Ex vivo graft purging and expansion of autologous blood progenitor cell products from patients with Multiple Myeloma

Running Title: Novel purging of myeloma autograft products.

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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 CD138⁻20⁺ B cells (highly clonogenic immature cells), and bortezomib to target CD138⁺ cells (normal and differentiated myeloma plasma cells), followed by co-culture with allogeneic mesenchymal stem cells (MSC) from normal donors. After 7 days of co-culture, non-adherent cells were removed and cultured in the absence of MSC for an additional 7 days. Then, efficacy of purging (removal of CD138⁻20⁺ 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 CD138⁻20⁺(19⁺) cells present in the initial products were depleted more than 3 and 4 logs, respectively based on 10⁶ flow acquisition events, and to levels below the limit of detection by PCR. In contrast TNC, CD34⁺ cell and colony-forming cell numbers were increased by approximately 12-20, 8 and 23 fold, respectively. Overall, ex vivo treatment of apheresis products with rituximab, bortezomib and co-culture with normal donor MSC depleted mature and immature myeloma cells from clinical aphereses, while expanding the normal hematopoietic progenitor cell compartment.
Introduction

High-dose chemotherapy (HDC) with autologous peripheral blood progenitor cell (PBPC) support is widely considered an effective therapy for selected patients with myeloma (1-2). However, tumor relapse constitutes the single most important cause of treatment failure. Relapse may result from insufficient eradication of residual disease by HDC and/or reinfusion of myeloma tumor cells with the PBPC autograft. While the consequence of reinfusing malignant cells remains unclear, one prospective study has suggested that the presence of myeloma cells in PBPC grafts correlates with poor outcome after transplantation (3). However, a different study appears to contradict these observations (4). Separate retrospective analyses of the European and North American transplant registries have shown that patients who received HDC followed by infusion of PBPC from a twin presented fewer relapses and improved event-free survival (EFS) compared to matched patients who received an autograft (5-6). These observations suggest that the higher relapse rate seen after autologous transplantation may be due, at least in part, to the presence of reinfused malignant cells. Thus, purging myeloma cells present in autologous PBPC products represents a valid strategy aiming at reducing relapses.

Previous randomized clinical trials targeting the CD34 antigen, which is expressed by normal progenitors but not by most myeloma cells (7), did not improve outcome after transplant, despite reducing myeloma contamination of the transplanted product by up to 3 logs (8-9). The negative results of these trials are perhaps consistent with observations that CD34+ cells in the blood of myeloma patients carry disease-specific IgH VDJ gene rearrangements (10).
The failure of CD34⁺ selection to effectively purge tumor cells demonstrates 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⁺CD20⁻(19⁻) phenotype and an extensive ability to replicate and generate differentiated progeny expressing CD138⁺ (13-16). These CD138⁺CD20⁻(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 demonstrated 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 co-culture with normal allogeneic donor mesenchymal stem cells (MSC) (20). Such co-culture 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 co-culture with MSC might prove effective by killing mature CD138⁺ and immature CD138⁺CD20⁻ myeloma cells, while preserving (and possibly increasing) the content of normal hematopoietic progenitors. Here we report the development of this multi-step strategy.
MATERIALS AND METHODS

Cell Lines

Myeloma cell lines RPMI-8226, ARP-1 and NCI-H929 were obtained from American Type Culture Collection (Manassas, VA) and cultured at 37°C, 5% CO₂ in air, fully humidified atmosphere in RPMI medium (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone, Logan, UT, USA).

Initial Treatment of Peripheral Blood Progenitor Cell (PBPC) 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., Gibbstown, NJ) and washed in CliniMACS buffer (Miltenyi Biotech, Auburn, CA) containing 0.5% human serum albumin (Baxter Healthcare, Deerfield, IL) and DNAse to reduce aggregate formation (50 µg/ml Dornase ALFA Pulmozyme, Genentech, Inc., San Francisco, CA). 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 CliniMACS CD138 MicroBeads (both Miltenyi Biotech, Auburn, CA). CD138⁺ cells were used as a positive control for flow cytometry. The CD138 depleted PBPC cells were cultured overnight in CellGro® SCGM medium (CellGenix™, Antioch, IL).
containing 10% (v/v) FBS prior to use (Fig. 1). Later experiments were performed without CD138⁺ cell removal to demonstrate 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, Auburn, CA) and CliniMACS CD34 Reagent (Miltenyi Biotech, Auburn, CA). Isolated CD34⁺ cells were cultured overnight in CellGro® SCGM medium containing 10% (v/v) FBS.

**Rituximab Treatment of PBPC from Myeloma Patients and Normal Donor CD34⁺ Cells.**

Rituximab (Genentech, Inc., San Francisco, CA) 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®, Lawrenceville, GA) as a source of complement (13).

**Bortezomib Treatment of Myeloma Cell Lines, CD138⁺ Myeloma Patient PBPC and Normal Donor CD34⁺ Cells.**

Bortezomib (Millenium Pharmaceuticals Inc, Cambridge, MA) 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 nM) for 16 or 24 hrs (12).
Combined Rituximab and Bortezomib Treatment of PBPC 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 compliment) for 24 hours with Bortezomib (20 nM) added for the last 16 hours of incubation.

Ex Vivo Culture Conditions

After drug treatment, cells were washed and co-cultured 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, Antioch, IL) supplemented with 10% (v/v) FBS and containing 100 ng/ml each of Granulocyte Colony-Stimulating Factor (G-CSF, Neupogen Filgrastim, Amgen, Thousand Oaks, CA) and Stem Cell Factor (SCF), thrombopoietin (TPO) and Flt-3 ligand (all from CellGenix™ USA, Antioch, IL ) (Fig. 1). Normal donor MSC were provided by Angioblast Systems, Inc. (New York, NY). After 7 days, non-adherent cells were transferred to liquid culture (i.e., culture without MSC) in EVEM and incubated for an additional 7 days (14 days total). After 14 days, non-adherent cells were collected. TNC number was determined, CFC assay performed and the expression of CD138, CD20, CD19 and CD34 on the cells determined by flow cytometry. Samples were also taken for PCR analysis for comparison between pre-treatment, post treatment and post ex vivo culture samples (Fig. 1). Comparison was made with data from pre-treatment, post treatment and post ex vivo culture samples.

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, Madison, WI) (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 5x10\(^4\) cells/well. The normal PBPC CD34\(^+\) cells were cultured in EVEM. After cells were incubated with different concentrations (2.5–160 nM) of bortezomib for 16 or 24 hours at 37\(^\circ\)C in a 5% CO\(_2\) in air, fully-humidified atmosphere, MTS cell viability assays were performed in triplicate on post-treatment samples. Cell growth inhibition was calculated as follows:

Percent (%) growth inhibition = \[1 – (OD490 sample / OD490 control)] \times 100\]

**Flow Cytometric Analysis**

The presence of CD138\(^+\) or CD138\(^{-}\)20\(^+\) myeloma cells and CD34\(^+\)45\(^\text{dim}\) normal hematopoietic progenitors in pre-depletion, post-depletion, post-treatment and post-ex vivo culture samples was evaluated by flow cytometric analysis. Briefly, at each time point, 2 x10\(^6\) cells were washed with FACS buffer (DPBS + 1% goat serum), then stained with phycoerythrin (PE)-conjugated anti-CD138 (Miltenyi Biotec Inc., Auburn, CA) and fluorescein isothiocyanate (FITC)-conjugated anti-CD20 and allophycocyanin (APC)-conjugated anti-CD19 or PE-conjugated anti-CD34 and FITC conjugated anti-CD45, (all BD Biosciences Pharmingen, San Jose, CA). 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 0.5-1.0x10\(^6\) events were acquired for each sample using a FACSCalibur (Becton Dickinson, San Jose, CA, USA) as permitted by cell number available. Data acquisition and analysis was performed
using CELLQuest Pro software (Becton Dickinson, San Jose, CA). 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, San Jose, CA) (22-23).

**PCR**

*Clonality Assay*

The QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) was used to extract DNA from 10\(^6\) cell aliquots collected at appropriate points throughout the purging procedure. Comparison was made between with pre-treatment, 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, San Diego, CA) according to the manufacturer’s instructions. These kits utilize oligonucleotide primers and conditions developed and validated by the BIOMED-2 consortium (24). PCR reactions were performed in a 50 µl total volume containing 45 µl of master mix, 1.25 units AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) and 5 µl of DNA at a concentration of 100-400 µg/mL. Samples were subjected to a 7 minute incubation at 95\(^\circ\)C followed by 35 cycles of denaturation (95\(^\circ\)C, 45 sec), annealing (60\(^\circ\)C, 45 sec) and extension (72\(^\circ\)C, 90 sec). 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 manufacture’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 (CFC) 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 3,000–10,000 cells in 1 ml of methylcellulose medium containing hematopoietic growth factors (Methocult H4435, Stem Cell Technologies Inc., Vancouver, BC, Canada) in duplicate 35 myeloma dishes (Falcon). Cultures were incubated in a 37°C, 5%CO2 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+ PBPC.

The effect of bortezomib (2.5-160 nM) was investigated on RPMI-8226 and ARP-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 nM induced 80-90% growth inhibition in the myeloma cell lines (Fig. 2A and 2B), however, and it also markedly inhibited (80%) the growth of normal CD34+ cells (Fig. 2C).
In contrast, a lower dose of bortezomib (20 nM) for 16 hours was sufficient to induce 70-90% growth inhibition in myeloma cell lines, with only 20% growth inhibition in normal CD34+ cells. Because of the efficacy of a 20 nM bortezomib dose for 16 hrs 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 PBPC 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-hr incubation with rituximab at 20 µg/ml was shown to be effective against CD20+ myeloma cells (Fig. 2D) with only minimal toxicity against normal 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(+) 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 PBPC Products from Myeloma Patients.**

Bortezomib (20 nM, 16 hrs) effectively depleted CD138+ cells with no effect on CD20(+) cells (Fig 3A and 3B). Rituximab killed CD20(+) cells, but not CD138+ cells (Fig. 3C). The combination of rituximab (20 µg/ml, 24 hrs) and bortezomib (20 nM,
during the last 16 hrs 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 demonstrated by flow cytometry (presence of CD138⁺ and CD138⁻20⁺19⁺ 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 ± 0.9x10⁶ to 0.1 ± 0.1x10⁶ (Fig. 4C). In addition, rituximab reduced the number of CD20⁺(19⁺) cells from 7.0 ± 5.1x10⁶ to 0.01 ± 0.02x10⁶ (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 (<1:10,000) [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 CD20⁺(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, the 14 day
period of *ex vivo* culture was responsible for an approximately 20, 8 and 23-fold increase in TNC, CD34+ and CFC numbers from those originally input (Fig 5A and B).

**MACS depletion of CD138+ cells does not improve efficacy of purging.**

With the demonstrated 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. 6 A-C) and PCR data (Fig. 6 D-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 10^6 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 12-20, 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 \textit{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 multi-drug resistance which is characteristic of a more primitive cell phenotype (25). Rituximab has therefore been used to target this immature myeloma cell precursor.\textit{(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.

While it has been reported that myeloma cells exhibit high mortality in culture (26), \textit{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 upon these observations. Confirming these reports, our results indicate that \textit{ex vivo} culture with MSC further depleted myeloma cells while reconstituting, and even increasing, normal HPC by 8 fold. The first week of \textit{ex vivo} culture was performed 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 microenvironmental stem cell niche and the complex molecular 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 co-culture step may be an important benefit. MSC from myeloma patients present a distinct genetic and phenotypic profile that appears 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 co-culture, 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. Firstly, 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) (38), we did not perform multi-parametric 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.
Secondly, while the purging process appeared 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 mouse model is currently in development, it might ultimately fall to a clinical trial to address this concern.

In conclusion, our sequential purging strategy, including rituximab and bortezomib treatment followed by ex vivo culture, appears 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 perform well in a transplant setting. A clinical trial is planned to determine the safety of this purging strategy in myeloma patients.

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Figure Legends

Figure 1. Initial Experimental Design.
PBPC products of myeloma patients are thawed and washed and subjected to CD138-depletion by MACS. (The CD138-depletion step was ultimately found to be unnecessary and was subsequently removed) CD138-depleted PBPC are collected and rested overnight in culture at 37°C prior to treatment with rituximab (20 µg/ml) for 24 hours at 37°C and bortezomib (20 nM) during the last 16 hours of incubation. Cells are washed and cultured for 7 days with MSC in ex vivo expansion medium containing growth factors. After 7 days, non-adherent cells are transferred to liquid culture for an additional 7 days (14 days total). Non-adherent cells are collected and analyzed by: (i) flow cytometry for the presence/absence of CD34+ cells (providing a quantitative measure of normal hematopoietic progenitors) and CD138+ and CD13820+19+ 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).

Figure 2. Optimization of bortezomib and rituximab treatment against myeloma cell lines or PBPC, while minimizing any impact on normal CD34+ progenitor cells.
Bortezomib: Growth inhibition induced in RPMI-8226 and ARP-1 myeloma cell lines or CD34+ cells from a normal healthy PBPC product was measured when incubated with different doses (nM) of bortezomib for 16 hours (● & solid line) or 24 hours (▼ & dashed line) (n=3) (Figs 2A-C).
Rituximab: Representative data show the sensitivity of (i) CD138<sup>+</sup>(19)<sup>+</sup> myeloma cells from the patient’s PBPC or (ii) CD34<sup>+</sup> cells from a normal healthy PBPC product to a 24 hour incubation with 0, 5, 10 and 20 µg/ml rituximab (Figs 2D and E).

**Figure 3. Flow cytometry to reveal the effect of bortezomib or rituximab or both (in combination) on a representative PBPC product from a myeloma patient.**

Upper panels reveal CD138<sup>+</sup> and CD20<sup>+</sup> cells present in the PBPC after the various treatment conditions. Simultaneous use of a CD19 detection antibody (lower panel) confirms that CD20<sup>+</sup>(19<sup>+</sup>) cells are eradicated and that the absence of CD20<sup>+</sup> cells is not simply a consequence of blocking of the CD20 detection antibody by rituximab (anti-CD20). These data demonstrate that (i) bortezomib eradicates CD138<sup>+</sup> (mature myeloma) cells (Fig. 3B upper panel) and (ii) rituximab eradicates CD20<sup>+</sup>(19<sup>+</sup>) (immature myeloma) cells (Fig 3C). The combination of bortezomib and rituximab eradicates both CD138<sup>+</sup> (mature myeloma) and CD20<sup>+</sup>(19<sup>+</sup>) (immature myeloma) cells present in the PBPC (Fig. 3D).

**Figure 4. Efficacy of CD138<sup>+</sup> cell depletion, treatment with rituximab and bortezomib and ex vivo culture at depleting tumor in myeloma patient PBPC product.**

Fig. 4A: Representative flow cytometric data revealed that a thawed myeloma patient PBPC product contained 1.1% CD138<sup>+</sup> cells (Fig. 4A(i) upper panel) and 11.0% CD138<sup>-</sup>CD20<sup>+</sup>(19<sup>+</sup>) cells (Fig. 4A(i) lower panel). MACS CD138<sup>+</sup> depletion reduced the CD138<sup>+</sup> cell content of the PBPC from 1.1% to 0.3% (Fig. 4A(ii) upper panel). Combination treatment with rituximab and bortezomib further reduced the CD138<sup>+</sup> cell content of the PBPC to from 0.3% to 0.0% (Fig. 4A(iii) upper panel) and reduced the CD138<sup>-</sup>CD20<sup>+</sup>(19<sup>+</sup>) cell content of the PBPC from 8.8% to 0.1% (Fig. 4A(iii) lower panel). After
ex vivo culture, the CD138⁺ (Fig 4A(iv) upper panel) and CD138⁻CD20⁺(19⁺) cell (Fig. 4A(iv) lower panel) content of the PBPC was 0.0%.

Fig. 4B: Representative data show a unique clonal peak (indicative of cells expressing a unique immunoglobulin rearrangement and which provides a surrogate marker for tumor cells) at 278 base pairs (indicated by arrow). Tumor cells are detected by PCR in the original PBPC product [Fig. 4B(A)], after MACS CD138⁺ cell depletion [Fig 4B(B)] and after combined rituximab and bortezomib treatment [Fig. 4B(C)]. Only after ex vivo culture is the signal associated with tumor absent [Fig. 4B(D)]. It should be noted that the range of the y-axis in Fig. 4B(D) has been changed from 0-8000 arbitrary units [used in Figs 4B(A-C)] to 0-4000 arbitrary units which, although increasing the background ‘noise’ present, further emphasizes that any signal associated with tumor cells is absent.

Fig 4C: Absolute numbers of CD138⁺ (black bar) and CD138⁻CD20⁺(19⁺) cells (hatched bar) throughout the depletion process for 12 PBPC products from myeloma patients. (Data shown as mean ± SEM.)

**Figure 5. Impact of purging on TNC, CD34⁺ cell and CFC numbers.**

Attempts were made to optimize purging conditions to those that yielded maximal anti-tumor impact with minimal impact on normal hematopoietic progenitors. MACS depletion of CD138⁺ cells and treatment with bortezomib and rituximab was responsible for an approximately 50%, 60% and 43% reduction in TNC, CD34⁺ cell and CFC numbers, respectively from those originally input (Figs 5A and B). After the 14 day period of ex vivo culture an approximately 20, 8 and 23-fold increase, respectively in TNC, CD34⁺ cell and CFC numbers from those originally input was observed. (Data as mean ± SEM, n=12, *p<0.05)

**Figure 6. MACS depletion of CD138⁺ cells is not required for effective purging.**
Flow cytometric (Fig. 6 A-C) and PCR analyses (Fig 6 D-F) reveal that indicators of myeloma (CD138+ and CD138-20^19^ 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 Fig. 6 F was changed from 0-8000 arbitrary units (used in Figs 6 D-E) to 0-4000 arbitrary units which, although increasing the background ‘noise’ present, further emphasizes the absence of any signal associated with the presence of tumor cells.

**Figure 7. Removal of the MACS depletion step did not compromise TNC and CD34^+ cell recovery.**

While treatment with bortezomib and rituximab was responsible for an approximately 50% and 30% reduction, respectively in TNC and CD34^+ numbers in the PBPC (consistent with data from the purging procedure containing the MACS CD138^+ cell depletion step), the subsequent 14 day period of ex vivo culture increased the numbers of TNC and CD34^+ cells by approximately 12 and 8-fold, respectively over those originally input. (Data as mean ± SEM, n=4, *p<0.05)
1. Multiple Myeloma PBPC.


3. MACS.

4. CD138 negative (over night culture).

5. Addition of 20 µg/ml rituximab in the presence of 10% human AB serum.

6. Addition of 20 nM bortezomib.

At t=0 hrs

At t=8 hrs

At t=24 hrs

7. Drugs removed by washing and cells co-cultured in medium containing growth factors with mesenchymal stem cells (MSC).

8. Co-culture performed for 7 days.

9. After 7 days, non-adherent cells collected and diluted for liquid culture.

10. Liquid culture performed for an additional 7 days (14 days total).

11. Cultured cells analyzed:
   (a) CFC assay
   (b) Flow cytometry
      (i) CD34+
      (ii) CD138+
      (iii) CD138-20'+19'
   (c) PCR

At t=8 hrs

At t=24 hrs
Growth inhibition (%) of CD34+ (A), ARP-1 (B), and RPMI-8226 (C) in response to different concentrations of Bortezomib (nM) over 24 hrs.

Proportion of CD138-20+ cells and CFU-GM recovery (% of control) in response to different concentrations of Rituximab (µg/ml) over 24 hrs.
A

(i) Pre-depletion (ii) Post-depletion (iii) Post-treatment (iv) Post-culture

CD138+ 1.1% CD138+ 0.3% CD138+ 0.0% CD138+ 0.0%

CD138-20+19+ 11.0% CD138-20+19+ 8.8% CD138-20+19+ 0.1% CD138-20+19+ 0.0%

B

C

Absolute number of CD138+ or CD138-20+19+ cells (x10^6) (Mean±SEM)

Pre-depletion Post-depletion Post bortezomib Post bortezomib + rituximab Post bortezomib + rituximab + ex vivo culture
A. Without depletion

B. Post-treatment

C. Post-ex vivo culture

D. Without depletion

E. Post-treatment

F. Post-ex vivo culture
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