Original Research

Improved engraftment with minimal graft-versus-host disease after major histocompatibility complex-mismatched cord blood transplantation with photochemically treated donor lymphocytes

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Abstract

There is a significant risk of severe graft-versus-host disease (GVHD) and graft failure after unrelated umbilical cord blood transplantation (CBT) if donor–recipient pairs are mismatched at major histocompatibility complex (MHC) loci. To mitigate these risks after MHC-mismatched CBT, we infused psoralen-treated, photochemically inactivated, mature donor T-lymphocytes with MHC (H2-haplotype) mismatched murine donor fetal near-term peripheral blood (FNPB) cells after sublethal irradiation. We analyzed the rates of donor engraftment, GVHD and long-term survival in H2 haplotype disparate (C57BL/6 [H-2^b/Thy1.1] → AKR [H-2^k/Thy1.2]) recipient mice. We observed inconsistent donor engraftment after transplantation with cord blood alone, but superior engraftment and long-term survival after FNPB transplantation supplemented with psoralen-treated donor T-lymphocytes. Additionally, there was fatal GVHD after FNPB co-infusion with untreated donor T-lymphocytes, but minimal GVHD after FNPB supplemented with psoralen-treated donor T-lymphocytes transplantation. Donor MHC^{high}/c-Kit$^+$/lineage$^-$/CD34$^-$ stem cells were noted in the recipient bone marrow compartment following co-infusion of photochemically inactivated T-cells with FNPB. Despite the non-myeloablative preparation before FNPB infusion, complete hematological recovery was delayed until 50–60 d after transplantation. We observed that co-transplantation of psoralen-treated donor T-lymphocytes with FNPB facilitated durable engraftment of donor hematopoietic stem cells in the marrow and splenic compartments with complete but delayed recovery of all hematopoietic lineages. This CBT model establishes the possibility of ensuring donor engraftment across a MHC barrier without severe GVHD.

Keywords: cord blood transplantation, major histocompatibility complex barrier, photochemically treated T-cells, engraftment, graft-versus-host disease

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Introduction

The utilization of umbilical cord blood as an alternative source of hematopoietic cells for allogeneic transplantation has expanded as a result of successful transplantation outcomes in children and adults with hematological disorders.[1–4] The principal reason for this increased utilization is the ability to elicit successful transplantation outcomes despite human leukocyte antigen (HLA) disparities among donor–recipient pairs.[5] Cord blood contains sufficient hematopoietic progenitor cells to support engraftment and also contains T-lymphocyte populations with a naïve phenotype that is expressed as a reduced allo-reactivity associated with a lower incidence of graft-versus-host disease (GVHD).[6,7] However, it is still important to optimize HLA matching between donor–recipient pairs, which tend to constrain the wider use of cord blood transplantation (CBT). In addition, there is a potential of graft rejection as a result of the limiting quantity of hematopoietic stem cells harvested in typical cord blood collections and HLA disparity.[8,9] Even when the hematopoietic cell dose in the cord blood unit is optimized, there is a greater risk of

graft rejection following allogeneic CBT compared with marrow or peripheral blood stem cell transplantation.

Several cellular therapies have been investigated to promote engraftment after mismatched CBT. These methods include co-transplantation of mesenchymal stem/stromal cells,[10–12] administration of purified haploidentical $CD34^+$ cells,[13] the administration of chemokines such as stromal derived factor-1[14] and a CD26 antagonist, Diprotin A.[15] We have investigated an alternative strategy that employs photochemically modified donor T-lymphocytes to promote engraftment after major histocompatibility complex (MHC)-mismatched CBT (major H2 and minor [non-H2] histocompatibility barriers) following a sublethal preparative regimen. The rationale for this approach follows from observations that murine cord blood is naturally deficient in T-lymphocytes, which facilitate engraftment through an allogeneic effect and which are required for the graft-versus-malignancy effect. We hypothesized that it might be possible to ensure engraftment without a significant risk of severe GVHD after MHC-mismatched CBT by utilizing 'add-backs' of amotosalen (S-59)-treated T-lymphocytes with donor cord blood.

S-59 is a water soluble, non-toxic, synthetic psoralen that has been used for pathogen inactivation in platelet and plasma products.[16–18] It has also been used in the creation of a new class of vaccines using microbes.[19] S-59 forms covalent mono-adducts and cross-links between the pyrimidine bases of the DNA and RNA upon illumination with long wavelength ultraviolet (UVA) light. When treated with nanomolar concentrations of S-59, photochemically treated T-lymphocytes demonstrate near-complete abrogation of lymphocyte proliferation even in the presence of strong mitogens.[20] This effect significantly decreases the ability of photochemically treated T-lymphocytes to cause GVHD. However, photochemical treatment does not cause cell death; thus, T-lymphocytes continue to secrete cytokines such as interleukin 2 after photochemical treatment, which makes photochemical inactivation a potent immunomodulatory tool in facilitating engraftment after allogeneic transplantation.[21]

To improve engraftment kinetics, after fully MHC mismatched murine fetal near-term peripheral blood transplantation (FNPBT), we administered the stem cell infusion with 'add-backs' of S-59-treated donor T-lymphocytes. Our results indicate that complete donor chimerism is feasible after H2 haplotype disparate CBT without significant GVHD, using sublethal radiation as the conditioning regimen. We speculate that this 'proof of principle' is sufficiently encouraging to warrant further trials of this methodology.

Materials and methods

Mice

Mice of 8–12-week-old wild-type C57BL/6 ($H\text{-}2^b$/CD45.2, Thy1.2, CD8.2) and AKR/J ($H\text{-}2^k$/CD45.2, Thy1.1, CD8.1) were purchased from The Jackson Laboratories (Bar Harbor, ME, USA) and/or inbred at the animal facility of Children's Hospital Oakland Research Institute (CHORI) (Oakland, CA, USA). These animals were fed standard fat chow and maintained in micro-isolator cages. Mice were maintained in a pathogen-free room after transplantation. All procedures were performed in compliance with National Institute of Health guidelines for animal welfare. The animal protocols and experiments were conducted after obtaining approval from the animal welfare committee at CHORI.

Submyeloablative radiation regimen

Twenty-four hours before transplantation, recipient mice (AKR/J) received 500 cGy of total body ionizing radiation from the RS-2000 X-ray biological irradiator (160 kV, 4.2 kW, radiation dose 96 rads/mt) (RadSource Technologies, Inc, Alpharetta, GA, USA), administered in a single fraction.

Donor stem cell preparation

Pregnant C57BL/6 female mice were euthanized by CO_2 asphyxiation between gestational age E 18–21 d. The abdominal skin was cleaned with 70% ethanol, the uterus exposed and opened by cesarean section, and each fetus was extracted and wiped with clean gauze to minimize the possibility of maternal blood contamination. Fetal near-term peripheral blood (FNPB) was collected from the neck veins following decapitation. FNPB was pooled from multiple litters as necessary to collect an adequate donor nucleated cell dose. The donor nucleated cells were enumerated by an Advia 120 Hematology system (Siemens Healthcare Diagnostics, Deerfield, IL, USA) with multispecies software, and the hematopoietic stem cell content was estimated and viability confirmed by fluorescent monoclonal antibody (mAb) staining for CD117 (c-Kit), Lineage and Sca-1 (KLS) antigen markers (Miltenyi Biotec, Auburn, CA, USA). Before transplantation, the FNPB hematopoietic cell preparation was suspended with the donor maternal T-lymphocytes in RPMI-1640 (Hyclone, Logan, UT, USA) to a final volume of 200–250 μL, as indicated below.

Donor T-lymphocyte preparation

Maternal C57BL/6 spleens were used to create single-cell suspension in RPMI-1640 (Hyclone), supplemented with 2 mmol/L L-glutamine, 10% fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich, St Louis, MO, USA), 2 mmol/L Na pyruvate and 50 μmol/L β-mercaptoethanol. The mononuclear cells were isolated by density gradient centrifugation over Ficoll-Paque Plus (Amersham Pharmacia Biotech, Piscataway, NJ, USA). T-lymphocytes were isolated from unselected mononuclear cell populations by positive CD90 (Thy 1.2) magnetic bead separation over two sequential magnetic columns (Miltenyi Biotec). Transplantation experiments were performed with selected T-lymphocyte populations that exceeded 95% purity, based upon cell-surface marker (CD3) expression. Graded doses of T-lymphocytes were used in all the experiments and standardized for optimum effect.

S-59 treatment of T-cells

Following T-lymphocyte collection and purification, the cells were suspended in 5 mL of phosphate-buffered saline/5% fetal bovine serum at a concentration of $1-20 \times 10^6$ cells per plate in 60×15 mm Petri dishes. The cells were then treated with a targeted concentration (1–10 nmol/L) of S-59 (Cerus Corporation, Concord, CA, USA), placed on the Cole Parmer UVA trans-illuminator device (Cole Parmer, Vernon Hill, IL, USA) and exposed to a dose of 3 J/cm^2 over five minutes (with gentle agitation after 2.5 min). The selected number of photochemically treated T-lymphocytes was then re-suspended in RPMI with the FNPB cells. Controls were generated by treating donor T-lymphocytes with UVA radiation in the absence of S-59 and by collecting untreated donor T-lymphocytes. T-lymphocytes with and without S-59 treatment were seeded in parallel into triplicate wells of 24-well culture plates containing phytohemagglutinin (PHA) or mouse CD3/CD28 Dynabeads (Invitrogen, Carlsbad, CA, USA), and incubated for 24–48 h at 37°C in 5% CO_2. The cells were then harvested and assessed for expression of activation markers CD25 and CD69 by flow cytometry, using mAbs conjugated to peridinin chlorophyll protein (perCP). To assess the proliferation potential after photochemical treatment, graded doses of T-lymphocytes ($1-20 \times 10^6$) were suspended in triplicate wells of 96-well culture plates. After 48 h of incubation with PHA, Cell titer 96 Promega Reagent (Promega, Madison, WI, USA) was added to each well. The cells were incubated for another four hours with the reagent and the absorbance at 490 nm was measured by a 96-well ELISA plate reader, after adjusting for background absorbance in the triplicate control wells.

Hematopoietic progenitor cell assay

Lineage depleted bone marrow hematopoietic progenitor cells were seeded into 24-well plates containing methylcellulose (MethoCultGF M3434; Stemcell Technologies, Vancouver, BC, Canada). These cells were co-cultured with allogeneic T-cells (both S-59 treated and not) and the cultures were maintained at 37°C in a humidified incubator at 5% CO_2, and colonies counted with an inverted microscope at 7 and 14 d.

Transplantation, assessment of GVHD and survival

Following sublethal irradiation, recipient mice (AKR/J) were transplanted with varying cell doses of FNPB suspended in RPMI with and without S-59-treated donor T-lymphocytes via tail vein infusion. Three experimental transplant cohorts included group 1: FNPB alone; group 2: FNPB with untreated donor T-lymphocytes; and group 3: FNPB with S-59-treated T-lymphocytes. After transplantation, the mice were monitored daily for the 30 d and twice weekly thereafter for signs of distress and evidence of GVHD, as described previously by Cooke *et al.*[22] Briefly, the animals were scored for various parameters that included weight loss, posture, fur texture, skin integrity and activity, with each criterion graded on a score of 0–2 and the sum of all five scores used as the clinical GVHD index. The mice exhibiting signs of severe GVHD (>6) were euthanized and the gut, liver and skin were evaluated for GVHD by histopathology. Tissues were placed in 10% buffered formalin and hematoxylin and eosin sections were made. A pathologist reviewed histopathology sections in a blinded manner to assess for GVHD.

Analysis of hematopoietic engraftment

Following transplantation, the animals were euthanized and peripheral blood, spleen and marrow donor chimerism levels were analyzed at predetermined time points. Bone marrow cells were isolated and stained with mAbs which included allophycocyanin-conjugated anti-mouse c-Kit, phycoerythrin (PE)-conjugated anti-mouse CD34 and anti-mouse lineage marker cocktail (biotin-conjugated mAbs against mouse CD5, CD11b, CD45/B220, Anti-Gr-1, Anti-Ter-119; Miltenyi Biotec). Secondary staining with anti-biotin flourescein isothiocyanate (FITC) was performed. Recipient spleen and bone marrow cells were isolated and stained with mAbs against H-2^k (FITC-conjugated) and H-2^b (PE-conjugated) (the H2 haplotypes of recipient and donor, respectively). To analyze the donor lineage-specific hematopoietic cells in the bone marrow, cells were stained with FITC-conjugated mAbs to anti-mouse CD8, CD4 (T-cells), B220 (B-cells), Anti-Gr-1 (granulocyte) and Ter-119 (erythrocyte) (BD pharmingen, San Jose, CA, USA). Flow cytometric analysis was conducted after hypotonic lysis of the cell suspension. Rat isotype controls, which resulted in less than 1% positively stained cells, were used. Multicolor FACS Caliber flow cytometer (BD Biosciences, San Jose, CA, USA) and FlowJo software were used in the data analysis.

Hematologic parameters

Hematological parameters, in recipient animals, were assessed by ADVIA 120 Hematology System (Siemens), using multispecies software. Hemoglobin, white blood cell (WBC) count and platelet count were assessed at predetermined time points.

Statistical analyses

Values were expressed as mean $\pm$ standard error of the mean. Survival data in each group were generated using Kaplan–Meier lifetime survival probability methodology and log-rank (Mantel–Cox) test using the PRISM software (SAS Institute, Cary, NC, USA). The proportion of mice in each group with donor chimerism and GVHD were compared and analyzed using χ^2 2×2 analyses and Fisher's exact test when required. P value <0.01 was considered significant due to multiple comparison groups with different doses of FNPB. The statistical significance in the mean weight, mean hemoglobin, WBC count and platelet count was analyzed by the two-tailed Student's t-test of means, assuming unequal variance. We considered a P value <0.05 as statistically significant.

Results

Photochemical treatment decreases T-lymphocyte proliferation without abrogating activation marker expression

Following magnetic separation and negative isolation and/or positive selection of C57BL/6 maternal splenocytes, a donor T-lymphocyte ($CD3^+$) population with >95% purity was obtained. These T-lymphocytes were treated with a broad range of exposures to S-59 and UVA (Figures 1a and b). There was an inverse relationship of T-lymphocyte proliferation to higher concentrations of S-59. T-lymphocyte proliferation was negligible at 4, 5 and 10 nmol/L concentrations of S-59. The inhibitive effect was

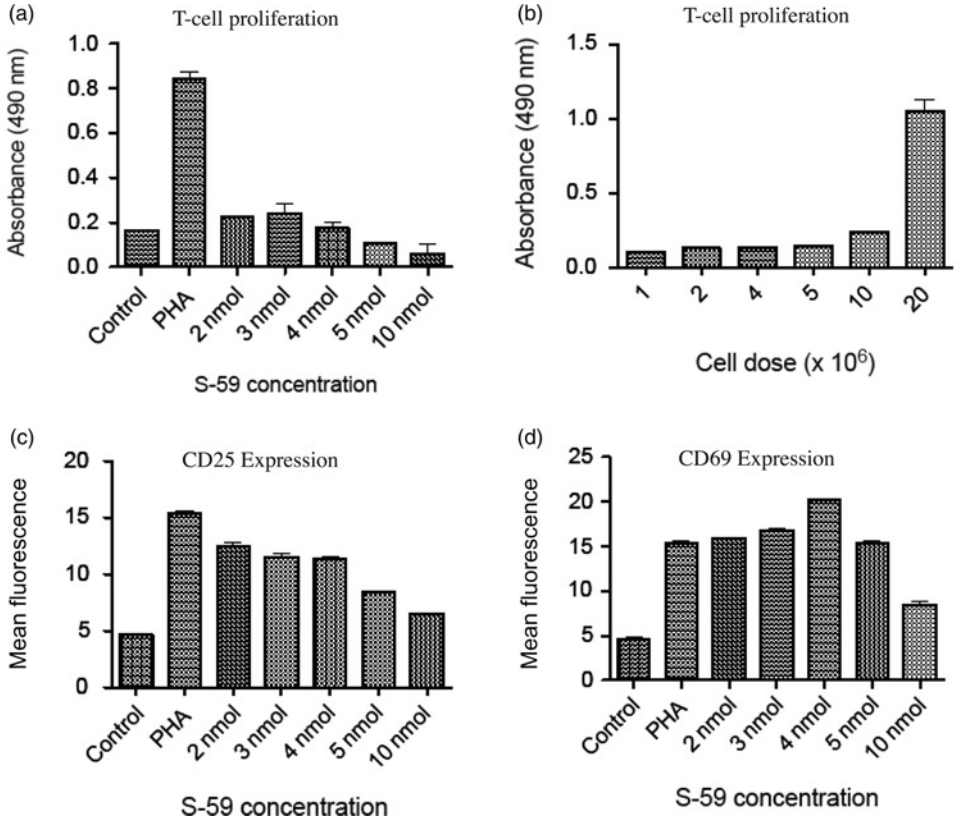

Figure 1 *In vitro* mouse T-lymphocyte proliferation and function after photochemical treatment. (a) Mouse T-lymphocyte proliferation: T-cells isolated from maternal spleen were seeded into triplicate wells at a concentration of 10^6 T-cells/200 μL. Control showing T-lymphocyte proliferation following ultraviolet (UVA) exposure alone. Positive control (phytohemagglutinin [PHA]) consists of cells treated with PHA and UVA for five minutes. (b) Proliferation of graded doses of T-cells following incubation with 4 nmol/L S-59 + UVA in the presence of PHA (bars represent mean value of triplicate samples ± SEM). Effect of S-59 treatment on T-lymphocyte surface antigen expression (c–d). Mouse T-lymphocytes were stained with fluorescent monoclonal antibody to activation markers CD25 (c) and CD69 (d) following stimulation by PHA. Negative control wells (Control) contained untreated cells exposed to UVA alone. Positive control (PHA) consisted of cells stimulated with PHA and exposed to UVA alone. Test wells consisted of cells with varying concentration of S-59 and UVA, after stimulation with PHA. The control and test wells containing varying doses of S-59 per 10^6 cells were incubated for 48 h, and mean fluorescence from the histogram peaks was measured by flow cytometry (as described in the Materials and methods section). Each column with error bars represent mean fluorescence of triplicate samples ± SEM

Table 1 Characteristics of C57BL/6 fetal near-term peripheral blood

	C57BL/6 strain
Litter size	6–12 (mean, 9)
Approximate volume of blood from each fetus	10–40 μL (mean, 25 μL)
Total number of nucleated cells from each litter	$1.32–8 \times 10^6$/mL (mean, 4.66×10^6/mL)
Total number of nucleated cells per fetus	$1.6–6.2 \times 10^5$ cells/fetus (mean, 4.22×10^5 cells/fetus)
T-lymphocyte concentration in FNPB ($CD3^+$ cells)*	0.1–0.55% of total nucleated cells (mean, 0.43%)
$CD117^+$ positive (c-Kit $^+$) cells in FNPB†	0.5–2.8% of total nucleated cells (mean, 1.35%)
c-Kit^+/Lin^-/Sca-1^+ (KLS) cells in FNPB	0.2–1.2% of total nucleated cells
$CD34^+$ KLS cells	90 (±1.2)% of KLS cells

FNPB, fetal near-term peripheral blood
*Expressed as percentage of the total nucleated cells
†Percentage of the total nucleated cells, expressed as range and mean

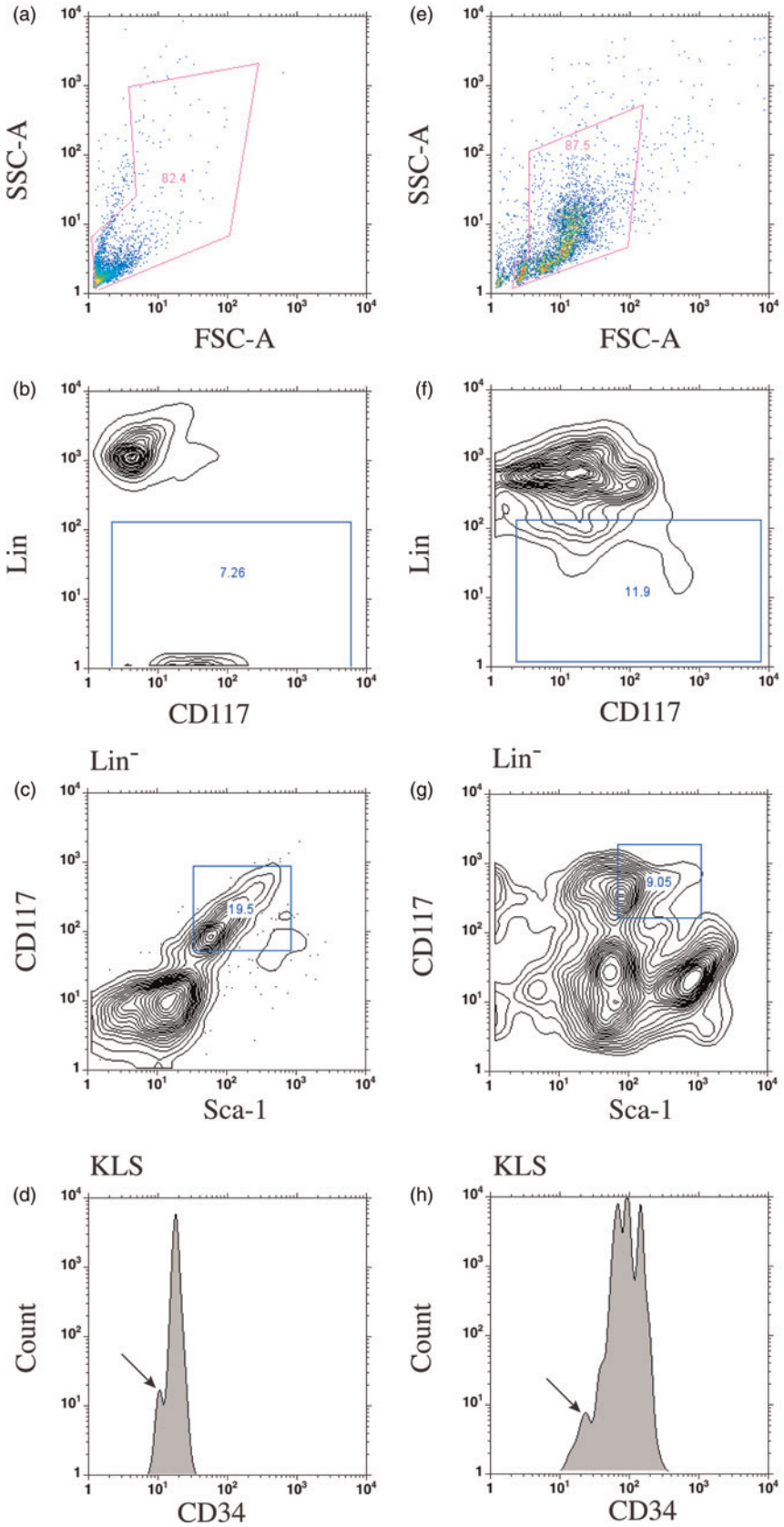

Figure 2 Phenotypic analyses of murine fetal near-term peripheral blood (FNPB) and bone marrow (BM). Representative flow cytometric (FACS) analysis profiles of FNPB (left panels): (a) forward scatter (FSC-A) and side scatter (SSC-A) pattern of FNPB; (b) CD117 (c-Kit) and lineage negative (lin^-) fraction in FNPB; (c) c-Kit^+, Lin^-, Sca-1^+ (KLS) fraction in FNPB; and (d) the CD34 population of the KLS fraction of FNPB. The arrow (→) denotes the CD34 negative (approximately 10%) of the gated population. Comparative FACS profiles of BM (right panels): (e) FSC-A and SSC-A pattern of BM; (f) CD117 (c-Kit) and lineage negative (lin^-) fraction in BM; (g) c-Kit^+, Lin^-, Sca-1^+ (KLS) fraction in BM; (h) the CD34 population of the KLS fraction of FNPB. The arrow denotes the $CD34^-$ fraction (approximately 7%) (A color version of this figure is available in the online journal)

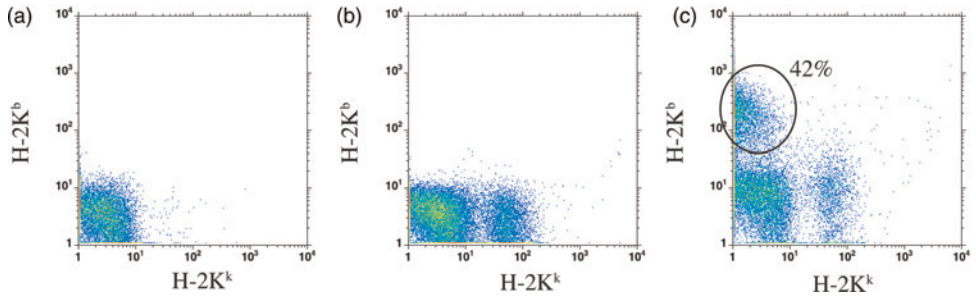

Figure 3 Chimerism analysis after fetal near-term peripheral blood (FNPB) transplantation in the absence of donor T-lymphocytes. Representative flow cytometric (FACS) analysis following cord blood transplantation (donor C57BL/6 FNPB [H-2^b] in recipient AKR mice [H-2^k]). Donor chimerism in bone marrow was determined 60 d after transplantation. The recipient bone marrow cells were stained with fluorescent monoclonal antibody to H2 haplotype antigen (FITC-conjugated H-2^k and PE-conjugated H-2^b). (a) Negative transplantation control, (b) marrow chimerism in a recipient receiving 3×10^6 donor FNPB nucleated cells and (c) 6×10^6 donor FNPB nucleated cells (A color version of this figure is available in the online journal)

sustained at T-lymphocyte doses as high as 10×10^6 cells (Figure 1b). Expression of T-cell activation markers by flow cytometry (CD25 [IL2 receptor] and CD69 [early activation marker]) showed that the S-59-treated T-lymphocytes expressed these markers optimally at a S-59 concentration of 4–5 nmol/L, when expanded by CD3/CD28 dynabeads or following PHA incubation (Figures 1c and d).

FNPB is deficient in T-lymphocytes

We obtained FNPB from C57BL/6 strains, which is characterized in Table 1. The T-lymphocyte content was negligible (0.1–0.55%) when compared with adult bone marrow ($4.9 \pm 0.83\%$) of the same species. In some experiments, we pooled FNPB from multiple litters to accommodate the targeted nucleated cell dose required for the cord blood dose escalation studies. Since murine FNPB contained only trivial quantities of T-lymphocytes, it was possible to escalate the nucleated cell dose by combining litters from timed pregnancies without significantly altering the donor T-lymphocyte content. The KLS cell population in FNPB ranged from 0.2% to 1.2% of the nucleated cells, of which 10% were $CD34^-$ (Figure 2 and Table 1). Only a small fraction of FNPB cells stained with annexin V prior to transplantation. There was no evidence of fetal thymocyte contamination of the peripheral blood when the decapitation technique was used (data not shown).

Graft rejection after sublethal total body irradiation and MHC-mismatched transplantation with $<6 \times 10^6$ nucleated cells

To target a sublethal dose of irradiation, the recipients were conditioned with total body irradiation (TBI) that ranged from 300 to 600 cGy in 50 cGy increments. Recovery of hematological parameters and weight were observed over a period of six months post-transplantation. We observed that a dose of 600 cGy was uniformly lethal ($n = 10$), but when a dose of 550 cGy was administered, 80% of the animals survived (4 of 5). None of the mice that received 500 cGy died of bone marrow aplasia and full hematological recovery was observed by 40 d after administration of the

Table 2 Characteristics of transplanted mice

Group	*n*	FNPB cells	T-cells	S-59 (nmol/L)	% donor cells*	GVHD	*P*
1a	8	0.5×10^6	–	–	0.5, 0.8, 1, 0.22, 0.54, 0.11, 0.38, 0.52	None	
1b	7	1×10^6	–	–	2, 0.82, 0.22, 0.85, 1.2, 1.6, 0.32	None	
1c	8	3×10^6	–	–	0.34, 0.56, 1.43, 1.88, 0.86, 1.32, 0.48, 1.24	None	
1d	8	6×10^6	–	–	1, 0.5, 42, 2, 3, 0.8, 3.2, 1	12.5%	
1e	6	10×10^6	–	–	1.8, 1.62, 0.34, 1.6, 0.44, 0.8	None	
2a	4	1×10^6	3×10^6	–	1.3, 1.85, 1.2, 1.66	None	
2b	6	3×10^6	3×10^6	–	90, 1.2, 0.5, 93, 2.2, 95	50%	0.545†
2c	2	3×10^6	9×10^6	–	93.5, 95	100%	
3a	4	0.5×10^6	1×10^6	4	1.08, 1.2, 1.11, 1.85	None	
3b	6	3×10^6	1×10^6	4	0.5, 3.5, 2.2, 2.8, 3.1, 1.2	None	
3c	6	3×10^6	3×10^6	4	1.2, 3.5, 0.5, 55, 2.3, 3.8	None	0.428‡
3d	8	3×10^6	9×10^6	4	90, 92, 98, 94.5, 2, 96, 3.2, 97	12.5%	0.0069§
3e	6	6×10^6	3×10^6	4	93, 82, 2, 1, 3, 90	None	0.244¶

Each group had 20–24 mice (*n* indicates the number of mice in each group evaluated at day 60 post-transplantation)

*Numbers represent data for individual mice (significant chimerism was defined as greater than 5% nucleated donor cells in the recipient bone marrow). The results show donor chimerism analyses done 60 d after FNPB transplantation. FNPB indicates fetal near-term peripheral blood. GVHD denotes the percentage of recipient animals in each group with graft-versus-host disease, analyzed both by clinical and histopathological means at 60-d time point. All *P* values were computed by comparing the donor engraftment between various groups

†*P* value when compared with group 3c

‡*P* value when compared with the group receiving 3×10^6 FNPB alone (group 1c)

§*P* value when compared with the group 1c

¶*P* value when compared with group 1d

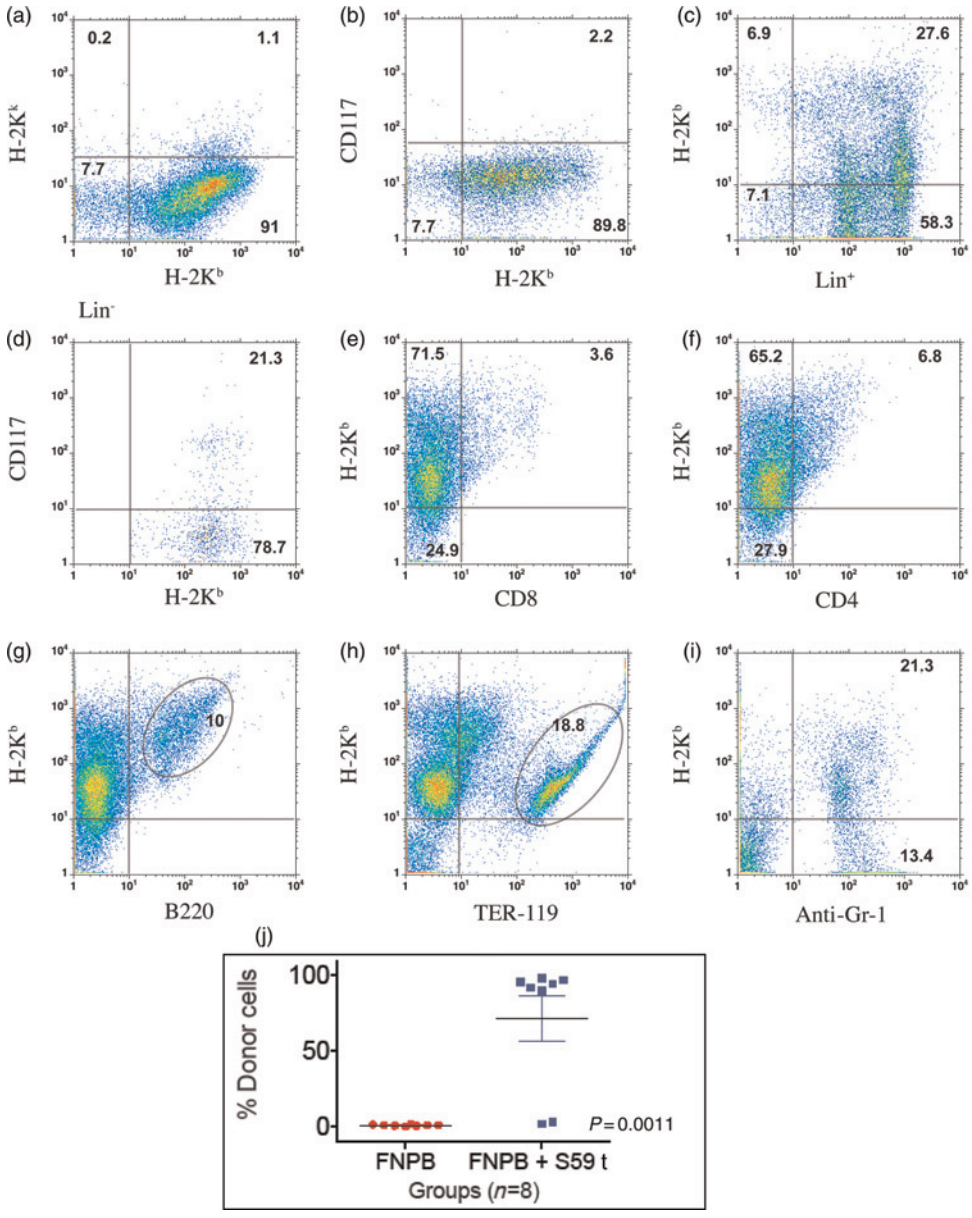

Figure 4 Chimerism analysis after fetal near-term peripheral blood (FNPB) transplantation with S-59-treated T-lymphocytes. Representative cell lineage flow cytometric (FACS) analyses of marrow cells performed 60 d after transplantation following submyeloablative irradiation. Recipient AKR (H-2^k) bone marrow cells were analyzed after transplantation of C57BL/6 (H-2^b) 3×10^6 FNPB + 9×10^6 S-59-treated donor T-lymphocytes. The bone marrow cells were stained with monoclonal Abs against mouse H2 haplotype. (a) Marrow donor chimerism by expression of MHC-class 1 antigen (recipient AKR [H-2^k] and donor C57BL/6 [H-2^b]), (b) H-2^b and CD117 (c-Kit) expression in the recipient marrow cells, (c) H-2^b and Lin$^+$, (d) H-2^b and CD117 expression in the CD34$^-$ and lineage$^-$ fraction of marrow cells, (e) H-2^b and CD4, (f) H-2^b and CD8, (g) H-2^b and B220, (h) H-2^b and erythroid, and (i) Gr-1 and H-2^b. Percentages of the various subsets are depicted in the graphs. (j) Scatter plot depicting donor chimerism fraction in recipient mice after transplantation of 3×10^6 FNPB cells alone and 3×10^6 FNPB with 9×10^6 S-59 T cells, 60 d after transplantation. The latter group shows evidence of donor chimerism in an 'all or none' manner (A color version of this figure is available in the online journal)

sublethal irradiation (data not shown). All the animals that received 500 cGy TBI had transient weight loss (<20%), ruffling of fur coat and decreased activity, which resolved by four weeks after transplantation.

We performed a total nucleated cell (TNC) dose escalation of donor C57BL/6 FNPB ranging from 0.5 to 10×10^6 donor TNC after conditioning with 500 cGy TBI. The donor chimerism was evaluated in the splenic and bone marrow compartments by flow-cytometric analysis, 30 and 60 d after transplantation. While most recipient mice lacked donor engraftment after FNPBT, one of eight recipient animals showed evidence of donor engraftment after the administration of 6×10^6 nucleated donor cells (Figure 3c). Escalating the FNPB cell dose to 10×10^6 TNC did not improve the engraftment rate (Table 2). Based upon these results, it was determined that a minimum of 6×10^6 allogeneic FNPB cells were required but not necessarily sufficient for donor engraftment after sublethal TBI preparation. Autologous reconstitution was commonly seen in this group after transplantation. Most recipient mice survived after transplantation and remained healthy through 100 d after transplantation (97.1% survival).

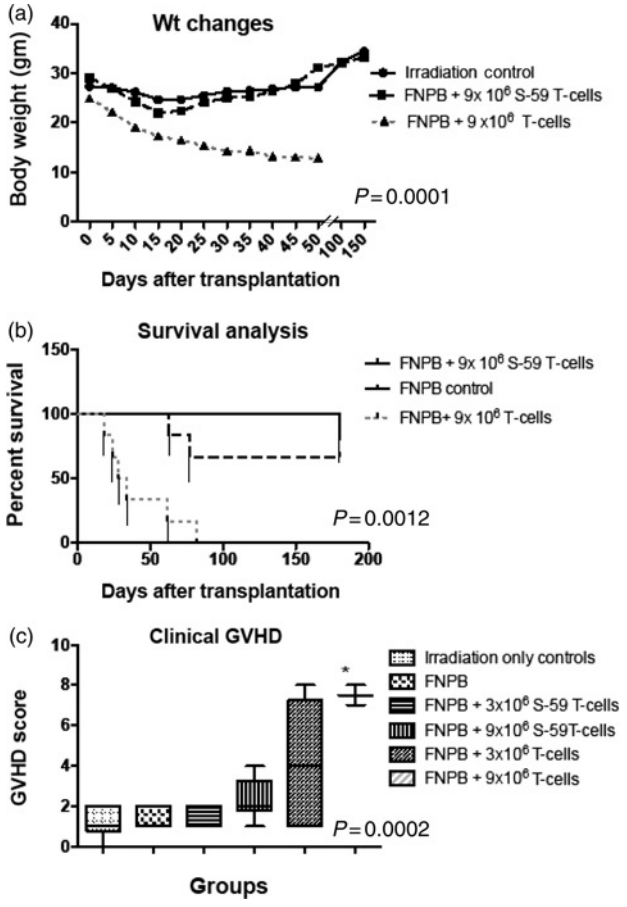

Figure 5 Changes in body weight, survival and clinical graft-versus-host disease (GVHD) following transplantation. AKR recipient mice ($n = 6$) after transplantation with C57BL/6 fetal near-term peripheral blood (FNPB) alone, FNPB with untreated donor T-lymphocytes and FNPB with S-59-treated T-lymphocytes. (- - ▲- -) 3×10^6 FNPB cells + 9×10^6 untreated T-lymphocytes. (- -■ - -) 3×10^6 FNPB cells + 9×10^6 S-59 treated T cells. (—●—) 3×10^6 FNPB cells. (a) Weight over time after transplantation. (b) Survival over time (percentage) after transplantation. (c) Clinical GVHD score (mean) in different groups of mice following transplantation

Transplantation with S-59-treated T-lymphocytes improves donor engraftment without significant GVHD

We also conducted experiments to evaluate engraftment after FNPBT with photochemically-treated, MHC-mismatched donor T-lymphocytes. Transplantation with 3×10^6 FNPB and 3×10^6 mature S-59-treated donor T-cells showed evidence of engraftment in one of six recipient mice, although consistent high-level donor chimerism was not observed in this experimental group (Table 2). To determine if a larger number of donor S-59-treated T-lymphocytes were required to ensure engraftment, we administered incrementally larger doses of S-59-treated donor T-lymphocytes with a fixed number of FNPB cells (3×10^6). While there was no improvement after transplantation with 6×10^6 S-59-treated donor T-lymphocytes, there was significant improvement in hematopoietic engraftment when 9×10^6 S-59 donor T-cells were administered compared with the animals that received FNPB alone (75% had donor engraftment, $P = 0.007$). Significantly improved donor chimerism was also observed when compared with the group that received a lower dose of 3×10^6 S-59-treated T-lymphocytes (Figure 4j). Doubling the FNPB nucleated cell dose to 6×10^6 (at 3×10^6 S-59 donor lymphocyte dose) did not significantly improve the engraftment rate (50% had engraftment, $P = 0.244$), when compared with transplantation of FNPB alone. In addition, the co-infusion of S-59-treated T-lymphocytes permitted a three-fold reduction in the size of the FNPB donor graft necessary to ensure donor engraftment compared with recipient animals that received only FNPB.

We observed nearly complete donor H2 haplotype expression in the hematopoietic stem cells from bone marrow (Figures 4 a–d). A similar pattern of donor c-Kit expressing cells was observed in the spleen. We showed the presence of donor MHC class I positive cells in the recipient marrow, which were $lin^-/CD34^-$ and $c\text{-}Kit^+$ (Figure 4d). The larger fraction of Lin^+ cells in the bone marrow compartment showed representation of lineage cell-surface markers specific for donor lymphocyte, myeloid and erythroid cells (Figure 4e–i). There was significant survival advantage in animals that received the S-59-treated donor T-lymphocytes during the observation period that extended through six months after transplantation (Figure 5b). Long-term donor engraftment (>100 d) also was noted in these mice.

Decreased incidence of GVHD after transplantation with S-59-treated T-lymphocytes

To evaluate the impact of photochemical treatment on GVHD, we transplanted 3×10^6 C57BL/6 FNPB donor cells with 3×10^6 and 9×10^6 S-59-treated and-untreated C57BL/6 $CD3^+$ cells, respectively, into AKR recipients. GVHD was observed in 50% of the mice that received 3×10^6 unmanipulated donor T-lymphocytes, whereas no GVHD was observed in the mice that received 3×10^6 S-59-treated T-lymphocytes. In the group transplanted with 9×10^6 donor T-lymphocytes, the incidence of GVHD was 100% and 12.5% in the untreated T-lymphocyte and S-59-treated T-lymphocyte recipient mice, respectively (Figures 5a and c). Severe GVHD (clinical GVHD score >6) with weight loss and diarrhea occurred in the group that received untreated donor T-lymphocytes (Figures 5a and c). In animals that received untreated donor T-lymphocytes, there was prominent involvement of the liver, although the skin and gut were less severely affected. Also, the engrafted mice that received unmanipulated donor T-lymphocytes exhibited a clinical GVHD score of 6–8 approximately 2–3 weeks after transplantation, which was correlated with organ toxicity by histopathology. These mice had significant lymphocytic infiltration near the portal triad (Figure 6h). Transient weight loss with recovery to baseline weight occurred in the group that received S-59-treated T-lymphocytes 30–40 d after transplantation (Figure 6a). The histopathology showed minimal damage to the skin, liver and gut after co-infusion of S-59-treated T-lymphocytes (Figures 6d–i).

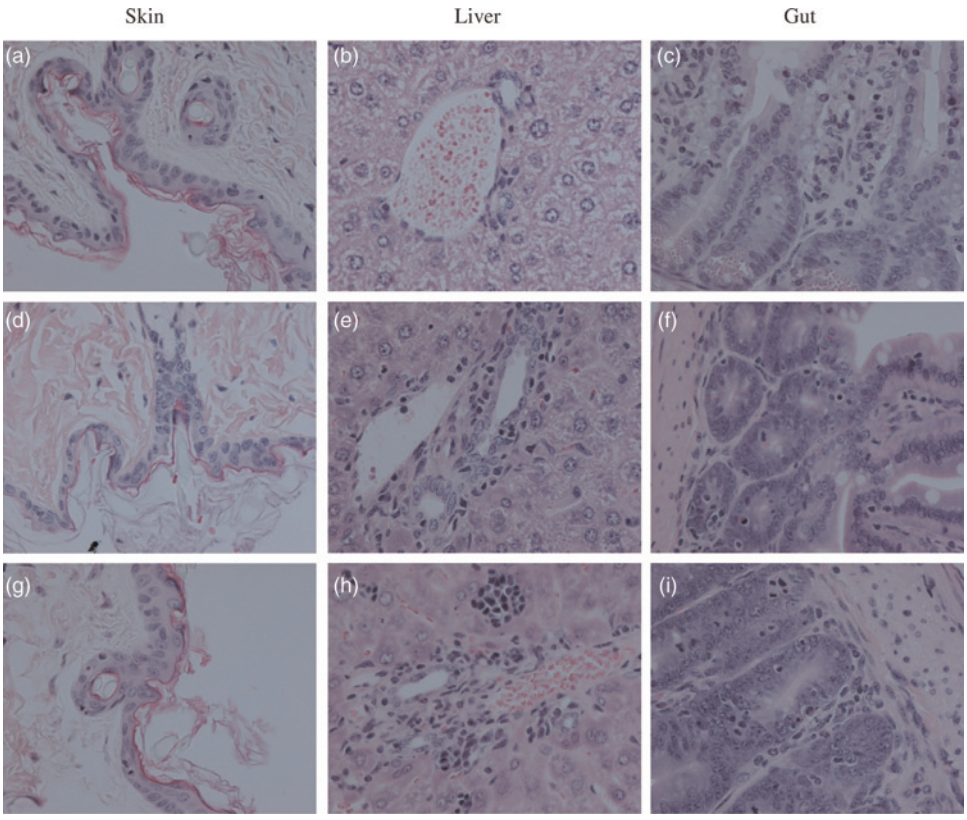

Figure 6 Histopathology sections of skin, liver and gut after transplantation with fetal near-term peripheral blood (FNPB). Microscopic examination of hematoxylin/eosin stained sections of skin, liver and gut (all panels ×400 magnification) in AKR recipient mice after MHC-mismatched FNPBT. Graft-versus-host disease (GVHD) was assessed 60 d after FNPBT. Panels (a–c) shows histopathology of (a) skin, (b) hepatocytes/portal triad and (c) intestinal villi and crypts following transplantation of 3×10^6 nucleated cells. The histopathology following transplantation of 3×10^6 nucleated cells and 9×10^6 S-59-treated T-cells are shown in panels d–f. (d) Dermis and epidermis, (e) portal triad and (f) intestine. In panels g–i, histopathology sections are shown after FNPBT with 3×10^6 nucleated cells and 9×10^6 untreated T-lymphocytes. (g) Skin, (h) liver and (i) intestine (A color version of this figure is available in the online journal)

Hematological recovery after transplantation

There was a delay in red blood cell, white blood cell and platelet recovery (Figures 7a–c) after FNPBT with S-59-treated donor T-lymphocytes, compared with control animals that received FNPB or irradiation alone. Control mice had pancytopenia approximately 10 d after transplantation, with full recovery by two months after transplantation. While the platelet and white blood cell levels started to recover by 30 d after transplantation in the group that received FNPB and 3×10^6 S-59-treated donor T-lymphocytes, red blood cell and hemoglobin recovery was delayed until 60 d after transplantation. This delay also was longer in the group that received 9×10^6 S-59 T-lymphocytes. In addition, neutrophil and platelet levels recovered 150 d after transplantation in these mice. Significant decreases ($P = 0.0001$) in the peripheral blood counts (Figure 7a–c) also were observed in the group that received 9×10^6 unmanipulated donor T-lymphocytes, culminating in death approximately 80 d after transplantation. There was evidence of increased colony-forming unit formation following direct co-culture of S-59-treated T-cells with hematopoietic progenitor cells (Figures 8 A and B). Approximately 6–7-fold increase in colony formation units of all lineages were noted in the presence of allogeneic T-cells.

FNPBT with unmanipulated donor T-lymphocytes results in engraftment but decreased survival due to severe GVHD

We evaluated the utility of unmanipulated T-lymphocytes in facilitating donor engraftment after sublethal TBI and FNPBT in a MHC H2 disparate model. There was no evidence of engraftment after transplantation with a donor nucleated cell dose of 1×10^6/mouse. However, near complete donor chimerism (>90%) was noted in 50% of the mice that received FNPB and unmanipulated donor T-lymphocytes at cell doses of 3×10^6 and 3×10^6, respectively. Long-term survival was 50% in this group. Increasing the T-lymphocyte dose to 9×10^6 was associated with 100% mortality, approximately 80–90 d after CBT. At necropsy, some of the animals that received 9×10^6 donor T-lymphocytes showed evidence of significant bone marrow aplasia and peripheral blood cytopenias (see Figure 7a–c).

Discussion

We have shown that it is possible to perform murine CBT across a major histocompatibility barrier after sublethal irradiation and generate durable donor chimerism by co-infusion of S-59-treated donor T-lymphocytes. Our initial

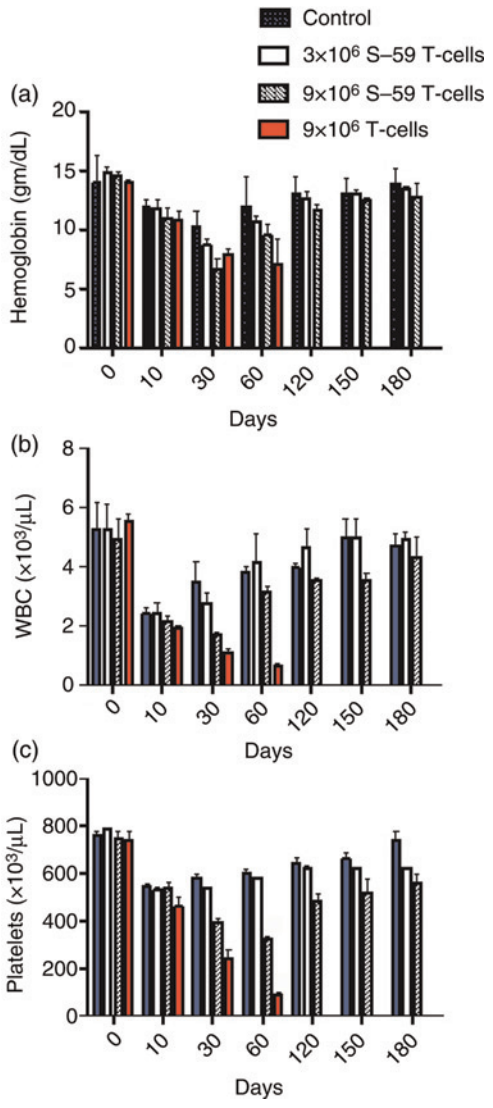

Figure 7 Hematological recovery after transplantation. Hematological recovery is depicted after transplantation with fetal near-term peripheral blood (FNPB) alone, FNPB with untreated donor T-lymphocytes and FNPB with S-59-treated donor T-lymphocytes. Controls consisted of AKR mice that received 500 cGy total body irradiation. (a) Hemoglobin over time after transplantation. (b) WBC count over time after transplantation. (c) Platelet count over time after transplantation. None of the group that received FNPB and 9×10^6 untreated T-lymphocytes survived longer than 82 d after transplantation. The column and error bars depict the mean values $\pm$ SEM (A color version of this figure is available in the online journal)

experiments using FNPB alone with sublethal irradiation demonstrated very inconsistent donor engraftment after transplantation. In addition, most of the animals that received FNPB with untreated donor T-lymphocytes died of GVHD and marrow aplasia by 80–90 d after transplantation. At necropsy, most of these animals with complete donor chimerism had significant lymphocytic infiltration in the liver (Table 2). This was most evident in the animals that received the highest donor T-lymphocyte dose (9×10^6). This complication was abrogated by infusing S-59-treated donor T-lymphocytes with donor cord blood, and it was possible to elicit durable donor engraftment in a majority of the MHC-mismatched recipient mice. The probability of engraftment was influenced primarily by the dose of donor T-lymphocytes administered, as the dose of FNPB administered (beyond a minimum threshold dose) had little impact on engraftment (Table 2: group 3c versus group 3e, $P = 0.545$). Our data also show that without the photochemically treated donor T-lymphocytes, engraftment was sporadic and unreliable across this MHC barrier. In addition, engraftment occurred for the most part without significant GVHD.

Our findings are similar to those published after T-lymphocyte-depleted bone marrow transplantation with photochemically treated donor T-lymphocytes.[21,23] The donor MHC class I expressing cells (H-2K^b) in the recipient marrow included a cellular population that expressed c-Kit$^+$, Lineage$^-$ and Sca-1$^+$ (KLS), which was CD34$^-$, and these cells have long-term self-renewal potential and the ability to generate multilineage hematopoietic cells.[24] Despite the presence of these progenitor hematopoietic cells in the marrow, we observed a delay in recovery after transplantation from peripheral blood cytopenias, particularly in the mice that received the highest dose (9×10^6) of S-59-treated T-cells. This delay in hematological recovery might have been related to a dual effect of radiation-induced injury to the bone marrow and limited GVHD directed at host marrow stromal cells. This is supported by the observation that recipient mice that received S-59-treated T-lymphocytes had mild hepatic GVHD that resolved spontaneously over time. Moreover, there was no evidence of direct toxicity of S-59 on hematopoietic stem cells, as demonstrated in the *in vitro* colony forming assay (Figures 8A and B).

Previously, multilineage engraftment with limited GVHD was observed after *in utero* transplantation with T-lymphocyte depleted donor bone marrow and S-59 treated, presensitized donor T-lymphocytes.[5] The potential mechanism for the engraftment-facilitating activity of S-59-treated T-lymphocytes is not known, but it could be related to the retention of cytotoxic or 'veto' activity, which might inhibit or even eliminate residual host T-cytotoxic function after sublethal conditioning. In addition, infusion of a large number of donor photochemically treated T-lymphocytes may overcome the donor-specific cytotoxicity of host immune cells by induction of anergy among cytotoxic host T-lymphocytes. This may occur by contact-dependent and-independent mechanisms, thereby permitting the survival of donor hematopoietic stem cells.[25,26] This notion is supported by the fact that a three-fold increase in dose of photochemically treated T-lymphocytes improved engraftment significantly (75% versus 16.66%, $P = 0.007$) when these were infused with a static dose (3×10^6) of FNPB cells.

Chen *et al.*[27] demonstrated that it is possible to achieve engraftment in a MHC disparate allogeneic setting with as few as 1×10^6 FNPB cells per mouse following lethal irradiation (18% of the mice engrafted). The different threshold doses for FNPB used in our experiments were based on the data published previously,[27] where administration of 1×10^6 allogeneic full-term fetal blood cells from a 20-day-old C57BL/6 fetus rescued 13% of lethally irradiated BALB/c mice that survived more than 100 d. With the use of submyeloablative radiation regimen, this dose

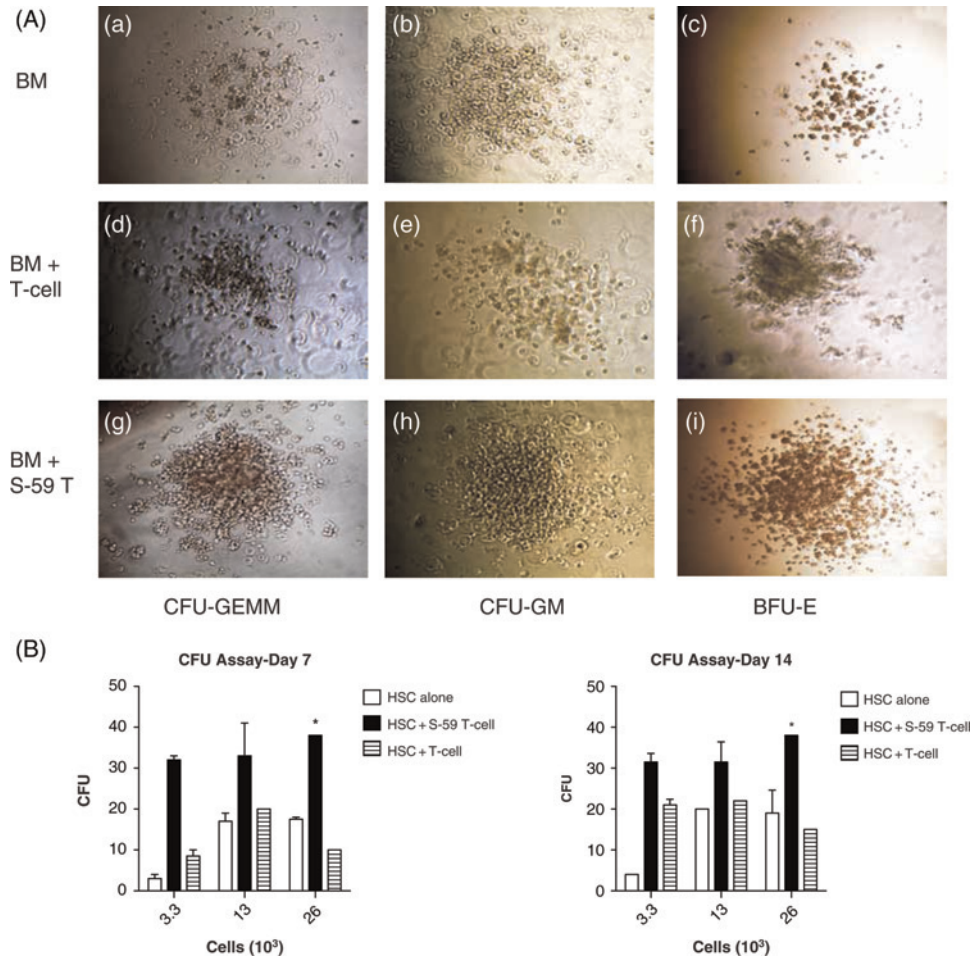

Figure 8 Impact of S-59-treated T-cells on hematopoietic colony formation. (A) Lineage depleted bone marrow stem (BM) cells were cultured with 10^4 allogeneic T-cells (with and without S-59 treatment) in methylcellulose media. Photomicrographs of the various colony forming units were taken on day 7 using an inverted microscope at $\times$100 (a, b, d, e, g, h) and $\times$200 (c, f, i) magnification. Colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM), Burst-forming unit - erthyroid (BFU-E) and colony-forming unit granulocyte, macrophage (CFU-GM), in cultures containing BM alone (a, b, c); BM with unmanipulated T-cells (d, e, f) and BM with S-59-treated T-cells (g, h, i). (B) Lineage-depleted BM stem cells were cultured with S-59 treated and untreated T cells and the cells were harvested on day 7 (left panel) and 14 (right panel) and counted. Allogeneic CD3 T-cells were plated along with the hematopoietic stem cells (HSCs) and fold increase from the original number of cells were calculated relative to day 0 ($n = 2$–3 independent experiments). $^*P$ value < 0.001 (A color version of this figure is available in the online journal)

was insufficient to achieve significant chimerism in our experiments as shown in Table 2. However, escalating the nucleated cell dose to 6×10^6 resulted in donor stem cell engraftment, in the context of submyeloablative preparative regimen. Even though increasing the donor nucleated cell dose did not increase the engraftment rates statistically, the radioprotective and engraftment effect was diminished after FNPBT compared with traditional T-lymphocyte depleted bone marrow transplantation in H2 (major) antigen and non-H2 (minor) antigen disparate settings.[21,23] For this reason, previous experiments using murine FNPB for transplantation invariably utilized parenteral to F1 hybrid models or included lethal irradiation in the conditioning regimen[28] More recently, Waller *et al.* demonstrated that adding irradiated whole splenocytes to T-lymphocyte depleted allogeneic bone marrow after lethal irradiation promoted engraftment in MHC mismatched recipients without causing GVHD. They also observed better long-term survival with donor-derived hematopoiesis.[25] These experiments showed survival and long-term repopulation by donor hematopoietic stem cells were dependent on the irradiated splenocyte T-lymphocyte dose. However, in these instances, lethal irradiation was also required for successful engraftment. Here, we have shown that sublethal irradiation is sufficient for engraftment across a MHC barrier when facilitated by photochemically treated donor T-lymphocytes. This important advance also has the potential to improve the safety profile of clinical transplantation across an HLA barrier.

The vital role played by T-lymphocytes in the pathogenesis of GVHD and on engraftment after unmanipulated and T-cell depleted bone marrow transplantation has been studied extensively.[29] Unfortunately, the benefit of a reduced risk of GVHD after TCD marrow transplantation is often achieved at the cost of higher rates of graft failure, opportunistic infections and recurrent malignancy.[30,31] The less efficient engraftment after TCD can be overcome in part by increasing the nucleated marrow hematopoietic

stem cell dose.[32] However, in CBT it is challenging to increase the hematopoietic stem cell dose due to limitations in collection and *ex vivo* expansion.[33–35] Hence, our method of adding back photochemically treated donor T-lymphocytes has the potential to overcome a significant limitation of mismatched donor CBT, which is to ensure engraftment without significant GVHD.

Even minor antigenic differences can stimulate GVHD in murine transplantation models, and as few as 3×10^4 T-lymphocytes are sufficient to cause lethal GVHD.[36,37] To mitigate the risk of GVHD, T-lymphocyte depletion by selective and non-selective methods has been employed after mismatched bone marrow transplantation.[38–45] Co-transplantation of $\gamma\delta$ donor T-cells in large numbers with T-cell depleted bone marrow grafts also have improved engraftment after major histocompatibility mismatched transplantation, without significant GVHD.[46] We did note minimal hepatic GVHD following infusion of higher dose (9×10^6) of S-59-treated T-cells. It could possibly be mediated by small numbers of T-cells that might have escaped psoralen treatment. But the clinical GVHD and long-term survival of the animals in this group were comparable to controls receiving FNPB infusate alone.

The uniqueness of our experiments lie in the use of FNPBT, as a surrogate for cord blood stem cell transplantation, after submyeloablative preparative regimen, and the use of photochemically treated T-cells to overcome MHC barrier. In our major and minor antigen (H2 and non-H2) mismatched transplantation using sublethal irradiation, the goal of transplantation was to achieve partial or full donor chimerism sufficient to ameliorate hematological and other genetic disorders in the absence of lethal irradiation. The lower engraftment rates observed in our FNPB alone transplant model might be secondary to the sublethal conditioning regimen that permitted the survival and proliferation of host cytotoxic rejection factors. However, engraftment was augmented by S-59-treated T-lymphocytes, and an optimal cell dose was defined that ensured engraftment without fatal GVHD. We show here that high doses of S-59-treated T-lymphocytes were able to overcome rejection factors and tolerize the host after a sublethal conditioning regimen. The efficiency of higher doses of S-59-treated T-cells in creating durable complete donor chimerism, when combined with fetal peripheral blood stem cells were confirmed in our replicate experiments. The factors conferring to the ability of S-59-treated T-lymphocytes to accomplish engraftment requires further investigation. However, these data suggest that our FNPBT model using S-59-treated T-lymphocyte 'add-back' may contribute a potential immunomodulatory tool in both animal and human systems and may be especially useful in the treatment of clinical hemoglobinopathies, where a non-myeloablative regimen to decrease the toxicity of transplantation could optimize the risk/benefit considerations of this treatment.

Author contributions: BK designed and performed experiments, analyzed data and wrote the paper. MM, CH, HG and SS performed some experiments; LN analyzed data, JEH designed the research; MCW and FAK designed the research, analyzed the data and wrote the paper.

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