Title: Stemness of B cell progenitors in multiple myeloma bone marrow

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Statement of equal author contribution: KB and NP contributed equally to this work.

Running Title: Clonotypic B cell progenitors in multiple myeloma marrow

Keywords: multiple myeloma; B cell progenitors; bone marrow; stemness

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Conflicts of Interest: The authors declare no competing financial interests and reported no potential conflicts of interest.

Word count: 4541

Total Number of Figures and Tables: 6

Supplement Fig: 3
Statement of Translational Relevance

Due to a clonal relation among B cells and malignant plasma cells, it has been postulated that progenitors represent a tumor-initiating compartment in multiple myeloma. Herein, we report stem cell-like phenotype, frequency, progenitor functional assays, and drug sensitivity of clonotypic B cells in a large series of myeloma patients’ bone marrow, expanding previous knowledge based primarily on myeloma cell lines. We suggest that bone marrow B cell differentiation stages may be involved in disease origination and/or progression in myeloma. Research studies of putative progenitor stem cell-like cells in myeloma may lead to novel treatments to eradicate myeloma minimal residual disease reservoir. Understanding survival mechanisms of clonotypic B cell progenitors may expand potential clinical applications of current anti-myeloma approaches.
Abstract

Purpose: In myeloma, B cells and plasma cells show a clonal relationship. Clonotypic B cells may represent a tumor-initiating compartment or cancer stem cell responsible for minimal residual disease in myeloma.

Experimental Design: We report a study of 58 patients with myeloma at time of diagnosis or relapse. B cells in bone marrow were evaluated by multicolor flow cytometry and sorting. Clonality was determined by light chain and/or immunoglobulin chain gene rearrangement PCR. We also determined aldehyde dehydrogenase activity and colony formation growth. Drug sensitivity was tested with conventional and novel agents.

Results: Marrow CD19+ cells express a light chain identical to plasma cells and are therefore termed light chain restricted (LCR). The LCR B cell mass is small in both newly diagnosed and relapsed patients (≤1%). Few marrow LCR B cells (~10%) are CD19+/CD34+, with the rest being more differentiated CD19+/CD34- B cells. Marrow LCR CD19+ B cells exhibit enhanced aldehyde dehydrogenase activity versus healthy controls. Both CD19+/CD34+ and CD19+/CD34- cells showed colony formation activity, with colony growth efficiency optimized when stroma-conditioned medium was used. B cell progenitors showed resistance to melphalan, lenalidomide, and bortezomib. Panobinostat, a histone deacetylase inhibitor, induced apoptosis of LCR B cells and CD138+ cells. LCR B cells are CD117, survivin, and Notch positive.

Conclusions: We propose that antigen-independent B cell differentiation stages are involved in disease origination and progression in myeloma. Further investigations of myeloma putative stem cell progenitors may lead to novel treatments to eradicate the potential reservoir of minimal residual disease.
Introduction

The cancer stem cell model predicts that the entire tumor mass emerges from a small proportion of proliferating cells with self-renewal capacity. Early evidence has shown that a small fraction of multiple myeloma (MM) cells is capable of colony formation originating from a single malignant cell in both mice and humans (1). Previous work has identified CD19+ B lymphocytes that express the corresponding light chain of myeloma plasma cells in the peripheral blood of myeloma patients referred to as clonotypic B cells (2). The clonal relation among B cells and malignant plasma cells has also been confirmed by molecular methods (3). Clonotypic B cells in myeloma bone marrow have been described in few reports, although not studied in depth (3, 4).

Attempts have been made to study the relationship between clonotypic CD19+ B cells and MM cancer stem cell. Putative MM cancer stem cell have been debated in the literature as being the phenotype of a pre-switch and/or a post-switch MM B cell. Evidence for pre-switch B cell progenitors with stem cell-like properties has been described in CD34+/CD19- cell subsets, which have resulted in xenograft myeloma in immuno-compromised hosts (5). An immature B cell subset characterized for the co-expression of CD19+/CD34+/CD11b+ in peripheral blood has been shown to have a clonal relation with the original patient’s malignant plasma cells (6), although this has not been consistently identified (7). The existence of a B pre-switch isotype species clonally related to myeloma has also been identified in bone marrow (3). B cells expressing clonotypic IgM (clgM) cells (pre-switch) in myeloma patients have been shown to be correlated with poor patient outcome (8)(8); however, additional work from the same group has shown that myeloma may originate from a single class switch event in clgM cells with ongoing mutations in the post-switch progeny, suggesting that clgM cells may
not be involved in disease progression (9). It has been also suggested that memory B cells represent a clonotypic remnant that is only partially transformed and may not be involved in maintaining tumor, as B cells express early myeloma-specific oncogenes but they lack late oncogene K-RAS mutations (10).

DNA sequencing revealed somatically hyper-mutated but homogeneous Ig heavy-chain genes, suggesting that, in myeloma, clonal proliferation may occur in cells that have already passed through phases of somatic hypermutation (post-switch) (11). Clonotypic B cells have been identified in the CD19+/CD38+/CD56+/monotypic Ig light-chain blood compartment, characteristic of late-stage B or pre-plasma cells (12-14). Malignant CD19- plasma cells have been reported as aneuploid, whereas cells expressing CD19 were diploid, supporting the concept that myeloma is a disease process mediated by a self-replicating late compartment of B-cell ontogeny (15). A long-term proliferating compartment in myeloma has been described in cells lacking expression of syndecan-1 and CD34 (CD138-/CD34-), characterized by a CD19+/CD27+/CD20+ phenotype, suggesting that myeloma may arise from the self-renewing memory B cell compartment (4, 16).

Controversy arises as both cell subsets, B cells (CD138-/CD19+) and plasma cells (CD38+/CD45-), have been shown to reproduce myeloma in xenograft models (4, 16, 17). Mature plasma cells have been shown to have proliferative and stem cell-like properties. Plasma cells include a subset of proliferating cells present within CD45bright cells; this subset has been postulated to be a growth fraction in myeloma (18). CD138+ cells have been shown to contain a stem cell-like side population with high proliferation index that is sensitive to lenalidomide (19). Clonogenicity of CD138+ cells has been shown with dendritic cells as co-culture support (20).
The clinical significance of monoclonal CD19+ cells remains to be determined. Myeloma patients have been reported to have increased numbers of circulating B cells, with numbers significantly increased after relapse (12, 21). In contrast, CD19+ blood myeloma cells were not significantly different from median levels shown in normal controls, although CD19+ cell levels convened an improved survival (22). CD19+ cells are not eliminated by any conventional or high-dose chemotherapy regimen (12, 21, 23), although they were undetectable after allogeneic transplant in one patient (23).

In this study, we purified light chain restricted (LCR) CD19+ B cells using multi-parameter flow cytometry and cell sorting and confirmed that bone marrow B cells from myeloma patients are clonally related to malignant plasma cells. Herein, we showed that marrow CD19+/CD34+ and more differentiated CD19+/CD34- B cell samples exhibited a stem cell-like aldehyde dehydrogenase positive (ALDH+) phenotype, which were able to grow colonies in colony formation assay (CFC), suggesting that an antigen-independent B cell maturation stage may be involved in disease origin. We confirmed chemoresistance of B cell progenitors to conventional myeloma agents and showed that panobinostat, a novel histone deacetylase inhibitor, exhibits activity against progenitor and mature plasma cells.

**Materials and Methods**

*Patient Specimens*

Human bone marrow and peripheral blood were obtained, with Institutional Review Board approval, by aspiration from the posterior iliac crest of patients with multiple myeloma either at time of diagnosis or at time of clinical evidence of disease relapse. All...
human participants provided written informed consent. Bone marrow mononuclear cells were isolated by Ficoll-Hypaque gradient purification (Mediatech-Cellgro, Manassas, VA) and kept in MEM (Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (FBS) (Omega Scientific, Tarzana, CA) and 1% penicillin/streptomycin (Invitrogen) at a concentration of 2 x 10^6 cells/mL until use within 18 hours from bone marrow collection. The HS5 human cell line was obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 (Invitrogen) supplemented with 10% FBS. HS5 cells were passaged for less than 3 months before renewal from frozen early-passage stocks. Cells were regularly screened for Mycoplasma using a MycoAlert Mycoplasma Detection Kit (Lonza).

Compounds

Bortezomib (Fisher Scientific, Pittsburgh, PA) and panobinostat (LBH 589; Novartis, Basel, Switzerland) were reconstituted in DMSO and stored at -20°C until use. Bortezomib was used at 10 nM for 48 hours. Panobinostat was used at 100 nM for 24 hours. Melphalan (Sigma/M2011) was reconstituted in acid-ethanol and stored at -80°C until use (33 mM). Melphalan was used at 25 µM for 24 hours. Apoptotic-induced cell death was determined by flow cytometry using annexin V-PE and 7-amino actinomycin-D. The percent specific cell death was calculated as follows: [((experimental apoptosis - spontaneous apoptosis)/(100 - spontaneous apoptosis)) x 100.

Flow Cytometric Acquisition and Sorting

Characterization of the progenitor population was performed using a series of multiple color antibody panels containing up to 7 colors. The panels included 1) CD138-APC,
CD14-FITC, kappa-PE or lambda-PE, CD34-PECy7, CD19-PacBlue; 2) CD138-APC, CD27-FITC, kappa-PE or lambda-PE, CD34-PECy7, CD19-PacBlue, CD20-PerCP-Cy5.5; 3) CD138-APC, CD56-FITC, kappa-PE or lambda-PE, CD34-PECy7, CD19-PacBlue; 4) CD138-APC, CD34-FITC, kappa-PE or lambda-PE, CD45-PECy7, CD19-PacBlue; 5) CD138-APC, CD38-FITC, kappa-PE or lambda-PE, CD34-PECy7, CD19-PacBlue; 6) CD138-APC, CD14-FITC, kappa-PE or lambda-PE, CD34-PECy7, CD19-PacBlue CD117-PerCP-Cy5.5; and 7) CD138-APC, Notch-1-biotin, streptavidin-FITC, kappa-PE or lambda-PE, CD34-PECy7, CD19-PacBlue; CD138-APC, survivin-AF488, kappa-PE or lambda-PE, CD34-PECy7, CD19-PacBlue. All antibodies were obtained from BD Biosciences (San Jose, CA) except CD19-PacBlue (Invitrogen), Notch-1-biotin (eBiosciences, San Diego, CA), and survivin-AF488 (Cell Signaling Technologies, Danvers, MA). A minimum of 3 x 10⁵ cells were acquired. All panels included a viability marker, Live/Dead Fixable Yellow Dead Cell Stain kit (Invitrogen). All analyses were performed using Flowjo software (Treestar). Samples were acquired on a LSRII (BD, Franklin Lakes, NJ) equipped with 488, 532, 633, and 405 nm excitation lasers.

To determine ALDH activity of bone marrow mononuclear cells, we used Aldefluor (Stem Cell Technologies, Vancouver, BC), per manufacturer’s instructions. Activated Aldefluor reagent was added to freshly isolated cells; 30 minutes later, cells were transferred to a tube containing the inhibitor, diethylaminobenzaldehyde (DEAB). Samples were incubated at 37°C for 1 hour and subsequently stained with CD138-APC, kappa-PE or lambda-PE, CD34-PE-Cy7 (BD Biosciences), and CD19-Pacific Blue (Invitrogen). A minimum of 1 x 10⁶ cells were acquired for ALDH analyses.
For sorting, freshly isolated bone marrow mononuclear cells were stained at 10 x 10^6 cells/mL with CD138-APC, CD14-FITC, kappa-PE or lambda-PE, CD34-PE-Cy7, CD19-Pacific Blue, and a viability marker, Live/Dead Fixable Yellow Dead Cell Stain kit (Invitrogen). Samples were acquired and sorted using a FACSAria-SORP (BD, Franklin Lakes, NJ) equipped with 488, 640, 407, 561, and 355 nm excitation lasers. To ensure that only live single cells were collected, we used FSC-W versus FSC-H and SSC-W versus SSC-H plots to exclude doublets or cell aggregates. Dead cells were excluded by gating the cells negative for the viability marker. Cells were then gated on CD14-negative cells to exclude monocytes. Finally, cells positive for the light chain of the patient were then gated, and clonotypic cells were sorted into the following populations: 1) LCR CD138+, 2) LCR CD138-CD19+CD34-, 3) LCR CD138-CD19+CD34+, 4) LC-CD138-CD19-CD34+, 5) LCR CD138-CD19+CD27+, and 6) LCR CD138-CD19+CD27-. We recovered 4 x 10^5 to 2 x 10^6 cells using the above phenotype restrictions, with purity of the sorted populations shown to be >95% (Supplemental Figure 1). For LCR CD138-CD19+CD34+ cells, purity was not checked after sorting because a low number of cells were recovered.

**Immunoglobulin Gene Rearrangement Detection**

Whole genomic DNA was extracted and amplified from 3 x 10^4 to 1 x 10^5 sorted subpopulations using the REPLI-g Mini kit (Qiagen, Valencia, CA). Sorting gating strategy was first based on light chain expression (Kappa or Lambda expression) and sorted based on phenotype as follows: A = CD138+/light chain+ and B = CD138-/CD19+/light chain+. To determine immunoglobulin heavy chain rearrangements, the amplified genomic DNA was further amplified by PCR using fluorescently labeled primers from the IGH gene rearrangement assay kit (InVivoscribe Technologies, San Diego, CA) targeting the joining region (J) and the three conserved framework regions.
between V and J within the IGH gene, as per manufacturer’s instructions. To determine immunoglobulin kappa/lambda light-chain gene rearrangement, fluorescently labeled primers targeting V\(\kappa\) – J\(\kappa\) and V\(\kappa\) – Kde for kappa and conserved regions within VI1-3 and JI1-3 regions that flank the complementarity determining region 3 for lambda were used (InVivoscribe). No template and amplification controls were run in each test. The resulting PCR products were separated and detected by capillary electrophoresis on the ABI 3130xl, using GeneMapper 4.0 software (Applied Biosystems).

**Immunoglobulin Gene Rearrangement Sequencing**

The resulting PCR products, as described above, were run in a 2% agarose gel. Clonal bands within the valid assay range were cut and gel extracted using the MinElute kit (Qiagen) and sequenced on an Applied Biosystems 3130XL genetic analysis system using the primers provided in the IGH Somatic Hypermutation kit. Sequences were analyzed using VBASE and ClustalW2 multiple sequence alignment.

**Colony Formation Assays**

Each flow cytometric sorted population was resuspended in IMEM (Mediatech-CellGro) + 2% FBS (Stem Cell Technologies, catalog #07700) and plated in triplicate in 35-mm\(^2\) dishes with methylcellulose containing 5% PHA (Methocult H4533, Stem Cell Technologies) per the manufacturer’s instructions at a concentration of 1000 cells/mL for CD34+ cells and at least 100,000 cells/mL for other populations. Conditioned medium from HS5 cells was added where noted, in place of IMEM + 2% FBS. For cultures containing cytokines, IL-2 (50 U/ml), IL-6 (10 ng/ml), IL-10 (50 ng/ml), IL-15 (20 ng/ml), and IL-21 (100 ng/ml) (R&D Systems, Minneapolis, MN) were added. Cultures were grown at 37°C-5% CO\(_2\) with a water bath, and colony growth was assessed 14-21 days later. Colony morphology was assessed by the Wright-Giemsa staining method using
the Hema 3 Stat Pack (Fisher Scientific) in cytospins slides examined with immunofluorescence microscopy or flow cytometry.

Results

Clonotypic B Cells in Multiple Myeloma Bone Marrow

Clonotypic B cell progenitors may contain the putative myeloma cancer stem cell, and represent a malignant, drug-resistant compartment responsible for disease relapse. Characterization of this population(s) is necessary to improve our management of the disease and patient outcomes. We evaluated marrow cells from 58 myeloma patients for expression of plasma cell and B cell progenitor surface markers by multicolor flow cytometry and identified distinctive marrow populations based on CD34, CD19, and syndecan-1 (CD138) expression. Plasma cells were shown to be positive for CD138 and negative for CD34 and CD19. Among cells negative for CD138, hematopoietic stem cells (HSC) or multi-potent progenitors are CD34+/CD19-, and B cells progenitors encompass CD34+/CD19+ or CD34-/CD19+ sub-populations (24, 25). A minority of the CD19+ marrow cells exhibited a CD34+ phenotype, but most had a differentiated, CD34-, B cell phenotype. In order to exclude monocyte contamination, we applied a strict, low SSC gate on CD138- cells (Supplemental Figure 1). In blood of myeloma patients (n = 8), we detected very low numbers of circulating plasma cells and a few differentiated CD19+ B cells, but no CD34+/CD19+ cells (Figure 1A).

To assess for expression of the myeloma clonotype in marrow B cells, we tested initial patients (n=8) for kappa or lambda light-chain expression using an intra-cytoplasmic and a surface staining protocol. As expected, cytoplasmic stain was brighter than the surface
stain, but percentages of light-chain cells positive by either assay were comparable (data not shown). No light-chain expression was detected in the CD34+/CD19- or the CD34/CD19/CD138-triple negative marrow cells (Figure 1A). Malignant plasma cells expressed either kappa or lambda light chain. Although the CD19+ light chain stain was perhaps skewed in the same direction as the malignant plasma cells, all patients who were tested also had some CD19+ cells that expressed the opposite light chain (Figure 1A).

To determine whether marrow CD19+ cells that expressed a surface light chain identical to the plasma cells were clonally related to the myeloma clone, we tested flow-sorted LCR B cells and plasma cells for the Ig heavy- and light-chain gene clonal rearrangements. Fluorescently labeled PCR products were tested by capillary gel electrophoresis; results showed a clonal population of cells yielding the same prominent amplified product within the expected size in both LCR B cell progenitors and plasma cells (Figure 1B). Furthermore, both myeloma populations showed identical sequences for IGH somatic hypermutation, confirming the same malignant clone (Supplemental Figure 2). Therefore, we focused on LCR B cells as they are clonally related to myeloma with the understanding that a proportion of these cells correspond to normal B cell precursors.

It has been postulated that B cell numbers are indicative of myeloma burden. In our study, we observed a very low proportion of LCR B cells in the marrow of newly diagnosed patients (0.7 ± 0.5%; n = 29) or patients with clinical relapse (0.5 ± 0.4%; n = 29) (Figure 1C) as previously shown in blood of myeloma patients (22).
We next assessed the kappa/lambda light-chain ratio of bone marrow CD138-/CD19+ B cells progenitors. As shown in Figure 1D, in newly diagnosed patients, the mean ratio was 1.5 ± 0.6 and 1.02 ± 0.4 for kappa- and lambda-restricted patients, respectively. For relapsed patients, the results were similar (1.25 ± 0.5 for kappa and 1.3 ± 0.5 for lambda patients) and comparable to those shown in healthy donors. Based on these results, commonly used criteria for plasma cell (CD138+) LC restriction (kappa LCR> 4.0 or lambda LCR <0.5) may not be applicable to malignant B cell progenitors.

**Stem-Like Phenotype of Clonotypic B Cell Progenitors in Multiple Myeloma Bone Marrow**

We first evaluated the phenotype of LCR CD19+ cells with emphasis on known B cell progenitors and malignant plasma cell antigens, as presented in Table 1 (n = 7). LCR CD34+/CD19- were characterized by aberrant CD27 and CD20 expression and low levels of CD10 and CD56 markers. ALDH is one of a family of enzymes involved in several detoxifying pathways. Elevated ALDH expression has recently been used to identify a rare stem cell-like population in normal hematopoietic cells and in several tumor types, including leukemia, brain, colon, and breast cancer (26-29) and in multiple myeloma (16). To determine whether myeloma bone marrow B progenitors contain a high ALDH-expressing stem cell-like population, we used the aldefluor reagent to test for ALDH activity. For each of the tested patients (n = 8), a control sample was run containing a population of cells with fluorescence that is inhibited by the ALDH inhibitor DEAB. High ALDH activity was detected in a mean of 3.05% (0.09-7.26 %) and 2.94% (0.01-6.9%) of LCR CD34+/CD19+ and CD34-/CD19+ B cells, respectively, and in 8% (1.3-15.3%) of CD34+/CD19- hematopoietic progenitors. ALDH activity was diminished in CD34+/CD19- progenitor cells from myeloma patients and increased in myeloma...
LCRCD19+/CD34+ or CD34- cells when compared to healthy subjects subpopulations (Figure 2A).

We investigated Notch and survivin expression in LCR B cells as they are involved in HSC and/or malignant plasma cell survival. B cell progenitors were characterized by high Notch-1 (90 ± 6%) and survivin (97 ± 2%) expression levels (Figure 2B) in all patient samples analyzed (n = 4). Expression of Notch-1 and survivin in healthy bone marrow (n = 3) was over 90% in CD19-/CD34+, CD19+ progenitors, and CD138+ cells (data not shown). In addition, we examined tyrosine-protein kinase c-Kit (CD117) expression levels in B progenitor cells. Historically, normal B cell progenitors in humans are CD117 negative, findings confirmed in healthy bone marrow (data not shown). B cell malignancies are characterized as having either negative (B-ALL, lymphoid crisis CML) or positive CD117 expression (myeloma, B-diffuse large cell lymphoma) (30, 31). We identified aberrant CD117 expression (n = 3) in a mean of 20.5% (range 11-25) of CD19+/CD34+ cells (n = 3), in 7.8% (range 2.8-17%) of CD19+/CD34- B cells (n = 7), and in 4.2% (range 0-15) of CD138+ cells (n = 6) (Figure 2B). Our results showed that CD19 progenitors co-expressed c-Kit in all samples, whereas only 2/6 patient samples co-expressed CD117/CD138. The functional role of Notch, survivin, and c-Kit on clonotypic B cell progenitors in myeloma remains to be determined.

**Colony Formation Assay of Clonotypic B Cell Progenitors in Multiple Myeloma**

To test the hypothesis that B cells of myeloma patients are enriched for CFC, we isolated marrow cells by flow sorting. During the initial experiments, the gating strategy was selected to compare colony formation in methylcellulose by CD34 and light-chain-restricted CD19+ cells (gate i: kappa or lambda restriction as plasma cell of each patient;
gate ii: CD138-/CD19+ or CD138+/CD19-). The sorting purity was greater than 98% in the majority of samples, excluding the possibility of contaminating cells (Supplemental Figure 3). Sub-populations were grown for 14 days in colony formation assays on methylcellulose, supplemented with 5% lymphocyte-conditioned medium to favor lymphoid differentiation. CD34 cells from myeloma patient bone marrow (n = 5) were highly efficient, requiring 1000 cells/plate to successfully grow colonies (Figure 3A). On the other hand, a minimum of 100,000 cells/plate were needed for colony formation derived from B cells with a colony efficiency estimated in 1 in 25,000 cells. CD138+ were unable to form colonies (n=8) despite increased input number (300,000-1,000,000 cells/plate); however, a few isolated cells persisted in culture. Cells harvested on day 14 are lympho-plasmacytoid (Figure 3A). As shown in Figure 3B, a small fraction of CD34+/CD19- cells are able to terminally differentiate into CD138+ cells (8 ± 2%); as opposed to LCR CD19+ B cells that differentiated into CD138+ cells (80 ± 5%).

Colony formation of sorted CD34+/CD19+ and CD34-/CD19- B cells was tested in three myeloma patients, with both cell subsets able to grow colonies. Furthermore, B cells, regardless of CD27 expression, successfully grew colonies. Data collected from these studies are shown in Figure 3C. In summary, LCR B cell progenitors in myeloma are able to grow colonies and differentiate into mature plasma cells (CD138+) regardless of CD34 and/or CD27 expression.

Interactions of Hematopoietic Progenitors and Clonotypic B Cell Progenitors in Multiple Myeloma With Stroma-Conditioned Medium

It has been well established that the tumor microenvironment creates a protective niche that supports myeloma growth (32). In addition, stroma secrete multiple cytokines that
support proliferation and differentiation of HSC and committed progenitors. Flow sorted CD34+/CD19- cells from myeloma patients were grown in methylcellulose supplemented with conditioned medium harvested from HS5 stroma grown for 48 hours (stroma-CM). CFC potential was compared using cytokines known to support plasmablast differentiation (33). Colony efficiency was slightly improved using either stroma-CM or a combination of recombinant human-IL-2, IL-6, IL-10, IL-15, and IL-21. Similar results were obtained when either IL-15 or IL-21 was omitted from the cytokine cocktail (data not shown). Cells harvested from day 14 colonies are CD19+ (35% to 50%), and very few terminally differentiated into CD138+ cells (3-10%) (Figure 4A). We next tested whether CFC efficiency of LCR CD19+ cells could be enriched in the presence of stroma-CM. Stroma-CM significantly improved colony number (3-fold) and size of each individual colony with an estimated colony efficiency of 1:10,000 cells (Figure 4B).

Apoptosis Resistance of Clonotypic B Cell Progenitors in Multiple Myeloma Bone Marrow

We next isolated bone marrow cells by flow sorting to test chemo-sensitivity of LCR CD19+ cells in myeloma patients. B cells exhibit relative resistance to melphalan, compared to plasma cells, as determined by annexin V/7-AAD staining (n = 3) (Figure 5A). B cells were less sensitive to apoptosis mediated by bortezomib (n = 4) (Figure 5B). Lenalidomide did not target LCR CD19+ cells (Figure 5C). We next tested the role of a hydroxamic acid-derived histone deacetylase inhibitor (LBH589, panobinostat), which has been shown to induce in vitro apoptosis in myeloma plasma cells (34) and acute lymphocytic lymphoblasts (35). Panobinostat (100 nM for 24 hours) activity against B cell progenitors in all treated patients (n = 5) was comparable to CD138+ cells (Figure 5D).
Discussion

Within the heterogenic cancer cell population, it is hypothesized that only a small subset of neoplastic cells is capable of extensive proliferation and differentiation leading to tumor development. In myeloma, the self-renewal compartment has been described within early B clonotypic lymphocytes or in memory B cells with the caveat that even CD34+/CD19-(5) and mature CD138+/CD38+/CD45- have been shown to reproduce myeloma in xenograft models (17).

In our study, we provide evidence of the existence of clonotypic LCR B cells, confirmed by molecular studies, in bone marrow from a large series of adult patients, in agreement with previous reports (3, 36, 37). Our analyses of discrete stages of B cell differentiation by antigen expression patterns in myeloma paralleled normal B cell development, with a few possible exceptions. Based on combined assessment of the CD19 and CD34 antigens, we showed the existence of clearly defined populations among LCR B cells. Both CD34+/CD19+ and CD34+/CD19+ expressed light chain in all tested patients, suggesting aberrant light-chain processing in myeloma clone and/or aberrant expression of CD34 in mature CD19+ cells. If one assumes CD34+/CD19+ as a more undifferentiated population, the relative number of B cell subsets increased from more immature cells to more differentiated B cells, indicating that malignant B cell differentiation parallels normal B cell development. Multi-parameter flow cytometry provides a powerful tool to elucidate different stages of B cell development. In our study, we used a very strict gate criterion to exclude CD14+ cells, thus avoiding monocyte contamination due to non-specific coating of M-protein to monocyte cell surfaces that is not completely washed off during specimen processing and/or unspecific binding of...
monoclonal antibodies, as previously described (38). Our gating strategy, however, excluded myeloma monocytoid B cells characterized by a higher SSC/FSC, which are detected with specific anti-CD19 monoclonal antibody, thus explaining differences in results versus a previous report that used less stringent criteria (39).

The overall number of CD19+ B cells in myeloma patients is comparable to that shown in healthy individuals (40). Clonotypic B cell compartment size in newly diagnosed patients or in myeloma patients with clinical evidence of relapse remains constant, as previously shown in blood (22). CD34+/CD19+ cells were not detected in circulation, suggesting that these cells reside in the marrow niche. CD34+/CD19+ expressed the TNF receptor CD27, expressed in normal memory B cells, and to a lesser extent CD20, denoting possible differentiation and/or aberrant expression. CD27 has been reported to be expressed in progenitor B cells in other B cell malignancies; however, its role in malignant growth remains to be defined (41, 42). Work by Sanz et al (24) suggested a dual B cell development pathway where pro-B cells (CD34+/CD19+/CD10+) are preceded by either a pre-pro B cell (CD34+/CD19+/CD10-) or a early B cell/common lymphoid progenitor (CD34+/CD19-/CD10+) and formerly described in B-cell acute lymphocytic leukemia (43). In multiple myeloma B progenitors, we identified both CD10-negative and -positive subpopulations within CD34+/CD19+ cells, potentially mimicking two distinct pathways. Stroma co-culture differentiation studies could help to track the order of emergence of B-cell differentiation stages in multiple myeloma.

To study “stemness” of LCR B cells, we assessed stem cell-like phenotype and/or evaluated stem cell function by ALDH enzymatic activity and carried out in CFC assays. Myeloma CD19-/CD34+ multi-potent progenitors generated polyclonal CD138+ cells in methylcellulose culture, suggesting that early non-committed CD19-/CD34+ cells in
myeloma are not involved in clonal B cell development. LCR B cells exhibited significantly increased ALDH activity versus that shown in healthy donors, and CFC assays showed activity in all stages of B cell differentiation in myeloma. In addition, hematopoietic stroma-derived cytokines support CFC growth of malignant B progenitors (44). In summary, these finding suggest that a subset of marrow CD19+/CD34+ cells and CD19+/CD34- B cells in myeloma are enriched for cells with a cancer stem cell-like phenotype and/or function.

The cancer stem cell hypothesis postulates that this compartment is intrinsically more resistant to therapy than other tumor cells, constituting a minimal residual disease reservoir responsible for disease relapse. Successful therapy must therefore eliminate these cells, which is hampered by their high resistance to commonly used treatment modalities. In our study, we showed that LCR B cells are relatively resistant to agents commonly used to target CD138+ cells (melphalan, bortezomib, and lenalidomide) as previously shown (4, 16). Panobinostat has been shown to be a potent growth inhibitor against resistant B lymphoblast-promoting histone hyperacetylation and cell growth gene regulation (35). Ongoing panobinostat clinical trials have reported encouraging results in myeloma (45, 46). In this study, we provide information suggesting a potential role of panobinostat to target clonotypic B cells. Strategies to optimize panobinostat-induced apoptosis in synergy with other drugs remain to be explored.

The clinical relevance of targeting cancer stem cell-associated surface markers has been previously shown (47). CD117 seems to be aberrantly expressed in LCR B cells compared to normal B cell progenitors. We hypothesize that, while c-Kit expression is preserved in clonotypic B cell progenitors as they differentiate into mature malignant plasma cells, its expression is lost on the majority of myeloma patients. Further studies
in a larger series of patients is necessary to confirm these results and more importantly to explore both the functional role of c-Kit and the clinical utility of novel tyrosine kinase inhibitors. Treatment of patients with anti-CD20 antibody failed to achieve clinical response (39, 48), suggesting the existence of myeloma CFC progenitors that are CD20 negative, as we have shown. Notch and survivin are expressed in all B cell development stages. Novel treatment strategies with monoclonal antibodies, inhibitors, and/or vaccines to target these surface proteins should be explored before drawing any conclusions on the functional role of these molecules on myeloma B cell progenitors (49, 50).

Cancer chemotherapy is deemed successful if it reduces tumor burden and induces apoptosis of "cancer cells." The challenge remains to identify all cellular components involved in the organized hierarchy of heterogeneous cell populations within myeloma. LCR B cells are relatively rare populations that have been shown to grow colonies in CFC assays and that are chemo-resistant, suggesting they potentially fit the cancer stem cell definition. Targeting clonotypic B cell progenitors in addition to inducing apoptosis of terminally differentiated plasma cells by novel treatment strategies may reduce disease recurrence and may improve long-term survival rates in myeloma.

Acknowledgements: The authors would like to thank Christine Simonelli, Robin Lavaron and Ashley Durand for coordinating bone marrow procurement. We would like to thank Francisca Beato, Herman Hernandez, the Flow Cytometry Core and the Molecular Core at Moffitt Cancer Center for technical support.
Grant Support: LEP was supported by research grants from the National Heart Lung and Blood Institute (K08-Career development award 2005-2010) and from the National Cancer Institute (R21CA152345 2010-2012).

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Tables:

Table 1. Phenotypic characterization of clonotypic B cells progenitors in multiple myeloma bone marrow. Multicolor flow cytometry for detailed phenotypic description of bone marrow sub-populations in multiple myeloma patients.

<table>
<thead>
<tr>
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<th>CD138-/CD34+/CD19+ (%)</th>
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<tr>
<td></td>
<td>mean (range)</td>
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Figure Legends:

Figure 1. Phenotype of bone marrow B cell populations in multiple myeloma. (A) Multicolor flow cytometric analysis of marrow cells gated based on SSC/FSC properties to avoid granulocytes and monocytes contamination (left top panel). CD14+ cells were excluded to avoid additional monocytes contamination (Supplement Figure 1). Plasma cells were identified as CD138+ (left middle panel). CD138-/low SSC were further analyzed based on CD19 and CD34 expression, with the same gating strategy applied.
to peripheral blood (PB) cells (right panels). Kappa and lambda light chain (LC) expression levels in cell surface were tested in bone marrow subpopulations (right panels) compared to fluorescence minus one (FMO) controls. (B) Immunoglobulin (Ig) gene rearrangement of a representative lambda positive patient. DNA extracted from sorted CD138+/lambda+ and CD138-/CD19+/lambda+ cells followed by PCR amplification using primers targeting all three Ig heavy chain (Ig HC) frameworks (FR) and Ig lambda light chain. PCR products were separated and detected by capillary electrophoresis. Peaks shown fall within acceptable ranges for each primer (FR1: 290-360 bp, FR2: 235-295 bp, FR3: 69-129 bp, and lambda:135-170bp). As a negative control, we used polyclonal DNA, per manufacturer’s instructions. (C) Percentages of light chain restricted (LCR) CD138-/CD19+ clonotypic cells in whole marrow in patients at time of diagnosis (n = 23) or in patients at time of relapse (n = 21) in kappa (left) and lambda LCR myeloma (right). Statistical analysis was performed with Student t test. (D) Light chain restriction of CD19+ cells in bone marrow was determined based on the kappa-to-lambda ratio. Graph compares kappa restricted (left) and lambda restricted (right) patients at diagnosis or at relapse compared to healthy marrow cells.

Figure 2. Stem cell-like phenotype of clonotypic B cell progenitors in multiple myeloma bone marrow. (A) Representative contour plots of ALDH activity of CD138-/CD34+/CD19- multipotent progenitors, comparing CD138- cells gated first based on light chain (kappa or lambda) similar to plasma cells of each individual patient, thus termed light chain restricted (LCR) and CD138+ cells (top panels). As a negative control, an aliquot of aldefluor-stained cells was immediately quenched with a specific ALDH inhibitor (DEAB) (bottom panels). Scatter plot shows percentages of cells with increased ALDH activity within bone marrow subpopulations (n=7). Statistical analysis was performed with Student t test. (B) Representative contour plots showing FACS labeling.
with Notch-1, survivin, and CD117 (c-Kit) expression gated based on FMO controls (not shown).

**Figure 3. Colony formation assay of clonotypic B cell progenitors in multiple myeloma bone marrow.** (A) Comparison of colony development in methylcellulose (MC) supplemented with PHA stimulated-5% lymphocyte-conditioned medium (PHA-LCM). Bone marrow (BM) cells were gated and sorted as described in Design and Methods. MC cultures were established with CD138-/CD19-/CD34+ (1,000 cells) or LCR CD138-/CD19+ (100,000 cells) and CD138+ cells (300,000 cells). Colonies were scored after 14 days of culture. Graph shows results (mean ± SD) from 5 myeloma patients. Colonies were harvested from each group for morphologic evaluation (hematoxylin-eosin stain, Zeiss Axiovert inverted microscope, magnification x40). (B) Sorted CD138-/CD19-/CD34+ or CD138- LCR CD19+ and CD138+ cells were cultured for 14 days. After induction stage, developing colonies or CD138+ cells that remained in culture were harvested for FACS analysis. Representative contour plots show CD138 expression on input cells before culture (day 0) and on harvested cells (day 14). (C) Colony counts in MC PHA-LCM cultures for 14 days with LCR BM cells, sorted based on phenotype as indicated. Results (mean ± SD) shown colony numbers of 3 myeloma patients/experiment.

**Figure 4. Colony formation cell assay of myeloma CD138-/CD34+/CD19- multipotent progenitors or clonotypic B cells in the presence of marrow-conditioned medium.** (A) Quantification of hematopoietic progenitors in methylcellulose (MC) colony assays with sorted CD138-/CD34+/CD19- cells (n = 3). Culture was supplemented with 5% PHA-LCM in all culture conditions (baseline); and with either HS5 stroma conditioned medium (stroma-CM) or recombinant human IL-2 IL-6, IL-10, IL-15 and IL-21. Graph represents colony output of triplicate experiments from 3 myeloma
patients at 14 days. Representative contour plots show phenotype (CD34, CD19, and CD138) of plucked cells from MC culture. CD138 cells in each culture condition were analyzed for light chain expression. Histogram shows kappa (solid line) and lambda (dotted line) light chain (LC) versus fluorescence minus one (FMO) controls (gray shade). (B) Sorted LCR CD138-/CD19+ cells were grown in MC PHA-LCM (baseline) or in the presence of stroma-CM. Colonies were scored at day 14 of culture. Images show representative colonies grown in each condition (Zeiss Axiovert Inverted Microscope, magnification 5X).

Figure 5. Drug sensitivity of clonotypic B cell progenitors in multiple myeloma bone marrow. (A) Apoptotic response of myeloma patients’ bone marrow (BM) cells after treatment with melphalan (25 µM) or acid-ethanol (control) for 48 hours (n = 3). Multipotent progenitors were sorted based on CD138-/CD19-/CD34+ expression. To isolate CD138+ or LCR CD19+ cells, BM was first gated on surface LCR corresponding to each patient’s plasma cells (kappa or lambda). Apoptosis was determined with annexin V-PE and 7-AAD staining. Percentage of each population is indicated in each quadrant of representative contour plots. (B) Apoptosis responses to treatment of BM subpopulations with bortezomib (10 nM) or DMSO (control) for 48 hours. Graph represents mean ± SD of percent specific apoptosis determined by flow cytometry (n = 3). (C) Apoptosis responses to treatment of BM subpopulations with lenalidomide (10 µM) or DMSO (control) for 48 hours. Graph represents mean ± SD of percent specific apoptosis determined by flow cytometry (n = 3). (D) Apoptosis responses to treatment of BM subpopulation with panobinostat (100 nM) or DMSO (control) for 24 hours. Graph represents mean ± SD of percent specific apoptosis determined by flow cytometry (n = 5).
Figure 1

Heavy Chain

CD19+

Relative Fluorescence

CD138+

Relative Size

CD19+

Heavy Chain Polyconal DNA control

CD138+

Relative Fluorescence

CD138+

Relative Size

CD19+

Relative Fluorescence

CD138+
Figure 1

Light Chain (Lambda)

CD138+

CD19+

Relative Size

Relative Fluorescence

B
Figure 1

C

% LCR CD138-/CD19+ cells

\( p = 0.0721 \)

\( p = 0.0984 \)

\( \kappa \) LCR Myeloma

\( \Lambda \) LCR Myeloma
Figure 1

Kappa Restricted

Lambda Restricted
Figure 2

A

CD138+/CD19-/CD34+ CD19+/CD34+ CD19+/CD34- CD138+

LCR CD138-

Aldefluor

Aldefluor + DEAB

SSC

ALDH

% ALDH +

Myeloma
Healthy Donor

p<0.01

p<0.05

p<0.05

CD19-/CD34+ CD19+/CD34+ CD19+/CD34- CD138+

LCR CD138-
Figure 2

B

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Notch-1

| SSC                         |                      |                          |                           |                        |
| 250K                        | 150K                | 100K                     | 50K                       | 0                      |
| 200K                        | 150K                | 100K                     | 50K                       | 0                      |
| 150K                        | 100K                | 50K                      | 0                         | 0                      |
| 200K                        | 150K                | 100K                     | 50K                       | 0                      |
| 250K                        | 150K                | 100K                     | 50K                       | 0                      |

Survivin

| SSC                         |                      |                          |                           |                        |
| 250K                        | 150K                | 100K                     | 50K                       | 0                      |
| 200K                        | 150K                | 100K                     | 50K                       | 0                      |
| 150K                        | 100K                | 50K                      | 0                         | 0                      |
| 200K                        | 150K                | 100K                     | 50K                       | 0                      |
| 250K                        | 150K                | 100K                     | 50K                       | 0                      |

CD117

| SSC                         |                      |                          |                           |                        |
| 250K                        | 150K                | 100K                     | 50K                       | 0                      |
| 200K                        | 150K                | 100K                     | 50K                       | 0                      |
| 150K                        | 100K                | 50K                      | 0                         | 0                      |
| 200K                        | 150K                | 100K                     | 50K                       | 0                      |
| 250K                        | 150K                | 100K                     | 50K                       | 0                      |
Figure 3

A

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B

CD138+

Day 0

Day 14

CD138-

C

# of colonies/10^6 cells

\[
\begin{array}{c|c|c}
\text{CD19+/CD34+} & 100 & 1000 \\
\text{CD19+/CD34-} & 10 & 100 \\
\text{LCR CD138-} & 1 & 1 \\
\end{array}
\]
Figure 5

A) Melphalan

B) Bortezomib

C) Lenalidomide

D) Panobinostat
Clinical Cancer Research

Stemness of B cell progenitors in multiple myeloma bone marrow

Kelly Boucher, Nancy Parquet, Raymond Widen, et al.

Clin Cancer Res  Published OnlineFirst September 17, 2012.

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