Title

Haploidentical donor T cells fail to facilitate engraftment but lessen the immune response of host T cells in murine fetal transplantation

Journal

British Journal of Haematology, 126(3)

ISSN

0007-1048

Authors

Chen, Jeng Chang
Chang, Ming Ling
Lee, Hanmin
et al.

Publication Date

2004-08-01

Peer reviewed

bjh research paper

Haploidentical donor T cells fail to facilitate engraftment but lessen the immune response of host T cells in murine fetal transplantation

Jeng-Chang Chen,[1,2] Ming-Ling Chang,[3,4] Hanmin Lee[1] and Marcus O. Muench[5]

[1]Fetal Treatment Center, Department of Surgery, University of California, San Francisco, CA, USA, [2]Department of Surgery, Chang Gung Children's Hospital, Taoyuan, Taiwan, [3]Liver Center, Department of Medicine, University of California, San Francisco, CA, USA, [4]Department of Hepatology, Chang Gung Memorial Hospital, Taoyuan, Taiwan, and [5]Department of Laboratory Medicine, University of California, San Francisco, CA, USA

Received 4 March 2004; accepted for publication 27 April 2004

Correspondence: Marcus O. Muench, PhD, University of California, 513 Parnassus Avenue, Room HSW-901B, San Francisco, CA 94143-0793, USA. E-mail: muench@itsa.ucsf.edu

Summary

The effects of donor T cells, or their $CD8^+$ subset, on engraftment and tolerance induction in fetal transplantation were evaluated using an F_1-into-parent mouse-model that does not permit a graft-*versus*-host effect. Gestational day 13 C57BL/6 ($H\text{-}2K^b$) fetuses were transplanted with $B6D2F_1$ ($H\text{-}2K^{b/d}$) light density bone marrow cells (LDBMC) containing 1–2% T cells, T-cell depleted bone marrow cells (TDBMC, <0·1% T cells), or TDBMC with enriched $CD8^+$ T cells (CD8). Chimaerism levels in the peripheral blood, spleen and bone marrow were usually below 0·2% in all groups, indicating that T cells do not improve engraftment without a graft-*versus*-host effect. A significant, but transient, wave of donor cells was seen in the peripheral blood at 1 month of age in the CD8 and LDBMC groups. Relatively high levels of chimaerism (<17%) were sometimes detected in the peritoneal cavities of recipients. T-cell tolerance specific to donor cells was evaluated in mixed lymphocyte cultures. The CD8 and LDBMC groups had significantly lower T-cell responses than untransplanted controls. These findings indicate that *in utero* transplantation of haploidentical donor $CD8^+$ or $CD3^+$ cells can help to lessen the immune response of host T cells towards donor cells. The persistence of donor cells in the peritoneal cavity also correlated with tolerance induction.

Keywords: CD8, engraftment, fetus, T cells, tolerance induction.

In utero transplantation (IUT) of haematopoietic stem cells holds considerable promise as an approach for the management of many congenital haematological and metabolic disorders (Flake *et al*, 1996; Flake & Zanjani, 1997; Muench & Bárcena, 2004). It has successfully created long-term, multilineage, allogeneic or even xenogeneic haematopoietic chimaeras in select animal models without the requirement of histocompatibility matching, immunosuppression or myeloablation (Flake *et al*, 1986; Harrison *et al*, 1989; Zanjani *et al*, 1992, 1994). However, clinical experience with IUT in most circumstances has been unsatisfactory because of limited engraftment. Thus, the future of this approach depends upon developing successful strategies to either improve engraftment *in utero* or induce donor-specific tolerance allowing for subsequent postnatal therapies.

T lymphocytes present in the marrow inoculum can facilitate engraftment, often at the expense of graft-*versus*-host disease (GVHD) in postnatal bone marrow transplantation (Kernan *et al*, 1986; Martin, 1992) as well as in IUT (Bhattacharyya *et al*, 2002). Studies have been undertaken to determine which subsets of T cells are beneficial for engraftment and to determine whether these T cells can be separated from those responsible for GVHD. It was reported that donor $CD8^+$ T cells appeared to be responsible for abrogating rejection as well as generating GVHD in postnatal allogeneic bone marrow transplantation (Martin, 1993; Martin *et al*, 1998). It was also found that donor $CD8^+$ cells could both induce GVHD and facilitate engraftment in the IUT setting of a totally allogeneic mismatched mouse model (Bhattacharyya *et al*, 2002). It was suggested that the graft-*versus*-host effect of donor $CD8^+$ T cells aided stem cell engraftment by generating space *in utero*, at the cost of potentially severe GVHD. Besides a direct cytotoxic effect on host cells, donor T cells can also produce cytokines, which

  doi:10.1111/j.1365-2141.2004.05040.x

may aid engraftment, and their presence in the circulation may alter the response of the host's immune system to the donor cells. Using an F_1-into-parent transplantation model, that does not permit a graft-*versus*-host effect, the current study was aimed at evaluating the role of T cells and their $CD8^+$ subset on stem cell engraftment and/or the induction of donor-specific tolerance in mice.

Materials and methods

Recipient and donor mice

An F_1-into-parent transplantation model was used: C57BL/6 (H-2^b) recipients and B6D2F_1 (C57BL/6 × DBA/2, H-$2^{b/d}$) donors. C57BL/6 (8 weeks of age) mice were purchased from Charles River (Wilmington, MA, USA) or Simonsen Laboratory (Gilroy, CA, USA). B6D2F_1 (8–20 weeks of age) mice were either purchased from the above companies or obtained from a breeding programme at Howard Hughes Medical Institute at University of California, San Francisco (UCSF). All animals were housed in the Animal Care Facility at UCSF under the standard federal guidelines and with the approval of the UCSF Committee on Animal Research. Recipient females were caged with males in the afternoon and checked for vaginal plugs the following morning. The day when the plug was observed was designated as day 0 of the pregnancy.

Preparation of enriched donor $CD8^+$ cells

Donor (B6D2F_1) splenocytes were harvested by passage through 70 μm cell strainers (Becton Dickinson and Co., Franklin Lakes, NJ, USA) and depleted of red cells using ACK buffer, pH 7·2–7·4, consisting of 0·15 mol/l NH_4CL, 1·0 mmol/l $KHCO_3$ and 0·1 mmol/l Na_2EDTA (Sigma Chemical Co., St Louis, MO, USA). The splenic leucocytes were treated with biotinylated anti-CD4, anti-CD19, anti-CD24, anti-MHC class II (I-A/I-E) and anti-Gr1 monoclonal antibodies (BD Biosciences Pharmingen, San Diego, CA, USA). $CD8^+$ T cells were then enriched by negative depletion using streptavidin-coated magnetic Dynabeads (Dynal Biotech Inc., Lake Success, NY, USA).

Preparation of donor bone marrow cells

Adult bone marrow cells (BMC) from B6D2F_1 mice were harvested by flushing the tibias and femurs with phosphate-buffered saline containing 0·3% bovine serum albumin (PBS/BSA) using a 26-gauge needle. Light-density bone marrow cells (LDBMC) were obtained by layering the cells over NycoPrep 1·077A and centrifuging at 600 × ***g*** for 25 min. For obtaining T-cell depleted bone marrow cells (TDBMC), LDBMC were first treated with biotinylated anti-CD3 monoclonal antibody (BD Biosciences Pharmingen) and then depleted magnetically of $CD3^+$ cells using streptavidin-coated Dynabeads.

In utero transplantation

Mice were grouped by different transplant preparations as shown in Table I. All the donor cells were freshly injected within 3 h after preparation. Time-dated pregnant mice (C57BL/6) were subcutaneously anaesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) on day 13 of gestation. The uterus was exposed through a vertical laparotomy. A 60 μm glass micropipette with bevelled tip was used to inject the transplant inoculum in 5 μl of Roswell Park Memorial Institute (RPMI) 1640 medium. The tip of the micropipette was inserted through the uterine wall into the peritoneal cavity of each fetus to deliver donor cells into the fetuses. Then the abdomen was closed using two layers of 4-O silk suture. After the operation, all mice were housed in an undisturbed room without bedding changes until the pups were 1 week old. Pups were weaned at 3 weeks of age.

Analyses of chimaerism

Peripheral blood was harvested from the tail tip at the age of 1, 2 and 3 months. Recipients were killed between 3 and 5 months of age for obtaining splenocytes, BMC and peritoneal cells. Peritoneal cells were harvested first by flushing the peritoneal cavity with 10 ml PBS/BSA using a syringe with an 18-gauge needle. Subsequently, BMC were harvested by flushing the tibias and femurs with PBS/BSA. At last, spleens were removed under sterile conditions, washed with PBS/BSA

Table I. Characteristics of the donor cell inoculum and survival statistics of the three experimental groups.

Group	CD8	LDBMC	TDBMC
Bone marrow graft (per pup)	1×10^6 TDBMC	1×10^6 LDBMC	1×10^6 TDBMC
Percentage of $CD3^+$ cells in BMC (%)	<0·1	1–2	<0·1
$CD8^+$ cells added (per pup)	$3·5–5·0 \times 10^5$ (70–75% $CD8^+$)	No	No
Number of fetuses injected	89	72	80
Survival rate at 1 month of age	13·48% (12/89)	15·28% (11/72)	11·25% (9/80)

BMC, bone marrow cells; LDBMC, light-density bone marrow cells; TDBMC, T-cell depleted bone marrow cells.

and dissociated by passage through 70 μm cell strainers. Samples were depleted of red cells using ACK buffer.

Prior to staining, cells were first incubated with culture supernatant containing anti-mouse FcγII/FcγIII antibody (Clone 2·4G2; American Type Culture Collection, Manassas, VA, USA), and then stained with anti-H-2K^b fluorescein isothiocyanate (FITC) (BD Biosciences Pharmingen) and anti-H-2K^d phycoerythrin (PE) (BD Biosciences Pharmingen). A negative control for each sample consisted of anti-H-2K^b FITC and mouse IgG2a PE (BD Biosciences Pharmingen) to define background staining. In some cases, cells were further stained with anti-H-2K^d PE and either anti-CD4, CD8 FITC, biotinylated anti-CD19, biotinylated anti-Gr1 or biotinylated anti-F4/80 (Caltag Laboratories, Burlingame, CA, USA). FITC-conjugated streptavidin (Caltag Laboratories) was used in conjunction with the biotinylated monoclonal antibodies. One hundred thousand events were acquired for analysis after gating out dead cells using propidium iodide. The level of chimaerism was derived by subtracting the background percentage (defined by double positive for anti-H-2K^b FITC and mouse IgG2a PE) from the percentage of donor cells in recipients (defined by double positive for anti-H-2K^b FITC and anti-H-2K^d PE). Tissues from untransplanted C57BL/6 mice were processed and stained by the protocols described above as an additional control.

Mixed lymphocyte reaction

T-cell tolerance was evaluated by measuring mixed lymphocyte reaction (MLR) using 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) labelling. The responses of CD3$^+$ cells were quantified by flow cytometry as described (Chen *et al*, 2003). Responder cells were red cell-depleted splenocytes from the recipients and stimulator cells were irradiated splenocytes from C57BL/6 (syngeneic), B6D2F$_1$ (donor strain) and FVB/N (third-party) mice. Splenocytes from untransplanted C57BL/6 mice were used as the control responders. Mixed lymphocyte cultures were harvested after 6 d. The number of recipient daughter T cells generated was recorded and directly compared among the experimental (B6D2F$_1$), third-party (FVB/N) and control (C57BL/6) groups. A relative simulation index (SI) was calculated as follows: $SI = (E_{BDF1} - ME_{C57BL/6})/(E_{FVB/N} - ME_{C57BL/6})$ where E_{BDF1} represents the number of events of recipient daughter T cells responsive to B6D2F$_1$ stimulators; $ME_{C57BL/6}$ represents the mean number of events of recipient daughter T cells responsive to syngeneic C57BL/6 stimulators, and $E_{FVB/N}$ represents the number of events of recipient daughter T cells responsive to third-party FVB/N stimulators.

Data presentation and statistical methods

The outcomes of the transplants are reported as the range of the results or as the median, to reduce the effects of extremes and outliers. The data are presented as box plots, and show the 10th (lower bar), 25th (box bottom), 50th (median-bar in box), 75th (box top) and 90th (upper bar) percentiles. Circles in the box plots indicate outlying data points between 1·5 and 3 box lengths from the upper or lower edge of the box. Extremes, shown as an asterisk, were more than 3 box lengths from the upper or lower edge of the box.

The chi-square test was used to determine the significance of differences in the survival rates among the transplant groups. The nonparametric Mann–Whitney *U*-test was used to measure the significance of differences between two transplant groups and the nonparametric Kruskal–Wallis test was used to determine the significance of the effects of a measured parameter among multiple transplant groups. The independent-samples *t*-test was used to compare proliferation of T cells stimulated by the different stimulator cells. Results were considered significantly different when $P < 0·05$.

Results

Graft characteristics and recipient survival

Fetal mice were transplanted with either of three different preparations of donor cells (Table I). The enriched CD8$^+$ population contained <1·5% of CD4$^+$ cells and 70–75% CD8$^+$ cells. LDBMC contained 1–2% CD3$^+$ cells and TDBMC had <0·1% CD3$^+$ cells.

For each group, 10 pregnant mice were operated upon and a total of 241 fetuses were injected (Table I). Most newborns were cannibalized by their mothers within the first week after birth. The actual number of live births was not always determined because such a census was believed to agitate the mothers resulting in cannibalization of the newborns. There were no significant differences in survival at 1 month of age among the three groups ($P = 0·764$, chi-square test). One mouse in the TDBMC group died by the age of 2 months.

Chimaerism levels in the peripheral blood of recipients

Chimaerism in the peripheral blood was examined at 1, 2 and 3 months of age. Chimaerism levels in the blood were usually less than 0·2% (Fig 1A). Nonetheless, the CD8 and LDBMC groups had significantly higher numbers of donor cells in the peripheral blood at 1 month of age than background measurements made using untransplanted mice ($P = 0·007$ and 0·048, respectively). In contrast, there were no significant differences between the TDBMC and untransplanted groups at 1, 2 and 3 months of age ($P \geq 0·142$).

The chimaerism levels of the CD8 and LDBMC groups dropped by 2 months of age, resulting in no significant differences when compared with the untransplanted mice. Two of 12 recipients in the CD8 group still had 0·10–0·15% donor cells in the peripheral blood at 3 months of age, allowing for a more detailed analysis of lineage composition. The donor cells consisted of CD8$^+$ T cells, CD19$^+$ B cells and Gr1$^+$ myeloid cells (Fig 2). The lack of donor CD4$^+$ T cells suggested these cells were derived from mature cells or committed progenitors rather than donor stem cell engraftment.

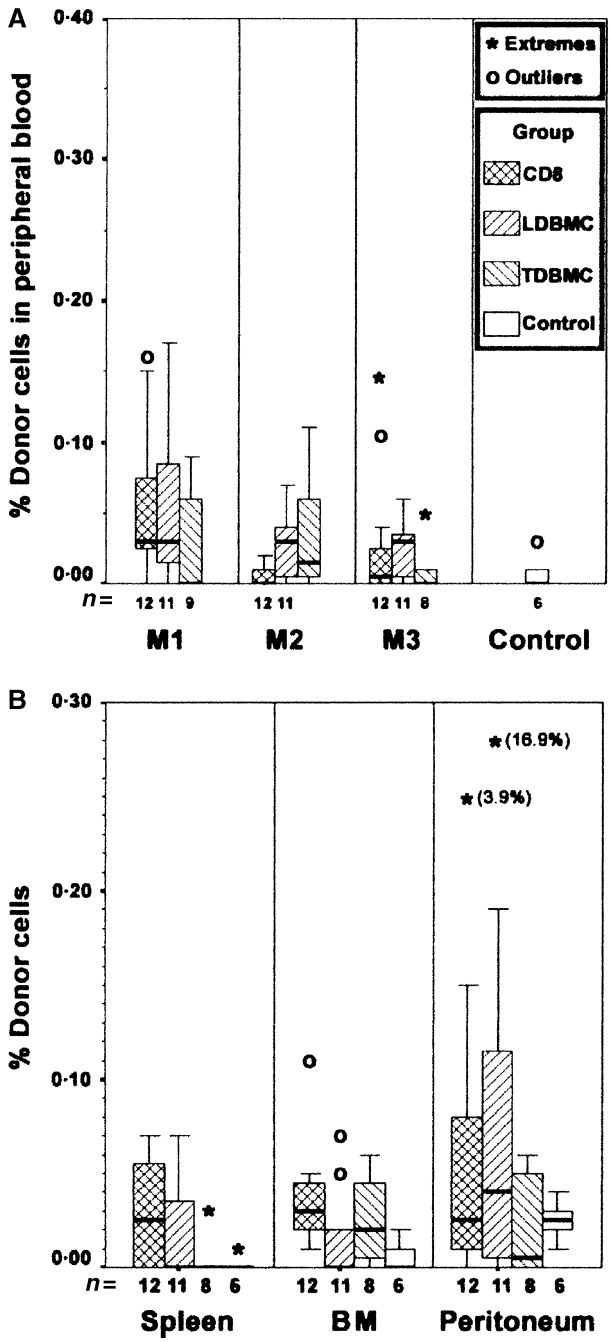

Fig 1. Levels of donor cells in recipients after IUT of various donor cell preparations. (A) The levels of donor cells in the peripheral blood of recipients at the ages of 1 (M1), 2 (M2) and 3 (M3) months are shown. (B) The levels of donor cells in the spleens, bone marrows and peritoneal cavities of recipients at 3–5 months of age are shown. Chimaerism levels are shown using box plots. Chimaerism levels outside the scale are shown in parentheses.

Chimaerism levels in the spleen, bone marrow and peritoneal cavity

Initial engraftment levels were measured in two mice from the CD8 group, 10 d after IUT (at about 3 d of age). There were no detectable donor cells in the peripheral blood, but high levels of donor cells were present in the spleen (0·79%, both mice) and bone marrow (1·51% and 0·65%). $CD8^+$ represented 4·6–38·0% of the donor cells detected in the spleen and bone marrow. Moreover, the donor $CD8^+$ cells represented a significant portion of the total number of $CD8^+$ cells detected in the recipients (Fig 3), indicating that donor T cells can contribute significantly to the neonatal immune system.

By 3–5 months of age, the levels of donor cells were below 0·15% in the spleen and bone marrow. Only the CD8 group showed a significant level of chimaerism in the bone marrow compared with the untransplanted control group ($P = 0·001$, Fig 1B). Two mice from the CD8 group, with the highest levels of donor cells in the peripheral blood, also presented with high levels of donor cells in the bone marrow (0·10–0·15%), which were mostly $CD8^+$ cells (Fig 2) and a small number of $Gr1^+$ cells (data not shown). In addition, the engrafted donor cells in the spleen consisted of $CD8^+$ (Fig 2) and $CD19^+$ cells (data not shown).

Despite the low levels of donor cells in the blood, bone marrow and spleen, some recipients in the CD8 and LDBMC groups displayed high levels of donor cells in their peritoneal cavities (Fig 1B). The median percentage of donor cells in the peritoneal cavities of TDBMC recipients was 0·05% (range 0–0·06%), whereas the median percentages of donor cells for the CD8 and LDBMC recipients was fivefold and eightfold higher, respectively. In addition, the persistence of donor $CD8^+$ cells was observed in the peritoneal cavities of two mice with higher peripheral chimaerism in the CD8 group (Fig 2).

Analysis of T-cell tolerance

To ascertain whether the chimaerism in our recipients was sufficient to induce T-cell tolerance, T cells from the spleens of 3–5-month-old recipients were tested for their proliferative response in mixed lymphocyte cultures. Untransplanted C57BL/6 mice served as controls. A proliferative response to third-party stimulators was observed in all the mice tested. There were five recipients (three in CD8 group, one in LDBMC group and one in TDBMC group) that showed T-cell specific tolerance to donor stimulators. Their T cells were unresponsive to the donor stimulators but responsive to the third-party stimulators when compared with the response to syngeneic stimulators (e.g. Fig 4A; CD8 no. 7; $P = 0·572$ and $<0·001$, respectively). Seven recipients (two in CD8 group, four in LDBMC group and one in TDBMC group) were found to be hyporesponsive to donor stimulators. Their T-cell responses to donor stimulators was significantly more than to syngeneic stimulators but significantly less than to the third-party stimulators (e.g. Fig 4A; CD8 no. 11; $P = 0·033$ and 0·001, respectively). In three recipients (one in the untransplanted controls and two in TDBMC group), hyper-responsive T cells were observed. The T cells from these mice responded significantly more vigorously to donor stimulators than to the third-party stimulators. The other recipients appeared to

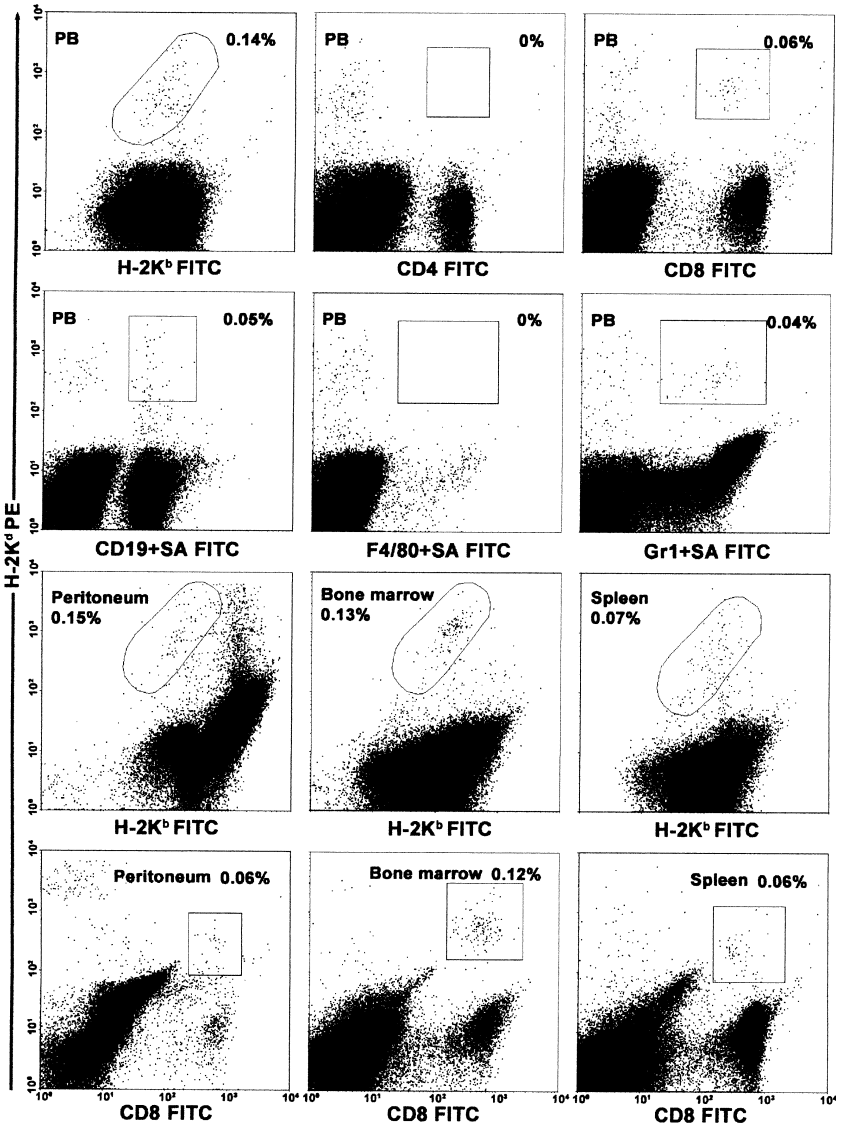

Fig 2. Flow cytometric analysis of chimaerism. Donor cells from the peripheral blood (PB) were analysed at 3 months of age from a recipient of the CD8 group. The lineage composition of the donor cells consisted of $CD8^+$, $CD19^+$ and $Gr1^+$ cells. $CD4^+$ lymphocytes were noticeably absent. The chimaerism of the peritoneal cavity, bone marrow and spleen was examined at 4 months of age, indicating that donor $CD8^+$ cell were a major component of the donor cells.

be equally responsive to donor and third-party stimulators (e.g. Fig 4A, CD8 no. 10; $P = 0{\cdot}282$).

The relative T-cell responses for the different groups were evaluated by SI (Fig 4B). The SI differed significantly among the four groups ($P = 0{\cdot}015$, Kruskal–Wallis test). As indicated by the Mann–Whitney *U*-test, the CD8 and LDBMC groups had significantly lower SI than the untransplanted control group ($P = 0{\cdot}015$ and $0{\cdot}003$ for the CD8 and LDBMC groups, respectively). The SI of the TDBMC group did not differ significantly from the control group ($P = 0{\cdot}107$). These results indicated that the presence of T cells, including only the $CD8^+$ subset of T cells, in the donor inoculum aided in lessening host T-cell response specific to the donor cells.

A link between tolerance and chimaerism levels was also sought by comparing the levels of chimaerism in the different tissues between tolerant and non-tolerant mice. For these analyses, tolerant animals included those animals that were deemed tolerant or hyporesponsive by the statistical analyses described above. Non-tolerant mice included the remaining animals that had a normal response or were hyper-responsive. There were no significant differences between tolerant and non-tolerant animals in the levels of donor cells in the peripheral blood during the first 3 months of life. Neither was there any significant difference in the frequency of donor cells in the bone marrow at the time of killing. There were, however, a greater number of donor cells in the spleens of tolerant animals ($P = 0{\cdot}001$). Splenic chimaerism in tolerant animals ranged from 0·00 to 0·07% (median = 0·03%), whereas in the non-tolerant animals it ranged from 0·00 to 0·05% (median = 0·00%). The presence of donor cells in the peritoneal cavity was also increased in tolerant mice ($P < 0{\cdot}001$). In the tolerant mice, the peritoneal chimaerism ranged from 0·04% to 16·89% with a median of 0·08%. Donor cells represented 0·00% to 0·07% of peritoneal cells, with a median 0·01%, in non-tolerant mice. Thus, all tolerant mice had at least a small frequency of donor cells in their peritoneal cavity and tended

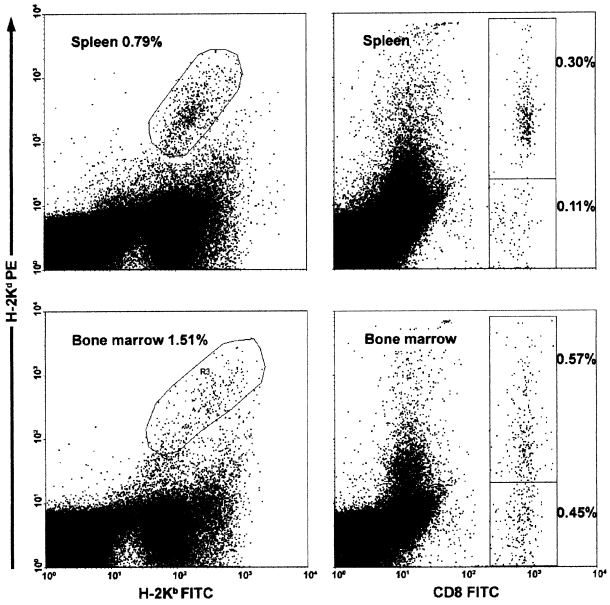

Fig 3. Presence of donor cells in a neonate. A recipient in the CD8 group was analysed 10 d after transplant for the presence of donor cells in the spleen and bone marrow (oval regions, left column). Donor $CD8^+$ cells (upper rectangular regions, right column) were also detected along with host $CD8^+$ cells (lower rectangular regions, right column) in the spleen and bone marrow. Percentages indicate the portion of cells found in the respective regions among all live cells.

to have a small percentage of donor cells in their spleens as well.

Discussion

The observation that T-cell-depleted allografts have a higher rate of graft failure has drawn attention to T cells as facilitators of bone marrow engraftment. The risks and benefits of T cells in IUT are incompletely understood. Clearly, fetal demise because of GVHD will ensue when too many T cells are transplanted (Zanjani *et al*, 1982; Bhattacharyya *et al*, 2002). However, no clear threshold has been established for the dose of T cells that can be tolerated by a human fetus. Moreover, beneficial effects of T cells have been suggested in some studies (Bhattacharyya *et al*, 2002). In this study, we used an F_1-to-parent murine model to elucidate the role of total $CD3^+$ or $CD8^+$ T cells in facilitating tolerance induction and engraftment in the IUT settings so that the role of donor T cells in shaping the development of the immune response could be studied separately from any graft-*versus*-host effect.

The presence of T cells in grafts resulted in higher short-term chimaerism but did not support an increase in durable haematopoietic engraftment. Donor $CD8^+$ cells were readily detectable in the spleen and bone marrow of neonatal mice. Donor cells were also detected in the peripheral blood at 1 month of age in mice transplanted with grafts containing T cells. However, by 2 months of age, peripheral blood chimaerism declined to near minimum detection levels in all

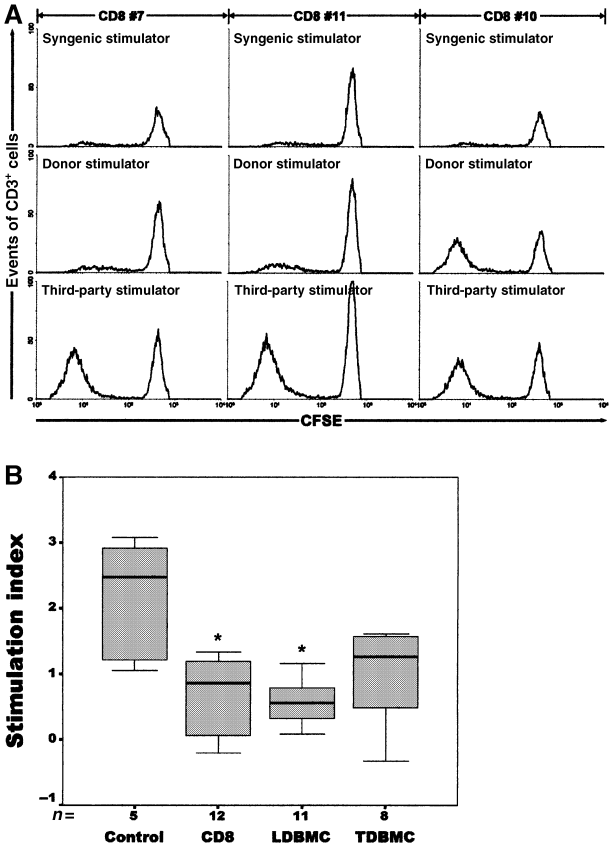

Fig 4. Measurement of T-cell specific response of recipients to donor cells. (A) T-cell specific mixed lymphocyte reaction was measured by carboxyfluorescein diacetate succinimidyl ester labelling. Representative histograms show recipients that were tolerant (CD8 no. 7), hyporesponsive (CD8 no. 11) and normally responsive (CD8 no. 10) to the cells of donor origin. (B) Box plots of the SI for the different transplant groups. The CD8 and light density bone marrow cells groups appeared to have lower SI than the untransplanted control group. Asterisks indicate $P < 0{\cdot}05$ compared with the control group.

groups. Some exceptions were observed, such as two recipients in the CD8 group that had 0·10–0·15% donor cells in the peripheral blood at 3 months of age. These donor cells did not appear to be the result of stem cell engraftment as only $CD8^+$, $CD19^+$ and a few $Gr1^+$ cells were detected but no $CD4^+$ donor cells. The lack of donor $CD4^+$ T cells argued against stem cell engraftment. Carrier *et al* (2000) also observed the persistence of donor $CD3^+$ T cells after IUT. The low levels of durable chimaerism seen in our study are similar to a number of previous reports (Carrier *et al*, 1995, 1997; Hajdu *et al*, 1996; Kim *et al*, 1998; Milner *et al*, 1999; Oppenheim *et al*, 2001). However, high levels of chimaerism have been observed with fully allogeneic IUT in which donor $CD8^+$ T cells could effectively facilitate engraftment (Bhattacharyya *et al*, 2002). Likewise, postnatal donor lymphocyte infusion after prenatal tolerance induction has resulted in high levels of engraftment (Hayashi *et al*, 2002). As donor T cells did not increase stem cell engraftment in our F_1-into-parent transplantation model we can conclude that a T-cell mediated graft-*versus*-host effect

is probably responsible for the improved engraftment. Localization of the donor T cells within the haematopoietic tissues, as observed in neonates in this study, positioned these cells to aid haematopoietic engraftment by a cytotoxic mechanism that generates space for donor stem cells.

Some mice in this study displayed a surprisingly high level of peritoneal chimaerism at 3–5 months of age. High levels of donor cells in the peritoneum did not necessarily correlate with donor cell levels in the other tissues. Without a consistent presence of donor cells in the other tissues examined, the origin of donor cells in the peritoneum is most likely to be the expansion of donor BMC *in situ* rather than the egress of mature donor cells from haematopoietic stem cells engrafted in the bone marrow. Thus, an intriguing possibility is that the donor cells in various recipients' tissues might migrate from the peritoneal cavity after their differentiation and maturation. This could explain the presence of $Gr1^+$ myeloid cells observed in mice several months after IUT but without further evidence of stem cell engraftment.

Despite low chimaerism levels, T-cell tolerance was observed in some mice, suggesting that marked stem cell engraftment is not necessary for prenatal tolerance induction. The donor-specific MLRs were reduced significantly in the LDBMC and CD8 groups, suggesting a helpful role for donor T cells (total $CD3^+$ or enriched $CD8^+$ cells, respectively) in attenuating the immune response of host T cells to the donors. The levels of donor cells were higher in the peritoneum and spleen of tolerant or hyporesponsive animals, whereas there was no difference in chimaerism levels in the bone marrow. There were also no differences between non-tolerant animals and the tolerant/hyporesponsive mice in the levels of donor cells in the peripheral blood during the first 3 months of life. These findings indicate that tolerance required lasting chimaerism, because all tolerant mice had at least some donor cells in their peritoneum at the time of killing and blood chimaerism measured at the earlier time points did not correlate with tolerance.

Several studies have suggested that transplantation with unpurified sources of stem cells might be the best for creating chimaerism and inducing tolerance. One group transplanted a large number of highly purified c-kit^+/Lin^- or Sca^+/Lin^- cells but failed to induce tolerance in mice despite microchimaerism (Donahue *et al*, 2001; Sefrioui *et al*, 2002). On the contrary, sensitization was observed resulting in the accelerated rejection of skin grafts. This prenatal sensitization, in association with low levels of donor $CD3^+$ cells, was also suggested in this study, as evidenced by two recipients in the TDBMC group that displayed hyper-reactivity of host T cells against donor cells. Additionally, a high dose of highly purified $CD90^+$ $CD34^+$ stem cells (0·03% $CD3^+$ cells) also failed to induce T-cell tolerance in a human fetus with chronic granulomatous disease (Muench *et al*, 2001). Successful tolerance induction has been reported in mice with minimally purified or unpurified grafts (Carrier *et al*, 1995; Hajdu *et al*, 1996; Kim *et al*, 1998; Hayashi *et al*, 2002). Tolerance induction has also been indicated in IUT for human α-thalassaemia with $CD34^+$ stem cells containing 1% $CD3^+$ cells (Hayward *et al*, 1998). Taken together, these results raise the possibility that donor T cells might play a role in lessening the immune response of host T cells.

Acknowledgments

This work was supported by grant CMRPG32094 (JCC) from Chang Gung Children's Hospital, Taoyuan, Taiwan and by NIH grant DK59301 (MOM). Drs Jeng-Chang Chen and Ming-Ling Chang were visiting scholars at the University of California, San Francisco from Taiwan during the period of 16 October 2000 to 15 October 2002.

We thank Rong-Hua Lu for his technical support in fetal injection, and Drs Alicia Bárcena, Michael R. Harrison and Yuet-Wai Kan for their suggestions, helpful discussions and support. We also wish to thank Paul Dazin for assistance with flow cytometry. We also thank Drs Akihiko Hara, KuoJen Tsao and Linda Flebbe-Rehwaldt for providing us with assistance in the care and breeding of the mice.

References

Bhattacharyya, S., Chawla, A., Smith, K., Zhou, Y., Talib, S., Wardwell, B. & Cowan, M.J. (2002) Multilineage engraftment with minimal graft-versus-host disease following in utero transplantation of s-59 psoralen/ultraviolet a light-treated, sensitized T cells and adult T cell-depleted bone marrow in fetal mice. *Journal of Immunology*, **169,** 6133–6140.

Carrier, E., Lee, T.H., Busch, M.P. & Cowan, M.J. (1995) Induction of tolerance in nondefective mice after in utero transplantation of major histocompatibility complex-mismatched fetal hematopoietic stem cells. *Blood*, **86,** 4681–4690.

Carrier, E., Lee, T.H., Busch, M.P. & Cowan, M.J. (1997) Recruitment of engrafted donor cells postnatally into the blood with cytokines after in utero transplantation in mice. *Transplantation*, **64,** 627–633.

Carrier, E., Gilpin, E., Lee, T.H., Busch, M.P. & Zanetti, M. (2000) Microchimerism does not induce tolerance after in utero transplantation and may lead to the development of alloreactivity. *Journal of Laboratory and Clinical Medicine*, **136,** 224–235.

Chen, J.C., Chang, M.L. & Muench, M.O. (2003) A kinetic study of the murine mixed lymphocyte reaction by 5,6-carboxyfluorescein diacetate succinimidyl ester labeling. *Journal of Immunological Methods*, **279,** 123–133.

Donahue, J., Gilpin, E., Lee, T.H., Busch, M.P., Croft, M. & Carrier, E. (2001) Microchimerism does not induce tolerance and sustains immunity after in utero transplantation. *Transplantation*, **71,** 359–368.

Flake, A.W. & Zanjani, E.D. (1997) In utero hematopoietic stem cell transplantation. A status report. *Journal of American Medical Association*, **278,** 932–937.

Flake, A.W., Harrison, M.R., Adzick, N.S. & Zanjani, E.D. (1986) Transplantation of fetal hematopoietic stem cells in utero: the creation of hematopoietic chimeras. *Science*, **233,** 776–778.

Flake, A.W., Roncarolo, M.G., Puck, J.M., Almeida-Porada, G., Evans, M.I., Johnson, M.P., Abella, E.M., Harrison, D.D. & Zanjani, E.D.

(1996) Treatment of X-linked severe combined immunodeficiency by in utero transplantation of paternal bone marrow. *New England Journal of Medicine*, **335,** 1806–1810.

Hajdu, K., Tanigawara, S., McLean, L.K., Cowan, M.J. & Golbus, M.S. (1996) In utero allogeneic hematopoietic stem cell transplantation to induce tolerance. *Fetal Diagnosis and Therapy*, **11,** 241–248.

Harrison, M.R., Slotnick, R.N., Crombleholme, T.M., Golbus, M.S., Tarantal, A.F. & Zanjani, E.D. (1989) In-utero transplantation of fetal liver haemopoietic stem cells in monkeys. *Lancet*, **2,** 1425–1427.

Hayashi, S., Peranteau, W.H., Shaaban, A.F. & Flake, A.W. (2002) Complete allogeneic hematopoietic chimerism achieved by a combined strategy of in utero hematopoietic stem cell transplantation and postnatal donor lymphocyte infusion. *Blood*, **100,** 804–812.

Hayward, A., Ambruso, D., Battaglia, F., Donlon, T., Eddelman, K., Giller, R., Hobbins, J., Hsia, Y.E., Quinones, R., Shpall, E., Trachtenberg, E. & Giardina, P. (1998) Microchimerism and tolerance following intrauterine transplantation and transfusion for α-thalassemia-1. *Fetal Diagnosis and Therapy*, **13,** 8–14.

Kernan, N.A., Collins, N.H., Juliano, L., Cartagena, T., Dupont, B. & O'Reilly, R.J. (1986) Clonable T lymphocytes in T cell-depleted bone marrow transplants correlate with development of graft-v-host disease. *Blood*, **68,** 770–773.

Kim, H.B., Shaaban, A.F., Yang, E.Y., Liechty, K.W. & Flake, A.W. (1998) Microchimerism and tolerance after in utero bone marrow transplantation in mice. *Journal of Surgical Research*, **77,** 1–5.

Martin, P.J. (1992) Determinants of engraftment after allogeneic marrow transplantation. *Blood*, **79,** 1647–1650.

Martin, P.J. (1993) Donor CD8 cells prevent allogeneic marrow graft rejection in mice: potential implications for marrow transplantation in humans. *Journal of Experimental Medicine*, **178,** 703–712.

Martin, P.J., Akatsuka, Y., Hahne, M. & Sale, G. (1998) Involvement of donor T-cell cytotoxic effector mechanisms in preventing allogeneic marrow graft rejection. *Blood*, **92,** 2177–2181.

Milner, R., Shaaban, A., Kim, H.B., Fichter, C. & Flake, A.W. (1999) Postnatal booster injections increase engraftment after in utero stem cell transplantation. *Journal of Surgical Research*, **83,** 44–47.

Muench, M.O. & Bárcena, A. (2004) Stem cell transplantation in the fetus. *Cancer Control*, **11,** 105–118.

Muench, M.O., Rae, J., Barcena, A., Leemhuis, T., Farrell, J., Humeau, L., Maxwell-Wiggins, J.R., Capper, J., Mychaliska, G.B., Albanese, C.T., Martin, T., Tsukamoto, A., Curnutte, J.T. & Harrison, M.R. (2001) Transplantation of a fetus with paternal Thy-1(+)CD34(+)cells for chronic granulomatous disease. *Bone Marrow Transplantation*, **27,** 355–364.

Oppenheim, S.M., Muench, M.O., Gutierrez-Adan, A., Moyer, A.L., BonDurant, R.H., Rowe, J.D. & Anderson, G.B. (2001) Hematopoietic stem cell transplantation in utero produces sheep-goat chimeras. *Blood Cells, Molecules and Diseases*, **27,** 296–308.

Sefrioui, H., Donahue, J., Srivastava, A.S., Gilpin, E., Lee, T.H. & Carrier, E. (2002) Alloreactivity following in utero transplantation of cytokine-stimulated hematopoietic stem cells: the role of recipient CD4(−) cells. *Experimental Haematology*, **30,** 617–624.

Zanjani, E.D., Lim, G., McGlave, P.B. Clapp JF, Mann LI, Norwood TH, Stamatoyannopoulos G. (1982) Adult haematopoietic cells transplanted to sheep fetuses continue to produce adult globins. *Nature*, **295,** 244–246.

Zanjani, E.D., Pallavicini, M.G., Ascensao, J.L., Flake, A.W., Langlois, R.G., Reitsma, M., MacKintosh, F.R., Stutes, D., Harrison, M.R. & Tavassoli, M. (1992) Engraftment and long-term expression of human fetal hemopoietic stem cells in sheep following transplantation in utero. *Journal of Clinical Investigation*, **89,** 1178–1188.

Zanjani, E.D., Flake, A.W., Rice, H., Hedrick, M. & Tavassoli, M. (1994) Long-term repopulating ability of xenogeneic transplanted human fetal liver hematopoietic stem cells in sheep. *Journal of Clinical Investigation*, **93,** 1051–1055.