Published in final edited form as:
Leukemia. 2017 February ; 31(2): 382–392. doi:10.1038/leu.2016.211.

Differentiation stage of myeloma plasma cells: biological and clinical significance

B Paiva[1], **N Puig**[2], **MT Cedena**[3], **BG de Jong**[4], **Y Ruiz**[3], **I Rapado**[3], **J Martinez-Lopez**[3], **L Cordon**[5], **D Alignani**[1], **JA Delgado**[1], **MC van Zelm**[4,6], **JJM Van Dongen**[4], **M Pascual**[1], **X Agirre**[1], **F Prosper**[1], **JI Martín-Subero**[7], **M-B Vidriales**[2], **NC Gutierrez**[2], **MT Hernandez**[8], **A Oriol**[9], **MA Echeveste**[10], **Y Gonzalez**[11], **SK Johnson**[12], **J Epstein**[12], **B Barlogie**[12], **GJ Morgan**[12], **A Orfao**[13], **J Blade**[14], **MV Mateos**[2], **JJ Lahuerta**[3], and **JF San-Miguel**[1] **on behalf of GEM (Grupo Español de MM)/PETHEMA (Programa para el Estudio de la Terapéutica en Hemopatías Malignas) cooperative study groups**

[1]Clinica Universidad de Navarra, Centro de Investigacion Medica Aplicada (CIMA), IDISNA, Pamplona, Spain [2]Hospital Universitario de Salamanca, Instituto de Investigacion Biomedica de Salamanca (IBSAL), Centro de Investigación del Cancer (IBMCC-USAL, CSIC), Salamanca, Spain [3]Hospital 12 de Octubre, Madrid, Spain [4]Department of Immunology, Erasmus MC, University Medical Center, Rotterdam, The Netherlands [5]Hospital Universitario y Politécnico La Fe, Valencia, Spain [6]Department of Immunology and Pathology, Monash University, Melbourne VIC Australia [7]Unidad de Hematopatología, Servicio de Anatomía Patológica, Hospital Clínic, Universitat de Barcelona, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain [8]Hospital Universitario de Canarias, Tenerife, Spain [9]Institut Català d'Oncologia, Institut Josep Carreras, Hospital Germans Trias I Pujol, Badalona, Spain [10]Hospital de Donostia, San Sebastian, Spain [11]Hospital Universitario Josep Trueta, Girona, Spain [12]Myeloma Institute for Research and Therapy, University of Arkansas for Medical Sciences, Little Rock, AR, USA [13]Servicio General de Citometría, Centro de Investigación del Cancer (IBMCC-USAL, CSIC), IBSAL and Department of Medicine, Universidad de Salamanca, Salamanca, Spain [14]Hospital Clínic, IDIBAPS, Barcelona, Spain

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-

Correspondence: Professor JF San Miguel, Clinica Universidad de Navarra; Centro de Investigacion Médica Aplicada (CIMA), Av. Pio XII 36, Pamplona 31008, Spain. sanmiguel@unav.es.

Conflict of Interest
The authors declare no conflict of interest.

Europe PMC Funders Author Manuscripts

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; $P = 0.005$) and overall survival (HR: 2.1; $P = 0.006$). 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 (for example: 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 harbouring 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 Tumour 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* down-regulation 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

Europe PMC Funders Author Manuscripts

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.

Materials and 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 criteria16 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 years; 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 200 μl of ethylenediaminetetraacetic acid-anticoagulated BM aspirated samples from newly diagnosed MM patients were immunophenotyped using two different eight-colour 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)/APCH7): (i) CD45/CD138/CD38/CD56/β2microglobulin/CD27/CD19/cyKappa/cyLambda; (ii) CD45/CD138/CD38/CD28/CD27/CD19/CD117/CD81 following the EuroFlow guidelines17 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 eight-colour MoAb combination (PacB/PacO/FITC/PE/PerCP-Cy5.5/PE-Cy7/APC/APCH7) with CD45/CD138/CD38/CD56/CD27/CD19/CD117/CD81 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 eight-colour MoAb combination was used to characterize the BMPC compartment of the 20 healthy individuals. In five out of the former 20 cases, an additional eight-colour MoAb combination (BV421/BV510/FITC/PE/PerCP-Cy5.5/PE-Cy7/APC/APCH7) with CD138/CD27/cyIgM+cyIgA/cyIgA+cyIgG/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 an FACSCantoII flow cytometer (Becton Dickinson – BD – San Jose, CA, USA) 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 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.20 Primers and probes were designed to specifically amplify the intronRSS-Kde rearrangements (coding joint) and the corresponding signal joint using TaqMan-based real-time quantitative PCR from DNA isolated from FACS-sorted cell subsets.20 The real-time quantitative PCR mixture of 25 μl contained TaqMan Universal MasterMix (Applied Biosystems, Waltham, MA, USA), 900 nM of each primer, 100 nM of each FAM-TAMRA-labelled probe, 50 ng of DNA and 0.4ng BSA, and was run on the ABIPRISM 7700 sequence detection system (Applied Biosystems).20

Cell cycle analyses

The proliferation index of different normal PC subsets according to CD19 − CD81 expression was analysed in BM samples from five healthy individuals using five-colour staining for nuclear DNA and four cell surface antigens (CD19–PacB/CD45–PacO/CD38–FITC/CD81–PE) as described elsewhere.21

Single-cell multidimensional phenotyping

Bone marrow aspirates from healthy individuals ($n = 10$) were immunophenotyped using four different eight-colour 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 analysed 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.22–24

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 six newly diagnosed MM patients was analysed including the corresponding germline samples. DNA was extracted from cells using AllPrep DNA/RNA Micro Kit, Qiagen (Hilden, Germany). 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, Waltham, MA, USA) 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, with 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, Bergisch Gladbach, Germany), as described elsewhere.25 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 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, Irvine, CA, USA) for bisulfite conversion of 500 ng genomic DNA. Bisulfite-converted DNA was hybridized onto the HumanMethylation 450 K BeadChip kit (Illumina, San Diego, CA, USA). Data from the 450 k Human Methylation Array were analysed as described previously.26

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 analysed 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 $P < 0.05$.The SPSS software (version 20.0; IBM, Armonk, NY, 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 BMPCs 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 years, their frequency progressively increased from younger to older individuals (Figure 1a). Accordingly, there was a significant ($P \leqslant 0.006$) 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_2M normal BMPCs. As expected, the majority of BM PCs were in G_0G_1 (data not shown), but while the relative distribution of all three $CD19^+CD81^+$, $CD19^-CD81^+$ and $CD19^-CD81^-$ subsets in G_0G_1 was inside the normal ranges described above, there were virtually no $CD19^-CD81^-$ PCs in S-phase/G2M (Figure 1b; $P = 0.03$). 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 κ-deleting recombination excision circle 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 ($P = 0.04$), 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 ($P = 0.08$). Additionally, there was a trend ($P = 0.07$) 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 mean fluorescence intensity 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 (that is: $CD19^{-}CD81^{-}$); conversely, 86 out of the 225 patients (38%) displayed intermediate-differentiated myeloma PCs (that is: $CD19^{-}CD81^{+}$), whereas only seven cases (3%) showed clonal PCs for which the normal counterpart would correspond to the less-differentiated BMPC subset (that is: $CD19^{+}CD81^{+}$). 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 ($P = 0.07$) for higher frequencies of cytogenetic abnormalities (that is: 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%; $P = 0.03$). Upon investigating if the differentiation stage of myeloma PC clones influenced patients' prognosis, we noted that progression-free survival and overall survival 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 b). The treatment arm had no impact in patients' outcomes according to PC differentiation (data not shown). Multivariate analysis of baseline 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 progression-free survival and overall survival were PC differentiation and FISH cytogenetics (Table 3). Accordingly, the differentiation stage of clonal PCs continued to be prognostically relevant for progression-free survival and overall survival when the analysis was restricted to cytogenetically defined standard-risk cases (Figures 2c and d), 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, $P = 0.04$). Accordingly, 30/40 (75%) patients displayed the same differentiation stage during baseline and MRD monitoring (16 corresponding to the fully differentiated PC

subset (that is: $CD19^{-}CD81^{-}$) and 14 to the intermediate stage (that is: $CD19^{-}CD81^{+}$)), whereas 10/40 (25%) patients showed clonal selection of PCs with altered differentiation upon therapy-induced selective pressure (Figure 3b). Namely, eight cases with fully differentiated phenotypes at diagnosis showed intermediate stage chemoresistant clonal PCs after therapy; conversely, the remaining two 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 favour 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 epidermal growth factor receptor among less-differentiated tumour 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 tumour 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 (that is: $CD19^{+}CD81^{+}$; $n = 8$) displayed 39 deregulated genes as compared to cases with intermediate-differentiation (that is: $CD19^{-}CD81^{+}$; $n = 33$) (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 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, among others (Supplementary Excel File 2). The comparison between patients with less- vs fully differentiated (that is: $CD19^{+}CD81^{+}$ 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.27 In MM, it has recently been suggested that a progenitor organization exists within clonal PCs that recapitulates maturation stages between B-cells and PCs, and may contribute to *in vitro* chemoresistance.28 However, there is no accurate knowledge on the myeloma PC differentiation pathway, nor how these correlate with patients' clinical behaviour; 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 harbouring 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 (for example: in response to measles and mumps vs influenza viruses)29 is consistent with specific survival patterns among unique PC subsets, with long-lived PCs being responsible for maintaining such antibody titres for a life-span of several years or decades.30 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 confirmation31 of the regulatory role of CD81 over CD19 within the B-cell co-receptor,14,32–34 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 *et al.*12 and Mei *et al.*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 space35 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 signalling down-stream of the CD28 Vav motif that regulates BLIMP1.36 CD56 is likely contributing to stronger PC adhesion to BM stromal niches. Most interestingly, pathological PCs in MM displayed a similar phenotypic behaviour as compared to normal PCs, and patients with fully differentiated clones also showed higher expression of CD28 and CD56, as well as $CD38^{low}$. The fact that mature $CD19^-CD81^-$ normal BMPCs are absent in infants aged 5–7 months13 but progressively accumulate later in life as shown here, is also a remarkable coincidence with the fact that monoclonal gammopathy of undetermined significance 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 tumour PC differentiation, we hypothesized that their regulation was under epigenetic grounds. Thus, we analysed 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 analysed has been previously observed,37 and underlines that the function of DNA methylation is genomic context dependent.38

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.28,39 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 BMPCs from healthy individuals.12 Importantly, MM patients harbouring 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, patients' 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 harbouring 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 (for example: 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 (for example: anti-CD19 CAR T-cells40 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.41,42 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 (that is: MRD) and inferior survival.43–46 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.22 Hence, we showed that therapeutic pressure may lead to *in vivo* selection of specific PC subsets, and that in approximately one-fourth of MM patients such clonal selection favoured 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 *in vivo*,47 by which PCs can transition from mature into more immature 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. Thus, 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 (for example: survival of an MRD-positive patient displaying immature PC clones may be poorer than other MRD-positive cases).23,48

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors acknowledge all the participants of the Spanish Myeloma Group. This study was supported by the Cooperative Research Thematic Network grants RD12/0036/0048, RD12/0036/0058, RD12/0036/0046, RD12/0036/0068, RD12/0036/0069, and RD12/0036/0061 of the Red de Cancer (Cancer Network of Excellence); Instituto de Salud Carlos III, Spain, Instituto de Salud Carlos III/Subdirección General de Investigación Sanitaria (FIS: PI060339; 06/1354; 02/0905; 01/0089/01-02; PS09-/01897/01370; PI13/01469, PI14/01867, G03/136; Sara Borrell: CD13/00340 and CD12/00540); Fundació La Marató de TV3 (20132130-31-32) and Asociación Española Contra el Cáncer (GCB120981SAN). The study was also supported internationally by the International Myeloma Foundation (IMF) Junior Grant, the Black Swan Research Initiative of the IMF, the Multiple Myeloma Research Foundation research fellow award, the Qatar National Research Fund (QNRF) Award No. 7-916-3-237, Marie Curie (LincMHeM-330598), the AACR-Millennium Fellowship in Multiple Myeloma Research (15-40-38-PAIV), Leukemia Research Foundation and the European Research Council (ERC) 2015 Starting Grant.

References

1. Dimopoulos M, Kyle R, Fermand JP, Rajkumar SV, San Miguel J, Chanan-Khan A, et al. Consensus recommendations for standard investigative workup: report of the International Myeloma Workshop Consensus Panel 3. Blood. 2011; 117:4701–4705. [PubMed: 21292778]
2. Flores-Montero J, de Tute R, Paiva B, Perez JJ, Bottcher S, Wind H, et al. Immunophenotype of normal vs. myeloma plasma cells: Toward antibody panel specifications for MRD detection in multiple myeloma. Cytometry B Clin Cytom. 2015; 90:61–72. [PubMed: 26100534]
3. Mateo G, Montalban MA, Vidriales MB, Lahuerta JJ, Mateos MV, Gutierrez N, et al. Prognostic value of immunophenotyping in multiple myeloma: a study by the PETHEMA/GEM cooperative study groups on patients uniformly treated with high-dose therapy. J Clin Oncol. 2008; 26:2737–2744. [PubMed: 18443352]
4. Paiva B, Gutierrez NC, Chen X, Vidriales MB, Montalban MA, Rosinol L, et al. Clinical significance of CD81 expression by clonal plasma cells in high-risk smoldering and symptomatic multiple myeloma patients. Leukemia. 2012; 26:1862–1869. [PubMed: 22333880]
5. Bataille R, Jego G, Robillard N, Barille-Nion S, Harousseau JL, Moreau P, et al. The phenotype of normal, reactive and malignant plasma cells. Identification of 'many and multiple myelomas' and of new targets for myeloma therapy. Haematologica. 2006; 91:1234–1240. [PubMed: 16956823]
6. Paiva B, Almeida J, Perez-Andres M, Mateo G, Lopez A, Rasillo A, et al. Utility of flow cytometry immunophenotyping in multiple myeloma and other clonal plasma cell-related disorders. Cytometry B Clin Cytom. 2010; 78:239–252. [PubMed: 20155853]
7. Rawstron AC, Orfao A, Beksac M, Bezdickova L, Brooimans RA, Bumbea H, et al. Report of the European Myeloma Network on multiparametric flow cytometry in multiple myeloma and related disorders. Haematologica. 2008; 93:431–438. [PubMed: 18268286]
8. Shapiro-Shelef M, Calame K. Regulation of plasma-cell development. Nat Rev Immunol. 2005; 5:230–242. [PubMed: 15738953]
9. Cobaleda C, Schebesta A, Delogu A, Busslinger M. Pax5: the guardian of B cell identity and function. Nat Immunol. 2007; 8:463–470. [PubMed: 17440452]
10. Kozmik Z, Wang S, Dorfler P, Adams B, Busslinger M. The promoter of the CD19 gene is a target for the B-cell-specific transcription factor BSAP. Mol Cell Biol. 1992; 12:2662–2672. [PubMed: 1375324]
11. Cocco M, Stephenson S, Care MA, Newton D, Barnes NA, Davison A, et al. In vitro generation of long-lived human plasma cells. J Immunol. 2012; 189:5773–5785. [PubMed: 23162129]
12. Halliley JL, Tipton CM, Liesveld J, Rosenberg AF, Darce J, Gregoretti IV, et al. Long-lived plasma cells are contained within the CD19(-)CD38(hi)CD138(+) subset in human bone marrow. Immunity. 2015; 43:132–145. [PubMed: 26187412]

13. Mei HE, Wirries I, Frolich D, Brisslert M, Giesecke C, Grun JR, et al. A unique population of IgG-expressing plasma cells lacking CD19 is enriched in human bone marrow. Blood. 2015; 125:1739–1748. [PubMed: 25573986]
14. van Zelm MC, Smet J, Adams B, Mascart F, Schandene L, Janssen F, et al. CD81 gene defect in humans disrupts CD19 complex formation and leads to antibody deficiency. J Clin Invest. 2010; 120:1265–1274. [PubMed: 20237408]
15. Barrena S, Almeida J, Yunta M, Lopez A, Fernandez-Mosteirin N, Giralt M, et al. Aberrant expression of tetraspanin molecules in B-cell chronic lymphoproliferative disorders and its correlation with normal B-cell maturation. Leukemia. 2005; 19:1376–1383. [PubMed: 15931266]
16. Durie BG, Harousseau JL, Miguel JS, Blade J, Barlogie B, Anderson K, et al. International uniform response criteria for multiple myeloma. Leukemia. 2006; 20:1467–1473. [PubMed: 16855634]
17. van Dongen JJ, Lhermitte L, Bottcher S, Almeida J, van der Velden VH, Flores-Montero J, et al. EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes. Leukemia. 2012; 26:1908–1975. [PubMed: 22552007]
18. Perez-Andres M, Paiva B, Nieto WG, Caraux A, Schmitz A, Almeida J, et al. Human peripheral blood B-cell compartments: a crossroad in B-cell traffic. Cytometry B Clin Cytom. 2010; 78(Suppl 1):S47–S60. [PubMed: 20839338]
19. Berkowska MA, Driessen GJ, Bikos V, Grosserichter-Wagener C, Stamatopoulos K, Cerutti A, et al. Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. Blood. 2011; 118:2150–2158. [PubMed: 21690558]
20. van Zelm MC, Szczepanski T, van der Burg M, van Dongen JJ. Replication history of B lymphocytes reveals homeostatic proliferation and extensive antigen-induced B cell expansion. J Exp Med. 2007; 204:645–655. [PubMed: 17312005]
21. Paiva B, Paino T, Sayagues JM, Garayoa M, San-Segundo L, Martin M, et al. Detailed characterization of multiple myeloma circulating tumor cells shows unique phenotypic, cytogenetic, functional, and circadian distribution profile. Blood. 2013; 122:3591–3598. [PubMed: 24072855]
22. Paiva B, Corchete LA, Vidriales MB, Puig N, Maiso P, Rodriguez I, et al. Phenotypic and genomic analysis of multiple myeloma minimal residual disease tumor cells: a new model to understand chemoresistance. Blood. 2016; 127:1896–1906. [PubMed: 26755711]
23. Paino T, Paiva B, Sayagues JM, Mota I, Carvalheiro T, Corchete LA, et al. Phenotypic identification of subclones in multiple myeloma with different chemoresistant, cytogenetic and clonogenic potential. Leukemia. 2015; 29:1186–1194. [PubMed: 25388955]
24. Paiva B, Corchete LA, Vidriales MB, Garcia-Sanz R, Perez JJ, Aires-Mejia I, et al. The cellular origin and malignant transformation of Waldenstrom macroglobulinemia. Blood. 2015; 125:2370–2380. [PubMed: 25655603]
25. Usmani SZ, Crowley J, Hoering A, Mitchell A, Waheed S, Nair B, et al. Improvement in long-term outcomes with successive Total Therapy trials for multiple myeloma: are patients now being cured? Leukemia. 2013; 27:226–232. [PubMed: 22705990]
26. Agirre X, Castellano G, Pascual M, Heath S, Kulis M, Segura V, et al. Whole-epigenome analysis in multiple myeloma reveals DNA hypermethylation of B cell-specific enhancers. Genome Res. 2015; 25:478–487. [PubMed: 25644835]
27. Zeijlemaker W, Gratama JW, Schuurhuis GJ. Tumor heterogeneity makes AML a ‘moving target’ for detection of residual disease. Cytometry B Clin Cytom. 2014; 86:3–14. [PubMed: 24151248]
28. Leung-Hagesteijn C, Erdmann N, Cheung G, Keats JJ, Stewart AK, Reece DE, et al. Xbp1s-negative tumor B cells and pre-plasmablasts mediate therapeutic protea-some inhibitor resistance in multiple myeloma. Cancer Cell. 2013; 24:289–304. [PubMed: 24029229]
29. Amanna IJ, Carlson NE, Slifka MK. Duration of humoral immunity to common viral and vaccine antigens. N Engl J Med. 2007; 357:1903–1915. [PubMed: 17989383]
30. Manz RA, Thiel A, Radbruch A. Lifetime of plasma cells in the bone marrow. Nature. 1997; 388:133–134. [PubMed: 9217150]

31. Vences-Catalan F, Kuo CC, Sagi Y, Chen H, Kela-Madar N, van Zelm MC, et al. A mutation in the human tetraspanin CD81 gene is expressed as a truncated protein but does not enable CD19 maturation and cell surface expression. J Clin Immunol. 2015; 35:254–263. [PubMed: 25739915]
32. Cherukuri A, Carter RH, Brooks S, Bornmann W, Finn R, Dowd CS, et al. B cell signaling is regulated by induced palmitoylation of CD81. J Biol Chem. 2004; 279:31973–31982. [PubMed: 15161911]
33. Cherukuri A, Shoham T, Sohn HW, Levy S, Brooks S, Carter R, et al. The tetraspanin CD81 is necessary for partitioning of coligated CD19/CD21-B cell antigen receptor complexes into signaling-active lipid rafts. J Immunol. 2004; 172:370–380. [PubMed: 14688345]
34. Shoham T, Rajapaksa R, Boucheix C, Rubinstein E, Poe JC, Tedder TF, et al. The tetraspanin CD81 regulates the expression of CD19 during B cell development in a postendoplasmic reticulum compartment. J Immunol. 2003; 171:4062–4072. [PubMed: 14530327]
35. Tokoyoda K, Hauser AE, Nakayama T, Radbruch A. Organization of immunological memory by bone marrow stroma. Nat Rev Immunol. 2010; 10:193–200. [PubMed: 20154734]
36. Rozanski CH, Utley A, Carlson LM, Farren MR, Murray M, Russell LM, et al. CD28 promotes plasma cell survival, sustained antibody responses, and BLIMP-1 upregulation through its distal PYAP proline motif. J Immunol. 2015; 194:4717–4728. [PubMed: 25833397]
37. Kulis M, Heath S, Bibikova M, Queiros AC, Navarro A, Clot G, et al. Epigenomic analysis detects widespread gene-body DNA hypomethylation in chronic lymphocytic leukemia. Nat Genet. 2012; 44:1236–1242. [PubMed: 23064414]
38. Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nat Rev Genet. 2012; 13:484–492. [PubMed: 22641018]
39. Chaidos A, Barnes CP, Cowan G, May PC, Melo V, Hatjiharissi E, et al. Clinical drug resistance linked to interconvertible phenotypic and functional states of tumor-propagating cells in multiple myeloma. Blood. 2013; 121:318–328. [PubMed: 23169779]
40. Garfall AL, Maus MV, Hwang WT, Lacey SF, Mahnke YD, Melenhorst JJ, et al. Chimeric antigen receptor T cells against CD19 for multiple myeloma. N Engl J Med. 2015; 373:1040–1047. [PubMed: 26352815]
41. Matsui W, Huff CA, Wang Q, Malehorn MT, Barber J, Tanhehco Y, et al. Characterization of clonogenic multiple myeloma cells. Blood. 2004; 103:2332–2336. [PubMed: 14630803]
42. Peacock CD, Wang Q, Gesell GS, Corcoran-Schwartz IM, Jones E, Kim J, et al. Hedgehog signaling maintains a tumor stem cell compartment in multiple myeloma. Proc Natl Acad Sci USA. 2007; 104:4048–4053. [PubMed: 17360475]
43. Puig N, Sarasquete ME, Balanzategui A, Martinez J, Paiva B, Garcia H, et al. Critical evaluation of ASO RQ-PCR for minimal residual disease evaluation in multiple myeloma. A comparative analysis with flow cytometryBlood. 2014; 28:391–397.
44. Paiva B, Gutierrez NC, Rosinol L, Vidriales MB, Montalban MA, Martinez-Lopez J, et al. High-risk cytogenetics and persistent minimal residual disease by multiparameter flow cytometry predict unsustained complete response after autologous stem cell transplantation in multiple myeloma. Blood. 2012; 119:687–691. [PubMed: 22128143]
45. Martinez-Lopez J, Lahuerta JJ, Pepin F, Gonzalez M, Barrio S, Ayala R, et al. Prognostic value of deep sequencing method for minimal residual disease detection in multiple myeloma. Blood. 2014; 123:3073–3079. [PubMed: 24646471]
46. Rawstron AC, Gregory WM, de Tute RM, Davies FE, Bell SE, Drayson MT, et al. Minimal residual disease in myeloma by flow cytometry: independent prediction of survival benefit per log reduction. Blood. 2015; 125:1932–1935. [PubMed: 25645353]
47. 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; 11:7599–7606. [PubMed: 16278377]
48. Paiva B, van Dongen JJ, Orfao A. New criteria for response assessment: role of minimal residual disease in multiple myeloma. Blood. 2015; 125:3059–3068. [PubMed: 25838346]

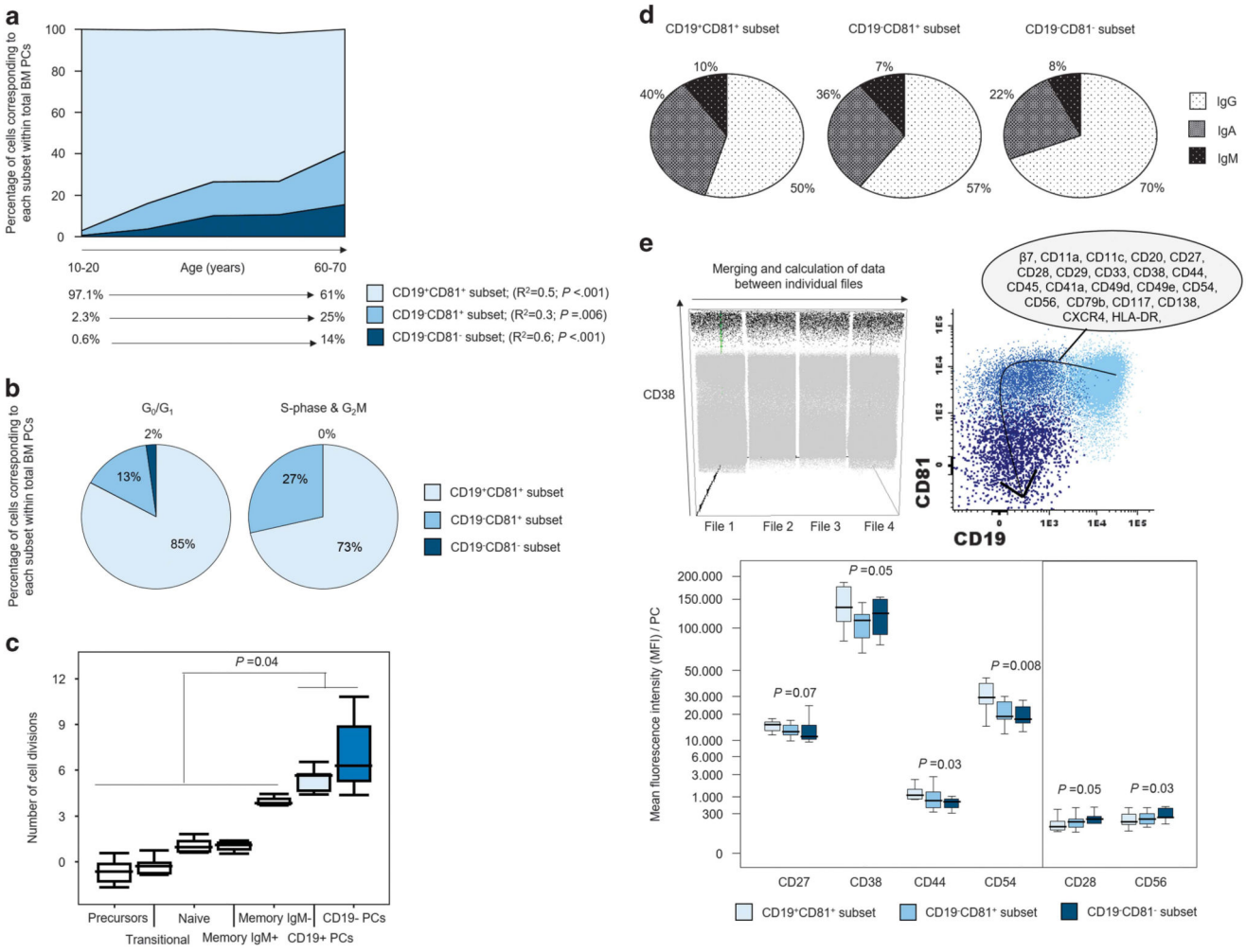

Figure 1.

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 G_0/G_1 and S-phase/G_2M 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 κ-deleting recombination excision circles. 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, cyIgG$^+$ PCs were defined as those showing reactivity in the PE channel (cyIgA+cyIgG) but not in the FITC channel (cyIgM +cyIgA), whereas cyIgA$^+$ PCs were defined as those showing (diagonal) double-staining in the FITC+PE channels; cyIgM$^+$ 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 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 four different eight-colour 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 eight-colour MoAb combination, this included data about those antigens that were measured directly on it and antigens that 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 neighbour’ 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 analysed 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 amounts of antigen mean fluorescence intensity expression per BM PCs; the line in the middle and vertical lines correspond to the median value and both the 10th and 90th percentiles, respectively.

Europe PMC Funders Author Manuscripts

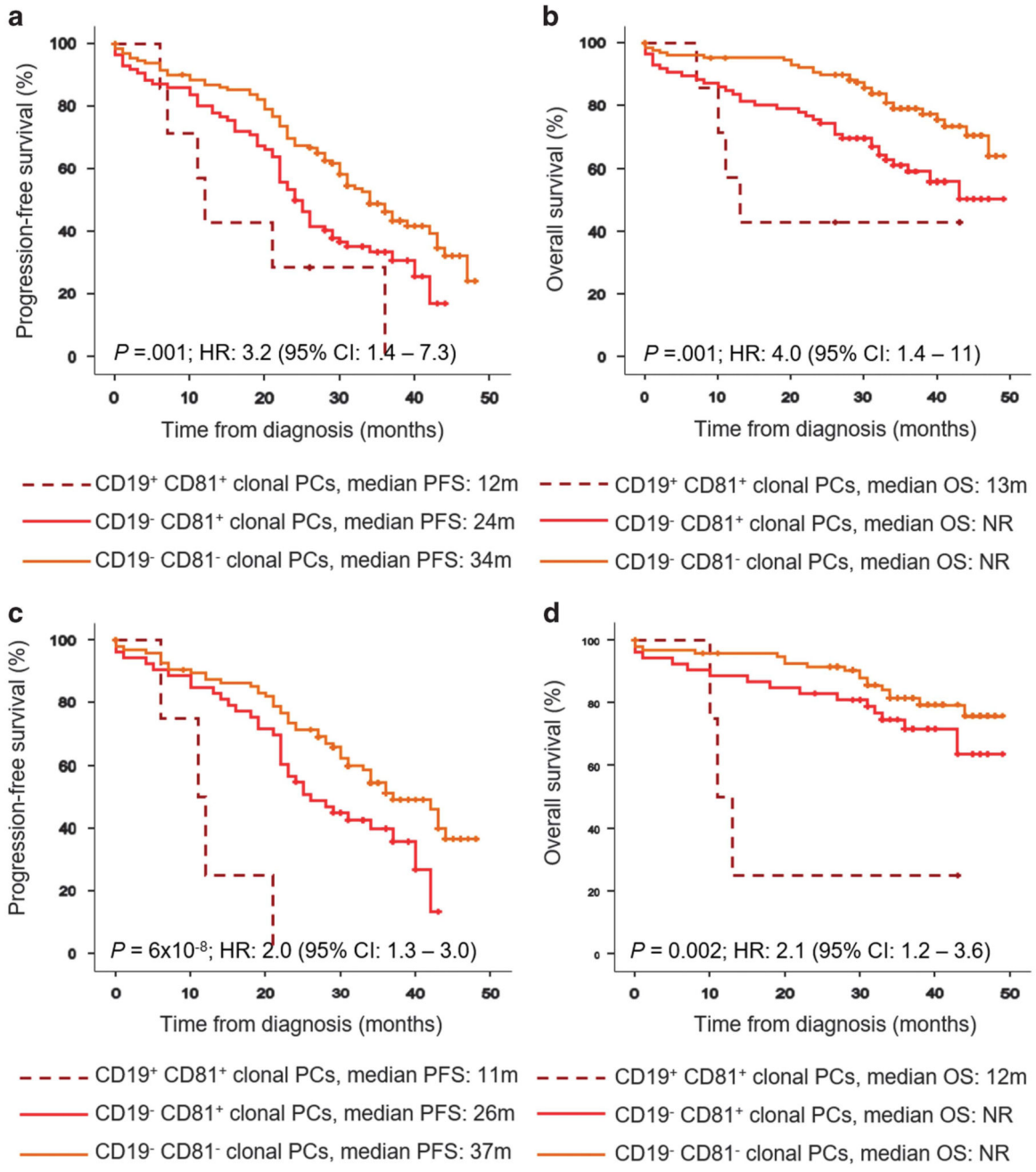

Figure 2.

Multiple myeloma (MM) patients' survival according to the differentiation stage of myeloma PC clones. Panels **a** and **b** show progression-free survival (PFS) and overall survival (OS) for 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-differentiated ($CD19^{-}CD81^{+}$) 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 18 courses ($n = 113$). The median follow-up of the series was 3 years. Panels **c** and **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)).

Europe PMC Funders Author Manuscripts

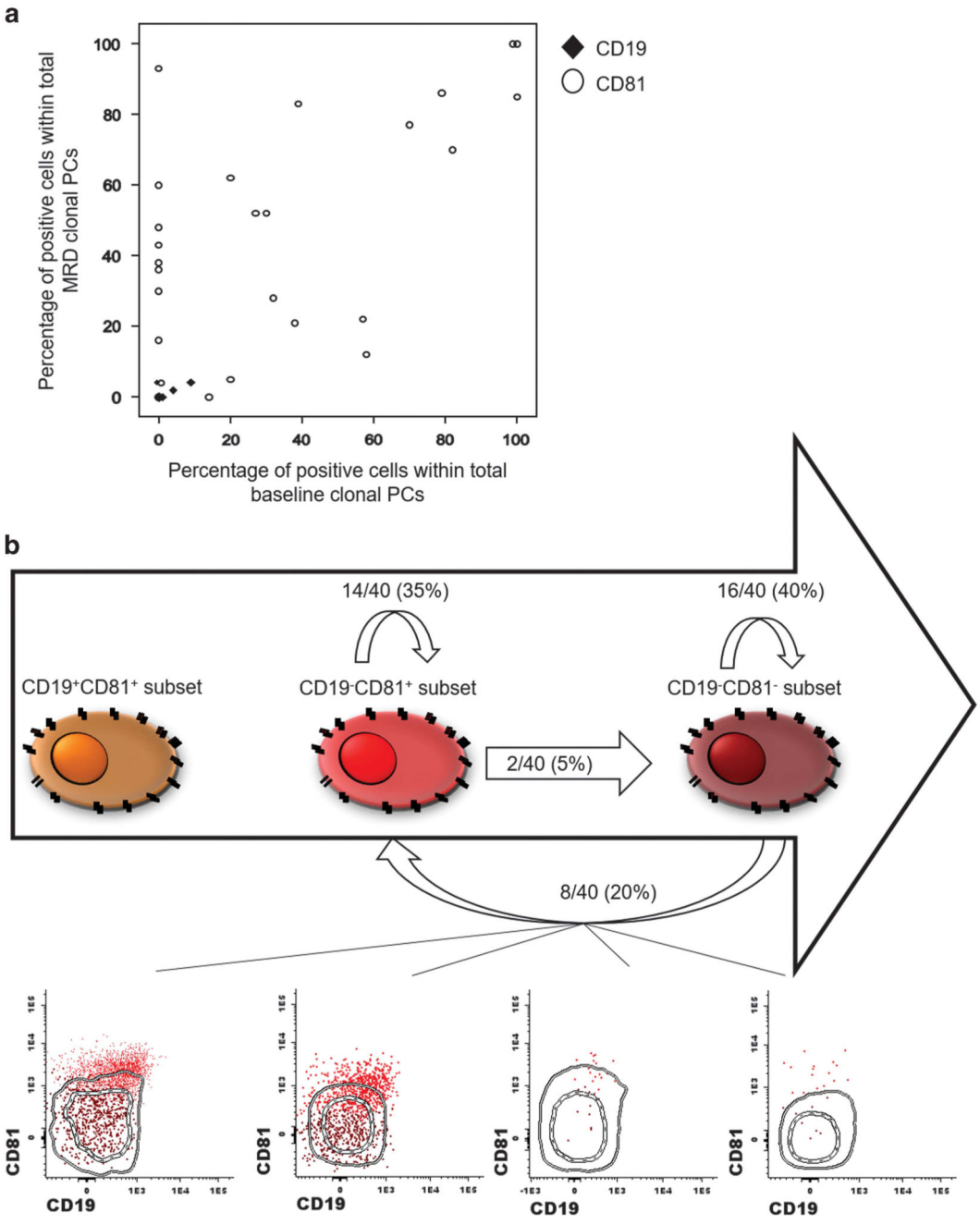

Figure 3.

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 analysed 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 vs 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 four out of the eight patients that evolved from baseline more differentiated (that is: $CD19^{-}CD81^{-}$) into intermediate-differentiated (that is: $CD19^{-}CD81^{+}$) chemoresistant PC clones after therapy, ordered from left to right, denoting 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, three out of the eight cases with fully differentiated phenotypes at diagnosis showing intermediate stage chemoresistant clonal PCs after therapy attained CR, and so did one out of the two patients transitioned from a $CD19^{-}CD81^{+}$ into a $CD19^{-}CD81^{-}$ phenotype.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

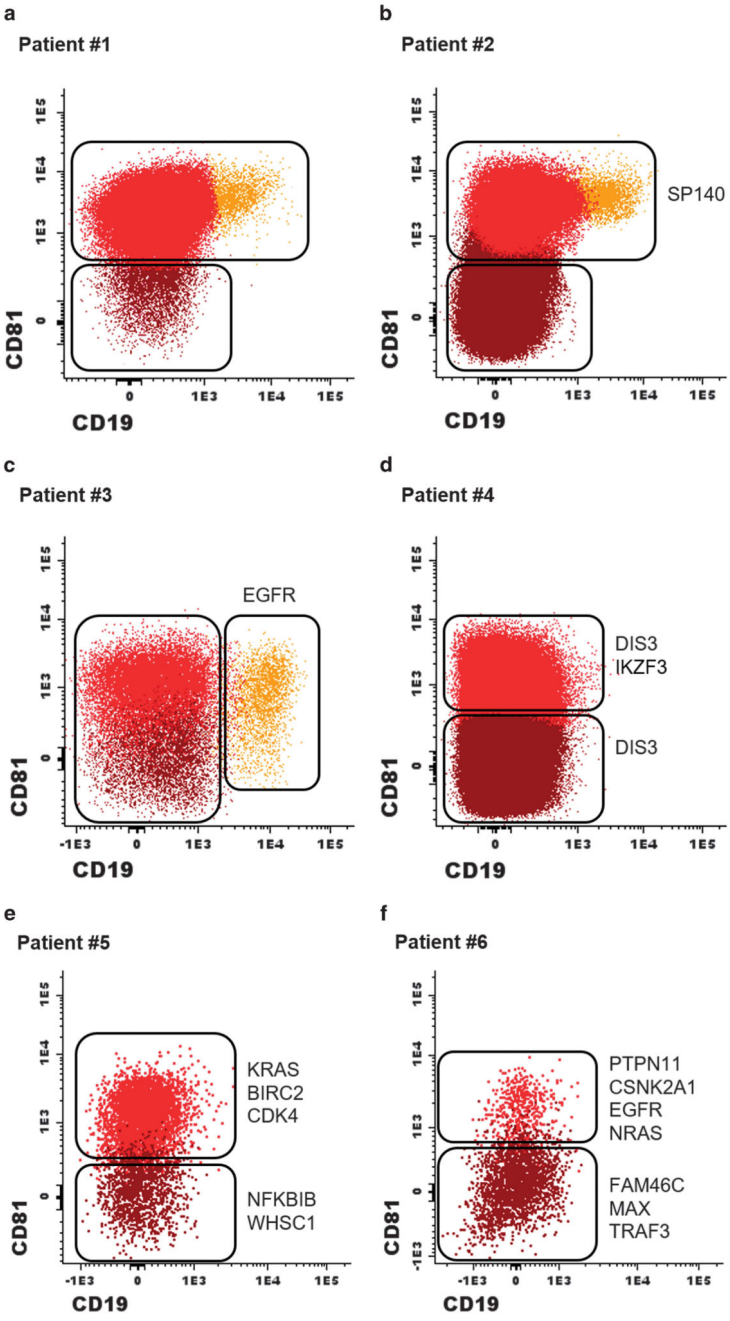

Figure 4.

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.

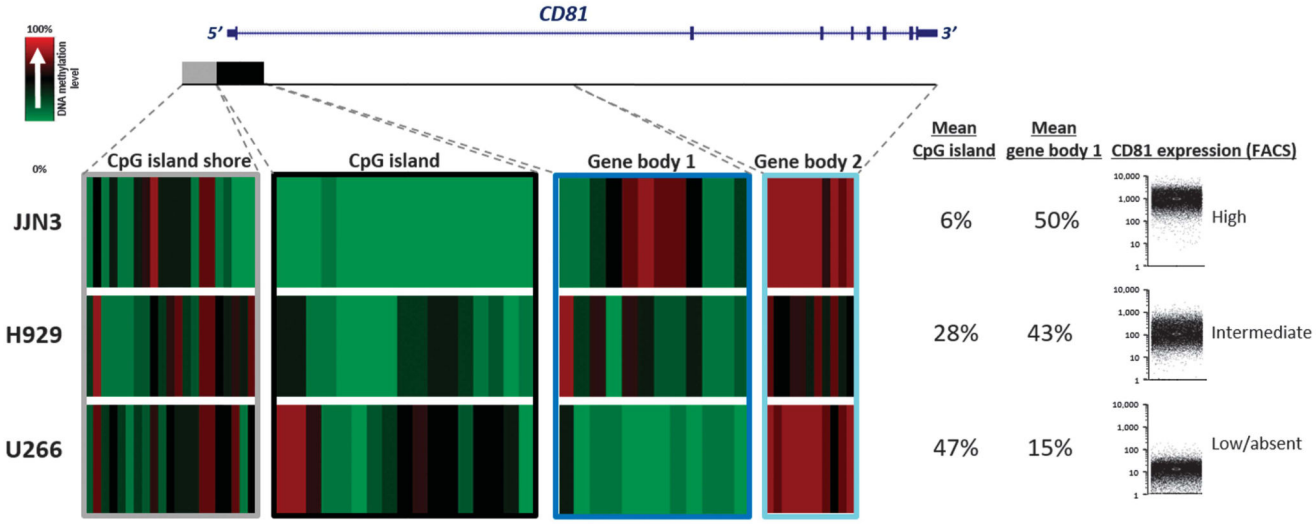

Figure 5.

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, JJN3, MM1S, MM1R, MM144, U266, U266-DOX4, U266-LR7, SJR and MGG) and identified five cell lines positive for CD81 (RPMI-8226, RPMI-LR5, NCI-H929, OPM-2, JJN3) 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, JJN3 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.

Europe PMC Funders Author Manuscripts

Table 1

Phenotypic features of patients with less-differentiated (that is, $CD19^{+}CD81^{+}$), intermediate-differentiated (that is: $CD19\text{-}CD81^{+}$) vs more-differentiated (that is, CD19-CD81-) plasma cell clones among newly diagnosed multiple myeloma patients ($n = 225$)

% of cases within subgroup	$CD19^{+}CD81^{+}$ (%)	$CD19\text{-}CD81^{+}$ (%)	$CD19\text{-}CD81^{-}$(%)	*P*-value
$CD38^{low}$	67	52	67	0.09
$CD138^{low}$	33	31	31	0.99
CD27+	33	43	50	0.48
$CD28^{+}$	17	20	35	0.04
$CD45^{+}$	67	50	29	0.003
$CD56^{+}$	77	76	75	0.85
$CD117^{+}$	67	24	41	0.009

Europe PMC Funders Author Manuscripts

Table 2

Cytogenetic characteristics of patients with less-differentiated (that is: $CD19^{+}CD81^{+}$) and intermediate-differentiated (that is: $CD19\text{-}CD81^{+}$) vs more-differentiated (that is: $CD19\text{-}CD81^{-}$) plasma cell clones among newly diagnosed multiple myeloma patients ($n = 169$)

Genetic abnormality	PCs differentiation subset			*P*-value
	$CD19^{+}$ $CD81^{+}$ (%)	CD19-$CD81^{+}$ (%)	CD19-$CD81^{-}$ (%)	
Any	25	62	72	0.07
t(4;14)	0	24	17	NS
t(11;14)	0	68	36	0.03
t(14;16)	0	0	11	NS
+1q	25	41	54	NS
del(13q)	0	49	51	NS
del(17p)	0	13	8	NS
High-risk FISH	0	23	19	NS

Abbreviation: NS, not significant.

Europe PMC Funders Author Manuscripts

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

	PFS		OS	
	HR	P	HR	P
Age (< 75 vs ≥ 75 years)	1.3	0.21	2.7	0.001
ISS	1.2	0.50	1.9	0.16
Interphase FISH cytogenetics (standard- vs high-risk)	1.9	0.003	2.7	0.001
PC differentiation stage	1.7	0.005	2.1	0.006

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