Proteasomal adaptations underlying carfilzomib-resistance in human bone marrow plasma cells

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Abstract

Donor-specific antibodies (DSAs) have a deleterious effect on allografts and remain a major immunologic barrier in transplantation. Current therapies to eliminate DSAs are ineffective in highly human leukocyte antigen (HLA)-sensitized patients. Proteasome inhibitors have been employed as a strategy to target bone marrow plasma cells (BMPCs), the source of long-term antibody production; however their efficacy has been limited by poorly defined drug resistance mechanisms. Here, we performed transcriptomic profiling of CD138^{+} BMPCs that survived *in vivo* desensitization therapy with the proteasome inhibitor carfilzomib to identify mechanisms of drug resistance. The results revealed a genomic signature that included increased expression of the immunoproteasome, a highly specialized proteasomal variant. Western blotting and functional

studies demonstrated that catalytically active immunoproteasomes and the immunoproteasome activator PA28 were upregulated in carfilzomib-resistant BMPCs. Carfilzomib-resistant BMPCs displayed reduced sensitivity to the proteasome inhibitors carfilzomib, bortezomib and ixazomib, but enhanced sensitivity to an immunoproteasome-specific inhibitor ONX-0914. Finally, *in vitro* carfilzomib treatment of BMPCs from HLA-sensitized patients increased levels of the immunoproteasome β5i (PSMB8) catalytic subunit suggesting that carfilzomib therapy directly induces an adaptive immunoproteasome response. Taken together, our results indicate that carfilzomib induces structural changes in proteasomes and immunoproteasome formation.

1. INTRODUCTION

Proteasome inhibitors (PIs) have revolutionized treatment of the PC malignancy multiple myeloma (MM) changing MM from a uniformly fatal disease with limited life expectancy to a chronically manageable disease with substantially enhanced survival (1–3). Recently, PIs have been employed as a means to target non-transformed PCs that mediate autoimmune disease and HLA-sensitization in organ transplantation, where pathogenic antibodies (Abs) play a major role. Hence, PIs now provide the foundation as a therapeutic strategy to safely and effectively treat a number of PC-related human diseases (4).

Kidney transplantation is the treatment of choice for patients with end stage kidney disease (ESRD) as is associated with improved patient survival and better quality-of-life (5–7). It is now clear in kidney transplantation that Ab-mediated rejection (AMR) is the major cause of renal allograft loss and HLA Abs also promote AMR in heart, lung, and pancreas transplantation (8–10). HLA Abs, that arise from pregnancy, blood transfusion, or prior transplantation, present a significant and often impenetrable barrier to kidney transplantation, leading to increased morbidity and mortality (11, 12). Current desensitization therapies do not impact Ab production nor eliminate BMPCs (13–16). Transplantation across HLA barriers, through preconditioning with intravenous immune globulin (IVIg), alone or combined with plasmapheresis, or the CD20 monoclonal Ab rituximab provides short-term reductions in HLA Ab levels (17, 18). B-cell depletion with rituximab was shown to reduce preformed HLA Abs in only 10% of treated patients and failed to target BMPCs. Thus, allosensitization represents an urgent and unmet medical need in organ transplantation as current desensitization methods fail to provide durable effects on HLA Ab responses. Desensitization strategies that target BMPCs and lead to durable responses would allow highly sensitized patients to receive a donor organ.

BMPCs exhibit exceptionally high levels of immunoglobulin synthesis that is accompanied by the accumulation of endoplasmic reticulum stress. As a result, BMPCs are highly susceptible to agents that disrupt protein homeostasis, such as proteasome inhibition (19, 20). Bortezomib is a reversible PI that effectively targets malignant PCs and is used to treat newly diagnosed and relapsed/refractory MM. Carfilzomib is an irreversible, epoxyketone PI that functions similarly to bortezomib primarily through inhibition of proteasomal chymotrypsin-like (ChT-L) activity. Carfilzomib selectively inhibits the proteasome through a binding mechanism distinct from that of bortezomib, has a short half-life, and a lack of off-target effects which may explain the favorable safety profile compared to bortezomib.

Proteasomes are composed of four stacked heptameric rings in which non-identical, but structurally-related, α subunits form the two outer rings, and distinct β subunits form the two inner rings (21). Proteasomes can exist in a constitutive c20S form that exhibits peptide-hydrolyzing activities conferred by β1, β2 and β5 catalytic subunits (Supp. Fig. 1). c20S proteasome catalytic subunits can be substituted by the IFN-γ-inducible β1i, β2i and β5i catalytic subunits, encoded by *PSMB9, PSMB10* and *PSMB8*, to form immunoproteasomes (i20S proteasomes) (22, 23). c20S proteasomes are capped at either or both ends by a 19S regulatory particle (RP) while i20S proteasomes are capped by a heptameric PA28α/β activator.

We and others have used PIs as a means to eliminate long-standing HLA Abs in transplant candidates, a process termed desensitization. Studies by our group and others have demonstrated that bortezomib consistently and substantially reduced HLA Ab levels in transplant candidates beyond that achieved with IVIg-based regimens (24–28). However, dose-limiting toxicity from peripheral neuropathy and the emergence of drug resistance has limited bortezomib efficacy in desensitization. Carfilzomib is clinically devoid of peripheral neuropathy but is also limited therapeutically by poorly defined chemoresistance mechanisms. Here, we hypothesized that carfilzomib treatment may induce proteasomal structural adaptations that contribute to drug resistance. We investigated the *in vivo* and *in vitro* effects of carfilzomib on BMPCs isolated from HLA-sensitized patients. Genomic analyses coupled with biochemical and functional assays, uncovered proteasomal adaptations that up regulate i20S proteasome levels and activity to reveal a novel mechanism of chemoresistance within the context of HLA-desensitization.

2. MATERIALS AND METHODS

2.1 BM aspiration and isolation of BMPCs.

BM was collected from the iliac crest using an aspiration needle under sterile conditions from patients enrolled in University of Cincinnati IRB-approved clinical trial NCT02442648. Patients were defined as HLA-sensitized if the calculated panel reactive Ab was >30% following single antigen bead (SAB, One Lambda, Canoga Park, CA) analysis on a Luminex® platform (Luminex, Austin, TX). Patient demographic features and clinical characteristics are provided in Supp. Table 1. Aspirates were diluted with sterile PBS, passed through a 100 uM filter to remove debris, layered onto 15 mL Ficoll-Paque plus (density 1.077 g/mL) and centrifuged at 445 x g. The pellet was removed, PBS added and centrifuged at 300 x g. To remove platelets, the pellet was resuspended in 50 mL PBS, centrifuged at 200 x g for 10 min and supernatant removed. The BM mononuclear fraction was then resuspended in PBS containing 0.5% BSA, 2 mM EDTA (buffer A), mixed with CD138 microbeads for 15 min at 4°C, and washed in buffer A. A CD138 column (Miltenyi Biotec, San Diego, CA) was equilibrated with buffer A at 4°C in the presence of a magnetic field, the suspension applied and the unbound CD138⁻ fraction collected. The column was then removed from the magnetic field, placed in a sterile eppendorf tube and the CD138⁺ fraction collected (Supp. Fig. 2). Live CD138⁺ cells were isolated to >95% purity as indicated by trypan blue exclusion and flow cytometry.

2.2 Co-culture of BMPCs with BMSC media

BMPCs were isolated from BM biopsies performed prior to and after *in vivo* carfilzomib treatment of highly HLA-sensitized patients on protocol NCT02442648 (Supp. Fig. 3). CD138+ BMPCs were co-cultured with conditioned media from the human HS.5 BMSC line (ATCC, Manassas, VA) supplemented with APRIL and IL-6 for indicated times. HS.5 cells were grown to confluency and conditioned media was removed and centrifuged at 3,000 x g for 10 min.

2.3 Transcriptomic analysis of pre- and post-carfilzomib treated BMPCs

Total RNA was analyzed using an Ilumina® TruSeq® RNA Access Library kits (NGS, Illumina, San Diego, CA) platform (30 million reads/sample) with paired-end 75 bp sequencing to evaluate the effects of carfilzomib on gene expression. Strongly and differentially expressed gene sets were clustered and evaluated using enrichment and prior knowledge-based network analyses using ToppGene Suite (toppgene.cchmc.org/).

2.4 HLA Single antigen bead (SAB) assays

Patient serum, BM mononuclear cells, CD138+ and CD138- cells were incubated in 100 uL BMSC conditioned media at 37šC for 72 h. Serum or culture supernatants were then used for SAB assays. SAB assays were performed by incubating serum or culture supernatant with purified HLA antigen beads, washing, and a fluorescent-conjugated anti-human IgG Ab added, followed by analysis on the Luminex® platform. The platform generated a semi-quantitative output for each bead expressed as mean fluorescence intensity (MFI). The coefficient of variation for SAB assays was consistently between 5-8%.

2.5 Isolation of proteasomes from BMPCs

Proteasomes were isolated using agarose-immobilized 20S α7 (PSMA7) subunit-specific Ab (Enzo Life Sciences, Farmingdale, NY) that allows for rapid isolation of proteasome complexes from biological samples with high integrity and without genetic modification of the proteasome which is not feasible with patient-derived samples. Cells were washed with PBS, lysates prepared in non-denaturing buffer and incubated with Abs to PSMA7 at 4°C overnight with protein A sepharose. The slurry was washed with PBS and resuspended in non-denaturing loading buffer for native gels. The method maintains proteasome integrity, avoids steps that denature the proteasome as well as high salt buffers that dissociate proteasome-associated subunits.

2.6 SDS and native polyacrylamide gel electrophoresis

BMPCs isolated from healthy individuals, HLA-sensitized patients and HLA-sensitized patients treated with carfilzomib were lysed in buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β −glycerophosphate, 1 mM Na_3VO_4 and 1 <g/ml leupeptin). Lysed cells were incubated for 10 min at 4šC, centrifuged at 3,000 x rpm for 5 min and supernatant removed. SDS sample buffer was added, samples boiled for 5 min and loaded onto 4-12% bis-tris-polyacrylamide gels. Individual proteasome complexes isolated from patient BMPCs were resolved using 3-8% gradient native gel electrophoresis with tris-borate-EDTA buffer (TBE, 89 mM Tris

base, 89 mM boric acid, 2 mM EDTA, pH 8.3) as running buffer. Samples from healthy individuals, HLA-sensitized patients or HLA-sensitized patients that had received carfilzomib therapy were mixed with 4x loading buffer (100 mM Tris pH 8.0, 20% glycerol). Electrophoresis was carried out at 4°C in TBE running buffer with 0.5 mM DTT, 0.5 mM ATP and 2 mM $MgCl_2$ added.

2.7   Western blotting

Proteins were transferred to PVDF membranes using a Bio-Rad mini-protean transfer system containing a buffer of 25 mM Tris base, 192 mM glycine, 0.1% SDS. Proteins were transferred for 6h at 40V at 4°C. Membranes with transferred proteins were blocked with a 1:1 mixture of Li-COR blocking buffer and PBS. Membranes were then incubated in blocking solution for 1 h and primary Abs (1:1000) applied for 16 h at 4°C. Membranes were washed and incubated with secondary Abs (IR Dye® 800CW goat anti-mouse IgG (H +L) or IR Dye 680CW donkey anti-rabbit IgG (H+L), and imaged using an Odyssey Li-Cor platform. Antibodies used were rabbit anti-PSMB5 polyclonal (Abcam, ab90867), rabbit monoclonal to anti-PSME1 (EPR10967, Abcam, ab155091), rabbit monoclonal to anti-PSME2 (EPR14931, Abcam, ab183727), rabbit anti-LMP2 polyclonal (PSMB9, Abcam, ab42987), rabbit anti-PSMB10 polyclonal (Abcam, ab77735), rabbit anti-LMP7 monoclonal (EPR14482, PSMB8, Abcam, ab180606), rabbit anti-PSMA6 polyclonal (Cell Signaling 2459), rabbit anti-PSMB1 polyclonal (Abcam, ab196623), rabbit anti-PSMB2 polyclonal (Abcam, ab140426), mouse anti-Rpn5 monoclonal (PSMD12, clone H3, Santa Cruz Biotechnology, sc-398279), mouse anti-α-tubulin monoclonal (DM1a, Abcam, ab7291), and rabbit anti-histone 3 polyclonal (Abcam, ab1791).

2.8   *In situ* proteasome activity overlay assay

Proteasomes (5ug/sample) were loaded onto native gels run as indicated above. Gels were incubated with either the pan-constitutive proteasome substrate Suc-LLVY-MCA (100uM) or the i20S proteasome substrate Ac-ANW-AMC (100uM) at 37°C for 15 min. *In situ* fluorescence was recorded using an Odyssey Li-Cor imaging platform.

2.9   Statistical methods

All bioassays were performed in triplicate. The arithmetic mean of a set of observed data was determined summation of the numerical values of each observation divided by the total number of observations. Error bars represent the standard deviation of the triplicate values relative to the arithmetic mean. Supervised hierarchical clustering by normalized expression of the UPS genes further indicated that a number of genes encoding catalytic, structural or regulatory subunits of the proteasome were upregulated in post-treatment samples. The ratio of individual gene expression in BMPCs isolated from HLA-sensitized patients post-treatment relative to that pre-treatment was calculated on an individual patient basis. A p-value cutoff of 0.05 was considered statistically significant (Fisher's exact test). The ratio of gene products on western blots was compared using densitometric analysis on an Odyssey imaging platform. R version 3.5.0 was then used for paired tests to compare pre- vs. post-treatment blots by 2-sided analysis. In Fig. 6, given that n=8, a non-parametric Wilcoxon signed rank test was used.

3. RESULTS

3.1 Co-culture with BMSC conditioned media extends the longevity of BMPCs

The short half-life of human BMPCs *in vitro* has significantly limited study of their growth and survival (30–33). Recent studies have shown that BMPCs cultured *in vitro* under well-defined conditions display a remarkable robustness, extended survival and produce immunoglobulins without contact with BM cells, if specific growth factors, e.g., APRIL (a proliferation-inducing ligand), IL-6, IL-12, and nutrients are provided. We and others have investigated the capacity of primary BM-derived stromal cells to maintain PC viability *in vitro*. PCs purified from BM died rapidly when plated in media, but a subpopulation of PCs survived and secreted high levels of Ab for up to 4 weeks when co-cultured with stromal cells (33). Importantly, the longevity of BMPCs from HLA-sensitized patients was extended upon co-culture with BMSC conditioned media supplemented with APRIL (200ng/mL) and IL-6 (10ng/mL). CD138$^+$ cell survival was >50% at 6 days when cultured in BMSC conditioned media while the majority of CD138$^+$ cells cultured in RPMI media alone were no longer viable at 3 days (Supp. Fig. 4).

3.2 BMPCs secrete HLA Abs that are reflective of profiles in patient serum

We then sought to determine whether CD138$^+$ cells were a major source of HLA Ab production and whether these cells produced class I and II HLA Ab profiles similar to those present in the serum of HLA-sensitized patients. BM biopsies were obtained from HLA-sensitized patients, the BM mononuclear cells isolated and the CD138$^+$ cells separated from the CD138$^-$ cells by positive selection. The BM mononuclear fraction, CD138$^+$ and CD138$^-$ PCs were then co-cultured with BMSC conditioned media for 72 h. Culture supernatants were removed and the levels of class I and II HLA Abs in the supernatants determined. Representative SAB assay histograms from an individual HLA-sensitized patient indicated that the CD138$^+$ BMPCs, but not the CD138$^-$ cells, produced class I and II Abs with similar hierarchical specificities to that seen in serum from the same patient (Fig. 1). Similar results were obtained using serum and cell fractions from two other HLA-sensitized patients to indicate that the CD138$^+$ BMPCs are a major source of class I and II HLA Abs (Supp. Fig. 5A, B). Plots then generated to demonstrate that Ab levels in CD138$^+$ culture supernatants correlated with the serum levels of HLA class I and class II Abs from the same three patients (Supp. Fig. 6A and 6B).

3.3 RNA-seq reveals proteasome genes upregulated in BMPCs after carfilzomib therapy

We initially sought to determine whether *in vivo* carfilzomib therapy reduced the relative number of BMPCs present in patient BM biopsies. The total number of live PCs recovered from patient BM was determined using trypan cell exclusion prior to and after *in vivo* carfilzomib. We detected a profound depletion in the percent of viable PCs recovered from patient BM, relative to the total number of nucleated cells recovered in that BM, following carfilzomib treatment. *In vivo* carfilzomib therapy depleted 60-70% of the BMPCs in HLA-sensitized patients (Fig. 2A).

We also sought to identify genes involved in BMPC survival following *in vivo* carfilzomib therapy. RNA-seq analysis was performed to detect genes significantly deregulated in

CD138^{+} BMPCs isolated from HLA-sensitized patients after *in vivo* carfilzomib therapy relative to their expression level prior to therapy. A total of 2,024 differentially expressed transcripts that corresponded to 1,241 genes (1043 upregulated, 198 down regulated) were identified. Following *in vivo* therapy with carfilzomib, we found that the ten most upregulated genes in BMPCs were: *MUC17, ZNF645, VN1R1, JAKMIP3, SH3TC2, MUC4, MAPT-IT1, SMCO3, NMNAT2,* and *MSL3P1* and the ten most down regulated genes were *SATL1, PYGM, RPRM, ARHGEF25, SYNC, ABO, NPIPA2, SH3RF1, MROH6,* and *OR1J4* (Supp. Table 2).

Kyoto Encyclopedia of Genes and Genomes (KEGG) and Ingenuity Canonical Pathway (IPA) analyses of differentially expressed genes implicated a number of molecular pathways and components that were upregulated in CD138^{+} BMPCs that survived carfilzomib therapy (Fig. 2B). Notably, these included a significant number of genes within the ubiquitin-proteasome system (UPS) differentially expressed with statistical significance between pre-treatment samples relative to post-treatment samples (Fig. 2C). We focused on genes within the UPS since this pathway is well known to undergo substantial perturbations following proteasome inhibition. The ten most up or down regulated genes within the UPS are shown in Supp. Table 3 and the ten most up- or down regulated proteasome/immunoproteasome genes are shown in Supp. Table 4. The ratio of individual gene expression in BMPCs isolated from HLA-sensitized patients post-treatment relative to that pre-treatment was calculated on an individual patient basis. The analysis revealed that *PSMB8*, which encodes the β5i catalytic subunit in i20S proteasomes, was the most significantly upregulated proteasome-associated gene in BMPCs isolated post-carfilzomib therapy (Fig. 2C). In addition, the FDA-approved PIs bortezomib, carfilzomib and ixazomib also demonstrate inhibitory effects on multiple catalytic subunits in addition to the proteasome β5 catalytic subunit encoded by *PSMB5*. *PSMB8* is a particularly attractive target since it also encodes an immunoproteasome catalytic subunit that is also inhibited by carfilzomib. *PSME1* and *PSME2,* which encode the proteasome activator PA28α/β subunits, were also upregulated following carfilzomib therapy (Supp. Fig. 7).

We also looked at the expression of known PC survival factors as well as cytokine and chemokine expression. Importantly, the expression of the APRIL, IL-6 and BAFF receptors was not significantly changed in BMPCs isolated following *in vivo* carfilzomib. Interestingly, a number of cytokine and chemokines and receptors were deregulated in BMPCs after carfilzomib, regardless of whether they are known to substantially affect PC survival (Supp. Table 5).

3.4 β5i and PA28 are upregulated in BMPCs upon *in vivo* carfilzomib therapy

To extend the aforementioned transcriptomic findings, lysates were prepared from BMPCs isolated from individual HLA-sensitized patients before and after *in vivo* carfilzomib therapy. Western blotting indicated that β5i (PSMB8), PA28α and PA28β were significantly upregulated in BMPC lysates after carfilzomib therapy, relative to that observed prior to therapy, while other proteasome catalytic subunits β1, β1i, β2, β2i, and β5 were not significantly changed (Fig. 3).

3.5 β5i incorporation into catalytically active i20S proteasomes upon carfilzomib therapy

We next sought to determine whether *PSMB8* upregulation upon carfilzomib therapy resulted in increased incorporation of the β5i subunit into i20S proteasomes. Proteasomes were affinity-purified from the BMPCs of healthy individuals and HLA-sensitized patients either prior to or after carfilzomib therapy and separated by native gel electrophoresis (33–35). Western blots were normalized using the proteasome structural subunit PSMA6 (Fig. 4A) and Rpn5 was used to detect 19S RPs in proteasome preparations from erythrocytes, liver and BMPC samples (Fig. 4B). Spleen has the greatest level of i20S proteasome expression compared to other organs and 20S spleen proteasomes were nearly exclusively i20S proteasomes lacking 19S RPs. Western blots indicated that β5i levels were greater in HLA-sensitized dialysis patient BMPC proteasome preparations and elevated further in preparations from BMPCs that survived *in vivo* carfilzomib therapy (Fig. 4C).

3.6 i20S proteasome activity is increased in BMPCs upon *in vivo* carfilzomib therapy

The HLA sensitized patients treated with carfilzomib also differed from healthy controls in that they were on dialysis. Therefore, we also evaluated proteasome catalytic activity in healthy controls in addition to HLA-sensitized dialysis patients before and after *in vivo* carfilzomib therapy. Proteasomes were isolated from the CD138$^+$ BMPCs of a single patient, separated by native gel electrophoresis and incubated with the pan-proteasome Ch-T-L substrate Suc-LLVY-MCA (Fig. 4D). Results indicated that Ch-T-L activity was increased in 20S proteasomes from the BMPCs of HLA-sensitized patients compared to BMPCs from healthy adults and further increased in proteasomes from BMPCs that survived carfilzomib therapy. Ac-ANW-AMC is a fluorogenic substrate preferentially cleaved by i20S proteasomes and is not hydrolyzed by c20s proteasomes (36). Ac-ANW-AMC hydrolysis by proteasomes isolated from healthy donor BMPCs was low but was significantly increased in proteasomes isolated from HLA-sensitized patient CD138$^+$ BMPCs (Fig. 4E). Ac-ANW-AMC hydrolysis by proteasomes from BMPCs of carfilzomib treated HLA-sensitized patients was even greater. Results indicated that β5i was indeed incorporated into i20S proteasomes and there was a shift toward i20S proteasome-specific activities within the proteasome pool of CD138$^+$ BMPCs that survived carfilzomib. Similar results were obtained using BMPCs from two additional HLA-sensitized patients (Supp. Fig. 8).

3.7 Carfilzomib reduces production of class I and II Abs by BMPCs cultured *in vitro*

Patient BMPCs were cultured *in vitro* with BMSC media either alone or in the presence of carfilzomib. Following 72 h of culture, the supernatants were removed and used for SAB assays (Fig. 5A, B). Results indicated that carfilzomib treatment *in vitro* reduced the level of most individual class I and II HLA Abs generated in the culture supernatants by 60-70%.

3.8 Carfilzomib treatment *in vitro* induces an increase of β5i (PSMB8) in BMPCs

CD138$^+$ BMPCs were isolated from eight individual HLA-sensitized patients, co-cultured with BMSC conditioned media, and either left untreated or treated with carfilzomib. Lysates were prepared and analyzed by western blotting to determine the effect of carfilzomib on the levels of the proteasome β5 and β5i subunits. Western blotting indicated a significant

increase in β5i in lysates from the patients that were analyzed (Fig. 6A). In contrast, little change was observed in the β5 subunit (Fig. 6B).

3.9 BMPCs that survive carfilzomib therapy are sensitive to immunoproteasome inhibition

BMPCs that had been isolated from three patients prior to and after *in vivo* carfilzomib therapy were treated with individual PIs and the number of apoptotic cells then measured using annexin V-FITC (Fig. 7). BMPCs isolated from patients prior to carfilzomib therapy were relatively sensitive to the proteasome inhibitors bortezomib, carfilzomib and ixazomib. In contrast, BMPCs isolated after *in vivo* carfilzomib therapy were more sensitive to ONX-0914 (Fig. 7).

DISCUSSION

Major progress has been made in understanding PC biology over the past decade, and more recently with respect to elucidating transcriptional regulatory networks that underlie B cell differentiation and PC generation in immune responses. Our prior experience in treating Ab-mediated rejection and in desensitization in transplantation, has led us to realize that plasmablasts are exquisitely sensitive to bortezomib therapy, whereas BMPCs demonstrate considerable, yet partial resistance. This realization led us to begin to explore the basis for the heterogeneity within BMPC populations following PI therapy. We reasoned that understanding drug resistance would enable development of more effective targeting of BMPCs. This rationale underpinned the present study. A key first step in the study was to determine whether BMPCs represented a major source of HLA Abs, as suggested in the existing literature. The similarities in HLA Ab level hierarchy between patient serum and that produced *in vitro* by BMPCs supported the concept that long-term HLA Ab production is derived from BMPCs, thereby supporting our selection of this cellular population to focus the present studies.

RNA-seq and biochemical analyses revealed that the β5i subunit, β5i-specific catalytic activity and i20S proteasomes were substantially upregulated in BMPCs isolated from HLA-sensitized dialysis patients following carfilzomib therapy. Transcriptomic studies of BMPCs before and after *in vivo* carfilzomib treatment revealed an unforeseen plasticity of the UPS and proteasomes within Ab-producing BMPCs. Unexpectedly, we found that i20S proteasomes are upregulated in response to carfilzomib, and were characterized by both upregulation of both β5i catalytic activity and also PA28 activator subunit expression (*PSME1* and *PSME2*). These observations suggest that specific targeting of β5i activity, and also potentially preventing PA28 association with the core 20S particles, represent potential avenues for future PI-base therapies that enhancing BMPC depletion. These studies are unique in that they investigate for the first time, the effects of *in vivo* carfilzomib treatment on human BMPCs and outline a unique adaptive mechanism that may limit clinical effectiveness of PI therapy.

An important question that we began to explore was whether PI resistance was predetermined, i.e., that specific BMPC subpopulations existed prior to therapy that would demonstrate PI resistance, or whether it arose as a result of BMPC adaptation upon drug

treatment. Interestingly, *in vitro* treatment of cultured BMPCs for 24 h induced responses similar to those observed following in vivo treatment, indicative of an adaptive response. The results are consistent with the known reduced sensitivity of i20S proteasomes to agents that target the c20S proteasome. Modulation of proteasome catalytic subunit gene expression suggests proteasomal adaptation as a new chemoresistance mechanism that promotes the survival of BMPCs. β5i alterations were also reliably accompanied by PA28 alterations. While the function of PA28 is not fully elucidated, association with i20S proteasomes suggests that it may also potentially contribute to chemoresistance.

i20S proteasomes have been proposed as a potential new target for suppressing autoimmune and inflammatory processes (37–43). Importantly, the β5i-selective inhibitor ONX0914 has shown to suppress autoreactive immune responses in mouse models of arthritis and diabetes (44). Furthermore, β5i inhibition prevented experimental colitis (45), Hashimoto's thyroiditis (46) and graft-versus-host disease in an MHC-matched minor histocompatibility antigen-disparate murine model (47). In addition to providing irreversible proteasome inhibition, carfilzomib displays a distinct, advantageous adverse-event profile compared to bortezomib for the treatment of MM patients. Understanding the mechanisms of resistance to proteasome inhibition will not only allow better use of PIs, but may also facilitate rational design of more effective synergistic drug combinations for clinical trials to reduce the BMPCs responsible for alloantibody production. Alternatively, the present study suggests that serial treatment with constitutive followed by immunoproteasome-specific inhibitors may provide an effective alternative to concomitant/ simultaneous therapy. While elimination of autoreactive PCs by c20S PIs represents a viable strategy for Ab-mediated diseases, our study reveals that targeting i20S proteasomes may further enhance PC depletion and abrogate HLA Ab production.

Our studies suggest that the development of effective long-term desensitization treatments will require genomic and functional studies to uncover mechanisms of chemoresistance. As shown here, bulk RNA-transcriptomic analyses revealed alterations in the UPS that occur during the initiation and progression of HLA-sensitization and/or upon carfilzomib therapy, and this knowledge can now be exploited for molecular diagnostics and the development of novel desensitization therapies. In so doing, we highlight the value of clinical trials that integrate genomic analyses to guide the use of currently available drugs and enable development of new targeted therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS:

DSA	Donor-specific antibodies

BMPCs	bone marrow plasma cells
Abs	antibodies
PIs	proteasome inhibitors
MM	multiple myeloma
ESRD	end stage renal disease
HLA	human leukocyte antigen
AMR	antibody-mediated rejection
IVIg	intravenous immunoglobulin
FDA	Federal Drug Administration
IRB	Institutional Review Board
NCT	National Clinical Trial
KEGG	Kyoto Encyclopedia of Genes and Genomes
IPA	Ingenuity Canonical Pathway Analysis
UPS	ubiquitin-proteasome system
SAB	single antigen bead

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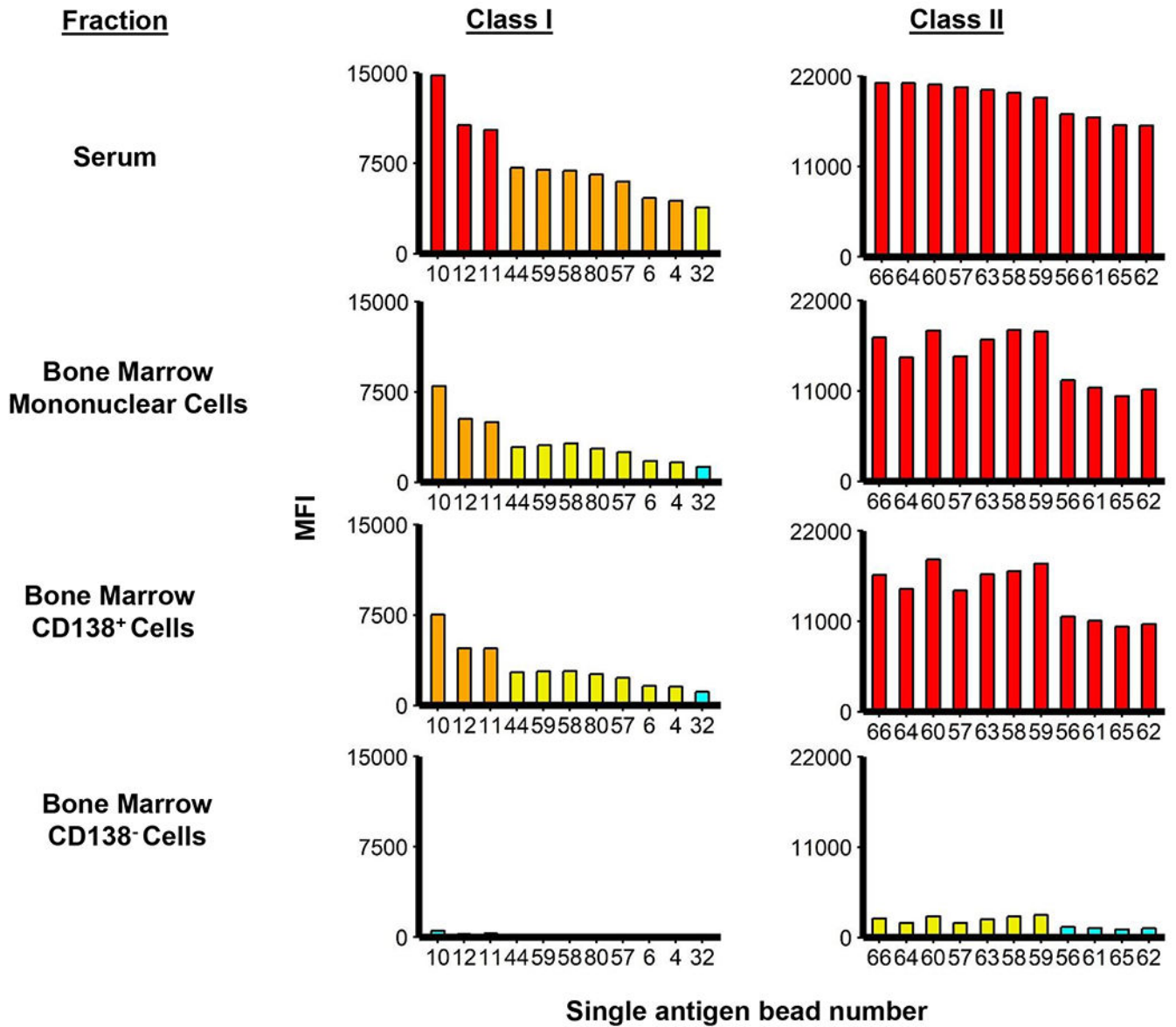
Figure 1.
CD138^{+} BMPCs from HLA-sensitized patients produce class I and II Abs at levels comparable to those detected in patient serum. Class I and II Ab levels were detected, identified and titered in serum from HLA-sensitized patients and culture supernatants from BM cell fractions using the single antigen bead (SAB) assay (LABScreen®; One Lambda, Canoga Park, CA) assay on a Luminex platform (Austin, TX). BM mononuclear cells (20,000/assay), CD138^{+} (100,000/assay) and CD138^{−} PCs (100,000/assay) from that same patient were incubated in 100 uL BMSC conditioned media supplemented with APRIL and IL-6 at 37°C for 72 h. Serum and cell culture supernatants were removed and 20 uL was used for SAB assays (48, 49). A representative histogram of the Luminex™ SAB results to determine the levels of class I and II HLA in patient serum and cell fractions is shown.

Similar results were observed using serum and cells from two additional HLA-sensitized patients (see Supp. Figure 5A, B).
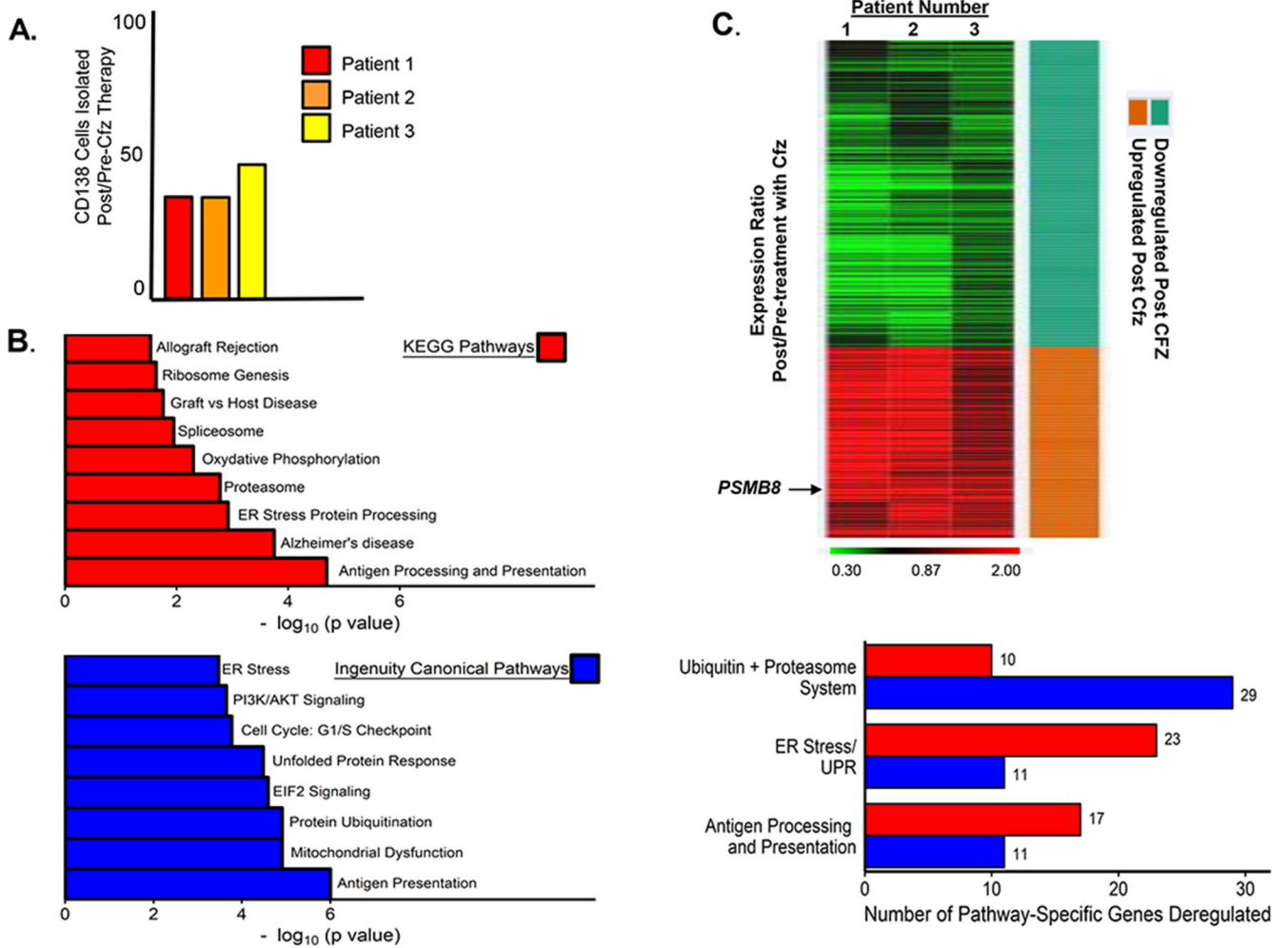
Figure 2.
Effect of *in vivo* carfilzomib therapy on gene expression in patient BMPCs. **A.** Proportion of live CD138+ cells recovered as a function of total bone marrow nucleated cells prior to and after *in vivo* carfilzomib therapy. Shown is the relative percent of live PCs recovered from the BM biopsies of three individual patients. Cell viability was determined using the trypan blue exclusion assay. Live PCs and the total number of nucleated BMMF cells isolated from individual patient BM biopsy were determined prior to and after *in vivo* carfilzomib therapy. Shown is the ratio of live CD138+PCs recovered from each patient post/pre-carfilzomib treatment. **B.** Unsupervised and supervised pathway analyses were performed to compare the genes differentially expressed in CD138+ PCs isolated from patients pre- and post *in vivo* carfilzomib therapy. KEGG and Ingenuity canonical pathway analyses were performed to identify the most significantly deregulated genes and pathways between BMPCs isolated pre- and post-carfilzomib therapy. Shown are the number of genes determined by either KEGG or Ingenuity canonical pathway analyses to be significantly deregulated in patient BMPCs following *in vivo* carfilzomib therapy. KEGG analysis generated an in-depth understanding of the differentially-expressed genes at the metabolic and functional levels, gene ontology functional annotation and KEGG pathway enrichment analysis were

performed using DAVID (**D**atabase for **A**nnotation, **V**isualization and **I**ntegration **D**iscovery) http://david.abcc.ncifcrf.gov/. Canonical pathway analysis was performed using the Ingenuity Pathway Analysis tool (IPA, Ingenuity® Systems, www.ingenuity.com) employed using the DEGs. A p-value cutoff of 0.05 was considered statistically significant (Fisher's exact test). **C.** Heat map depicting the genes down- or upregulated in each of the three HLA-sensitized patients following *in vivo* carfilzomib therapy. BMPCs were isolated from patients prior to and after *in vivo* carfilzomib therapy and then analyzed by RNA-seq. The heat map indicates genes that demonstrated a fold change ([Post]/[Pre]) greater than 1.5 in all three patients. Green indicates genes down regulated and red indicates those upregulated. A p-value cutoff of 0.05 was considered statistically significant (Fisher's exact test).
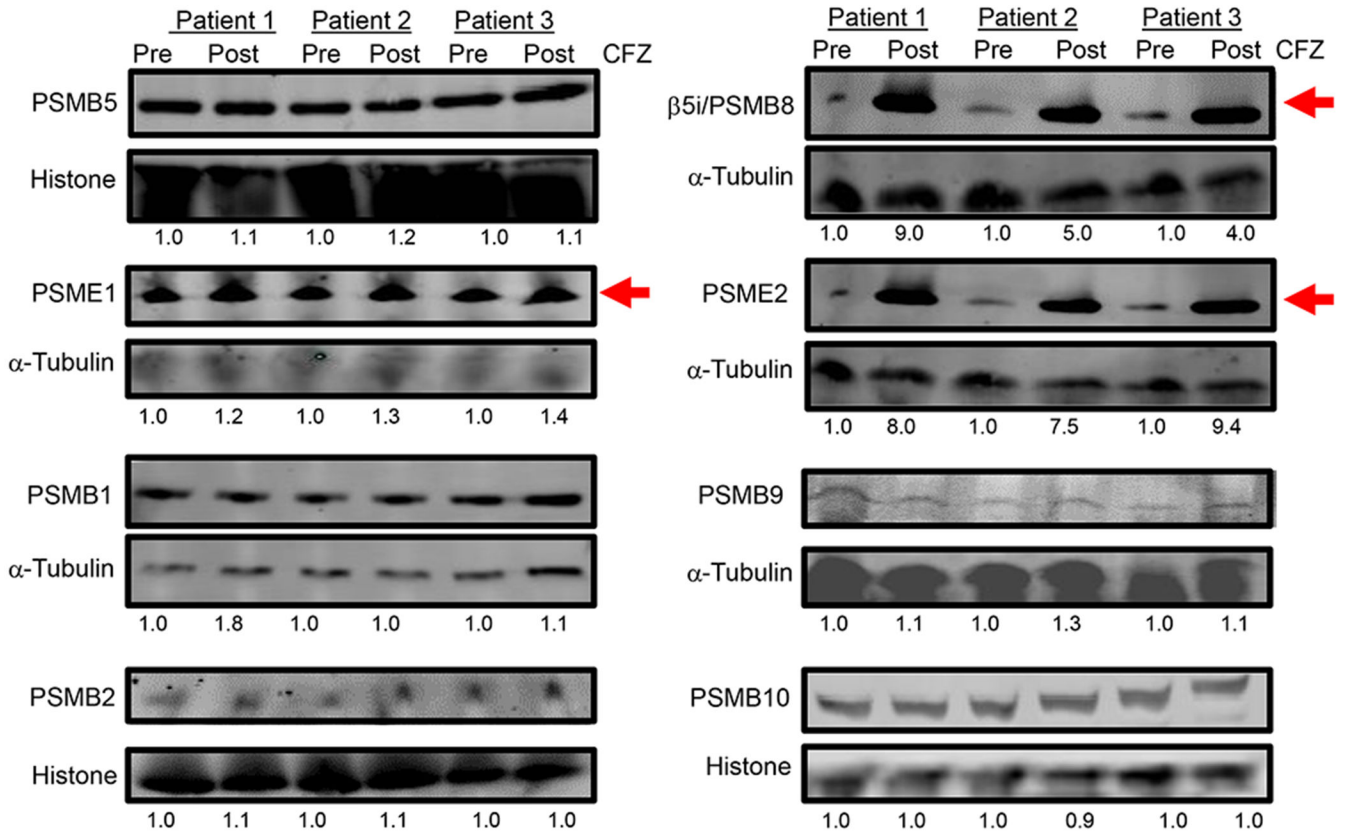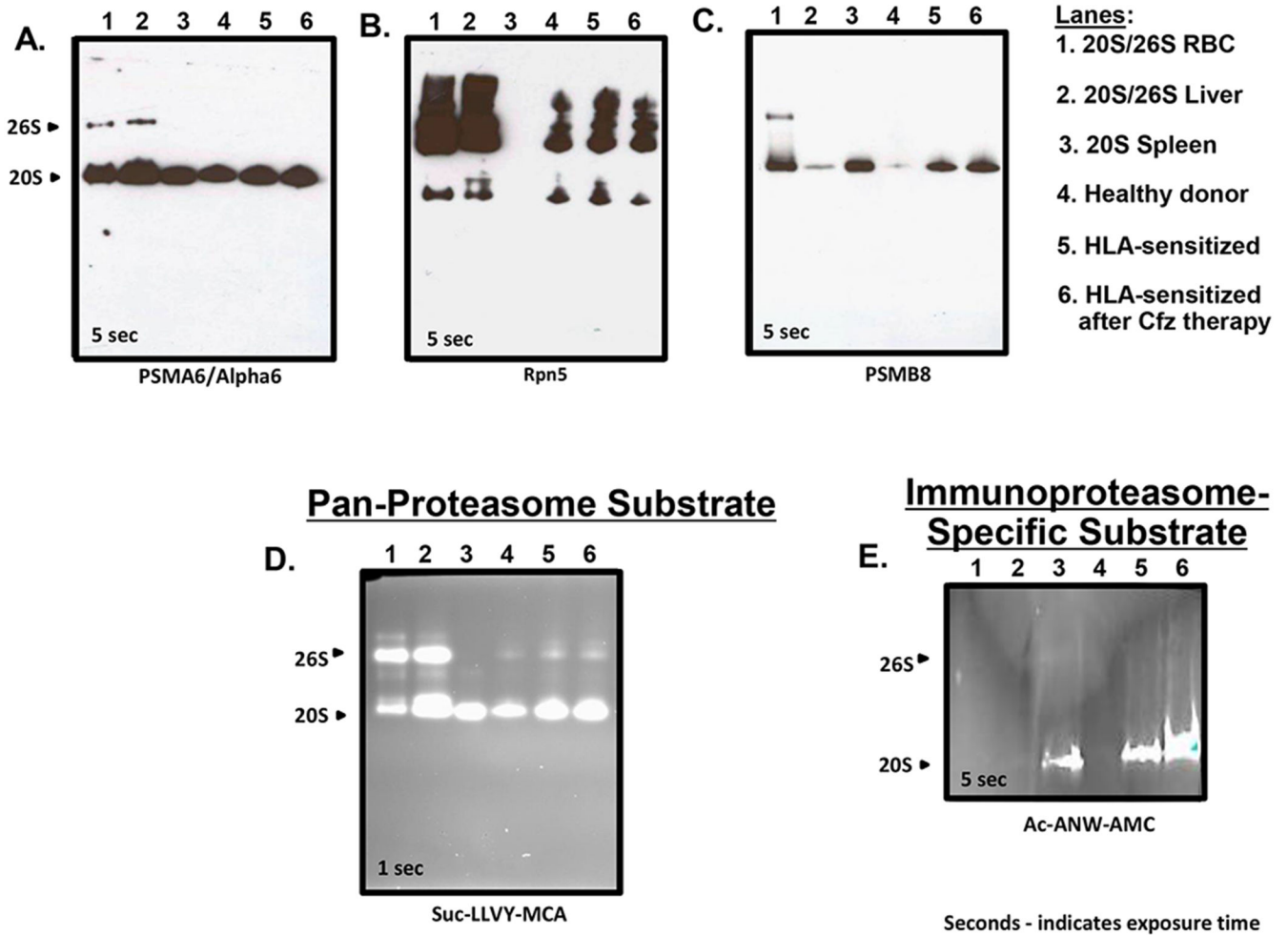
Figure 3.
Effect of *in vivo* carfilzomib therapy on proteasomal catalytic and activator subunits in patient BMPCs. Western blot of c20S proteasome and i20S proteasome catalytic subunits and the PA28 activator subunits (PSME1 and PSME2) in BMPC lysates from HLA-sensitized patients isolated before or after *in vivo* carfilzomib therapy. Ratio represents the relative intensity of a band in lysates isolated after carfilzomib therapy relative to the loading control. Results shown are from three individual patients.

Figure 4.
Native gel electrophoresis to detect structural and functional changes within proteasomes isolated from patient BMPCs. A. Samples were run on 3-8% Bis-Tris protein gels (Invitrogen) for 45 V at 4°C overnight and subjected to western blotting using alpha6 (clone MCP20). Western blot using a proteasome α6 subunit-specific Ab against proteasomes isolated from human erythrocytes, liver or spleen (lanes 1-3). Samples from the PCs of a healthy adult (lane 4) or BMPCs from a single HLA-sensitized patient prior to and after *in vivo* carfilzomib therapy (lanes 5 and 6). Samples (5ug each) were loaded onto native gels and electrophoresed as described above. **B.** Western blot using a proteasome Rpn5 subunit-specific Ab with the same samples electrophoresed as above. **C.** Western blot using a proteasome β5i (PSMB8) subunit-specific Ab with the same samples electrophoresed as above. **D.** Activity overlay assay using the pan-proteasome fluorogenic substrate Suc-LLVY-MCA. **E.** Activity overlay assay using the i20S proteasome substrate Ac-ANW-AMC. Shown are representative western blots and overlay assays observed using lysates from a single patients. Similar results were observed using BMPCs isolated from two additional HLA-sensitized patient (Supp. Fig. 8).
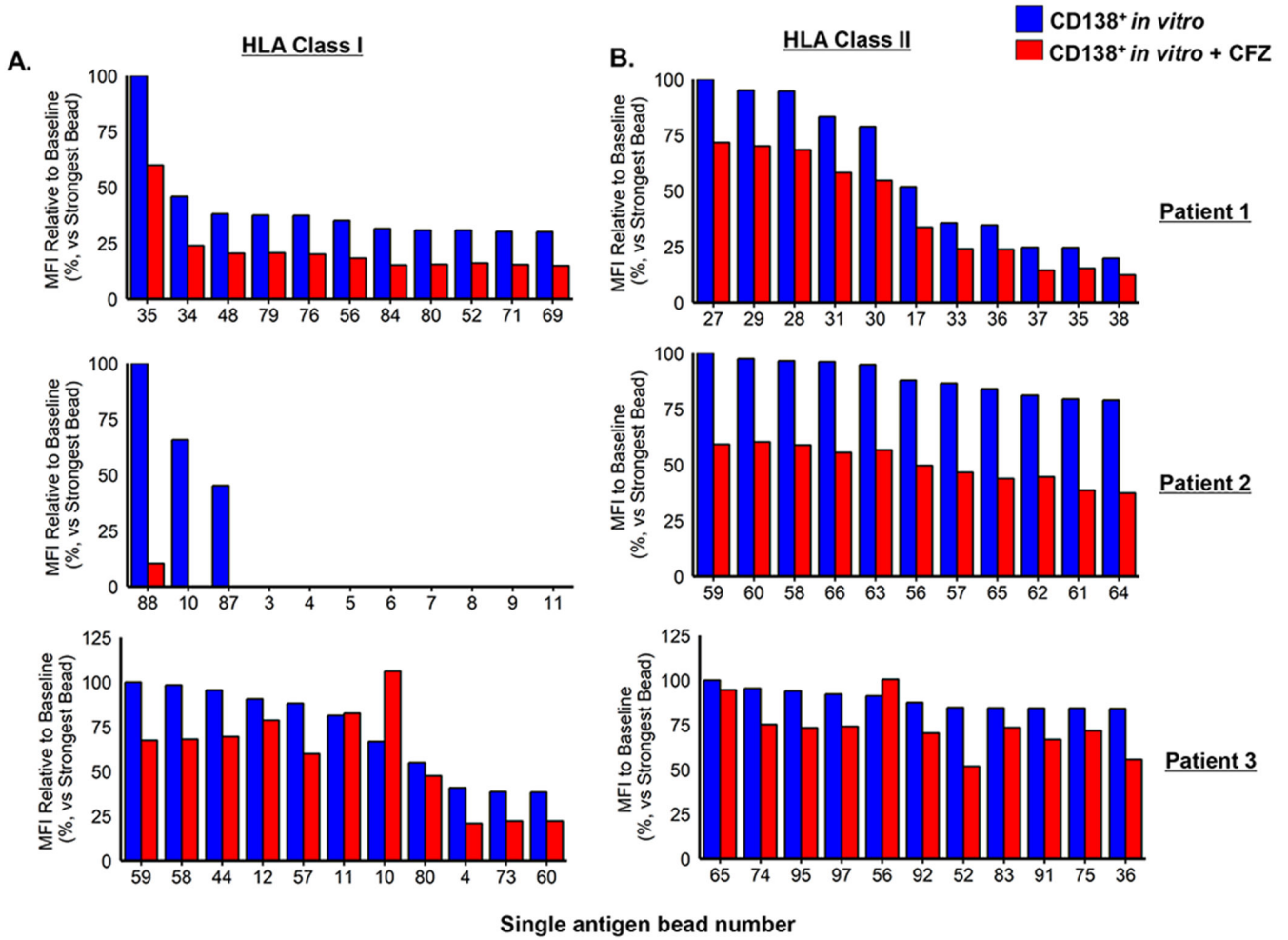
Figure 5.
Effect of carfilzomib treatment *in vitro* on the production of class I (**A**.) and II (**B**.) Abs by BMPCs from HLA-sensitized patients. Patient BMPCs (100,000 cells) were co-cultured in 100uL BMSC conditioned media for 72 h. Cell suspensions were centrifuged for 3 min at 3,000 rpm, culture supernatants removed and individual class I and II Ab levels measured using the SAB assay. Culture media was incubated alone as a negative control. Serum isolated from the whole blood of HLA-sensitized patients was used to measure the levels of individual class I and II Abs.
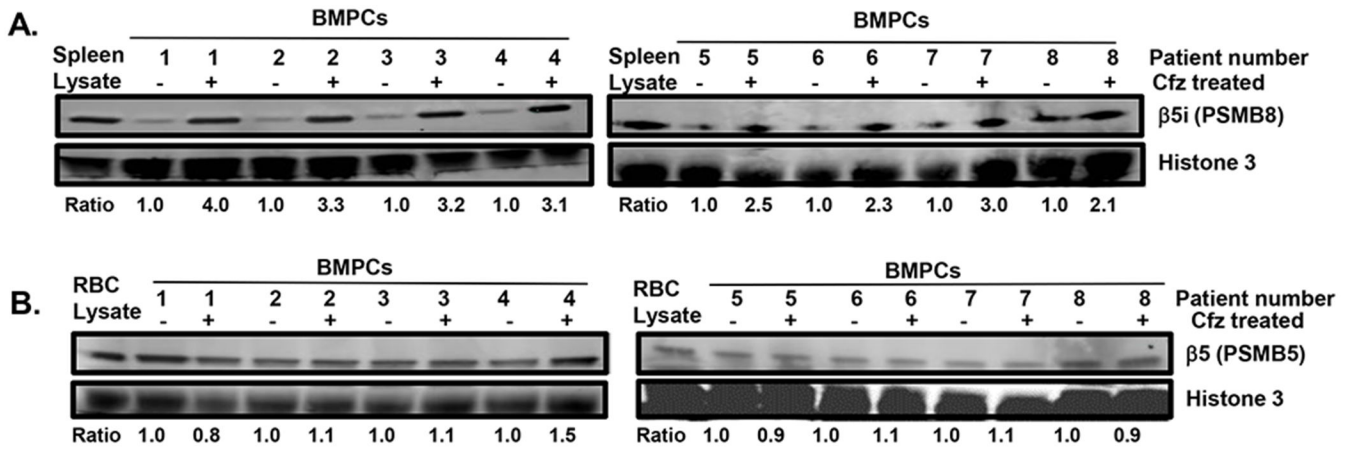
Figure 6.
Effect of *in vitro* carfilzomib treatment on β5i levels in BMPCs. Patient BMPCs (100,000 cells) were co-cultured with BMSC conditioned media for 72 h in the presence or absence of carfilzomib for 72 h. Cells were removed, pelleted and lysates prepared in 50 uL celLytic non-denaturing buffer. Histone 3 was used as a loading control. Lysates were separated on 4-12% bis-tris gels, transferred to PVDF membranes and probed using Abs to proteasome subunit β5i (PSMB8) (**A**) and β5 (PSMB5) (**B**). Erythrocytes (RBCs) contain constitutive 20S proteasomes and do not contain i20S proteasomes. RBC lysates was used as positive control to detect constitutive 20S proteasomes. Ratio indicates the relative intensity of a given proteasome subunit in lysates of that individual patient prior to and after carfilzomib treatment (N=8).
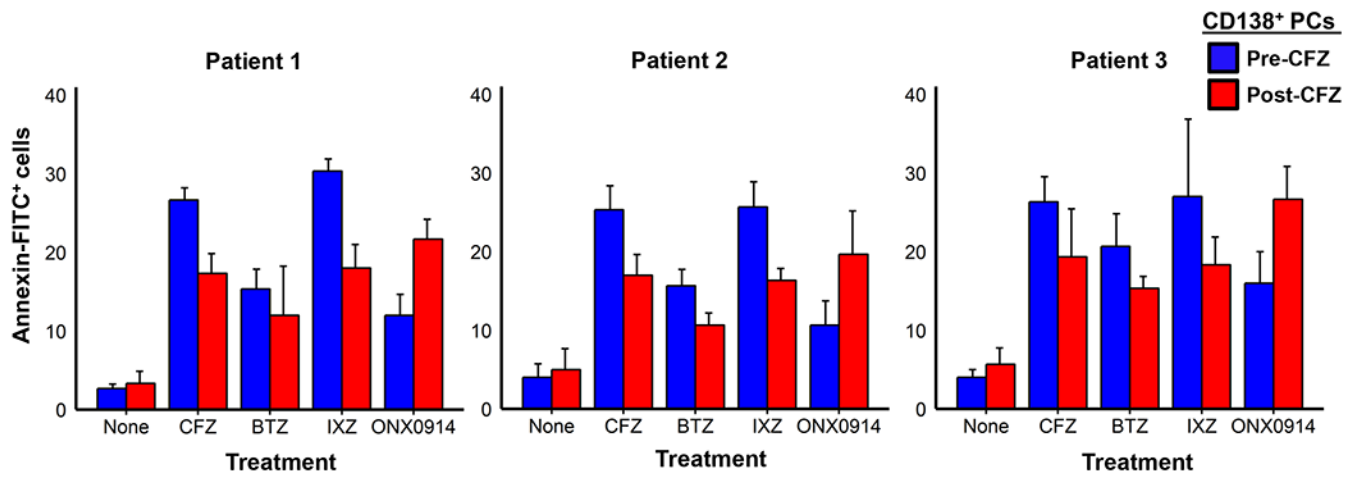
Figure 7.
Effect of different PIs on BMPCs isolated before and after *in vivo* carfilzomib therapy. Cells were incubated with the PIs bortezomib, carfilzomib or ixazomib (10 nM) or ONX-0914 (100 nM) for 16h and then stained to detect apoptotic cells using the annexin V-FITC apoptosis detection kit. Shown are representative results from three HLA-sensitized patients.