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Induction of Chimerism in Rhesus Macaques through Stem Cell Transplant and Costimulation Blockade-Based Immunosuppression

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A strategy for producing high-level hematopoietic chimerism after non-myeloablative conditioning has been established in the rhesus macaque. This strategy relies on hematopoietic stem cell transplantation after induction with a non-myeloablative dose of busulfan and blockade of the IL2-receptor in the setting of mTOR inhibition with sirolimus and combined CD28/CD154 costimulation blockade. Hematopoietic stem cells derived from bone marrow and leukopheresis products both were found to be successful in inducing high-level chimerism. Mean peripheral blood peak donor chimerism was 81% with a median chimerism duration of 145 days. Additional immune modulation strategies, such as pre-transplant CD8 depletion, donor-specific transfusion, recipient thymectomy or peritransplant deoxyspergualin treatment did not improve the level or durability of chimerism. Recipient immunologic assessment suggested that chimerism occurred amidst donor-specific down-regulation of alloreactive T cells, and the reappearance of vigorous T-mediated alloreactivity accompanied rejection of the transplants. Furthermore, viral reactivation constituted a significant transplant-related toxicity and may have negatively impacted the ability to achieve indefinite survival of transplanted stem cells. Nevertheless, this chimerism-induction regimen induced amongst the longest-lived stem cell chimerism reported to date for non-human primates and thus represents a platform upon which to evaluate emerging tolerance-induction strategies.

Key words: chimerism, costimulation, non-human primate, nonmyeloablative, tolerance, transplant

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Introduction

In murine transplantation, the induction of mixed hematopoietic chimerism is a potent method of promoting transplant tolerance (1). Early strategies resulted in full donor chimerism, and although tolerance ensued, significant immunodeficiencies occurred when performed between major histocompatibility complex (MHC) disparate transplant pairs (2–4). This incited interest in establishing mixed chimerism, which could potentially increase immunocompetence (4). Initial approaches involved near-complete, non-selective donor T-cell depletion (1,5). More recently, an emphasis on selective T-cell deletion/inactivation using transient blockade of costimulatory pathways has produced mixed chimerism in mice (1,6,7). Costimulation blockade induces selective deletion of peripheral donor-reactive cells (6–9), which, when combined with the maintenance of tolerance by thymic deletion of emerging donor-reactive T cells (6,7,10,11), allows long-term acceptance of MHC disparate grafts.

Given its potential importance to clinical transplantation, the translation of sustained chimerism to pre-clinical non-human primate (NHP) models has been actively pursued. Kawai and colleagues used a combination of total body irradiation, local thymic irradiation, anti-thymocyte globulin (ATG), anti-CD154 and cyclosporin to promote acceptance of donor bone marrow (12–18). Additionally, select examples in human transplantation support the potential utility of chimerism-induction-based acceptance of solid organ transplants (19–22). In NHP, these strategies achieved donor chimerism, although its durability was relatively shorter than in mice (13,14,16–18). Despite an incomplete understanding of the underlying mechanisms, these studies provide proof of concept that even transient chimerism can lead to tolerance to solid organ allografts.

The induction of sustained chimerism after hematopoietic stem cell transplant (HSCT) remains a significant goal

after non-myeloablative transplant. Successful induction of sustained chimerism has two major potential clinical applications: first, in organ transplantation, in order to promote robust donor-specific tolerance with freedom from chronic immunosuppression; and second, in non-malignant hematologic disease and inborn errors of metabolism, where such an induction is capable of curing disease (23–26). Correcting hematologic and genetic abnormalities will require durable macrochimerism and thus, these diseases are not amenable to transient chimerism-induction. Herein, we describe a T-cell costimulation blockade-based regimen in NHP that reliably induces high-level chimerism that persists for as many as 196 days post-transplant in the setting of ongoing immunosuppression. While the risk-benefit ratio for selected patients severely affected with hematologic disease may support a strategy requiring chronic immunosuppression, further advances will be required for chimerism and tolerance-induction for organ transplantation.

Methods

Experimental animals

This study utilized rhesus macaques from either the Yerkes National Primate Research Center or the NIH-sponsored NHP colony at Yemassee, SC, managed by Alphagenesis, Inc. All animals were treated in accordance with IACUC regulations. Donor-recipient pairings were based on MHC typing (27–29) to maximize disparity at both class I and class II loci.

Sustained chimerism-induction strategy

The standard chimerism-induction strategy (abbreviated 'CoBBS') is shown in Figure 1. It consisted of a pre-transplant busulfan infusion (10 mg/kg Busulfex, ESP Pharma, Clinton, NJ), followed by immune modulation with: (i) Two doses of the anti-IL2-receptor antibody basiliximab (Novartis, Basel, Switzerland, 0.3 mg/kg) within the following treatment windows: first, day –1 or day 0; and second, day 3 or day 5. (ii) Blockade of CD28 signaling with the belatacept fusion protein (20 mg/kg/dose, supplied by Bristol Myers Squibb, NY) and CD154 blockade with the H106 monoclonal antibody (20 mg/kg/dose, supplied by Bristol Myers Sqibb), each infused on the following days relative to transplant: Belatacept on day −1, +3, 15, 29, 43, 57, 71, 85, 99, 113, 127, and H106 on day −1, +1, 3, 6, 8, 10, 15, 29, 43, 57, 71, 85, 99, 113, 127. (iii) Sirolimus (Wyeth-Ayerst, Madison, NJ) was dosed daily beginning at day −3 and continued through day 77–100 post-transplant (Table 1), to maintain troughs of 5–15 ng/mL.

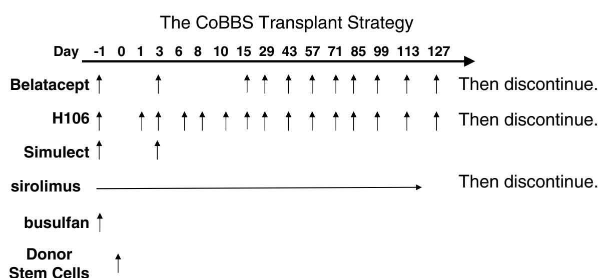

Figure 1: Transplant strategy: Our standard chimerism-induction strategy included a single non-myeloablative dose of busulfan given on day −1 relative to the transplant and induction with two peritransplant doses of the IL2-receptor blocking monoclonal antibody basiliximab, and maintenance therapy with anti-CD28/anti-CD154 CoB (belatacept and H106) and sirolimus. This strategy, referred to as 'CoBBS', when accompanied by a stem cell infusion on day 0 routinely produced mixed chimerism in transplant recipient animals. While the treatment with CoB and sirolimus ideally continued until day 100–127 after stem cell transplant, the clinical condition of the transplant often necessitated early withdrawal of one or more of these agents, as indicated in Table 1.

Modifications to CoBBS

CD8 depletion

CD8 depletion occurred after OKT8 treatment (5 mg/kg) on days −5, −3, 0, 2, 4, 7, 9, 11, 14 relative to bone marrow transplant (BMT).

Donor-specific transfusion

A 6 mL/kg whole blood transfusion was given 1 week prior to BMT. After donor-specific transfusion (DST), belatacept and H106 infusions were given on days −6, −4 and −1 and sirolimus was begun on day −6.

Thymectomy

Recipient animals were surgically thymectomized (as previously described (30)) 14–21 days prior to BMT.

Deoxyspergualin

Recipient animals were treated with the LF15-0195 analog of deoxyspergualin (DSG) (Richman Chemical, Lower Gwynedd, PA) (31,32) dosed at 0.3 mg/kg subcutaneously on days −1 through 14, followed by a second 14 day course (0.3 mg/kg/dose) beginning either on day 79 (animal RDp-8) or 91 (animal RVf-8).

HSCT

Bone marrow

Bone marrow was harvested from the vertebral column, long bones and pelvis of terminal donors and then centrifuged at 1500 RPM for 8 min prior to resuspension in RPMI plus 0.2 mg/mL DNAse (Sigma) for 45 min at 37°C. Finally, it was recentrifuged, filtered through a 70μM filter and resuspended in sterile PBS plus heparin (3 u/mL).

Peripheral blood stem cell infusion

Leukopheresis was performed after donors received 8 days of G-CSF (100 μg/kg, Amgen, Thousand Oaks, CA). The femoral vein was catheterized (9F Duo Flow catheter, Medcomp, Harleysville, PA) prior to leukopheresis with a Cobe Spectra (Gambro BCT, Lakewood, CO) using the auto-PBSC settings and human albumin priming. The leukopheresis circuit was anticoagulated with sodium citrate and the animal concomitantly infused with Calcium

Table 1: Characteristics of transplant recipients, hematopoietic stem cell products and transplant outcomes

Animal ID	Treatment	Cell dose/kg ($\times 10^9$)	Peak chimerism %	Chimerism duration (days)	Day of sirolimus d/c	Indication of sirolimus d/c	Day of CoB d/c	Indication for CoB D/C
RAf-7	CoBBS	5.4	0	0	90	Diarrhea, dehydration	112	Per protocol
RHf-7	CoBBS	4.8	nd	nd	9	Herpes B reactivation	9	Herpes B reactivation[3]
RUn-7	CoBBS	5.3	65	63	9	Herpes B reactivation	112	Per protocol
RYj-7	CoBBS	4.7	100	174	62	CMV	112	Per protocol[4]
RRi-8	CoBBS	7.2	90	189	78	CMV	127	Per protocol
RUi-8	CoBBS	2.7	60	161	78	CMV	127	Per protocol
RWb-8	CoBBS	5	90	119	77	CMV	127	Per protocol
CW7B	CoBBS[1]	2.1	90	133	90	CMV	180	Per protocol
RVq-8	CoBBS[1,2]	2	99	165	161	Animal euthanized	188	Animal euthanized[5]
RAi-7	CoBBS + CD8 depletion	nd	95	196	98	Colitis, dehydration	98	Colitis, dehydration
RMf-7	CoBBS + CD8 depletion	5.9	70	175	97	Per protocol	112	Per protocol
RUh-7	CoBBS + CD8 depletion	6.8	100	36	36	Herpes B reactivation	28	Herpes B reactivation[6]
RBg-7	CoBBS + CD8 depletion + DST	2.5	30	133	100	Per protocol	140	Per protocol
RTd-7	CoBBS + CD8 depletion + DST	2.7	20	77	100	Per protocol	140	Per protocol
RGy-6	CoBBS + CD8 depletion + DST	3.7	20	77	70	Per Protocol	42	Per protocol
RSk-7	CoBBS + thymectomy	2.6	60	133	100	Per protocol	112	Per protocol
RUu-7	CoBBS + thymectomy	4	20	49	100	Per protocol	112	Per protocol
RVu-7	CoBBS + thymectomy	4.9	15	56	38	Acute illness	100	Per protocol
CG8B	CoBBS + DSG	5.6	60	56	60	Severe diarrhea	55	Severe diarrhea[7]
RDp-8	CoBBS + DSG	3.5	100	84	86	CMV	71	CMV[8]
RVf-8	CoBBS + DSG	4.8	90	112	100	Per protocol	85	Per protocol

[1]For CW7B and RVq-8, chimerism induced after a transplant of peripheral blood stem cells rather than from a bone marrow product.
[2]RVq-8 initially received a leukopheresis stem cell product that was inadequate to induce chimerism: While the original stem cell product was produced after mobilization with 10 μg/kg GCSF, subsequent leukopheresis products were produced after donor stem cell mobilization with 100 μg/kg GCSF. RVq-9 was thus given a second transplant. All measurements with RVq-8 are relative to the second, successful stem cell infusion.
[3]Animal was euthanized due to complications of Herpes B reactivation.
[4]Animal died from acute renal failure and campylobacter sepsis.
[5]Animal was euthanized in the setting of pancytopenia and a GI bleed after loss of chimerism.
[6]Animal was euthanized due to complications of Herpes B reactivation.
[7]Animal died from bacterial typhlitis.
[8]Animal was euthanized due to complications of CMV reactivation.

Gluconate (2 g per 4 h). iSTAT (Abbott Point-of-Care, East Windsor, NJ) chemistry and hematology analysis was performed to assure calcium homeostasis. Hypotension was treated either with volume or vasopressors (dopamine, maintaining diastolic blood pressure >30 mmHg). Typically, six harvests (using a 5 mL harvest volume and 7 mL chase volume) were collected and infused into the recipient without further processing.

Analysis of the hematopoietic stem cell product

Both bone marrow and leukopheresis products were analyzed for the following: (i) total nucleated cell dose, (ii) $CD34^+$ cell dose, (iii) $CD3^+$ T-cell dose, (iv) $CD4^+$ T-cell dose, (v) $CD4^+/CD25^+$ T-cell dose, (vi) $CD8^+$ T-cell dose and (vii) B-cell dose. This analysis was performed either by automated CBC or by flow cytometric analysis with anti-CD34 (clone 563), anti-CD3 (clone SP34), anti-CD4 (clone SK3), anti-CD8 (clone SK1), anti-CD25 (clone 3G10) and anti-CD20 (clone L27), all antibodies from Pharmingen, San Jose, CA.

Chimerism monitoring

Donor Mamu A*01, A*03/04, A*08 and/or B*01 MHC alleles were monitored by real-time SybrGreen PCR (ABI, Foster City, CA) (29,33–35) or for the *SRY* gene in male-to-female transplantation (36). Sixty nanograms of genomic DNA were used in a 20 μL reaction, containing 200 mM dNTPs, 0.1 U AmpliTaq DNA polymerase and XU-AmpERASE-UNG. Reactions were amplified for 40 cycles using a 7900HT Sequence Detection Systems (Applied Biosystems, Foster City, CA). To construct a standard curve, 60 nanogram mixtures of donor and recipient

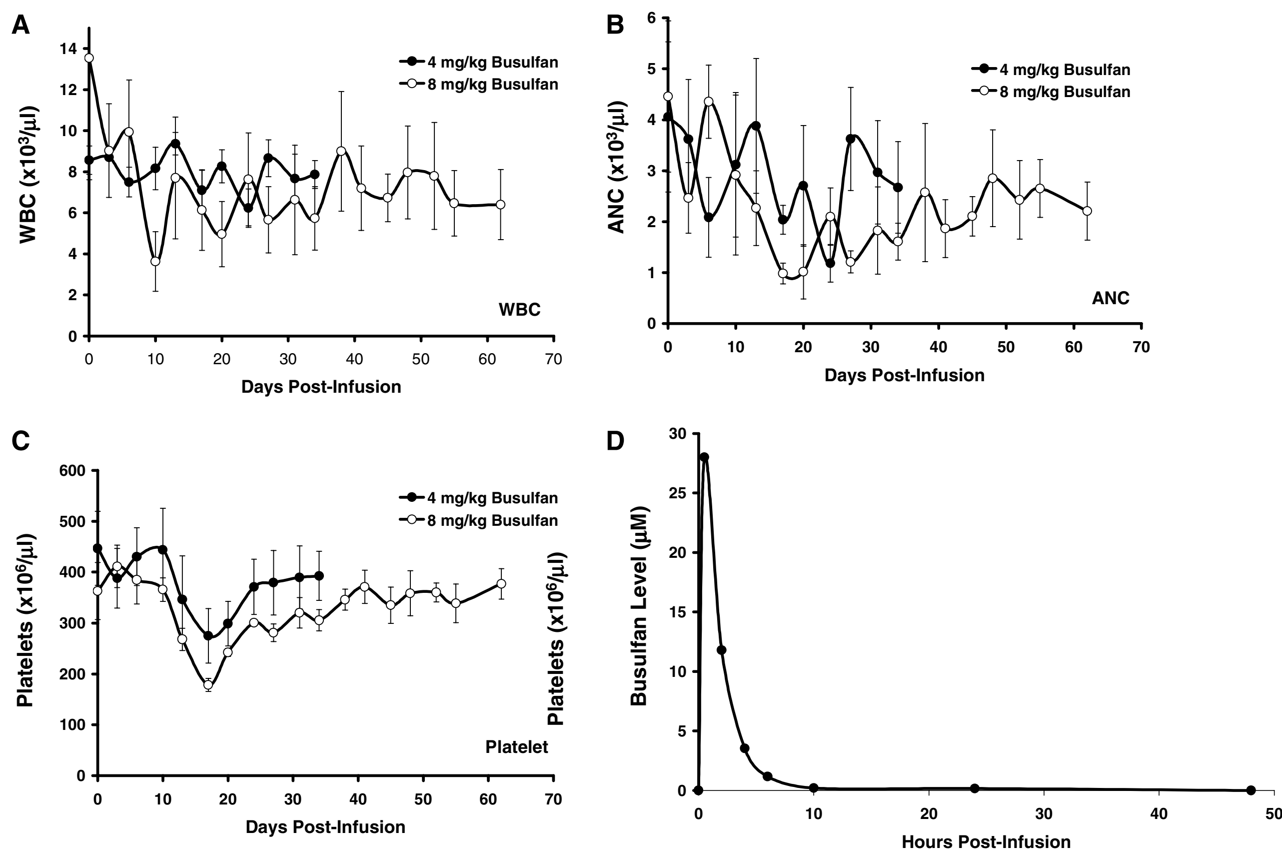

Figure 2: Pre-transplant infusion of 10 mg/kg busulfan achieves myelosuppression but not myeloablation and is lymphocyte sparing. (A–C) Effect of dosing with either 4 mg/kg or 8 mg/kg busulfan on WBC count (Figure 2A), ANC (Figure 2B) and platelet count (Figure 2C). Shown is the mean ±SEM (n = 3). (D) A single 10 mg/kg busulfan dose is cleared from the peripheral blood within 10 h after infusion. After the busulfan infusion, serial blood draws were collected and analyzed at the Emory University clinical laboratory for serum busulfan levels. Shown is the clearance of busulfan over time in a single representative animal (of three animals in which this pharmacokinetic analysis was performed). (E–H) Analysis of hematopoiesis in four animals receiving a single 10 mg/kg busulfan infusion. Serial WBC (Figure 2E), ANC (Figure 2F), platelet counts (Figure 2G), hemoglobin concentration (Figure 2H) and absolute lymphocyte counts (Figure 2I) were performed either using a standard veterinary CBC analyzer and manual differential counting (Figure 2E–H) or FACScan flow cytometer (Figure 2I). Shown are the results of these analyses in four animals separately treated with 10 mg/kg busulfan. Units on these measurements are as follows: WBC: $\times 10^3/\mu L$, ANC: $\times 10^3/\mu L$, platelets: $\times 10^6/\mu L$, Hb: g/dL, absolute lymphocyte counts: $\times 10^3/\mu L$.

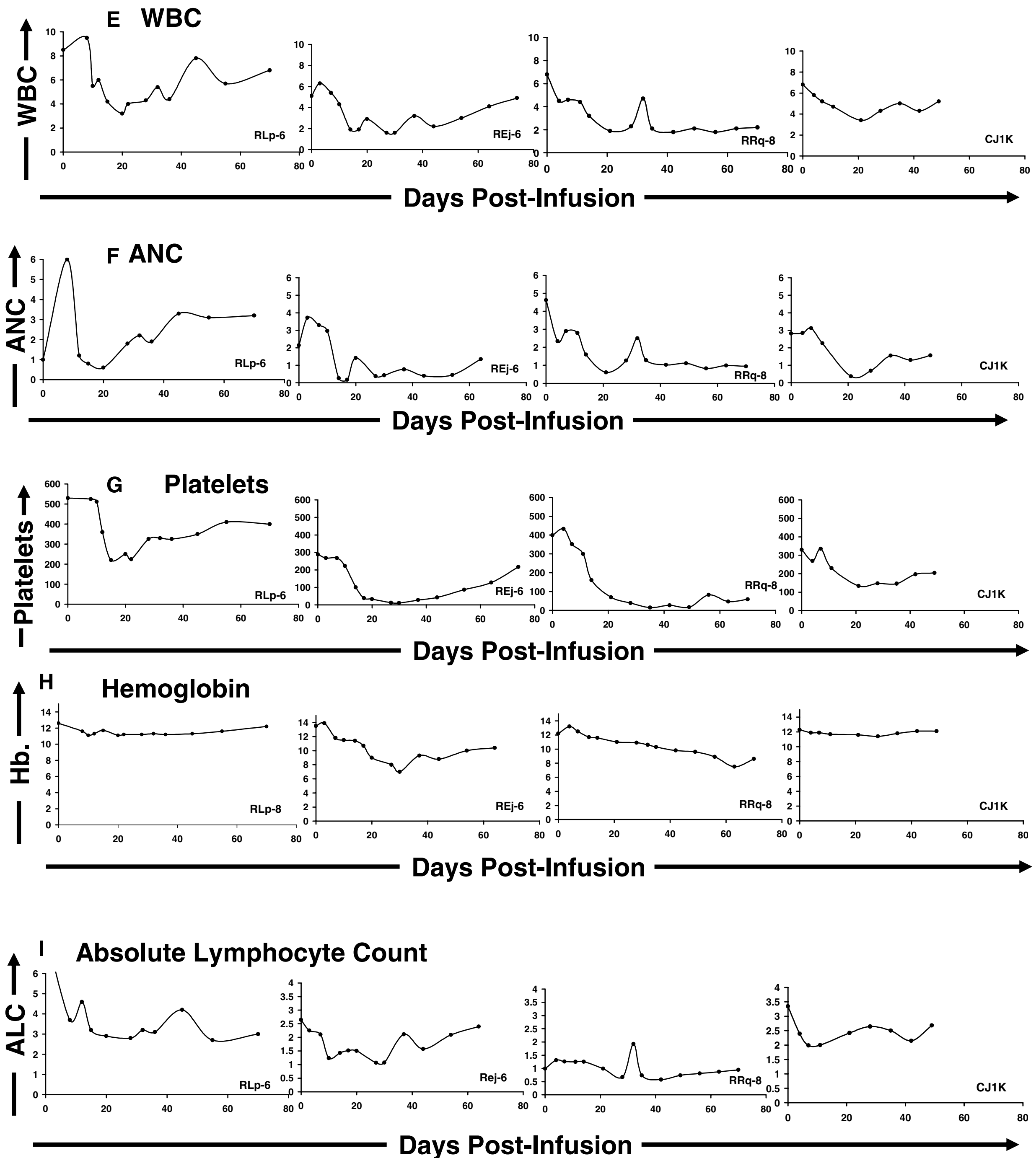

Figure 2: Countinued.

DNA (representing 5%, 10%, 50% and 100% donor DNA) were prepared and used as PCR templates.

Lineage chimerism determination

T cell, B cell and granulocyte lineages were sorted using a Miltenyi MACS system (Miltenyi, Auburn, CA) or a FACSAria cell sorter (Becton Dickinson, San Diego, CA) after labeling with CD3 (T cells, clone SP34), CD20 (B cells, clone L27) or CD14 (granulocytes, clone M5E2). Sorted populations were routinely purified to >95% purity (data not shown). After sorting, chimerism was determined as described above.

Measuring anti-donor T-cell alloreactivity using CFSE-MLR

Recipient peripheral blood lymphocytes (PBL) were enriched for T lymphocytes by depletion of antigen presenting cells by binding to anti-mouse IgG-Dynal Beads (Invitrogen, Carlsbad, CA) after labeling with anti-CD20 and anti-HLA-DR antibodies (Pharmingen). The enriched T-cell fraction was labeled with 5μM CFSE (Molecular Probes, Eugene, OR) and 2 $\times 10^5$ were incubated for 5 days at 37°C with 2×10^5 donor PBL. Flow cytometric analysis of APC-labeled anti-CD4 and anti-CD8 antibodies (clones SK3 and SK1, respectively) and CFSE was performed. The CD8-APC antibody labels T cells with a greater fluorescence intensity than does the CD4-APC and, therefore, both populations could be monitored simultaneously.

CMV monitoring

The number of cytomegalovirus (CMV) copies/mL of whole blood was determined by real-time PCR using TaqMan chemistry. To determine the viral load, 7.5 μL template genomic DNA was placed into a 50 μL PCR reaction using the TaqMan Universal Master Mix (Applied Biosystems). Reactions were amplified for 40 cycles using the default conditions on the ABI 7900HT Sequence Detection System. Sample threshold cycles were converted to quantities per PCR reaction by a standard curve consisting of dilutions of plasmid DNA. Copies of virus/reaction were converted to copies/mL blood by multiplying by the conversion factor of 66.7, the ratio of the amount of DNA analyzed to the amount of DNA in 1 mL of whole blood. The primers used were previously described (28,29).

CMV prophylaxis and treatment regimen

Weekly CMV PCR-based surveillance was performed and a prophylaxis strategy was developed including weekly infusions of cidofovir (5 mg/kg i.v.) from day 21 post-transplant until day 90 and oral valganciclovir (12 mg/kg) thereafter. If CMV reactivation was detected, treatment included twice daily ganciclovir (6 mg/kg/dose subcutaneously). Once CMV PCR became negative, ganciclovir treatment was continued twice daily for an additional week, decreased to once daily for 2 weeks, then discontinued and oral valganciclovir was resumed.

Results

Strategy for the establishment of long-term multilineage chimerism in rhesus macaques

Our standard transplant strategy (referred to as CoBBS for Costimulation Blockade/basiliximab/sirolimus) is shown in Figure 1. As shown in the figure, our protocol employed preparation with a single non-myeloablative dose of busulfan. To determine optimal busulfan dosing, a dose-response analysis was performed (Figure 2A–I). While 4 mg/kg and 8 mg/kg produced only modest decreases in the total white blood cell (WBC) count, absolute neutrophil count (ANC) and platelet count (Figure 2A–C), a single 10 mg/kg dose of busulfan produced more substantial cytopenias while still allowing count recovery without stem cell rescue. Figure 2E–I shows that while individual variation existed, 10 mg/kg busulfan resulted in an ANC nadir at ~21 days, which ranged from 0.171×10^3/μL–0.608×10^3/μL. The platelet count nadir exhibited a wider range, from 11×10^6/μL to 231×10^6/μL. While one animal (RRq-8) displayed prolonged thrombocytopenia, the other three animals exhibited less extensive decreases in platelet count. Neither the hemoglobin concentration nor the absolute lymphocyte count fell significantly (Figure 2H,I), consistent with the myelospecificity of busulfan. As shown in Figure 2D, the 10 mg/kg busulfan dose cleared rapidly from the peripheral circulation, reaching undetectable levels after 10 h. Figure 3 shows that in a representative animal who received 10 mg/kg busulfan along with HSCT, significantly attenuated cytopenias resulted (nadir ANC was 1.5×10^3/μL, 14 days post-transplant and nadir platelet count was 163×10^6/μL, 28 days post-transplant), consistent with rapid hematologic replenishment after HSCT.

CoBBS-mediated chimerism-induction using leukopheresis-derived stem cells

In addition to BMT, we developed a leukopheresis-based strategy for harvesting sufficient stem cells from the peripheral blood of living NHP. Living donors potentially increase our ability to perform post-transplant immune monitoring (on freshly isolated donor tissues) and, in the future, solid organ transplants after chimerism-induction. Figure 4A shows that both bone marrow and leukopheresis products yielded significant transplant innocula, with mean nucleated cell doses of 6.7×10^9 for bone marrow and 8.2×10^9 for leukopheresis (n = 3). Both had similar $CD34^+$ cell percentages (bone marrow, 7.4 ± 2.3% $CD34^+$ cells; leukopheresis, 10 ± 4.9% CD34+ cells). The stem cell-like $CD34^+/CD38^-$ fraction was enriched in the bone marrow, (3± 1% $CD34^+$/CD38– cells), while leukopheresis products possessed 0.8 ± 0.2% $CD34^+$/CD38– cells. Both were infused without fractionation, so that recipient animals also received differentiated hematopoietic cells. As shown in Figure 4A, bone marrow had significantly fewer T cells (5.4 ± 1%) than leukopheresis (16.6 ± 5.3%), and CD4 and CD8 T cells were similarly reduced (1.8 ± 0.7% vs. 7.7 ± 2.2% $CD4^+$ T cells and 3.3 ± 0.7% vs. 7.6 ± 2.1% $CD8^+$ T cells, respectively), as were B cells (7 ± 0.5% vs. 13 ± 3.5%, respectively). Leukopheresis products also possessed increased numbers of $CD4^+CD25^+$ cells (3.5 ± 2% compared with 0.23 ± 0.1% in bone marrow). Figure 4B shows that the leukopheresis-derived HSCT was as efficient as BMT in producing donor chimerism (peak chimerism levels of 86% compared to 80%, respectively).

Seven recipients received BMT + CoBBS; two received leukopheresis product HSCT + CoBBS. One recipient was not evaluated as he was terminated after 1 week due to Herpes B reactivation. As shown in Figure 5 and Table 1,

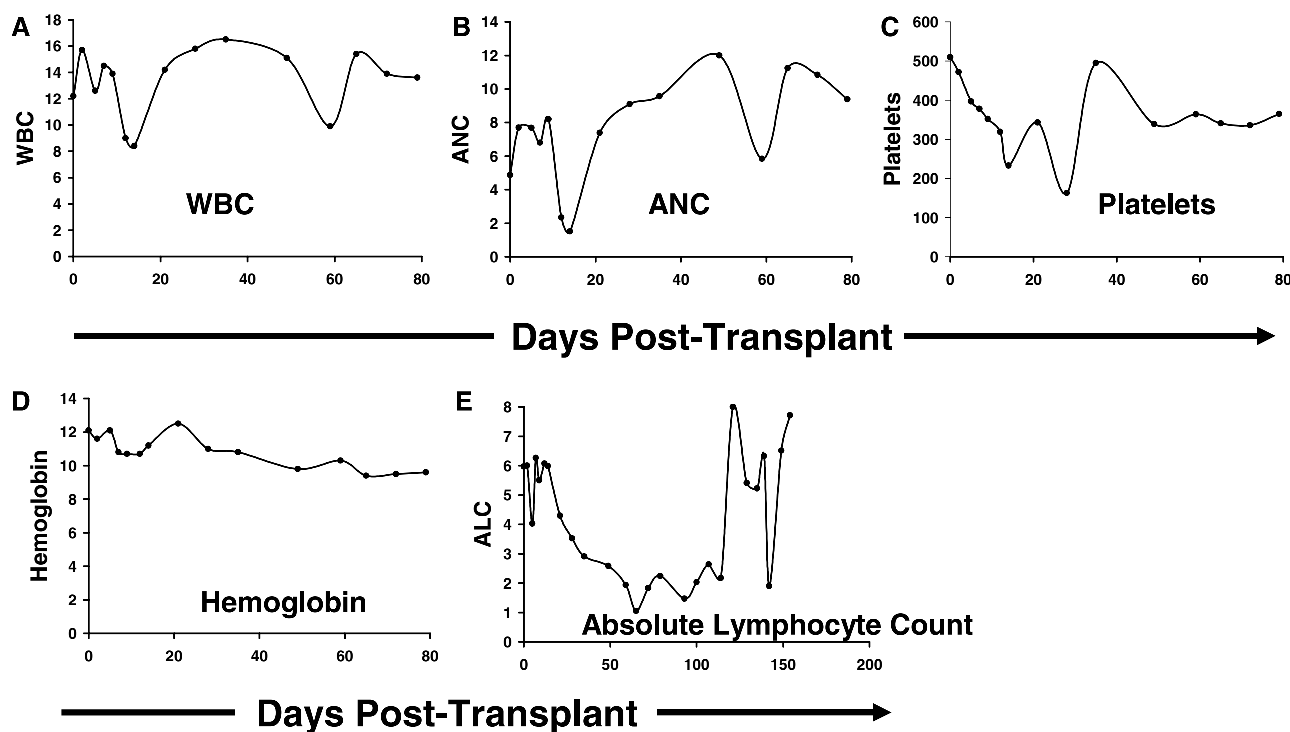

Figure 3: Analysis of hematopoiesis in a representative animal receiving 10 mg/kg busulfan in the context of hematopoietic stem cell transplantation and CoBBS immunomodulation. Shown is the WBC (Figure 3A, $\times 10^3/\mu L$), ANC (Figure 3B, $\times 10^3/\mu L$), platelet count (Figure 3C, $\times 10^6/\mu L$), hemoglobin (Figure 3D, g/dL) and absolute lymphocyte count (Figure 3E, $\times 10^3/\mu L$) for a single animal, representative of the eight evaluable animals treated with the CoBBS transplant regimen.

CoBBS resulted in high-level peripheral blood donor chimerism in seven of the eight evaluable recipients. Recipient RAf-7 (Table 1 and Figure 5A) never achieved chimerism despite adequate bone marrow dose (5.4×10^9 nucleated cells/kg vs. $5 \pm 1 \times 10^9$ nucleated cells/kg for other transplants). While the cause of this early, isolated lack of engraftment remains unknown, all sixteen subsequent transplants reported in this study did result in chimerism. All seven chimeric animals sustained high levels for the duration of post-transplant treatment with both costimulation blockade (CoB) and sirolimus. Recipient RYj-7 remained chimeric when he died from acute renal failure and campylobacter sepsis on day 200 post-transplant, despite sirolimus discontinuation on day 62 and CoB discontinuation on day 112. The remaining six chimeric animals lost chimerism after either sirolimus discontinuation (Run-7, RWb-8, CW7B, RVq-8, Table 1 and Figure 5B,F–H, respectively) or CoB discontinuation (RRi-8, RUi-8, Table 1 and Figure 5D,E, respectively). As shown in Figure 5I, chimerism existed in multiple sites, including bone marrow, spleen, and lymph nodes. However, despite significant granulocyte chimerism (55%), T-cell chimerism was much reduced (2.9%, Figure 5J). In clinical BMT, a lack of significant T-cell chimerism increases the potential for rejection (37–40), as occurred in these transplants. Despite the lack of indefinite chimerism-induction, CoBBS led to greater chimerism durability than previously reported (13,17,18,41). While other regimens have led to <50 days duration of mixed-chimerism (17), donor cells were present in our transplants for a median of 119 days, and extended as far as 196 days post-transplant.

Modifications to the standard chimerism-induction strategy did not improve the level or durability of chimerism

Based on evidence from murine tolerance-induction models (7,11,32,42,43), we evaluated four ancillary therapies: CD8 depletion, DST, recipient thymectomy, and peritransplant NF-kappa-B inhibition to determine their effects on chimerism duration (see Figures 6A–E and Table 1).

Peritransplant CD8 depletion was performed in three transplants, resulting in >95% CD8 depletion as measured by flow cytometry, which lasted until day 28 post-transplant (Figure 6A–B). Animal RUh-7 (Table 1 and Figure 6A) developed Herpes B-reactivation-related complications necessitating sacrifice at day 35, despite high (80%) chimerism. In the other two animals, high-level chimerism developed (mean peak chimerism 82%) and was maintained for the length of therapy, but ultimately waned. Recipient RAi-7 (Table 1 and Figure 6A) lost chimerism by day 200, after both sirolimus and CoB were discontinued at day 98. Recipient RMf-7 (Table 1 and Figure 6A) lost chimerism at day 175, after both sirolimus and CoB were discontinued (days

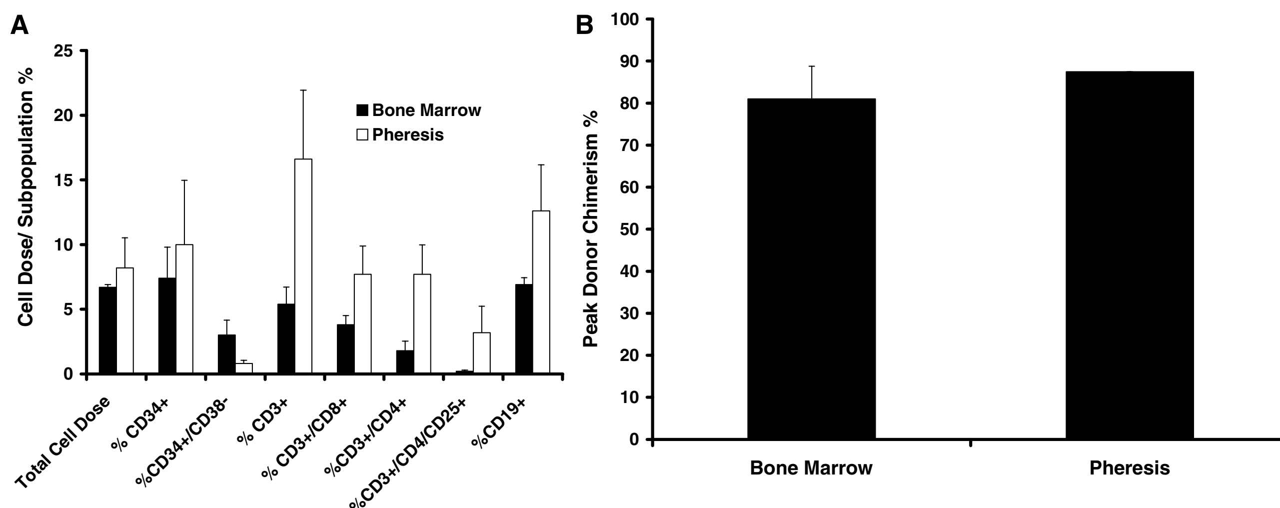

Figure 4: Leukopheresis represents a viable alternative to terminal bone marrow harvest for producing mixed chimerism after non-myeloablative transplant in the setting of the CoBBS transplant strategy. (A) Comparison of the components of bone marrow or leukopheresis products. Cell dose: $\times 10^{-9}$. Each parameter is shown as the mean ±SEM, n = 3 separate stem cell donors. (B) Comparison of the peak % peripheral blood donor chimerism from bone marrow (left-hand panel, mean ± SEM n = 3) or leukopheresis (right-hand panel, mean, n = 2) transplants with the CoBBS transplant strategy.

97 and 112, respectively). Thus, transient depletion of CD8 T cells did not significantly improve chimerism stability, nor result in immune tolerance after withdrawl of immunosuppression.

Given others' results demonstrating salutary effects of DST in prolonging renal allograft survival (44,45), and previously-documented tolerogenic effects of prior exposure to donor antigen in the presence of T-cell CoB (7,11), we tested the ability of peritransplant DST to improve chimerism-stability. As shown in Table 1 and Figure 6C, while animals treated with DST developed chimerism, the level was lower than without DST (25% vs. 82%), and was not stabilized (recipient RTd-7 lost chimerism before either sirolimus or CoB discontinuation and recipient RBg-7 lost chimerism after sirolimus discontinuation but before CoB discontinuation, Figure 6C).

Regimens including thymic irradiation have resulted in chimerism and tolerance to donor kidney allografts in NHP (13,14,16–18). Given these results, we determined whether recipient thymectomy would increase CoBBS-induced chimerism stability. As shown in Figure 6D, while thymectomized animals all developed chimerism, it ultimately waned. Thus, recipient RUu-7 lost chimerism before either sirolimus or CoB was discontinued, and recipient RVu-7 lost chimerism after sirolimus was discontinued but before CoB was discontinued. Only RSk-7 remained chimeric until both sirolimus and CoB were discontinued. Tolerance to the BMT did not develop, implicating the pre-existing population of recipient T cells in rejection, as thymectomy would eliminate the development of newly emergent donor-reactive T cells.

Finally, we tested the effect of NF-kappa-B inhibition with DSG on chimerism-induction. DSG has been shown to have adjunctive tolerogenic activity in mice and to prolong renal and islet allograft survival in NHP (32,46–52). The LF15-0195 DSG analog has increased stability and biological activity as an immunosuppressant compared to parent DSG (31). The results of the addition of LF15-0195 are shown in Figure 6E. While two of five animals were sacrificed due to viral reactivation in the immediate post-transplant period and were thus unevaluable (data not shown), the remaining three animals developed chimerism (mean 82%). One of these animals (CG8B) also died prematurely (of bacterial typhlitis) at day 60 post-transplant, at which time he showed 45% chimerism. The remaining animals both showed chimerism, however, one recipient (RDp-8) lost chimerism after CoB discontinuation and the other (RVf-8) lost chimerism after both sirolimus and CoB were discontinued. Thus, LF15-0195 did not impact the stability of chimerism nor induce immune tolerance.

Transplant rejection was associated with the reappearance of donor-reactive T cells

Chimerism ultimately waned despite achievement of unprecedented high chimerism levels and despite recipient thymectomy, suggesting that tolerance to donor antigens in the preexisting pool of T cells was not achieved. As the data in Figure 7 support, mixed-chimerism was initially accompanied by the inhibition of proliferation of donor-reactive T cells as measured by an alloreactive CFSE-based MLR assay. Rejection was accompanied by the reappearance of proliferation, consistent with a T-mediated mechanism of rejection. These data, in combination with the

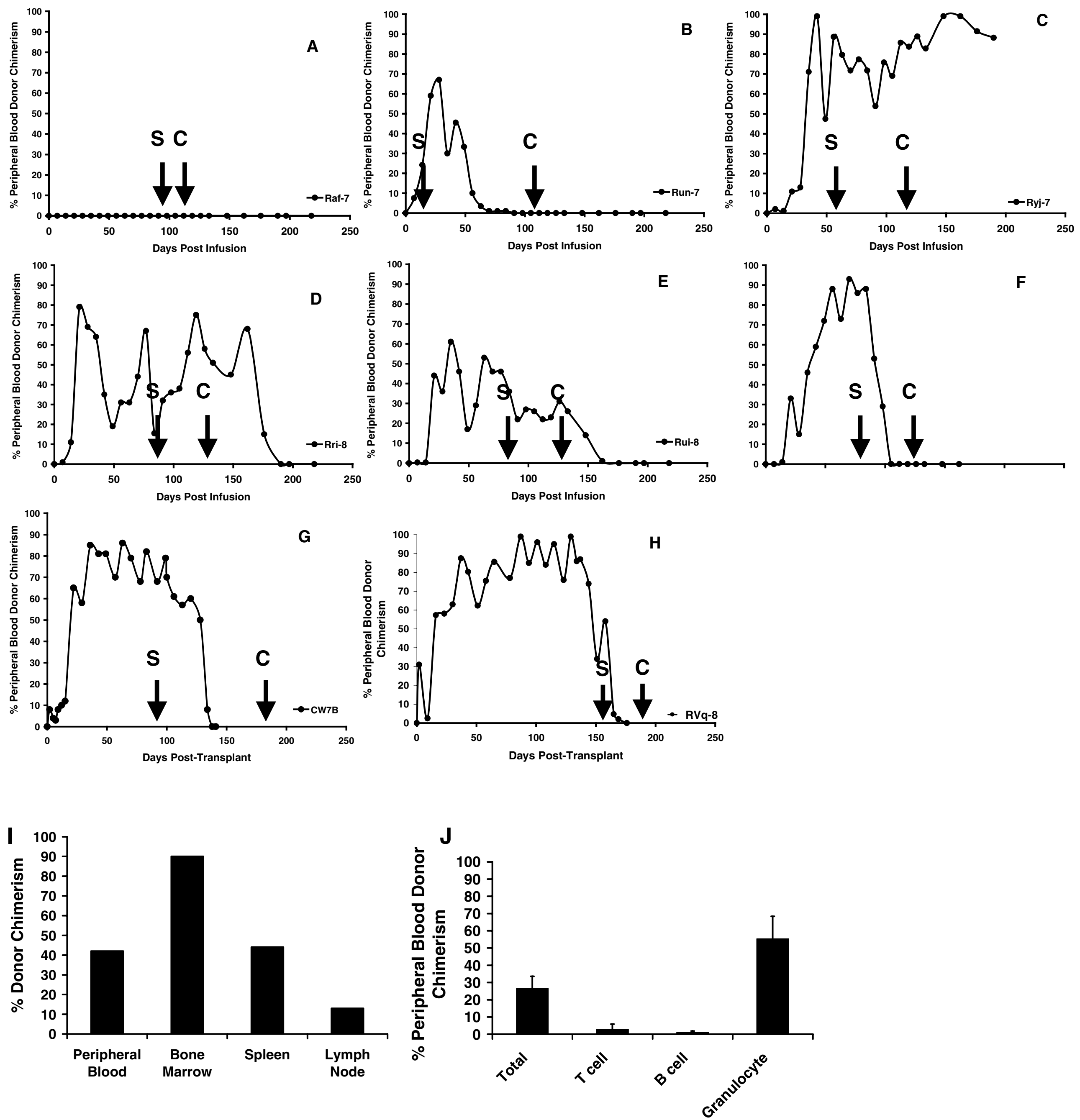

Figure 5: Peripheral blood donor chimerism in animals transplanted in the context of the CoBBS immunomodulation regimen. (A–H) Percent peripheral blood donor chimerism was determined as described in Methods for animals treated with the CoBBS regimen. As shown in Table 1, all recipients received a bone marrow transplant, except for CW7B and RVq-8, who received leukopheresis transplants. Chimerism is shown for each individual animal over time, with the date of discontinuation of sirolimus (S arrow) or CoB (C arrow) shown superimposed on each chimerism graph. (I) Chimerism in the bone marrow, spleen and lymph nodes was determined for one representative animal after its demise from viral sepsis. Chimerism analysis was performed by real-time PCR, as detailed in Methods. (J) Lineage-specific analysis reveals higher levels of myeloid than lymphoid chimerism. T cells, B cells and granulocytes were sorted as detailed in Methods and then the percent donor chimerism was determined by real-time PCR as detailed in Methods. Shown is the mean ± SEM for six separate lineage-chimerism determinations.

thymectomy data, support the conclusion that CoBBS-based HSCT failed to delete or permanently tolerize the preexisting pool of donor reactive T cells.

Viral reactivation

Transplanted animals developed significant complications involving primary viral infection and viral reactivation (Table 1). While Herpes B-positive animals could be excluded as transplant participants, the ubiquity of CMV-positivity rendered CMV an important pathogen, just as in clinical transplantation. Given the importance of CMV reactivation to recipient health and graft stability, we prospectively monitored CMV after transplant and instituted CMV prophylaxis with cidofovir and valganciclovir and treatment with ganciclovir. In animals whose clinical situation either did not improve on antiviral therapy or was grave (intractable diarrhea, weight loss, dehydration), immunosuppression (usually sirolimus) also was weaned (see Table 1 for indication for discontinuation either sirolimus or costimulation blockade and Figures 5 and 6). Figure 8 shows the temporal relationship of CMV reactivation, immunosuppression withdrawal, and the persistence of donor chimerism in 12 transplanted animals. In many recipients (Figure 8C,D,I,K,L), transplants were rejected in the context of high CMV titers. Likewise, transplant recipients were also at risk for persistent viremia despite

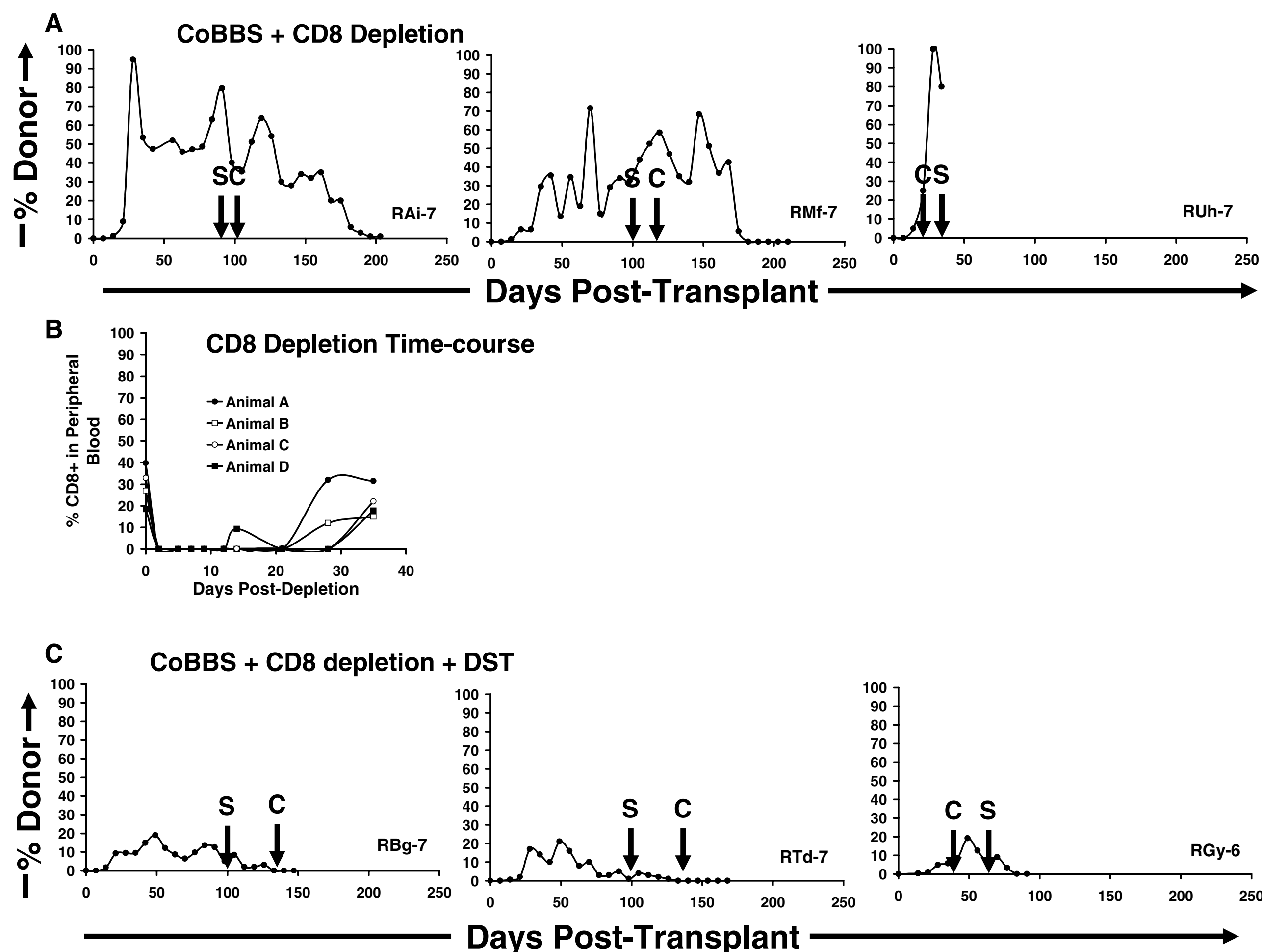

Figure 6: Chimerism in the setting of CoBBS plus additional therapies: (A) CoBBS + CD8 depletion, as detailed in Methods. This chimerism is shown for each individual animal over time, with the date of discontinuation of sirolimus (S arrow) or CoB (C arrow) shown superimposed on each chimerism graph. (B) Time-course of CD8 deletion after treatment with the OKT8 antibody as described in Methods. The percent of CD8+ cells in the peripheral blood for four individual animals is shown. (C) CoBBS + CD8 depletion + donor-specific transfusion (DST) as detailed in Methods. This chimerism is shown for each individual animal over time, with the date of discontinuation of sirolimus (S arrow) or CoB (C arrow) shown superimposed on each chimerism graph. (D) CoBBS + thymectomy as detailed in Methods. This chimerism is shown for each individual animal over time, with the date of discontinuation of sirolimus (S arrow) or CoB (C arrow) shown superimposed on each chimerism graph. (E) CoBBS + DSG treatment, as detailed in Methods. This chimerism is shown for each individual animal over time, with the date of discontinuation of sirolimus (S arrow) or CoB (C arrow) shown superimposed on each chimerism graph.

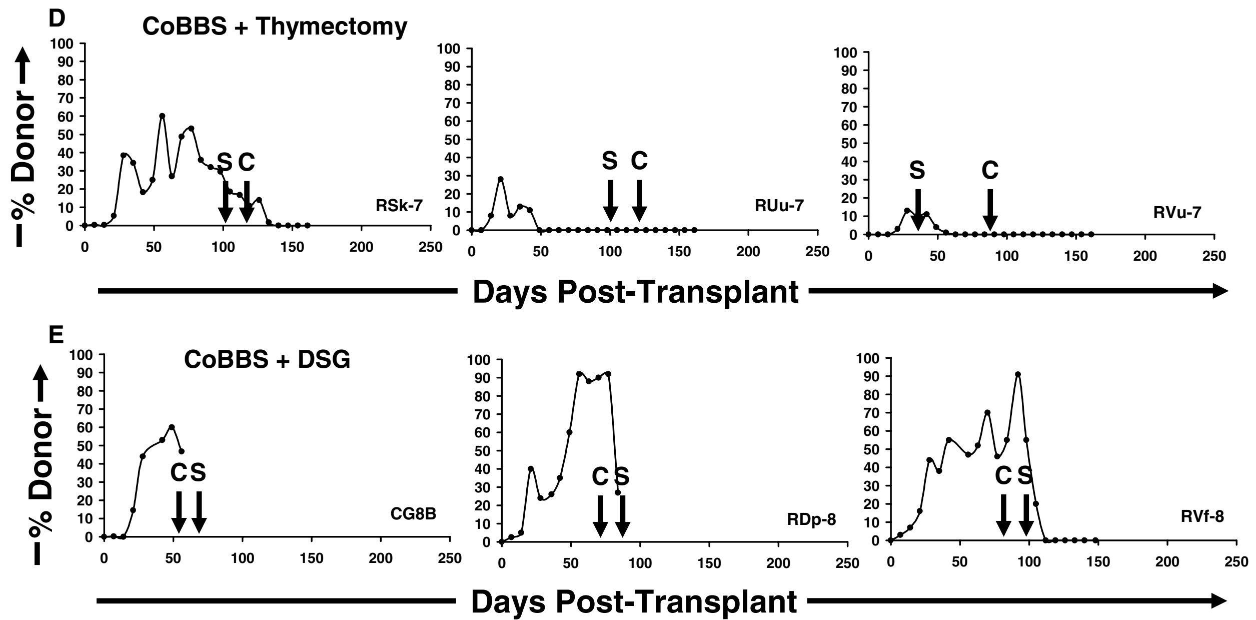

Figure 6: continued.

anti-viral therapy. Thus, while mixed-donor chimerism was present, animals were unable to clear virus despite antiviral therapy (Figure 8C,D,I,K,L). However, upon transplant rejection, they were able to clear CMV from their blood. Other animals (Figure8 A,B,E–H,J) showed a natural history of viral infection and chimerism that appeared more independent of each other, demonstrating the variability in the interactions between these interrelated phenomena.

Discussion

In mice, the induction of stable mixed hematopoietic chimerism is a well-established strategy for producing immune tolerance after transplant (4,7,11,16,53–58). Case reports of patients freely accepting solid organ transplants when these organs are received from a previous bone marrow donor also support the contention that hematopoietic

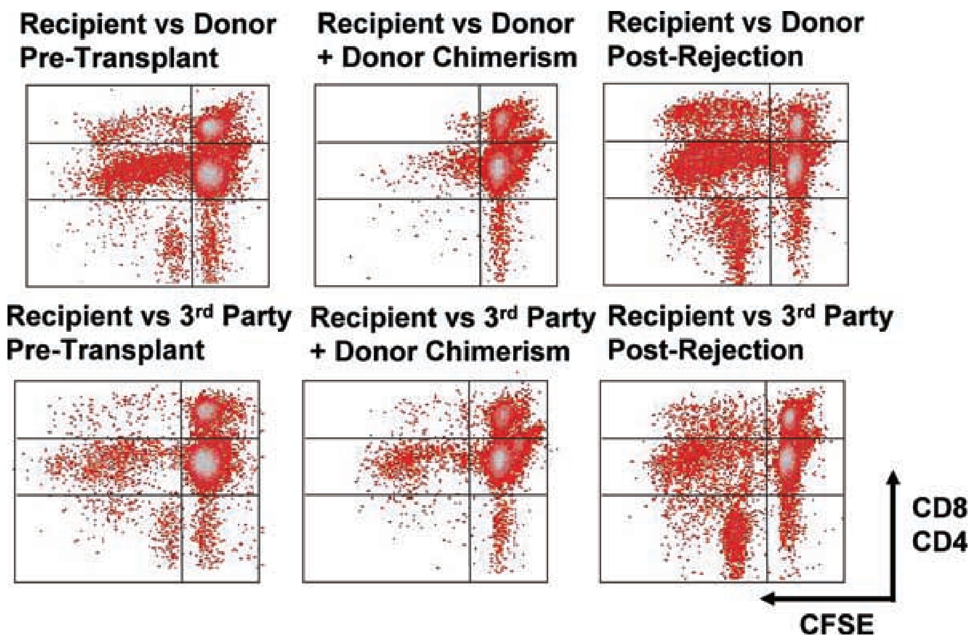

Figure 7: Chimerism is accompanied by donor-specific down-regulation of T-cell proliferation, and rejection is accompanied by the reappearance of anti-donor T-cell alloreactivity. Top row: recipient vs. donor, either pre-transplant, peritransplant or post-rejection. Bottom row: recipient vs. third party, either pre-transplant, peritransplant or post-rejection. X-axis: CFSE, Y-axis: brighter population: $CD8^{+}$, less-bright population: $CD4^{+}$.

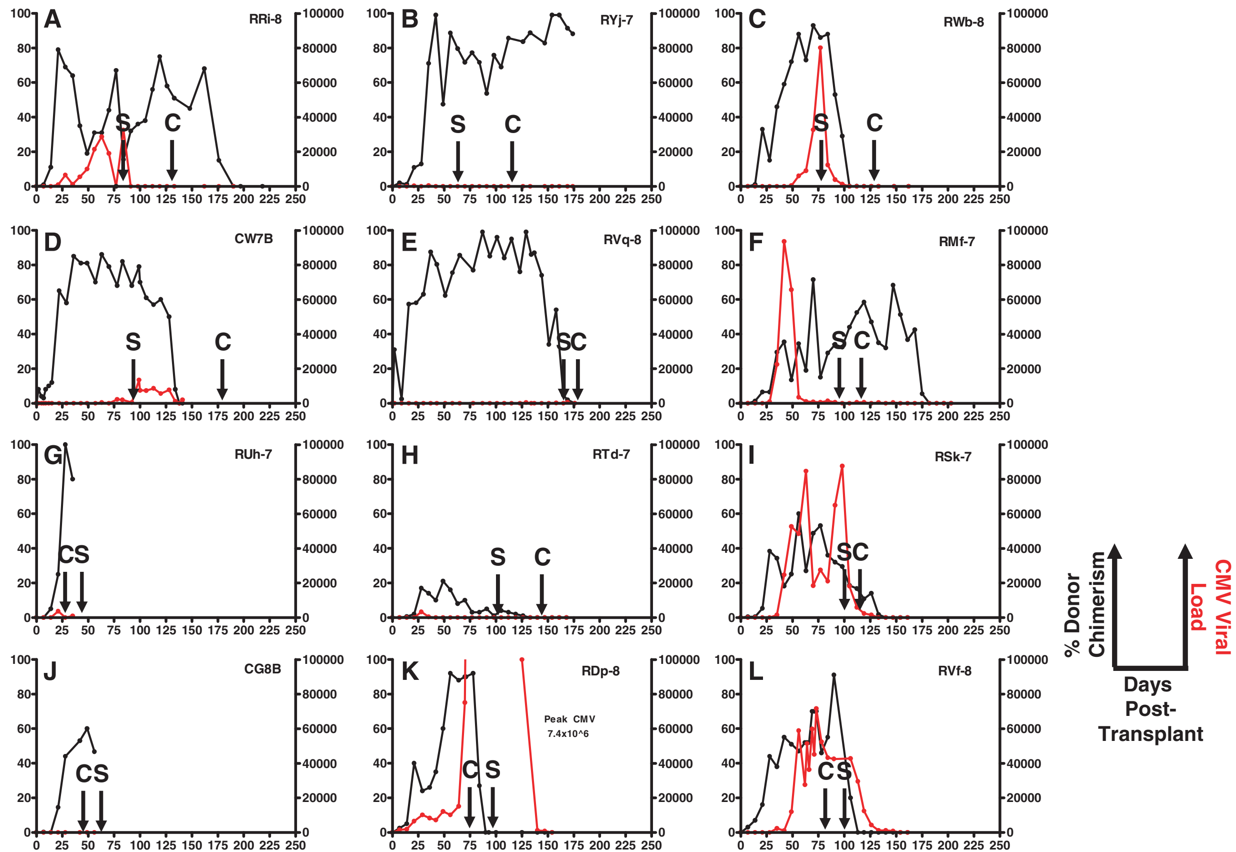

Figure 8: Correlation of CMV reactivation, chimerism and immunosuppression withdrawal. Shown are 12 animals in which CMV was monitored prospectively by real-time PCR as detailed in Methods. The time-course of CMV reactivation (red) and chimerism (black) is shown. The date of discontinuation of sirolimus (S arrow) or CoB (C arrow) are superimposed on these two curves for each animal analyzed.

chimerism induces immune tolerance (59). However, the ability to intentionally produce sustained chimerism after non-myeloablative HSCT has been challenging to achieve in NHP and in patients. The potential application of chimerism-induction strategies after non-myeloablative HSCT include both immune tolerance for solid organ transplants and/or HSCT for non-malignant hematologic disease, such as sickle cell disease, β-thalassemia and immunodeficiency syndromes (24–26,40,60–64). Given the organ toxicity faced by these patients, the ability to reproducibly create sustained chimerism after HSCT with minimally myeloblative conditioning represents an important unmet clinical need. We describe a strategy that routinely produced high-level mixed-chimerism in an NHP model of HSCT that was often stable for the duration of immunomodulation. While RBC chimerism was not measured (given the DNA-based chimerism assays), our previous work has shown that for hemoglobinopathies, transplant produces significantly higher RBC chimerism than WBC chimerism (based on the reduced life-span of diseased RBCs compared to healthy donor RBCs) (24). In murine studies, WBC chimerism as low as 5–10% produced a phenotypic cure (24), predicting that CoBBS-induced chimerism would be adequate to correct hemoglobinopathies. This strategy may thus be applicable to the most severely affected patients, for whom the risk:benefit ratio may favor long-term therapy with immunosuppressive agents over the ongoing morbidities of their primary disease. However, the ability to apply this regimen clinically may be limited by the significant immunosuppression that accompanied these CoBBS-based transplants between fully MHC mismatched pairs. Ongoing experiments are designed to test the extent to which the immunosuppressive agents or the degree of MHC mismatching contributed to the observed immunosuppression in the transplant recipients.

Our chimerism-induction strategy (Figure 1) was based on a large body of evidence supporting the potential efficacy of CoB pathways, and the mTOR inhibitor sirolimus for inducing immune modulation and graft acceptance (7,14,18,56,65–71). This strategy produced chimerism in eight of nine evaluable recipients, which was stable for the duration of immunomodulation in seven of eight an-

imals. While chimerism eventually waned, the ability to produce chimerism with this calcineurin-free regimen may form the basis for the development of new treatment options, if this could be achieved without serious infectious complications.

In NHP solid organ transplantation, DST and DSG have been shown to prolong allograft survival (12–14, 17,18,44,45,47,49–52,72–74). However, in this transplant series, the inclusion of DST, the DSG analog LF15-0195, or transient CD8 depletion did not increase either the level or the stability of donor chimerism. Our results with LF15-0195 are especially notable, as DSG-mediated inhibition of allograft rejection has been strongly implicated in the success of NHP renal transplantation (51,52,74), and in tolerizing the heterologously immune memory T-cell populations that develop after repeated viral exposure in murine transplantation (32). While our results are consistent with those found in the most recent human renal transplantation trials, where the addition of DSG did not improve allograft survival (75), the effect of DSG may be regimen-dependent, and thus, a role for DSG may be apparent in selected transplant settings.

The lack of efficacy of thymectomy in improving chimerism stability leads us to conclude that the loss of chimerism was due to a failure of the CoBBS regimen to induce tolerance in the existing population of donor-reactive T cells. These results further distinguish the macaque model from murine models of costimulation-blockade-resistant rejection, in which thymectomy resulted in prolonged allograft survival, thus supporting a role for new thymic emigrants as mediators of CoB-resistant rejection (43). The specific T-cell subpopulations (naïve, effector-, central-memory) that ultimately caused rejection have not yet been determined. Furthermore, the possibility that suboptimal dosing or timing of the immunosuppressive reagents was cause for graft failure also must be considered. The development of tools and approaches to analyze whether the failure of tolerance-induction occurred primarily in the memory or naïve subpopulations and to assess the efficacy of dose/duration strategies will be of considerable importance to future pro-tolerogenic transplant strategies.

Viral reactivation was a significant clinical obstacle in this transplant series. Both Herpes B and CMV were noted in this series (as shown in Table 1), and other viral infections (SV40 and adenovirus) have occurred in other ongoing CoBBS-based transplant series (data not shown) indicating that CoBBS-based immunosuppression may confer a broad susceptibility to viral reactivation and primary infection, especially in the setting of MHC disparate transplantation. The associations between CMV reactivation and loss of chimerism also are consistent with work in murine and clinical models suggesting that prior viral exposure or chronic viral infection can impede engraftment and tolerance-induction (76–80) and that fully MHC disparate chimerism may lead to significant defects in both T-cell immunity and recovery from viral infection (Koehn, Larsen et al., manuscript submitted). These two phenomena have important implications for transplant regimens involving fully MHC disparate donors and recipients and underscore the importance of ongoing investigations into the impact that MHC-matched transplants will have on future immune reconstitution.

Our findings advance the ability to produce long-lasting chimerism in NHP, establish the efficacy of both bone marrow-derived and leukopheresis-derived stem cells in producing chimerism, and that an immunosuppressive regimen based on CD28/CD154 blockade could produce chimerism lasting as long as 196 days despite full donor:recipient MHC disparity. Furthermore, they show that a busulfan-based preparative regimen can be employed to induce mixed-chimerism-induction without full marrow ablation. However, significant challenges to the translation of these results to clinical transplantation remain; in particular, the lack of indefinite durability of chimerism, the current lack of permanent deletion or down-regulation of donor-reactive T cells, and the risks of viral reactivation. The risk of viral reactivation was substantial, and may have arisen from either the fact that these transplants were performed between donors and recipients that were fully MHC disparate, from the CoBBS immunosuppressive strategy or from the busulfan preconditioning regimen (or from a combination of these factors). Thus, while costimulation blockade/busulfan based transplant will avoid the some of the specific risks associated with radiation-based regimens, the comparative toxicity and safety of these two types of approaches deserves close comparison, to determine which is more feasible for translation to the clinic. In addition, supplementary therapies, such as adoptive immunotherapy with donor lymphocytes or infusion of pro-tolerogenic T-cell populations may be required to increase the durability of chimerism. Furthermore, tolerance-induction and protective immunity may benefit from increasing degrees of relatedness and MHC-matching between donors and recipients in the setting of CoBBS-based immunomodulation. Ultimate success in producing stable mixed chimerism and robust donor-specific tolerance in the setting of preserved immunity may lie in the combination of several of these strategies.

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