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A Phased Desensitization Protocol With Rituximab and Bortezomib for Highly Sensitized Kidney Transplant Candidates

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Background. Desensitization protocols comprising plasmapheresis, IVIGs, and rituximab and/or bortezomib have allowed for successful kidney transplantation in some highly HLA-sensitized patients with end-stage renal disease. However, the optimal combination of these therapies and their proper timing remains entirely unknown. We propose a phased desensitization strategy using rituximab followed by bortezomib as a safer method. **Methods.** Three sensitized kidney transplant candidates who could not be desensitized using our conventional protocol, which consists of a single rituximab dose combined with plasmapheresis, were enrolled in this study. When IgM^{+} $CD27^{-}$ naive B cells reappeared but IgM^{+} $CD27^{+}$ memory B cells remained undetectable in their peripheral blood, the patients were treated with 1 cycle of bortezomib followed by plasmapheresis. **Results.** After bortezomib treatment, patients' donor-specific anti-HLA antibodies (DSA) values were decreased, and cross-match tests were consistently negative. All 3 patients underwent living donor kidney transplantation. They showed immediate renal function, and both DSA and non-DSA were undetectable during the observation period. Neither antibody-mediated rejection nor severe acute cellular rejection was encountered in these patients after transplantation. **Conclusions.** The present cases suggest that a phased use of rituximab and bortezomib can safely desensitize highly sensitized kidney transplant candidates.

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Sensitization to HLAs is a significant obstacle to kidney transplantation and a risk factor for antibody-mediated rejection.[1] Recently developed desensitization protocols comprising plasmapheresis, IVIG, and rituximab and/or more novel agents including bortezomib can decrease antibody (Ab) levels against allogeneic HLAs in some highly HLA-sensitized patients with end-stage renal disease, resulting in successful kidney transplantation.[2-5] However, the optimal combination of such therapies and their proper timing remains entirely unknown.

A history of pregnancy, transfusion, or organ transplantation occasionally causes severe sensitization against HLA.[1] In such sensitized patients, both memory B cells responding to donor-specific HLA and plasma cells secreting anti-HLA Abs are targets for desensitization intended to persistently eliminate anti-HLA Abs. It is well known that shortly after treatment with rituximab, an anti-CD20 monoclonal Ab (mAb), a depletion of naive B cells in circulating blood is achieved.[6] At long-term follow-up, a reduction of $CD27^{+}$ memory B cells in the blood and bone marrow has also been observed.[7] This may inhibit the rapid renewal of precursors of anti-HLA Ab secreting cells. Although plasma cells, terminally differentiated $CD20^{-}$ B cells that secrete Abs, are resistant to rituximab, short-lived plasma cells likely exhaust their lifespans shortly after rituximab treatment.[8] In cases where short-lived plasma cells exclusively produce donor-specific HLA Abs (DSA), desensitization should be complete after rituximab treatment and sequential plasmapheresis. However, in cases where long-lived plasma cells are also responsible for DSA production, an additional therapy, such as bortezomib, a proteasome inhibitor with demonstrated apoptotic properties against plasma cells,[9] might be required to complete desensitization against allogeneic HLA. Because the simultaneous or sequential use of rituximab and bortezomib

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may cause hypogammaglobulinemia, administering both agents with a time lag may be safer. Hence, we propose a phased desensitization strategy using rituximab followed by bortezomib for highly sensitized kidney transplant candidates (Figure 1).

METHODS

Study Design and Desensitization Protocol

This study was conducted with informed consent using a protocol approved by the institutional review board of the

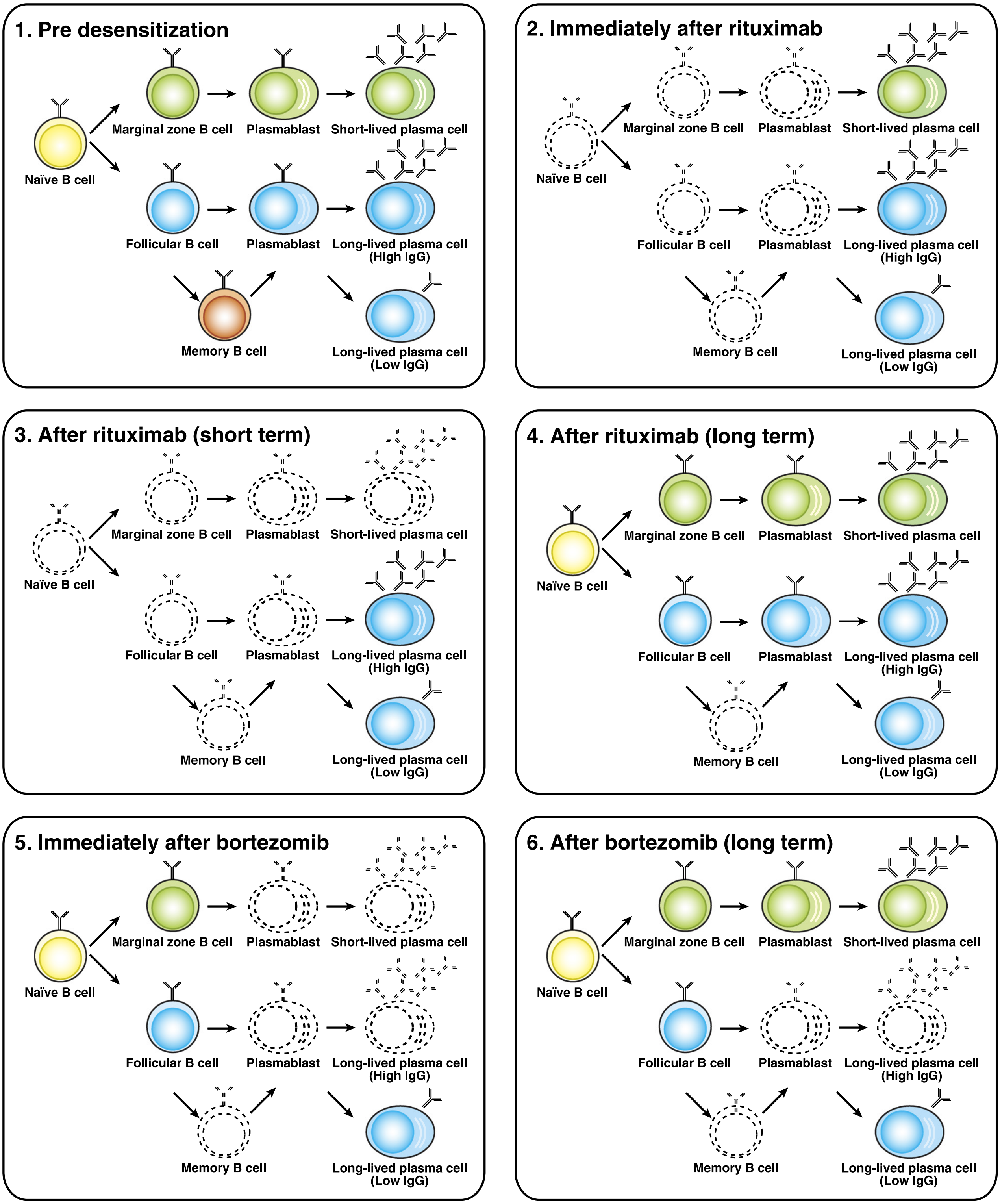

FIGURE 1. Concept for a phased desensitization strategy using rituximab followed by bortezomib for highly HLA-sensitized kidney transplant candidates. In cases where short-lived plasma cells exclusively produce DSA, desensitization should be complete after rituximab treatment and sequential plasmapheresis. However, in cases where long-lived plasma cells are also responsible for DSA production, additional therapy with bortezomib may be required in order to complete desensitization against allogeneic HLA.

TABLE 1A.

Baseline Characteristics

Parameters	Patient 1	Patient 2	Patient 3
Age, y	58	61	24
Sex	Male	Female	Male
Original kidney disease	Glomerulonephritis	Diabetic nephropathy	Glomerulonephritis
Sensitizing event	Previous transplant	Pregnancy	Previous transplant
Living donor relationship	Wife	Husband	Father
Donor age, y	56	63	58
HLA mismatches	Full mismatch	Full mismatch	One haplotype identical
ABO compatibility	Incompatible	Identical	Incompatible
T-CDCXM	Positive	Negative	Positive
T-FCXM	Positive	Positive	Positive
ICFA-I	Positive	Positive	Positive
ICFA-II	Negative	Negative	Negative
Luminex SA test result			
SA reactivities to HLA class I	11	16	7
SA reactivities to HLA class II	6	0	7
Donor-specific alloantibody	HLA-A2	HLA-B7	HLA-B52
(MFI)	(18694.12)	(11110.51)	(20763.28)

T-CDCXM, T-cell complement-dependent cytotoxicity cross-match; ICFA-I, ICFA class I; ICFA-II, ICFA class II; SA, single antigen; MFI, mean fluorescence intensity.

Hiroshima University Hospital (no. 156). The kidney transplant candidates, who had positive T-cell flow cytometry cross-match (T-FCXM) or immunocomplex capture fluorescence analysis (ICFA) class I results, received our conventional desensitization protocol as follows; that is, they received a single dose of rituximab (375 mg/m^2) combined with 3 double-filtration plasmapheresis (DFPP) sessions, followed by low doses (100 mg/kg per day) of IVIG (DFPP/low-IVIG).[10] Tacrolimus (target trough level: 5-10 ng/mL) or cyclosporine A (target trough level: 80-100 ng/ml) and mycophenolate mofetil (MMF, 20 mg/kg per day) were started 1 week before the DFPP/low-IVIG treatment. Three patients, in whom cross-match tests remained positive despite 3 DFPP/low-IVIG sessions, underwent the phased desensitization protocol. In these patients, the proportion of peripheral blood B cell subsets was determined at 3-month intervals. After verifying the absence of IgM$^+$ CD27$^+$ memory B cells and the presence of CD19$^+$ IgM$^+$ CD27$^-$ naive mature B cells in the peripheral blood, they received 1 cycle of bortezomib (1.3 mg/m^2, days 1, 4, 8, and 11), as established in the treatment of multiple myeloma,[11] followed by DFPP/low-IVIG. Dexamethasone 20 mg was added on the day of bortezomib administration as well as the following day.

B Cell Phenotype Analyses

For B cell phenotyping, peripheral blood mononuclear cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-IgM; phycoerythrin-conjugated anti-CD5, anti-CD19, anti-CD20, or anti-CD27; and allophycocyanin-conjugated anti-CD38 mAbs. For plasma cell identification, peripheral blood mononuclear cells were stained with fluorescein isothiocyanate–conjugated anti-IgM, phycoerythrin-conjugated anti-CD19, and allophycocyanin-conjugated anti-CD38 mAbs. Dead cells were excluded from the analysis by light-scatter and/or propidium iodide staining. Flow cytometric (FCM) analyses were performed on a FACSCalibur (BD Biosciences, Mountain View, CA).

Cross-Matching and Ab Detection

The complement-dependent cytotoxicity cross-match and the T-FCXM were performed as previously reported.[10] As an alternative cross-match test, ICFA was performed according to the manufacturer's protocol (WAKFlow HLA Ab class I&II, Wakunaga Pharmaceutical Co., Ltd., Japan). A screening test dedicated to detecting antibodies against HLA class I and class II in addition to MHC class I–related chain A was performed on a Luminex platform (LABScan 100 Flow Analyzer; Luminex Corporation, Austin, TX) using LABScreen Mixed (One Lambda, Canoga Park, CA) according to the manufacturer's protocol. Anti-HLA single antigen reactivity was detected on the Luminex platform using LABScreen Single Antigen assays (One Lambda). The results were recorded as mean fluorescence intensity. Mean fluorescence intensity values greater than 1000 were considered positive. Calculated panel reactive antibodies (CPRA) were computed using the United Network for Organ Sharing's CPRA calculator to evaluate the extent of a recipient's sensitization. The antiblood type isoagglutinin titers for IgM and IgG were serially measured as previously reported.[12]

Immune Monitoring

To evaluate the immune reactivity of these patients, we evaluated the antidonor T-cell response by performing mixed lymphocyte reactions (MLR) assay with the intracellular carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeling technique (CFSE-MLR assay). The CFSE-MLR assay allows the quantification of cell proliferation, which is a responses to allogeneic stimuli, and the simultaneous determination of the phenotype of the proliferating cells by using multiparameter FCM. CD4$^+$ and CD8$^+$ T-cell proliferations and stimulation indexes were quantified by using a previously reported method.[10]

RESULTS

Patient Characteristics

Consistent with the enrollment criteria, all the 3 patients had positive T-FCXM and ICFA class I test results before desensitization (Table 1A). In fact, in 2 of the 3 patients, the results were strongly positive for T-cell complement-dependent cytotoxicity cross-match (81-100% lysed cells), and both patients were ABO-incompatible living donor kidney transplant candidates. These 2 patients had previously received a living donor kidney transplant, but did not undergo an allograft nephrectomy before retransplantation.

Desensitization Outcomes

In accordance with the concept of this study, after rituximab treatment, we waited for the recovery of naive mature B cells in the peripheral blood. The recovery of mature B cells was defined as the presence of more than 1% of IgM$^+$ CD19$^+$ B cells among the peripheral blood lymphocytes (to ensure statistical significance, data on 30,000 lymphocytes were collected for each sample). Even after the reappearance of IgM$^+$ CD19$^+$ B cells, IgM$^+$ CD27$^+$ memory B cells remained undetectable in the peripheral blood of all patients until kidney

TABLE 1B.

Desensitization and Posttransplant Outcomes

Parameters	Patient 1	Patient 2	Patient 3
Duration between RIT and BTZ treatment, mo	20	9	12
Percentage of naïve B cells in PBMCs at BTZ, %	8.3	1.0	1.9
ICFA-I (index)			
Predesensitization	116	6.8	8.1
Postdesensitization	1.9	1.1	1.1
Immunosuppressant	TAC	CsA	TAC
Posttransplant follow up, mo	48	28	18
eGFR at latest follow-up, mL/min per 1.73 m^2	57.0	51.0	55.0

The eGFR of each participant was calculated based on age and serum creatinine value (S-Cr) according to the new Japanese equation as follows: eGFR (mL/min per 1.73 m^2) = 194 × Age-0.287 × S-Cr-1.094 (if female × 0.739).

RIT, rituximab; BTZ, bortezomib; PBMC, peripheral blood mononuclear cell; eGFR, estimated glomerular filtration rate; CsA, cyclosporine A.

transplantation (**Figure S1, SDC**, http://links.lww.com/TXD/A2). The mean time interval between rituximab treatment and bortezomib initiation was 13.7 months (Table 1B). Both drugs were tolerated well with no adverse events. After bortezomib administration followed by 3 DFPP/low-IVIG sessions, patients' DSA values and CPRA were decreased (Figure 2) and cross-match tests including the FCM and ICFA were consistently negative (Table 1B).

We previously reported that a subset of $CD19^+$ IgM^+ $CD5^+$ B-1 cells exclusively responds to blood group A carbohydrate antigens, and these cells need to be depleted in advance to successfully accomplish adult ABO-incompatible organ transplantation.[13,14] After rituximab/bortezomib/PP, the DSA values of all 3 patients were decreased and cross-match test results, including the FCM and ICFA, were consistently negative (Table 1B). Because the IgM^+ $CD19^+$ B cells that reappeared after rituximab treatment contained IgM^+ $CD5^+$ B-1 cells, 2 candidates for ABO-incompatible kidney transplantation additionally received a single dose of rituximab

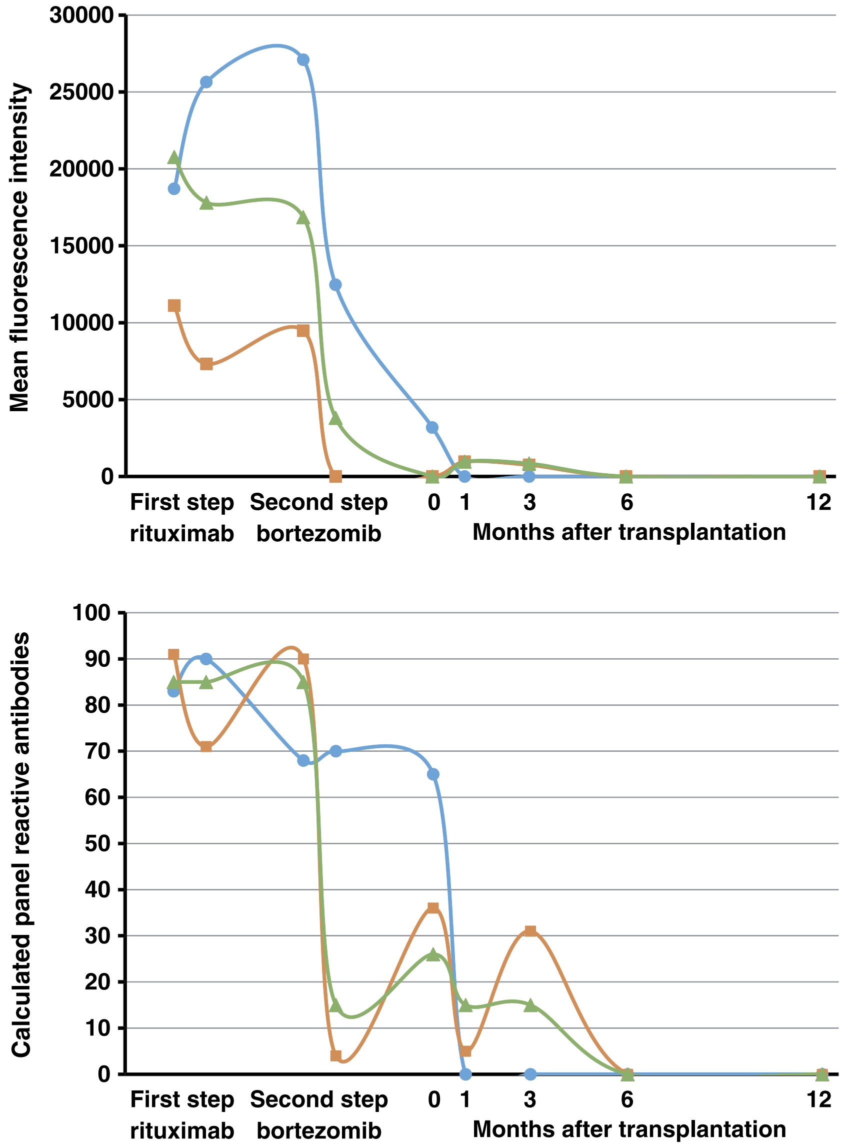

FIGURE 2. Kinetics of preexisting DSA and CPRA. The CPRA were computed using UNOS' CPRA calculator to evaluate the extent of a recipient's sensitization. After transplantation, each patients' DSA (specific for HLA-A2 in patient 1; HLA-B7 in patient 2; and HLA-B52 in patient 3) and CPRA were undetectable. Each line represents a single patient. Blue lines, patient 1; orange lines, patient 2; and green lines, patient 3.

($375\ mg/m^2$) 2 weeks before transplantation, despite negative cross-match test results. The interval between the final administration of bortezomib and the second administration of rituximab in these 2 patients was 19 weeks and 10 weeks, respectively. These time lags were determined by verifying the replenishment of $CD19^+$ $CD38^+$ plasma cells in patients' peripheral blood (data not shown). The kinetics of anti-A Ab titers are shown in **Figure S2**, http://links.lww.com/TXD/A2. No infectious episodes were encountered during desensitization in all patients.

Posttransplantation Outcomes

The induction immunosuppression protocol comprised tacrolimus or cyclosporine A, MMF, and methylprednisolone, but did not include basiliximab (Table 1B). The doses of the 3 agents were comparable to those used in nonsensitized cases. Patients displayed immediate renal function, and DSA was persistently undetectable after transplantation (Table 1B). At 48, 28, and 18 months into their follow-up, respectively, the patients are entirely healthy. Serum creatinine values were 1.04, 0.96, and 1.36 mg/dL, respectively, at the latest follow-up and renal allograft biopsies conducted within 1 year demonstrated no evidence of rejection, microvascular inflammation, or allograft glomerulonephritis. The Ab titers specific for cytomegalovirus and varicella zoster virus remained above the level of protection during the observation period (**Figure S3, SDC**, http://links.lww.com/TXD/A2), whereas both DSA and non-DSA were undetectable even at the latest follow-up (**Table 1B** and **Figure S4, SDC**, http://links.lww.com/TXD/A2).

DISCUSSION

To date, several aggressive protocols have been developed to eliminate Abs against allogeneic HLAs in patients with a positive cross-match.[15,16] Mounting evidence indicates that high-dose IVIG has a limited ability to reduce HLA Abs, but a few centers reported success with high-dose IVIG plus rituximab.[16] However, the overall experience in multiple centers has shown high antibody-mediated rejection rates, particularly in patients with the highest degrees of HLA sensitization.[17] Recent experiences with plasma cell-targeted therapies based on bortezomib in addition to rituximab are relatively small in number but may represent an important alternative to nondeletional strategies using IVIG.[5,18]

There have been reports of patients with persistent B-cell dysfunction even after the completion of rituximab treatment for B-cell lymphomas and autoimmune conditions, although the incidence is rare.[19] To avoid either persistent immune dysfunction or hypogammaglobulinemia, identifying immune recovery via periodic monitoring is prudent before starting additional immunomodulatory therapy with bortezomib. It has been reported that mature B cells recover more rapidly, returning to baseline by 6 months, whereas memory B cells remain low at 2 years after rituximab treatment.[6] Hence, the period after rituximab treatment characterized by the presence of mature B cells but the absence of memory B cells may represent an appropriate therapeutic window for the subsequent bortezomib treatment (Figure 1). The reappeared mature B cells may promptly replenish plasma cells producing immunoglobulin without specificity for HLA even after the bortezomib-induced destruction of preexisting plasma cells, possibly leading to a competent immune-defensive activity. Consistent with the previous observation,[6] IgM^+ $CD27^+$ memory B cells remained undetectable in the peripheral blood during observation even after the reappearance of IgM^+ $CD19^+$ B cells (**Figure S1, SDC**, http://links.lww.com/TXD/A2). Because it is entirely unknown whether B lymphopoiesis in the peripheral blood reflects B lymphopoiesis in secondary lymphoid tissues, such as the spleen and lymph nodes, the validity of this provisional definition of B cell recovery remains to be elucidated in future studies.

Alloantigen-reactive T cells are thought to play a key role in the production of DSA. In this study, we evaluated the antidonor T-cell response by using CFSE-MLR assay. Owing to the continuous administration of CNI and MMF during desensitization, the antidonor T-cell responses in all the 3 patients were decreased in the CFSE-MLR assay compared with those before desensitization (**Figure S5, SDC**, http://links.lww.com/TXD/A2). The effective suppression of alloantigen-reactive T cells might be a prerequisite for successful desensitization for highly sensitized cases. If all-reactive T cells (probably memory T cells) are still in the activated state when B cells reappear after rituximab treatment, the newly formed B cells are possibly resensitized. Hence, our phased desensitization protocol with rituximab and bortezomib might be effective in cases with well-suppressed T-cell responses. To address this speculation, future studies are needed.

One of the striking findings in this series might be the minimal impact of the phased desensitization protocol on Abs specific for varicella zoster virus and cytomegalovirus (**Figure S3, SDC**, http://links.lww.com/TXD/A2) despite its remarkable depleting effects on both DSA and non-DSA. Such a differential ability of proteasome inhibition to affect DSA and not Abs against viruses may be due to individual plasma cell activity.[20] It is likely that bortezomib will have a differential effect on plasma cells that produce high amounts of anti-HLA Abs compared with those that produce low amounts of Abs specific for various viruses. Apart from such a speculative mechanism, other unknown mechanisms may also exist.

Although cross-match test results were consistently negative in this series, 2 recipients of kidney allografts from ABO-incompatible donors received additional rituximab treatment because the reappeared IgM^+ $CD19^+$ B cells contained IgM^+ $CD5^+$ B-1 cells, which potentially respond to blood group A/B antigens. However, the necessity of this additional treatment remains to be elucidated, as it is unknown whether those newly formed IgM^+ $CD5^+$ $CD27^-$ B-1 cells are already equipped with the specificity for A/B antigens.

In conclusion, the present cases suggest that a phased use of rituximab and bortezomib can safely desensitize highly HLA-sensitized kidney transplant candidates, warranting a further larger scale prospective study.

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