Supplemental Materials and Methods

Animals, kidney transplantation, and monitoring

Male, outbred rhesus macaques *(Macaca mulatta,* Alpha Genesis, Yemassee, SC) were tested to ascertain specific pathogen free status. Donor–recipient pairs were selected on the basis of full MHC class I mismatch and maximal MHC class II mismatch by 454 sequencing (WNPRC, Madison, WI; **Supplemental figure 1**). Skin grafts (1inch diameter) were swapped between paired animals and evaluated by daily visual inspection. Necrosis of greater than 50% of the transplanted skin surface was defined as rejection. Heterotopic renal allotransplantation was performed as previously described using the same donor–recipient pairs (kidney swap) [30]. Medications, procedures, housing, and maintenance were approved by the Emory and Duke University Institutional Animal Care and Use Committee, and were conducted in accordance with Yerkes National Primate Research Center (YNPRC) or Duke Laboratory Animal Resources (DLAR) and the National Institutes of Health guidelines.

Immunosuppression agents

Desensitization regimens: Sensitized animals were treated with belatacept (20mg/kg, twice weekly, IV), and 2C10 (20mg/kg, twice weekly, IV), or belatacept/2C10/bortezomib (1.3mg/m^2, twice weekly, IV) over four weeks for desensitization. Control animals did not receive any drug treatment prior to kidney transplantation.

Study groups (by desensitization): Five animals were assigned to the untreated control group, four of which have been previously reported [30]. Three animals were assigned to receive belatacept (20mg/kg, IV) and 2C10 (20mg/kg, IV, Nonhuman Primate Reagent Resource, Boston, MA). Three animals received belatacept and 2C10 with bortezomib (1.33mg/m^2, IV) or carfilzomib (20mg/m^2, IV).

Transplantation regimen: All renal allograft recipients received induction with 0.3mg/kg basiliximab IV on post-transplant day 0 and 4; 0.05mg/kg tacrolimus IM BID (adjusted later to obtain a trough level of 8–12ng/mL); 15mg/kg SC or 30mg/kg PO MMF; 125mg methylprednisolone IV (tapered daily with IM dose). 60mg/kg SQ ganciclovir was given for prophylaxis and therapy of rhesus cytomegalovirus reactivation (over 10,000 copies/ml).

Flow cytometric analysis and detection of alloantibody

Peripheral blood cells (obtained by femoral venipuncture), lymph node cells (obtained by axillary or inguinal lymph node biopsy), and bone marrow cells (obtained by bone marrow aspiration at

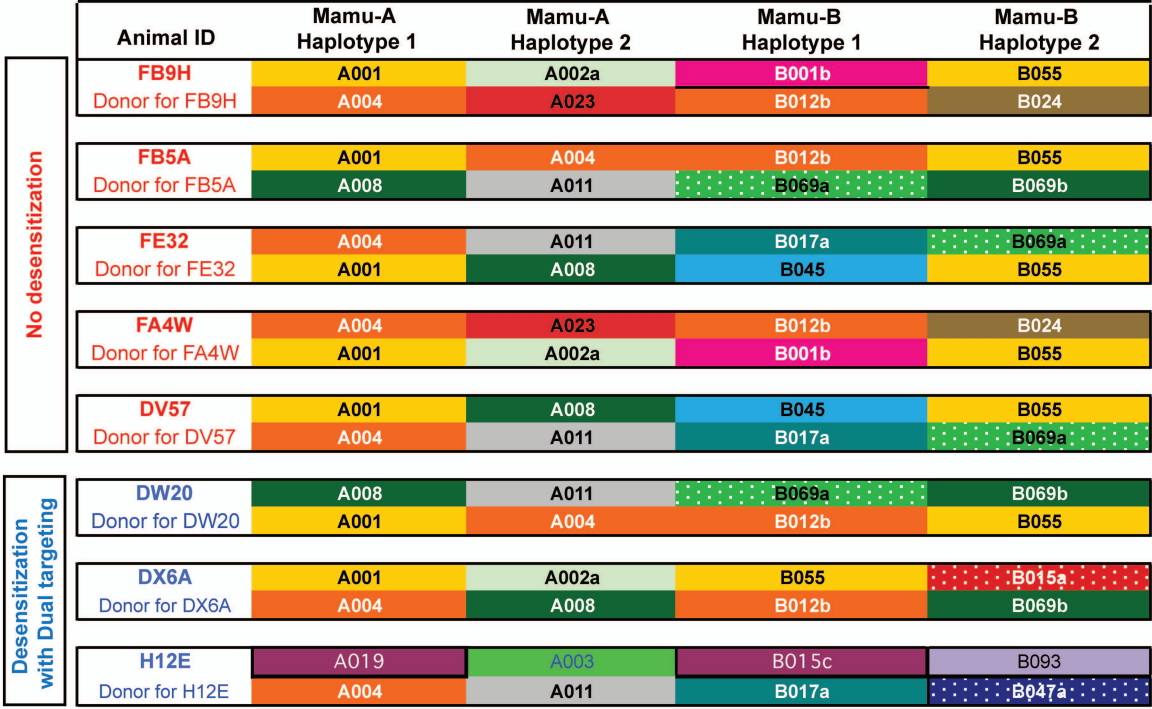
iliac crest) were isolated by Ficoll method using 5 mL of lymphocyte separation medium (Mediatech Inc., Manassas, VA) per sample. Washed cells were stained for live/dead cell using Aqua dead cell stain kit (Life Technologies, Eugene, OR) and then with antibodies: CD3, CD4, CD8, CD20, CD25, CD27, CD28, CD95, PD-1, CXCR5, ICOS, IgD, IgG, IgM, Ki67, and FoxP3. Donor-specific alloantibody was assessed by flow crossmatch from serially collected recipient serum samples with donor PBMCs as previously described [32-34]. The DSA level was expressed as MFI ratio compared to the pre-transplant mean fluorescence intensity (MFI) value. Samples were collected with an LSRII flow cytometer (BD Biosciences, San Jose, CA) or BD LSR FORTESSA X-20 (BD Bioscience) and analyzed using FlowJo software 9.9. (Tree Star, Ashland, OR).

Histology, immunohistochemistry and quantitative image analysis

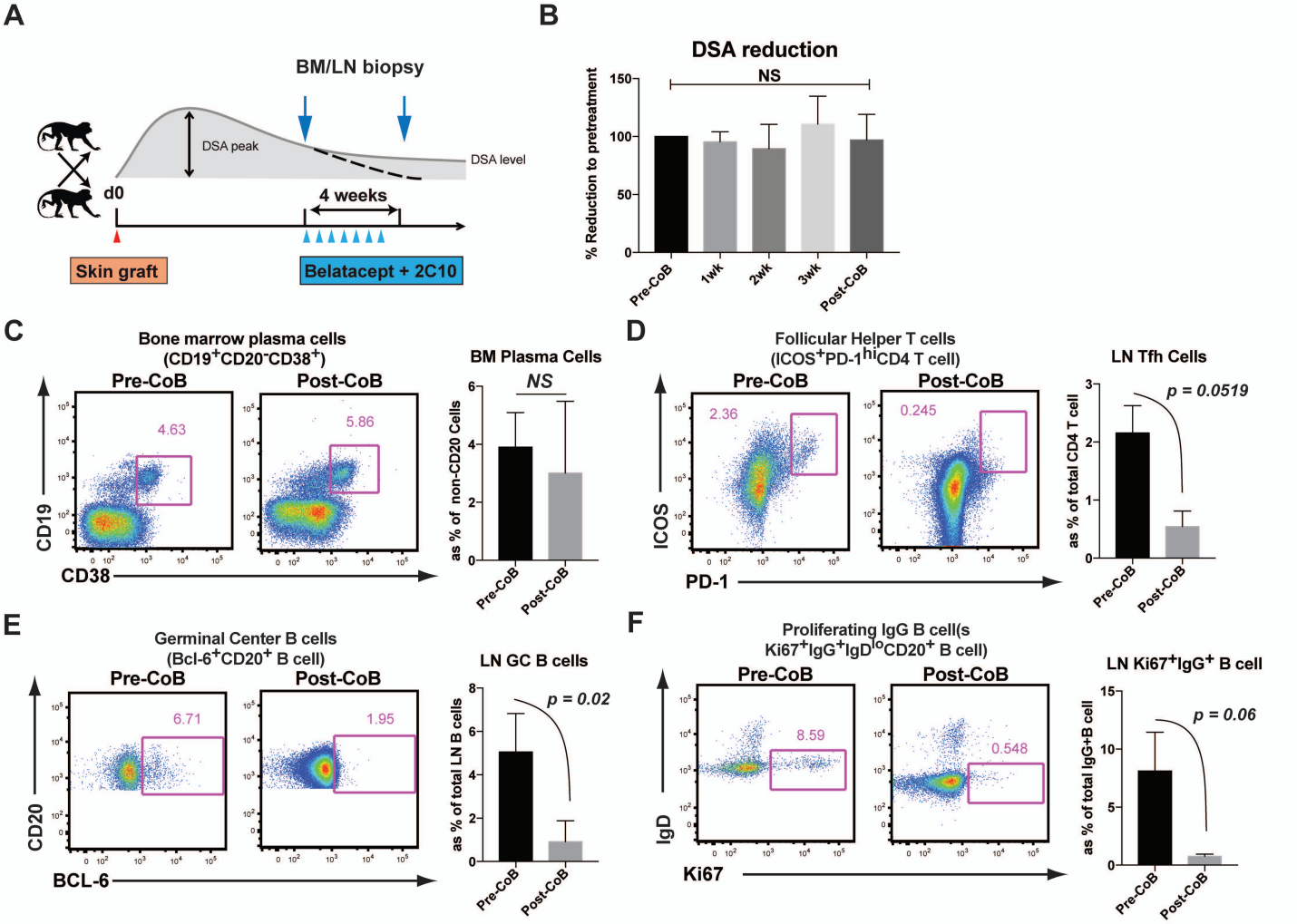
Routine light microscopy on H&E and PAS stained sections were performed on paraffin-embedded tissues obtained from biopsy or necropsy. Immunohistochemical evaluation with anti-human C4d (American Research Products, Waltham, MA) was performed as previously described [33]. The degree of rejection was evaluated blindly by a pathologist (A.B.F.) according to the Banff 2007 criteria [35], taking into consideration recent Banff updates [36]. Quantitative image analysis was performed as described in previous studies [33]. We immunostained using human Ki67 (clone MM1, Vector, Burlingame, CA), CD20 (Thermo Scientific, Rockford, IL), and CD3 (clone CD3-12, AbD Serotec, Raleigh, NC), appropriate secondary antibodies (Jackson ImmunoResearch, West Grove, PA) and nucleus dye (Hoechst 33342, Invitrogen, Carlsbad, CA). All images were acquired with an Axio Imager Z1 microscope (Zeiss) using 20x objectives. Mean fluorescence intensities of Ki67, CD3 and CD20 were analyzed using AxioVs40 V4.8.1.0 program (Zeiss) and Image J1.43u (NIH).

Statistics

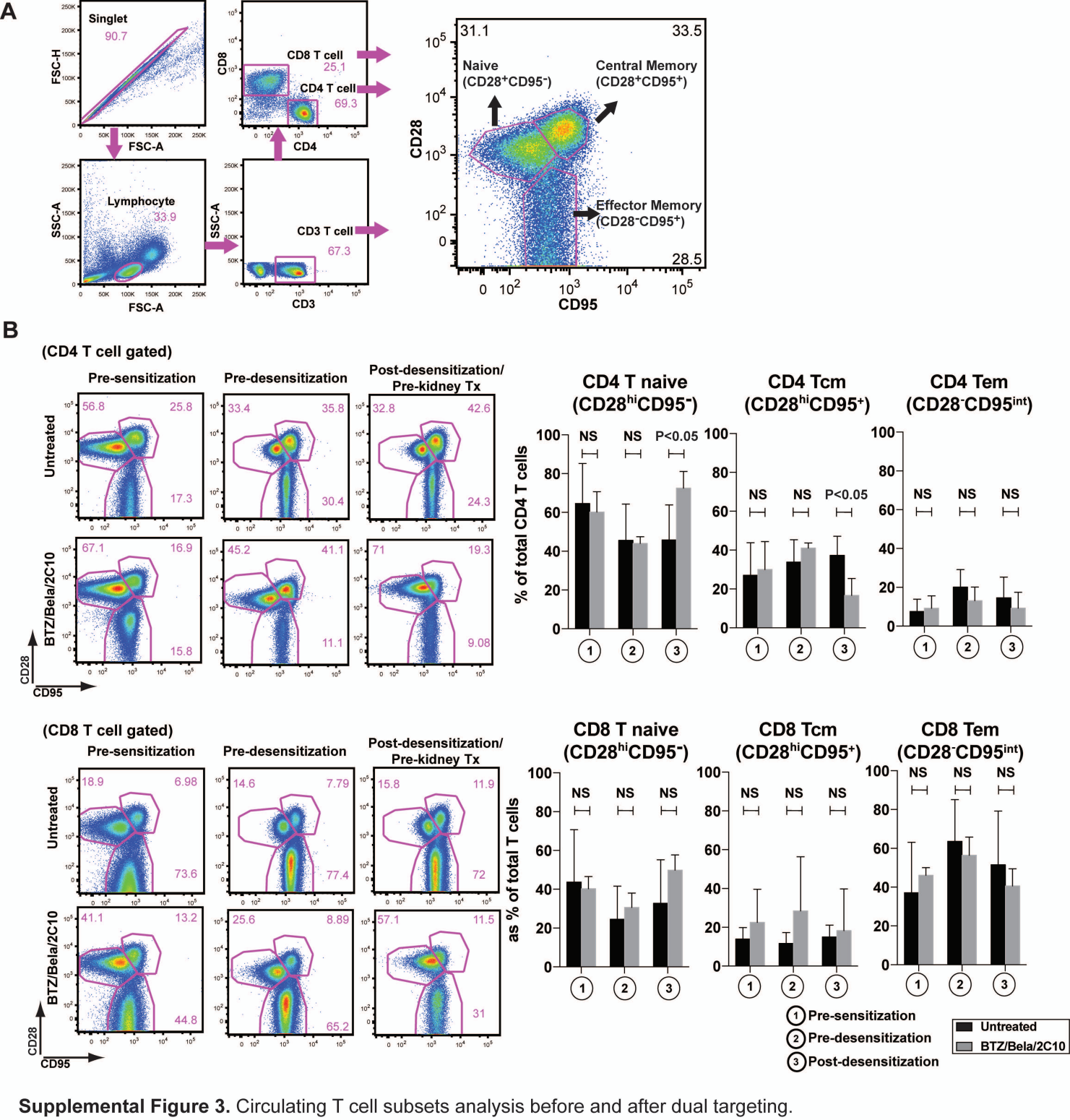
Experimental variables were analyzed by Prism statistical analysis program (GraphPad Software 7.0, San Diego, CA) using the Kaplan-Meier method and log-rank test for differences in graft survival. Sample comparisons of same animals were achieved by paired t-test and student t-test for others. Error bars represent the mean ± SD in all bar graphs. P values less than 0.05 were considered statistically significant.



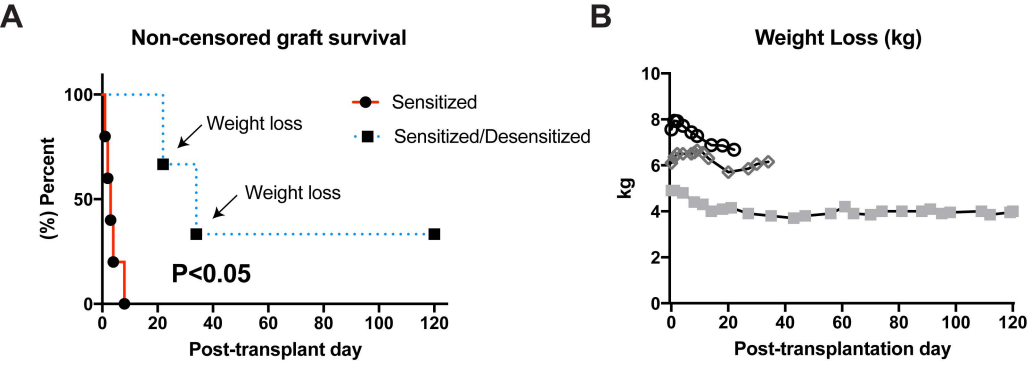
Supplemental Figure 1. MHC class I Mamu-A and Mamu-B haplotypes from skin/kidney donor and recipient rhesus macaques used in the study.



Supplemental Figure 2. Costimulation blockade with the combination of belatacept and anti-CD40 mAb (2C10) reduced follicular helper T cells (Tfh), germinal enter B (GC-B) cells, and proliferating IgG B cells in the lymph node but showed limited efficacy on plasma cells (PC) in the bone marrow.



Supplemental Figure 3. Circulating T cell subsets analysis before and after dual targeting.



Supplemental Figure 4. Non-censored graft survival and post-transplant weight loss.