Belatacept-Based Maintenance Immunosuppression Controls the Post-Transplant Humoral Immune Response in Highly Sensitized Nonhuman Primates

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Key Points
- Belatacept-based maintenance immunosuppression prevents antibody-mediated rejection and enables long-term kidney allograft survival in sensitized nonhuman primate recipients.
- Post-transplant belatacept prevents the rebound of follicular helper T cells, class-switched B cells, and antibody-secreting cells.
- Additional belatacept with tacrolimus increases the risk of viral reactivation and post-transplant lymphoproliferative disease.

Abstract
Preexisting donor-specific antibodies (DSA) to MHC antigens increase the risk of antibody-mediated rejection (AMR) in sensitized transplant recipients and reduces graft survival. Pretreatment desensitization with costimulation blockade and proteasome inhibition has facilitated transplantation in our preclinical nonhuman primate (NHP) model. However, long-term graft survival is limited by rebound of DSA after transplantation. In this study, we performed kidney transplants between highly sensitized, maximally MHC-mismatched NHPs (n=14). At kidney transplantation, primates received T cell depletion with rhesus-specific anti-thymocyte globulin (rhATG; n=10) or monoclonal anti-CD4 and anti-CD8 antibodies (n=4). Maintenance immunosuppression consisted of belatacept and tacrolimus (n=5) or belatacept and rapamycin (n=9) with steroids. Rebound of DSA post–kidney transplantation was significantly reduced compared with maintenance immunosuppression with tacrolimus, mycophenolate, and steroids. Protocol lymph node biopsy specimens showed a decrease in germinal center activity, with low frequencies of T follicular helper cells and class-switched B cells after kidney transplantation. Combined belatacept and rapamycin was superior in controlling viral reactivation, enabling weaning of ganciclovir prophylaxis. Tacrolimus was associated with increased morbidity that included cytomegalovirus and parvovirus viremia and post-transplant lymphoproliferative disorder. All primates in the tacrolimus/belatacept group failed discontinuation of antiviral therapy. Overall, belatacept-based immunosuppression increased AMR-free graft survival by controlling post-transplant humoral responses in highly sensitized NHP recipients and should be further investigated in a human clinical trial.

Introduction
Organ transplantation successfully resolves end stage organ failure (1–3). Current standard-of-care immunosuppressive strategies involving a calcineurin inhibitor (CNI), typically tacrolimus, have substantially improved short-term outcomes; CNIs are commonly used with mycophenolate and steroid for maintenance immunosuppression (4,5). However, the same regimen has lower success in sensitized patients with donor-specific antibody (DSA) (6,7). Patients with DSA, therefore, have significantly lower likelihood of receiving a deceased donor kidney transplant or kidney-paired donation compared with candidates who are nonsensitized (8–10). Persons may become sensitized to foreign human leukocyte antigen (HLA) by transplants, transfusions, or pregnancies (11), and their breadth of sensitization is commonly defined by the presence of anti-HLA antibodies against the national donor pool, expressed as calculated panel reactive antibody (cPRA) (12). Highly sensitized candidates for kidney transplantation with cPRA >98% represent approximately 7% of the waitlist, and sensitized patients with cPRA between 20%–98%
represent approximately 25% of the renal transplant waiting list (13). Unfortunately, the numbers of sensitized patients are increasing on the kidney, heart, and lung transplant waiting lists and the associated prolonged waiting time leads to increased waitlist mortality (14).

Desensitization regimens have enabled HLA-incompatible transplantation. Most desensitization protocols are involve plasmapheresis and/or intravenous immune globulin given before and after transplantation (15–17). Rituximab has shown some benefit in patients who are high risk with high DSA titers, previous transplants, or repeat HLA mismatches (18). However, these treatments have not been shown to reduce rebound or de novo antibody production after kidney transplantation (19). Proteasome inhibitors (PI), which target highly metabolically active antibody-producing cells have been tested for the treatment and prevention of antibody-mediated rejection (AMR) with some success (20,21). Nevertheless, outcomes after HLA-incompatible transplantation vary, with 1-year graft loss rates as high as 19% in recipients with positive cytotoxic crossmatches, as shown in a recent multi-institutional study (22). Such recipients have higher rates of AMR and shorter graft survival due to the presence of DSA and immunologic memory (6,7,23) with rebound of DSA usually occurring once PI therapy stops.

We have shown that desensitization with PI and costimulation blockade (CoB) significantly prolongs graft survival via reducing bone marrow (BM) plasma cells (PCs) and preventing a compensatory germinal center (GC) response (24,25). However, the desensitization treatment has been limited in its durability, and animals eventually experienced rebound of DSA and AMR (26,27). Given that CTLA4-Ig (either belatacept or abatacept) inhibits T follicular helper (Tfh) cell participation in antibody responses (28,29), we hypothesized that belatacept-based maintenance immunosuppression after desensitization would promote long-term graft survival in recipients with high sensitization by continuously suppressing the humoral immune response.

**Materials and Methods**

**Sensitized Nonhuman Primate Kidney Allograft Transplantation Model**

Fourteen Indian-origin male rhesus macaques (RMs) were enrolled in this study. The study animals were obtained from breeding colonies at Alpha Genesis Inc. (Yemassee, SC). Maximally MHC class I and II–mismatched pairs of nonhuman primates (NHPs) (Supplemental Table 1) were sensitized to each other with two sequential full-thickness skin transplants, performed 8 weeks apart, as described previously (30). Twelve weeks after the second skin transplant, performed 8 weeks apart, as described previously (30). After desensitization, swapping kidney transplantation with contralateral native nephrectomy was performed. Axillary and inguinal lymph node (LN) biopsies and BM biopsies from the iliac crest were performed before and after desensitization and 30 and 90 days post–kidney transplantation. During the desensitization phase, primates were sedated once per week for intravenous infusions and blood collection. After kidney transplantation, primates were routinely sedated twice per week for physical examination and blood collections. The study end point was defined as clinical evidence of acute AMR with renal allograft failure (e.g., rising creatinine, reduced urine output, and edema). All animal care and procedures were conducted in accordance with the National Institute of Health (NIH) guidelines and were approved by the Institutional Animal Care and Use Committee.

**Immunosuppressive Drug Regimens**

Immediately before kidney transplantation, primates received induction therapy with rhesus-specific anti-thymocyte globulin (rhATG; RRID, AB_2716327; NIH NHP Reagent Resource [NHPRR], Worcester, MA) at a dose of 20 mg/kg divided in five even dosages given daily between the day of transplant and post-transplant day 4. Alternatively, anti-CD4 (RRID, AB_2716322; NHPRR) and anti-CD8 mAbs (RRID, AB_2716320; NHPRR) were used for T cell depletion. Maintenance immunosuppression consisted of belatacept, methylprednisolone (Pfizer, New York, NY), and tacrolimus (Astellas Pharma, Northbrook, IL) or rapamycin (Alfa Aesar, Tewksbury, MA), to which primates were randomized. All primates were on an immunosuppression weaning protocol as outlined below. Methylprednisolone was started on the day of kidney transplantation at a dose of 15 mg/kg daily intramuscularly (IM) and tapered to 0.5 mg/kg daily by day 5 after kidney transplantation. Methylprednisolone was discontinued on post-transplant day 150. Tacrolimus was administered IM twice daily, dose adjusted to maintain trough levels of 8–12 ng/ml. Rapamycin was given IM once per day, also dose adjusted to maintain trough levels of 8–12 ng/ml. Tacrolimus or rapamycin were given until post-transplant day 180 and primates maintained on belatacept monotherapy thereafter. Trough level of tacrolimus and rapamycin was measured twice weekly with 1-cc whole blood (EDTA tube) from Duke University Health System Clinical Laboratories (Duke University) and Emory Medical laboratory (Emory University, respectively). Belatacept was given at a dose of 20 mg/kg on post-transplant days 0, 14, and 42, and every 4 weeks thereafter. We reduced the dose to 10 mg/kg on post-transplant day 210 and discontinued belatacept on day 270 after kidney transplantation. We monitored the renal allograft function daily by assessing the urine output and, at least twice per week, by serum chemistry analysis (VDL laboratory, Duke University). Clinically suspected acute cellular rejection (ACR) episodes (e.g., rise in creatinine, reduced urine output, and edema) were treated with methylprednisolone 125 mg/kg IM daily for 3 days, followed by 75 mg/kg IM daily for 3 days and 25 mg/kg IM daily for 3 days.

**Collection and Processing of Tissues from RMs**

Blood was collected in EDTA tubes for PBMCs and plasma and clot tubes for serum collection via femoral vein stick. PBMCs were obtained by density-gradient centrifugation using 90% separation media from Sigma. Peripheral LN biopsies (four sites, left/right axillary and inguinal LNs) were taken before and after pharmacologic desensitization, 1 and 3 months after kidney transplantation, and at
necropsy. LNs were either put in 10% buffered formalin and then embedded in paraffin, snap frozen with optimal cutting temperature compound, or ground over 70- and then embedded in paraffin. LNs were either put in 10% buffered formalin or taken when necessary. Spleen and transplanted kidney grafts were collected at necropsy. Grafts were digested in collagenase H (Sigma-Aldrich) for 30 minutes at 37°C.

**Histology, Diagnosis, and Pathologic Grading**

Renal biopsy and necropsy specimens were fixed in 10% neutral buffered formalin, paraffin embedded, and sectioned; we performed standard hematoxylin and eosin (H&E) and Periodic acid–Schiff staining of all allograft tissue samples. An experienced transplant pathologist (A.B.F.) evaluated and scored the histology specimens, according to Banff criteria (31–35), in a blinded fashion. Peripheral LN specimens were also fixed in 10% neutral buffered formalin, paraffin embedded, sectioned, and stained with H&E to visualize GCs. Post-transplant lymphoproliferative disorder (PTLD)-affected samples (i.e., BM, LN, liver, etc.) were fixed in 10% neutral buffered formalin, paraffin embedded, sectioned, stained with H&E, and evaluated by Jeffrey Everitt (veterinary pathologist), and the findings were confirmed by a hematopathologist. The images were either captured under a light microscope or scanned with an Aperio ScanScope XT (Aperio Technologies Inc., Vista, CA) and then captured with the ImageScope (Aperio Technologies).

**Monitoring of the Allogeneic Immune Response with Polychromatic Flow Cytometry**

We have previously described our standard method for detection of DSA in the serum (25). Briefly, serum samples for analysis were collected throughout the study period. Recipient serum was incubated with donor PBMCs. IgG (1:50 dilution) DSA was measured by flow cytometric crossmatch on a BD LSRFortessa (BD Biosciences, San Jose, CA) and analyzed using FlowJo software version 10 (Tree Star, Ashland, OR). For NHP cells, single-cell suspensions of LNs, BM, and PBMCs were stained for various lymphocyte population markers at the indicated time points. All NHP cells were stained using LIVE/DEAD Fixable Blue Dead Cell Stain kit (Thermo Scientific) for 20 minutes at room temperature before surface or intracellular staining. NHP cells were then surface stained with CD4 PerCP-Cy5.5 (clone L200; BD Biosciences), CD27 PerCP-eFlour710 (clone 323; Invitrogen), CD45RA FITC (clone L48; BD Biosciences), IgD FITC (Polyclonal; Southern Biotech), CCR7 Alexa Fluor 700 (clone 150503; BD Biosciences), IgG Alexa Fluor 700 (clone G18-145; BD Biosciences), CD20 Alexa Fluor 700 (clone 2H7; BD Biosciences), CD25 APC (clone CD25-3G10; Invitrogen), CD8 APC-Cy7 (clone RPA-T8; BD Biosciences), CD127 APC (clone eBioRDR5; eBioscience), CD20 APC-Cy7 (clone 2H7; BioLegend), CD19 APC (clone CB19; Abcam), PD-1 APC (eBioJ105; eBioscience), CD3 V500 (clone SP34-2; BD Biosciences), CD14 BV510 (clone M5E2; BD Biosciences), CD95 eFlour 450 (Clone D2X2; eBioscience), IgM PacBlue (clone MMH-88; BioLegend), CD8 PacBlue (clone RPA-T8; BD Biosciences), CD28 PE-Cy7 (clone CD28.2; eBioscience), ICOs PE-Cy7 (clone C298.4A; BioLegend), Ki-67 PE (clone B56; BD Biosciences), CD25 PE (clone 4E3; Miltenyi Biotec), CD38 PE (clone OKT10, RRID, AB_2716320; NHPRR), and CXCRC5 PE (clone MUSUBEE; eBioscience) for 30 minutes at 4°C. For intracellular staining, cells were fixed and permeabilized using the eBioscience FoxP3/Transcription Factor Staining Buffer Set (Thermo Scientific) and stained intracellularly with Ki-67 PerCP-CY5.5 (clone B56; BD Biosciences) and FoxP3 Alexa Fluor 488 (clone 259D; BioLegend) for 30 minutes at 4°C. All data were acquired on a BD LSRFortessa (BD Biosciences) using the BD FACSDiva software version 9.0 (BD Biosciences) and analyzed using FlowJo software version 9 or 10 (FlowJo LLC).

**Statistical Analysis**

Statistical analyses were performed using GraphPad Prism software version 8.4 (GraphPad Software, San Diego, CA). Survival data were plotted using the Kaplan–Meier method and the log-rank test was performed to determine statistical significance. Variation in the T cell subsets was determined by two-way ANOVA with a Tukey post-test. Statistical comparisons between different groups were performed using the t test, and values of P<0.05 were considered statistically significant.
**Results**

**Belatacept and Carfilzomib Decrease the Preformed Humoral Response**

Five pairs of maximally MHC-mismatched RMs (Supplemental Table 1) were sensitized to each other with two full-thickness skin transplants. Primates received weekly belatacept and carfilzomib infusions for 4 weeks (Figure 1A), as previously reported (26). Treatment caused a significant reduction of both class I and class II DSA (Figure 1B), concomitant reductions of LN Tfh cells (PD-1hi,ICOS1 or PD-1,ICOS1,CD41) and circulating Tfh-phenotype cells (cTfh, PD-1,ICOS1,CD41) (Figure 1C). As expected, the treatment did not alter the general B cell population in the LN, but reduced CD201 B cells in the circulation. We also observed a significant reduction of IgD1 IgG1 class-switched B cells in the LN and a similar trend of a reduction of fewer class-switched B cells in the blood (Figure 1D). We evaluated ASCs using ELISPOT to measure PCs in the BM, LN, and blood. As shown in Figure 1E, ASC frequencies in these immune compartments were significantly diminished. During desensitization, the absolute number of T cells was not significantly altered by the treatment (data not shown). However, we observed a significant reduction of central memory T cells and increased frequency of naive T cells in the blood for both CD41 and CD81 T cell subsets (Figure 1F). Belatacept/carfilzomib desensitization caused a significant reduction of circulating and LN FoxP31 regulatory T cells (Figure 1G). Taken together, pretransplant belatacept and carfilzomib treatment reduced humoral immune components, such as Tfh cells, PCs, B cells, and DSA, and also altered the T cell repertoire toward more naive phenotypes.

**Belatacept-Based Maintenance Immunosuppression Enables Long-Term Kidney Allograft Survival**

After desensitization, ten primates were randomized 1:1 to maintenance immunosuppression with either (1) belatacept, tacrolimus, and steroids; or (2) belatacept, rapamycin, and steroids. There was no difference in preformed DSA levels between the two groups (Supplemental Figure 1A). All primates received rhATG. Immunosuppressants were gradually discontinued after kidney transplantation (Figure 2A). Belatacept-based maintenance immunosuppression significantly prolonged graft survival compared with previously reported animals (27) and enabled long-term allograft survival for >6 months in three of five animals in the belatacept/tacrolimus (bela/tac) group (Figure 2B). Renal biopsy specimens from 1 and 6 months post-transplant showed low AMR (g+ptc) scores for these animals (Figure 2C, Table 1). Compared with conventional triple immunosuppression (tacrolimus/mycophenolate/steroid) (26,27), the addition of belatacept significantly prolonged graft survival and reduced the incidence of late AMR after desensitization (Supplemental Figure 1B). However, despite overall success, two animals (H65G and H99F) showed severe anemia and PTLD. For animals in the belatacept/rapamycin (bela/rapa) group, we observed three cases of early graft rejection, despite therapeutic rapamycin trough levels (8–12 ng/ml; Figure 2, D and E), mainly due to ACR (Supplemental Table 2) within the first 2 months post-kidney transplantation, whereas two out of five animals had long-term graft survival with excellent kidney function (Table 1). Switching induction from rhATG to anti-CD4/CD8 mAbs abolished early ACR in bela/rapa group (Supplemental Figure 2, A and B). Induction with mAbs induced significantly more CD8 T cell depletion with slower repopulation kinetics compared with rhATG (Supplemental Figure 2C). These animals also did not show AMR while on bela/rapa immunosuppression (Supplemental Figure 2, D and E). Class II DSA rebound was more frequent in the bela/tac group, whereas no rebound of class I and class II DSA was seen with the bela/rapa group despite three early graft losses (Figure 2F). The peak post-transplant class II DSA level was significantly higher in the bela/tac group than the bela/rapa group (Figure 2F).

To assess the overall benefit of additional belatacept for maintenance immunosuppression, we analyzed post-transplant outcomes from combined groups of sensitized NHP recipients (n=46) who received CoB and PI as desensitization with tacrolimus-based (group 2, n=17) or belatacept-based (group 3, n=14) maintenance immunosuppressive regimens (Supplemental Table 3). All animals received cytolytic induction with either rhATG or CD41/CD8 mAbs. Multivariant analysis revealed that pre-transplant desensitization with CoB and carfilzomib (group 2 and 3) significantly prolonged graft survival compared with animals without desensitization (group 1, n=15). Finally, the addition of belatacept to the maintenance immunosuppression significantly prolonged graft survival (group 3 versus group 2; mean survival time=127.1 versus 66.2 days; P=0.03; Figure 2G) and recipient survival (173.5 versus 77 days; P=0.007) in the 6-month study timeframe.

**Belatacept Controls the Post-Transplant GC Response**

Reduction of post-transplant AMR and DSA rebound suggested continuous modulation of PC generation, including B cell activation via Tfh cells by belatacept. To explore this concept, we examined the Tfh cell, B cell, and PC populations. All animals showed a significant reduction of lymphocytes after rhATG treatment, characterized by both T cell and B cell depletion (Figure 3A). T cells did not repopulate to baseline levels until discontinuation of maintenance immunosuppression, whereas B cells reached baseline within 3 months after rhATG treatment. Similar to humans treated with thymoglobulin (36–38), animals maintained a prolonged CD41-dominant T cell lymphopenia (Supplemental Figure 3A). CD1 T cell frequency slowly decreased, whereas memory subsets increased, during T cell repopulation (Supplemental Figure 3B). Belatacept in combination with tacrolimus or rapamycin suppressed the Tfh cell population (CD41,ICOS1,PD11) within LNs. This suppression was associated with lower proliferation of class-switched B cells (CD201IgGIgD1), measured by Ki-67 (Figure 3B). Circulating Ki-671 B cell and cTfh cell populations also declined in peripheral blood. We observed no evidence of expansion of CXCR51 and CXCR51 ICOS1 PD11 CD41 T cells or proliferating B cells. However, COST1 PD11 CD41 T cells increased (Supplemental Figure 3C). Because ICOS1 PD11 CD41 T cells increased in the setting of AMR (Supplemental Figure 3D), the increase in this subpopulation may be due to AMR. Tfh cells were not significantly elevated compared with pretransplant until 6 months post-transplantation (Figure 3C). ASCs in BM, LNs, and PBMCs also showed no increases in frequency during this period (Figure 3D). Likewise,
desensitization and desensitization before transplantation. (B) T and B cell flow cytometry crossmatch (TFXM and BFXM, respectively) of all animals at pre- and post-desensitization. (C) Representative flow cytometry plots for PD-1^hiICOS^ and PD-1^ICOS^- Tfh cells in gated CD4^+ cells and collated data for lymph node (LN) and circulating Tfh populations. (D) Flow cytometric analysis of LNs and circulating B and IgG^+IgD^- class-switched B cell populations collected pre- and post-desensitization. The absolute circulating CD20^+ B cell counts were calculated by multiplying the frequency with the absolute lymphocyte count. (E) Representative ELISPOT images at pre- and post-desensitization from bone marrow (BM), LN, and blood. Collated data for frequencies of ASCs. (F) Flow cytometric analysis of CD4 and CD8 T cell subsets defined by CD28 and CD95 surface markers. Central memory T cell (Tcm) were defined as CD28^+CD95^-T effector memory cells (Tem) were defined as CD28^- and CD95^+. Naive T cells (Tnaive) were defined as CD28^+ and CD95^- . (G) Flow cytometric analysis of circulating and LN T regulatory cells (Tregs) defined as CD4^+CD25^+FoxP3^+ T cells. All data were compiled from all rhesus macaques (n=10) that were later randomized into two different treatment groups (red, belatacept and tacrolimus, versus blue, belatacept and rapamycin). Error bars represent mean±SD. **P<0.01, ***P<0.001, two-tailed paired t test. MFI, mean fluorescence intensity; Rapa, rapamycin; Tac, tacrolimus; Tx, transplant.

post-transplant LNs showed a prolonged dissolution of GCs (Figure 3E, Supplemental Figure 3E). Taken together, belatacept with tacrolimus or rapamycin continuously downmodulated the Tfh cell-driven GC response and PC generation.

**Belatacept and Tacrolimus Immunosuppression Increases the Risk for Viral Infections**

After initial weight loss, primates regained their body weight after kidney transplantation. Primates in the bela/rapa group regained weight faster than primates in the bela/tac group (β=0.08 versus β=0.16; P<0.001; Figure 4A). The slower weight gain in the bela/tac group correlated with the higher morbidity of this regimen compared with rapamycin. All primates in the bela/tac group (n=5) experienced cytomegalovirus (CMV) reactivation during the study period, frequently meeting threshold for treatment (10,000 copies/ml). In contrast, no CMV reactivation occurred in the bela/rapa group (n=5). The post-transplant peak CMV titer was higher in belatacept with tacrolimus compared with rapamycin (Figure 4B). We attempted discontinuation of CMV prophylaxis in all primates with low viral titers during the second month after transplant. Bela/tac-treated primates experienced an immediate elevation of CMV viral load, requiring restarting CMV prophylaxis. In contrast, CMV prophylaxis was discontinued successfully in all bela/rapa-treated primates (Figure 4C). We encountered two severe cases of simian parvovirus infection in bela/tac-treated animals (H65G and H72F) leading to therapy-resistant anemia treated with multiple transfusions. No anemia or simian parvovirus infection/reactivation occurred in the rapamycin group (Figure 4D). We observed two cases of PTLD in the
Belatacept-based maintenance immunosuppression controls the post-transplant humoral response and enables long-term graft survival in highly sensitized nonhuman primate recipients. (A) Schematic of the experimental design and immunosuppression regimen. A representative renal pathology at 1 and 6 months post-transplantation from animals treated with belatacept/tacrolimus/steroid and those treated with belatacept/rapamycin/steroid (showing no evidence of acute cellular rejection or overt glomerulopathy). (B) Kaplan–Meier curve for the individual contribution of graft survival, with censoring of data for recipient who died for other reasons than rejection. The right panel shows the Kaplan–Meier curve for the composite end point of recipient and graft survival. *P<0.05. Bela, belatacept; Bx, biopsy; DSA, donor-specific anti-HLA antibody; MFI, mean fluorescence intensity; mmf, mycophenolate mofetil; Rapa, rapamycin; rhATG, rhesus-derived anti-thymocyte globulin; Tac, tacrolimus; Tx, transplant.

**Rebound of the Humoral Immune Response on Belatacept Monotherapy**

To evaluate whether belatacept alone can maintain stable graft function without additional immunosuppression, tacrolimus and rapamycin were discontinued 6 months after kidney transplantation (postoperative day 180). Four out of five animals experienced graft rejection, with gradual decline of graft function under belatacept monotherapy (mean survival time=42.5 days after weaning; Figure 5A). All four animals showed mixed rejection (ACR and AMR) at necropsy (Figure 5B, Table 2). One animal (bela/tac group, H48J) maintained normal kidney function under belatacept monotherapy, but rejected after immunosuppression was completely withdrawn. Serial biopsy specimens revealed the gradual development of ACR and AMR under belatacept monotherapy (Supplemental Figure 5). Interestingly, after discontinuation of tacrolimus or rapamycin, primates also showed a rapid repopulation of total lymphocytes, including T and B cells, to baseline levels under belatacept monotherapy. DSA rebounded in all animals with class II antibody populations during belatacept monotherapy (Figure 5D).

In one animal (H99F), an enlarged LN obstructed the transplant ureter (Supplemental Figure 4A). In the other animal (H65G), PTLD was diagnosed postmortem with numerous areas of large pleomorphic atypical lymphoid cells in the liver and BM (Figure 4E, Supplemental Figure 4).

Figure 2. | Belatacept-based maintenance immunosuppression controls the post-transplant humoral response and enables long-term graft survival in highly sensitized nonhuman primate recipients. (A) Schematic of the experimental design and immunosuppression regimen. (B) Kaplan–Meier curve of the 6-month graft survival of both study groups and a control group of animals without desensitization. (C) Representative renal pathology at 1 and 6 months post-transplantation from animals treated with belatacept/tacrolimus/steroid and those treated with belatacept/rapamycin/steroid (showing no evidence of acute cellular rejection or overt glomerulopathy). (D) Serum creatinine levels of each individual study animal of both treatment groups. (E) Mean tacrolimus and rapamycin trough levels throughout the study period. Dashed lines mark the goal trough level of 8 ng/mL.

Graft Survival (Death censored/rejection free)
Table 1. Banff scoring of early (at 1–3 months) and late (at 6 months) kidney allograft biopsy specimens

| Name   | Postoperative day | Treatment | Tubulitis | Intimal Arteritis | Interstitial Inflammation | Total Inflammation | Cortical Inflammation | Allograft Glomerulitis | Cortical Interstitial Fibrosis | Cortical Tubular Atrophy | Allograft Glomerulopathy | Mesangial Matrix | Vascular Fibrous Intimal Thickening | Arteriolar Hyalinosis | Peritubular Capillary Inflammation | Diagnosis a |
|--------|-------------------|-----------|-----------|------------------|---------------------------|------------------|-----------------------|------------------------|---------------------------|------------------------|----------------------|----------------|-------------------------------|----------------------|-----------------------------|----------------|
|        |                   |           |           |                  |                           |                  |                       |                        |                           |                        |                      |                 |                               |                      |                            |                |
| Early biopsies |
| H65G   | 28                | T         | 0         | 0                | 0                         | 0                | 0                     | 0                      | 0                         | 0                      | 0                    | 0                | 0                | 0                | No evidence of ACR                 |
| H72F   | 27                | T         | 0         | 0                | 0                         | 0                | 0                     | 0                      | 0                         | 0                      | 0                    | 0                | 0                | 0                | No evidence of ACR, interstitial fibrosis and tubular atrophy with inflammation |
| H99F   | 86                | T         | 0         | 0                | 0                         | 0                | 1                     | 0                      | 0                         | 0                      | 0                    | 0                | 0                | 0                | No evidence of ACR                 |
| H48F   | 28                | R         | 1         | 0                | 0                         | 1                | 1                     | 0                      | 1                         | 1                      | 0                    | 0                | 0                | 0                | No evidence of ACR, interstitial fibrosis and tubular atrophy with inflammation |
| Late biopsies |
| H72F   | 183               | T         | 0         | 0                | 0                         | 0                | 0                     | 0                      | 0                         | 0                      | 0                    | 0                | 0                | 0                | No evidence of ACR                 |
| H87X   | 200               | T         | 1         | 0                | 0                         | 0                | 0                     | 1                      | 0                         | 0                      | 0                    | 0                | 0                | 0                | No evidence of ACR, interstitial fibrosis and tubular atrophy with inflammation |
| H99H   | 181               | T         | 1         | 0                | 0                         | 0                | 0                     | 1                      | 0                         | 0                      | 0                    | 0                | 0                | 0                | No evidence of ACR, interstitial fibrosis and tubular atrophy with inflammation |
| H48F   | 183               | R         | 1         | 0                | 0                         | 0                | 1                     | 1                      | 1                         | 0                      | 0                    | 0                | 0                | 0                | No evidence of ACR, interstitial fibrosis and tubular atrophy with inflammation |
| H48F   | 217               | R         | 1         | 0                | 1                         | 1                | 2                     | 0                      | 2                         | 1                      | 0                    | 0                | 0                | 0                | “Borderline changes” “suspicious” for ACR |

T, tacrolimus; ACR, acute cellular rejection; R, rapamycin.

*ACR is judged according to recent Banff criteria requiring ≥interstitial inflammation grade 1 for a diagnosis of “borderline changes” “suspicious” for ACR (35).
Figure 3. Continued administration of belatacept suppresses post-transplant humoral response. (A) Absolute lymphocyte count and absolute CD20⁺ B cell and CD3⁺ T cell counts after induction therapy with rhATG. All available samples were analyzed from postoperative day (POD) 0, 1, 4, 7, 14, 28, 42, 56, 84, 112, 140, and 168 (n=5–10). P value was calculated on the basis of comparison to POD 0 (paired or unpaired t test). (B) Flow cytometric analysis of peripheral LN single-cell suspensions, sampled prior and 1 and 3 months after transplantation. LN Tfh cells were defined as CD4⁺ ICOS⁺ PD1hi T cells and proliferating class-switched B cells were defined as CD20⁺ IgG⁺ IgD⁻ Ki-67⁺ cells. Both study groups were grouped for this analysis, but individual primates of each group are identified through the following color code: red, tacrolimus group; blue, rapamycin group. (C) Flow cytometric analysis of post-transplant circulating Tfh cells defined as CD4⁺PD-1⁺ICOS⁺ T cells (red, tacrolimus group; blue, rapamycin group). (D) Representative ELISPOT images at pre-desensitization, post-desensitization, and 1- and 3-months post-transplantation from BM, LN, and blood. Frequencies of ASCs are expressed as spots per million. (E) Representative hematoxylin and eosin images of LN biopsy specimens from pre-desensitization, post-desensitization, and 1- and 3-months after kidney transplantation. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Bela, belatacept; GC, germinal center; Rapa, rapamycin; Tac, tacrolimus; Tx, transplant.
dominance (Figure 5E). Consistent with rebound of DSA, flow cytometric analysis of LNs at necropsy revealed the recovery of GC activity with a significant increase in Tfh cells and Ki-67+ class-switched B cells compared with pre-weaning (Figure 5F). In summary, in sensitized renal transplant recipients, belatacept used alone failed to control post-transplant alloimmune responses (both T cell and antibody mediated) and was unable to maintain stable graft function in the absence of tacrolimus or rapamycin.

Discussion

In MHC-incompatible kidney transplantations between maximally MHC-mismatched allosensitized NHPs, pretransplant desensitization with belatacept and carfilzomib downregulates humoral immune responses, altering Tfh and B cell activation and reducing PCs and DSA levels with GC contraction (Figure 1). However, the humoral response gradually reappears after kidney transplantation with conventional CNI-based immunosuppression and recipients developed AMR in our previous studies (26,27). The use of belatacept as maintenance immunosuppression after kidney transplantation enabled long-term kidney allograft survival in a highly sensitized NHP transplant model (Figure 2) by controlling Tfh cells, PCs, and DSA development (Figure 3). Given the sample size and the unexpectedly observed subject morbidity, including PTLD, viral complications, and ACR, it is difficult to state which drug combination (bela/tac versus bela/rapa) is superior, with both regimens having advantages and disadvantages (Figure 4). However, the performed meta-analysis showed a benefit of belatacept-based maintenance immunosuppression over conventional triple immunosuppression (Figure 2). Interestingly, after weaning tacrolimus or rapamycin, we observed breakthrough humoral immune responses under belatacept monotherapy with subsequent graft rejection (Figure 5). This may reflect a synergistic role of CNI or mTOR inhibitors and belatacept on the Tfh cell–driven post-transplant GC response.

Previously, we described desensitization of allosensitized NHPs with PI or CoB. Targeting either pathway alone did not achieve successful desensitization (24,39), and our iterative trials in sensitized NHP models led us to pretransplant desensitization using both CoB and PI (24,25,27). These regimens deplete BM PCs and suppress GC activity simultaneously, which results in reduction of DSA. This approach has also been translated into the clinic with a small series of four highly sensitized (cPRA>99%) heart transplant recipients, of which three were able to find a suitable donor after the desensitization due to a marked reduction of class I and class II anti-HLA antibodies (40). Others have used the combination of bortezomib and belatacept for the treatment of AMR (41). Six patients with active AMR were treated and DSA levels were reduced to low or undetectable levels during a follow-up of up to 30 months. Despite potential of belatacept and carfilzomib desensitization (24–26), highly sensitized animals eventually developed AMR with concomitant rebound of GC activity on a standard CNI-based maintenance immunosuppression (25–27). The most suitable maintenance immunosuppression for these recipients with high chance of AMR has, therefore, yet to be determined and prompted this study. Due to their contributions to antibody responses, Tfh cells became a
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Belatacept alone fails to downmodulate post-transplant alloimmune response and both T and B cells break through under belatacept monotherapy. (A) Kaplan–Meier curve of the graft survival and serum creatinine levels after 180 days on belatacept monotherapy and off all immunosuppression after day 270. Red lines indicate that the study animal was previously on tacrolimus and blue lines indicate that the primate was previously on rapamycin. (B) Representative hematoxylin and eosin image of animals at euthanasia from both study groups. Acute cellular rejection (ACR) score was calculated on the basis of Banff gradings: intimal arteritis (v), tubulitis (t), and interstitial inflammation (i). Antibody-mediated rejection (AMR) score was calculated on the basis of Banff gradings: allograft glomerulitis (g) and peritubular capillaritis (ptc). The ACR grade is given on the image; in addition, varying degrees of glomerulopathy were also present (also depicted). (C) Mean absolute lymphocyte count, absolute CD3+ T cell count, and CD20+ B cell count show repopulation to pre-transplant baseline levels under belatacept monotherapy. (D) Selected time points showing CD4+ T cell frequency of memory subsets signifi-
cantly reduced by the addition of T cell depletion and/or transient CNI or rapamycin. (E) Donor-specific antibody (DSA) kinetics measured with T and B cell flow crossmatch (TFX and BFX, respectively) after discontinuation of tacrolimus and rapamycin. (F) Analysis of peripheral LN ICOS+ PD1hi Tfh cells and proliferating Ki-67+ IgG+ IgD– class-switched B cells, which show a rebound of germinal center activity.

new target for antibody-mediated autoimmune diseases. Abatacept treatment reduced cTfh cells and production of autoantibodies in patients with primary Sjögren syndrome (42) and rheumatoid arthritis (43,44). In kidney transplantation, belatacept-based maintenance immunosuppression has also been shown to decrease levels of preexisting anti-HLA antibodies and to prevent the emergence of de novo DSA (the BENEFIT and BENEFIT-EXT trials) (45,46). These clinical observations are consistent with preclinical work demonstrating the role of Tfh cells in the generation of DSA (47,48) and other works showing the effect of CTLA4-Ig in DSA production in organ transplantation (49,50). Recent data also suggest that the rate of early acute rejection, also termed as CoB-resistant rejection, can be significantly reduced by the addition of T cell depletion and/or transient CNI or rapamycin (51–53). In this study, we showed the addition of belatacept in the post-transplant immunosuppression maintained the low frequencies of Tfh cells, class-switched B cells, and antibody-producing cells after kidney transplantation. As a result, graft survival was significantly improved compared with our previously reported studies (25–27,30), in which recipients received tacrolimus-based conventional triple immunosuppression.

Although CoB is known to suppress humoral immune responses, sufficient control of the post-transplant humoral response required the combination of belatacept and tacrolimus/rapamycin in our model, because all animals in the study experienced a rebound of GC response, including Tfh cells and DSA, after discontinuing tacrolimus or rapamycin under belatacept monotherapy. The breakthrough Tfh cells under both tacrolimus-based immunosuppression or belatacept monotherapy suggest heterogeneous Tfh populations with different susceptibility to these two agents. Interestingly, nonsensitized or minimally sensitized human kidney transplant recipients can be weaned to belatacept as sole maintenance immunosuppression and avoid rejection (53). The difference may be due to the higher immunologic barrier our model presents (maximal MHC mismatch, repeated sensitizations). Another concern for belatacept in combination with
| Name  | Postoperative day | Treatment | Tubulitis | Intimal Arteritis | Interstitial Inflammation | Total Inflammation | Cortical Inflammation | Allograft Glomerulitis | Cortical Interstitial Fibrosis | Cortical Tubular Atrophy | Allograft Glomerulopathy | Mesangial Matrix | Vascular Fibrosis | Intimal Thickening | Arteriolar Hyalinosis | Peritubular Capillary Inflammation | Diagnosis* |
|-------|------------------|-----------|-----------|------------------|--------------------------|-------------------|---------------------|----------------------|--------------------------|---------------------|----------------------|----------------|----------------|----------------|----------------|-----------------------------|-------------|
| H72F  | 228              | T         | 3         | 0                | 2                        | 2                 | 3                   | 3                    | 1                        | 1                   | 0                    | 0              | 0              | 0              | 3              | ACR, type 1B; findings also suspicious for AMR |
| H87X  | 223              | T         | 3         | 3                | 2                        | 2                 | 3                   | 2                    | 1                        | 1                   | 0                    | 0              | 3              | 0              | 2              | ACR, type 3 (multifocal arterial fibrinoid necrosis with mural inflammation and aneurysmal change); findings also suspicious for AMR and/or TMA with arteriolar fibrin |
| H99H  | 203              | T         | 3         | 3                | 3                        | 3                 | 3                   | 3                    | 2                        | 3                   | 3                    | 3              | 0              | 3              | 2              | ACR, type 3 (multifocal arterial fibrinoid necrosis with mural inflammation and aneurysmal change); findings also suspicious for AMR and/or TMA with glomerular and arteriolar fibrin |
| H49F  | 236              | R         | 3         | 0                | 3                        | 3                 | 3                   | 3                    | 2                        | 1                   | 3                    | 3              | 0              | 0              | 3              | ACR, type 1B; chronic transplant glomerulopathy with hemosiderin deposition and glomerulitis, s/o CAMR and/or TMA; tubular injury with oxalate crystals |
| H48J  | 292              | R         | 2         | 3                | 2                        | 3                 | 3                   | 1                    | 1                        | 1                   | 3                    | 3              | 2              | 0              | 3              | ACR, type 3 (focal arterial fibrinoid necrosis with mural inflammation); findings are at least suspicious for AMR |

T, tacrolimus; AMR, antibody-mediated rejection; TMA, thrombotic microangiopathy; R, rapamycin; CAMR, chronic active AMR; s/o, associated with.

*AMR is judged according to recent Banff criteria requiring g+ptc ≥2 for a diagnosis of “suspicious” for AMR.
conventional immnosuppression should be the morbidity of the regimen, which is often neglected when drug regimens are initially explored in NHP studies. Lifelong immnosuppression, although required to prevent allograft rejection, can trigger opportunistic infections and cancer. Currently, infection is one of leading causes of death in kidney transplantation (54,55). Single-center analyses have shown that tacrolimus-based immnosuppression, and especially high serum trough levels, are significant independent risk factors for Epstein–Barr virus-associated PTLD and a negative prognostic factor for post-PTLD survival (56,57). Animals treated with belatacept and tacrolimus showed a strong association with CMV viremia, which did not permit stopping CMV prophylaxis and had a high incidence of PTLD. On the contrary, belatacept with rapamycin demonstrated an additional benefit in preserving antiviral immunity, which allowed discontinuation of CMV prophylaxis. Although the combination of tacrolimus and belatacept is not used in clinic as a maintenance immnosuppression as suggested in this study, the similar immnosuppressive milieu has been created in tacrolimus to belatacept conversion. During the conversion process, patients are often treated with CNI together with belatacept in an overlapping manner. On the basis of our data, it is not too surprising that a CNI to belatacept trial was terminated due to high incidence of PTLD (58). On the other hand, our findings are consistent with a number of clinical observations that mTOR inhibitors show better outcomes with regard to CMV disease compared with CNI-based immnosuppression (59–61), perhaps due to the immunostimulatory effect of mTOR inhibitors on memory CD8+ T cell response to pathogens (62–64). This mechanism could augment anti-CMV immune responses in our animals. We also showed that a more profound induction agent could reduce acute rejection under belatacept and rapamycin maintenance therapy. Therefore, rapamycin may provide an attractive immnosuppressive option for patients who are sensitized in the setting of virus-related comorbidities.

Our study showed the advantages and limitations of both regimens. However, our conclusions are limited by the small numbers of animals per group with unexpected outcomes (e.g., PTLD and viral complications), which make it hard to interpret the efficacy of our approaches on graft survival. Furthermore, the control group was not properly set up in the study but relied on a previously reported group; this is partially due to the national shortage of RMs because of the coronavirus disease 2019 pandemic. Additionally, repeating a similar control group is not appealing in the NHP study considering financial pressure. The latter issue was partially resolved by analyzing accumulated data with similar treatment reported previously (25–27,30). Although these meta-analyses revealed the potential benefit of adding belatacept to the maintenance immnosuppression, it is also important to integrate pertinent comorbidities to provide thorough information related to the new therapeutic approaches. Given the published clinical experience and our data in this stringent NHP model, belatacept-based maintenance immnosuppression after desensitization may be an attractive therapeutic approach to managing highly sensitized human transplant recipients but needs to be executed with caution.

Disclosures

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Author Contributions

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Data Sharing Statement

The primary data that support the findings of this study are available from the corresponding authors upon reasonable request.

Supplemental Material

This article contains the following supplemental material online at http://kidney360.asnjournals.org/lookup/suppl/doi:10.34067/KID.0001732022/-/DCSupplemental.

Supplemental Table 1. MHC class I (Mamu-A and -B) and II (Mamu-DRB, -DQB, and -DPB) haplotypes of all rhesus macaque pairs used in the study.
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