
BM and display unique morphological, transcriptomic and phenotypic features
consistent with increased differentiation as compared to CD19+ PCs (12,13);
accordingly, affinity for viral antigens to which healthy individuals had not been
exposed for more than 40 years have been exclusively detected among CD19−
BMPCs. (12) Such observations open new research areas to further investigate the
features of specific normal and pathological PC subsets according to their
differentiation. (12) Thus, reinforced by the recent confirmation (32) of the regulatory
role of CD81 over CD19 within the B-cell co-receptor (33-36), we decided to investigate
if the CD19-CD81 pattern of expression could help to further dissect unique PC
differentiation subsets. Our results are consistent with those reported by Halliley (12)
and Mei (13) and show that in healthy individuals, CD19+ BMPCs are less proliferative
and are enriched in IgG secreting cells, as compared to the CD19+ subset. The notion
that CD19+ BMPCs are more differentiated than the positive subset was further
confirmed in our study after demonstrating that the former have higher replication
history. However, we also showed that CD19+ BMPCs can be further dissected into
CD19+CD81+ and CD19+CD81− subsets, and that the latter represent the most
differentiated compartment among total BMPCs.

Longevity of PCs in BM is restricted by competition for niche space (37) and in
this competitive model, PC intrinsic features likely contribute to determine their life span
by controlling PC function and niche affinity. (11) Here, CD28 and CD56 expression
was found to be progressively increased from less- CD19+CD81+ into more-
differentiated CD19−CD81− and CD19−CD81+ BMPCs; accordingly, long-term humoral
immunity has been reported to depend on the PC-intrinsic function of CD28 signaling
downstream of the CD28 Vav motif that regulates BLIMP1. (38) CD56 is likely
contributing to stronger PC adhesion to BM stromal niches. Most interestingly,
pathological PCs in MM displayed a similar phenotypic behavior as compared to
normal PCs, and patients with fully-differentiated clones also showed higher expression
of CD28 and CD56, as well as CD38low. The fact that mature CD19+CD81− normal
BMPCs are absent in infants aged 5 to 7 months (13) but progressively accumulate
later in life as shown here, is also a remarkable coincidence with the fact that MGUS
and MM typically develop in the elderly, and that more than half of the patients (59%)
display PC clones that phenotypically overlap with fully-differentiated normal PCs.
Since loss of CD19 and CD81 expression was observed in both normal and tumor PC
differentiation, we hypothesized that their regulation was under epigenetic grounds.
Thus, we analyzed DNA methylation levels around the CD81 gene [in its upstream
CpG island shore region, CpG island, gene body region close to CpG island (Gene
Body 1) and the rest of gene body (Gene Body 2) in three MM cell lines with variable levels of CD81 expression (Figure 5). While no differences in DNA methylation in the CpG island shore and Gene Body 2 regions were observed, the methylation levels in the CpG island and Gene Body region 1 showed a clear correlation with CD81 expression, suggesting that these regions contain regulatory elements that control CD81 expression. Accordingly, methylation in the CpG island and CD81 expression were inversely correlated. In contrast, levels of DNA methylation in the Gene Body region 1 were positively correlated with gene expression. This dual pattern of negative and positive association between gene expression and DNA methylation depending on the region analyzed has been previously observed (39), and underlines that the function of DNA methylation is genomic context dependent.(40)

The notion that PCs represent the terminally-differentiated end-stage of the B-cell lineage has likely contributed to a deficiency in knowledge about the levels of phenotypic plasticity and maturation of clonal PCs in MM.(29,41) Here, we show that up to 41% of MM patients display at diagnosis PC clones corresponding to less differentiated normal PC counterparts, including 3% corresponding to the more immature CD19'CD81' subset. Most interestingly, the latter maintain high expression of genes typically related to mature B-cell stages such as PAX5, CD20, CD79b, VPREB3, TNFSF8 and CCND1 as revealed by comparing their GEP against that of PCs obtained from patients with intermediate- (CD19'CD81') and fully-differentiated phenotypes (CD19'CD81'). These results suggest that the proposed phenotypic differentiation model of myeloma PCs is corroborated at the molecular level, similarly to what has been recently shown in normal BM PCs from healthy individuals.(12) Importantly, MM patients harboring less-differentiated PC clones had dismal outcome with a median survival of approximately 1 year. Accordingly, the differentiation status of clonal PCs emerges as a new and independent prognostic marker in MM, complementary to patients' cytogenetic profile. In fact, patient' characterization according to PC differentiation status allowed the identification of a subset of cases with dismal survival albeit standard-risk cytogenetics. It should be noted that the small number of cases harboring less-differentiated PC clones limits the robustness of the statistical comparison between groups (particularly regarding survival analyses), and these results should be reproduced in larger series of patients (eg.: GEM2005MENOS65 and GEM2005MAS65 clinical trials; Supplementary Figure 1). That notwithstanding, the availability of multiple novel and effective drugs combined with the advent of high-throughput (cellular and molecular) techniques, may help to identify small patient subgroups with a unique biology that could benefit from tailored
treatment (e.g.: anti-CD19 CAR T-cells (42) for cases with less differentiated myeloma PCs).

The identification of more immature cancer (stem) cells has been historically pursued to justify unexplainable relapses, particularly among patients achieving CR.(43,44) However, relapses among MM patients in CR are now better understood and predicted with the advent of MRD monitoring, which have shown an intrinsic correlation between the persistence of residual clonal PCs after therapy (i.e.: MRD) and inferior survival.(45-48) Here, we used a novel approach to understand ultra-chemoresistance by performing in individual patients, longitudinal comparisons between clonal diversity according to PC differentiation at diagnosis vs. MRD.(23) Accordingly, we showed that therapeutic pressure may lead to \textit{in vivo} selection of specific PC subsets, and that in approximately one-fourth of MM patients, such clonal selection favored less-differentiated PC subclones. Thus, further studies are warranted to establish a clear relationship between the extent of PC differentiation and their chemoresistant potential. On a different note, these results may also reflect previously unknown levels of cellular plasticity \textit{in vivo} (49), by which PCs can transition from immature into more mature stages (and vice-versa) upon therapeutic pressure; the observations that CD81 expression is epigenetically regulated together with the lack of a clear pattern of accumulating mutations in FACS-purified immature vs. mature PCs subclones from individual patients, would support such phenomenon of cellular plasticity. Establishing the temporal acquisition of mutations and genetic abnormalities in less vs more differentiated PC clones should be investigated in future studies. Interestingly, these findings also unravel that detailed characterization of the MRD PC compartment might be as informative as more conventional MRD quantitation to predict patients’ outcome (e.g.: survival of an MRD-positive patient displaying immature PC clones may be poorer than other MRD-positive cases).(24,50)

In summary, we shed new light into normal and tumor PC plasticity, with the identification of three well-defined differentiation subsets in both healthy individuals and MM patients, respectively. The demonstration that tumor PC differentiation might be related to unique chemoresistant, molecular and mutation profiles, highlights its importance in the prognostication and monitoring of MM patients.

“Supplementary information is available at Leukemias’s website”
Acknowledgments: The authors want to acknowledge all the participants of the Spanish Myeloma Group.

References


(49) Yaccoby S. The phenotypic plasticity of myeloma plasma cells as expressed by dedifferentiation into an immature, resilient, and apoptosis-resistant phenotype. Clin Cancer Res 2005 Nov 1;11(21):7599-7606.

Figure 1. Bone marrow (BM) normal plasma cell (PC) subsets according to the CD19-CD81 expression axis. (A) Age-related changes in the distribution of BM normal PC subsets. The percentage of the CD19⁺CD81⁺, CD19⁺CD81⁻ and CD19⁻CD81⁻ subsets within total BM PCs from each healthy donor (n=20) was determined, and median values per subset for each age decade are represented by light, intermediate and dark blue areas, respectively. Linear regression between individuals’ age and the respective percentage for each PC subset is also shown. (B) Proliferative potential of the different BM normal PC subsets. The percentage of the CD19⁺CD81⁺ (light blue), CD19⁺CD81⁻ (intermediate blue) and CD19⁻CD81⁻ (dark blue) subsets within total BM PCs from healthy donor (n=5) in G₀/G₁ and S-phase/G₂M phases of the cell cycle is shown. (C) Quantification of the replication history of progressively maturing BM B cell and PC subsets from healthy individuals using KRECs. The line in the middle and vertical lines correspond to the median value and both the 10th and 90th percentiles, respectively, for the ΔCT between the coding joint and the signal joint in FACS-sorted B cell precursors, transitional, naïve and memory B cells, CD19⁺ and CD19⁻ PCs from BM samples of healthy individuals (n=5). (D) Immunoglobulin (Ig) heavy chain isotype distribution of the different BM normal PC subsets. After PC identification according to their bright CD38 and CD138 expression and unique scatter characteristics, cylg⁺ PCs were defined as those showing reactivity in the PE channel (cylgA+cylgG) but not in the FITC channel (cylgM+cylgA), whereas cylgA⁺ PCs were defined as those showing (diagonal) double-staining in the FITC+PE channels; cylgM⁺ PCs were defined by reactivity in the FITC channel but not in PE. The percentage of cytoplasmic IgG, IgA and IgM is shown within the respective CD19⁺CD81⁺, CD19⁺CD81⁻ and CD19⁻CD81⁻ subsets. (E) Immunophenotypic protein expression profiles (iPEP) of the different BM normal PC subsets. Due to the existence of five parameters measured in common for each aliquot (CD38, CD45, CD19, forward light scatter –FSC- and sideward light scatter –SSC-), it was possible to define the PC compartment in each aliquot and fuse the different data files corresponding to the 4 different 8-color MoAb combinations studied per sample into a single data file containing all information measured for that sample, using the merge function of the Infinicyt software. For any single PC in each 8-color MoAb combination, this included data about those antigens that were measured directly on it and antigens which were not evaluated directly (“missing values”) for that cell in the corresponding tube it was contained in. Then, the calculation function of the Infinicyt software was used to fill in the “missing values”, based on the “nearest neighbor” statistical principle, defined by the unique position of individual PCs the multidimensional space created by the five common (backbone) parameters (FSC, SSC, CD38, CD45 and CD19). Ultimately, the expression of all 23
phenotypic markers could be analyzed at the single PC level, and compared between PCs clustering into the specific CD19⁺CD81⁺, CD19⁻CD81⁺ and CD19⁻CD81⁻ subsets. Markers differentially expressed between the CD19⁺CD81⁺ (light blue), CD19⁻CD81⁺ (intermediate blue) and CD19⁻CD81⁻ (dark blue) subsets within BM normal PCs from healthy individuals (n=10). Notched boxes represent the 25th and 75th percentile values of the ratio between the amounts of antigen MFI expression per paired MRD / diagnostic BM clonal PCs; the line in the middle and vertical lines correspond to the median value and both the 10th and 90th percentiles, respectively.

**Figure 2.** Multiple myeloma (MM) patients’ survival according to the differentiation stage of myeloma PC clones. Panels A & B show progression-free survival (PFS) and overall survival (OS) of the overall series of MM patients (n=225) grouped according to the differentiation stage of clonal plasma cells (PCs) at diagnosis: more differentiated (CD19⁻CD81⁻), intermediate (CD19⁻CD81⁺) and less differentiated (CD19⁺CD81⁺). Patients treatment consisted of either nine identical induction cycles with bortezomib, melphalan, prednisone (VMP) followed by other nine cycles of lenalidomide plus low-dose dexamethasone (Rd; n=112), or alternating cycles of VMP and Rd for up to eighteen courses (n=113). The median follow-up of the series was 3-years. Panels C & D show PFS and OS in patients with standard-risk cytogenetics [n=154; all those cases without t(4;14), t(14;16) and/or del(17p13)].

**Figure 3.** Therapeutic selection at the MRD stage of myeloma PC subclones defined according to their differentiation stage. (A) Correlation between the percentage of CD19 (black squares) and CD81 (open circles) positive plasma cells (PCs) within total baseline (x axis) vs. MRD (y axis) clonal PCs in longitudinal bone marrow samples from 40 multiple myeloma (MM) patients analyzed at diagnosis and after therapy. (B) Schema showing the frequency of patients following specific clonal dynamics according to the differentiation stage of myeloma PCs from diagnosis to the MRD stage. Representative bivariate dot plot histograms illustrating the patterns of CD19 versus CD81 expression in clonal PCs at diagnostic (represented by lines corresponding to one and two SD) and at the MRD stage (red dots) corresponding to 4 out of the 8 patients that evolved from baseline more differentiated (i.e.: CD19⁻CD81⁻) into intermediate-differentiated (i.e.: CD19⁻CD81⁺) chemoresistant PC clones after therapy, ordered from left to right according from high to low MRD levels, are also shown. Twelve out of the 30 patients displaying the same differentiation stage during baseline and MRD monitoring attained CR, 3 out of the 8 cases with fully-differentiated phenotypes at diagnosis showing intermediate stage chemoresistant clonal PCs after
therapy attained CR, and so did 1 out of the 2 patients transitioned from a CD19^+CD81^+ into a CD19^+CD81^‐ phenotype.

**Figure 4. Distinct PC differentiation subsets within individual patients show different mutation profiles.** Clonal plasma cells (PCs) corresponding to the intermediate- (CD19^+CD81^+) and more-differentiated (CD19^+CD81^‐) subsets were FACS-sorted from patients #1, #2, #4, #5, and #6 (A, B, D, E and F) for mutation analysis using a targeted-sequencing panel covering 77 genes; in patient #3 (C), mutations were investigated in less differentiated (CD19^+CD81^+) vs. intermediate (CD19^+CD81^‐) and more differentiated (CD19^+CD81^‐) PC clones.

**Figure 5. The sequential CpGs measured by HumanMethylation450 BeadChip for the CD81 gene.** We investigated the expression levels of CD19 and CD81 in a large panel of MM cell lines (RPMI-8226, RPMI-LR5, NCI-H929, OPM-2, JNJ3, MM1S, MM1R, MM144, U266, U266-DOX4, U266-LR7, SJR and MGG) and identified 5 cell lines positive for CD81 (RPMI-8226, RPMI-LR5, NCI-H929, OPM-2, JNJ3) in the absence of CD19; all the others exhibited no expression for both CD19 and CD81 (data not shown). Afterward, under the hypothesis that loss of CD81 expression could be due to epigenetic regulation of the CD81 gene, we investigated the DNA methylation profile of CD81 in the NCI-H929, JNJ3 and U266 cell lines (the first two positive for CD81 and the third negative). Accordingly, we observed an inverse correlation between DNA methylation levels in the CpG island region of the CD81 gene and the protein (antigen) expression level of CD81 in the three MM cell lines. Interestingly, the DNA methylation levels in the CpG island region were also inversely correlated with the DNA methylation levels in the gene body region of CD81. These results indicate that an epigenetic mechanism of DNA methylation plays an important role in the regulation of CD81 expression. The mean of the DNA methylation levels of the CpGs located in the CpG Island or gene body region of CD81 are also shown.
Table 1. Phenotypic features of patients with less-differentiated (i.e.: CD19+CD81+), intermediate-differentiated (i.e.: CD19’CD81’) vs. more-differentiated (i.e.: CD19’CD81’) plasma cell clones among newly-diagnosed multiple myeloma patients (n=225).

<table>
<thead>
<tr>
<th>(%) of cases within subgroup</th>
<th>CD19+CD81⁺</th>
<th>CD19’CD81⁺</th>
<th>CD19’CD81⁻</th>
<th>P-value</th>
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<tr>
<td>CD38⁺</td>
<td>67%</td>
<td>52%</td>
<td>67%</td>
<td>.09</td>
</tr>
<tr>
<td>CD138⁺</td>
<td>33%</td>
<td>31%</td>
<td>31%</td>
<td>.99</td>
</tr>
<tr>
<td>CD27⁺</td>
<td>33%</td>
<td>43%</td>
<td>50%</td>
<td>.48</td>
</tr>
<tr>
<td>CD28⁺</td>
<td>17%</td>
<td>20%</td>
<td>35%</td>
<td>.04</td>
</tr>
<tr>
<td>CD45⁺</td>
<td>67%</td>
<td>50%</td>
<td>29%</td>
<td>.003</td>
</tr>
<tr>
<td>CD56⁺</td>
<td>77%</td>
<td>76%</td>
<td>75%</td>
<td>.85</td>
</tr>
<tr>
<td>CD117⁺</td>
<td>67%</td>
<td>24%</td>
<td>41%</td>
<td>.009</td>
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Table 2. Cytogenetic characteristics of patients with less-differentiated (i.e.: CD19^+CD81^+), and intermediate-differentiated (i.e.: CD19^CD81^+ vs. more-differentiated (i.e.: CD19^CD81^-) plasma cell clones among newly-diagnosed multiple myeloma patients (n=169).

<table>
<thead>
<tr>
<th>Genetic abnormality</th>
<th>PCs differentiation subset</th>
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<tr>
<td></td>
<td>CD19^+ CD81^+</td>
<td>CD19^- CD81^+</td>
<td>CD19^- CD81^-</td>
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<tr>
<td>None</td>
<td>25%</td>
<td>62%</td>
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<td>.07</td>
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<tr>
<td>t(4;14)</td>
<td>0%</td>
<td>24%</td>
<td>17%</td>
<td>NS</td>
</tr>
<tr>
<td>t(11;14)</td>
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<td>68%</td>
<td>36%</td>
<td>.03</td>
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<td>49%</td>
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<td>del(17p)</td>
<td>0%</td>
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<td>8%</td>
<td>NS</td>
</tr>
<tr>
<td>High-risk FISH</td>
<td>0%</td>
<td>23%</td>
<td>19%</td>
<td>NS</td>
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Table 3. Multivariate analyses including baseline disease features with univariate significant effect on PFS and/or OS of newly diagnosed elderly myeloma patients included in the GEM2010MAS65 trial.

<table>
<thead>
<tr>
<th></th>
<th>PFS</th>
<th></th>
<th>OS</th>
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<tr>
<td></td>
<td>HR</td>
<td>P</td>
<td>HR</td>
<td>P</td>
</tr>
<tr>
<td>Age (&lt;75 vs. ≥75 years)</td>
<td>1.3</td>
<td>.21</td>
<td>2.7</td>
<td>.001</td>
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<tr>
<td>ISS</td>
<td>1.2</td>
<td>.50</td>
<td>1.9</td>
<td>.16</td>
</tr>
<tr>
<td>Interphase FISH cytogenetics</td>
<td>1.9</td>
<td>.003</td>
<td>2.7</td>
<td>.001</td>
</tr>
<tr>
<td>(standard- vs. high-risk)</td>
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<td></td>
<td></td>
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<tr>
<td>PC differentiation stage</td>
<td>1.7</td>
<td>.005</td>
<td>2.1</td>
<td>.006</td>
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</table>

PFS: progression-free survival; OS: overall survival; ISS: International Staging System; FISH: Fluorescence In Situ hybridization; High-risk FISH: t(4;14), t(14;16) and/or del(17p13); PC: plasma cell