High Level Allogeneic Chimerism Achieved by Prenatal Tolerance Induction and Postnatal Non-Myeloablative Bone Marrow Transplantation.

William H. Peranteau¹, Satoshi Hayashi¹, Michael Hsieh¹, Aimen F. Shaaban¹, and Alan W. Flake¹

¹The Children’s Institute for Surgical Science, The Children’s Hospital of Philadelphia, 34th Street and Civic Center Blvd. Philadelphia, PA, 19104-4399, USA,

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Address Correspondence to:

Alan W. Flake, M.D.
The Children’s Institute for Surgical Science
The Children’s Hospital of Philadelphia
34th Street and Civic Center Blvd.
Philadelphia, PA 19104-4399
Office (215) 590-3671
FAX (215) 590-3324
e-mail Flake@email.chop.edu

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Abstract

Clinical application of allogeneic bone marrow transplantation (BMT) has been limited by toxicity related to cytoreductive conditioning and immune response. In utero hematopoietic stem cell transplantation (IUHSCTx) is a non-ablative approach that achieves mixed chimerism and donor specific tolerance, but has been limited by minimal engraftment. We hypothesized that mixed chimerism achieved by IUHSCTx could be enhanced after birth by non-myeloablative total body irradiation (TBI) followed by same donor BMT. To test this hypothesis, mixed chimerism was created by IUHSCTx in an MHC mismatched strain combination. After birth, chimeric animals received non-myeloablative TBI followed by transplantation of donor congenic BM cells. Our results show that: (1) low level chimerism after IUHSCTx can be enhanced to high level chimerism by this strategy; (2) enhancement of chimerism is TBI dose dependent; (3) the mechanism of TBI enhancement is via a transient competitive advantage for non-irradiated HSC; (4) engraftment observed in the tolerant, fully allogeneic IUHSCTx recipient is equivalent to a congenic recipient; and (5) host reactive donor lymphocytes are deleted with no evidence of GVHD. This study supports the concept of prenatal tolerance induction to facilitate non-myeloablative postnatal strategies for cellular therapy. If clinically applicable, such an approach could dramatically expand the application of IUHSCTx.
Introduction

In utero hematopoietic stem cell transplantation (IUHSCTx) is a non-myeloablative approach to achieve mixed hematopoietic chimerism and donor specific tolerance\(^1\). Although engraftment of allogeneic or xenogeneic HSC, with resultant long-term, multilineage chimerism has been achieved\(^2,3\), engraftment in most circumstances has been minimal, i.e. well below what would be considered therapeutic for most target diseases\(^4,9\). In contrast, high level engraftment has been achieved after IUHSCTx in experimental\(^10-13\) and clinical circumstances\(^14,15\) where a competitive or survival advantage exists for donor cells. Thus it appears that competition from a normal host hematopoietic compartment is the primary barrier to successful application of IUHSCTx.

One strategy to circumvent the failure to achieve high level engraftment by IUHSCTx is to take advantage of the donor specific tolerance associated with mixed chimerism and increase levels of chimerism into the therapeutic range after birth by a non-myeloablative postnatal regimen. It has been observed in syngeneic mice that low-dose irradiation followed by syngeneic BMT confers a competitive advantage for the non-irradiated cells and results in markedly enhanced levels of chimerism relative to syngeneic BMT alone\(^16,17\). We hypothesized that our tolerant chimeras created by IUHSCTx would be immunologically similar to syngeneic animals, and that the competitive advantage conferred on non-irradiated cells would result in a marked enhancement of chimerism with conversion of low level mixed chimerism, to high level chimerism. We tested this strategy in a fully allogeneic murine model of IUHSCTx.

Materials and Methods

**Mice.** Balb/c (H-2K\(^d\), CD45.2, I-E\(^+\), *MtV*-6\(^+\)), C57BL/6 (referred to as B6 – H-2K\(^b\), CD45.2, I-E\(^-\), *MtV*-6\(^-\), GFP\(^-\) and B6Ly5.2 (H-2K\(^b\), CD45.1, I-E\(^-\), *MtV*-6\(^-\), GFP\(^-\)) mice were purchased from Charles River. B6Pep3b (H-2K\(^b\), CD45.1, I-E\(^-\), *MtV*-6\(^-\)), SJL/J (H-2K\(^s\), CD45.1, I-E\(^-\), *MtV*-6\(^-\)) and CBA/J mice were purchased from Jackson Laboratories.
C57BL/6TgN(act-EGFP)OsbY01 (H-2K^b, CD45.2, I-E^d, Mtv-6, GFP^+) mice were kindly provided by Dr. Okabe, Osaka University, Genome Information Research Center and are referred to as B6GFP in this manuscript. Animals were housed in the Laboratory Animal Facility of the Abramson Research Center at the Children’s Hospital of Philadelphia. The experimental protocols were approved by the Institutional Animal Care and Use Committee at the Children’s Hospital of Philadelphia and followed guidelines set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Donor bone marrow harvest and T-cell depletion.**

Adult BM was harvested from 6-8 week old donors after sacrifice by flushing the tibias and femurs with Ca^{++}/Mg^{++} free phosphate buffered saline (PBS, Gibco, Rockville, MD). Flushed BM was passed through 26 guage needle several times to form a single cell suspension and then filtered through a 70-µm nylon mesh filter and layered over Ficoll (Histopaque 1077, Sigma). After centrifugation at 600g for 15 minutes, the light density mononuclear cell (LDMC) layer was carefully removed and washed with PBS. CD3^+ T cell depletion was performed by incubation of washed cells with FITC-conjugated, anti-CD3 mAb (Pharmingen, San Diego, CA) followed by incubation with anti-FITC microbeads (Miltenyi Biotec, Auburn, CA) and subsequent passage through a Vario MACs magnetic cell sorter (Miltenyi Biotec, Auburn, CA). Flow cytometry, performed on a FACScan (Becton Dickinson, Mountain View, CA), was used to ensure that CD3^+ cells constituted less than 0.5% of the donor bone marrow after depletion. Cells were counted prior to transplant and >95% viability confirmed by trypan blue exclusion.

**In utero bone marrow transplantation.**

Fetuses of time dated pregnant mice were injected at days 13-14 of gestation as previously described^7,8. All fetuses were injected intraperitoneally with a 100-µm (outside diameter) beveled glass micropipette and received 5x10^6 cells in 5 µL of PBS. Control animals of
each recipient strain received 5µl of PBS rather than donor cells. Pups were weaned at 3 weeks of age.

**Postnatal transplant conditioning.**

Peripheral blood of mice transplanted in utero was analyzed by flow cytometry at 4 or 8 weeks of age to identify chimeric mice. Chimeric mice were then subjected to either 0 cGy, 82.5 cGy, 138 cGy or 276 cGy of TBI. PBS injected control mice were subjected to 276 cGy of TBI prior to TCD BMT. Naïve B6 control mice were subjected to 82.5 cGy or 276 cGy of irradiation. TBI was delivered as a single dose by a Gammator M-38 cesium 137 irradiator at a rate of 276 cGy/minute unless otherwise stated.

**Postnatal bone marrow transplantation.**

Six to eight hours after irradiation the mice received donor congenic (B6Pep3b or B6Ly5.2) BM cells. 30x10^6 TCD BM cells, resuspended in 100 µL of PBS, were injected via the lateral tail vein into the chimeric and control mice at either 4 or 8 weeks of life.

**Split dose irradiation and duration of irradiation effect studies.**

Chimeric mice created by IUHSCTx that were used to analyze the duration of irradiation effect on engraftment as well as the effect of split dose irradiation on engraftment received a postnatal transplant conditioning regimen consisting of either 0 cGy or 82.5 cGy TBI at 4 weeks of life as described above. Six to 8 hours after irradiation the mice received a postnatal BMT by lateral tail vein injection consisting of 30 x 10^6 TCD B6Ly5.2 BM cells. 4 weeks after the initial postnatal transplant, at 8 weeks of age, the mice received a second transplant. Mice used to study the duration of irradiation effect on engraftment received the second transplant without any pre-transplant conditioning. Mice used to study the effects of split dose irradiation on engraftment received 82.5 cGy TBI 6-8 hours prior to the second transplant. In all mice, the second postnatal transplant consisted of 30 x 10^6 TCD B6GFP BM cells, resuspended in 100µl of PBS.

**Competitive repopulation assay.**
A competitive repopulation assay was performed as described by Harrison et al.\textsuperscript{18} to assess the mechanism of the enhancing effect of TBI on engraftment. Briefly, 8-10 week old B6Ly5.2 mice received either 0 cGy or 276 cGy of TBI. The mice were subsequently sacrificed within 1-2 hours following TBI and bone marrow was harvested from the tibia and femurs. Either 7.5 x 10\textsuperscript{6} irradiated B6Ly5.2 BM cells or the same dose of non-irradiated cells were injected in competition with 7.5 x 10\textsuperscript{6} non-irradiated B6GFP BM cells via the lateral tail vein into lethally irradiated (950cGy TBI) B6 mice to create an experimental group and control group respectively. Peripheral blood chimerism levels were assessed by flow cytometry at 2 months and 5 months post transplant. Additionally, at 4 months post transplant, multilineage engraftment of the donor cell populations was assessed by flow cytometry for lymphoid and myeloid markers.

**Monoclonal Antibodies used for Flow Cytometric Analysis.**

Fluoresceine isothiocyanate (FITC)-conjugated monoclonal antibodies included antibodies against H-2K\textsuperscript{b}, CD45.1, and Vβ3. Phycoerythrin (PE)-conjugated antibodies included antibodies against H-2K\textsuperscript{d}, CD45, and CD45.1. For lineage analysis biotinylated antibodies against CD3, B220, CD11b and Gr1 were developed with streptavidin-cytochrome or streptavidin-PE. Non-specific Fc\textgamma receptor binding was blocked by the monoclonal antibody against mouse Fc\textgamma receptor 2.4G2. Conjugated monoclonal antibodies with irrelevant specificities served as negative controls. Propidium-Iodide staining was used to exclude dead cells in dual color flow cytometry. All antibodies were purchased from Pharmingen (San Diego, CA) and flow cytometry was performed on a FACScan (Becton Dickinson, Mountain View, CA).

**Blood Sampling and Flow Cytometric Analysis.**

Chimerism levels were assessed in recipients of in utero transplantation at 4 or 8 weeks of life prior to receipt of postnatal transplants. Chimerism levels were again assessed every week in all mice after receipt of a postnatal transplant for the first 8 weeks, followed by
every other week until 16 weeks post transplant and then monthly until sacrifice. Lineage analysis of engrafted donor cells was performed at 8 and/or 24 weeks after postnatal transplant. Vβ3 T cell receptor (TCR) analysis was performed at 28 weeks after postnatal transplant. For each analysis approximately 200 µL of peripheral blood was collected in heparinized capillary tubes via retro-orbital vein puncture and diluted to 10 ml with heparinized PBS. The sample was layered over a Ficoll (Histopaque 1077, Sigma) gradient. The LDMCs were collected after centrifugation at 600 g for 15 minutes and subsequently washed in PBS. Forward angle and 90-degree light-scatter properties were used to distinguish lymphocytes, monocytes, and granulocytes in peripheral white blood cells. Dual color flow cytometry was used in most of the studies to distinguish donor and host cells, and to determine percentages of lineage specific donor cells. Three color flow cytometry was used in the studies utilizing B6GFP mice to distinguish between the three congenic populations by using PE-conjugated anti-CD45.1, biotin-conjugated anti-H-2Kb developed with streptavidin-cytochrome to provide a common background on which chimerism was evaluated, and the natural fluorescence of GFP. For lineage evaluation biotin-conjugated lineage specific antibodies were used and developed with streptavidin cytochrome. For analysis of Vβ3 TCR three color staining with FITC-conjugated anti-Vβ3, anti-CD45.1 PE and anti-CD3 biotin antibodies developed with streptavidin-cytochrome was performed. A minimum of ten thousand events were analyzed for each determination.

**Assessment of hematological parameters.**

Naïve Balb/c mice were irradiated with 0 cGy, 82.5 cGy or 276 cGy of irradiation at a rate of 276 cGy/minute from a cesium 137 irradiator. 100 µL of peripheral blood was collected in heparinized tubes by retro-orbital vein puncture prior to irradiation and at days 2, 4, 8, 16 and 23 post irradiation. Hematological parameters (white blood count (WBC), hemoglobin (Hgb) and platelets) were assessed with a HemaVet CBC machine (Mascot, CDC Technologies, Oxford, CT).
Graft versus host disease (GVHD) assessment. Mice were weighed prior to receipt of a postnatal transplant and weighed weekly following the transplant. Mice were also monitored for clinical signs of GVHD including runting, fur loss and serositis. Skin biopsies were taken from representative mice receiving TBI conditioning and postnatal transplants from the four irradiation dose groups. The biopsies were stained with hematoxylin and eosin and examined by light microscopy for evidence of GVHD.

Assessment of donor specific tolerance by mixed lymphocyte reaction (MLR).

Splenocyte responder cells harvested from allogeneic chimeric mice created by IUHSCTx were subjected to mixed lymphocyte culture by standard methods. Briefly, splenic LDMCs were cultured at 37° in 5% CO2 for 3 days in triple wells containing 2 x 10^5 responders with 5 x 10^5 stimulators (irradiated with 30Gy) in RPMI 1640 medium (Life Technologies, Grand Island, New York) supplemented with 10% fetal calf serum (Life Technologies, Grand Island, New York), 50 mM 2-mercaptoethanol (Sigma, St. Louis, Missouri) and antibiotics (penicillin, 100 U/ml; streptomycin, 100 mg/ml) (Life Technologies, Grand Island, New York). Cells were then pulsed with 3H-thymidine and collected approximately 24 hours later.

Statistical methods.

Data is graphically represented as the mean of the respective group ± 1 standard deviation. Statistical comparisons between groups were performed with the Student’s t-Test for two samples assuming unequal variances. A two tailed p value of ≤ 0.05 was considered significant.

Results
Low dose TBI does not depress hematologic parameters in normal mice.

The doses of TBI chosen for this study were well below those considered to be lethal in mice (i.e. 950 to 1100 cGy). To assess the hematologic effects of the doses of TBI utilized in this study, routine hematologic parameters were assessed in non-transplanted normal mice. No decrease in total WBC count, Hgb, or platelets was observed with the lowest irradiation dose (82.5 cGy), while only slight decreases that returned to normal by 23 days after irradiation were seen with the highest irradiation dose (276cGy) (Figure 1). This data confirms that the doses of TBI utilized in this study constitute very minimal or non-myeloablative conditioning.

IUHSCTx results in allogeneic mixed hematopoietic chimerism and donor specific tolerance.

Flow cytometry for H-2K^b and H-2K^d allowed for the clear assessment of donor chimerism in recipients of IUHSCTx (Figure 2A). IUHSCTx of 5 x 10^6 B6 TCD BM cells into Balb/c recipients resulted in moderate levels of allogeneic mixed hematopoietic chimerism that were stable for up to 32 weeks after transplant (Figure 2B). The levels of chimerism present in this study were consistently associated with donor specific tolerance as assessed by MLR (Figure 2C). Skin grafts were not performed on the experimental
Figure 2. IUHSCTx results in stable long-term low level chimerism and induces donor specific tolerance. (A) Representative dot plot of peripheral blood discriminating donor and host cells using antibodies specific for H-2K^b and H-2K^d. (B) Levels of chimerism remain stable for greater than 6 months. (C) MLR was performed using responder splenocytes from chimeric mice, created by IUHSCTx and Balb/c and B6 controls. Splenocyte responders were mixed with host (Balb/c), donor (B6) and third party (CBA) stimulator cells and the SI was calculated. B. CPM = Baseline counts per minute (CPM) in control cultures without stimulator cells. Stimulation indices (SI) were calculated by dividing mean CPM from responses against host (self) (Balb/c), donor (B6) or third party (CBA/J) by mean Baseline CPM. * p<0.001, # p>0.05 when compared to B6 or Balb/c control.

animals in this study to avoid any possible effects on chimerism. The MLR data is from our experimental animals pre-TBI and TCD BMT and confirms the presence of donor specific tolerance. In general, there is good correlation between a non-reactive MLR and skin graft tolerance after IUHSCTx in this model^7,8.
IUHSCTx followed by postnatal low-dose TBI and congenic TCD BMT results in high level allogeneic donor chimerism in tolerant recipients.

Levels of donor cell engraftment were significantly enhanced in tolerant recipients by low-dose TBI and congenic TCD BMT. This effect was consistent and reproducible with enhancement of chimerism in 100% of irradiated experimental chimeric mice. Enhancement of chimerism was irradiation dose dependent, with increases in chimerism reaching a plateau at 10-12 weeks after TBI conditioned BMT and remaining stable for at least 32 weeks (Figures 3A and 3B). The kinetics of enhancement, and the

Figure 3. IUHSCTx followed by a postnatal low dose TBI/BMT regimen results in high levels of allochimerism. Chimeric mice after IUHSCTx received one of four doses of TBI followed by a postnatal TCD BM transplant with cells congenic (B6Pep3b) to the allogeneic prenatal donor at 4 (A) or 8 (B) weeks of age. Control mice were 4 week old naïve Balb/c males that received 276 cGy of TBI followed by tail vein infusion of 30 X 10^6 TCD B6 BM cells. At all time points there is no difference between chimerism levels in mice boosted at 8 and 4 weeks of age (p>0.05) with the exception of the two marked points (*). Levels of chimerism were statistically different between each irradiation dose with the exception of the # marked point where p>0.05.
final level of engraftment were not significantly different between animals undergoing TBI and BMT at 4 weeks or 8 weeks after birth suggesting that this approach to postnatal enhancement of chimerism is not age dependent. No engraftment was observed in control mice injected prenatally with PBS who received the highest dose of TBI and postnatal TCD BMT confirming that prenatal tolerance induction is required for postnatal allogeneic engraftment with this regimen.

**Enhanced chimerism is stable and multilineage confirming HSC engraftment.**

Phenotypic analysis of peripheral blood chimerism was performed at 8 and 24 weeks after TBI and TCD BMT to assess multilineage engraftment. Donor cell chimerism was multilineage in all irradiation groups after TBI and TCD BMT with no significant differences, as shown in Figure 4. Analysis of multilineage engraftment at 8 and 24 weeks post TBI and TCD BMT at 4 (A) or 8 (B) weeks of age. Donor lineage analysis for myeloid (Gr-1 and Mac-1) and lymphoid (CD3 and B220) markers is expressed as a percentage of the level of total donor chimerism. Controls consist of normal lineage composition in PB of naïve B6Pep3b mice. Those levels denoted with a * demonstrate no significant difference (p>0.05) compared to controls.
change over time (Figure 4). The chimerism is balanced with a lineage profile similar to that seen in naive B6Pep3b control animals. Stable multilineage chimerism for greater than 6 months supports the engraftment of long-term reconstituting cells or HSC.

**Increased engraftment following low-dose TBI and TCD BMT is due to the non-irradiated postnatal innoculum.**

The postnatal use of TCD BM congenic to the prenatally transplanted cells allows for serial analysis of which donor population is responsible for the increase in engraftment seen following TBI and TCD BMT. Figure 5 demonstrates the engraftment profile of pre and postnatally derived donor cells in the 4 week old recipients (8 week data was similar -

**Figure 5. Analysis by CD45 isoforms of the pre and postnatal transplanted donor cells with TBI doses of 0cGy (A), 82.5cGy (B), 138cGy (C) or 276cGy (D).** Prenatal and postnatal donor cell chimerism is represented on the y axis as the percent of total donor chimerism for which each donor group is responsible.
data not shown). It is clear that the postnatal non-irradiated cells are entirely responsible for the increase in engraftment at all irradiation levels.

**Low-dose TBI followed by TCD BMT enhances engraftment by creating a competitive advantage for non-irradiated donor cells.**

The ability to determine which donor population is responsible for the increase in engraftment at the different irradiation doses lends insight into the mechanism by which TBI followed by TCD BMT augments engraftment. Comparison of Figure 5A, which represents chimerism levels in mice that received no pre-transplant TBI to figures 5B, 5C and 5D, which represent mice that received pre-transplant TBI, demonstrates that the postnatal non-irradiated donor cell population is responsible for the entire increase in donor cell chimerism. This is in contrast to mice that did not receive pre-transplant TBI, in which the time required for the postnatal transplant population to represent the majority of the total donor chimerism is longer and more gradual (Figure 5) with no increase in total levels of donor chimerism (Figure 3). Additionally, even at 32 weeks in this group, there remains a significant prenatal donor cell contribution (>20% vs <5% for the TBI conditioned mice) to the level of total donor cell chimerism. The reason that the postnatal donor cells ultimately predominate even in the non-irradiated control is presumably due to the relative excess of stem cells transplanted (5 million pre versus 30 million post) in keeping with findings in the non-myeloablated syngeneic model in which the ultimate level of engraftment represents the fraction of donor HSC present in the total HSC compartment.

To confirm the implicated mechanism of competitive advantage, we compared the repopulating capacity of cells that received the same doses of irradiation in a competitive repopulation assay. Competition of 7.5 x 10⁶ BM cells, that had received 276 cGy *in vivo*, against 7.5 x 10⁶ non-irradiated congenic BM cells in a lethally irradiated congenic recipient results in reconstitution of hematopoiesis with > 85% of PB derived from non-irradiated donor cells and < 15% from irradiated donor cells (Figure 6). This is in contrast to the approximately 50:50 reconstitution observed when the same number of non-irradiated cells
of the same two congenic strains are competed. The greater than 5 fold advantage of non-irradiated cells over irradiated cells in the competitive repopulation assay continues to be seen 4 months post transplant at which time multilineage engraftment of both donor populations is demonstrated supporting an irradiation effect on the long-term repopulating HSC.

**The competitive disadvantage conferred by irradiation on host cells is transient.**

The finding that enhanced chimerism levels following TBI and TCD BMT were stable up to 6 months post transplant raised the question of the duration of the irradiation effect on host HSC. If irradiation permanently impaired the competitive capacity of the host HSC, then one would expect that a similar enhancement of engraftment would occur with additional transplants with non-irradiated cells at later time points. If on the other hand, the impairment in host HSC competitive capacity is transient, late TCD BMT would have little
or no enhancement effect. In fact, introduction of the same dose of non-irradiated cells 4 weeks after TBI does not result in a significant contribution to chimerism by this cell population (Figure 7). It is important to note in this experiment however that at the time of the second transplant, there is approximately 40% donor chimerism from the first transplant of non-irradiated cells. Thus, detection of any persistence of irradiation effect might be blunted by donor vs. donor competition. Nevertheless, we would expect to see some evidence of advantage since the majority of hematopoiesis is derived from irradiated host cells. This argues for a transient effect of TBI on the host HSC allowing expansion of the donor HSC population, resulting in a stable fractional increase in postnatal donor derived hematopoiesis.

**Split dose irradiation and re-transplantation provides better enhancement of engraftment with less total TBI.**

Previous studies have indicated that split doses of irradiation, separated by a time interval that allows for recovery of the irradiated host, results in decreased toxicity compared to single higher doses of irradiation. We sought to evaluate the effect on enhancement of donor chimerism of split dose irradiation, using our lowest TBI dose, with TCD BMT following each dose of TBI. A system of three congenic donors, (B6, B6Ly5.2 and...
B6GFP) was used to assess the contribution of each pre- or postnatal population to chimerism enhancement after split dose TBI and TCD BMT. The administration of split dose TBI, separated by a 4 week interval, each followed by TCD BMT results in significant enhancement of donor cell chimerism (Figure 8A).

Comparison of levels of chimerism achieved following split dose TBI (82.5 cGy x 2) and two TCD BMTs (30x10^6 TCD BM cells x 2) to the levels of chimerism achieved following single higher doses of TBI (138 cGy or 276 cGy) and a single TCD BMT (30x10^6 TCD BM cells) demonstrates that the recipients of split dose TBI and TCD BMT ultimately achieve levels of chimerism (Figure 8B).

**Figure 8. Split dose TBI allows equivalent enhancement of engraftment at a lower total TBI dose.** (A) Chimeric mice after IUHSCTx received 82.5 cGy TBI at 4 and 8 weeks of life and were transplanted with 30x10^6 B6Ly5.2 TCD BM cells and 30x10^6 B6GFP TCD BM cells 6-8 hours after each TBI dose respectively (Split dose TBI/BMT x 2). Chimerism levels were compared to levels in mice which received a single irradiation dose of 82.5 cGy TBI at 4 weeks of age and were transplanted with 30x10^6 B6Ly5.2 TCD BM cells and 30x10^6 B6GFP TCD BM cells 6-8 hours after TBI at 4 weeks of life (TBI/BMT x 2) as well as chimerism levels in mice which received a single dose of 138 cGy or 276 cGy TBI at 4 weeks of age and were transplanted with 30x10^6 B6Pep3b TCD BM cells 6-8 hours after irradiation (TBI/BMT). (B) The composition of total donor chimerism of the “Split Dose TBI/BMT x 2” group was assessed by flow cytometry for GFP as well as CD45.1 and H-2K^d.
equivalent to the single dose 276 cGy group despite receiving a significantly lower dose of TBI (165cGy vs 276cGy). One possible explanation for the similar levels of engraftment between these groups despite less TBI is the fact that the recipients of split dose TBI and TCD BMT receive twice as many HSC as the recipients of a single dose of TBI and TCD BMT. Nevertheless, as the clinical objective would be to reduce the total dose of TBI received, and its potential morbidity, this would represent a viable clinical strategy. Figure 8B demonstrates that in the split dose irradiation group, the majority of chimerism results from the non-irradiated donor cells reconfirming their competitive advantage.

**Absence of GVHD or other apparent toxicity in high level chimeras.**

To assess for GVHD or other clinical toxicity mice were serially weighed once per week and closely observed. No chimeric mice demonstrated signs of GVHD, i.e. runting, serositis, or fur loss. In addition, the experimental mice in both the 4 and 8 week groups all demonstrated weight gain equivalent or superior to non-chimeric irradiated controls or chimeric non-irradiated controls (Data not shown). Finally, histologic assessment of skin, intestine, spleen, and liver after sacrifice was normal and revealed no evidence of GVHD (histology not shown).

**IUHSCTx and postnatal TBI with TCD BMT results in near complete deletion of host reactive donor lymphocytes.**

To assess the potential for GVHD in this system and to define the mechanism for its absence we assessed the fate of host reactive donor T-cells in high level chimeric animals using the mammary tumor virus (Mtv) super-antigen system. In this system, as applied to expressed antigens in our strain combinations, Vβ3TCR expressing thymocytes undergo clonal elimination in the presence of appropriate presentation of Mtv6 superantigen in association with MHC ClassII I-E. B6Pep3b strain mice (Mtv6−, I-E−) normally express high numbers of Vβ3TCR+ T-cells whereas Balb/c strain mice (Mtv6+, I-E+) normally clonally delete Vβ3TCR+ cells. Chimeric mice after IUHSCTx alone as well as from all
three radiation groups exhibited near complete deletion of Vβ3TCR+ cells. Whereas B6Pep3b mice normally have 3.75 ± 0.5% Vβ3TCR+ cells, all of the chimeric mice had less than 0.5% Vβ3TCR+ cells detectable in peripheral blood at 28 weeks of age, consistent with the development of donor derived T-cells in the Balb/c host thymic environment with clonal deletion of host reactive cells. This supports the absence of GVHD and the minimal potential for its occurrence with this postnatal enhancement approach.

**Figure 9.** Enhancement of chimerism in a mixed chimera created by IUHSCTX is equivalent to that observed in naïve congenic recipients. Direct comparison of chimerism levels following TBI and BMT in allogeneic chimeras created by IUHSCTX with those following congenic transplantation of a naïve host (A) keeping the recipient strain constant (SJL->B6 and B6Ly5.2 ->B6) or (C) keeping the donor strain constant (B6-> Balb/c and B6->B6Ly5.2). Analysis of multilineage engraftment in these strain combinations was performed at 24 weeks post transplant (B). For all graphs, p>0.05 when comparing congenic to allogeneic strain combination chimerism levels and multilineage engraftment levels unless indicated by *.
The enhancement of engraftment observed in fully allogeneic mixed chimeras created by IUHSCTx is equivalent to that observed in naïve congenic recipients.

To directly test our hypothesis that a mixed chimera produced by IUHSCTx would demonstrate equivalent engraftment to that observed in a congenic strain combination in which the same low dose TBI and TCD BMT regimen was applied we compared the effect of TBI and TCD BMT in allogeneic chimeras produced by IUHSCTx with postnatal TBI and TCD BMT in naïve congenic mice. The experiment was performed utilizing strain combinations in which the recipient was the same (SJL->B6 vs. B6Ly5.2->B6) or in which the donor was the same (B6->Balb/c vs. B6-> B6Ly5.2). Our results demonstrate that the kinetics and ultimate level of donor cell engraftment are equivalent in allogeneic or congenic systems (Figures 9A and C). Chimerism is multilineage at 24 weeks after TCD BMT with similar levels of myeloid and lymphoid engraftment. Although significant differences in levels of donor cells were present within lineages between allogeneic and congenic strain pairs, the levels of lineage engraftment were similar to donor strain controls (Figure 9B). This data argues against any MHC or MHC linked barrier to engraftment in a fully allogeneic strain combination once tolerance is achieved by IUHSCT.

Postnatal enhancement of engraftment after IUHSCTx by this strategy does not appear to be strain specific.

We have noted considerable strain dependence for the level of engraftment after IUHSCTx in the murine model. For instance, in the two allogeneic strain combinations used in this study, IUHSCTx alone of 5 x 10⁶ TCD BM cells results in levels of engraftment of 3-10% in B6->Balb/c chimeras and only around 2% in SJL/J->B6 chimeras. To determine whether the ability to enhance engraftment using TBI and TCD BMT was also strain dependent we compared this regimen in the two fully allogeneic strain combinations (Figure 10). In this experiment we achieved equivalent postnatal enhancement
of engraftment in these two strain combinations suggesting that once tolerance is achieved by IUHSCTx, enhancement of engraftment with this regimen is not strain specific.

**Figure 10. Chimerism enhancement is not strain specific.** Chimerism levels were assessed following the same low dose TBI/BMT transplant regimen in chimeras created by IUHSCTx in two different fully MHC mismatched strain combinations. B6:Balb/c chimeras and SJL/J:B6 chimeras, formed by IUHSCTx, were transplanted with 30x10^6 TCD B6Pep3b and SJL/J BM cells respectively at 8 weeks of life 6-8 hours after receiving either 82.5 cGy or 276 cGy of TBI. p>0.05 for all chimerism values when comparing the two strain combinations with the exception of those indicated by *.

**Discussion**

Cytoreductive conditioning utilizing lethal irradiation or cytotoxic drugs has traditionally been utilized to overcome allogeneic barriers in BMT. The conditioning regimen has been considered necessary for two primary reasons; to suppress the host immune system; and to create “space” in the host BM for engraftment of transplanted HSC. Recently, the requirement for myeloablation to engraft donor HSC has been challenged by a number of observations. Observations in the non-myeloablated, syngeneic mouse model have supported the ability to engraft donor cells with no conditioning in the absence of an immune barrier. In addition, in tolerant systems or immunosuppressed systems, minimally or non-myeloablative regimens have successfully achieved engraftment. However, the primary obstacle to the application of non-myeloablative approaches in clinical BMT remains the immune system. The requirement for immune
suppression is fundamental to the success of clinical non-myeloablative approaches and application across HLA barriers has been limited by graft failure, toxicity and GVHD\(^{27-29}\). In contrast, IUHSCTx can achieve donor specific tolerance without the need for immunosuppression. Studies in transgenic mice have documented the role of the fetal thymus in self-recognition and the determination of repertoire of response to foreign antigen\(^{30-32}\). The end result of this process is deletion of T-cell clones with high affinity for self-antigen, and preservation of a T-cell repertoire against foreign antigen. The introduction of allogeneic cells prior to completion of this process by IUHSCTx also results in clonal deletion of alloreactive T-cells and we have documented that the presence of clonal deletion in the murine model is dependent upon the level of donor chimerism. In the presence of “microchimerism” where donor cells are only detectable by PCR, tolerance is inconsistent and when present is associated with only partial clonal deletion and anergy of residual donor reactive host cells\(^{7,8}\). This form of tolerance is relatively weak, and can be “broken” by postnatal administration of antigen. In the presence of higher levels of chimerism (> 1-2%) deletional tolerance is consistently achieved\(^{33,34}\) and can be enhanced by postnatal administration of additional donor BM.

In our previous efforts to enhance engraftment, multiple transplants of high doses of donor cells in the first week of life resulted in small but sustained increases in engraftment, analogous to observations in the non-myeloablated syngeneic model\(^ {35}\). However, the ultimate levels of mixed chimerism remained too low to be therapeutic for most potential target diseases. The primary difference in this study relative to our previous efforts is the addition of low dose TBI prior to TCD BMT. The addition of low-dose TBI as a non-toxic conditioning regimen was suggested to us by the study of Stewart et.al.\(^ {19}\) in which the syngeneic non-myeloablation model was modified by exposure of the host to low dose TBI (100 cGy). In that study, syngeneic male donor cells showed high levels of engraftment in female recipients despite transplantation of relatively low numbers of cells. Irradiation of donor cells resulted in markedly reduced engraftment capacity. In agreement with that study,
our results confirm that the primary mechanism of low-dose TBI in the sustained enhancement of engraftment is reduction of the competitive capacity of host HSC relative to non-irradiated donor cells, rather than by the initial engraftment of an increased number of HSC from the primary donor inoculum. This statement is supported in this study by three observations: 1) the minimal degree of myelosuppression observed after the doses of irradiation utilized; 2) the impact of irradiation on the competitive capacity of donor cells demonstrated in the competitive repopulation assay; and 3) the contribution of postnatal non-irradiated donor cells relative to prenatal donor cells (that are irradiated by virtue of their presence within the host) to the ultimate level of donor cell engraftment. The most compelling support comes from the competitive repopulation assay. In this assay the repopulating capacity of irradiated and non-irradiated cells were directly compared with all other variables being equal. It is clear from our data that even low dose irradiation that appears to have a minimal effect on other tissues, creates a reduction in the competitive capacity of HSC. The mechanism of the reduction in competitive capacity in irradiated cells is not determined by this study. Possibilities include a reduction in self replication or proliferative capacity of HSC, alterations in normal cell cycling, or toxicity inducing cell death. In any case, the effect allows the relative expansion of an initially limited number of non-irradiated engrafted HSC into the host hematopoietic compartment establishing a steady state with a higher ratio of donor HSC to host HSC. The effect appears to be transient as supported by our observation that no significant enhancement of engraftment could be seen in mice that received a second postnatal transplant without TBI conditioning 4 weeks after receiving low dose TBI.

We observed no GVHD in this study. Susceptibility to GVHD is in part dependent on the absence of host anti-donor immune response. In previous studies in which we attempted to enhance chimerism in allogeneic chimeras created by IUHSCTx by postnatal transplantation of high doses on non-TCD BM, we have observed GVHD but not loss of chimerism. We were therefore concerned that GVHD might occur after BMT in our
recipients made tolerant by IUHSCTx. To avoid GVHD we utilized TCD BM (< 0.5% CD3+ cells) which significantly reduced exposure to mature donor T-cells. The other source of potential GVHD would be donor HSC derived T-cells with anti-host alloreactivity that escape clonal deletion during maturation in the recipient. Our data demonstrating near complete deletion of relevant Vβ TCR clones suggests that, at least in the mouse, few if any host reactive donor cells evade deletion mechanisms. This is in agreement with our studies in the mouse model which document high efficiency clonal deletion of donor anti host T-cells after IUHSCTx alone. These results suggest that GVHD should not be a problem as long as the number of mature donor T-cells transplanted is kept to a minimum.

How does this approach compare to postnatal minimally myeloablative regimens reported to achieve mixed chimerism in fully allogeneic systems? There are a number of postnatal approaches that have achieved varying levels of donor chimerism across major MHC barriers. One study achieved levels of stable PB chimerism of 20 to 90% utilizing a conditioning regimen of 550 to 850 cGy of TBI and “megadoses” of TCD BM (200 x 10^6 cells). In another study, the combination of sublethal host TBI (400 cGy) and administration of anti-CD154 antibody achieved > 99% donor PB chimerism at 6 weeks after transplant with associated donor specific tolerance. Wekerle et.al. recently demonstrated that host conditioning with anti-CD154 and CTLA4 Ig in combination with high dose (200 x 10^6 cells) BMT has been shown to result in an engraftment efficiency of 64% but with stable multilineage chimerism levels of only 2 to 3%. Sykes et.al. utilized anti-CD4 and anti-CD8 antibodies in combination with local thymic irradiation (700 cGy) and high dose (174-200 x 10^6 cells) BMT to achieve an engraftment efficiency of 70% with stable levels of PB chimerism of 40%. Finally, it has been demonstrated that apoptotic leukocytes can enhance allogeneic bone marrow engraftment. In this system 49% of mice receiving 600cGy of TBI, 1 x 10^6 BM cells and 5 x 10^6 apoptotic leukocytes engrafted with chimerism levels of 92-93%, 45-50 days after transplant. Similar protocols have been successfully applied in leukocyte antigen matched swine and haploidentical or HLA
matched human studies\textsuperscript{26,43} although generally more intensive regimens are required than those utilized in mice. Our approach differs fundamentally from all of the above, in that it achieves T-cell deletion by IUHSCT\textsubscript{x}, a non-toxic approach. We have demonstrated consistent and stable, high level, multilineage donor chimerism, across full MHC barriers without GVHD in two strain combinations. In addition, this has been achieved with a practical number of donor cells and levels of TBI that are associated with no significant myeloablation or other apparent toxicity.

Our success in this study strongly supports the general strategy of prenatal tolerance induction to facilitate postnatal cellular or organ transplantation. The convergence of a number of technologies provides a compelling rationale for further development of prenatal therapies. Advances in maternal screening, molecular diagnosis, the human genome project, and microarray technology make it highly likely that in the near future most human genetic diseases will be diagnosed early in gestation, either from fetal cells or fetal DNA in the maternal circulation. The diagnosis of disorders amenable to cellular therapy early in gestation will provide an optimal opportunity for prenatal stem cell therapy as one option for effected families. In addition, genetic disorders in which organ failure or the need for treatment by non-hematopoietic stem cells could be anticipated would potentially benefit from prenatal tolerance induction. Although, clinical application of IUHSCT\textsubscript{x} has been limited thus far by minimal engraftment, and the need for a selective advantage for donor cells, the ability to enhance engraftment after birth to near complete donor chimerism, using a non-toxic approach, would dramatically expand the application of IUHSCT\textsubscript{x}. 
References


6. Harrison MR, Slotnick RN, Crombleholme TM, Golbus MS, Tarantal AF, Zanjani ED. In-utero transplantation of fetal liver haemopoietic stem cells in monkeys. Lancet. 2:1425-1427, 1989


11. Blazar BR, Taylor PA, Vallera DA. In utero transfer of adult bone marrow cells into recipients with severe combined immunodeficiency disorder yields lymphoid progeny with T- and B-cell functional capabilities. Blood. 86:4353-4366, 1995


17. Stewart FM, Zhong S, Lambert JF, Colvin GA, Abedi M, Dooner MS, McAuliffe CI, Wang H, Hsieh C, Quesenberry PJ. Host marrow stem cell potential and


22. Brecher G, Ansell JD, Micklem HS, Tjio JH, Cronkite EP. Special proliferative sites are not needed for seeding and proliferation of transfused bone marrow cells in normal syngeneic mice. Proc Natl Acad Sci USA. 79:5085-5092, 1982


34. Shaaban AF, Milner R, Kim HB, Flake AW. High-level allogeneic hematopoietic chimerism following prenatal stem cell transplantation requires negative
selection of donor thymocytes mediated by host antigen presentation cells. Exp Hematol. 27 Supple 1:347, 1999


36. Shaaban AF, Milner R, Hayashi S, Peranteau WJ, Flake AW. Tolerance induced by in utero hematopoietic stem cell transplantation is required to facilitate higher levels of engraftment with post-natal booster transplants. Blood 96 Suppl. 2 of 2:306b Abstract 5068, 2000


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Aimen F Shaaban, William H Peranteau, Satoshi Hayashi, Michael Hsieh and Alan W Flake