**Ex Vivo Graft Purging and Expansion of Autologous Blood Progenitor Cell Products from Patients with Multiple Myeloma**

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**Abstract**

Autologous peripheral blood progenitor cell (PBPC) transplantation is the treatment of choice for selected myeloma patients. However, tumor cells contaminating the apheresis product are a potential source of relapse. Here we report a sequential purging strategy targeting mature and immature clonogenic myeloma cell populations in the autograft. Thawed PBPC products of myeloma patients were treated with rituximab to kill CD138+/CD20+ B cells (highly clonogenic immature cells), and bortezomib to target CD138+ cells (normal and differentiated myeloma plasma cells), followed by coculture with allogeneic mesenchymal stem cells (MSC) from normal donors. After 7 days of coculture, nonadherent cells were removed and cultured in the absence of MSC for an additional 7 days. Then, efficacy of purging (removal of CD138+/CD20+ and CD138+ cells) was assessed by flow cytometry and PCR. We used our *ex vivo* purging strategy to treat frozen aphereses from 16 patients. CD138+ and CD138+/CD20+(19+) cells present in the initial products were depleted more than 3 and 4 logs, respectively based on 10^6 flow-acquisition events, and to levels below the limit of detection by PCR. In contrast, total nucleated cell (TNC), CD34+ cell, and colony-forming cell numbers were increased by approximately 12 to 20, 8-, and 23-fold, respectively. Overall, *ex vivo* treatment of apheresis products with rituximab, bortezomib, and coculture with normal donor MSC depleted mature and immature myeloma cells from clinical aphereses while expanding the normal hematopoietic progenitor cell compartment. *Cancer Res; 71(14); 5040–9. ©2011 AACR.*
The failure of CD34+ selection to effectively purge tumor cells shows the need for the development of new purging strategies, specifically ones that target relevant tumor cells. CD138 is expressed by normal and differentiated myeloma plasma cells. The proteosome inhibitor bortezomib can be used to kill cells that express CD138 (11–12). However, a separate myeloma population that does not express CD138 exists. It is a highly clonogenic immature cell population with a CD138−20+(19+) phenotype and an extensive ability to replicate and generate differentiated progeny expressing CD138+ (13–16). These CD138−20+(19+) tumor cells are effectively killed by the anti-CD20 antibody rituximab (13). Thus, bortezomib and rituximab provide the ability to purge both mature and immature clonogenic tumor populations. In addition to the use of these agents, our group (and others) has previously showed that a period of ex vivo culture can selectively deplete tumor cells in autografts for chronic myelogenous leukemia (17–19). The loss of normal primitive hematopoietic progenitors in the autograft associated with ex vivo culture and differentiation can be overcome by coculture with normal allogeneic donor mesenchymal stem cells (MSC) (20). Such coculture conditions not only deplete tumor cells, but preserve, and in most cases increase the numbers of normal hematopoietic progenitors present in the graft providing a potentially better product for subsequent transplantation.

Therefore, we hypothesized that a purging strategy for PBPC products from patients with myeloma that used a combination of rituximab and bortezomib and a period of ex vivo culture with MSC might prove effective by killing mature CD138+ and immature CD138−20+ myeloma cells while preserving (and possibly increasing) the content of normal hematopoietic progenitors. Here we report the development of this multistep strategy.

Materials and Methods

Cell lines
Myeloma cell lines RPMI-8226, ARP-1, and NCI-H929 were obtained from American Type Culture Collection and cultured at 37°C, 5% CO2 in air, fully humidified atmosphere in RPMI medium (Gibco) supplemented with 10% (v/v) FBS (HyClone).

Initial treatment of peripheral blood progenitor cell products
Cryopreserved PBPC products were obtained from G-CSF-mobilized normal donors (N = 4) and myeloma patients (N = 16) in accordance with a University of Texas M. D. Anderson Institutional Review Board (IRB)-approved protocol. All patients and donors gave their informed consent for these experiments.

Myeloma PBPC products were thawed in the presence of a DNase to reduce aggregate formation (55 U/mL Benzonase, EMD Chemicals, Inc.) and washed in CinitMACS buffer (Miltenyi Biotech) containing 0.5% human serum albumin (Baxter Healthcare) and DNase to reduce aggregate formation (50 μg/mL Dornase ALFA Pulmozyme, Genentech, Inc.). Mononuclear cells were isolated by density separation (Histopaque-1077, Sigma).

In initial experiments, CD138+ cells were removed from the PBPC by magnetic activated cell sorting (MACS) according to the manufacturer’s instructions using the MidiMACS device and CinitMACS CD138 MicroBeads (both Miltenyi Biotech). CD138+ cells were used as a positive control for flow cytometry. The CD138 depleted PBPC cells were cultured overnight in CellGro SCGM medium (CellGenix) containing 10% (v/v) FBS prior to use (Fig. 1). Later experiments were carried out without CD138+ cell removal to show that this step was not required for effective tumor cell purging.

For initial optimization experiments requiring normal CD34+ cells, apheresis products from normal donors were subjected to CD34 selection by MACS using the MidiMACS device according to the manufacturer’s instructions (Miltenyi Biotech) and CinitMACS CD34 Beads (Miltenyi Biotech). Isolated CD34+ cells were cultured overnight in CellGro SCGM medium containing 10% (v/v) FBS.

Rituximab treatment of peripheral blood progenitor cells from myeloma patients and normal donor CD34+ cells
Rituximab (Genentech, Inc.) was used to treat CD138 depleted PBPC from myeloma patients and, for the purpose of assessing toxicity against normal hematopoietic cells, CD34+ cells from normal donors. Cells were incubated for 24 hours at 37°C, 5% CO2 in air, fully humidified atmosphere in CellGro SCGM medium containing 10 or 20 μg/mL rituximab with 10% human AB serum (Atlanta Biologicals) as a source of complement (13).

Bortezomib treatment of myeloma cell lines, CD138+ myeloma patient peripheral blood progenitor cells and normal donor CD34+ cells
Bortezomib (Millenium Pharmaceuticals Inc.) was used to treat myeloma cell lines (RPMI-8226, ARP-1, and NCI-H929), CD138 depleted cells from the PBPC of myeloma patients and, for the purpose of assessing toxicity against normal hematopoietic cells, CD34+ cells from normal donors. Cells were treated with a range of concentrations of bortezomib (2.5–160 nmol/L) for 16 or 24 hours (12).

Combined rituximab and bortezomib treatment of peripheral blood progenitor cells from myeloma patients
Following single drug optimization studies, CD138 depleted cells or total PBPC from myeloma patient samples were incubated with 20 μg/mL rituximab in the presence of 10% human AB serum (source of complement) for 24 hours with Bortezomib (20 nmol/L) added for the last 16 hours of incubation.

Ex vivo culture conditions
After drug treatment, cells were washed and cocultured for 7 days at 37°C in a 5% CO2 in air, fully humidified atmosphere with normal donor bone marrow-derived MSC (20) in “Ex Vivo Expansion Medium” (EVEM): CellGro SCGM (CellGenix USA)

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supplemented with 10% (v/v) FBS and containing 100 ng/mL each of granulocyte colony-stimulating factor (G-CSF, Neupogen Filgrastim, Amgen) and stem cell factor (SCF), thrombopoietin (TPO), and Flt-3 ligand (all from CellGenix USA; ref. 17). Normal donor MSC were provided by Angioblast Systems, Inc. After 7 days, nonadherent cells were collected and analyzed by: (i) flow cytometry for the presence/absence of CD34+ cells (providing a quantitative measure of normal hematopoietic progenitors) and CD138+ cells (providing a quantitative measure of mature and immature myeloma cells), (ii) in vitro CFU-GM assay (providing a quantitative measure of normal hematopoietic progenitors) and (iii) PCR (providing a quantitative measure of tumor cells).

Viability assay

Cell viability was measured using the MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2h-tetrazolium]) assay (Promega; ref. 21). Cell lines (RPMI-8226, ARP-1, and NCI-H929) and human CD34+ cells from normal PBPC samples were cultured in triplicate in flat-bottomed 96-well plates at 5 × 10^4 cells/well. The normal PBPC CD34+ cells were cultured in EVEM. After cells were incubated with different concentrations (2.5–160 nmol/L) of bortezomib for 16 or 24 hours at 37°C in a 5% CO_2 in air, fully-humidified atmosphere, MTS cell viability assays were conducted in triplicate on post-treatment samples. Cell growth inhibition was calculated as follows:

\[
\text{Percent (\%) growth inhibition} = \left(1 - \frac{\text{OD}_{490 \text{ sample}}}{\text{OD}_{490 \text{ control}}}\right) \times 100
\]

Flow cytometric analysis

The presence of CD138+ or CD138−20+19+ normal hematopoietic progenitors in predépletion, post-depletion, post-treatment, and post–ex vivo culture samples was evaluated by flow cytometric analysis. Briefly, at each time point, 2 × 10^6 cells were washed with FACS buffer (DPBS + 1% goat serum), then stained with phycoerythrin (PE)-conjugated anti-CD34 (Miltenyi Biotec Inc.) and fluorocsein isothiocyanate (FITC)-conjugated anti-CD20 and allophycocyanin (APC)-conjugated anti-CD19 (BD Pharmingen). Samples of CD138+ cells (product of MACS CD138 depletion) were also stained and analyzed to confirm that CD138+ cells would be clearly detected if present.
A range of 0.5–1.0 × 10^6 events were acquired for each sample using a FACSCalibur (Becton Dickinson) as permitted by cell number available. Data acquisition and analysis was carried out using CELLQuest Pro software (Becton Dickinson). The sensitivity of flow cytometry allows the detection of one positive (tumor) event in 10,000 acquired events.

We evaluated the possibility that the failure to detect CD20^+ cells by the fluorescently-conjugated detection antibody might be incorrectly interpreted as cell killing and that these data might be a consequence of rituximab blocking access to the CD20 antigen by the detection antibody. To directly address this issue, samples were also stained with allophycocyanin (APC)-conjugated anti-CD19 (Becton Dickinson) (22–23).

**PCR**

**Clonality assay.** The QIAamp DNA Blood Mini Kit (Qiagen) was used to extract DNA from 10^6 cell aliquots collected at appropriate points throughout the purging procedure. Comparison was made between pretreatment, post-CD138 depletion, post-treatment, and post-ex vivo culture samples for the presence of amplified (tumor-associated) sequences. B-cell clonality was determined using the IgH Gene Clonality Assay or IGK Gene Clonality Assay kits (InvivoScribe Technologies) according to the manufacturer’s instructions. These kits utilize oligonucleotide primers and conditions developed and validated by the manufacturer's instructions. These kits utilize oligonucleotide primers and conditions developed and validated by the BIOMED-2 consortium (24). PCR reactions were carried out in a 50 μl total volume containing 45 μl of master mix, 1.25 units AmpliTaq Gold DNA polymerase (Applied Biosystems), and 5 μl of DNA at a concentration of 100 to 400 μg/mL. Samples were subjected to a 7-minute incubation at 95°C followed by 35 cycles of denaturation (95°C, 45 seconds), annealing (60°C, 45 seconds), and extension (72°C, 90 seconds). Each run included polyclonal, clonal, and no template controls.

**Capillary electrophoresis.** One μl PCR product, 0.5 μl ROX size standard, and 8.5 μl deionized formamide were mixed according to the manufacturer’s instructions (Applied Biosystems), heated at 95°C for 2 minutes and placed on ice for at least 1 minute before electrokinetic injection (20 seconds) into the ABI 3130XL Genetic Analyzer (Applied Biosystems). The size (units of fluorescence) and positions of peaks on the electropherogram were analyzed using GeneScan analysis software (Applied Biosystems).

** Colony-forming cell assay**

The number of CFC was assessed in unmanipulated PBPC samples and after CD138 depletion, rituximab and bortezomib drug treatment, and ex vivo expansion. Cells were plated at 3,000 to 10,000 cells in 1 mL of methylcellulose medium containing hematopoietic growth factors (Methocult H4435, Stem Cell Technologies Inc.) in duplicate 35 millimeter dishes (Falcon). Cultures were incubated in a 37°C, 5% CO_2_ in air, fully humidified atmosphere for a period of 2 weeks. Colonies were scored using an inverted microscope as clusters of >50 translucent cells.

**Results**

**Effect of bortezomib on myeloma cell lines and normal CD34^+ peripheral blood progenitor cells**

The effect of bortezomib (2.5–160 nmol/L) was investigated on RPMI-8226 and ARPP-1 myeloma cell lines (Fig. 2A and 2B) and on normal CD34^+ isolated from 3 healthy donor PBPC (Fig. 2C). A 24-hour incubation with bortezomib at 80 nmol/L induced 80% to 90% growth inhibition in the myeloma cell lines (Fig. 2A and B), however, and it also markedly inhibited (80%) the growth of normal CD34^+ cells (Fig. 2C).

In contrast, a lower dose of bortezomib (20 nmol/L) for 16 hours was sufficient to induce 70% to 90% growth inhibition in myeloma cell lines, with only 20% growth inhibition in normal CD34^+ cells. Because of the efficacy of a 20 nmol/L bortezomib dose for 16 hours against the myeloma cell lines and minimal impact against normal CD34^+ cells, this dose was adopted for further combination drug strategies.

**Effect of rituximab on myeloma peripheral blood progenitor cells and normal CD34^+ cells**

Rituximab has previously been shown to inhibit the growth of clonogenic CD20^+ myeloma cells *in vitro* at 10 μg/mL (13). We studied the effect of rituximab at 5, 10, and 20 μg/mL on CD20^+ myeloma cells from patient PBPC by flow cytometry and on hematopoietic progenitor cell numbers (CFU–GM) from CD34^+ cells isolated from normal donor PBPC. A 24-hour incubation with rituximab at 20 μg/mL was shown to be effective against CD20^+ myeloma cells (Fig. 2D) with only minimal toxicity against normal hematopoietic progenitor cells (HPC) (Fig. 2E). This dose and time was therefore chosen for subsequent combination drug strategies.

The use of a CD19 detection antibody confirmed that CD20^+ (19^+) cells were indeed killed by rituximab (Fig. 3C). This addresses any argument that the failure to detect CD20^+ cells might not be indicative of cell death, rather it may simply be a consequence of rituximab (anti-CD20 antibody) stereochemically blocking access of the CD20 detection antibody to the CD20 antigen.

**Efficacy of bortezomib alone, rituximab alone, and bortezomib and rituximab in combination on peripheral blood progenitor cell products from myeloma patients**

Bortezomib (20 nmol/L, 16 hours) effectively depleted CD138^+ cells with no effect on CD20^+ (19^+) cells (Fig. 3A and B). Rituximab killed CD20^+ (19^+) cells, but not CD138^+ cells (Fig. 3C). The combination of rituximab (20 μg/mL, 24 hrs) and bortezomib (20 nmol/L, during the last 16 hours of incubation) was effective at killing both CD138^+ and CD20^+ (19^+) cells present in the PBPC (Fig. 3D).

**Building on the efficacy of combination drug treatment with ex vivo culture**

The efficacy of a tumor-purging procedure that consisted of MACS CD138^+ depletion followed by treatment with a...
combination of rituximab and bortezomib and ex vivo culture was investigated in 12 myeloma patient PBPC products. The efficacy of each step of the tumor-purging procedure is showed by flow cytometry (presence of CD138⁺ and CD138⁻/CD20⁺ cells) and PCR (presence of cells expressing unique light chain (Kappa) immunoglobulin rearrangements in representative patient samples (Fig. 4A and B). Combined analysis of the 12 patient products subjected to
this purging strategy (Fig. 4C) revealed that the combination of CD138 MACS depletion and bortezomib treatment reduced the number of CD138+ cells from $1.8 \pm 0.9 \times 10^6$ to $0.1 \pm 0.1 \times 10^6$ (Fig. 4C). In addition, rituximab reduced the number of CD20+ (19+) cells from $7.0 \pm 5.1 \times 10^6$ to $0.01 \pm 0.02 \times 10^6$ (Fig. 4C). After a subsequent 2-week period of ex vivo culture, CD138+ and CD20+ (19+) cells were reduced to levels below the limit of detection by flow cytometry (<10,000) [Fig. 4A(iv)] and PCR [Fig. 4B(D)]. Thus, from the original PBPC input, an overall >3-log depletion of CD138+ and >4-log depletion of CD138−20+ (19+) tumor cells can be estimated.
Impact of purging on normal hematopoietic progenitor cells

With the efficacy of this purging strategy against tumor cells confirmed both by FACS (Fig 4A) and PCR (Fig 4B), the impact of the process on normal HPC was also investigated. Although MACS depletion of CD138⁺ cells and treatment with bortezomib and rituximab was responsible for an approximately 50%, 60%, and 43% reduction in TNC, CD34⁺, and CFC numbers, respectively from those originally input (5A and B). After the 14-day period of ex vivo culture an approximately 20-, 8-, and 23-fold increase, respectively in TNC, CD34⁺, and CFC numbers from those originally input was observed. (Data as mean ± SEM, n = 12, *P < 0.05).

Magnetic activated cell-sorting depletion of CD138⁺ cells does not improve efficacy of purging

With the shown efficacy of bortezomib against the CD138⁺ cells and with the goal of simplifying the purging procedure and facilitating its clinical application, we removed the MACS CD138⁺ cell depletion step. To this end, 4 myeloma PBPC were treated with bortezomib and rituximab without prior CD138⁺ MACS depletion to determine the efficacy of this approach. Further, in these 4 cases, the myeloma patients had been previously treated with bortezomib, allowing us to better mimic the clinical situation where the majority of myeloma patients will have received bortezomib as standard of care. The removal of the CD138⁺ MACS depletion step did not impact the efficacy of the tumor purging achieved as shown by representative flow cytometric (Fig. 6A–C) and PCR data (Fig. 6D–F). Although an approximate 50% and 30% reduction in TNC and CD34⁺ numbers, respectively, was still observed after treatment with bortezomib and rituximab (in the absence of the MAC depletion), the subsequent 14-day period of ex vivo culture produced an approximate 12- and 8-fold increase in TNC and CD34⁺ numbers, respectively, from those originally input (Fig. 7).

Discussion

Our results indicate that incubation of PBPC products of myeloma patients with rituximab and bortezomib followed by a period of ex vivo culture markedly depletes CD138⁺ and CD138⁺ 20⁺ cell populations. CD138⁺ and CD138⁺ 20⁺ (19⁺) cells present in the initial products were depleted by more than 3 and 4 logs, respectively, based on 10⁶ flow-acquisition events and to levels below the limit of detection by PCR, reflecting an effective purging of mature and immature myeloma tumor cells. Further, in the same PBPC samples the numbers of normal HPC as measured by TNC, CD34⁺ cell, and colony-forming cell numbers were increased by approximately 12 to 20-, 8-, and 23-fold, respectively.

Bortezomib and rituximab are important elements of our purging strategy. Bortezomib has been previously reported to inhibit CD138⁺ mature myeloma cells in vitro (13, 25). In keeping with these data, we have shown that the use of bortezomib markedly depletes the mature CD138⁺ myeloma cell population from apheresis products. However, bortezomib has little effect on CD138⁺ 20⁺ (19⁺) immature myeloma precursors. This may be consequence of multidrug resistance, which is characteristic of a more primitive cell phenotype (25). Rituximab has therefore been used to target this immature myeloma tumor cell precursor. (13, 25) In keeping with these data, we have shown that the use of rituximab markedly depletes the immature CD138⁺ 20⁺ (19⁺) myeloma cell population from the apheresis products of patients with myeloma.

Whereas it has been reported that myeloma cells exhibit high mortality in culture (26), ex vivo culture in the presence of hematopoietic growth factors has also been shown to not only maintain, but expand the numbers of normal HPC without supporting tumor cells (17, 27). Our data confirm and build on these observations. Confirming these reports, our results indicate that ex vivo culture with MSC further depleted myeloma cells while reconstituting, and even increasing, normal HPC by 8-fold. The first week of ex vivo culture was carried out in the presence of growth factors and MSC. It is thought that MSC positively impact HPC expansion by providing cellular and extracellular components of the micro-environmental stem cell niche and the complex molecular environment.
cues that direct hematopoietic stem cell self-renewal, proliferation, and differentiation (28–31).

The availability of normal allogeneic MSC (predisposed to support normal hematopoiesis) rather than having to use autologous MSC derived from the myeloma patient for the coculture step may be an important benefit. MSC from myeloma patients present a distinct genetic and phenotypic profile that seems to favor the survival and proliferation of myeloma cells. As such they might be predisposed to support the survival and proliferation of myeloma cells during coculture. The use of normal MSC might be, at least in part, responsible for the continued purging of tumor cells and support of normal HPC observed in our procedure (32–37).

In addition, our experiments show that the MACS CD138⁺ cell depletion, first thought to be an important component of the purging process, does not contribute substantially to its efficacy. The removal of the MACS CD138⁺ depletion step will facilitate the clinical development of our purging procedure.

We appreciate that these data need to be interpreted with caution. First, while most of the CD138⁺ plasma cells detectable in the bone marrow of myeloma patients are malignant (as opposed to earlier stages of the myeloma transformation process; ref. 38), we did not carry out multiparametric flow cytometry. This might reveal disease-specific aberrant phenotypes and allow differentiation between tumor and normal plasma cells present in these patients (39). Therefore, while the eradication of the CD138⁺ population by the use of bortezomib markedly depletes the malignant plasma cells, it also likely depletes the small number of normal plasma cells present. Any adverse impact of such a depletion on post-transplant immunity might only be ascertained in a clinical trial.

Second, while the purging process seemed to successfully deplete mature and immature components of the disease, it is possible that tumor cells were still present in the end product, albeit at levels below the levels of detection of flow cytometry and PCR. Evidence for potential residual tumor might only become apparent during in vivo studies. While an in vivo

Figure 6. MACS depletion of CD138⁺ cells is not required for effective purging. Flow cytometric (A–C) and PCR analyses (D–F) reveal that indicators of myeloma (CD138⁺ and CD138⁻/CD19⁺ cells for flow cytometry and the presence of the Ig Kappa light chain for PCR) were reduced to levels below those detectable by either technique after the purging procedure. The treatment of PBPC with bortezomib and rituximab followed by ex vivo culture was itself sufficient to eradicate tumor cells from the PBPC without the use of CD138⁺ MACS depletion. It should be noted that the range of the y-axis in (F) was changed from 0 to 8000 arbitrary units (used in D–E) to 0 to 4000 arbitrary units which, although increasing the background "noise" present, further emphasizes the absence of any signal associated with the presence of tumor cells.
Bortezomib and rituximab treatment followed by ex vivo culture, seems to effectively deplete mature and immature myeloma cells present in PBPC from myeloma patients. Further, in addition to effective tumor purging, another benefit of this procedure is the expansion of normal HPC. This we hypothesize will provide a tumor-free graft with a high hematopoietic reserve, which should ultimately carry out well in a transplant setting. A clinical trial is planned to determine the safety of this purging strategy in myeloma patients.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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In conclusion, our sequential purging strategy, including rituximab and bortezomib treatment followed by ex vivo culture, seems to effectively deplete mature and immature myeloma cells present in PBPC from myeloma patients.
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