Differential Effect of Bortezomib on HLA Class I and Class II Antibody

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Background. Bortezomib has been used to reduce HLA antibody in patients either before transplantation or as treatment for antibody-mediated rejection (AMR). Reports on its efficacy show mixed results. The mechanism of action of this agent is via proteasome inhibition. The primary route of synthesis of HLA class I molecules is dependent on peptide generation by the proteasome, whereas that of class II is not. We observed a differential effect of bortezomib on class I versus class II antibody and hypothesized that this was related to a reduced expression of class I HLA antigens.
Methods. The effect of bortezomib on HLA antibody levels was evaluated in 13 patients who were desensitized for incompatible renal transplantation. We calculated the percent difference in HLA antibody level before and after bortezomib treatment and the impact of bortezomib on HLA expression in lymphocytes of healthy control subjects.
Results. On average, the level of HLA class I donor-specific antibody (DSA) decreased by 32%, whereas that of class II DSA increased by 29%. In vitro bortezomib treatment of lymphocytes resulted in a mean decrease of 23% in MHC class I expression on B lymphocytes and no change (+1.08%) in MHC class II expression (P=0.0003). The amount of intracellular class I molecules was reduced by a mean of 29% with bortezomib.
Conclusion. These data indicate that bortezomib reduces HLA class I antibody more effectively than class II antibody. This difference may be due to the reduced expression of class I molecules resulting from treatment with this proteasome inhibitor.

Keywords: Bortezomib, HLA antibody, Renal transplantation, Proteasome inhibition, MHC molecules.

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Sensitization to HLA antigens is a barrier to both access to transplantation and to long-term graft survival (*1*). Approximately 30% of patients awaiting kidney transplantation have a PRA greater than 20%; 7% to 9% of those have a PRA greater than 80% and are the most difficult cases to transplant (*2*). Treatment protocols have been developed to eliminate or reduce circulating HLA antibody and allow transplantation to occur successfully. Most protocols involve reduction of donor-HLA specific antibody (DSA) via removal of the antibody by plasmapheresis or downregulation of antibody production with IVIg. These treatments are often augmented by depletion of B cells with a chimeric anti-CD20 antibody. However, these treatments do not remove the antibody-producing plasma cells (*3, 4*).

Bortezomib is a proteasome inhibitor that induces apoptosis of plasma cells and was used initially for treatment of multiple myeloma (*5*). More recently, bortezomib has been used in attempts to reduce HLA antibodies either before transplantation or as treatment for antibody-mediated rejection (AMR). Single-center reports on the use of bortezomib for either condition show mixed outcomes (*6*). In some cases, a significant reduction in HLA antibody was reported with improvement in graft function (*7–9*). In these earlier reports, HLA antibody levels were evaluated at the end of combined therapies that included bortezomib and other antibody-depleting treatments. Therefore, the change in antibody level observed could have been due to other treatments. Flechner et al. reported a mixed outcome in a cohort of 20 patients, with a variable decrease in HLA antibody that did not translate to improved renal function (*10*). Some other reports indicated no change in antibody strength (*11–13*), whereas two reports noted a greater change in HLA class I versus HLA class II DSA (*14, 15*). Perry et al. showed that bortezomib effectively depletes the bone marrow–derived plasma cells producing

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HLA-specific antibodies (*4*). These authors also showed that treatment with bortezomib completely blocked anti-HLA class I and class II antibody production in the supernatant of the cultured, bone marrow–derived plasma cells (*16*).

One possible explanation for the differences in reported outcomes may be related to the effect of this proteasome inhibitor on HLA antigen expression. The proteasome is important in the generation of the HLA class I-peptide complex (*17*). Biosynthesis and stable expression of HLA molecules require binding of peptides. Class I molecules are synthesized in the endoplasmic reticulum where they bind peptides that have been generated by proteasome digestion of intracellular proteins. In contrast, HLA class II molecules acquire peptides generated by proteolytic degradation of proteins in the endocytic pathway. Although bortezomib may eliminate plasma cells, it has a short half-life (*18*). In multiple myeloma studies, the mean inhibition of proteasome activity changed from greater than 90% after 5 min of bortezomib administration to 22% to 48% within 48 hr and 10% to 35% after 10 days posttreatment (*19*). Continued expression of mismatched donor HLA antigens may contribute to the activation of B cells and their transformation into plasma cells, particularly during AMR. Thus, a difference in the level of expression of class I versus class II molecules may contribute to a difference in the production of antibodies to HLA class I and HLA class II. We have examined the effect of bortezomib treatment on the level of HLA antibodies and on the expression of HLA antigens and present our results here.

RESULTS

Effect of Bortezomib Therapy on Levels of HLA Antibodies

Demographic data for the 13 patients evaluated in this study are given in Table 1. All 13 patients were refractory to plasmapheresis and IVIg treatments; therefore, bortezomib was later added to the treatment regimen. The 13 patients had antibodies to donor and nondonor HLA class I and class II antigens at the time of bortezomib treatment. Seven patients were previously transplanted ,and six were receiving their first transplant. AMR occurred in the presence of DSA and was confirmed by biopsy. Before the beginning of bortezomib treatment, the average MFI values for DSA were 7811 (1544–22,374) and 9855 (1065–24,372) for class I and class II antibodies, respectively. The average MFI values for non-DSA were 11,768 (1093–19,999) for HLA class I and 10,105 (2290–18,506) for class II.

The DSA courses for each of the 13 patients are shown in Figure 1. On average, the level of all HLA class I antibodies (DSA and non-DSA) decreased by 31% (±33%), but that of HLA class II antibodies increased by 23% (±57%). We observed a mean decrease of 32% (±33%) in HLA-DSA class I, whereas HLA-DSA class II increased by 29% (±65%) at the end of treatment (P=0.00105). Notably, all but 2 class I DSAs decreased, whereas 10 of 13 class II DSAs increased. DSA class I antibodies were not decreased effectively for two patients JH-6 and JH-7 (Fig. 1A). Three of 16 HLA class II antibodies specific for DR 7, DR13, and DR4 were only slightly decreased, whereas the remaining 13 HLA class II DSAs were increased at the end of treatment (Fig. 1B). The level of third party (non-DSA) antibodies decreased by 28% (±34%) for HLA class I and increased by 14% (±39%) for HLA class II (P=0.004) (Fig. 1C and D).

Ten of the 13 patients in this cohort received between two and four cycles of bortezomib. There was no additional change in the level of HLA class II antibody among the 10 patients that were treated with more than one cycle of bortezomib. Figure 2 shows the course of DSA and non-DSA of patient JH-4 during treatment. This patient's class I DSA (A24, A25, B18, B44, and Cw5) and class I non-DSA (A66)

TABLE 1. Patient demographics

Patient ID#	Sex	Race	No. Trplts[a]	Current donor type	Time from transplant to beginning of bortezomib treatment (months)[b]	No. of bortezomib cycles	Additional treatments[c]	Follow-up time[d] (mo)	Graft loss	Calculated PRA[e]
JH-1	M	B	2	DD	3	1	Eciluzumab	1	No	100%
JH-2	M	W	2	LUR	4	2		2	No	100%
JH-3	M	W	3	DD	4	4	Rituximab	9	No	41%
JH-4	M	B	1	DD	6	2		2	Yes	96%
JH-5	M	B	2	DD	8	2	Splenectomy	3	No	64%
JH-6	M	B	1	DD	24	2		2	No	37%
JH-7	M	W	1	DD	36	2	Rituximab	2	Yes	71%
JH-8	F	B	2	DD	36	1		1	No	90%
JH-9	M	B	1	DD	38	1		2	No	98%
JH-10	F	W	2	LR	84	2		2	No	94%
JH-11	F	W	3	DD	84	2		3	No	88%
JH-12	F	W	2	LUR	pre transplant + post (1)	4 pre+4 post	Splenectomy	3	No	100%
JH-13	F	B	1	LUR	pre transplant only	4		1	No	88%

[a] No. trplts: number of transplants includes current transplant.
[b] Time from transplant to beginning of bortezomib treatment: includes date of most current transplant to the date of the first bortezomib treatment.
[c] Agents or treatments added in addition to the standard protocol, which includes thymoglobulin, MMF, PP/IVIg and steroids.
[d] Time from bortezomib administration to end of HLA antibody evaluation—Patients JH-4 lost graft 3 months after this follow-up period, and Patient JH-7 lost graft approximately 5 months after this follow-up time.
[e] These are HLA antibodies that are strong enough to yield a positive flow cytometric crossmatch.

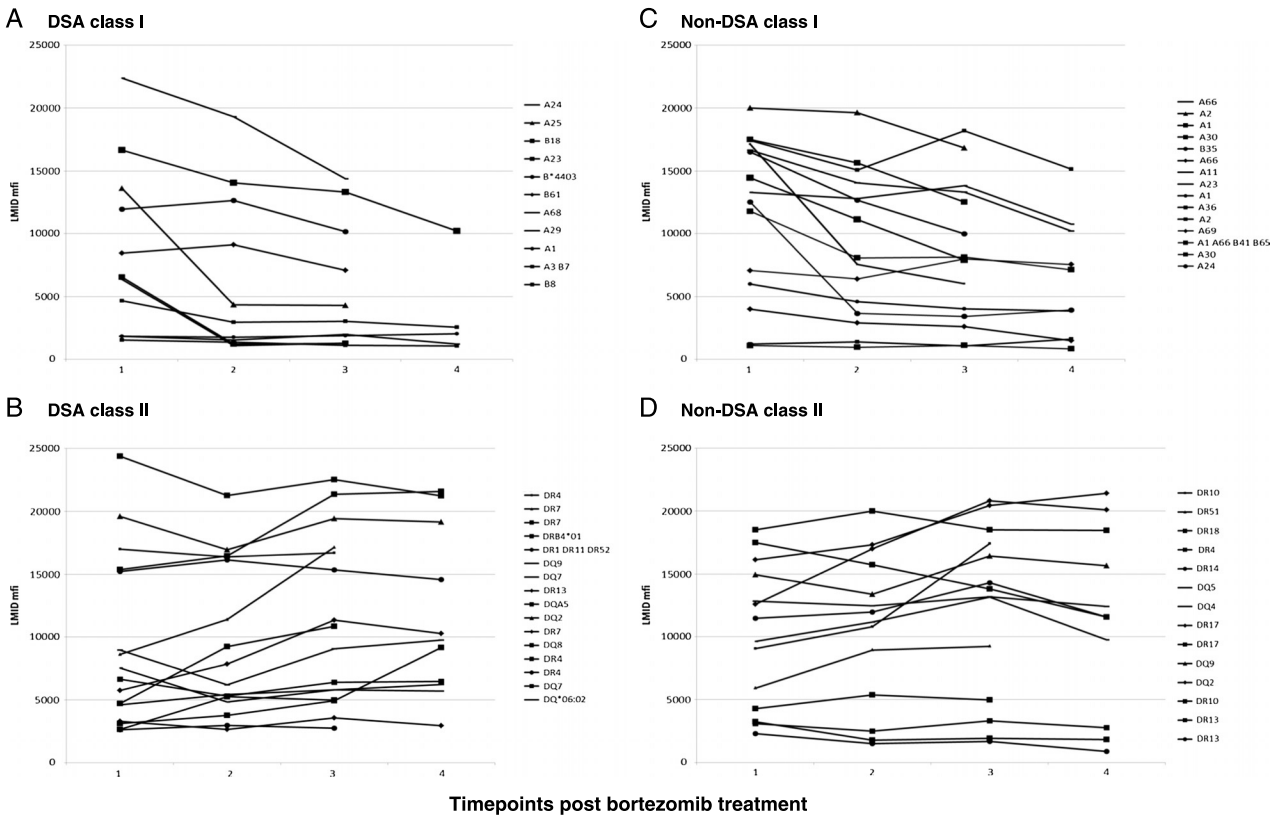

FIGURE 1. HLA antibody levels for 13 patients evaluated. The Y-axis indicates MFI values on the phenotype panels (LMID). The X-axis represents the time points post bortezomib treatment and include a baseline evaluation between 2 and 4 weeks before the beginning of bortezomib treatment, and time points approximately 1, 3, and 6 weeks posttreatment for each patient. A and C, The reduction of class I DSA (A) and class II non-DSA (C) following treatment with bortezomib for most study subjects. B and D, No reduction or increase in class II DSA (B) and class II non-DSA (D) during treatment.

were decreased by 74%. HLA class II DSA (DR4 and DR7) and class II non-DSA (DR10) remained unchanged after two cycles of bortezomib.

Early acute rejection episodes are reported to be more amenable to treatment; whereas late acute rejection, defined as occurring more than 3 months posttransplant, are more resistant to any type of antibody reduction therapy (*20–22*). We compared the HLA antibody reduction in groups treated for an AMR episode that occurred early after transplantation (n=3; average time from transplant=3.6±0.57 months) to those with AMR later after transplantation (n=10; average time from transplant=39±30 months). There was a 37.44% decrease in HLA class I and an 8% increase in HLA class II DSA in the early AMR group, whereas in the late AMR group, there was a 21% decrease in HLA class I DSA and a 29% increase in HLA class II DSA. Although we observed a greater decrease in DSA class I in the early AMR group, the data show that HLA class II antibody was not reduced by treatment with bortezomib even in this cohort that is considered more amenable to treatment. Unfortunately, the numbers are too small for a meaningful statistical analysis.

The Effect of Bortezomib on HLA Class I and Class II Expression

We treated lymphocytes from nine healthy donors with bortezomib and measured MHC class I and class II expression on treated and untreated lymphocytes. In bortezomib-treated cells, class I expression decreased 32% (±21%) and 23% (±13%) on T cells and B cells, respectively, whereas there was no change (+ 1.08%±4%) in class II expression on B cells (P=0.0003) (Fig. 3).

Effect of Bortezomib on HLA Biosynthesis

We performed intracellular staining of class I molecules to determine if the effect of bortezomib on class I expression was due to reduced biosynthesis of intact molecules or to interference with transport of the molecules to the cell surface. We compared the extent of intracellular staining of cells treated with BD Golgistop solution alone to that of cells treated with both BD Golgistop and bortezomib (Fig. 4). The results show a mean decrease of 29% (±12%) in intracellular MHC class I with lymphocytes treated with BD Golgistop and bortezomb compared with BD Golgistop alone, suggesting a reduction in the synthesis of stable, intact class I molecules.

DISCUSSION

Inhibition of the proteasome by bortezomib results in an increase in pro-apoptotic factors such as Bax and CHOP, which contribute to plasma cell apoptosis (*23*). The ability to eliminate these antibody-producing cells has made bortezomib an attractive drug for treatment of AMR in organ transplantation. In this current study, we report a difference in

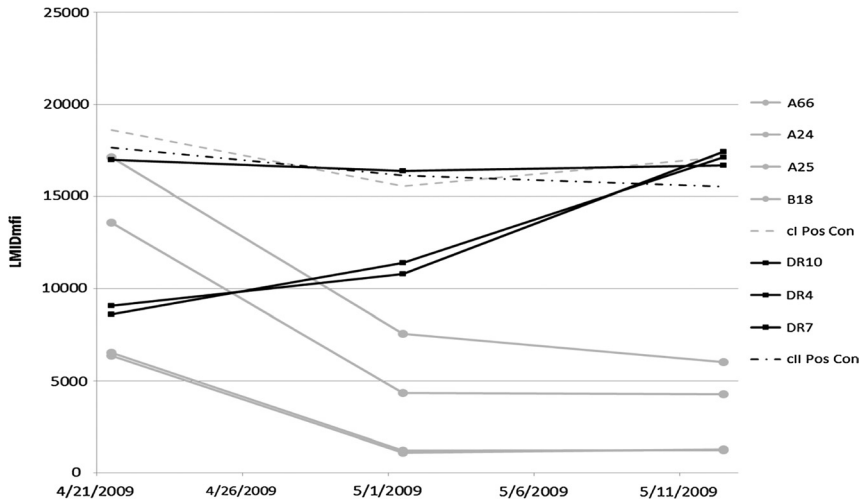

FIGURE 2. HLA antibody levels monitored in a selected study subject. Effect of bortezomib treatment on class I (gray) versus class II (black) HLA donor-specific and non–donor-specific antibodies for a selected study subject (JH-4). Patient JH-4 was treated with two cycles of bortezomib. The first cycle was administered between April 26, 2009, and May 1, 2009. The second cycle was between May 6, 2009, and May 11, 2009. HLA class I DSA (A24, A25, B18, B44, and Cw5) and class I non-DSA (A66) were decreased by 74% at the end of the second cycle, whereas HLA class II DSA (DR4 and DR7) and class II non-DSA (DR10) remained unchanged. The positive controls (dashed line) are plotted to control for run-to-run variation in the assay.

the effect of bortezomib on HLA class I and class II antibodies. Treatment with bortezomib resulted in a decrease in the levels of HLA class I antibodies but no change or an increase in HLA class II antibodies. This differential effect of bortezomib on HLA class I versus class II antibody has also been reported in two previous studies (*14, 15*).

When we divided our study group into early AMR and late AMR, bortezomib treatment did not reduce the level of HLA class II antibodies in either group, suggesting that the timing of treatment did not improve the drug's ability to affect class II antibodies. Repeated doses of the drug also failed to reduce HLA class II antibodies. Finally, we saw no difference in the elimination of strong versus weak antibodies. In all instances, bortezomib decreased HLA class I antibodies more effectively than HLA class II antibodies. We hypothesized that this differential effect on class I versus class II antibody was not due to plasma cell apoptosis only but rather was secondary to a reduction in the level of expression of class I but not class II antigen resulting from proteasome inhibition.

Bortezomib inhibits the proteasome that generates the peptides that are bound to newly synthesized class I molecules

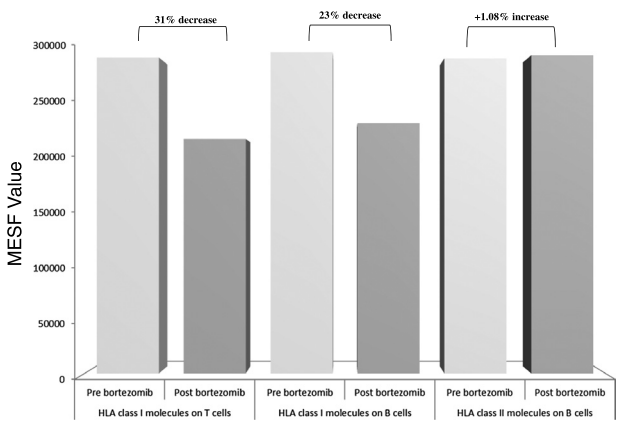

FIGURE 3. Cell surface staining of MHC molecules. Cells treated with bortezomib were stained with MHC class I and MHC class II-FITC labeled and compared with untreated cells. The mean change of a total of nine cells for MHC class I and MHC class II on T cells and on B cells was determined.

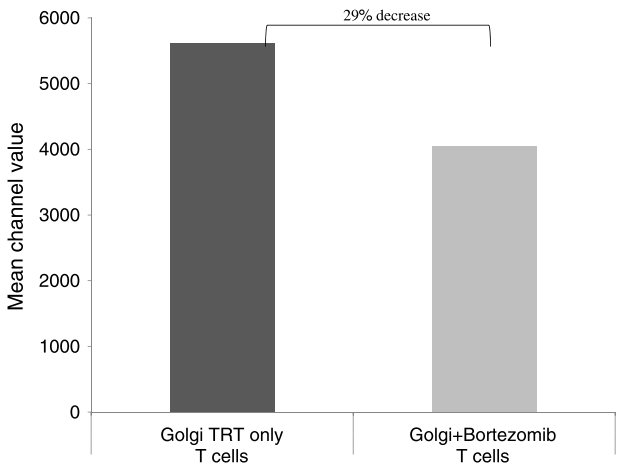

FIGURE 4. Intracellular staining of MHC class I molecule. The amount of intracellular MHC class I molecules were compared in cells treated with and without bortezomib. The mean change of four cells tested is represented. Cells from normal individuals were treated with BD GolgiStop solution with and without bortezomib. Cells were then fixed and permeabilized then stained with a PE labeled monoclonal antibody against HLA-ABC to visualize internalized MHC class I. Stained cells were analyzed on the Becton-Dickinson FACSAria.

and are necessary for their stable expression. In contrast, the peptide bound to class II molecules is not generated by the proteasome but is generated by proteolytic digestion of extracellular molecules and acquired in the endocytic pathway. In the absence of a stably bound peptide, the class I molecule is retained in the ER and is eventually degraded (*17, 24*). We have shown that in vitro treatment of cells with bortezomib reduces cell surface expression of HLA class I but not HLA class II molecules. We also demonstrated that borezomib treated cells did not show an accumulation of intracellular class I molecules when their exit from the Golgi was inhibited, whereas in the absence of bortezomib, these molecules did accumulate in the cell. This is comparable to the effect of viruses that interfere with peptide generation by the proteasome (*25, 26*). It is important to note that peptide generation to MHC class I can also occur via other routes including protein degradation by various proteases (*27*). Therefore, the limited reduction in surface MHC class I (32%) and intracellular MHC class I (29%) may be explained by the fact that the proteasome is not the only route of peptide acquisition by MHC class I. Alternatively, it might result from incomplete or temporary inhibition of the proteasome.

We have shown, previously, that desensitization with plasmapheresis and IVIg results in a greater reduction or elimination of class I DSA than of class II DSA, yet there was still a substantial reduction of class II DSA (*28, 29*). We propose that there is a secondary effect that results from the impact of bortezomib on HLA antigen expression. During AMR, expression of donor HLA might sustain activation of memory B cells and also provoke generation of new, donor-specific B cells. Then, inhibition of class I but not class II HLA antigen expression is likely to result in a differential activation of B cells resulting in a difference in the levels of class I versus class II antibody. Bortezomib is known to eliminate antibody-producing plasma cells, but it is short lived in the circulation (*18*). Therefore, increases in the levels of HLA class II antibody are likely the result of the generation of new plasma cells from activation of B cells. The sustained expression of HLA class II in the allogeneic graft during bortezomib treatment could result in ongoing activation of B cells specific for class II, whereas this would not occur as effectively for class I-specific B cells because of the reduced expression of class I.

In many of the previous studies, evaluation of the effect of bortezomib alone was hindered by simultaneous treatment with other agents. Our analysis differs from these studies as we assessed HLA antibodies after bortezomib was administered but before other treatments were delivered. In our study, we were careful to analyze antibody levels almost immediately after a dose of bortezomib (1–3 days posttreatment) and before the next treatment with plasmapheresis and IVIg. The different results reported by others could be due to examining the collective effects of multiple therapies.

Our study is limited by the small size of the cohort as only 13 patients were evaluated. Larger studies and/ or clinical trials are needed to define the patient populations, time points, antibody specificities, and bortezomib combination therapies that may provide more effective reduction of HLA antibodies. Despite the small study group size, our data clearly indicate a greater efficacy of bortezomib on class I antibodies; thus, we propose that the most effective use of bortezomib may be in patients whose DSA is specific, predominantly, for donor class I antigens.

MATERIALS AND METHODS

The study is a retrospective analysis of an IRB-approved database and treatment protocol.

Study Group

Between 2009 and 2011, thirteen patients who had undergone desensitization for HLA incompatible, living donor renal transplantation, received bortezomib, either pretransplant as part of the desensitization (n=2) to treat an episode of AMR (n=11) or both (n=1). The desensitization protocol consisted of alternate day single-volume plasmapheresis, low-dose (100 mg/kg body weight) hyperimmune anticytomegalovirus IgG (CMVIg), and immunosuppression consisting of tacrolimus, mycophenolate mofetil, steroids, and the following induction agents: thymoglobulin (n=10), basiliximab (n=1), rituximab and thymoglobulin (n=1), and rituximab (n=1). These patients were refractory to plasmapheresis and IVIg treatments, and bortezomib was later included to treat AMR. Patients received between one and four cycles of bortezomib. The number of cycles of bortezomib was based on a constellation of clinical factors including: response to therapy, toxicities, and general prognosis for the viability of the allograft. Patient JH-12 was treated with four cycles of bortezomib before transplant and four cycles post-transplant. Each cycle of bortezomib consisted of four doses of 1.3 mg/m^2 each given on days 1, 4, 8, and 11. Additional treatment for AMR included rituximab (n=2), eculizumab (n=1), and splenectomy (n=2). These additional agents were administered between 1 and 4 months before or after treatment with bortezomib.

Detection and Characterization of HLA Antibody

HLA antibody testing was performed on sera obtained before each dosage of bortezomib and 1 to 3 days following the end of bortezomib treatment. For patients who received more than one treatment, the overall change in antibody level was determined after the last cycle. Samples were treated to remove interfering factors (*30*). Sera were tested with multiplexed bead assays performed on the Luminex platform using phenotype (Immucor, Lifecodes, Stamford, CT) and single antigen (One Lambda Inc., Canoga Park, CA) panels. Characterization of HLA-A, B, Cw, DRB1, DRB3-5, DQA, DQB, and DPB antibodies was done by manual analysis performed by two or more individuals with expertise, working independently. HLA antibody was assessed with the phenotype panel unless a patient was broadly sensitized and the change in antibody strength could not be evaluated. In those cases, the DSA was determined from tests on a single antigen panel (n=2 patients). Importantly, the changes of HLA class I and class II antibodies were the same for both the phenotype and single antigen panels.

In Vitro Analysis of MHC Expression

In Vitro Bortezomib Treatment

Lyophilized bortezomib was rehydrated according to the manufacturer's instructions to a final concentration of 1.0 mg/mL (1 ng/μL). The concentration of drug comparable to patient dosing was calculated to be 0.38 μL per 1 mL of whole blood. Bortezomib treated and untreated aliquots of blood were incubated for 2 hr at 37°C.

Cell Surface Staining of MHC Molecules

Lymphocytes were isolated from both bortezomib treated and untreated blood. Lymphocyte subsets were identified by staining with monoclonal antibodies to CD3 and CD19 (BD Biosciences, San Jose, CA), and surface expression was assessed by staining for MHC class I (FITC mouse antihuman HLA-ABC; BD Biosciences, San Jose, CA) and MHC class II (FITC mouse antihuman HLA DR, DP, DQ; BD Biosciences). FITC mouse antihuman IgG mouse antihuman IgG were used as isotype controls (BD Biosciences). After incubation with the monoclonal antibodies, cells were washed three times using a 1% BSA enriched saline wash buffer, fixed, and analyzed on the Becton-Dickinson FACSCalibur.

Intracellular Staining of MHC Class I Molecules

Whole blood was treated with BD GolgiStop for 14 hr at 37°C, after which bortezomib was added to one half of the blood. After staining surface MHC molecules, lymphocytes were then incubated with 250 μL of BD fixation and permeabilization solution (BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit with BD GolgiPlug; BD Biosciences) for 20 min at 4°C and stained with a monoclonal antibody (PE mouse anti-human HLA-ABC;BD Biosciences) to visualize internalized MHC class I. Stained cells were analyzed using the Becton-Dickinson FACSAria. Class II staining was not performed because of lack of a suitable reagent for staining.

Statistical Calculations

Change in relative antibody levels were calculated as follows:

$$\text{Percent change} = [(\text{MFI Post Bortezomib} - \text{MFI Pre Bortezomib})/\text{MFI Pre Bortezomib}](100)$$

MFI values of test sera were normalized to account for run-to-run variability by dividing test sera values by the MFI value of the positive control.

Change in MHC expression was calculated as follows:

$$\text{Percent change} = [(\text{MCV Post treatment} - \text{MCV Pretreatment})/\text{MCV Pretreatment}](100)$$

Mean values, standard deviation, and paired *T* tests were used to compare differences; $P<0.05$ was considered statistically significant.

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