Creation of an MHC-defined Rhesus macaque colony: microsatellite-based pedigree determination and MHC typing

We have performed an immunogenetic analysis on all potential transplant pairs, which has included both family tree analysis and MHC haplotype determination via DNA microsatellite mapping. (*1–7*) To accomplish this, DNA was first purified from 1cc of citrate-anticoagulated blood. Eight multiplexed PCR reactions were performed to obtain genotype data for 41 markers, 15 of which spanned the MHC region. Details of markers, primers and multiplex groups are summarized in Table S1. PCR reactions were separated by capillary electrophoresis on ABI 3730 instruments (Applied Biosystems, Foster City, CA). MHC-based family trees were created by first determining parent-offspring relationships, and then MHC haplotype sharing by analysis of inheritance of microsatellites between parents and offspring. A typical family pedigree display is shown in Fig. S1. As expected, and shown in this figure, genetic analysis revealed the harem-mating style of rhesus macaque colonies, in which highly reproductively-competent males mate with multiple females, producing large numbers of offspring that are related through the patriline. In this depiction, the sire, RMc-4, is placed at the center of a circular family tree, the 19 different females that he had mated with placed at the edges of the circle, and the resulting offspring shown connected to the sire/dam mating lines. In addition, each of the MHC haplotypes of each of the two parents is symbolized as a color-coded bar. Thus, the two MHC haplotypes from the sire are depicted as either a black or a red bar, with the two MHC haplotypes of each of the dams also depicted by unique color-coded bars. The MHC haplotype of the offspring between each mating pair is thus depicted with the inheritance of either black or red from the sire as well as one of the two maternal MHC haplotypes. Transplant pairs that were half-siblings, and matched on one MHC haplotype were then chosen for analysis.

HSCT protocol

Hematopoietic stem cells (HSCs) were harvested either by leukopheresis as previously described (8) or by terminal bone marrow (BM) harvest by flushing from the bilateral femurs, humeri, and, in some cases, the vertebral column, and filtering using the MH2150 Bone Marrow Collection Kit from Bio Access Inc. (Baltimore, MD). The choice of stem cell product was made based on donor animal weight: Animals that weighed more than 7 kg were suitable for leukopheresis. Animals that weighed less than 7kg could not be safely or effectively leukopheresed, and were therefore used as terminal BM donors. For one of the transplant recipients (R.5), stem cells were initially planned to be collected by leukopheresis, but the harvest was converted to a terminal marrow harvest when the donor became clinically unstable during the leukopheresis procedure. Thus, recipient R.5 received a stem cell product which included both leukopheresis and BM-derived cells. The total transplanted nucleated cell doses (TNC), total $CD3^+$ cell doses and $CD34^+$ cell doses are described in detail in these supplemental methods and shown in Table 1.

Pre-transplant preparation and post-transplant supportive care

The pre-HSCT preparative regimen consisted of total body irradiation (TBI) of 8 Gy with lung shielding to 6 Gy, given in a single fraction, using a Varian Clinac 23EX (Varian, Palo Alto CA), with a dose rate of 0.5 Gy/min. The peri-transplant supportive care strategy included gut decontamination with non-absorbable oral antibiotics (neomycin sulfate (Teva Pharmaceuticals, Sellersville, PA, 500 mg orally, given daily) and polymyxin B (Ben Venue Laboratories, Inc, Bedford, OH, 1,000,000 U orally, given daily), which were begun on day $^-6$ relative to the transplant and continued until the ANC was greater than 500 cells/microliter. (9, 10) Empiric

systemic antibiotic prophylaxis with enorofloxacin (Baytril®, Bayer Healtchare, Shawnee Mission, KS, 7mg/kg IM daily) was begun on day $^{-}1$ and continued until the ANC was greater than 500 cells/microliter. Cytomegalovirus prophylaxis with cidofovir (Vistide®, Gilead, Foster City, CA, 5 mg/kg IV weekly) was begun on day 21. Antifungal prophylaxis with fluconazole (5mg/kg po, daily) was begun on day $^{+}14$ relative to the transplant. Leukoreduced (using an LRF10 leukoreduction filter, Pall Medical, East Hills, NY) and irradiated (2200 rad) platelet-rich plasma or whole blood was given for a peripheral platelet count of $\leq 50 \times 10^3$ per μL or a hemoglobin < 9g/dL, respectively, or if clinically significant hemorrhage was noted. Blood product support adhered to ABO antigen matching principles. Other than antimicrobial and blood product support, no parenteral nutritional support nor adjunctive treatment for symptoms of GvHD were given to transplant recipients. Thus, the survival shown for the treated cohort represents a minimum survival, which would be expected to be longer if animals were given nutritional and other supportive care measures.

Post-transplant immunosuppression

Post-transplant immunosuppression consisted of combined CD28/CD40-based **Co**stimulation **B**lockade and **S**irolimus ("CoBS" immunosuppression, Fig. 1A). CD28 costimulation blockade was with CTLA4Ig (Bristol Myers Squibb, Pennington, NJ), which was dosed at 20mg/kg on day $^{-}2$, 0, 1, 5, 8 and weekly thereafter, continuing for the length of analysis. CD40 blockade was with a non-depleting anti-CD40 antibody, 3A8 (obtained through Keith Reimann, of the Non-Human Primate Reagent Resource, Boston MA). Dosing of the anti-CD40 antibody was as follows: 20mg/kg/dose × 2 doses on day $^{-}2$ and day 0, 10mg/kg/dose × three doses on days 1, 5,8, and then maintenance dosing at 5mg/kg/dose twice weekly thereafter, continuing for the length of analysis. Sirolimus (LC laboratories, Woburn MA) was given in an intramuscular formulation, with serum trough levels measured at the Emory University Hospital laboratories, and targeted at 5–15 ng/mL).

Pharmacokinetic and pharmacodynamic analysis of CoBS immunosuppression

1: Sirolimus: As shown in Fig. 6C, most CoBS-treated recipients maintained sirolimus levels within the targeted range (5–15 ng/mL). However, one animal, R.7 had a prolonged period of subtherapeutic sirolimus, with levels ranging from 2–3ng/mL for approximately 20 days prior to sacrifice.

2: CTLA4Ig: The CTLA4Ig dosing schedule resulted in significant CTLA4Ig levels (measured by ELISA by Bristol Myers Squibb), with serum trough levels which ranged from 73–240 μg/mL (not shown) which was similar to the peak serum measurements achieved in clinical trials of CTLA4Ig for arthritis. (12)

3: 3A8 anti-CD40 monoclonal antibody: The 3A8 dosing strategy achieved partial blockade of CD40 as measured by flow cytometry (56–68% blockade of CD40 expression on $CD20^+$ B cells, not shown). ELISA analysis of the 3A8 mouse IgG2b isotype (Bethyl Laboratories, Montgomery TX) showed that median serum trough levels of 1 μM, 1.4 μM, 5.9 μM and 4.3 μM for R.4-R.7, respectively, were attained. These trough levels have been shown to inhibit both $CD4^+$ and $CD8^+$ alloreactivity by 50–75% as measured by mixed lymphocyte reaction (Larsen et al., unpublished). Although transplant recipients were exposed to the mouse-derived 3A8 antibody, ELISA analysis demonstrated that they did not develop Rhesus anti-mouse antibodies (RAMA),

while a control animal given the anti-mouse antibody without concomitant use of sirolimus and CTLA4Ig did develop RAMA (not shown).

Creation of a Rhesus macaque model of GvHD: GvHD clinical grading, criteria for euthanasia and study endpoints

(A) Clinical GvHD staging: Staging of primate GvHD was performed daily using a variation of the standard clinical staging scale (11), and is shown in Table 2 of the main manuscript. This evaluation was comprised of individual skin, liver and gastrointestinal clinical scores as well as a determination of animal activity.

Skin rash was delineated as no rash (score= 0), <25% body surface area (score= 1), 25–50% of body surface area (score= 2), >50% body surface area (score= 3), or with bullae (score= 4).

Liver GvHD was delineated as bilirubin <4-fold increased over baseline (score = 0), bilirubin 4–8-fold higher baseline (score = 1), bilirubin 8–20-fold higher than baseline (score = 2), bilirubin 20–50-fold higher than baseline (score = 3) and bilirubin >50-fold higher than baseline (score = 4).

Given constraints of animal care and caging, diarrhea could not be quantified. Therefore, a qualitative gastrointestinal GvHD score was given, which was defined as: no diarrhea, described as formed stool (score= 0), "mild" diarrhea, described as soft, loose stool, not responsive to antibiotics (score= 1), "moderate" diarrhea, described as intermittent liquid stool, not responsive to antibiotics (score= 2), "severe" diarrhea, described as persistent liquid stool, not responsive to antibiotics (score= 3), and "very severe" diarrhea, described as persistent, bloody liquid stool, not responsive to antibiotics (score = 4). To determine the final gastrointestinal GvHD score, the daily stool scores for the thirty days prior to an animal's death were averaged.

The activity score allowed animal well-being/disability to be quantified. Each morning, transplant recipients were scored for their activity level by a member of the veterinary staff. Normal activity, described as normal interaction with the caregiver, active movement throughout the cage, and normal response to stimulation and food was given a score of "3". Activity that was moderately below what was expected for the animals, including altered interactions with the caregiver, decreased movement in the cage and decreased responsiveness to stimulation and food was given a score of "2". Activity that was severely altered compared to what was expected for the animals, including significantly decreased responsiveness to the caregiver, significantly decreased activity in the cage, and lack of response to stimulation or food was given a score of "1". An animal that was unable to respond was given a score of "0".

(B) Criteria for euthanasia: According to Yerkes National Primate Center animal care guidelines, euthanasia decisions were made both on clinical criteria and based on weight loss. Weight loss alone was a trigger for euthanasia, with animals losing >25% of their pre-transplant weight, without signs of acute, easily reversible causes, being euthanized for excessive weight loss. For this study, no parenteral nutritional support was given, and only intermittent, low-calorie enteral feeds were supplied. Thus, weight-maintenance depended on an animal maintaining adequate appetite and intake. Megesterol acetate was given at a dose of 100–400mg daily to all transplant recipients to help maintain appetite. Other clinical criteria for euthanasia included significant

change in mental status, inability to maintain adequate fluid intake, pain not adequately controlled by analgesic therapy, and activity levels persistently below normal. Animals that were unresponsive were considered for immediate euthanasia unless a readily reversible cause was found and corrected with reversal of the clinical presentation.

(C) Study end-points: The primary end-point of this study was survival at 30 days, comparing untreated recipients and recipients treated with CoBS-immunosuppression using a log-rank test. No parenteral nutritional support nor rescue treatment for GvHD signs and symptoms were included in this study, such that the clinical endpoints were unaffected by adjunctive supportive measures. Two secondary end-points were evaluated: The secondary clinical end-point of this study was overall survival, comparing the untreated cohort to the treated cohort when analysis was continued beyond 30 days. The secondary mechanistic endpoint was the immunophenotypic analysis, determining the immune profile that correlated with GvHD, and with protection from disease.

Characteristics of transplanted cells from either bone marrow or leukopheresis products
As shown in Fig. S2A–C, all transplanted animals received similar doses of total nucleated cells (TNC). Bone marrow (BM) recipients received an average TNC dose/kg of 10.3×10^8 $^{+/-}$ 2.1×10^8, while animals receiving a leukopheresis product received an average TNC dose/kg of 9.1×10^8 $^{+/-}$ 4.4×10^8 (Fig. S2A, p= 0.45). Animals receiving BM allografts received more $CD34^+$ cells than those receiving leukopheresis products: (5.0×10^7 $^{+/-}$ 1.2×10^7 compared to 1.9×10^7 $^{+/-}$ 0.2×10^7 p <0.03, Fig. S2B). Conversely, animals receiving a BM transplant received an average $CD3^+$ T cell dose/kg of 3.9×10^7 $^{+/-}$ 1.4×10^7, while animals receiving a leukopheresis product received significantly more $CD3^+$ T cells/kg, with the average dose/kg being 21.0×10^7 $^{+/-}$$3.6 \times 10^7$ (p<0.01, Fig. S2C).

Flow cytometric analysis
(A) Phenotypic analysis: Multicolor flow cytometric analysis was performed on all transplant recipients, using the following leukocyte phenotypic characteristics: T cells: $CD3^+/CD20^-$; B cells: $CD20^+/CD3^-$; NK cells: $CD3^-/CD20^-/CD16^+/CD8^+$; $CD4^+$ T cells: $CD4^+/CD3^+/CD8^-/CD20^-$; $CD8^+$ T cells: $CD8^+/CD3^+/CD4^-/CD20^-$; Tregs: $CD3^+/CD4^+/FoxP3^+$; Naïve T cells (Tn): $CD28^+/CD95^-$ cells in either the CD4 or CD8 T cell subsets. Central memory T cells (Tcm) $CD28^+/CD95^+$ cells in either the CD4 or CD8 T cell subsets. Effector/Effector memory T cells: $CD28^-/CD95^+$ cells in either the CD4 or CD8 T cell subsets.(13) In addition, the level of CD127, Ki-67 and BCl-2 were determined on both $CD4^+$ and $CD8^+$ T cells, and the level of expression of CD25, CD127 and CD27 was determined on $CD4^+/FoxP3^+$ T cells. In addition to the relative percentages of each of these subpopulations, absolute numbers of each of the subpopulations were determined by calculations from the complete blood count and absolute lymphocyte count analysis. The sources and clones used for each of these antibodies are as follows: From BD Biosciences (San Jose CA) CD3, Clone SP34-2; CD8, Clone: RPA-T8; CD16, Clone 3G8; CD25, Clone M-A251; CD127, Clone Hil-7R–M21, BCl-2, Clone: Bcl-2/100. From eBioscience (San Diego CA): CD4, Clone OKT4; CD20, Clone: 2H7; CD27, Clone O323; CD28, Clone: CD28.2; CD95, Clone: DX2; CD127 Clone eBioRDR5; FoxP3, Clone: PCH101. From Dako (Glostrup, Denmark): Ki67, Clone Ki-67.

(B) CFSE MLR analysis: CFSE MLR assays were performed as previously described (14) either with unfractionated responder T cells or with responder T cells that were obtained after flow cytometric purification based on expression levels of CD28 and CD95. For CTLA4Ig inhibition experiments, cells were incubated with a increasing concentrations of CTLA4Ig (0.8 μM, 1.6 μM, 3.2 μM, 6.4 μM and 12.8 μM). A representative example showing the proliferation profile after treatment with 1.6 μM is shown in Fig. 6E, however, all of the CTLA4Ig concentrations that were tested resulted in similar inhibition of proliferation of $CD28^+$ T cells.

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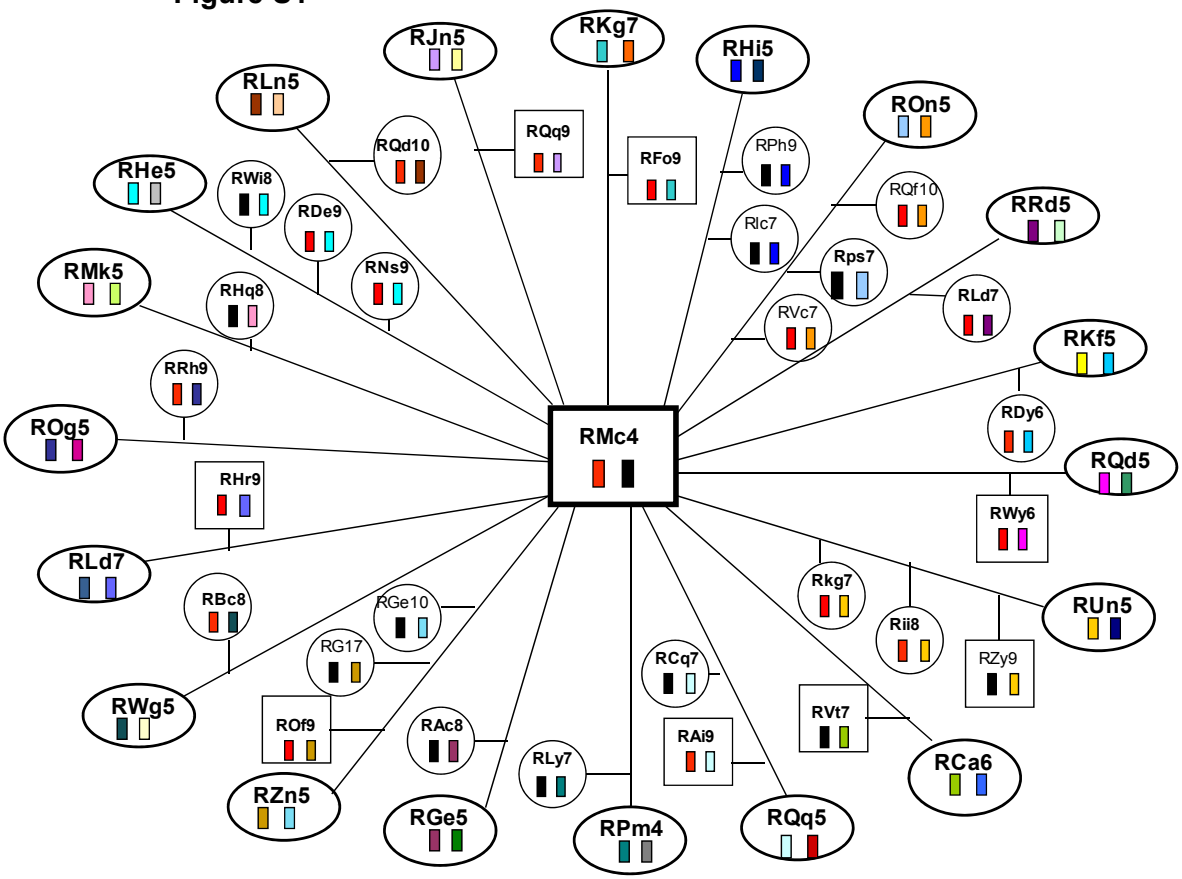
Locus [a]	Mmu # [b]	Dye	Multiplex[c]	Forward Primer (5'-3')	Reverse Primer (5'-3')[d]	µM in PCR	Size Range [e]
D1S548	1	NED	C1	GAACTCATTGGCAAAAGGAA	gttcttGCCTCTTTGTTGCAGTGATT	0.09	190-210
D2S1333	12	NED	C1	CTTTGTCTCCCCAGTTGCTA	TCTGTCATAAACCGTCTGCA	0.18	269-341
D3S1768	2	FAM	A2	GGTTGCTGCCAAAGATTAGA	CACTGTGATTTGCTGTTGGA	0.08	181-233
D4S2365	5	FAM	A2	AGTAATTCTTCAACTGCATCACC	ATGCCAAGGATGGTGAGTTA	0.16	117-151
D4S413	5	VIC	C2	TCTGAATATAGTGCTCCAGAAA	CAATCAGTGGGTTTTTGAA	0.31	271-307
D5S1457	6	NED	A2	TAGGTTCTGGGCATGTCTGT	gttcttTGCTTGGCACACTTCAGG	0.09	112-148
D6S501	4	NED	B2	GCTGGAAACTGATAAGGGCT	GCCACCCTGGCTAAGTTACT	0.06	160-196
D7S513	3	VIC	A1	AGTGTTTTGAAGGTTGTAGGTTAAT	ATATCTTTCAGGGGAGCAGG	0.13	185-249
D7S794	3	FAM	A1	ACCATACTCCTCAGCCTCCA	GTGTTCGGGTTCTCCAAAGA	0.09	108-140
D8S1106	8	FAM	C1	GCGGCATGTTTTCCTACTTT	TTCTCAGAATTGCTCATAGTGC	0.08	132-188
D9S921	15	PET	C1	CCTGGAGAATCTTGTGATGC	gttcttTCTTTCATGTTGGCTCCTGT	0.10	167-203
D10S1412	9	VIC	B2	TGCCTTAGCTCCTGCATACTGA	GGGACAGTTCTTCTCCCTCCA	0.06	154-166
D11S2002	14	VIC	B2	AGTAGTAGGAGGCCCCAAGG	CAAGCAATCCTCCCACCTTA	0.11	244-272
D11S925	14	VIC	A1	GCTCCTCCAGTAATTCTGTC	TTAGACCATTATGGGGGCAA	0.25	298-348
D12S364	11	FAM	B2	TTGGGAAAGTCGTTTTGCAT	TGAGACTCAAATCCCCTGGA	0.22	264-296
D12S67	11	PET	B2	GCAACAGTTTATGCTAAAGC	GCCTATGCAGTTCAAATCTA	0.47	105-244
D13S765	17	NED	A2	TGTAACTTACTTCAAATGGCTCA	TTGAAACTTACAGACAGCTTGC	0.12	196-272
D15S823	7	VIC	C1	GGCTTTGCATCCAGAATTTA	gtttcttCACTTCCAACACTGAGGATC	0.13	317-385
D16S403	20	PET	A1	GTTTTCTCCCTGGGACATTT	TATTCATTTGTGTGGGCATG	0.56	140-182
D17S1300	16	PET	A2	TAGTGTGTATATATGTATGCATGCA	ggataacaatttcacacaggTGCAGATATCTGTCTTTTGGC	0.22	224-328
D18S537	18	VIC	C1	TCCATCTATCTTTGATGTATCTATG	gttcttAGTTAGCAGACTATGTTAATCAGGA	0.14	162-178
D18S72	18	NED	B1	GCTAGATGACCCAGTTCCC	CTGCAGAAAGGTTACATATTCCA	0.18	302-344
D22S685	10	PET	C2	TTCTCAGTGGGGGAGGGAT	TGGAGTTTGATGTTTTTGAGAGAC	0.25	223-267
DXS2506	X	VIC	C1	GGAGAAATGGGGAGTAACTG	gttcttACACATGGCTGGCTAGCTT	0.09	258-296
MFGT21	8	FAM	B1	AACTTCAGTAAGATAAGGACC	CCTGAGGTCTGGACTTTAT	0.20	93-133
MFGT22	?	VIC	B1	CAACATAGAGAGATTCCATCTC	CGTTAAGTATGATGTTAGCTAG	0.25	94-128
MHC-linked							
D6S291	4	VIC	B1	CTCAGAGGATGCCATGTCTAAAATA	GGGGATGACGAATTATTCACTAACT	0.16	177-231
D6S2741	4	VIC	D	AGACTAGATGTAGGGCTAGC	CTGCACTTGGCTATCTCAAC	0.03	247-297
D6S2876	4	FAM	D	GGTAAAATTCCTGACTGGCC	GACAGCTCTTCTTAACCTGC	0.04	194-252
9P06	4	NED	E	CACTAACGATAGCTGATGAGCTTAAA	TGCACATCCCTGTATATCAAGC	0.11	175-191
D6S2883	4	NED	D	TGGAATCTCATCAAGGTCAG	TTGAAATTGATACTTTCCCAGTTCTC	0.03	112-152
MICA	4	NED	D	CCTTTTTTTCAGGGAAAGTGC	CCTTACCATCTCCAGAAACTGC	0.03	185-209
246K06	4	NED	E	GCCCAATAGCAAGCCAAGAA	TGGTGAGGGGATTTCTCTGAA	0.05	271-287
162B17A	4	VIC	E	ACAGCCTCACCAACACCTGA	CCCCTTCTCTCCCCAAAGAT	0.15	238-252
162B17B	4	FAM	E	GAAGATGTGCCCATTTCCAGA	TTTCCACCACTGCCTTCTCA	0.22	281-317
151L13	4	PET	E	AGGGCATCTCAGGCATTCAT	GGGGGAGGGATAGCATTAGG	0.03	300-326
MOG-CA	4	FAM	D	GAAATGTGAGAATAAAGGAGA	GATAAAGGGGAACTACTACA	0.19	107-137
268P23	4	FAM	E	TCAGAAATGTGAGAATAAAGGAGACA	TGAAGCATTGGAAGGCAAAA	0.09	148-156
222I18	4	VIC	E	GGAGGGAGGGAGAGAAAGTCA	GCCTCGGCACTCACACATTA	0.03	161-177
D6S276	4	NED	B1	TTCCAGTGTATACATCAATCAAATCA	GGGTGCAACTTGTTCCTCCT	0.28	195-245
D6S1691	4	FAM	B1	AGGACAGAATTTTGCCTC	GCTGCTCCTGTATAAGTAATAAAC	0.22	196-222

Table S1. Microsatellite marker panels used to establish pedigrees and MHC haplotypes. [a]: Names with D prefix correspond to human nomenclature for markers; [b]: Rhesus macaque chromosome number in Jan 2006 MSGC Merged 1.0/rheMac2 draft assembly (http://genome.ucsc.edu/cgi-bin/hgGateway); [c]: Multiplexes with same group letter (e.g. A1 and A2) were combined for electrophoresis; [d]: bases in lower case are tails added to primers; [e]: allele size range does not include tails.

Figure S1. Representative family tree showing both parentage analysis and MHC haplotype segregation
The sire is placed in the middle of the family tree, with all of the mating dams along the outside of the resulting circle. Offspring from individual mating pairs are depicted in the center, connected to both parents. MHC haplotypes are color coded for both the sire and the dams, and the segregation of the color-coded MHC genes is shown in the offspring.

Figure S2. Cell doses in transplanted animals
(A) Comparison of the total nucleated cell (TNC) dose that animals received from either a BM-derived stem cell product (black) or a leukopheresis-derived stem cell product (red). (B) Comparison of the $CD34^+$ cell dose that animals received from either a BM-derived stem cell product (black) or a leukopheresis-derived stem cell product (red). (C) Comparison of the $CD3^+$ cell dose that animals received from either a BM-derived stem cell product (black) or a leukopheresis-derived stem cell product (red).

Figure S1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RDe9
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RDy6
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RG17
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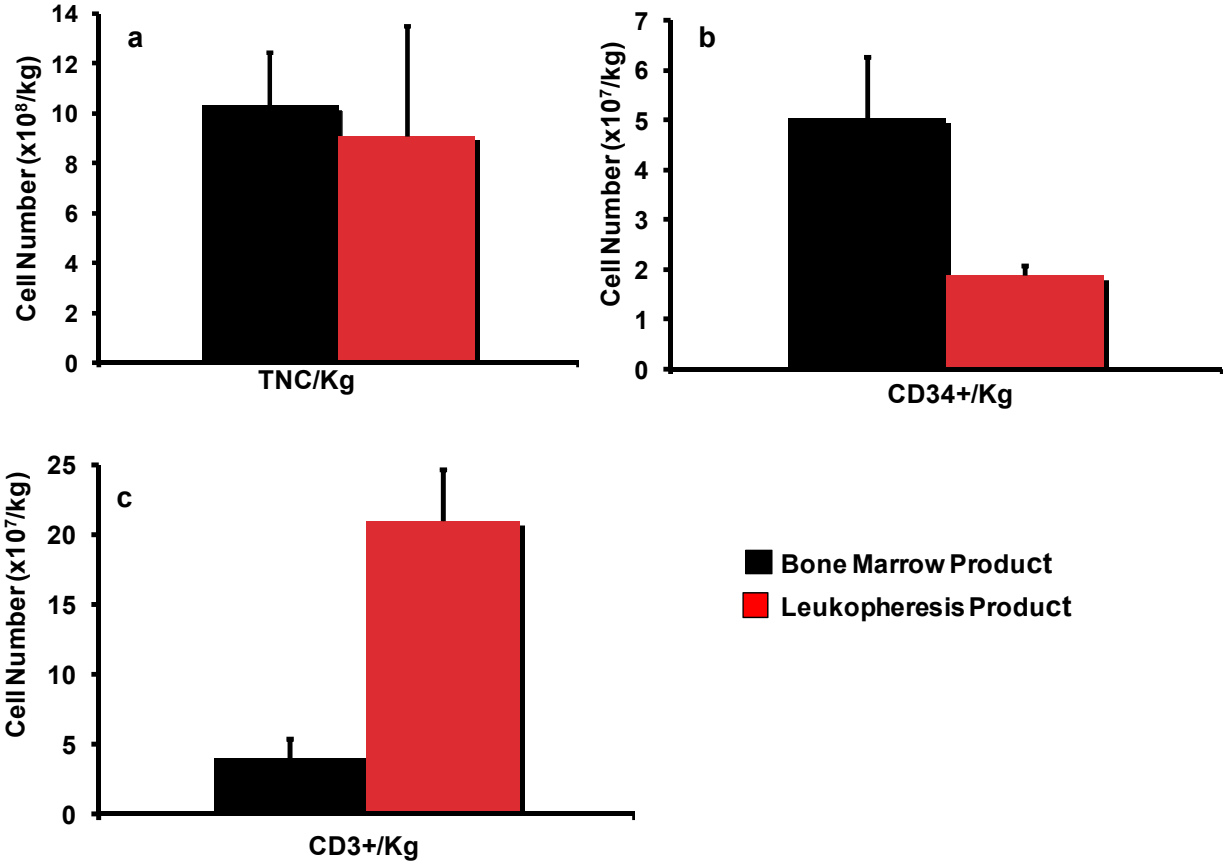
Figure S2


a
Cell Number (x10^8/kg)
14
12
10
8
6
4
2
0
TNC/Kg
b
Cell Number (x10^7/kg)
7
6
5
4
3
2
1
0
CD34+/Kg
c
Cell Number (x10^7/kg)
25
20
15
10
5
0
CD3+/Kg
Bone Marrow Product
Leukopheresis Product