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Successful Allogeneic Neonatal Bone Marrow Transplantation Devoid of Myeloablation Requires Costimulatory Blockade

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A significant number of nonmalignant, progressive childhood disorders respond to bone marrow transplantation (BMT). Toxic myeloablative pretreatment regimens, graft failure, and graft-vs-host disease complicate the utility of BMT for neonatal treatment. We recently demonstrated high-dose BMT in neonatal animals enables chimeric engraftment without toxic myeloablation. Reagents that block T cell costimulation (anti-CD40L mAb and/or CTLA-4Ig) establish tolerant allogeneic engraftment in adult recipients. Donor lymphocyte infusion (DLI) re-establishes failing grafts and treats malignant relapse via a graft-vs-leukemia response. In this study, we tested the hypothesis that combining these approaches would allow tolerant allogeneic engraftment devoid of myeloablation in neonatal normal and mutant mice with lysosomal storage disease. Tolerant chimeric allogeneic engraftment was achieved before DLI only in the presence of both anti-CD40L mAb and CTLA-4Ig. DLI amplified allografts to full donor engraftment long-term. DLI-treated mice either maintained long-term tolerance or developed late-onset chronic graft-vs-host disease. This combinatory approach provides a nontoxic method to establish tolerant allogeneic engraftment for treatment of progressive childhood diseases. The Journal of Immunology, 2003, 171: 3270–3277.
 CTLA-4Ig fusion protein (ATCC CRL-10762) was obtained from Chinese hamster ovary culture medium supernatant. Cells were grown in DMEM, 10% FBS, 0.2 mM proline, and 1 μM methotrexate. mAb and fusion protein were purified using T-Gel or protein A absorbent columns ( Pierce, Rockford, IL), according to the manufacturer’s recommendations.

**Strains of mice**

Three genetically marked strains that are H2b at the MHC locus were used as recipients of whole BM. B6.CAST-Gpi1α/Ei mice contain the genetic marker glucose phosphate isomerase type 1. B6.Cg-Ptprc-Gpi1α mice also have the Gpi1α isotype, but in addition express the CD45.1 CD45.2 isotype by tyrosine phosphatase. These mice can be distinguished from animals that have the CD45.2 isotype by flow cytometry. These two strains are henceforth referred to as B6-H2b (henceforth B6-MPS VII-H2b) animals (both are Gpi1b, CD45.2, and are normal for GUS expression). B6-H2b differs at the MHC from the recipient animals, but is matched for minor Ags (21). B6-MPS VII-H2b mice are completely matched to the recipients at the MHC and have multiple minor Ag mismatches (22).

**Transplantation and DLI**

Neonatal recipients were treated on day 2 after birth with 75 μg MR1 (anti-CD40L) and 10 μg CTLA-4Ig IP. BM was collected from femurs, tibia, and humeri of 8- to 12-wk-old donors, as described (8). Whole BM was injected at 1.5 × 10⁷ nucleated cells/100 μl into the superficial temporal vein (23) on day 2, after administration of costimulatory blockade. This differs from the classic experiments of Medawar and colleagues (24), who injected cells at birth. By day 2, neonatal losses from maternal neglect have subsided and recipients more easily tolerate infusion of cells. Co-stimulatory blockade was repeated on days 3, 4, and 8 after birth. For DLI, a single cell suspension of donor-matched spleen cells was prepared in PBS, passed through a 70-μm nylon filter, and treated with Gey’s lysis buffer to remove RBCs. Recipients were i.v. injected with 3 × 10⁷ viable leukocytes at either 22, 30, 43, or 87 days post-BM engraftment.

**Analysis of donor engraftment**

PBL was collected by retro-orbital puncture at 1 mo or just before DLI and at monthly intervals thereafter. For analysis by Gpi isotype, RBC and white blood cell populations were fractionated by centrifugation in hematocrit tubes, and isolates were separated by cellulose acetate electrophoresis, as described (25, 26). Strips were scanned with a Fujifilm Luminescent Image Analyzer, and percentages were determined using Image Gauge (v3.45) analysis software (Fujifilm Medical Systems, Stamford, CT). Three-color flow cytometry (FITC, allophycocyanin, and PE) was used to evaluate multilineage reconstitution. In Gpi 1A-CD45.1 recipients, nucleated PBL cells were labeled with conjugated CD45.1 (A201.7) and either CD11b (F4/80) plus Gr-1 (RB6-8C5) or CD45R (RA3-6B2) plus αβTCR (H57-597). The nucleated PBL cells from B6.MPS VII-H2b recipients were incubated with the fluoresceinlated GUS substrate Imagen Green C₁₂ FDGlCU (Molecular Probes, Eugene, OR), as described (4), followed by lineage marker Ab staining.

**Mixed lymphocyte reaction**

Proliferative assays for T cell function were performed as described (27). Splenic stimulators were irradiated at 20 Gy (120 Cs) and seeded at 5 × 10⁶ cells/well of a 96-well round-bottom plate. Nucleated cells were collected from 200 μl PBL per mouse and divided into each of four wells containing stimulator cells or just medium. Cells were incubated in 200 μl DMEM (Life Technologies, Rockville, MD) containing 5% FBS, 2-ME (5 × 10⁻⁵ M), HEPES (7.4 mM), folic acid (13.6 μM), asparagin (270 μM), t-ariginine (0.56 mM), Na-pyruvate (1 mM), t-glutamine (1.4 mM), and gentamicin (50 μg/ml). After 48 h at 37°C, 50 μl medium containing 1 μCi [³H]thymidine was added and cells were incubated an additional 15 h. Proliferation was measured by change in thymidine incorporation (stimulated sample – unstimulated medium alone) using a Wallac plate counter (Turku, Finland).

**Histology**

A portion of small intestine, liver, kidney, spleen, and skin was placed in Bouin’s fixative, embedded in paraffin, sectioned, and stained with H&E. For B6.MPS VII mice, the remaining portion of each tissue was placed in Tissue Tek OCT compound (Sakura Finetek, Torrance, CA), snap frozen, sectioned, and stained for GUS donor cells, as described (7).

**Table I.** Engraftment of fully allogeneic donor BM transplanted into neonatal recipients requires both MRI and CTLA-4Ig and amplifies with donor lymphocyte infusion

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DLI</th>
<th>Mean % Donor</th>
<th>SE</th>
<th>n</th>
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<tr>
<td>B6-H2b²MRI and CTLA-4Ig</td>
<td>None</td>
<td>8.6</td>
<td>±0.9</td>
<td>4</td>
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<tr>
<td>MRI and CTLA-4Ig</td>
<td>22 days</td>
<td>9.2</td>
<td>±0.7</td>
<td>6</td>
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<tr>
<td>MRI and CTLA-4Ig</td>
<td>30 days</td>
<td>9.6</td>
<td>±1.1</td>
<td>6</td>
</tr>
<tr>
<td>MRI and CTLA-4Ig</td>
<td>43 days</td>
<td>98.8</td>
<td>±0.6</td>
<td>5</td>
</tr>
<tr>
<td>MRI and CTLA-4Ig</td>
<td>87 days</td>
<td>100.0</td>
<td>±0.0</td>
<td>3</td>
</tr>
<tr>
<td>CTLA-4Ig</td>
<td>None</td>
<td>0</td>
<td>±0.0</td>
<td>3</td>
</tr>
<tr>
<td>CTLA-4Ig</td>
<td>43 days</td>
<td>0</td>
<td>±0.0</td>
<td>3</td>
</tr>
<tr>
<td>PBS</td>
<td>None</td>
<td>0</td>
<td>±0.0</td>
<td>5</td>
</tr>
<tr>
<td>C3-SW-H2b²MRI and CTLA-4Ig</td>
<td>None</td>
<td>3.6</td>
<td>±0.7</td>
<td>3</td>
</tr>
<tr>
<td>MRI and CTLA-4Ig</td>
<td>43 days</td>
<td>2.3</td>
<td>±0.4</td>
<td>4</td>
</tr>
<tr>
<td>CTLA-4Ig</td>
<td>None</td>
<td>6.1</td>
<td>±0.5</td>
<td>4</td>
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<tr>
<td>CTLA-4Ig</td>
<td>43 days</td>
<td>7.2</td>
<td>±0.6</td>
<td>4</td>
</tr>
<tr>
<td>PBS</td>
<td>43 days*</td>
<td>4.9</td>
<td>±0.8</td>
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</table>

* B6-H2b² neonatal recipients were injected i.p. 1 day after birth (day 2) with either MRI mAb plus CTLA-4Ig, CTLA-4Ig alone, or PBS, followed by 1.5 × 10⁷ whole BM cells injected i.v. from either B6-H2b² or C3-SW-H2b² donors. Costimulatory blockade was continued on days 3, 4, and 8. DLI recipients from each treatment group were given 3 × 10⁷ donor-matched spleen cells 22, 30, 43, or 87 days after the BM transplant. Means were calculated from percentage donor PBL data collected 3–12 mo postengraftment. n = Number of animals in each treatment group. v, Engraftment was obtained prior to DLI and was not amplified.
Results

Neonatal engraftment of MHC-disparate BM requires full costimulatory blockade

We first compared the ability of B6-H2kBM to engraft B6-H2b neonatal recipients either with PBS, CTLA-4Ig alone, or a combination of MR1 (anti-CD40L mAb) plus CTLA-4Ig (Table I). The donor strain MHC differs from B6-H2k at multiple antigenic loci and is matched for minor Ags. These strains share the H2 class I D loci. No engraftment was observed in the absence of costimulatory blockade or when CTLA-4Ig was used alone. MR1 alone also did not allow engraftment (data not shown). Using both MR1 and CTLA-4Ig, donor PBL chimerism averaged 8.6% ± 0.9 (±SE) when calculated from 3–12 mo posttransplantation (i.e., long-term repopulation). Results were compared with a similar experiment using C3.SW-H2k donor BM (containing multiple minor Ag disparities only) into B6-H2b recipients. In the latter, costimulatory blockade was not required, as noted by engraftment in PBS-treated controls (Table I). Inclusion of both reagents did not increase donor chimerism. In fact, recipients treated with CTLA-4Ig alone had slightly higher chimerism than animals treated with both MR1 and CTLA-4Ig. The differences in these treatments (3.6% ± 0.7 for MR1 and CTLA-4Ig vs 6.1% ± 0.5 for CTLA-4Ig alone) were statistically significant (p = 0.004).

Allogeneic chimerism can be amplified by DLI

We next examined the ability of DLI to amplify allogeneic donor chimerism. Mice engrafted with B6-H2kBM were injected with donor-matched spleen cells either 22, 30, 43, or 87 days post-BMT. DLI at 22 or 30 days did not amplify donor engraftment (Fig. 1A). DLI at 43 and 87 days rapidly expanded donor engraftment to 100% within 30–45 days (Table I and Fig. 1A). Amplified engraftment was maintained for the duration of the study (12 mo). Three recipients within the 43-day DLI group retained low-level host hemopoiesis. Host cells fluctuated between 0 and 4%, explaining why the mean percentage of donor contribution for this group was 98.8% ± 0.6 (Table I).

DLI was also tested in recipients of C3.SW-H2k BM to see whether multiple minor Ag differences would enable amplification. None of the animals engrafted with these cells amplified with DLI (Table I). When engraftment percentages were averaged

FIGURE 1. Costimulatory blockade leads to long-term, multilineage allogeneic engraftment, and DLI amplifies chimeric engraftment to full donor. A, Average percentage of donor PBL contribution over time when B6-H2kBM was transplanted into B6-H2b recipients with both MR1 and CTLA-4Ig costimulatory blockade. A total of 3 × 10^7 donor-matched lymphocytes was either not injected or injected 22, 30, 43, or 87 days post-BMT (see legend). B, B6-H2kBM transplanted into B6.MPS VII-H2b recipients with both MR1 and CTLA-4Ig costimulatory blockade. DLI was performed at 43 days post-BMT.

FIGURE 2. Flow cytometric analysis of PBL from B6.MPS VII-H2k recipients shows rapid multilineage expansion of donor cells post-DLI with a transient decline in donor B cells. A, Histogram of ImagenGene Green FDGlcU(GUS)-labeled PBL from B6.MPS VII-H2k recipients 1 day before DLI showing 5% donor engraftment. Open dotted line shows profile of B6.MPS VII-untreated control, and the open solid line shows a B6-H2k PBL control. B and C, Gated donor GUS^+ cells (5%) from A showing percentage of each indicated lineage within the donor PBL population. D, PBL from B6.MPS VII-H2k recipients 60 days post-BMT and 14 days post-DLI showing 99% donor engraftment. E and F, Percentage of lineage distribution within the donor GUS^+ cells shown gated in D. G, PBL from B6.MPS VII-H2k recipients 150 days post-BMT showing 98% donor engraftment. H and I, Percentage of lineage distribution within the donor GUS^+ cells shown gated in G.
(3–12 mo) for each treatment group, no significant differences were noted between mice with or without DLI. Recipients treated with both MR1 and CTLA-4Ig without DLI had a mean engraftment of 3.6% ± 0.7, and those with DLI had a mean of 2.3% ± 0.4 (p = 0.079). Recipients treated with CTLA-4Ig alone had a mean engraftment of 6.1% ± 0.5, and those treated with CTLA-4Ig and DLI had a mean of 7.2% ± 0.6 (p = 0.122). DLI in the engrafted PBS-treated controls also elicited no expansion. Clearly, greater allogeneic disparity (or prior stimulation with host minor Ags) is required for DLI amplification of donor cells, and DLI performed within 30 days of transplantation fails to amplify donor hemopoiesis.

Costimulatory blockade enables stable allogeneic engraftment in MPS VII recipients

Successful engraftment of normal recipients was followed by treatment of B6.MPS VII-H2b recipients using the same protocol. Donor BM was from B6.H2b mice, and DLI was performed at 43 days. PBL was analyzed by flow cytometry at 42 days, 60 days, and at monthly intervals thereafter. B6.MPS VII-H2b animals engrafted like normals (Fig. 1, A vs B) and had long-term multilineage expansion indicating hemopoietic stem cell engraftment (Fig. 2). Average percentage of donor engraftment at 42 days, just before DLI, was 5% ± 0.4 (Fig. 2A). Amplification post-DLI to a mean of 99% ± 0.4 donor occurred within ~14 days (Fig. 2D). Interestingly, the percentage of B cells within the donor population sharply decreased from 35% ± 1.3 at 1 day before DLI to 5% ± 0.6, 14 days following DLI (Fig. 2, C and F). The percentage of donor-derived T cells doubled in the same period, from 14% ± 0.8 to 27% ± 2.6 (Fig. 2, C and F). The donor monocye population also doubled, from 8% ± 0.6 to 15% ± 1.5 (Fig. 2, B and E). The granulocyte population increased, but to a lesser extent (from 39% ± 2.0 to 52% ± 3.2; Fig. 2, B and E). From 3–12 mo, the lineage distribution in the donor PBL population returned to percentages similar to those observed before DLI (Fig. 2, H and I). In summary, costimulatory blockade enables multilineage engraftment of allogeneic BM. DLI causes a transient decrease of donor-derived B cells accompanied by an increase in donor T cells, monocytes, and granulocytes during the overall expansion of donor cells into the PBL. The distribution of donor lineage-committed cells in the PBL returns to pre-DLI percentages 6 wk after DLI and remains stable long-term.

Costimulatory blockade induces tolerance in allogeneic neonatal BMT

We performed MLR with PBL from transplant recipients 4 and 12 mo postengraftment to determine whether long-term tolerance was obtained between donor and host. PBL was stimulated with irradiated splenocytes from B6-H2b (recipient), B6-H2b or C3.SW-H2b (donor), and B10.BR-H2k (third party). [3H]Thymidine was added after 48 h to measure proliferative response to the stimulators. When B6-H2b recipients were engrafted with B6-H2b BM, and DLI was not performed, no proliferative responses were observed when stimulated with recipient or donor Ags (Fig. 3A). When stimulated with novel third party Ags, a response was observed. These results were obtained at both 4 and 12 mo postengraftment, indicating long-term stable tolerance.

The neonatal B6-H2b recipients treated with both MR1 and CTLA-4Ig and transplanted with C3.SW-H2b donor BM without DLI did not show proliferative responses to either donor or host stimulator Ags, as measured by MLR at 4 and 12 mo (data not shown). Because minor Ags generally do not illicit a primary response by MLR, this demonstrated a lack of sensitization. Three of four recipients of C3.SW-H2b donor BM treated with CTLA-4Ig alone and no DLI also showed no proliferative responses at 4 and 12 mo. The fourth animal showed no antihost-stimulated proliferation at 4 mo, but did respond at 12 mo, indicating donor cells became sensitized to host at a late stage of engraftment. In summary, both MR1 and CTLA-4Ig (without DLI) are required to prevent sensitization of donor cells to host Ags.

DLI can break tolerance

B6-H2b recipients of B6-H2b BM amplified to 100% donor by DLI had mixed results in the MLR assay (Fig. 3B). One recipient (number 11) died at 3 mo postengraftment of unknown causes and was not evaluated by MLR (Table II). Animal number 6 had a relatively high proliferative response to recipient Ags (2459 Δcpm), and two others (numbers 8 and 12) had very minor antigenic responses that were just above background (602 ± 98) at
Histological analysis reveals DLI can induce chronic GVHD

B6-H2b animals engrafted with B6-H2b BM without DLI remained healthy throughout the analysis (Table II). None displayed any clinical signs of acute or chronic GVHD. Histologic examination of small intestine, liver, kidney, spleen, and skin did not identify any changes as compared with similarly aged controls (Fig. 4, A and B vs C and D, for intestine and kidney, respectively). B6-H2b mice treated with CTLA-4Ig alone rejected the B6-H2b BM, remained healthy throughout the study, and subsequently were not evaluated histologically. B6-H2b animals transplanted with C3.SW-H2b BM using both MR1 and CTLA-4Ig (no DLI) also remained disease free both clinically and histologically. When CTLA-4Ig was used alone to aid engraftment of C3.SW-H2b BM, one of four animals presented with erythematous ears at 8 mo postengraftment that resolved by 12 mo. This is the same animal that had antirecipient response by MLR (above). Histologically, this and one other animal of this group had chronic GVHD indicated by shortened and fused villi in gut, while liver, kidney, and spleen appeared normal (data not shown).

The B6-H2b recipients of B6-H2b BM treated with MR1, CTLA-4Ig, and 43-day DLI never displayed signs of acute GVHD. Animal 11 died unexpectedly at 3 mo with no signs of wasting, skin lesions, hair loss, or prolapse (Table II). Animal 5 looked frail before necropsy at 12 mo and had a large spleen. As described above, this animal spontaneously developed a lymphoid leukemia. Animal number 12 was the only animal of this group to develop clinical signs of chronic GVHD that included frail/hunched posture, hair loss, and erythematous ears. These signs appeared at 12 mo, just before necropsy. Histological examination of gut showed villus shortening and fusion (Fig. 4E), indicating GVHD. In addition, this animal had cystic kidneys (Fig. 4F), a finding not commonly found associated with GVHD. The remaining two animals of this treatment group remained completely free of GVHD both clinically and histologically (Fig. 4, G and H).

The B6-MPS VII-H2b recipients of B6-H2b BM amplified with 43-day DLI also never displayed signs of acute GVHD. Similar to number 11 above, animal number 40 died unexpectedly at 4 mo without signs of GVHD. At 5 mo, animal number 13 looked moribund, but did not have skin lesions or hair loss. Necropsy revealed enlarged, fluid-filled kidneys. Other visceral organs looked normal, with minor neutrophil infiltration in small intestine and bile duct. Animal number 41 became frail and started losing hair at month 4 (811 and 900 Δcpm, respectively). Interestingly, none of these animals had antirecipient reactions at 12 mo postengraftment. All but one (number 5) of these animals maintained healthy third party responses at both 4 and 12 mo. Animal number 5 was tolerant at 4 mo based on no recipient or donor responses coinciding with proliferation to third party Ag. During the last month postengraftment tested by FACS (month 12), this animal demonstrated 92% αβTCR-positive cells in its BM and 64% in spleen (data not shown), indicating lymphoid leukemia. No recipient, donor, or third party response was detected by MLR at 12 mo with animal number 5.

B6-MPS VII-H2b recipients of B6-H2b BM with full costimulatory blockade and 43-day DLI amplification to 100% donor did not maintain recipient Ag tolerance (Fig. 3C). Two separate experiments were performed. The first contained five B6-MPS VII-H2b recipients, and the second contained eight. At 4 mo, animals from both experiments proliferated in response to recipient (1837 Δcpm ± 290) and third party Ag (2036 Δcpm ± 496), and did not proliferate when stimulated with donor Ags (81 Δcpm ± 50). Only the first group of five animals was evaluated at 11 mo (the second group is still being monitored), and of these only three survived to be evaluated. The mean antirecipient response was 2864 Δcpm ± 2193, antidonor was 758 Δcpm ± 168, and third party response was 9369 Δcpm ± 1297. Two of these animals had low antirecipient and antidonor responses at 11 mo, while the third animal (number 41) had strong antirecipient proliferation. These MLR results of B6-MPS VII-H2b animals demonstrate a reduction of recipient tolerance post-DLI. Not all of the animals maintained the response to recipient Ags long-term.

Histological summary of normal B6-H2b and B6-MPS VII-H2b recipients treated with MRI and CTLA-4Ig followed by transplantation of B6-H2b BM ± 43-day DLI

Animal Survival (mo) Clinical Appearance Histology GVHD

<table>
<thead>
<tr>
<th>Animal</th>
<th>Survival (mo)</th>
<th>Clinical Appearance</th>
<th>Histology</th>
<th>GVHD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>Healthy/Normal</td>
<td>No DLI (B6)</td>
<td>Normal</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>Healthy/Normal</td>
<td>No DLI (B6)</td>
<td>Normal</td>
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<tr>
<td>3</td>
<td>12</td>
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<tr>
<td>4</td>
<td>12</td>
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<td>Normal</td>
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<tr>
<td>7</td>
<td>12</td>
<td>Healthy/Normal</td>
<td>No DLI (B6)</td>
<td>Normal</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>Frail, enlarged spleen</td>
<td>43-day DLI (B6)</td>
<td>Lymphoid leukemia</td>
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<td>6</td>
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<td>Normal</td>
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<td>No DLI (B6)</td>
<td>Normal</td>
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<tr>
<td>11</td>
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<td>Healthy/Normal prior to death</td>
<td>ND*</td>
<td>Gut, villus fusion &amp; shortening, neutrophil infiltration, necrosis</td>
</tr>
<tr>
<td>12</td>
<td>12</td>
<td>Frail, hairloss (at 12 mo), erythematous ears</td>
<td>43-day DLI (MPS VII)</td>
<td>Gut, neutrophil infiltration, Liver, bile duct infiltration</td>
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<tr>
<td>13</td>
<td>5</td>
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<td>No DLI (MPS VII)</td>
<td>Kidney, cystic</td>
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<td>Healthy/Normal</td>
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<td>Normal</td>
</tr>
<tr>
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<tr>
<td>40</td>
<td>4</td>
<td>Healthy/Normal prior to death</td>
<td>ND*</td>
<td>Gut, villus fusion &amp; blunting</td>
</tr>
<tr>
<td>41</td>
<td>12</td>
<td>Frail, hairloss (at 12 mo)</td>
<td>ND*</td>
<td>Kidney, cystic</td>
</tr>
</tbody>
</table>

*ND, No data.
12, similar to number 12 above. Intestine showed villus fusion and blunting (Fig. 4I); kidneys were cystic (Fig. 4J); and liver contained chronic portal bridging, was sclerosis-like, and had hepatitis (not shown). Donor GUS-positive cells (red staining) were noted lining the interior surface of the large kidney cyst (Fig. 4, J and K). The remaining animals, numbers 38 and 39, showed no clinical or histological signs of GVHD (Fig. 4, L, M, and N).

Discussion
A number of heritable disorders respond to BMT therapy. Many are progressive and require intervention either in utero or neonatally to prevent tissue malformation, CNS damage, and/or premature death. These include the thalassemias, immunodeficiencies, and LSD. Although in utero transplantation may be a viable approach
to tolerant engraftment of allogeneic BM without toxic ablative methods and host immune consequences (28), many patients are not diagnosed until after birth. In addition, transplantation in utero has a number of risks associated with the surgical procedure. Neonatal BMT currently entails toxic preparatory regimens that contribute to morbidity and mortality (29–32). These complications prevent treatment of nonmalignant disorders by BMT until no other options are available. Therefore, a nontoxic method of obtaining tolerant allogeneic neonatal engraftment would help treat a significant number of childhood diseases before irreversible establishment of disease sequela.

In the mouse, adult percentages of immune cells do not appear in PBL until 3 wk postbirth (33). Although neonatal immune effector cells appear few in number, these cells are fully capable of rejecting donor grafts. This was confirmed when we initially attempted high-dose allogeneic BMT (B6-H2b→B6-H2k) without costimulatory blockade. When we attempted to engraft MHC-mismatched allogeneic BM using MR1 mAb or CTLA-4Ig alone, we did not detect engraftment. It was not until both blockade reagents were included that MHC-disparate BM engrafted. Importantly, engraftment occurred in all animals of the treatment group and was maintained long-term. This is in contrast to a report of similarly treated adult recipients of completely mismatched BM, in which 5 of 15 animals were transiently engrafted and 1 was negative (9).

We also attempted MHC-matched, minor Ag-mismatched BM (C3.SW-H2k→B6-H2b) to examine the role of minor Ags during neonatal engraftment. All recipients engrafted regardless of whether costimulatory blockade reagents were used or not. When CTLA-4Ig was used alone, 1 of 4 animals developed mild chronic GVHD. These results indicate minor Ags, at least with the strain combinations used in this study, do not have a significant role in neonatal graft rejection. Dominant minor Ags in other strains (H60 and B6drefl) have been described that have a significant role in GVHD and may play a role in neonatal graft rejection (34, 35).

In neonatal mice, MHC class II macrophages and dendritic cells are found in thymus, are rare in spleen, and acquire Ag processing later in development (36). Neonatal B cells are immature and poorly up-regulate B7.2 upon ligation of B cell receptors (37). These observations, in addition to differences in cytokine signaling, are thought to explain the poor response to foreign Ag by neonates. During transplantation of adult BM into neonatal recipients, mature donor macrophages and B cells present foreign Ag to neonatal T cells directly. The mature donor APC express CD40 and B7.1–2, enabling full costimulation of neonatal T cells. This explains why neonatal T cells easily reject donor allogeneic BM, and why full costimulatory blockade is required to induce tolerance (9, 38–40).

The costimulatory blockade used in this study was only provided during early engraftment and most likely was involved in the prevention of initiating immune response. Establishment of long-term tolerance after high-dose BM injection and costimulatory blockade in adult mice was previously shown to be mediated by intrathymic clonal deletion (9, 41). The authors elegantly showed donor APC engraft host thymus, and along with host APC and thymic epithelia, present Ag to nascent host and donor T cells. Positive and negative selection mechanisms then delete donor- and host-reactive cells (9, 41). Extrathymic clonal deletion has also been reported as a mechanism of maintaining tolerance in adult mice after establishing chimeric engraftment with costimulatory blockade and low-dose irradiation (42). Taken together, it is reasonable to hypothesize that intrathymic and extrathymic clonal deletion also establish and maintain tolerance after high-dose allogeneic BM engraftment with costimulatory blockade in neonatal recipients.

Our earlier study of chimeric neonatal syngeneic engraftment demonstrated significant therapeutic benefit (4). Nevertheless, comparison with animals with complete donor GUS+ cell engraftment predictably shows further correction of disease due to increased enzyme contribution (43). Therefore, we used DLI to amplify allogeneic chimerism to full donor in the hopes of increasing therapeutic benefit without inducing GVHD (full analysis of treatment efficacy for MPS VII storage disease using this protocol will be published separately). We found that minor Ag disparities alone do not enable amplification of donor engraftment by DLI. In contrast, long-term stable amplification of donor chimeric BM with MHC antigenic disparities occurred with DLI at 43 and 87 days post-BMT. It is not clear why DLI failed to amplify at 22 and 30 days post-BMT. Perhaps the proportion of donor-derived T regulatory cells is very high during the initial stages of allogeneic engraftment in this model (44). Alternatively, the titer of costimulatory blockade reagents may still be at levels that inhibit reactivity of donor lymphocytes toward host BM cells.

The DLI-amplified results demonstrate for the first time complete donor allogeneic engraftment in neonatal recipients without any toxic pretreatment regimens. None of these recipients developed acute GVHD, although several animals developed milder, late onset, chronic GVHD. The most severe histological manifestation observed in this study was fluid-filled/cystic kidneys. In human patients with chronic GVHD, kidney pathology has been reported as membranous nephropathy (45–49), although it remains unclear whether this finding is a direct result of chronic GVHD. In a recent review of chronic GVHD in human patients, the involvement of kidney remains questionable and is not commonly reported (50).

Although still a concern, mild forms of chronic GVHD respond well to systemic immunosuppression and morbidity is low, although more severe forms are associated with high mortality (50). GVHD was not observed in animals with chimeric allogeneic grafts without DLI. In addition to being a nontoxic method to treat a multitude of nonmalignant diseases, high-dose BMT with costimulatory blockade is a safe method to establish donor tolerance before large organ transplantation.

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References